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13. Head wounds account for almost half of combat deaths and in peacetime traumatic brain injury costs the U.S. Military more than 60M dollars/year. We used the piston impact injury model to produce focal, cortical brain trauma in the anesthetized rat to gain insights into brain injury mechanisms and treatment strategies to help brain injured soldiers.

Widespread axonal damage occurred throughout the brain from the focal injury. Neurons cell bodies were often spared about the injury area but their axons and dendrites were severely damaged. Surviving neurons, thus, may be nonfunctional. Free fatty acids, diacylglycerols, and phosphiolipase A_2 were elevated widely throughout the brain not only acutely but up to 35 days after injury. Early elevations relate to the acute trauma; sustained elevations probably relate to brain recovery mechanisms. Improvement following brain trauma was biphasic. The early, rapid improvement represents recovery from the acute effects of trauma; the later slower phase possibly represents brain plasticity mechanisms. Neither the platelet activating factor inhibitor (BN52021) nor the calpain inhibitor (MDL 28170) improved the rate or extent of improvement following cortical impact injury.

Hypoxia or hypotension following brain injury significantly retarded recovery. Hypotension severely down regulated both immediate early genes and "recovery genes" seen with normal recovery.

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FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army. Much of the work presented is "in progress". Conclusions drawn from "in progress" work are our best understanding of the various biological processes based on current data. As more data becomes available in the future our conclusions may change.

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Michael E Carey MD 25 June, 1990
PI- Signature Date

TABLE OF CONTENTS

1 BACKGROUND

In combat the head receives 15% of all "hits" and head wounds account for almost half of all battlefield deaths. No basic change in either overall or neurosurgical mortality from brain wounds has occurred since World War II, Figure 1 [1].

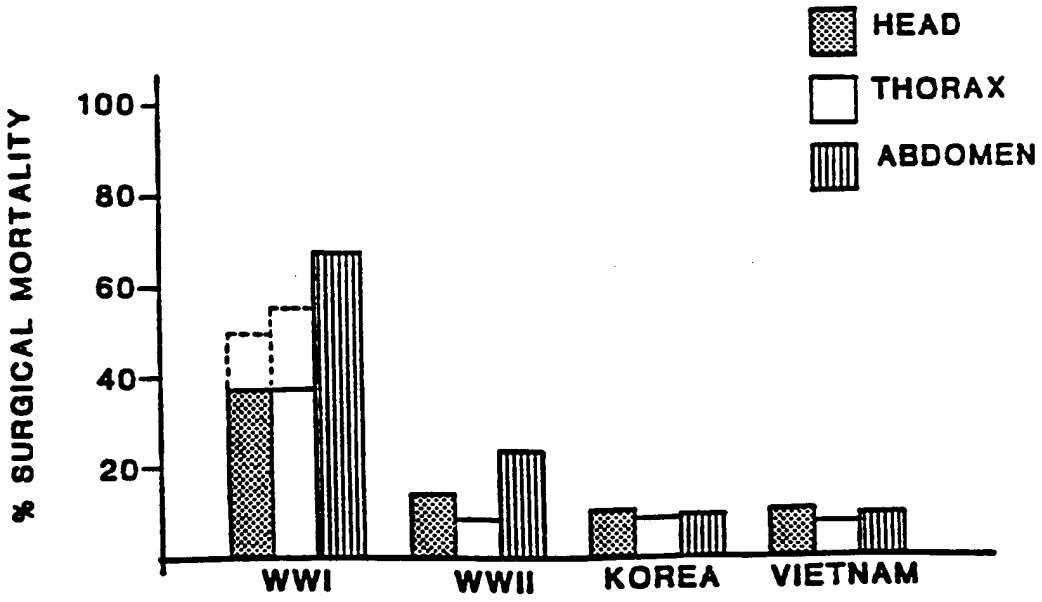


Figure 1: The postoperative mortality of head and chest wounds has remained relatively unchanged since World War II. The post operative mortality of abdominal wounds has not changed since Korea.

Approximately 7% of soldiers with brain wounds survive with varying degrees of neurologic residua [2]. While the percentage is small, the actual number of such individuals is quite substantial: about 34,000 from WWII and 4,000 from the Korean war. Whether a surviving brain-wounded soldier is retained on active duty and, thus, conserves the fighting strength will depend upon the severity of neurologic residua and the manpower needs of the Army. During WWII the British Army retained about 12% of the brain wounded [3]. Later about 70% of surviving British brain wounded soldiers were employed after their service, somewhat more than the 56% which U.S. investigators found 15 years after the Vietnam experience [4]. So the need for therapy to ameliorate combat-incurred brain damage is real, not just theoretical. If brain damage could be attenuated, more soldiers could be made available for continued service; those leaving the service could better perform their civilian jobs.

Brain injury causes a substantial loss of unit effectiveness in the Army during peacetime as well, and costs the Army a great deal of money. In fiscal 1992 and 1993 1,617 active duty Army personnel were hospitalized for head injuries. Over the subsequent 4 years direct medical costs for each of those who sustained a mild head injury averaged $2,600; for those sustaining a severe head injury average individual costs were $61,500. Considering all military services a total of 6,520 active duty personnel with head injuries were admitted to either military or civilian hospitals in 1992. Direct medical cost to the military was 41.9 million dollars [5,6], an average of $6,421 for each. The indirect additional costs to the military were considerably greater as trained individuals with unique skills obtained by service training were lost to active duty and the services had to train replacements at further additional cost. Thus, a better understanding of brain wounds and injury leading to improved treatment is in the Army's (and Navy's and Air Force's) interest both in wartime to conserve the fighting strength and in peacetime to prevent the costly loss of skilled personnel.

2 PURPOSE OF PRESENT WORK

The overall purpose of our research was to develop and establish a reproducible model of traumatic brain injury in the rat which would have relevance to soldiers with brain injury. We wished to characterize this injury histologically, neurochemically, and behaviorly and to test drugs to see whether behavioral deficits characteristic of our brain lesion could be ameliorated by drug therapy. We felt that a model that actually damaged brain (as occurs in real life) was optimal and, thus, basically used the model of Dixon and Lighthall [7,8,9].

It was important to make a neurologic lesion that would produce an easily testable and quantifiable deficit. Since humans often have a disabling contralateral hemiparesis after brain injury we decided to produce a left sided sensorimotor deficit in the rat by damaging the right sensorimotor cortex, Figure 2.

The Motor and Somatosensory Cortex of the Rat

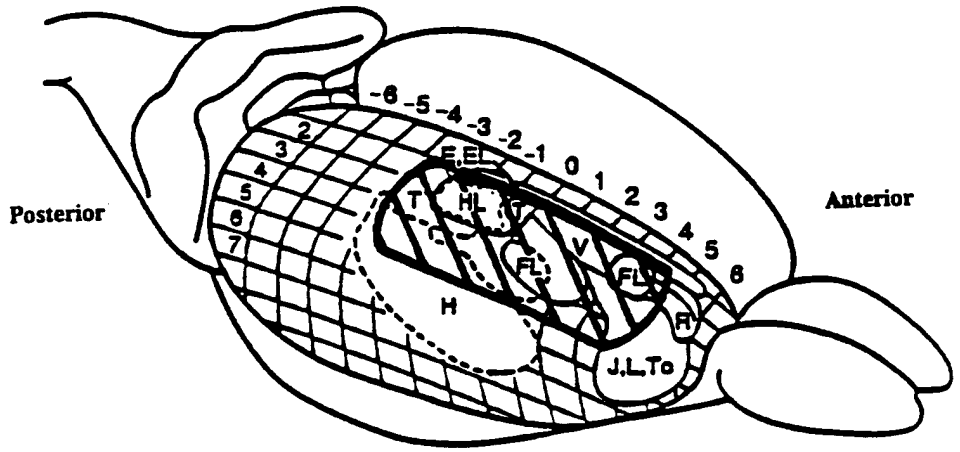


Figure 2: Areas of rat injured by our 4mm X 8mm piston impact tip. Motor areas (solid lines) and primary somatosensory areas (broken lines). FL= forelimb, HL=hindlimb, E=eye, EL=eyelid, V=vibrissae, T=trunk, R=rhinarium, J=jaw, L=lip, To=tongue, H=head

A. SPECIFIC METHOD OF PRODUCING RIGHT SENSORIMOTOR CORTICAL INJURY*

Overnight fasted rats were anesthetized with isoflurane (1.5%) with N_2O:O_2 /5:1. The rats were then placed in a stereotaxic instrument. Under sterile conditions we made a midline scalp incision and right sided craniectomy 5 mm wide by 9 mm long in each rat. The craniectomy began 1 mm lateral to the midline and extended 5 mm further laterally. It was placed 4.5 mm anterior and 4.5 mm posterior to the coronal suture (i.e. the craniectomy was 9 mm in length).

We then moved the rat and stereotaxic frame under a computer-interfaced injury device fitted with a 4 mm x 8 mm pneumatic piston impact tip which impacted the exposed dura at 5.2 m/sec, depressed it 1 mm for 300 msecs, and contused the underlying right sensorimotor cortex.

During the experiments all rats breathed spontaneously. Body and brain temperatures were maintained within normal limits by a homeothermic heating blanket and heat lamp. After injury bleeding was stopped, the scalp sutured, and the rat was placed in a warmed oxygenated chamber until fully awake. The animal was then returned to a holding cage and frequently observed up to four hours after injury. A comfort score was kept on all rats for several days after injury to document their well being. No rat ever appeared in distress. When the rat was eating and drinking, it was returned to its home cage in the AAALAC-approved animal facility supervised by 2 full-time veterinarians. Injury produced no significant effect on appetite or food and water consumption.

Uninjured, control rats were treated exactly the same way except for a slight modification in the craniectomy. The usual 5 mm x 9 mm craniectomy incorporates the coronal suture which adheres to the underlying dura. Even with utmost care, dissecting the coronal suture periosteum from the dura results in minor sensorimotor deficits clearly evident on our motor behavior tests. Such minor deficits are greatly overshadowed by the major sensorimotor deficits caused by piston impact so are unimportant in injured animals. These minor deficits, however, confounded behavioral testing in control, uninjured animals. To obviate this effect our control animals had two craniectomies. One extended from about .5 mm to 4 mm posterior to the coronal suture and the other .5

* All experiments in this report were evaluated and approved by the LSU IACUC

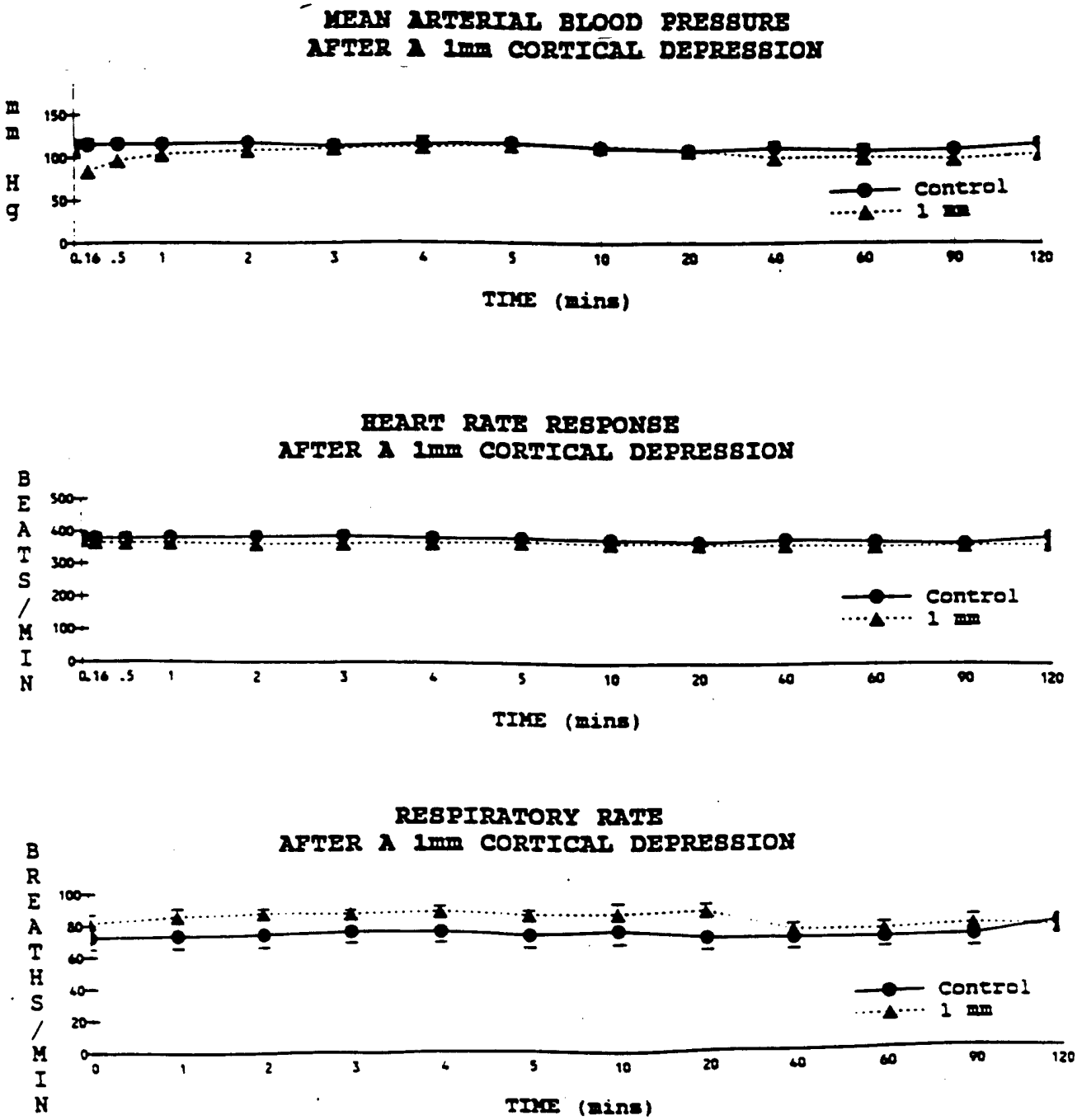
mm to 4 mm anterior to it. We did not remove the connective tissue which attaches the dura to the coronal suture line. Only a few control rats thus prepared exhibited any subsequent motor deficits. Such rats were excluded from our various studies.

