BRAIN CELLULAR IMMUNRESPONSE, C-*FOS* PROTEIN EXPRESSION, MOTOR AND COGNITIVE PERFORMANCE FOLLOWING CLOSED HEAD INJURY IN RATS

Ph.D. Thesis

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LIST OF ABBREVIATIONS IN THE TEXT

BBB: blood-brain barrier

CHI: closed head injury

CNS: central nervous system

Gd-DTPA: gadolinium-diethylenetriamine pentaacetat

ICP: intracranial pressure

IEGs: immediate-early genes

IR: immunoreactive

MHC: major histocompatibility complex

MRI: magnetic resonance imaging

TBI: traumatic brain injury

THH: trauma + hypoxia + hypotension

1. INTRODUCTION

An elevated intracranial pressure (ICP) subsequent to brain swelling is the single, most frequent cause of death in head injured patients. Analysis by the American Traumatic Coma Data Bank indicates that the probability of mortality and morbidity increases with time during elevated ICP levels above 20 mmHg [75]. Thus raised ICP therefore continues to be a prominent feature of severe traumatic head injury. Morphologic and magnetic resonance studies of traumatic brain injury (TBI) have provided compelling evidence that brain swelling is associated with axonal injury and a predominantly cellular edema formation, which begins within 1 h post injury and becomes dominant at 1-2 weeks post injury [4;76;81;93;105].

1.1. Cellular immunresponse following head injury

Although information concerning the process of cytotoxic damage in TBI is limited [8;9], there is some evidence of an inflammatory response in the pathogenesis of head injury; as such, brain swelling appears to be, in part, an inflammatory process [17;26;55;64]. The central nervous system (CNS) has often been considered an “immunologically privileged site”, this presumption being based on the absence of lymphatic drainage and the unique type of endothelium forming the blood-brain barrier (BBB). The data suggest that, even under normal conditions, there is a moderate traffic of hematogenous cells through the BBB [5;47;110]. In immune-mediated illnesses of the CNS, large quantity of the cells of the immune system is permitted to enter the brain [44;45;62;90;108]. These inflammatory cells capable of entering the CNS include T-cells and macrophages, whereas B-lymphocytes are less abundant and neutrophils are rarely detected.

There are two major subtypes of TBI: focal and diffuse damage. It is clear that cortical contusion, and particularly a penetrating head injury such as a stab wound damaging the BBB, leads to the recruitment of numerous circulating monocytes and white blood cells [49;50]. Since contamination of the contusion in open head injuries clearly visible, the contribution of inflammation to traumatic brain swelling in such injuries has never been questioned. Although potential roles of microglia activation and the release of mediators have been suggested, direct evidence of the cellular immune reactivity (and entry of immune cells into the brain) in diffuse TBI following induction of a closed head trauma has not been presented so far. Furthermore,

several papers proved that the contusional model elicits an entirely different immunological reaction pattern than does the diffuse brain injury model [15].

1.2. Induction of immediate-early genes (IEGs)

Following brain injury neurons degenerate, while surviving neurons may undergo neuritogenesis and synaptogenesis to reestablish the normal function. Although, information concerning the pathophysiology of cytotoxic damage in TBI is limited, the experimental evidence indicates, that a deterioration of the calcium homeostasis [4;41; 42;92]., the accumulation of free radicals, breakdown of the blood-brain barrier (BBB) and an excessive release of excitatory amino acids contribute to the parenchymal damage [28;87;94].. A few literature data describe the induction of immediate-early genes (IEGs) in TBI [1;6;16;24;41;92;94;95;96;112]. However, a comprehensive description of IEG induction is not to be found in the literature, although IEG expression is indicative of long-lasting phenotypic changes and cell death, too. One method that has recently been used to study the cellular response to brain injury is to measure the expression of IEGs [21;22;23;84]. The IEG c-*fos* has been identified as a proto-oncogene and assigned a role as a transcription factor and a marker of activated and injured neurons [87;102]. Similarly to other injuries, TBI, whether induced by fluid percussion or by impact acceleration, results in the transcription of c-*fos* gene and the subsequent expression and phosphorylation of the Fos protein [1;112]. Most studies characterizing the induction of IEGs in experimental models of TBI have been restricted to a short timescale and some representative brain regions, and little or no information is available on the whole brain and large-scale time-related dynamics of c-*fos* expression.

1.3. Neuropsychological dysfunction

Long- and short-term neuropsychological deficits after craniocerebral trauma remain a significant clinical problem, despite considerable efforts to understand and prevent these consequences. Good post-traumatic recovery of patients, with attainment of appropriate levels of behavior and cognition, is one of the primary aims of therapy. Behavioral outcome, therefore, represents a focus of both clinical and experimental research into novel therapeutic interventions.

Clinically, neuropsychological deficits are detected following head injury using many different protocols, each with its own merits to recommend [66;97]. In considering

neuropsychological deficits following experimental injury, such protocols are difficult to replicate in the laboratory. However, they are frequently approximated in the rodent using a battery of well-validated tests including the Morris water maze, the beam walk and beam balance tests, the inclined plane test and the monitoring of acute reflex suppression after injury. These tests have also been successfully used to investigate the benefit of novel therapies on neuro-psychological function following experimental head injury [73].

Shirotoni has previously demonstrated poor performance in a battery of behavioral tests after impact acceleration injury in the rodent [104]. However, the relationship between behavioral deficits and the severity of the impact acceleration injury has not been described and in particular it is not known whether the severity of an injury can predict the severity of resulting behavioral deficit with this model.

The other perspective to this question is how sensitive these behavioral tests are to experimental injury severity and, therefore, how responsive the tests are to subtle changes in injury gradation. This information is important for understanding behavioral data using the impact acceleration model of head injury, and is especially important when considering the effects of experimental therapeutic interventions. Effective therapies may make comparatively modest changes to the overall severity of the injury. If behavioral tests cannot show a gradation in the severity of deficit, which matches the severity of the injury, then few conclusions can be made about the effects of treatment.

2. OBJECTIVES

2.1. To assess the time-dependent changes in microglia activation and lymphocyte migration in an experimental model of diffuse TBI. With this aim, our first step was to provide evidence of the entry of lymphocytes into the brain; next, we attempted to describe the temporal characteristics of lymphocyte migration.

2.2. To follow the changes in c-*fos* expression during the first 24 h after TBI, and to compare the sensitivities of different brain areas on the basis of quantitative immunohistochemistry.

2.3. To assess behavioral and cognitive parameters in five groups of animals, exposed to differing severities of impact acceleration injury. Experimental end points included acute behavioral observations, the Morris water maze, the beam walk test, the beam balance test, and the animal's weight, as a measure of general health. The data were analyzed to assess whether there were graded behavioral deficits, which matched the gradation of injury severity.

3. MATERIAL AND METHODS

3.1. Time-dependent changes in microglia and lymphocyte activation

One-hundred adult male Wistar rats (weighing 320 to 350grams) were used in the study. The experiments were conducted in accordance with prevailing laws and ethical considerations. Written permission was obtained in advance from the University of Szeged Faculty Ethical Committee on Animal Experiments. The rats were initially anesthetized with isoflurane, then intubated and artificially ventilated with a gas mixture of N_2O (70%), O_2 (30%) and isoflurane (0.5-1.5%). The rectal temperature was monitored and the body temperature was maintained at 37 °C ± 0.5 °C by means of heat.

Impact acceleration injury

An impact acceleration head injury model was used to produce the closed head injury (CHI) [80]. A midline scalp incision was made, the skin and periosteum were reflected, and the skull was carefully dried. A round stainless steel disc was mounted on the skull with super glue. When the bonding agent was dry, the rat was positioned under a hollow Plexiglas tube, disconnected from the respirator, and a sectioned brass weight of 450 g was dropped from a height of 2 m onto the center of the metal disc. Under these experimental conditions, a mortality rate of 44% resulted with a low incidence of skull fracture [29;80]. The disc was used with a view to preventing skull fracture. After induction of the trauma, the rat was rapidly reconnected to anesthesia and artificially ventilated, and the wound was closed.

Schedule of the measurements

The rats (n=100) were separated into three groups: *group Ia:* unoperated controls (n=20); g*roup Ib:* sham-operated controls (n=20); g*roup II:* trauma (n=60).

At various times after TBI induction (5, 15, 30 or 45 min, or 1, 2, 3, 6, 12 or 24 h), the rats (6 at each survival time) were perfused transcardially with 500 ml of chilled 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

CD3 (T-lymphocyte) immunohistochemistry

Postfixed brains were routinely embedded in paraffin and 4 µm thick serial sagittal

sections were cut and air-dried on silanized slides. For the immunohistochemistry of CD3, the samples were dewaxed in xylene, and rehydrated in a series of decreasing concentrations of ethanol. The sections were submerged in a target retrieval solution of DAKO (code no. S1699), and boiled in a pressure-cooker for 5 min.

The specimens were preincubated in 10% normal swine serum, and processed for immunohistochemistry, using rabbit anti-human CD3 (DAKO; code no. N1580; prediluted form) overnight at room temperature. The sections were then reacted with DAKO EnVision+ (EnVision+, Peroxidase, Rabbit; code no. K4003) for 1 h. The immunereactions were developed in 3,3'-diaminobenzidine (Sigma) for 30 min, then slightly counterstained with hematoxylin (DAKO Automation Hematoxylin; code no. S3301), and finally covered with DePeX (Fluka).

Sections from the brain were analyzed semiquantitatively in each group. Ten sections from each brain were examined and the T-cells in the brain parenchyma were counted under a light microscope (E600; Nikon). The brain regions examined included the cortex, diencephalon, brain stem and cerebellum. The number of T-lymphocytes was assessed statistically by means of repeated measurement ANOVA.

CD11b (microglia) immunohistochemistry

The brains were dissected and postfixed for 16 h at 4 °C. Following postfixation, the brains were cryoprotected overnight in 30% sucrose-containing 1 M phosphate buffer, pH 7.4. The brain samples were cut at coronal planes on a freezing microtome. Serial sections of 24 µm thickness were collected in 0.01 M phosphate buffer containing 0.9% NaCl, supplemented with 0.1% sodium azide. Representative free-floating sections were pretreated in 3% H_2O_2 and in 0.5% Triton X-100 for 10 min each. The samples were treated in 10% normal swine serum for 1 h, and then processed for immunohistochemistry, using biotin-labeled mouse anti-rat CD11b antibody (Serotec; cat. no. MCA275B) at a dilution of 1:1000 at room temperature overnight. Next, specimens were incubated in a 1/1000 dilution of peroxidase-labeled streptavidin (Jackson Immunoresearch) for 1 h. Primary antibody binding was visualized with 3,3'-diaminobenzidine (Sigma) in the presence of nickel ammonium sulfate for 15 min. The sections were finally mounted on glass slides, air-dried and covered with DePeX .

3.2. Induction of immediate-early genes (IEGs)

A total of 143 adult male 320-350 g Wistar rats were used. The experiments were conducted in accordance with prevailing laws and ethical considerations. Written permission was obtained in advance from the University of Szeged Faculty Ethical Committee on Animal Experiments. The rats were initially anesthetized with isoflurane, then intubated and artificially ventilated with a gaseous mixture of N_2O (70%), O_2 (30%) and isoflurane (0.5-1.5%). Rectal temperature was monitored and body temperature was maintained at 37 °C ± 0.5 °C by means of a heating pad.

After the impact acceleration injury, each rat was rapidly reconnected to the anesthesia and artificially ventilated, and the skin wound was closed with sutures (trauma group, Group I, n=48). The sham-operated animals (Group IIa, n=16) underwent surgery and ventilation, but no CHI was applied. The control animals (Group IIb, n=16) were neither operated, nor ventilated.

C-*fos immunocytochemistry*

At various times after CHI induction (5, 15 or 30 min, or 1, 3, 6, 12 or 24 h), rats subjected to the trauma (6 animals at each time), together with sham-operated and control animals (2 at each time) were perfused transcardially with 500 ml cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for immunocytochemical processing as described below.

The brains were dissected and postfixed for 16 h at 4 °C. Following postfixation, the brains were cryoprotected overnight in 30% sucrose-containing 1 M phosphate buffer, pH 7.4. The brain samples were cut at coronal planes on a freezing microtome. Serial sections (24 µm thick) were collected in 0.01M phosphate buffer containing 0.9% NaCl, supplemented with 0.1% sodium azide.

Every fifth section was processed for routine c-*fos* immunocytochemistry. Tissue specimens were incubated first in an affinity-purified polyclonal antibody to c-*fos* at a dilution of 1:2000 (Santa Cruz Biotechnology, CA, USA), overnight, followed by biotin-labeled goat anti-rabbit IgG (1:400; Jackson ImmunoResearch, PA, USA) for 1 h. Finally, peroxidase-labeled streptavidin (1:1000, Jackson) was applied to the sections for 1 h. Tissue-bound peroxidase was developed with standard nickel-containing diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) solution for 15 min.

