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

Summary of 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

ICP: intracranial pressure

IEGs: immediate-early genes

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. 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.

Although information concerning the process of cytotoxic damage in TBI is limited, 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. 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. In immune-mediated illnesses of the CNS, large quantity of the cells of the immune system is permitted to enter the brain. 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. 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.

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, the accumulation of free radicals, breakdown of the BBB and an excessive release of excitatory amino acids contribute to the parenchymal damage. A few literature data describe the induction of immediate-early genes (IEGs) in TBI. 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. Similarly to other injuries, TBI results in the transcription of c-*fos* gene and the subsequent expression and phosphorylation of the Fos protein. 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.

Long- and short-term neuropsychological deficits after craniocerebral trauma remain a significant clinical problem, despite considerable efforts to understand and prevent these sequelae. 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 it. 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. 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 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.

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 rats 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).

Impact acceleration injury

An impact acceleration head injury model was used to produce trauma. After a midline scalp incision a round stainless steel disc was mounted on the skull with super glue, and a sectioned brass weight of 450 g was dropped from a height of 2 m onto the center of the metal disc. After induction of the trauma, the rat was rapidly reconnected to anesthesia and artificially ventilated, and the wound was closed.

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. The immunoreactions were developed in 3,3'-diaminobenzidine for 30 min, then slightly counterstained with hematoxylin, and finally covered with DePeX. Sections from the brain were analyzed semiquantitatively in each group. The number of T-lymphocytes was assessed statistically by means of repeated measurement ANOVA

CD11b (microglia) immunohistochemistry

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 at a dilution of 1:1000 at room temperature overnight. Next, specimens were incubated in a 1/1000 dilution of peroxidase-labeled streptavidin for 1 h. Primary antibody binding was visualized with 3,3'-diaminobenzidine in the presence of nickel ammonium sulfate for 15 min.

3. 2. Induction of immediate-early genes (IEGs)

A total of 143 adult male 320-350 g Wistar rats were used. 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.

Immunocytochemistry

At various times after CHI induction (5, 15 or 30 min, or 1, 3, 6, 12 or 24 h), rats subjectied to the trauma (6 animals at each time), together with sham-operated and control animals (2 at each time) were perfused transcardially for immunocytochemical processing. Every fifth section was processed for routine c-*fos* immunocytochemistry.

Quantitative analysis of c-fos immunoreactivity

C-*fos* immunoreactive cell nuclei displaying grayish-black staining were counted with the aid of a Nikon Eclipse 600 microscope equipped with a Polaroid DMC digital camera. The cell counts were analyzed by ANOVA.

3. 3. Assessment of neuropsychological dysfunction

One hundred Sprague-Dawley rats weighing 360g-385g were divided into five subgroups, which varied in the intensity of injury they received. Group 1 was exposed to Sham injury (n = 39); Group 2 received an injury from a 450g weight falling 1 m (n = 8); Group 3 was exposed to a 450g weight falling 2 m (n = 40); 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 10 min secondary insult of hypoxia and hypotension (n = 5). 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 all 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 that the animals were sacrificed.

Statistical analysis

All values are expressed as means±SEM, and the Student's 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. 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.

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, 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 at 2-3 h post injury, depending on the brain region examined.

During the next several hours, the number of T-cells decreased rapidly; at 24 h post injury no significant difference was observed in any region as compared with the controls. The white matter in the cerebrum, cerebellum and the brain stem displayed the highest T-cell concentration (1-2/mm^2). 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 h post injury.

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.

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.

In the *trauma group* 5 minutes post injury the c-fos immunoreactivity showed sharp increase in the thalamus (117.4 ± 15.4 /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 h after injury depending on the brain region. Reticular nucleus, hypothalamus, ependyma cells of the ventricles and two regions of the brain stem showed maximum intensity at 60 min, whereas cortex, corpus callosum, thalamus, hippocampus and region of anterolateral fasciculus in the brain stem at 180 min. During the next hours the c-fos immunoreactivity decreased gradually and 6 h post injury no significant difference was observed in any region as compared to the controls.

Region-dependent changes in c-fos immunoreactivity

The c-fos immunoreactivity 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 immunoreactivity did not show any changes following CHI in the cerebellum, and in the nuclei of the cranial nerves.

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-operated 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 (Group 5), was 35%.

Acute neurological assessment

In all the reflexes considered, the group exposed to THH, displayed the longest time for return of the reflexes after injury. In all groups, complex postural somatomotor function was delayed the longest, and the swiftest reflexes to return were the non-postural somatomotor reflexes. 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. 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.

Body weight

The mean weights of animals in Groups 1-5 were not significantly different prior to injury; the mean weight being 357g ± 8.7g. Mean weights fell over the first three post-trauma days in Groups 3, 4 and 5, and then began to rise again. There was a drop in weight on the first post-trauma day only in Group 2, and no weight change at all in Group 1. 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, until post-trauma day 13 in Group 3 and until post-trauma day 16 in Group 5.

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. There were only slight differences between Groups 3 and 4, and only a slight difference between Groups 1 and 2, until post-trauma day 13, after which the group injured over 1 m became the heaviest group.

4. 4.   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 appreciably differ 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. 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 and Group 5 appeared to perform worst of all. Animals in Group 3 showed a marked deficit, but significantly less than that seen in Group 4 or Group 5 and significantly greater than that seen in Group 2. Group 2 animals showed a deficit which, despite the proximity of the data points on the graph, was significantly greater than the Sham group.

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, 4 and 5 showed significantly lower scores. The most severely affected animals were in Group 5. Group 4 showed a significantly greater deficit than Group 3. The sham animals, and the animals injured over 1 m were not significantly different from each other, although both were significantly different from Group 3. Animals in Group 3, 4 and 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, 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.

Inclined plane performance

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

Morris water maze performance

The mean latency of the animals to find the hidden platform was calculated over post-injury days 14-18. A marked gradation in ability on this test is apparent according to injury severity. Group 5 animals performed least well, and had the worst improvement in ability, and the longest latency on the final day. Group 4 animals 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 performed better than Group 4 animals at all time points; however, they started out with a faster latency and then showed a similar rate of improvement. Group 3 and 4 animals showed similar, but significantly different, latencies on the final day of testing. There were no significant differences between Group 2 and 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 at these later time-points. Group 1 animals performed best on each day of the assessment; however, the rate of improvement was broadly comparable to that seen in Group 2, 3 and 4.

5. CONCLUSIONS

5.1.a.   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 h after trauma induction.

5.1.b.   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 are triggered by activated microglia and infiltrating lymphocytes.

5.2.a.   CHI induces transient increase of c-fos immunoreactivity 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 h after trauma.

5.2.b.   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.

5.2.c.   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.

5.3.a.   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.

5.3.b.   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.

5.3.c.   It is clear that the different tests have differing sensitivities to injury levels, which may partially explain the results seen. Consequently, the 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.

6.   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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