After injury the animals were treated and euthanized differently depending upon the particular study for which they were intended. Their treatment and mode of sacrifice for any particular study will be discussed in the appropriate sections.

B. PRELIMINARY STUDIES

In our preliminary studies we injured the anesthetized rats with 1mm, 2mm, and 3mm dural depressions. Right sensorimotor piston impacts resulting in 2 or 3mm dural depressions produced significant cardiorespiratory changes and invariably tore the dura. A 1mm dural depression produced a significant left sensorimotor defect but did not cause significant systemic physiologic changes. This impact caused a brief, mild hypotensive response (mean arterial blood pressure, MABP, 80mmHg < 30 secs) but neither heart nor respiratory rates were significantly affected, Figure 3.

Figure 3: Mean arterial blood pressure, heart rate and respirations following 1mm dural impact



Furthermore, blood gas and pH and blood glucose levels were also unaffected by this degree of injury (See Mid Contract Report June, 1995 for specific physiological details). A 1mm dural depression did not tear the dura and, thus, avoided brain

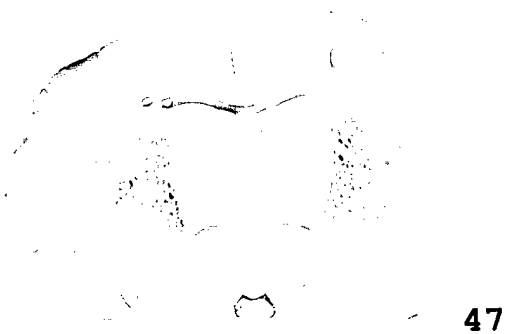
herniation out through dural tears with resultant local brain ischemia/hypoxia; any posttraumatic changes which occurred in the brain could be attributed to piston impact per se and not secondary ischemia or hypoxia from local or systemic events. **THUS, WE ELECTED TO MAKE THE 1MM DURAL DEPRESSION OUR STANDARD INJURY. ALL HISTOLOGICAL, NEUROCHEMICAL AND BEHAVIORAL STUDIES IN THIS REPORT ARE FROM ANESTHETIZED ANIMALS RECEIVING A PISTON IMPACT WHICH DEPRESSED THE DURA OVER THE RIGHT SENSORIMOTOR CORTEX 1mm.**

C. THE BASIC ANATOMIC AND NEUROBEHAVIORAL RESULTS FROM OUR STANDARD INJURY

Figure 4, depicting coronal brain sections from 3 control and 8 injured rats, shows that the amount of cortical mantle loss consequent to the piston strike was variable but the distribution and degree of cortical and subcortical fiber track degeneration (black areas) were quite constant from animal to animal. (See Appendix 1, pp158-163 for additional coronal sections)

Figure 5 shows that the neurobehavioral deficits produced by the sensorimotor injury were quite constant and reproducible in more than 60 rats over 4 years. We infer that the uniformity of the sensorimotor deficit and recovery pattern is because of the constancy of the fiber track degeneration caused by the cortical injury.

0.0 mm



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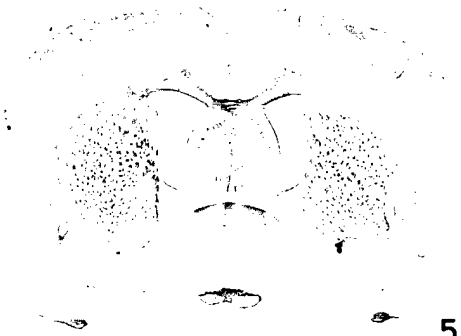

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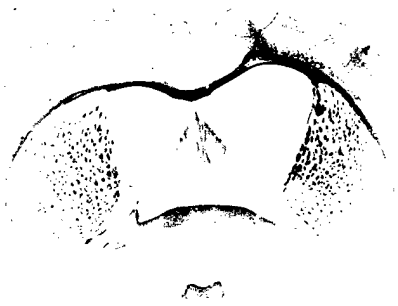
Control

Y

Control



42

Injured - 8 Wks

51

Injured - 8 Wks

54

Injured - 8 Wks

499

Injured - 8 Wks

500

Injured - 8 Wks

501

Injured - 8 Wks

Figure 4

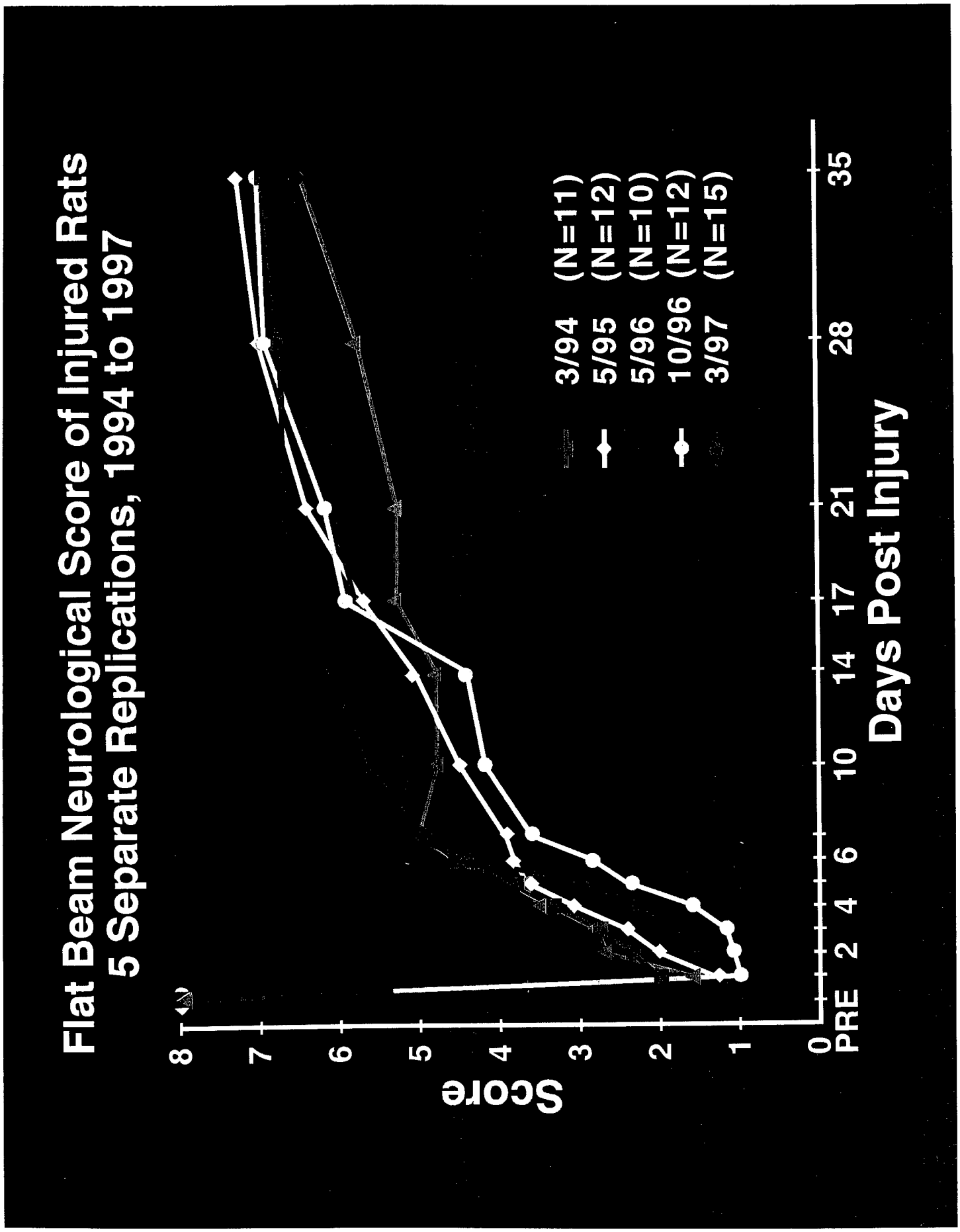

Flat Beam Neurological Score of Injured Rats
5 Separate Replications, 1994 to 1997
Score
Days Post Injury
PRE
2
4
6
10
14
17
21
28
35
0
1
2
3
4
5
6
7
8
3/94 (N=11)
5/95 (N=12)
5/96 (N=10)
10/96 (N=12)
3/97 (N=15)

Figure 5

3 SPECIFIC HISTOLOGIC STUDIES AND THEIR SIGNIFICANCE

Method of Animal Preparation and Evaluation of Histologic Studies

At the appropriate times after the standard injury all rats were deeply anesthetized by intraperitoneal (i.p.) pentobarbital (50 mg/kg). When deeply anesthetized each rat underwent transcardiac perfusion with 4% paraformaldehyde buffered with 0.067M sodium cacodylate with 4% sucrose. For hematoxylin and eosin (H and E) and luxol fast blue-cresyl violet stains done at LSU we removed the brains and sent them to the Department of Anatomy for processing: We have done histological evaluations of the brain from immediately to 8 weeks after injury. Since the "gold standard" for such basic morphological studies by neuropathologists has been the H and E stain, we chose this technique to demonstrate the basic posttraumatic morphological changes. These histologic sections were evaluated by Dr. James Nelson, a neuropathologist in the LSU Department of Pathology, and former head of Neuropathology for the AFIP. When brains were intended for fiber track degeneration studies (deOlmos cupric silver or amino cupric silver) or Golgi staining for dendrite morphology we decapitated the rats after fixation and sent the fixed heads to Neuroscience Associates, Knoxville, TN (fiber track degeneration studies) or Neurometrics, Columbus, OH (Golgi staining).

Results and Discussion

A. **Neuropathologic Studies** (Dr. James Nelson)

The H and E sections revealed the expected and classical changes of traumatic brain injury (Appendix 2 pp 164-177).

What has not heretofore been described with focal brain injury and what our laboratory has shown, however, is that two morphologic patterns of neuronal change appear to occur early after focal cortical injury. Just after impact there is a shrinkage of the cell body and nucleus, scalloping of the cell body, faint cytoplasmic eosinophilia, and the appearance of perineuronal vacuoles, Figures 6,7,8. The second pattern becomes evident at about 4 hours after impact and has the features of eosinophilic neuronal necrosis wherein the neuron nucleus is dark and clumped and the surrounding cytoplasm well stained with eosin. This morphology is characteristically seen in association with ischemic infarcts, Figures 9,10.