Quantitative analysis of c-fos immunoreactivity

C-*fos* immunoreactivity was observed in cell nuclei. Areas were selected from several regions of the brain: neocortex, subcortical white matter (corpus callosum), hippocampus (CA1 and hilum), thalamus (centromedian and reticular nuclei), putamen, hypothalamus (paraventricular nuclei), cerebellum and serial sections of the brain stem (medulla, pons and mesencephalon). The immunoreactive (IR) cell nuclei displaying grayish-black staining were counted with the aid of a Nikon Eclipse 600 microscope equipped with a Polaroid DMC digital camera (1600 x 1200 dpi in 8 bits) with 40x objective magnification, using the Image Pro Plus 4 morphometry program (Media Cybernetics, Silver Spring, MD). Following background subtraction, the threshold was adjusted so that pale-stained and deep-stained nuclei could be equally recognized by the counting program. The area of interest was the rectangular tissue area captured by the camera (0.05 mm^2). This measured value was used when the cell numbers were normalized to 1 mm^2. The cell counting was made blind of the treatment.

The cell counts were analyzed by ANOVA (*post-hoc* test: Bonferroni method). The statistical analysis was performed with the SPSS 9.0 computer program.

3.3. Assessment of neuropsychological dysfunction

Adult male Sprague-Dawley rats weighing 360g-385 g (meanis.d. 356g±8.7g) were examined before and after impact acceleration injury for both acute and long-term motor and cognitive deficits. Throughout the protocol they were housed in the same animal care facility with food and water available *ad libitum,* during a 12 h light/dark cycle. All animals used received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, 1985), and in compliance with the VCU Institutional Animal Care and Use Committee Regulations.

Experimental procedures

One hundred animals were divided into five subgroups, which varied in the intensity of injury they received, by altering either the weight of the plexor or its acceleration at contact with the pleximeter, as defined by the height fallen. Group 1 was exposed to sham injury (n = 39);

Group 2 received an injury from a 450 g weight falling 1 m, a 'mild' injury (n = 8); Group 3 was exposed to a 450g weight falling 2 m (n = 40), a 'moderate' injury; Group 4 was injured by a 500 g weight falling 2.1 m (n = 8) and Group 5 was exposed to a 450 g weight falling 2 m with a 10min secondary insult of hypoxia and hypotension (n = 5), 'severe' injuries. The rationale for adding a ten-minute insult of hypoxia and hypotension to Group 5 is the knowledge that clinical patients exposed to hypoxia or hypotension have both a higher mortality rate and a worse outcome [11;74;101].

Pre-operative behavioral training was undertaken on the two days prior to injury for the beam balance and beam walk tests. Baseline behavioral tests were completed on the day of injury for the beam balance, beam walk, and inclined plane tests. Post-operative testing started on the first day after injury for ail tests except the Morris water maze. Water maze testing was undertaken on post-operative days 14-18. General testing continued for 31 post-operative days, after which the animals were sacrificed.

Surgical preparation and injury

The methodology of impact acceleration injury is described in chapter 3.1.

In the group undergoing secondary insult, hypoxia and hypotension were induced by manipulation of anesthesia. Reduction of oxygen to 12% of pre-trauma values, and an increase in halothane to 2.5% resulted in hypotension of 30 ±5 mmHg and hypoxia of 47±5 mmHg O_2 as determined by a preliminary series of experiments [104;113]. Hypoxia and hypotension were sustained for 10 min, after which anesthesia was returned to pre-trauma configuration. All animals that died on impact or experienced skull fractures were excluded from the study. Animals considered as shams were exposed to the same experimental manipulations without either weight drop, hypoxia or hypotension.

Behavioral evaluations

A series of both acute and chronic behavioral evaluations were undertaken on the groups of animals as described above. Acute behavioral reflexes were measured in Groups 1, 2, 3 and 4 while chronic behavioral deficits were evaluated in Groups 1-5.

I. Acute behavioral suppression

For each of the following reflexes the length of time (in seconds) which it took for the reflex to return during recovery was noted.

1. Non-postural somatomotor functions were tested by recording the duration of the suppression of the corneal reflex, as assessed by lightly touching the cornea with a cotton-tipped applicator in order to elicit a blink reflex.
2. Postural somatomotor functions were tested by measuring the duration of suppression of hindpaw and tail flexion reflexes. The hindpaw reflex is assessed by applying a pinch to the paw and observing for a withdrawal response, the pinch being administered by blunt-ended tissue forceps. The tail flexion reflex was assessed in the same way by application of pressure to the proximal tail.
3. Complex postural somatomotor function was assessed by observing the duration of suppression of the head support, righting and escape responses. The head support suppression is the time taken for the animal to support its own head weight, and is a measure of nuchal muscle tone. The righting response is the animal's ability to return to a prone position when placed on its back. The escape response is defined as an organised locomotor activity away from a noxious stimulus, in this case a brief pinch to the tail again using blunt ended tissue forceps.

II. Chronic behavioral evaluation

Long term assessment of locomotor function was made using several tasks: beam-balancing, beam-walking, and inclined plane balancing. Cognitive processes and in particular visuospatial memory were assessed using the Morris water maze. Locomotor tests involved two days of pre-insult training, baseline testing on the day of insult and daily assessment starting 24 h post-insult, for 10 days, and on every third day after this until 31 days post-trauma. The water maze testing was undertaken on post-operative days 14-18, in order to avoid the confounding effects of transient motor deficits and as a correlate of the ability to learn a new task. In addition the weight of each animal was measured daily.

The beam balance test

The animal is placed on a suspended wooden beam (1.5 cm diameter), and the quality of balance is graded according to the following scale: 1, balances with steady

posture; 2, grasps beam, but steady; 3, grasps beam with lateral/medial balancing movements, generally unsteady; 4, falls in 10-60 sec; 5, falls in 0-10 sec; and 6, no attempt to balance at all. Daily assessment consisted of three trials from which the mean scores were taken. Baseline was considered as the assessment immediately prior to surgery, and the training consisted of repeated testing until the animal could balance for 60 sec with a score of 1 or 2. The task specifically assesses gross vestibulomotor function [72].

The beam walk test

This test assesses fine motor co-ordination [27]. The goal of the task is for the rats to traverse a wooden beam (2.5 cm diameter, 100 cm long), and enter a darkened goal box at the end, in order to escape a noxious stimuli. In this instance bright light and loud noise were used, which were terminated as soon as the rat entered the box. Four steel pegs (4 cm high, 3 mm diameter), were also placed at even intervals along the beam in order to increase the difficulty of the task. Performance equated to the animal's latency to traverse the beam. Daily testing consisted of three trials from which the mean latency was determined. The animal was allowed to remain in the box for 30 sec in between trials. Baseline was the latency immediately prior to surgery, and training consisted of repeatedly testing the animals until they could achieve latencies consistently less than 8 sec.

Motor ability

This was further tested using an inclined plane test [100], which consists of two rectangular plastic boards joined at one end by a hinge. One board serves as a base, the other as a movable plane. The rat is placed on the plane and raised until it is no longer able to balance and the angle at which this occurs is recorded. Daily testing consisted of three trials from which the mean was determined. There was no training necessary for this task.

The Morris water maze

The maze [88;89] was constructed from a 180 cm diameter and 60 cm high metal pool, filled with water to a depth of 28 cm. The water is made opaque with non-toxic white paint. A platform 10 cm diameter and 26 cm high is placed at a fixed point in the

water. Rats were given four consecutive trials on five consecutive days, starting from four equidistant points around the edge of the pool (north, east, south and west) in a random order. Rats were given a maximum of 120 sec to locate the hidden platform, and the latency time for each location recorded. If a rat failed to find the platform it was placed on the platform by the investigator. Each rat was left on the platform for 30 sec after every trial. The daily data consisted of the mean of the four trials. Testing occurred over five consecutive days post injury (days 14-18).

Statistical analysis

All values are expressed as means±SEM, and the Student's t-test was used to assess the significance of the differences between the groups.

4. RESULTS

4.1. Lymphocyte migration and microglia activation

T-cell entry into the CNS parenchyma

Control groups (Ia and Ib): In sections showing CD3 positivity, most T-lymphocytes were found in regions without a BBB such as the area postrema and the pineal gland (**Figure 1**). A few T-cells (0-0.05/mm^2) were also found in the brain parenchyma with an intact BBB, but this finding appeared consistent during the next 24 h.

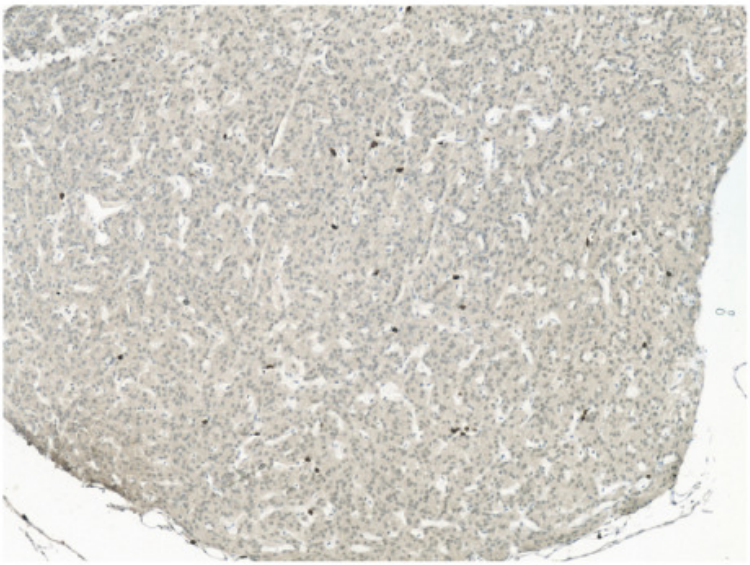

Figure 1: *Photomicrographs of CD3-positive T-lymphocytes in rat pineal gland, control group (x10)*

Trauma group (II): As expected, the number of infiltrating lymphocytes did not reveal any change in different brain regions during the first 30 min after TBI induction. Over the next few hours, however, the TBI-induced T-cell infiltration, displayed a biphasic pathophysiological response. The number of CD3-positive T-lymphocytes started to increase at 30 min post trauma and reached a maximum level at 45 min post injury. After a temporary decrease at 60 min, the number of CD3-positive T-lymphocytes began to increase again, reaching peak level (exceeding that observed at 45 min post-injury) at 2-3 hours post injury, depending on the brain region examined (**Figures 2-3**).

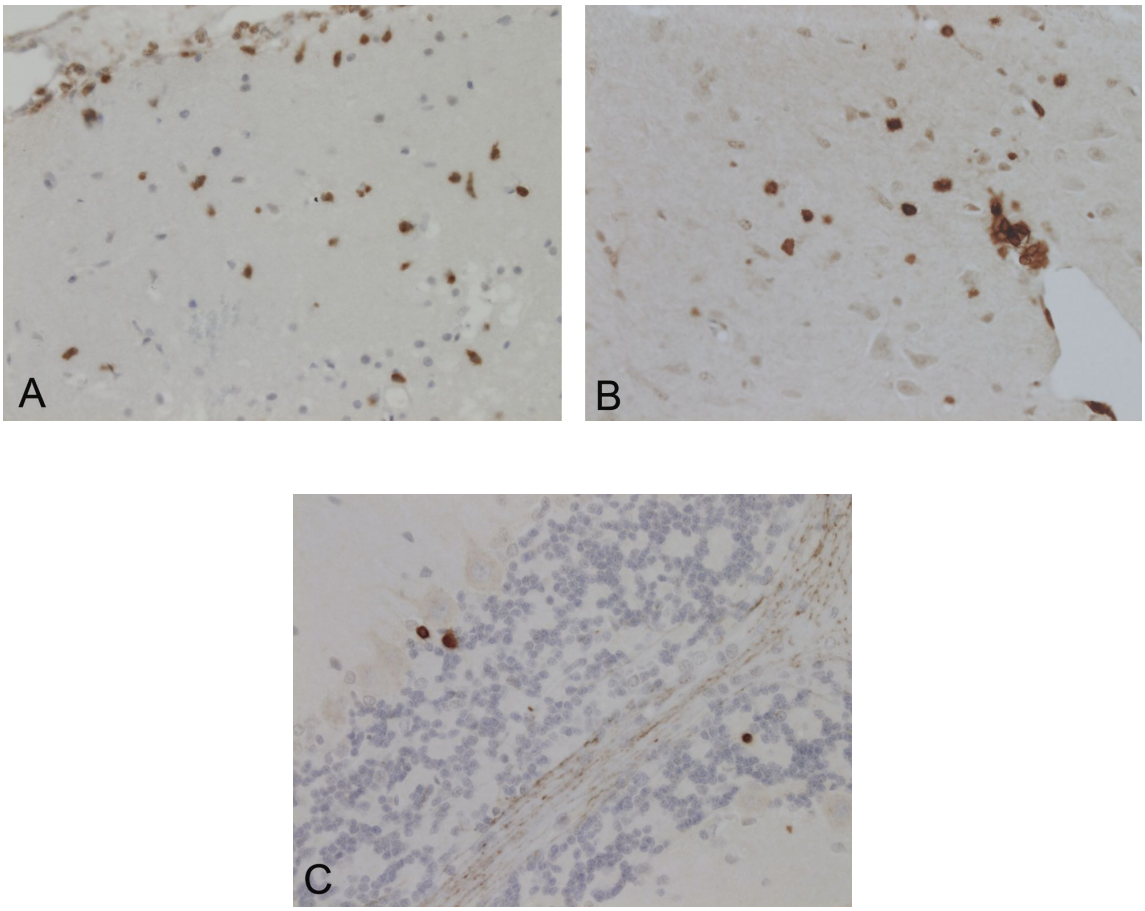

Figure 2: *Photomicrographs of CD3-immunoreactive T-lymphocytes in rat neocortex (**A**), brain stem (**B**) and cerebellum (**C**) (x40) 2 hours after closed head injury induction.*

During the next several hours, the number of T-cells decreased rapidly; at 24 hours post injury no significant difference was observed in any region as compared with the controls (**Figure 3**).