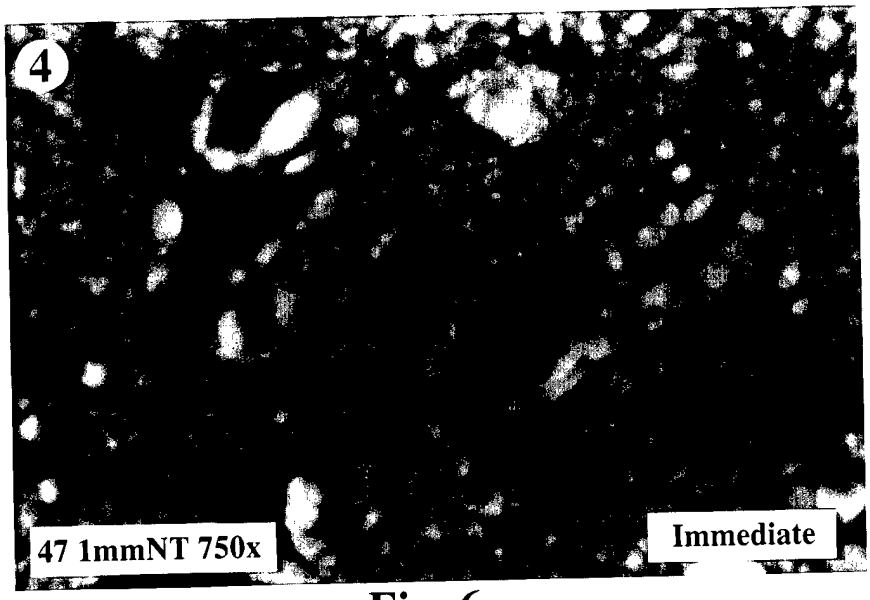


Fig. 6

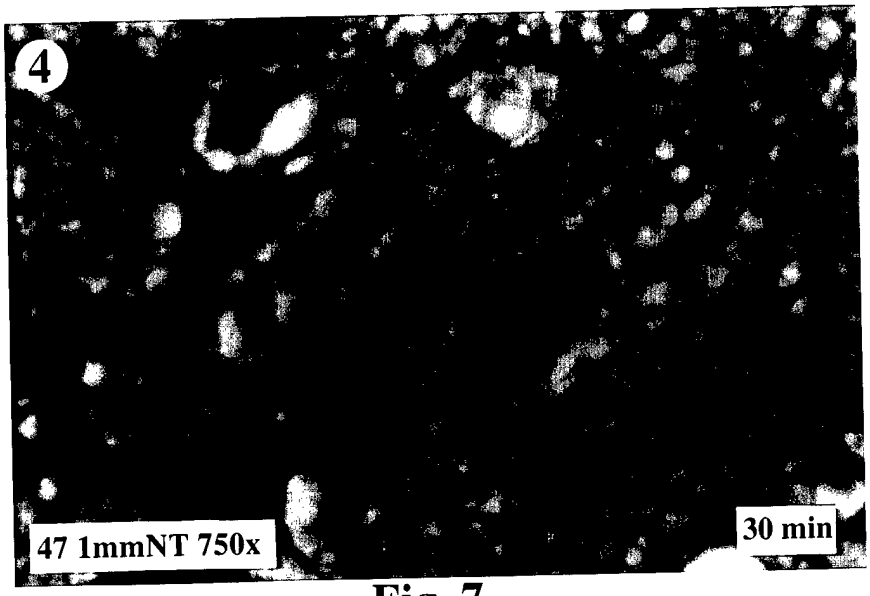


Fig. 7

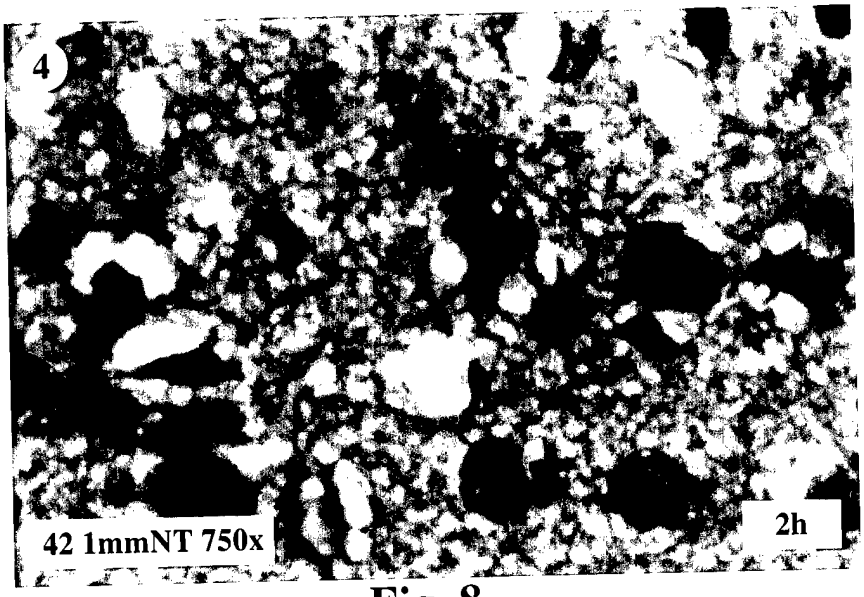


Fig. 8

Fig. 9

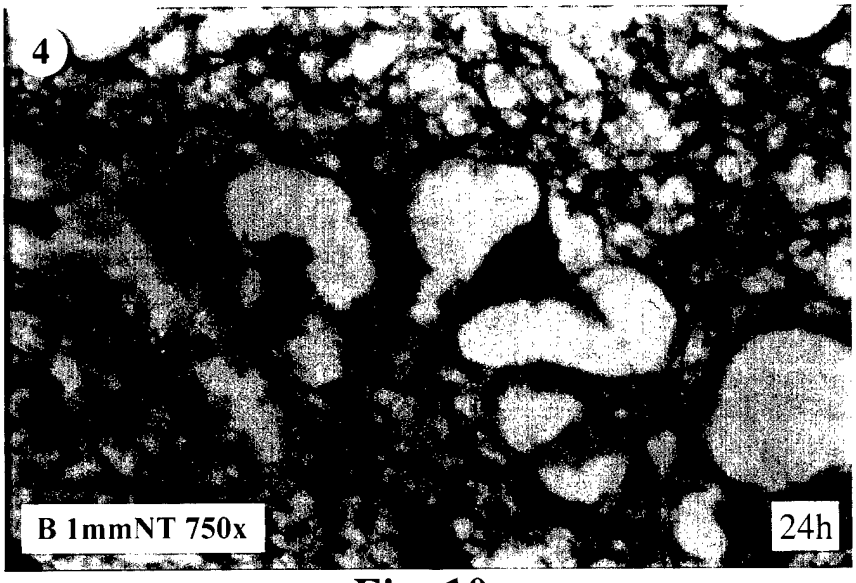


Fig. 10

The two histologic patterns suggest two different mechanisms of neuronal injury: early, a non-ischemic injury; later, an ischemic one. The early histologic picture, not associated with ischemia and occurring up to 4 hours post injury, correlates well with our early neurochemical findings of free fatty acid and diacylglycerol elevation which are not typical of those seen in ischemia. **Thus, our research provides both histological and neurochemical evidence that the earliest posttraumatic events associated with traumatic brain injury are non-ischemic in nature.** The ability to combine findings from our separate research groups (e.g. histology and neurochemistry) enhance the findings of each and demonstrates the advantage of a simultaneously conducted multidiscipline approach to the study of brain trauma.

B. Cortical Mantle, Other Cellular Changes, Dendrites, and Fiber Tracks (Dr. Murray Matthews and Dr. Michael Carey)

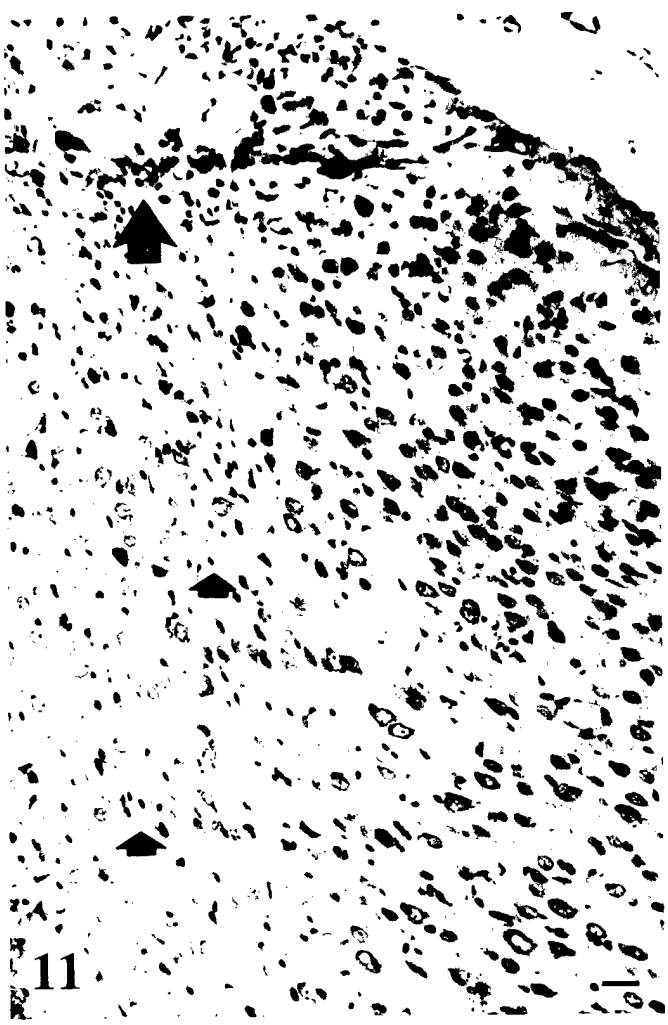
Cerebral Mantle After Injury

Figure 4 and Appendix 1 show that following injury the cerebral mantle at the impact site underwent a variable amount of loss possibly because of differing amounts of posttraumatic hemorrhage. Most often considerable cortical mantle remained; only occasionally was mantle loss severe. Brain under the medial and lateral piston edges appeared most severely affected and we infer that brain at the piston edges was subject to more destructive shear forces while brain directly under the piston face underwent a less destructive compression force. Because of the variability in cortical mantle loss with seemingly identical injuries we, in contrast to other investigators [10,11], do not feel that quantification of mantle loss provides a worthwhile estimate of either injury severity or drug neuroprotection.

Cortical Cells After Injury

After right cortical injury light microscopic studies of luxol fast blue- cresyl violet stained sections revealed a darkening and shrinking of neurons not only at the impact site, Figure 11, but many millimeters away in the contralateral cortex, Figure 12, and in the hippocampus, Figure 13.* Darkened shrunken neurons were seen within hours of injury throughout both cerebral cortices. Darkened neurons were noted throughout the cortices up to 8 weeks following the trauma but to a lesser extent.

* bar in Figures 11,12, and 13= 100μm



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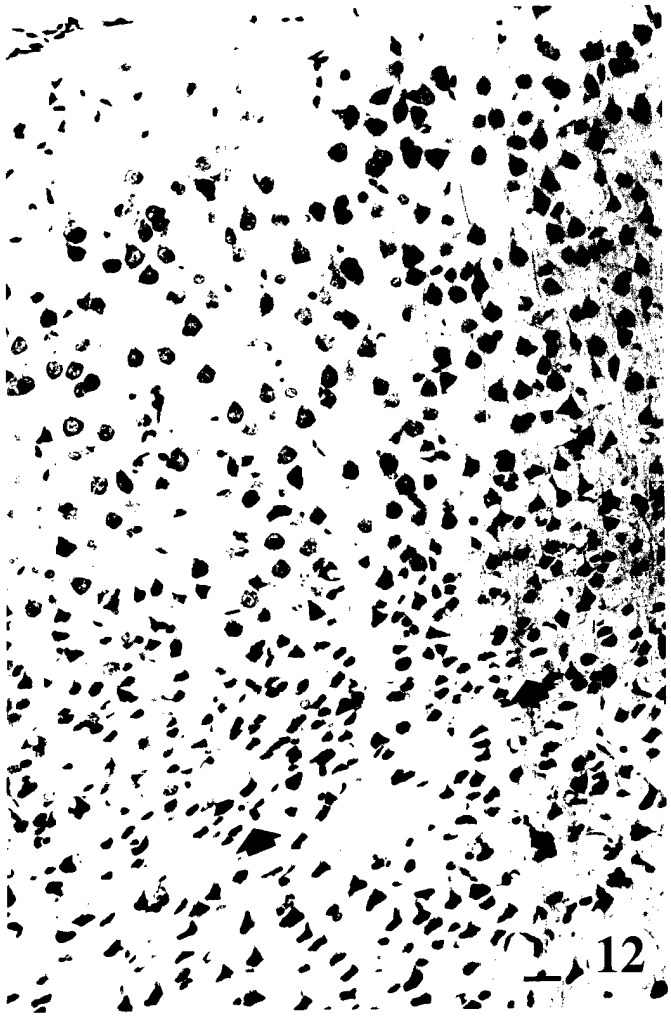

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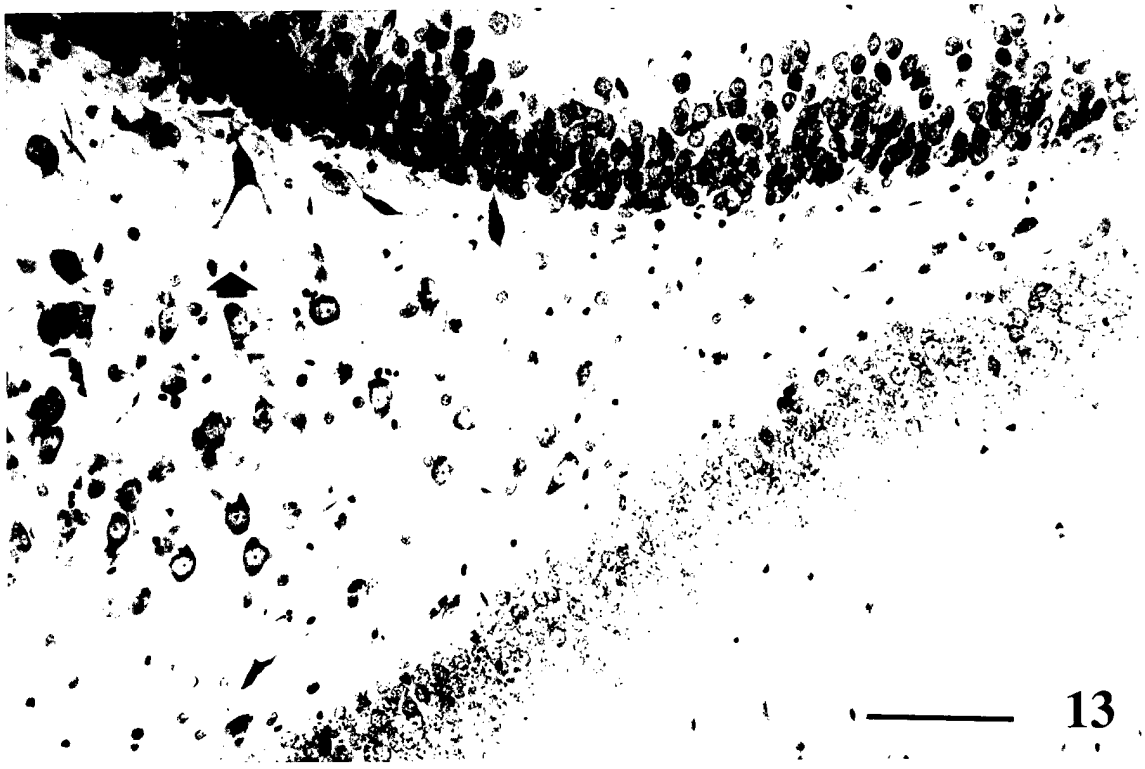
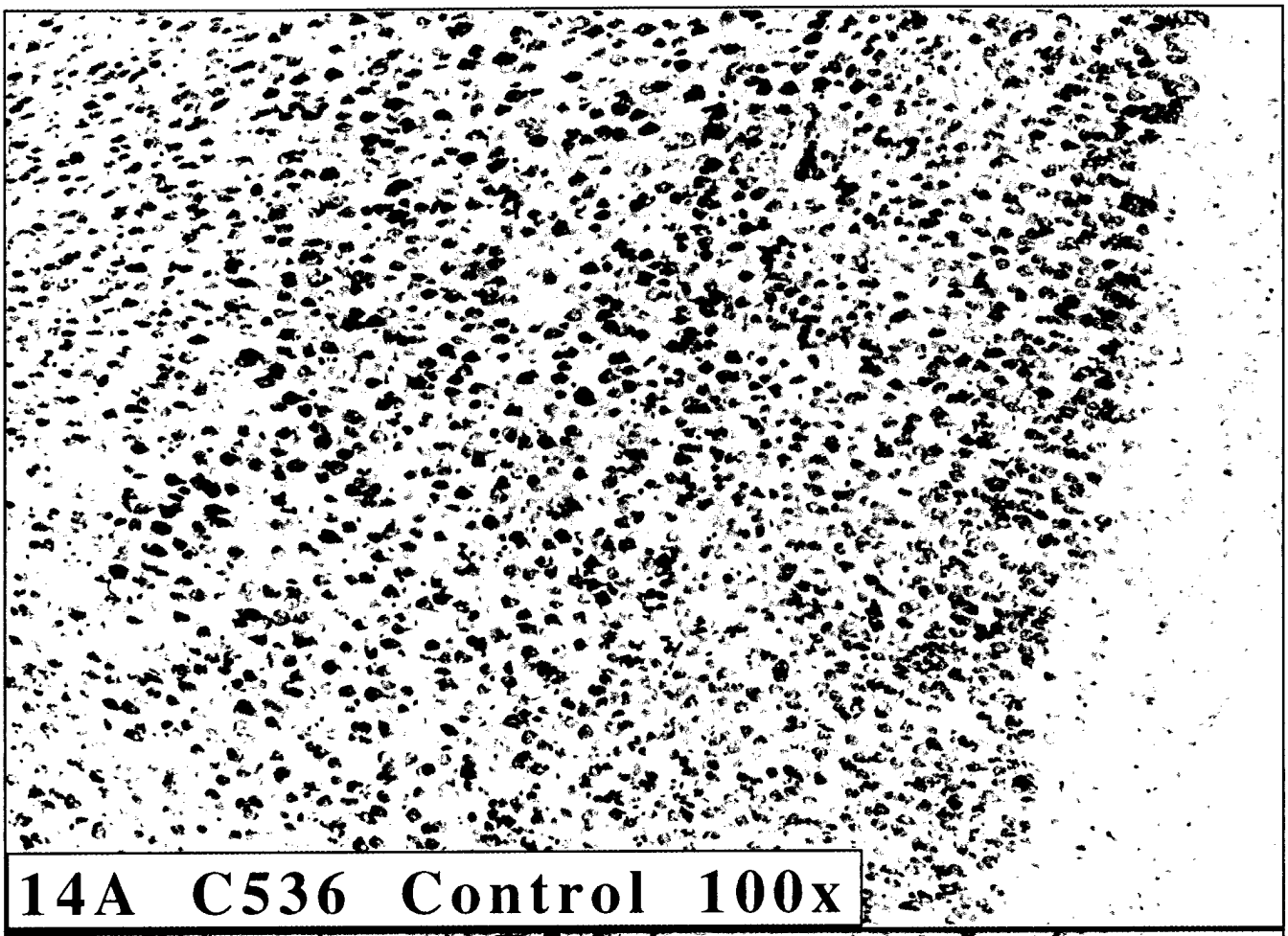
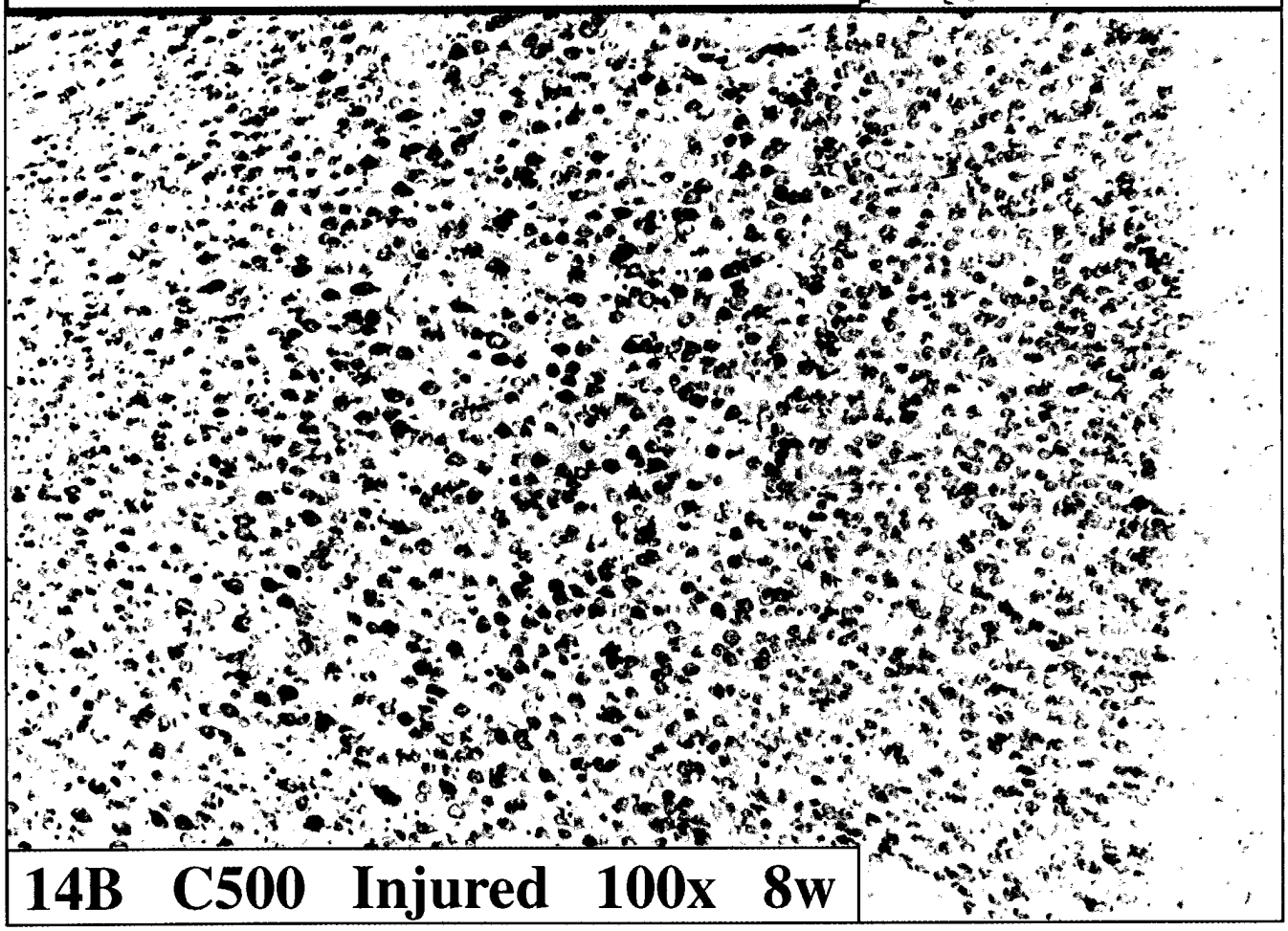


13

Why cells many millimeters from the focal injury site and even contralaterally exhibited damage is unclear. It is also unclear whether the shrunken darkened cells we observed represent injured cells going through some reparative process or dying cells. We infer that many cells did die because the cortical mantle thickness was reduced both on the side of injury and contralaterally with concomitant ventricular dilatation. Cortical cells could be damaged directly by shock and pressure waves associated with piston strike or by brain displacement. They could also be damaged indirectly owing to the acute loss of axons (a loss which this present study strongly suggests) projecting from the damaged sensorimotor cortex to their target cells distributed widely throughout both cerebral hemispheres. The loss of normal neural input might cause the target neurons to undergo metabolic or other changes reflected by chromatolysis [12,13,14,15]. Despite the cell darkening seen early after injury by luxol fast blue-cresyl violet, many cells in the cerebral cortex directly under the piston face appeared quite normal to thionine and H and E stains 8 weeks after injury, Figure 14. Figure 14A cells shows from a control, uninjured rat; 14B shows cells from cortex directly under the piston impact area 8 weeks after injury.



14A C536 Control 100x



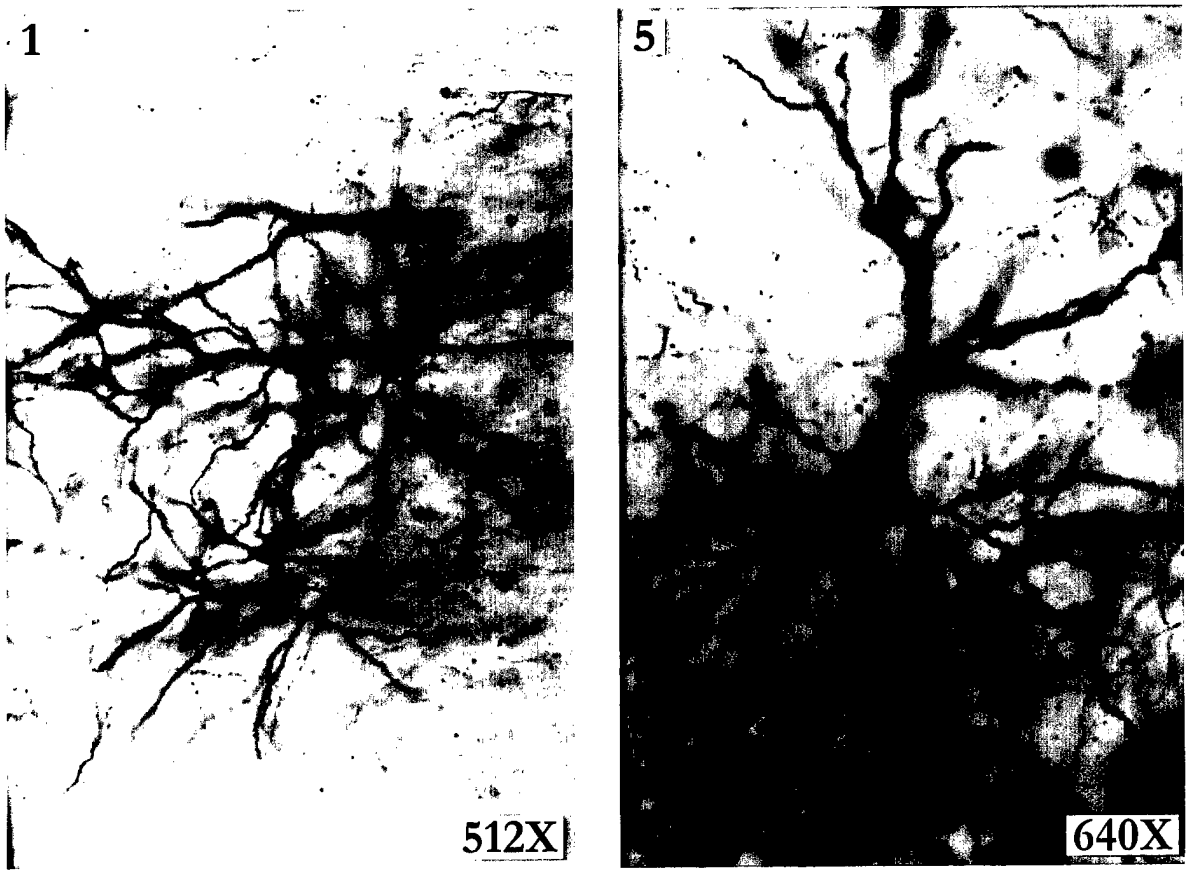
14B C500 Injured 100x 8w

Perhaps by 8 weeks the population of darkened shrunken cells had died and disappeared. The widespread and long lasting darkening and shrinking of neurons correlated with the observed axonal degeneration (see below in this chapter).

Dendrites After Injury

The dendritic array constitutes ~95% of a single neuron's total volume [16] and dendritic branching and spines reflect the integrity of brain circuits and brain activity. Learning and memory are closely correlated with dendritic integrity [17,18,19]. Dendritic branches and spines comprise the receptive antennae for impulses into a neuron and these postsynaptic structures reveal early neuronal damage by dendrite branch atrophy and/or spine loss. Because branches and spines are highly quantifiable one can precisely delineate not only neural damage but how effective a beneficial drug may be in minimizing or reversing traumatic neural damage [20].