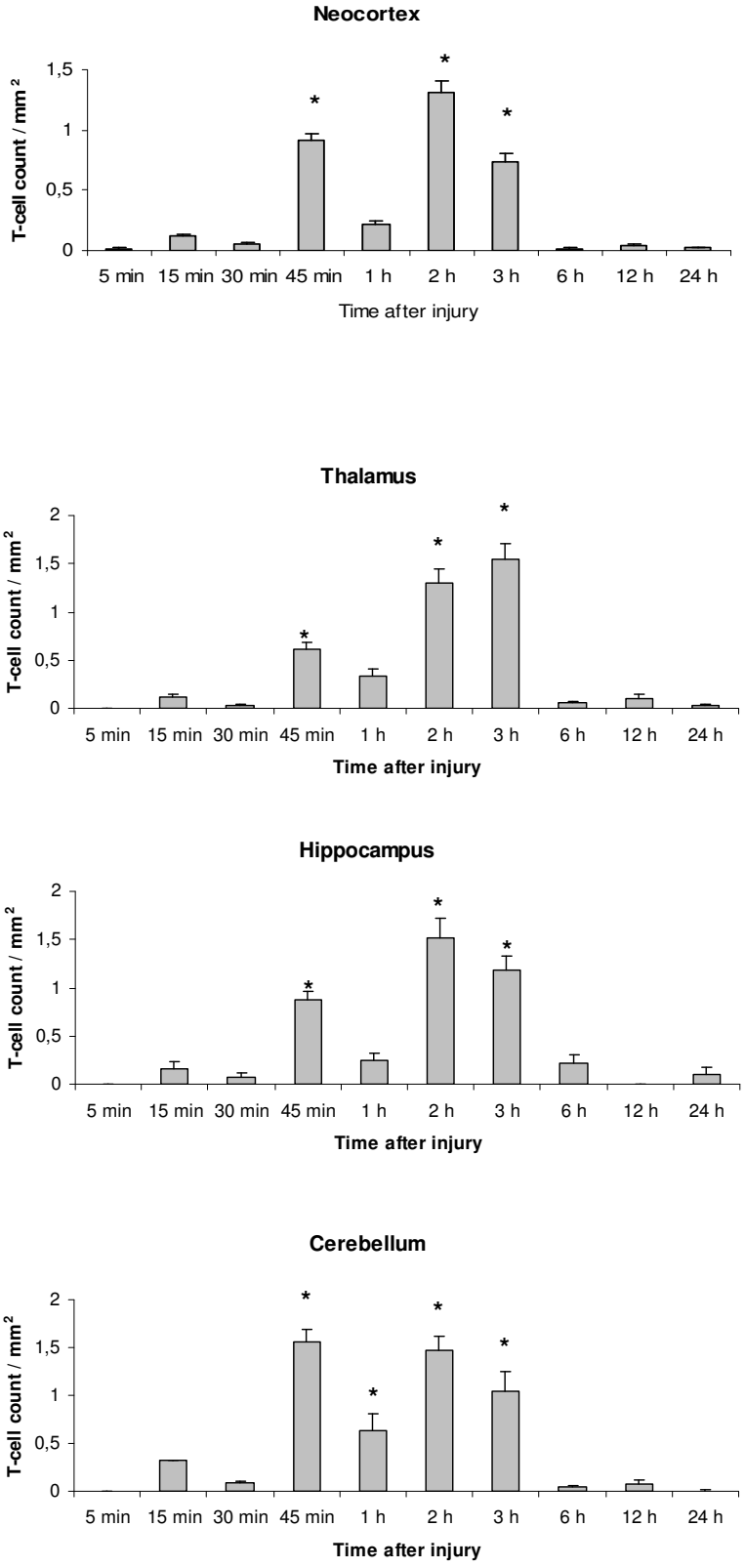

Figure 3: *Kinetics of presence of CD3-immunoreactive T-lymphocytes in different brain regions.*

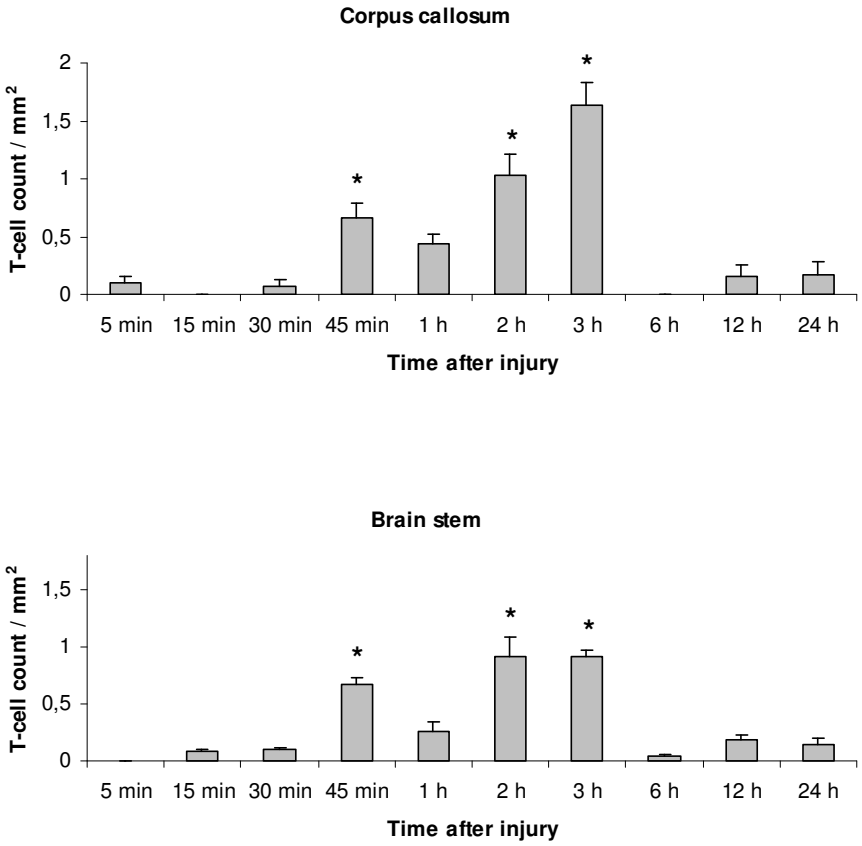

Figure 4: *Kinetics of presence of CD3-immunoreactive T-lymphocytes in the white matter and in the brain stem.*

The white matter in the cerebrum, cerebellum and the brain stem displayed the highest T-cell concentration (1-2/mm^2) (**Figure 4**).

In regions without a BBB (pineal gland and area postrema), the number of T-cells gradually increased to its peak value (40-45 cell/mm^2) at 6 hours post injury (**Figure 5**).

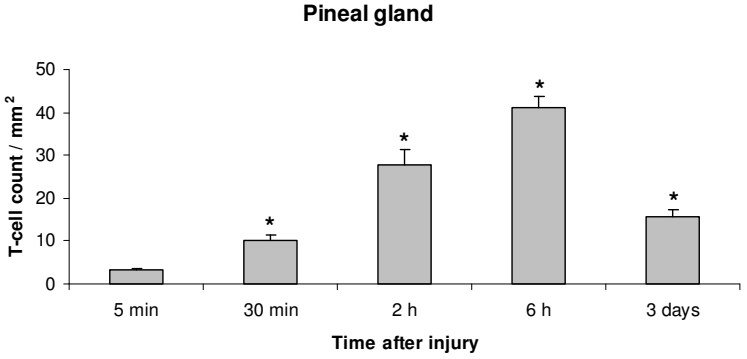

Figure 5: *Kinetics of presence of CD3-immunoreactive T-lymphocytes in rat pineal gland.*

Microglia activation

Coronal brain sections labeled with anti-CD11b antibodies were analyzed for microglia activation. One hour after TBI induction, the microglia proliferated and displayed a rapid transformation from resting to activated state. During this time, the cell body became hypertrophic, with long, branched and crenated processes. In the course of the next few hours, the shape of the microglial cells changed again, with a morphological transformation from the previous bipolar/ramified form into an activated amoeboid form. This amoeboid shape remained unchanged during the next 24 h (**Figures 6 and 7**).

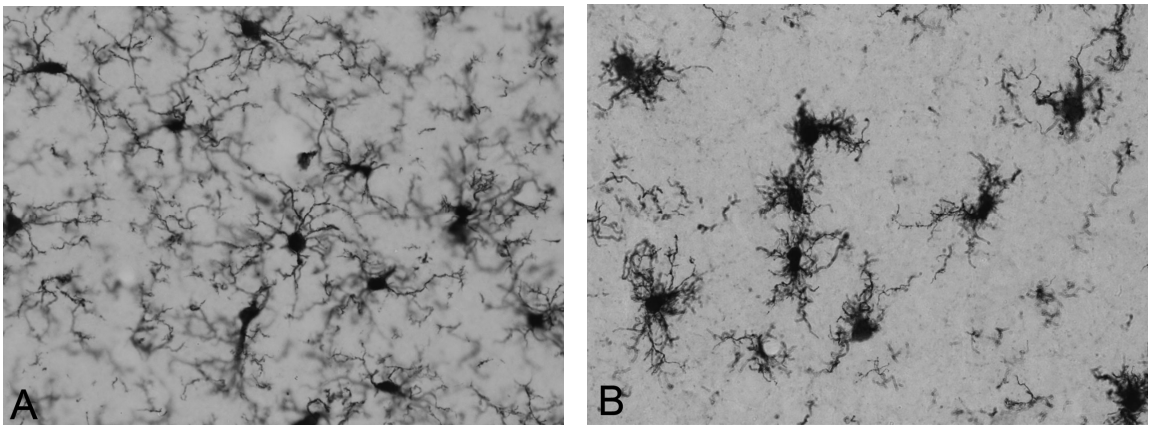

Figure 6: *Photomicrographs of CD11b-immunoreactive microglia in rat cerebral cortex.* ***A****: Resting microglia in neocortex.* ***B****: Activated ramified microglia in neocortex 1 hour after closed head injury induction (x60).*

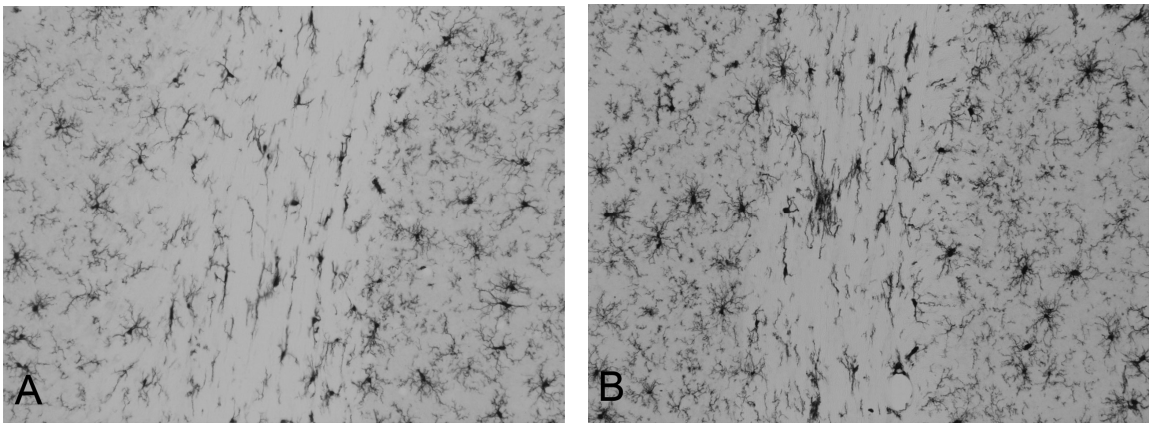

Figure 7: *Photomicrographs of CD11b-immunoreactive microglia in white matter.* ***A****: Resting microglia in corpus callosum.* ***B****: Activated ramified microglia in corpus callosum 1 hour after closed head injury induction (x20).*

4.2. Induction of immediate-early genes (IEGs)

Time-dependent changes in c-fos immunoreactivity

In general, the sham-operated group (C*ontrol group Ia*), did not differ from the control group (C*ontrol group Ib*), in any region. In both groups only a few cells had c-fos positive nuclei in the whole brain (6.8 ± 1.7 / mm^2 to 41 ± 5.8 / mm^2; mean±SE), except the hypothalamus, where significantly higher amount of stained cells (135.3 ± 19.3 / mm^2) were found. The c-fos staining did not change during the next 24 hours' period in any of the regions of the brain (**Figure 8-10.**).