Golgi stains 1 week, 3, and 4.5 months after injury revealed a very severe and persistent loss of normal dendrite architecture and dendritic spine density in and adjacent to the injury site, Figures 15,16,17,18. These photomicrographs dramatically demonstrate that afferent input to surviving neurons in or about the injury site is greatly impaired. We hypothesize that preservation or restitution of dendrites of damaged neurons would improve post injury function.



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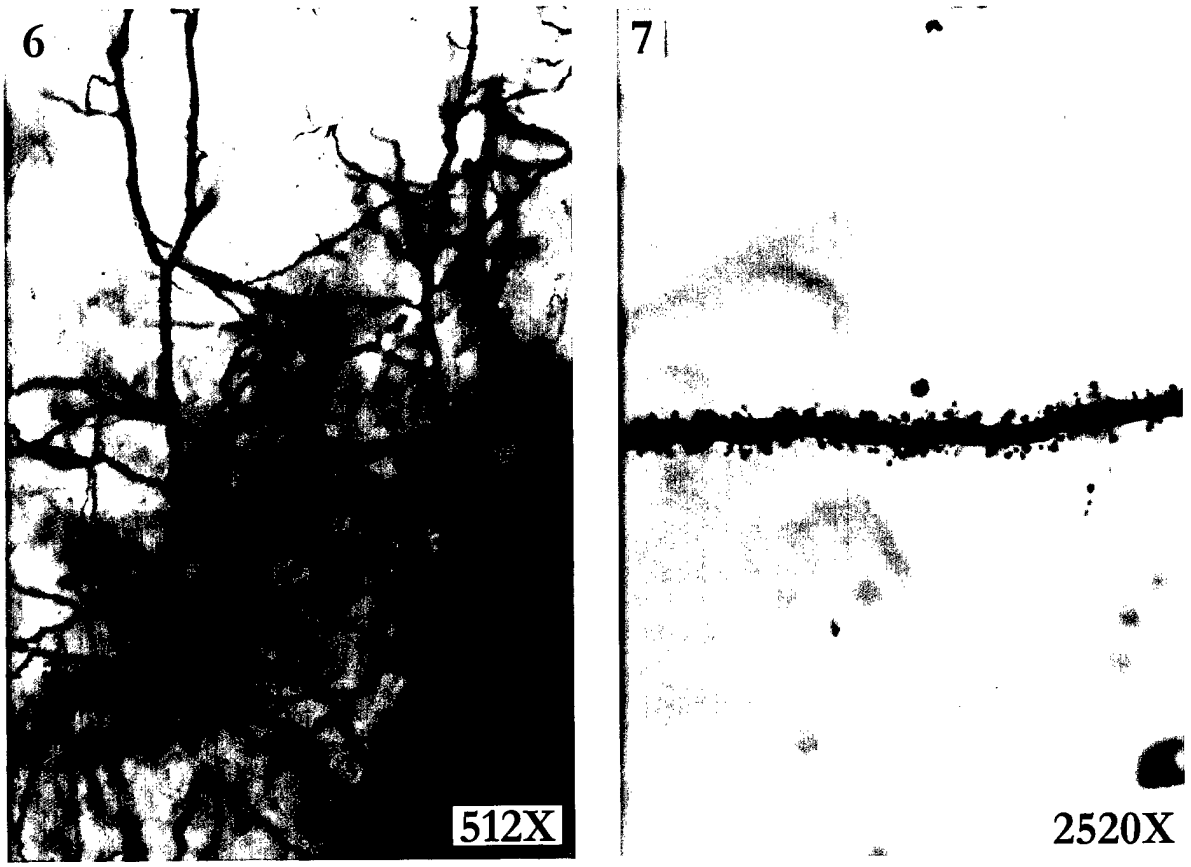
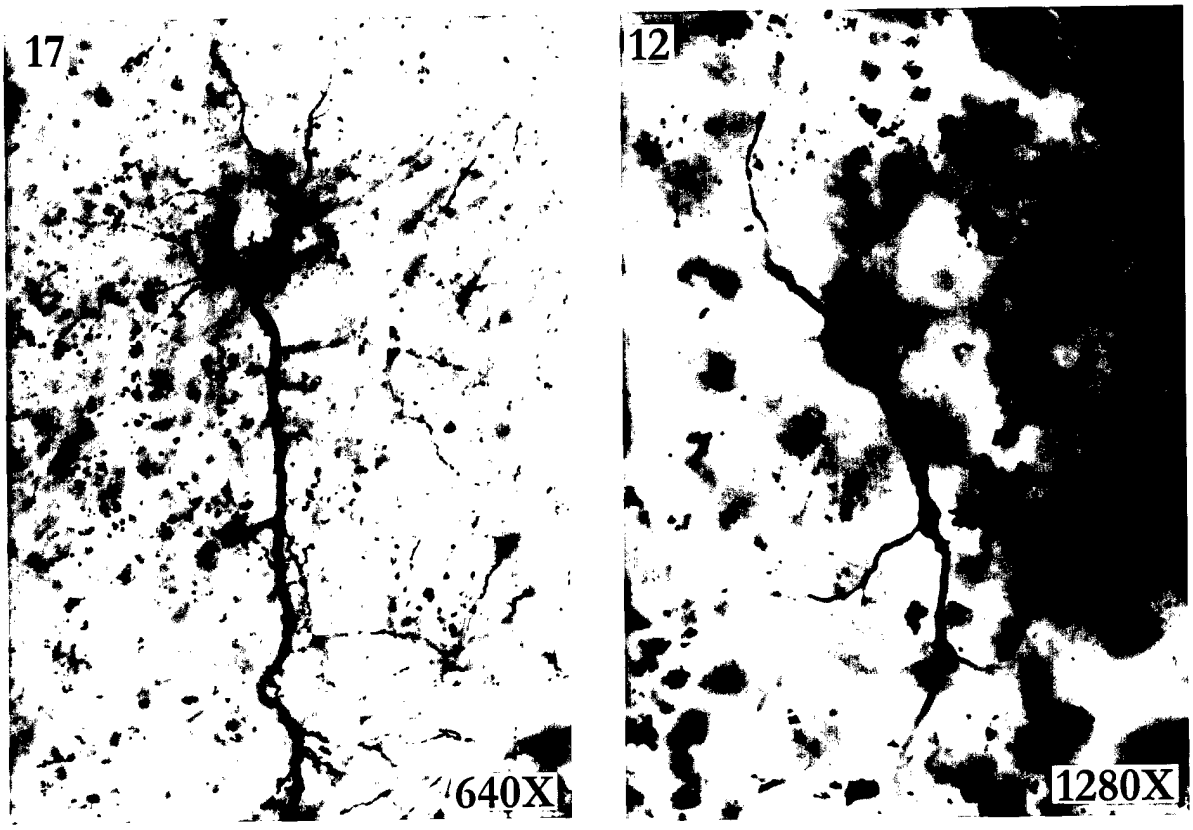


Fig. 15



6 Days After Injury

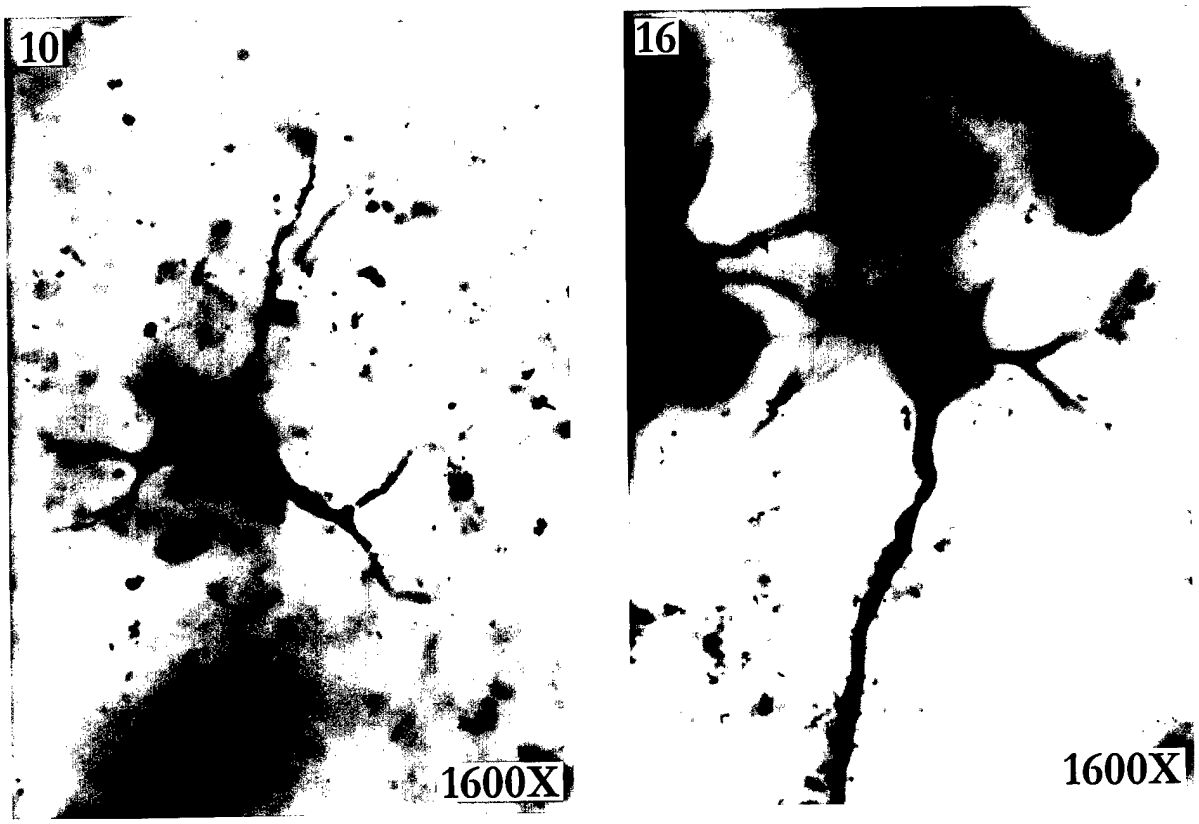
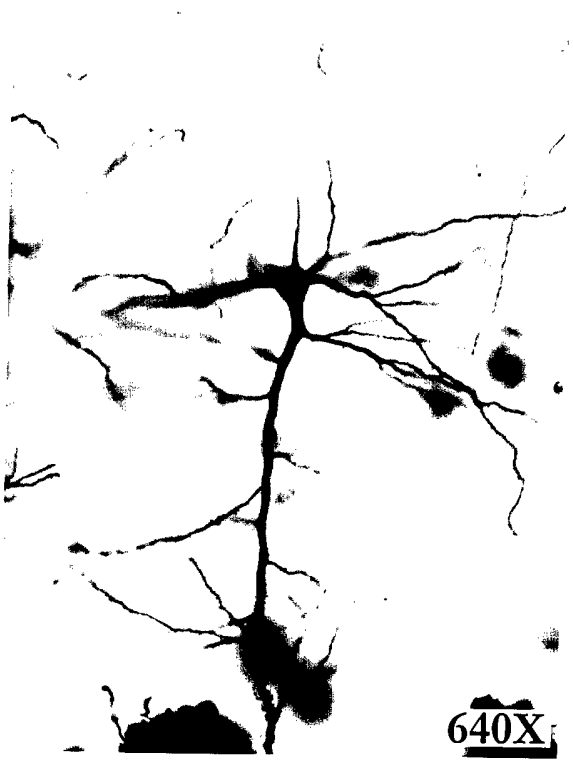