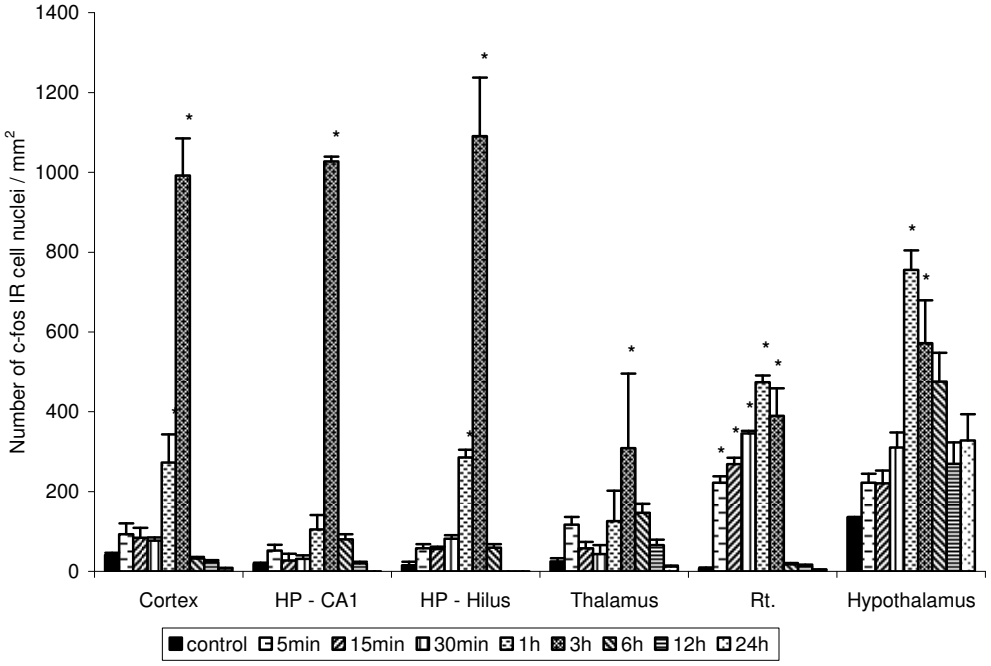

Figure 8: *Time course of c-fos IR changes in the supratentorial regions: Cortex; hippocampal formation: area CA1 (HP-CA1) and hilus (HP-Hilus); thalamus; thalamic reticular nucleus (Rt); and hypothalamus. Significant differences are indicated by asterisks (P≤0.001). The standard error of the mean is displayed at the top of the columns.*

In the *trauma group* five minutes post injury the c-fos IR showed sharp increase in the thalamus (117.4 ± 15.4 /mm^2) especially in the reticular nucleus (222.7 ± 22.5 /mm^2), whereas c-fos positive nuclei were rare in the other brain regions. During the next 15 min c-fos activity

remained unchanged. After 30 min post injury the number of the c-fos positive cells increased rapidly. This increase continued during the next hour and reached its maximum value between 1 and 3 hours after injury depending on the brain region. Reticular nucleus, hypothalamus, ependyma cells of the ventricles and two regions of the brain stem (corticospinal tract and the area postrema) showed maximum intensity at 60 minutes, whereas cortex, corpus callosum, thalamus, hippocampus (CA1 and HILUM) and region of anterolateral fasciculus in the brain stem at 180 minutes. During the next hours the c-fos IR decreased gradually and 6 hours post injury no significant difference was observed in any region as compared to the controls (**Figure 8-10.**).

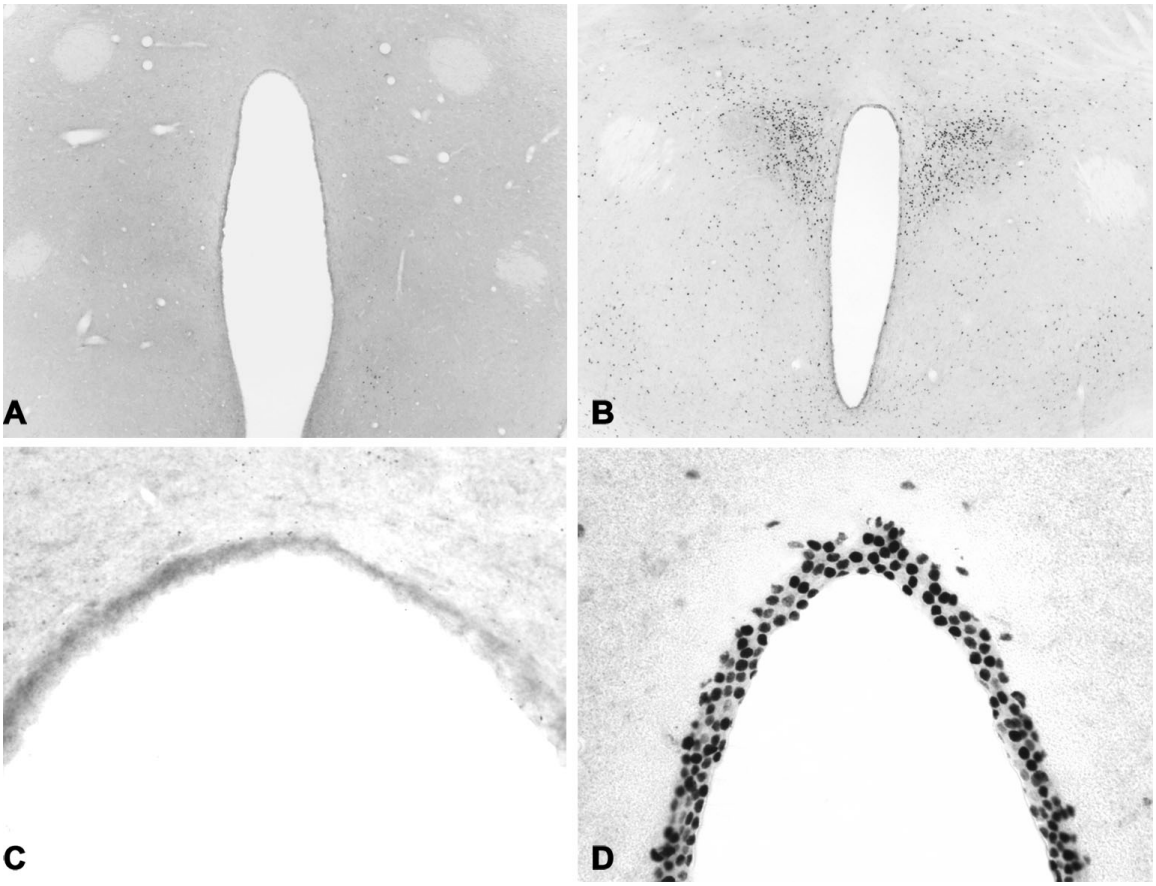

Figure 9: *C-fos expression in the paraventricular nucleus of the hypothalamus (x40), and in the nuclei of the ependyma cells of the third ventricle (x400). A, C: No immunoreactivity is present in the controls. B, D: Massive c-fos expression is present 1 h after CHI. C-fos immunoreactivity is seen as dark, punctuate labeling.*

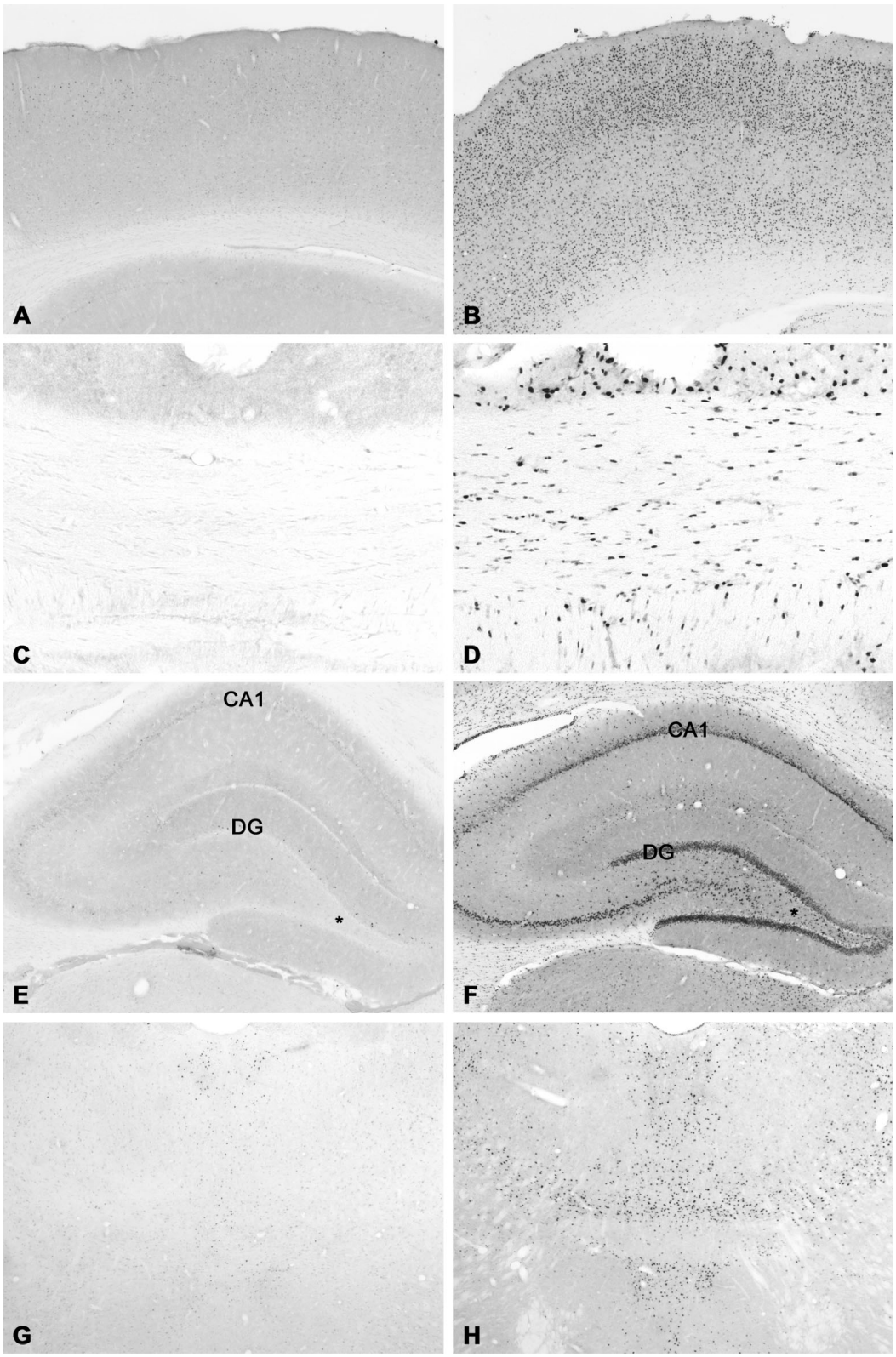

Figure 10: *C-fos expression in the rat neocortex (A,B; x40); corpus callosum (C,D; x200); hippocampal formation (E,F; x40) and thalamus (G,H; x40). A, C, E, G: No immunoreactivity is present in controls. B, D, F, H: Massive c-fos expression is present 3 h after CHI. C-fos immunoreactivity is seen as dark, punctuate labeling. The asterisk denotes the hilus of the dentate gyrus (DG) of the hippocampus.*

Region-dependent changes in c-fos immunoreactivity

The c-fos IR changes were not diffuse in the brain, rather displayed regional differences. The increase was highest in the corpus callosum (from 4.4 ± 1.3 /mm^2 to 317 ± 44.5 /mm^2), in the reticular nucleus (from 6.8 ± 1.7 /mm^2 to 474.8 ± 49.2 /mm^2), in the dentate hilum (from 15 ± 8 /mm^2 to 1090 ± 187. /mm^2) and in the neocortex (from 41.5 ± 5.8 /mm^2 to 992 ± 93 /mm^2). C-fos IR did not show any changes following CHI in the cerebellum, and in the nuclei of the cranial nerves (**Figure 8-10.**).