Fig. 16

4 Control 39

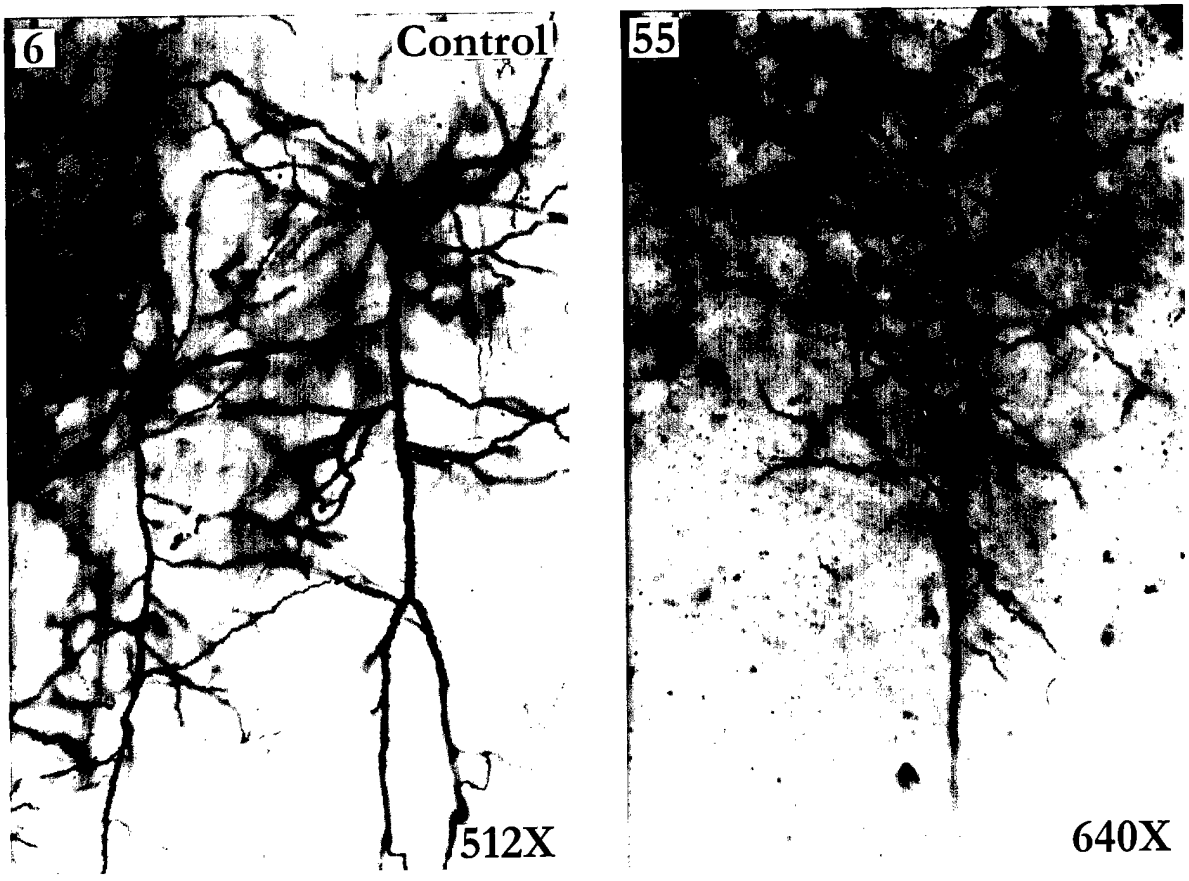


3 Months After Injury

38

36

Fig. 17



4.5 Months After Injury

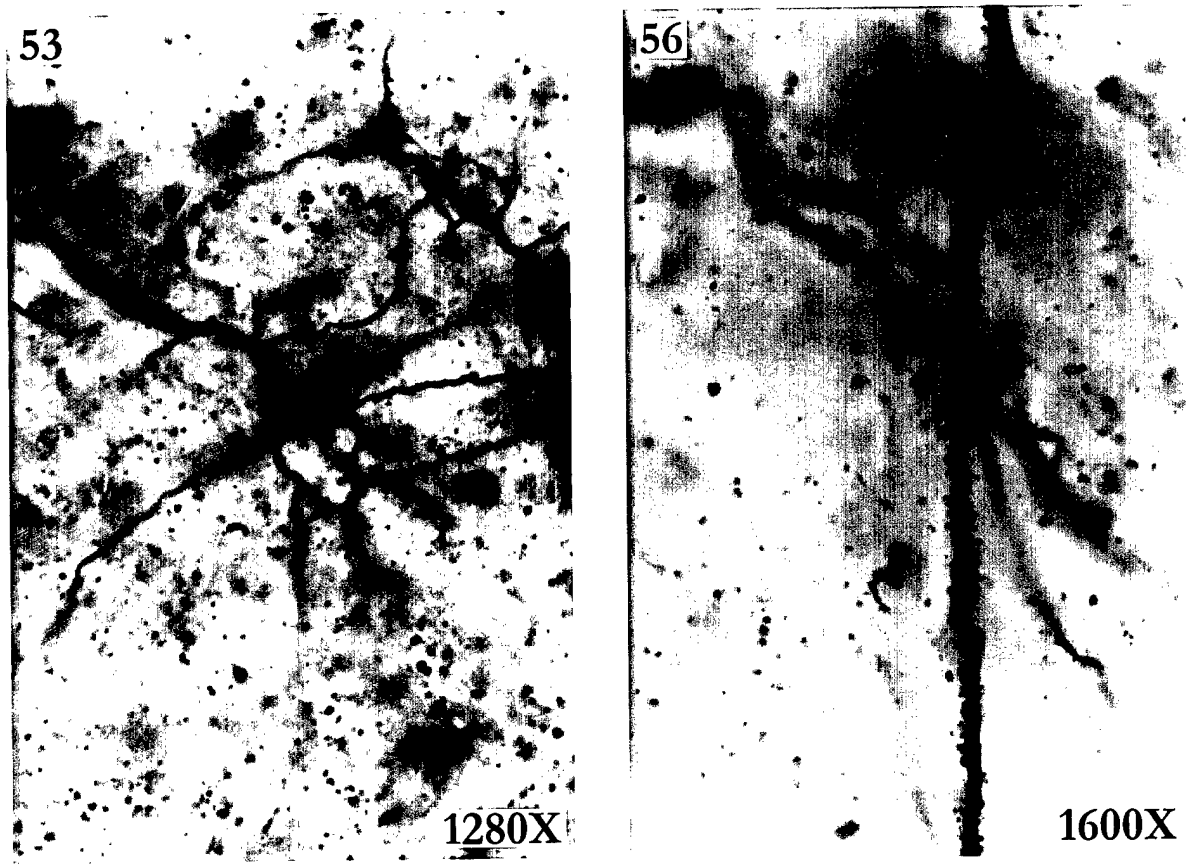
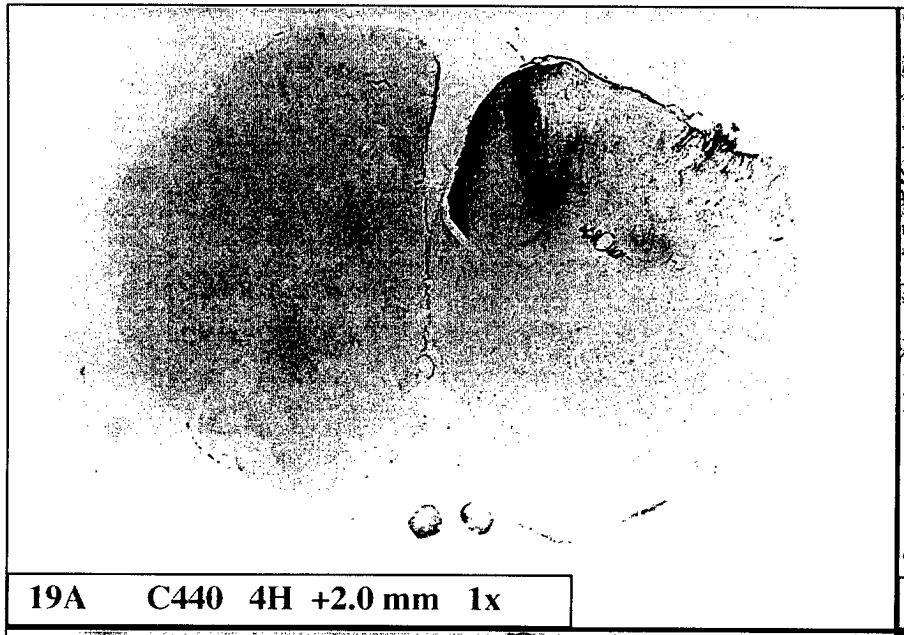


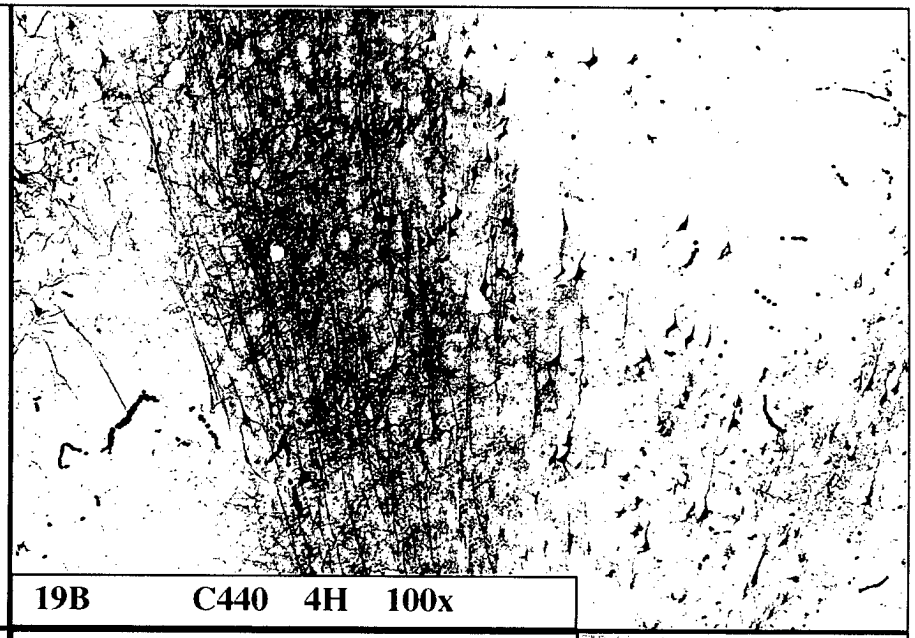
Fig. 18

Fiber Tracks After Injury

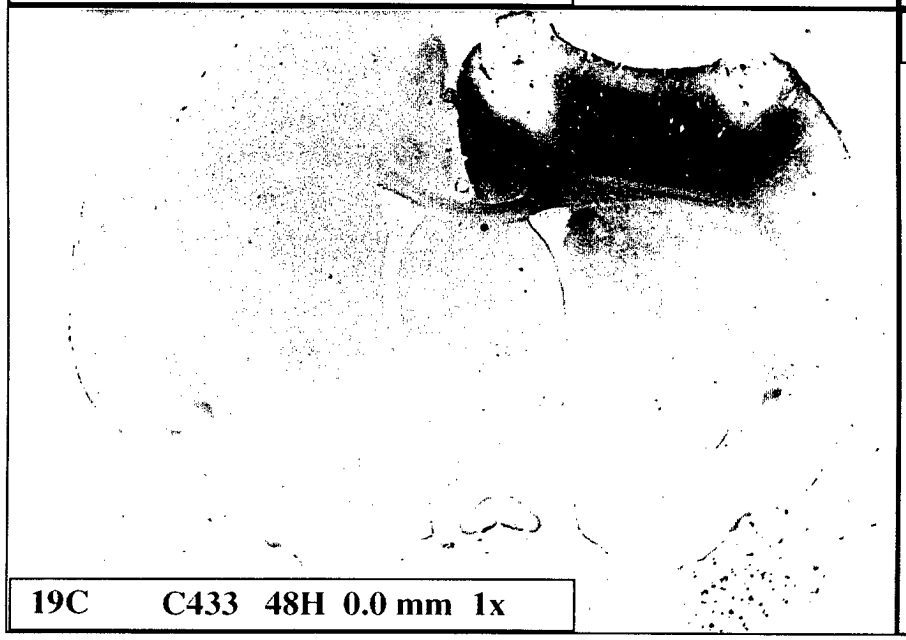
Using deOlmos silver stain techniques [21,22] it was evident that axon degeneration occurred within hours of injury, Figures 19 A and B. Forty-eight hours after injury damaged axons occupied the whole mantle under the area of piston strike as well as the corpus callosum, Figure 19C. Degenerating axons were also evident in the inferior portions of the vermis and the cerebellar white matter, Figures 20 A and B. At this time we noted no evidence of fiber track degeneration in the mesencephalon, Figure 19D.



19A C440 4H +2.0 mm 1x

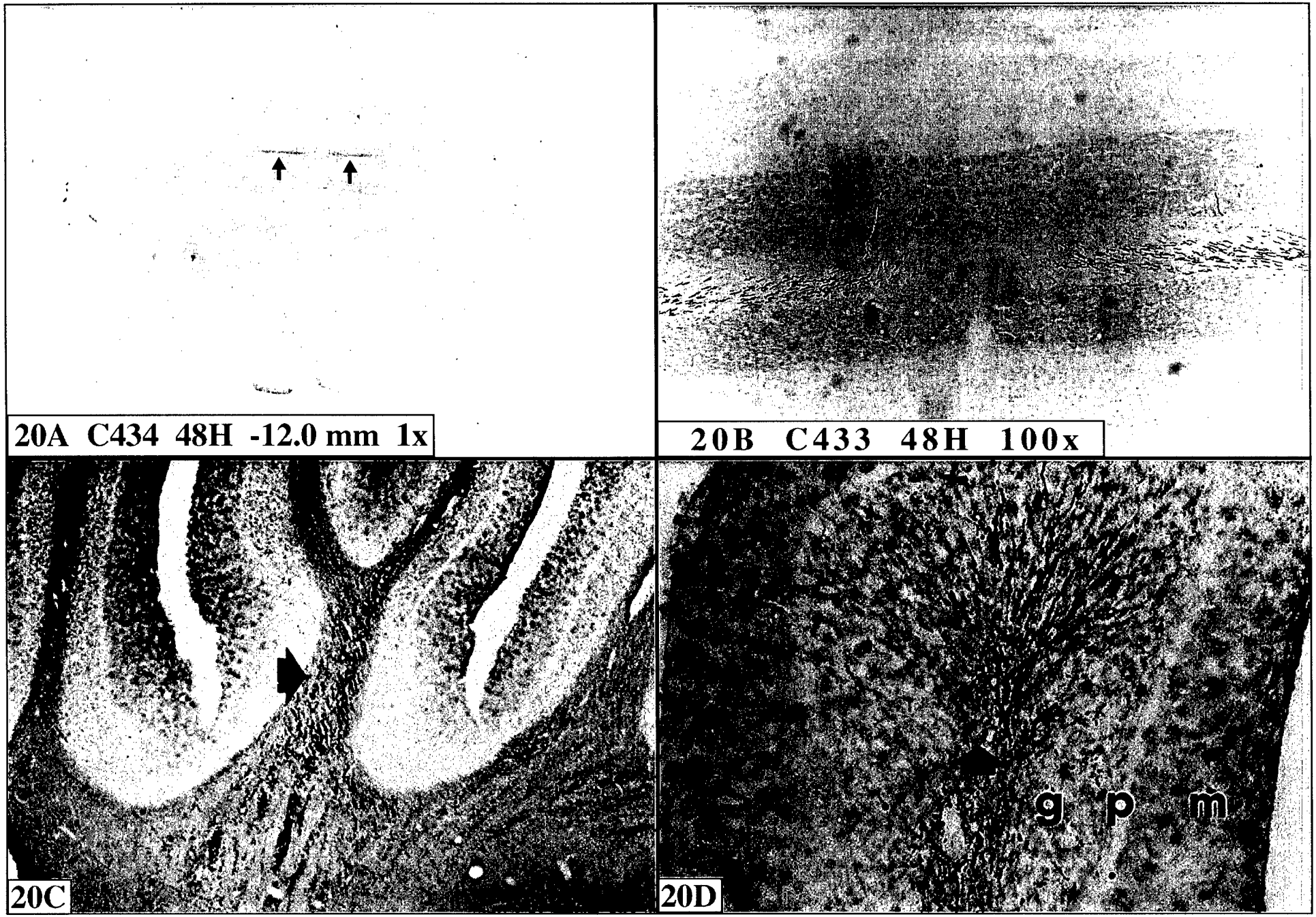


19B C440 4H 100x

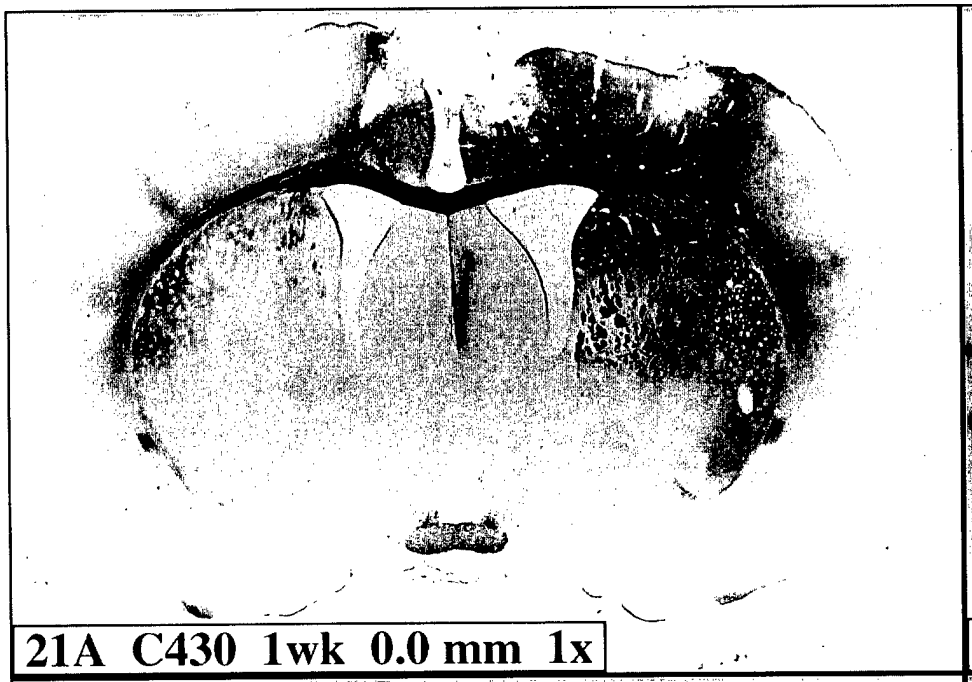


19C C433 48H 0.0 mm 1x

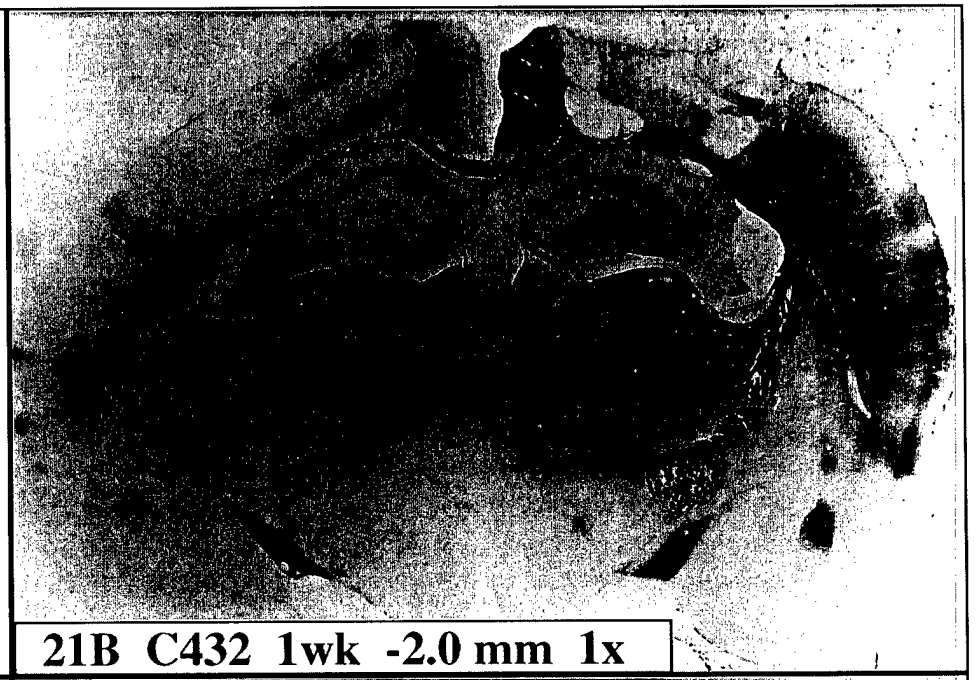
19D C433 48H -8.0 mm 1x


20A C434 48H -12.0 mm 1x
20B C433 48H 100x
20C
20D
g
p
m

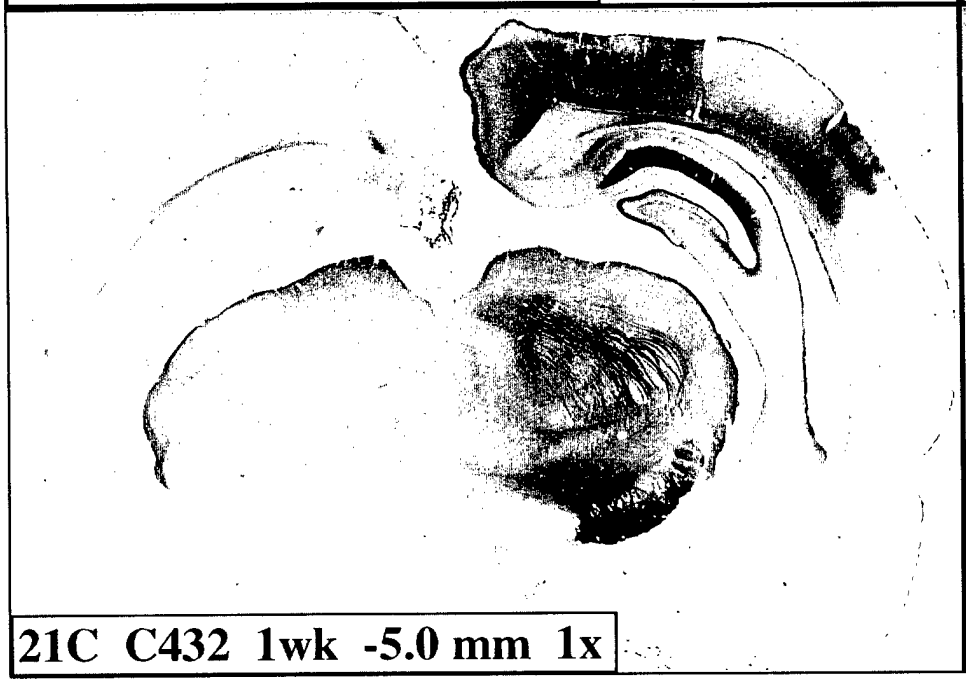
One week after injury evidence of axonal degeneration was flagrant with degenerating axons extending from the cortical impact area into the underlying white matter, Figure 21 A to D. Degenerating fibers also extended into ipsilateral parieto-occipital cortex and contralaterally via the corpus callosum into the opposite sensorimotor cortex, particularly in the midline. Projections to the ipsilateral, dorsolateral, and medial striatal areas showed extensive degeneration. Slight degeneration was also evident in the contralateral upper striatum. Numerous degenerating axons entered the internal capsule on the same side as the lesion and coursed into ipsilateral ventroposteromedial and ventroposterolateral thalamic nuclei. A substantial number of degenerating fibers also entered the contralateral thalamus. Considerable degeneration also occurred in the optic tracks. One week after injury degenerating axons were also seen within the ipsilateral peduncle and substantia nigra suggesting a possible terminal degeneration onto nigral neurons. The red nucleus and superior cerebellar peduncle also exhibited concentrations of silver stained fibers and the ipsilateral pyramid was invariably deeply stained, Figures 21B,C, and D. Within the cerebellum large numbers of degenerating axons were seen prominently in the vermal midline and extending into many folia. They appeared to terminate in the granule cell layer, Figures 20C and D and 21D.