4.3. Mortality, body weight, short- and long-term neuropsychological deficits

Mortality

All animals subjected to impact acceleration injury over 1 m (Group 2) survived, as did all the sham animals. The mortality for animals injured over 2 m (Group 3) was 10%, and over 2.1 m with a 500 g weight (Group 4), was observed to be 34%. Similarly the mortality in the trauma, hypoxia and hypotension group (THH) (Group 5), was 35%. These data demonstrate that increasing the weight in the impact acceleration injury and the height, over which it falls, even by small amounts, can result in a significantly higher mortality rate. Furthermore the addition of a 10 min period of hypoxia and hypotension has a similar effect, and the mortality rate for this group was very similar to the group exposed to the maximally increased weight and height. Consequently our assumption that Groups 2-5 represent increasing injury severity is valid, at least in terms of overall animal survival.

Acute neurological assessment

In all the reflexes considered, the group exposed to trauma, hypoxia and hypotension, displayed the longest time for return of the reflexes after injury (**Figure 11.**). In all groups, complex postural somatomotor function was delayed the longest (head support, righting and escape responses), and the swiftest reflexes to return were the non-postural somatomotor reflexes (corneal, hindpaw and tail movement). There were only slight differences between the animals injured over 1 m, as compared with the sham animals, which were not significant. The animals injured over 2 m (Group 3) and the

animals exposed to trauma, hypoxia and hypotension (Group 5) showed significantly longer latencies for reflex return compared to sham animals and animals injured over 1 m ($p < 0.00001$). Animals injured over 2 m showed a delay in reflex return intermediate between the trauma, hypoxia and hypotension animals and the sham injured animals, which was significantly different to both these groups ($p < 0.00001$ cf. Group 5, $p < 0.0001$ cf. Group 2).

Therefore, acute neurological reflex suppression is a sensitive measure of injury severity for the more severely injured groups of animals, but it is difficult to distinguish mildly injured animals (Group 2) from sham injured animals based on this data. The complex postural somatomotor functions are delayed by the greatest amount and show the greatest difference in values between the groups and, therefore, can be considered to be the most sensitive reflexes to consider.

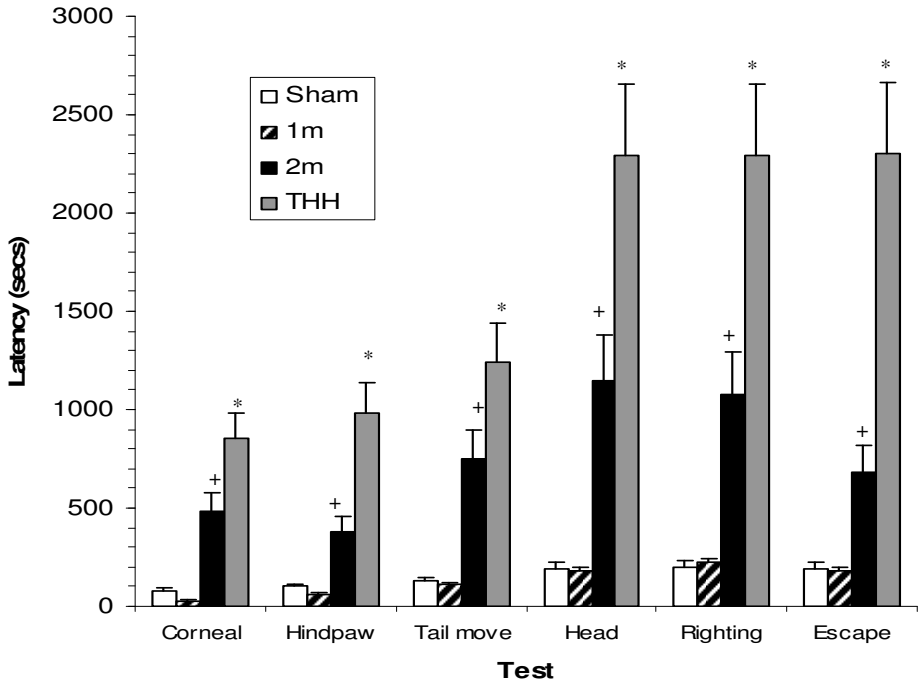

Figure 11: *Graph showing the latency for return of acute neurological reflexes after injury, in Groups 1, 2, 3 and 5. The reflexes considered are the corneal reflex, the hindpaw reflex, the tail flexion reflex, head support, righting reflex and escape response, as described in Materials and Methods.*

Body weight

The mean body weight of animals in Groups 1-5 were not significantly different prior to injury; the mean weight being 357 g± 8.7 g. Mean weights fell over the first three post-trauma days in Groups 3, 4 and 5, and then began to rise again (**Figure 12.**). There was a drop in weight on the first post-trauma day only in Group 2 (450 g weight over 1 m), and no weight change at all in Group 1 (sham). Baseline mean weight was surpassed by animals in Groups 1 and 2 by post-trauma day 3. Baseline mean weight was not surpassed until post-trauma day 10 in Group 4 (500 g weight over 2.1 m), until post-trauma day 13 in Group 3 (450 g over 2 m) and until post-trauma day 16 in Group 5 (trauma, hypoxia and hypotension).

On the final day of testing (Day 31), the animals weighed 119.9%, 125.9%, 115.0%, 115.7% and 111.7% of baseline mean weights, for groups 1 to 4 respectively. It can be seen that the consistently lightest animals over the 31 days are those in Group 5, the animals exposed to trauma, hypoxia and hypotension. There were only slight differences between Groups 3 and 4, and between Groups 1 and 2, until post-trauma day 13, after which the group injured over 1 m became the heaviest group *(p*< 0.001 cf. Sham).

Despite these differences in weight, the slope of the lines is very similar and, therefore, the rate of weight gain after injury is approximately the same in all the groups. Therefore any weight differences, in either absolute values or in terms of time taken to return to baseline, are likely to be differences in the degree of weight loss immediately after trauma and not to rate of weight gain *per se.* Consequently it is fair to say that weight gain in the chronic post-trauma period is not a sensitive marker of injury severity. However, the degree of weight loss in the 24 h succeeding trauma is related more uniformly to injury severity.

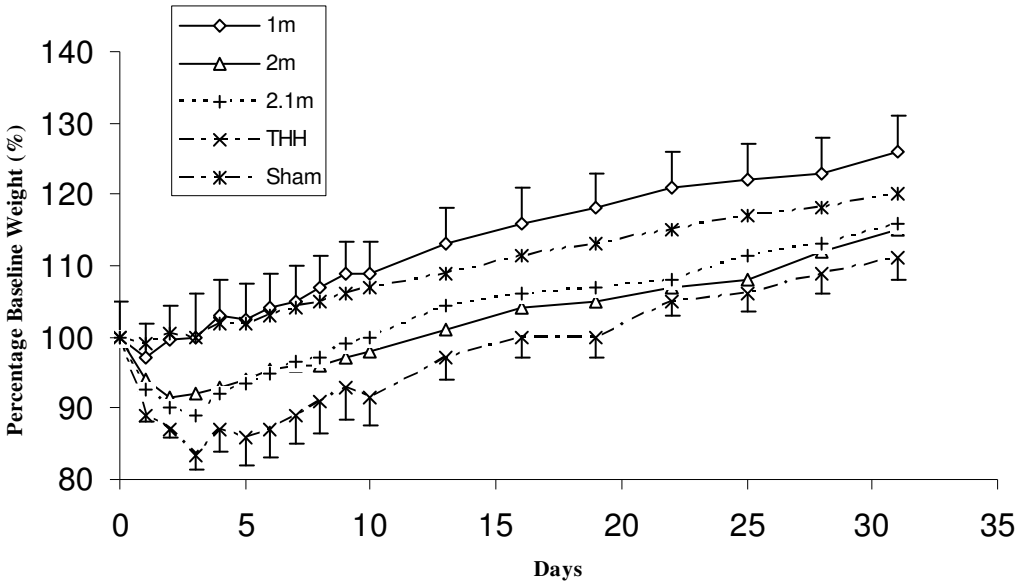

Figure 12: *Graph displaying the percentage body weight change in each of the experimental groups after injury. Data points represent the mean for the group on the indicated day; error bars where shown correspond to the standard deviation of the mean.*

Long term behavioral assessment

Beam walk assessment

The mean latency for entry into the goal box was recorded for all groups. The groups did not differ appreciably in their initial performance of the task. All traumatised animals showed significant deficits in their ability to complete the task on all days following trauma (**Figure 13.**). The differences were greatest on post-trauma day 1 and showed improvement over time, but without a complete return to baseline in any group. Animals in Group 4 (500 g over 2.1 m) and Group 5 (trauma, hypoxia and hypotension) appeared to perform worst of all. Animals in Group 3 (450g over 2 m) showed a marked deficit, but significantly less than that seen in Group 4 or Group 5 ($p < 0.00001$) and significantly greater than that seen in Group 2 ($p < 0.0001$). Group 2 animals showed a deficit which, despite the proximity of the data points on the graph, was significantly greater than the Sham group ($p < 0.00001$).

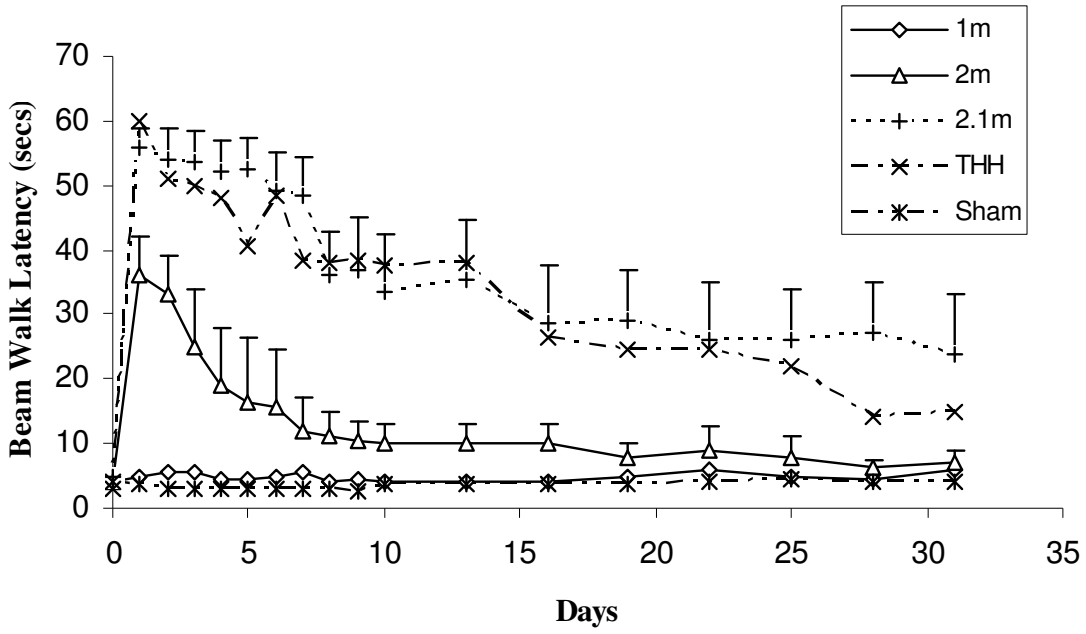

Figure 13: *Graph showing the mean beam walk latency for each experimental group both before and after trauma. Each data point correpsonds to the mean latency for that group on the indicated day; error bars where shown represent the standard deviation of the mean.*

All the groups of animals displaying deficits also showed marked improvement in the post-trauma period. The animals in Group 3 showed latency times only slightly elevated above baseline (2 sec) by post-trauma day 28, although these values were still significantly different from Sham ($p < 0.0001$). The animals in Group 4 and Group 5 showed some improvement, but there were still marked deficits in performance on the task by post-trauma day 31. The deficits in Group 4 (500 g over 2.1 m) and Group 5 (trauma, hypoxia and hypotension) seemed to closely parallel each other until the 25th day, after which Group 5 animals seem to show a slight increase in the rate of improvement.

These data demonstrate the sensitivity of this test in delineating injury severity. It appears to categorize three levels of deficit, mild, moderate and severe. The mild injury shows a slightly elevated latency throughout the entire post-traumatic period. The moderate group shows a large deficit immediately after trauma, followed by a rapid improvement in function, which begins to approach sham values within 31 days post-trauma. The severe category shows the most dramatic deficit immediately after injury, and despite some improvement in function with time, there is still a gross deficit 31 days post-

injury. The mild category corresponds to the Group 2 animals (450 g over 1 m), the moderate group corresponds to the Group 3 animals (450 g over 2 m), and the severe group corresponds to Group 4 (500 g over 2.1 m) and Group 5 (trauma, hypoxia, hypotension).

Consequently, the beam walk is very sensitive to changes in injury severity, and it can certainly distinguish mildly injured animals from sham. However, it is possible that there is a threshold for severity above which the test is no longer sensitive. This threshold will in part be determined by the conduction of the test, in that 60 sec is the maximum latency allowed before the animal is removed from the beam for another attempt. For example, two severely injured animals may have appreciably different levels of function, but if both are unable to complete the task within 60 sec, then they will score the same. Note, however, that this situation could only have applied on the first day post-trauma. For the remainder of the protocol mean values were lower than 60 sec, and yet the test was unable to distinguish between Group 4 and Group 5. It is, of course, possible that the 500 g over 2.1 m and the insult of 450 g over 2 m followed by hypoxia and hypotension represent broadly similar severities of injury.

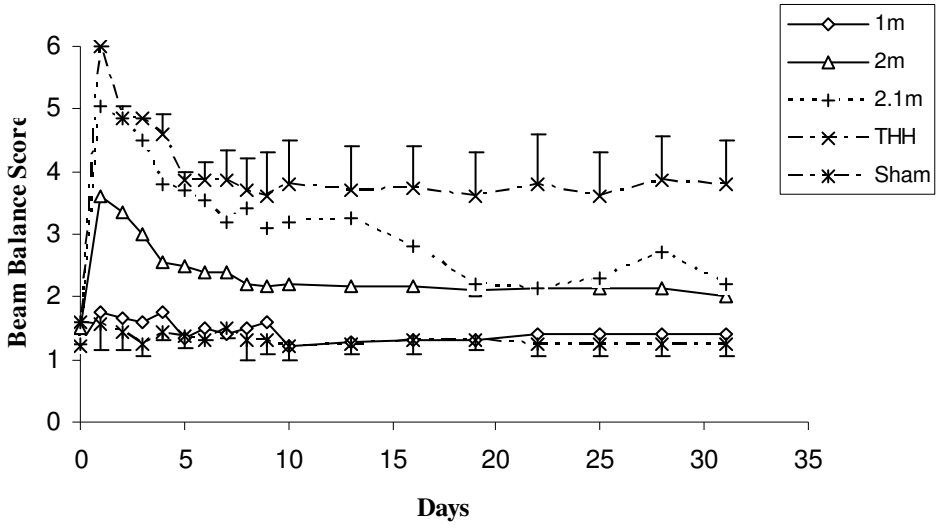

Figure 14*: Graph showing the mean beam balance score both before and after injury. Each data point corresponds to the mean score for that group on the indicated day; error bars where shown represent the standard deviation of the mean.*

Beam balance assessment

The groups of animals did not vary appreciably in their beam balance score before trauma. On post-trauma day 1 Group 3, Group 4 and Group 5 showed significantly lower scores (**Figure 14.**). The most severely affected animals were in Group 5 ($p < 0.00001$ c.f. both Sham and Group 4). Group 4 (500 g over 2.1 m) showed a significantly greater deficit than Group 3 (450 g over 2 m) ($p < 0.00001$). The sham animals (Group 1), and the animals injured over 1 m (Group 2) were not significantly different from each other, although both were significantly different from Group 3 ($p < 0.00001$). Animals in Group 3, Group 4 and Group 5 showed rapid improvements in scores after post-trauma day 1, although none returned to baseline within the period of assessment. By inspection, the initial rate of improvement in all three groups appeared to be comparable until post-trauma day 10. After this point animals in Group 5 made no further improvement. The animals in Group 3 also appeared to make only minor improvement after post-injury day 10. Animals in Group 4 (500 g over 2.1 m), however, showed a continuing improvement up to and including day 19, which made them less comparable with Group 5 animals and more comparable with Group 3 at the end of the study period.

Overall the results of this test demonstrate a graded response according to injury severity, with animals exposed to trauma, hypoxia and hypotension performing the worst of all the groups, and significantly worse compared with Group 4, However, Group 2 animals (450 g over 1 m) did not perform significantly worse than sham injured animals. These results are in contrast to those obtained for the beam walk test, where significant differences between Group *2* animals and sham were seen, compared with only minor differences between Group 4 and Group 5 animals. It appears that the beam walk test is more sensitive to differences in the less severely injured groups, whereas the beam balance test is more sensitive to differences between the more severely injured groups.

Inclined plane performance

The mean inclined plane angle at which the animals could support themselves was recorded for each group post-injury on the same days as the other tests. Interpretation of the data for this test is more difficult as there is less separation between the groups. However, it is clear that over the first five days post-trauma (**Figure 15.***)* Group 4 and Group 5 animals performed worst of all ($p < 0.00001$ c.f. sham) and animals in Group 3 (450 g over 2 m) performed better than animals in Group 4 and Group 5 ($p < 0.00001$), but still showed significant deficit compared with sham

($p<0.00001$). Animals in Group 2 (450g over 1 m) showed a smaller but still significant deficit compared with sham over the same period ($p< 0.00001$). After post-trauma day 5, the picture became more complicated. All groups remained significantly different from sham ($p< 0.0001$) until post-trauma day 19, at which point there were no discernible differences between any of the groups, except Group 4, which retained deficit ($p<0.0001$ c.f. sham). Between days 5 and 19, Group 4 and Group 5 still performed with the worst ability, and this was still significantly worse than Group 3 animals ($p< 0.00001$). Group 2 (450 g over 1 m) and Group 3 (450 g over 2 m) had very similar scores, but were still significantly different from each other ($p< 0.001$) on all but two days, and significantly different from sham on all the days up to day 19 ($p< 0.00001$).

Clearly, in this test, the injury severity does correlate with the severity of deficit as shown by the inclined plane test. However, this relationship is clearest over the first five days after injury; between days 5 and 19 post-injury, although all groups remain significantly different from sham, differences between the injured groups became less obvious. Finally after day 19, all groups became indistinguishable from each other, with no significant differences. The inclined plane test is possibly the least sensitive test examined over the 31 days; however, it is still capable of demonstrating differences between severity groups, and this ability is most profound over the first five days post-trauma.

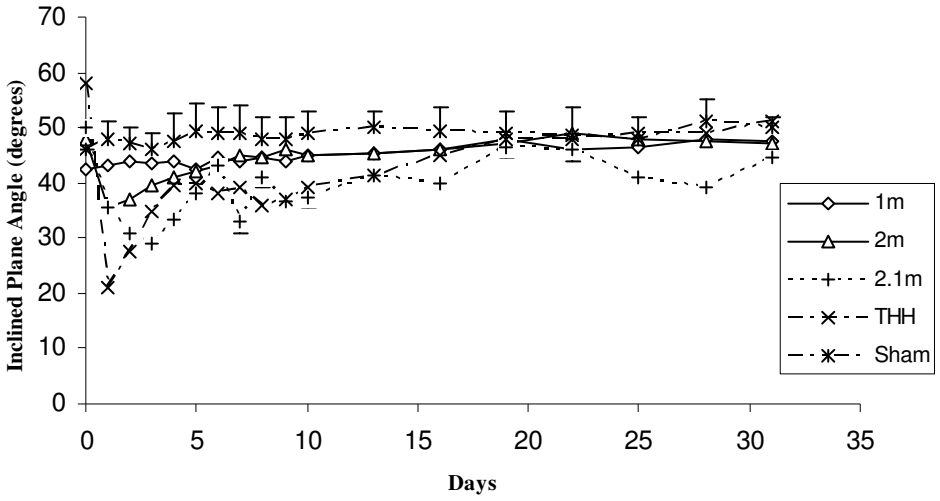

Figure 15: *Graph showing the mean inclined plane angle at which the animal became unable to balance. Each data point corresponds to the mean latency for that group on the indicated day; error bars where shown represent the standard deviation of the mean. All groups were significantly different from sham up to day 21; however, inter-group differences were only apparent over the first five days of testing*

Morris water maze performance

The mean latency of the animals to find the hidden platform was calculated over post-injury days 14-18 (**Figure 16.**). A marked gradation in ability on this test is apparent according to injury severity. Group 5 animals (trauma, hypoxia and hypotension) performed least well ($p < 0.00001$ c.f. sham), and had the worst improvement in ability, and the longest latency on the final day. Group 4 animals (500 g over 2.1 m) performed only slightly better than Group 5 initially, but demonstrate a much more dramatic reduction in latency and, therefore, by definition, improvement in ability over the course of the five days of testing. Group 3 animals (450 g over 2 m) performed better than Group 4 animals at all time points ($p< 0.00001$); however, they started out with a faster latency and then showed a similar rate of improvement. Group 3 and Group 4 animals showed similar, but significantly different ($p< 0.001$), latencies on the final day of testing. There were no significant differences between Group 2 and Croup 3 animals over the first three days of testing, and in fact, the mean latencies were remarkably similar. However, on the final two days of testing, the Group 2 animals continued to improve, whereas the Group 3 animals did not; Group 2 animals, therefore, performed significantly better ($p< 0.0001$) at these later time-points. Group 1 animals (Sham) performed best on each day of the assessment; however, the rate of improvement was broadly comparable to that seen in Group 2, Group 3 and Group 4.

The Morris water maze demonstrates a graded deficit according to injury severity with impact acceleration injury, although it does not distinguish between the groups injured with 450 g over 1 m and 2 m respectively (Group 2 and Group 3). There are necessarily two components to this test. Firstly, the absolute latencies, which are most important to consider on the first and last days. The gradation with injury severity on the first day is quite marked, and is less so on the final day. However, all the groups are still significantly different from each other on the final day of testing. The second parameter to consider is the rate of learning, as represented by the rate of change of latency. In this study all the groups improved at a broadly similar rate, except for Group 5 (trauma, hypoxia and hypotension), which improved with a markedly slower rate.

Consequently, the Morris water maze is a sensitive tool for examining differences in the severity of deficit after impact acceleration injury. Furthermore, it appears to be most sensitive to the superimposition of l0 min secondary insult of hypoxia and

hypotension, since with this paradigm not only were absolute latencies prolonged, but also the rate of improvement was markedly slowed.

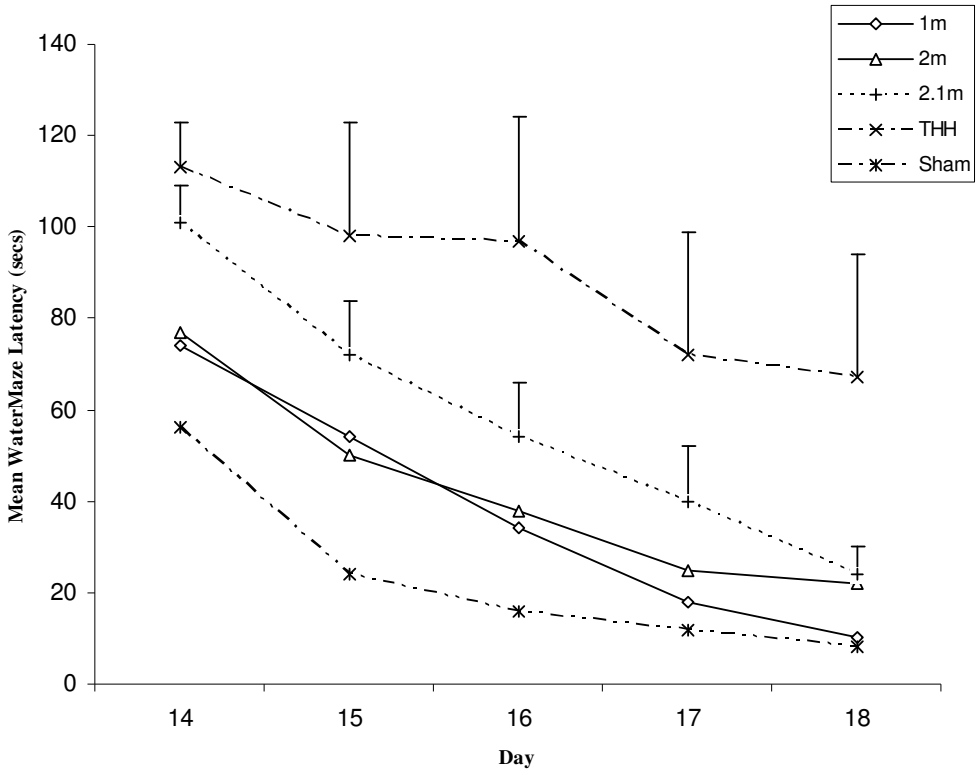

Figure 16: *Graph showing the mean Morris water maze latency for all experimental groups on post injury days 14-18. Each data point corresponds to the mean latency for that group on the indicated day.*

5. DISCUSSION

5.1. Cellular immunresponse following head injury

Induction of an inflammatory process in the CNS has been reported following brain trauma, but no study has been performed to assess the cellular immune reactivity in a *closed* TBI, so far. The present study revealed that severe diffuse TBI induces microglia activation and a transient biphasic T-cell infiltration of the brain parenchyma in all regions, beginning at 30 min post injury, peaking at 45 min and 3 hours after trauma induction. Despite the diffuse nature of the injury model applied, marked differences were detected between the various brain regions; the most severe alterations were detected in the white matter of the cerebrum, the cerebellum and the brain stem.

T-cell invasion in the brain

Until recently, little consideration was given to inflammation as a significant factor in the pathophysiology of secondary brain damage associated with CHI. The presence of astrocytes and microglia that embody the immune function, and the restrictive BBB, which is also known to possess immune functions, argue strongly for the unique immune status of the CNS [37;111]. It is now clear that the CNS is characterized by a partial immune privilege, in terms of downregulation and suppression of many aspects of the immune function in comparison with other organs. Almost all tissues contain strategically distributed antigen-presenting cells that constitutively express major histocompatibility complex (MHC) antigens and play a primary role in the initiation of the immune response. The concept of immune surveillance has been confirmed for almost all vertebrate tissues, with the noteworthy exception of the CNS. The immune status of the brain, however, has been re-evaluated recently, in order to learn more about the cellular autoimmune reactions that are suspected of playing a key role in the pathogenesis of a number of autoimmune diseases in the CNS [7;110]. Experiments with T-cell lines specific for CNS antigens have led to the surprising conclusions, that the CNS is routinely surveyed by activated T-lymphocytes that can cross the BBB, and that astrocytes play a major part in the initiation and subsequent regulation of the intracerebral immune response [46;68]. These results are in accordance with our findings of a significant number of T-lymphocytes in the control group in regions without a BBB, and a few

T-cells even in those regions which are protected by the BBB. Although the number of these T-cells appeared very low (0-0.05 cell/mm^2), the antigen recognition by these lymphocytes following a head injury might be crucial in terms of triggering the ensuing immune response.

Dynamics of lymphocyte migration

In our diffuse head injury model, we found a transient biphasic T-cell infiltration of the brain parenchyma in all regions, which begins 30 min post injury and reaches its maximum at 45 min and at 3 hours after trauma induction, indicating two different mechanisms of lymphocyte migration. In a previous study, in which the BBB was tested with a contrast agent (Gd-DTPA) in MRI, we demonstrated that the BBB opens at the time of the trauma and approaches closure at about 60 min post injury [3]. On the basis of these findings, we presume that the first surge of T-cell infiltration might be consistent with the BBB disruption, while the second and more pronounced surge at 3 hours post injury could be a result of T-cell activation. Since the BBB opening is transient, some of the lymphocytes might be trapped, while the others (circulating-T cells in the brain vessels) might not be able to penetrate the rapidly re-established BBB. This could be the explanation of the temporary decrease in T-cell number 1 h post injury.

The large number of T-cells seen in all brain regions during the second surge (at 3 hours following TBI induction) indicates the presence of specific targeting of the immune response, supporting specific connections between the CNS and the immune system. Although these surveying lymphocytes and the T-cell infiltration demonstrated during BBB disruption in our study are sufficient to produce activated T-lymphocytes, other features might also contribute to the further development of the cellular immune response. By what mechanism the T-cell activation is induced is still not fully understood, but the availability of T-cell lines specific for CNS antigens raises the fundamental question of which cell type(s) present the antigen in order to further activate the T-cells once they are within the CNS. There are two possible ways in which antigens can be processed and presented to the immune system by the CNS. First, by the two major antigen-presenting cell types: microglia and perivascular cells. However, these cells are remote from regional lymph nodes and few of them can trigger the activation of circulating lymphocytes in the CNS. Alternatively, antigens could be presented to lymphocytes in regional lymph nodes by the drainage of antigens themselves, or by the migration of antigen-presenting cells [7;64;111]. It has recently been shown that not only are there pathways for the lymphatic

drainage of interstitial fluid from the brain, but also the drainage of such proteins exerts a significant effect upon antibody production within the regional lymph nodes in the neck [58;59].

Since our investigations targeted at the cellular immune response were restricted to the first 24 h we could only assess the acute cellular response. Although the 3 h period of immune cell invasion demonstrated in our study is sufficient to trigger a cascade of events resulting in tissue specific inflammation and significant brain edema, other features, such as delayed BBB opening and astrocytic swelling might also contribute to the late edema, which begins a few hours post injury and exhibits a maximum effect between 3 and 8 days after trauma induction [3;81]. Further studies are necessary to determine the applicability of these factors to diffuse injury.

Time-dependent microglia activation and interaction between lymphocytes and microglia

The inflammation associated with CNS injury involves two major components: the activation of intrinsic microglia cells and the recruitment of bone marrow-derived inflammatory cells from the peripheral bloodstream [91;107].

It is generally accepted that microglia and peripheral monocytes respond to injury in various proportions, depending on the type and severity of the lesion. Chemical injuries to the brain appear to lead to a predominantly microglia cell inflammatory response, while direct stab wound injuries (with BBB disruption) involve mostly peripheral monocytes.

In our CHI model, we discerned a rapid transformation of the microglia; the cell body became hypertrophic, with long, branched and crenated processes during the first 60 min post injury. Although we focused exclusively on the microglia activation, the short duration of BBB damage and the microglia transformation observed within 60 min post injury suggest that predominantly microglia cells contribute to the induction of the immune response, while peripheral monocytes possibly play a role only in the eventual development of cystic cavities after the clearance of damaged tissue. This lends support to the report of microglia activation within 1 hour in a similar scenario, followed considerably later by macrophage and neutrophil infiltration in spinal cord, peripheral nerve and brain injury [15;25;57;60;103].

Lassmann et al. found both microglia cell processes and the soma of these cells in the perivascular astrocyte layer of the glia limitans reaching, the vascular basement membrane of

the BBB [63]. This microglia processes express class I and II MHC antigens, while the astrocytic processes remain unstained by these MHC-specific antibodies. Lassmann et al were able to demonstrate microglia processes in 4-13% of all vessel cross-sections, irrespective of their size and the type (artery, capillary or vein) of the vessel segment [62;63]. The presence of these immunocompetent microglia cell processes in the glia limitans, with the potential to function as antigen-presenting elements, could account for our observation that lymphocyte infiltration develops in the brain parenchyma 3 h following a closed head injury. Since microglia cells have been shown to produce inflammatory mediators (cytokines), to act as antigen-presenting cells for T-lymphocytes, our present observations of early microglia activation and lymphocyte infiltration further support the concept that these cells may play a fundamental role in the induction and maintenance of the inflammatory reactions in the CNS following a closed head injury [13;30;43]. On the other hand, while T-lympocytes and activated microglia may be equally key to ongoing neurodegenerative processes, they may also be secondary to other processes such as excitotoxicity and related mechanisms.

5.2. Induction of immediate-early genes (IEGs)

The present study examined the time-dependent changes of the inducible transcription factor c-fos in the whole brain following closed head injury. The principal findings are that severe diffuse head injury induces transient increase of c-fos in all brain areas except the cerebellum and some part of the brain stem which begins 30 minutes post injury and reaches its maximal value at 1-3 hours after trauma. Although, the injury was diffuse large differences have been found, that is the highest activity changes were restricted to the cortex, hippocampus (hilum, CA1), corpus callosum, the reticular nucleus and the hippocampal area. We will now discuss our results with regard to the functional role of the specific temporal and regional expression pattern.

Gene regulation and c-fos induction

Transient c-fos expression in the central nervous system was first observed after seizure activity and following noxious stimulation in spinal cord [20;52;87], and has been assigned a role as a functional marker of activated neurons. By forming heterodimers with members of the jun family, c-fos forms a transcription factor referred to as activator protein-1

(AP-1). Fos-related antigen-2 from AP-1 transcriptor factor family is induced in neurons in several models of injury. Its elevated expression lasts from days to months, corresponding to the severity, and results in long-term cellular changes in phenotype. This cascade is initially induced by phosphorylation of c-fos and jun by fos-regulatory kinase and c-jun N-terminal kinase, respectively, which alters the DNA-binding affinities of c-fos and members of the jun family. Some c-fos/jun complexes are stimulatory, while others are mostly inhibitory, on target gene expression [61]. In some cases, the effects of c-fos expression are protective [56], but they are also implicated in apoptosis [53]. Thus the subsequent activation of genetic programs for either cell death or for regeneration are initially similar, and possibly the same. An interchange between them is possible, but they would diverge at c-jun and c-fos. Therefore the presence of c-fos from the beginning of axonal injury up to the final stage of neurodegeneration could indicate either an involvement in ongoing regenerative efforts, or its final functional switch to a "killer" protein [42].

Time-dependent changes in c-fos activity

In the control groups only a few cells had c-fos positive nuclei in the whole brain, except the hypothalamus where significantly higher amount of immunoreactive cells (135.3 ± 19.3 / mm^2) were found. It could be explained by the stress of the experiment (induction of anaesthesia, intubation) because many neuroendocrin and behavioral responses induced by various forms of stress are highly correlated with an increase in c-fos expression [83].

The data in this report demonstrate for the first time the entire temporal course of c-fos activity changes following CHI from the induction of injury to as late as 24 h post injury. Our findings suggest that the IEG expression is changing with time after the traumatic insult and shows monophasic pathological response. During the first 30 minutes post injury c-fos activity remains unchanged (except the thalamus, where mild transient increase was observed). After 30 min the number of the c-fos positive cells increases rapidly. This increase continues during the next hour and reaches its maximum value between 1 and 3 h following injury, depending on the brain region. During the next hours the c-fos activity decreases gradually and 6 h post injury no significant difference is observed in any region as compared to the controls (Figure 1). Only a few results are available about temporal pattern of IEGs expression following CHI. Although these studies analyzed c-fos expression of a short duration, or in a particular time

point, these findings are in good agreement with our results. They reported increased activity between 5 min and 24 h post injury [1;21;24;92;112]. Although, a comparison of published results is complicated because of the use of various animal strains and trauma models, it should be noted that we observed no considerable increase in c-fos activity during the first 30 min and later than 6 h post injury. Since the level of c-fos mRNA begins to increase at 5 minutes after trauma the mild surge in c-fos activity in the thalamus observed in our model at 5 minutes post injury as well as the early c-fos activity changes observed by others may indicate a sensory stimulus-induced neuronal activation before the trauma and not the direct effect of the injury [16;41]. Although the results showed a regional variability as expected the observed time course of c-fos activity may be a valuable parameter for further investigations especially to test pharmacological therapies.

Regional differences in c-fos activity

Induction of c-fos has been reported following brain trauma, but no work is available in which direct comparison of regional pattern of IEG induction has been performed following closed head injury. This type of injury has usually been distinguished from other forms of brain trauma by its mechanism, namely it causes primarily axonal injury as a result of diffuse trauma [8;93]. As seen in Figure 10, the gross c-fos activity increase was not uniform in the brain (it was most pronounced in the cortex, white matter, thalamus, hypothalamus, hippocampus) and was not even localized at the site of the injury in contrast to other models of head injury. This observation conforms to the concept that trauma in this closed head injury model results in a generalized, diffuse injury and not a supratentorial focal brain lesion [3;29].

Several authors evaluated the c-fos expression surround the contusion and in other particular brain areas after various type of traumatic brain injuries [1;21;24;92;94;95;112]. They found increased c-fos activity mainly surrounding the site of the injury and in the ipsilateral hippocampal, thalamic and cortical area. Our results are consistent with these previous reports and support the behavioral and cognitive disturbances observed after human traumatic injuries. Raghupathi et al, similarly to our findings also demonstrated c-fos induction in the subcortical white matter and ependyma following fluid percussion injury. The presence of c-fos in these regions and the occurrence of reactive gliosis following head injury suggest that c-fos may participate in the neuron-glia interaction following TBI [14;95]. On the

other hand the transient blood-brain barrier opening and the significant water increase (0.5-1% in the cerebrum) during the first 90 minutes post injury observed in this model support to the hypotheses that closed head injury triggers regionally and temporally specific c-fos expression, which may be a common step in the cascade of events following CHI leading to cellular edema and brain swelling [3;4].

Finally it is important to analyze the insignificant change in c-fos activity in the cerebellum and most of the brain stem. The mild blood pressure changes (as a consequence of slight brain stem effect) seen in our closed head injury model is, however in contrast to the documented prolonged surge of arterial pressure following fluid percussion model explains the relatively low c-fos activity we found in the medulla [18].

Nevertheless, further investigations are necessary to identify the cause and the precise nature of the heterogeneous c-fos activity. In this regard, we must consider that all these measures of intensity may be influenced by the fact of the invasive nature of the method which does not allow each animal to serve as its own control.

5.3. Neuropsychological and neurobehavioral deficits after diffuse head injury

The aim of this part of study was to consider the motor and cognitive functions of four groups of animals with different severities of impact acceleration injury, in order to get evidence of a graded behavioral deficit which corresponded to the severity of injury. The severity of impact acceleration injury is thought to be dependent on the height and weight parameters of the model, and, therefore, varying heights of weight drop were considered ranging from 1 m to 2.1 m, and also a varying weight of either 450 g or 500 g. The superimposition of hypoxia and hypotension after injury is known to exacerbate the craniocerebral trauma [11;101] and, therefore, a group which experienced a 10min period of hypoxia and hypotension after trauma was included.

These data demonstrate that the impact acceleration model of head injury produces a transient loss of muscle tone, suppression of somatomotor reflexes, and long lasting deficits in motor and cognitive functioning. They also demonstrate that the severity of motor and cognitive deficit is very clearly graded according to the severity of injury.

Neuropsychological dysfunctions

Long- and short-term neuropsychological deficits after craniocerebral trauma remain a significant clinical problem, despite considerable efforts to understand and prevent such damage [2]. Good post-traumatic recovery of patients, with attainment of appropriate levels of cognition and behavior are a primary aim of therapy. Behavioral outcome is, therefore, a focus of research into novel therapeutic interventions. Memory deficits are one of the most persistent consequence following trauma [65].

Clinically, many different assessment protocols are used which have a wide variety of criteria ranging from overt neurological functioning to subtle neuropsychological tests of higher function. Some of the more common ones include: complete behavioral batteries, e.g. the Halstead-Reitan Neuropsychological Battery [98] or the Luria-Nebraska Neuropsychological Battery' [101]; specific tests of motor function, e.g. the finger tap test [98]; specific tests of memory, e.g. continuous recognition memory test [36]; tests of behavior, e.g. the neuro-behavioral rating scale [66]; and global tests such as the disability rating scale [97], which focuses on the patient's ability or disability to function.

A uniform feature of these tests is that they demonstrate significant deficits in patients with even mild trauma [10;66]. Deficits also appear to be graded according to the severity of the injury [12]. However, assessment of injury severity *per se* does not always represent actual severity of impact, rather categorisation relies on radiological or neurological features of the patient on admission. Consequently, it is not surprising that neurobehavioral deficits are correlated with these findings.

Neuropsychological deficits

Our study has demonstrated significant neurobehavioral deficits following impact acceleration injury. The effects of other models of craniocerebral trauma on motor and cognitive functioning have been examined in the rodent, using similar tests to those used in this study. Work with the fluid percussion injury [71;72;73] and cortical contusion model [35;48] have demonstrated similar deficits to those seen with the injury alone group in this study, namely a significant deficit in function in the first 7-10 days after injury followed

by a relatively rapid recovery to baseline after this. Dixon et al. [19] suggested that despite function returning to baseline, a period of covert decreased function exists, as evidenced by an increased sensitivity to pharmacological inhibition of behavioral function. Clinical data is in agreement with this, which would suggest that sub-clinical deficits can exist in patients with otherwise good recovery [32].

Experimental models are unique in that the severity of the injury can be controlled, and, therefore tests of motor and cognitive function can be accurately correlated with this. It is apparent both in this study and others that injury causes neurobehavioral deficits, the extent of which is correlated in injury severity. Lyeth et al. [69;70] examined differing grades of fluid percussion injury, and saw that increasing severity resulted in an increased severity of behavioral deficits. Prior to this study, it was not known whether the same was true with the impact acceleration model of head injury.

In these models, animals also showed improvement in function with time, which was apparent in all the tests considered. Furthermore, the severity of the injury seems to affect the rate of the functional improvement. Above a certain threshold of injury, particularly where a secondary insult has been introduced, a complete return to baseline is prevented. It is possible that given a study period longer than 31 days, a return to baseline could be affected. Therefore it must be acknowledged that the persistent deficits in this study may represent a prolongation of the recovery phase. However, in humans, long term and permanent deficits are seen following head injury [2]. Therefore it is not unreasonable to assume that if improvement has slowed to a halt, then this may correspond to a permanent deficit.

Laboratory tests of neuropsychological deficits

Human neuropsychological tests cannot be faithfully reproduced in a laboratory environment; however, they are frequently approximated in the rodent using the battery of well-validated tests utilised in this study. The Morris water maze [88;89] is a test of visuospatial memory in the rodent and is thought to be an analogue of declarative memory and hippocampal function in humans. However, a component of cognition exists, in that the animals must come to understand the task in order to learn. Testing is undertaken on days 14-18 post-trauma with the specific aim of reducing any component of

motor impairment upon the animals' performance. As well as absolute latency to complete the test on each day, it is important to consider the rate of change of latency, the parameter which most closely reflects 'learning'.

In this study, it was shown that the worst performance in the water maze latency was seen in the group exposed to trauma, hypoxia and hypotension. At each time point the rank of latencies corresponded to the rank of injury severity; however, the sham group and injured Groups 2-4 all started from different latencies, and all seemed to improve at comparable rates. The group experiencing trauma, hypoxia and hypotension not only began with the slowest latency, but also demonstrated a very poor rate of improvement. Consequently it is clear that the Morris water maze is sensitive to injury severity at all grades of injury, and it responds most dramatically to the superimposition of a secondary insult by showing a prolonged latency and a slower rate of improvement.

The beam walking test is an assessment of refined locomotor activity in the context of a learned avoidance task. Poor performance in this task can be attributed to significant motor impairment, or amnesia, since the task was learned pre-injury. The rats fell into three categories of response; some were unable to move, and clung to the bar, even falling off; others completed the task, but only very slowly, and other animals showed little or no compromise in function. This disturbance of function translates very elegantly into a change in the latency to complete the task. A clear gradation in latency on this task is seen which corresponds to injury severity; however, in this instance the task is less sensitive to the most severe injury groups, but shows much clearer differences between the mild and moderate injuries.

The reason why this test is more sensitive to the milder injuries remains an object for speculation. It is obvious from the other behavioral tests that there is a difference in severity between Group 4 and Group 5, therefore the data cannot be explained simply by a comparable injury in the two severe groups. It is more likely that there is a threshold for severity above which the test is no longer sensitive.

The beam balance test is a fairly crude assessment of motor and vestibular functioning. The assessment is based on a discrete score of ability to balance, as outlined previously. Despite the crude nature of this test, a gradient of observed deficit can be seen, which matches the injury severity. Group 5 (trauma, hypoxia and

hypotension) animals remained the most consistently poor performers. In contrast with the distribution of data in the beam walk test, the beam balance score clearly differentiates between Group 4 and Group 5 but, however, was not able to demonstrate any significant difference between Group *2* animals (450 g over 1 m) and Group 1 animals (sham). It is clear from the other data that a functional difference exists between these two groups, and therefore lack of difference on this test must be directly attributable to the sensitivity of the test.

In comparing the beam balance and beam walk data, it seems appropriate to consider the gain of the tests. It appears that under the paradigm of impact acceleration injury, the gain of the beam walk test is greater than the gain of the beam balance test. Consequently the beam walk test is very sensitive to injury severity, and is able to differentiate between mild injury levels, but is somewhat overwhelmed by severe injuries, such that subtle differences between severely injured groups are not apparent. The gain of the beam balance test on the other hand is lower and more capable of elucidating differences between severely injured groups, but is less sensitive, therefore, to differences in more mild injury levels.

The inclined plane test [100] is intended to objectively assess motor function, with no component of task learning. Injury severity broadly corresponds to test deficits. This is especially true in the first five days post-injury. However, between post-injury days 5 and 19 the test becomes less sensitive, although severe injuries can still be differentiated from mild and moderate ones. From day 19 onwards the groups become indistinguishable. This test is, therefore, possibly the least sensitive of all the ones utilised. This insensitivity may relate to several phenomena; firstly very few rats have pure motor deficits, if any, that are clinically transient, and usually not evident after post-injury day 14 in all but the most severely injured animals. Secondly, absolute values for the inclined plane test are rather user dependent.

Impact acceleration injury acutely causes a transient loss of muscle tone, reflex pathway suppression and dysfunction of more complex somatomotor responses. Acute reflex tests assess postural and non-postural somatosensory functioning. They are also adversely affected by trauma and this manifests as a delay in return of the reflex after discontinuation of anesthesia. It has been postulated that at least part of these effects are

mediated by activation of a discrete cholinergic inhibitory system located in the rostral pons, which may be related to the reticular activating system [38;69]. Necessarily though, the depth of anesthesia is important in this instance, and is an important source of inter-animal variation. This fact underlines the importance of consistent anesthesia protocols and also the advantage of using short-acting anesthetics.

The complex somatomotor reflexes exhibit the longest delay in return, and consequently the greatest sensitivity to injury severity. These tests were, in general, unable to distinguish between the Group 2 animals (450 g over 1 m) and sham animals. It is interesting to note that despite not demonstrating any significant deficits in reflex suppression immediately after trauma, this mild injury level is still capable of causing deficits within other longer term behavioral tests such as the beam walk test.

Neuropsychological deficits with impact acceleration injury

This study has, for the first time, delineated the effects of injury severity on motor and cognitive deficits in the impact acceleration model of head injury. Work in this laboratory has previously demonstrated behavioral deficits in this model [104]; however, it has not been previously reported what relationship the observed behavioral deficits have to the severity of impact acceleration injury, with or without a secondary insult of hypoxia and hypotension.

By necessity, the other perspective to this question is how sensitive the behavioral tests are to injury severity and how responsive the tests are to subtle changes in gradation of the injury. This information is important for understanding data obtained from behavioral studies using the impact acceleration injury, and is especially important when considering the effects of experimental therapeutic interventions on post-traumatic neuropsychological functioning. If behavioral tests are not graded in severity of deficit, matching the severity of the injury, then few conclusions can be made about improvement in function with therapeutic intervention.

The reasons underlying this observed neurobehavioral dysfunction is not clear. Several propositions have been put forward, which include diffuse axonal injury and widespread neurotransmitter release [72]. Although the neuropathological effects of impact acceleration include diffuse axonal injury, which would be expected to result in

disordered neuronal functioning, an alternative mechanism must be responsible for the extra degree of deficit seen in the group exposed to hypoxia and hypotension. It is possible that hypoxia and hypotension contributes to axonal damage or has an additional effect on neuronal survival.

This study has clearly demonstrated that the battery of tests routinely used to assess behavioral functioning in experimental head injury, respond very well to alterations in the severity of impact acceleration injury and, therefore, are appropriate tools to assess therapeutic intervention. Furthermore this study has underscored the relative differences in sensitivity between the tests and how the most relevant test may depend on the severity of injury being considered. For example, the beam walk test seems very sensitive to mild injury levels, but is overwhelmed by severe injuries. The beam balance test, acute reflexes and inclined plane tests (days 1-5) are more sensitive to the more severe injury levels. The Morris water maze, despite being sensitive to all injury levels, shows increased sensitivity to the superimposition of a secondary insult of hypoxia and hypotension.

6. CONCLUSIONS

6.1.1. A diffuse CHI induces T-cell infiltration of the brain parenchyma and rapid microglia transformation in all regions, which begins 30 min post injury and peaks between 45 min and 3 hours after trauma induction.

6.1.2. These results lead us to suggest that the acute response to a severe head trauma with early edema formation is likely to be associated with inflammatory events which might be triggered by activated microglia and infiltrating lymphocytes.

6.2.1. CHI induces transient increase of c-fos activity in all brain areas except the cerebellum and some part of the brain stem which begins 30 minutes post injury and reaches its maximal value at 1-3 hours after trauma.

6.2.2. Although, the injury was diffuse, considerable regional differences were found. The highest activity changes in the cortex, corpus callosum, reticular nucleus and the hippocampal area are in good agreement with the cognitive and memory deficits observed after human head injury.

6.2.3. The observed time course and regional differences in c-fos activity may be a valuable parameter for further investigations especially to test the effect of pharmacological or cellular therapies.

6.3.1. The findings support the applicability of using behavioral end-points to assess the impact acceleration model, in that a more severe injury does indeed result in worse behavioral functioning.

6.3.2. The study established the use of these standard behavioral tests as useful assessment tools for the efficacy of therapeutic interventions in this model. If graded injuries cause graded deficits, then changes in the response to trauma induced by treatment are more likely to be seen as reductions in severity. If a behavioral change following injury were an all-or-nothing response, effects of therapy may be missed.

6.3.3. It is clear that the different tests have differing sensitivities to injury levels, which may partially explain the results seen. Consequently the gold standard approach should be to include all these tests in any assessment protocol, and an estimate of injury severity should be used to help interpret the findings.

7. SUMMARY

The acute response to a severe head trauma with early edema formation is likely to be associated with inflammatory events which might be triggered by activated microglia and infiltrating lymphocytes. The immediate early gene c-*fos* to be a reliable marker for prolonged neuronal activity and a potential genetic marker of accommodation. It is difficult to overestimate the clinical significance of these observations, as the early and targeted treatment of patients with severe head injuries with immunosuppressive medication may result in a far more favorable outcome. The observed time course and regional differences in c-*fos* expression can be utilized in further investigations, especially to test the effects of various forms of pharmacological or cellular therapies. The neurophysiological data confirm that the tests considered, and the injury model used, provide a useful system for the consideration of potential therapies which might ameliorate motor and cognitive performance in diffuse brain injury.

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