21A C430 1wk 0.0 mm 1x



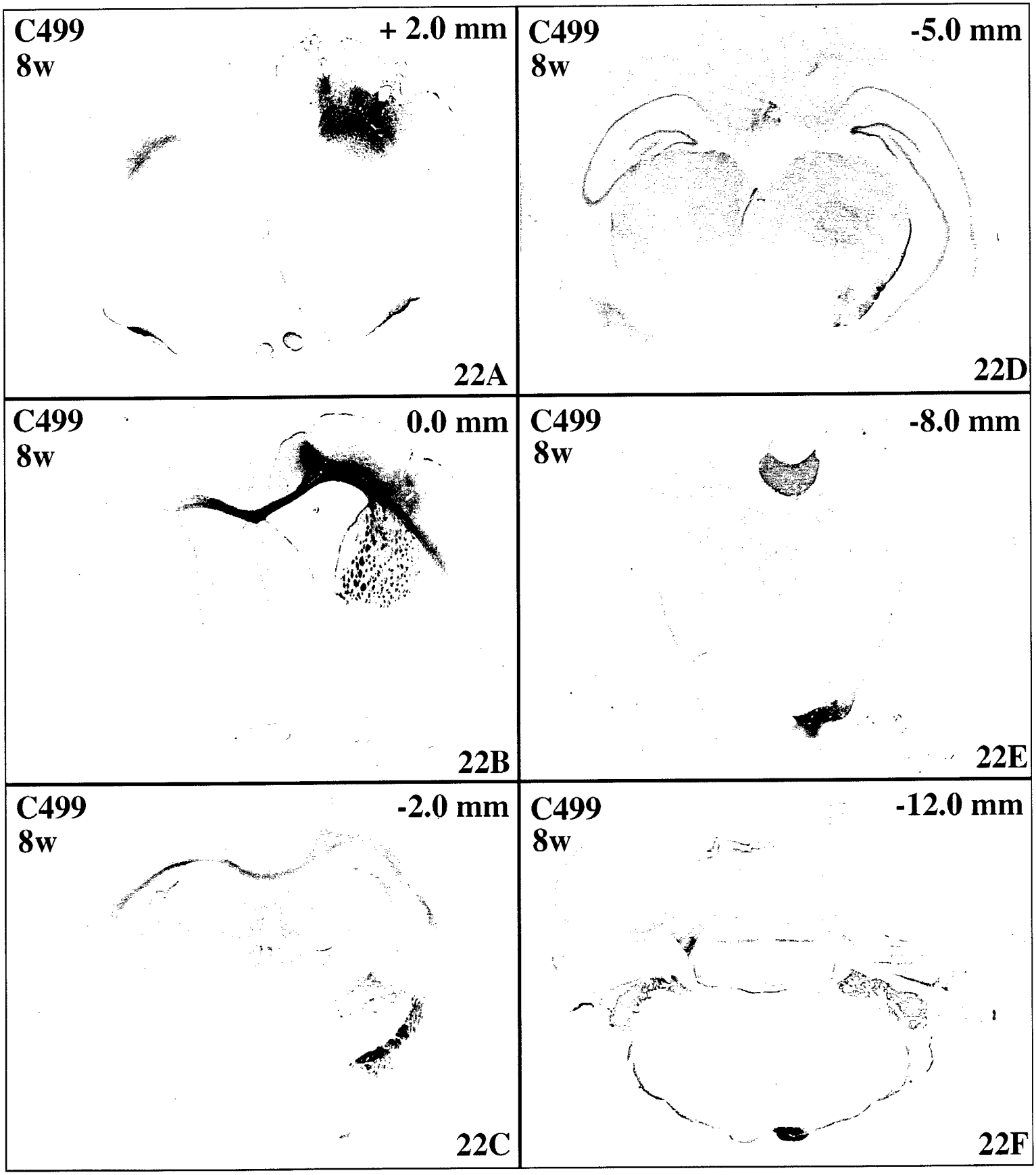
21B C432 1wk -2.0 mm 1x

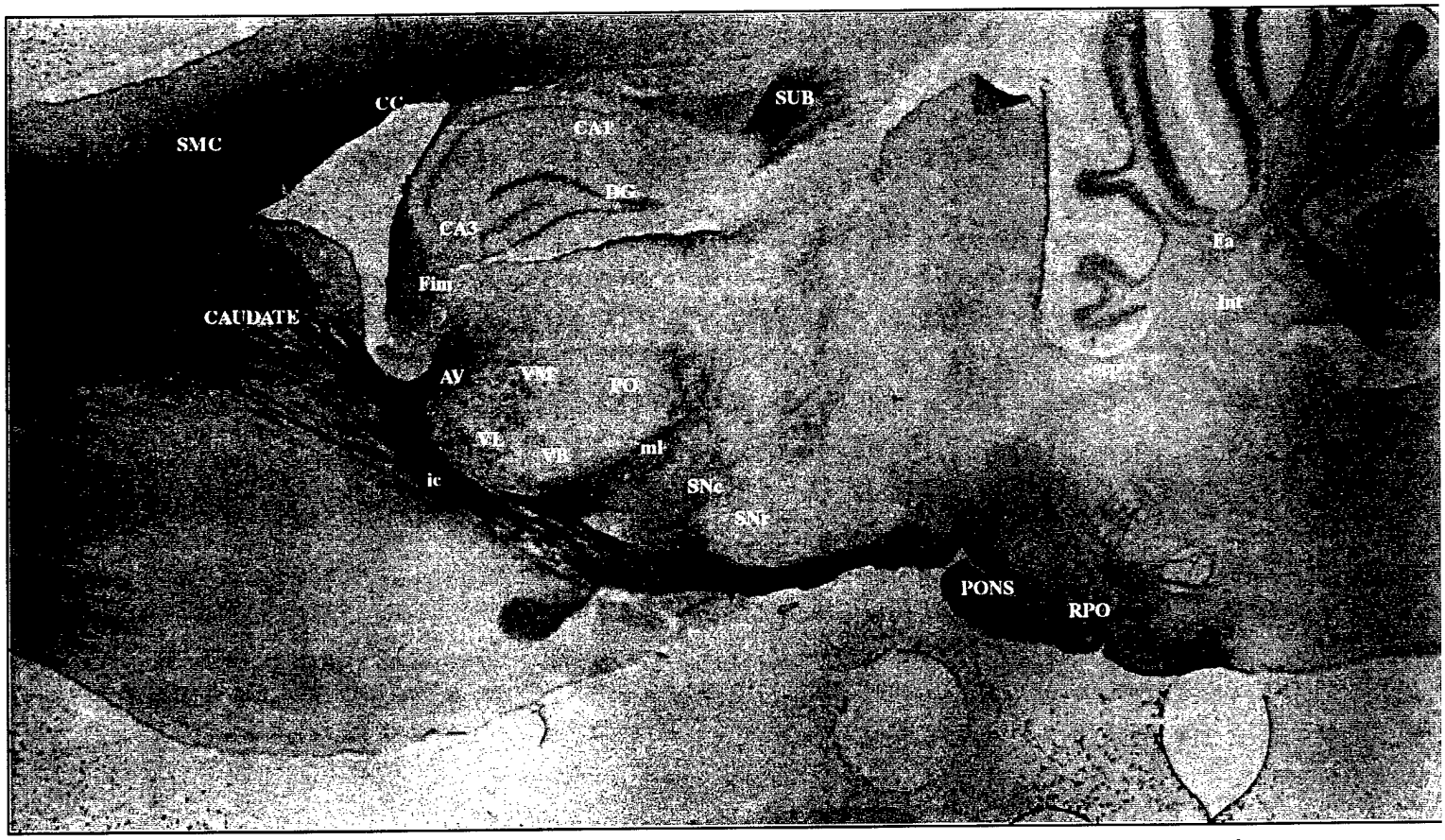


21C C432 1wk -5.0 mm 1x

21D C432 1wk -12.0 mm 1x

By 4 and 8 weeks the widespread evidence of axonal damage within both hemispheres had resolved, Figure 22 A to F, but persistent evidence of degeneration could be seen in the sensorimotor corticofugal fibers as well as the same subcortical structures noted at one week. Figure 23 graphically demonstrates the widespread axonal degeneration resulting from the focal right sensorimotor piston impact.


C499
8w
+ 2.0 mm
22A
C499
8w
-5.0 mm
22D
C499
8w
0.0 mm
22B
C499
8w
-8.0 mm
22E
C499
8w
-2.0 mm
22C
C499
8w
-12.0 mm
22F



23- Side of injury, 2.0 mm lateral to midline. Sagittal section demonstrating degenerating axons 8 weeks after injury. Cortical degeneration is most dense at region of impact (SMC). Cortical association fibers are also stained. Corticofugal fibers descend through caudate and brain stem to spinal cord. Degenerating fibers extend to fimbria, thalamus, substantia nigra, collicular region, reticular formation, and cerebellum; amino-cupric-silver technique (Neuroscience Associates, Knoxville, TN.).

Abbreviations

Thalamic Nuclei :

AV = anteroventral
VL = ventrolateral
VM = ventromedial
VB = ventrobasal
PO = posterior

Cerebellar Nuclei :

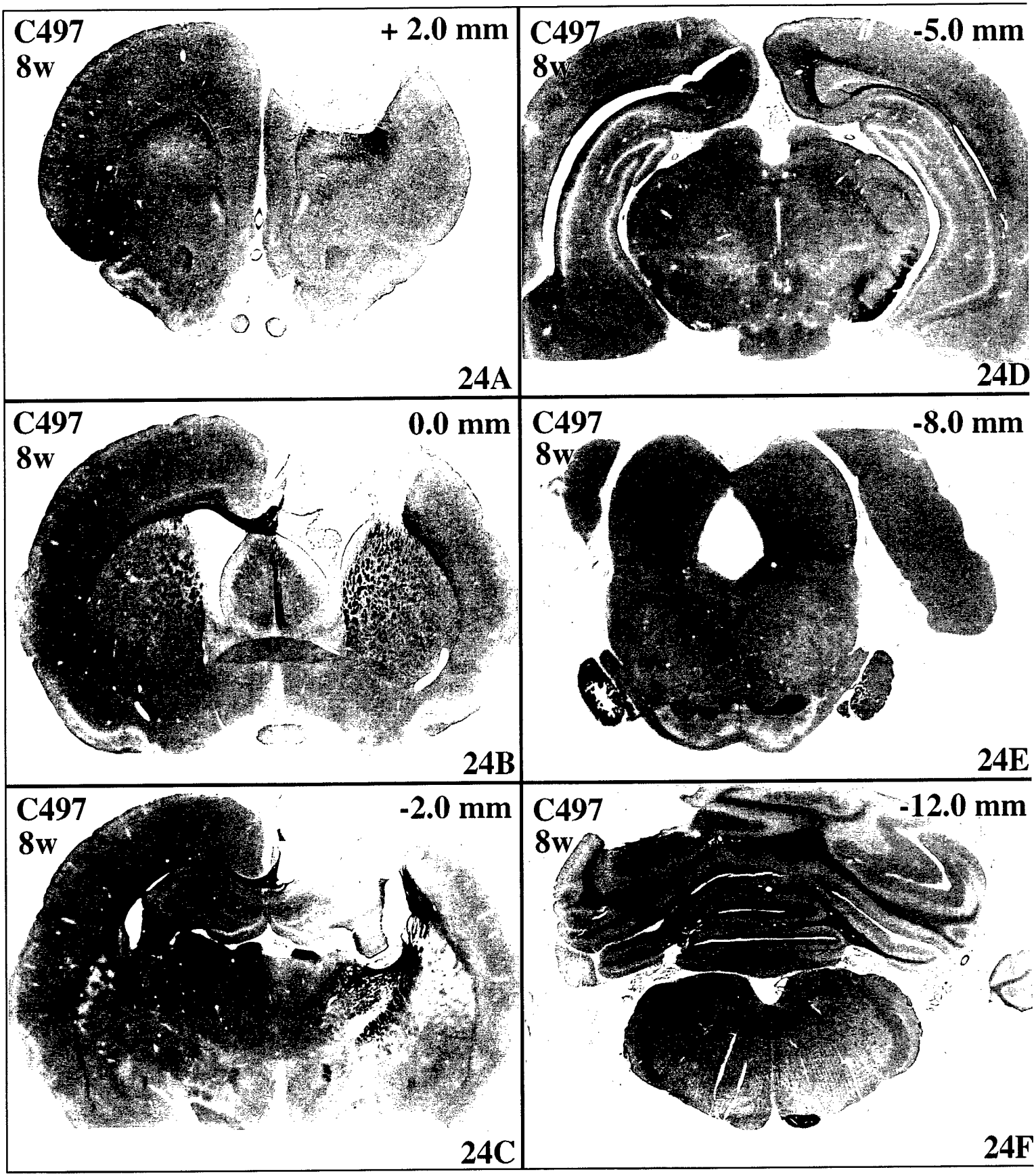
Fa = fastigial
Int = interpositus

Other Structures :

SMC = sensorimotor cortex
Fim = fimbria
Sub = subiculum
RPO = rostral periolivary N.
scp = superior cerebellar peduncle
ml = medial lemniscus
cc = corpus callosum
ic = internal capsule
SNc = substantia nigra compacta
SNr = substantia nigra reticulata

We could not ascertain whether all the darkened axons within the hemispheres evident at one week were fatally damaged so they could not be stained at 8 weeks or whether they recovered and were therefore not susceptible to silver impregnation at 8 weeks. The occurrence of bihemispheral free fatty acid and diacylglycerol changes indicative of cell membrane breakdown or remodeling up to 5 weeks after injury as well as posttraumatic cortical mantle loss and ventriculomegaly, which was often bilateral, Figure 4 suggest that bihemispheral tissue loss occurred following the right focal injury. So, perhaps, many of the darkened axons seen one week after injury did die off.

The extensive, bilateral axonal degeneration we observed in the cerebellum was unexpected and was seen in every injured animal to varying degrees. It appeared two days after cortical injury before any degenerating corticofugal fibers were seen in the corticospinal track within the mesencephalon or medulla and without fiber track degeneration being evident in the brachium conjunctivum as would occur if thalamocerebellar tracks were undergoing degeneration. Thus, the intracerebellar fiber track degeneration did not appear to be caused by retrograde transsynaptic extension from the thalamus or by anterograde transsynaptic mechanisms from pontine nuclei. Furthermore, in rats whose cortical sensorimotor areas were aspirated instead of impacted by the piston, the pattern of axonal degeneration in the cerebral hemispheres, striatum, and brain stem was similar to that observed after trauma but degenerating axons were virtually nonexistent in the cerebellum, Figure 24 A to F. Therefore, we conclude that the cerebellar axonal degeneration occurring in this trauma model is an effect of the cortical impact per se and not the result of transsynaptic degeneration. The piston striking the cerebral cortex might displace the brain caudally and force the inferior aspect of the cerebellum (where degeneration is prominent) against the occipital bone. This could have provoked axonal damage within the cerebellum either by direct axon shearing or by axolemmal injury and axoplasmic flow disruption within cerebellar axons. Despite extensive axon degeneration we were not able to detect cerebellar cellular damage. Specifically, Purkinje cell loss was not evident.

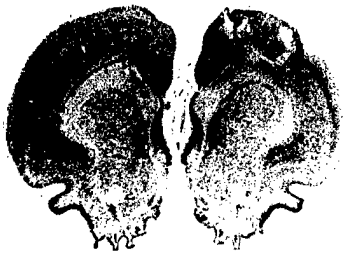

C497
8w
+ 2.0 mm
24A
C497
8w
-5.0 mm
24D
C497
8w
0.0 mm
24B
C497
8w
-8.0 mm
24E
C497
8w
-2.0 mm
24C
C497
8w
-12.0 mm
24F

Profound fiber track degeneration invariably occurred under the area of cortical injury despite remaining cortex, Figures 4 and 25. Figure 26, obtained by superimposing images from the deOlmos amino cupric silver axon degeneration stains onto the thioneine cellular stains of Figure 25, clearly reveals that though large numbers of cortical cells remain apparently intact, they are surrounded by degenerating afferent and efferent axons which project to and from these apparently intact cortical cells.

Control C536 +2.0

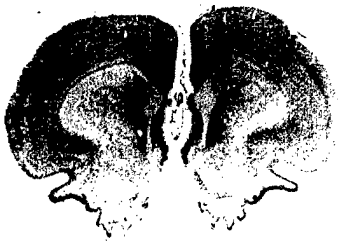
Control C536 +2.0

1 mm Inj C499 +2.0 8 Wks



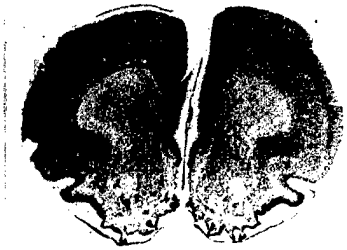
1 mm Inj C499 +2.0 8 Wks

1 mm Inj C500 +2.0 8 Wks

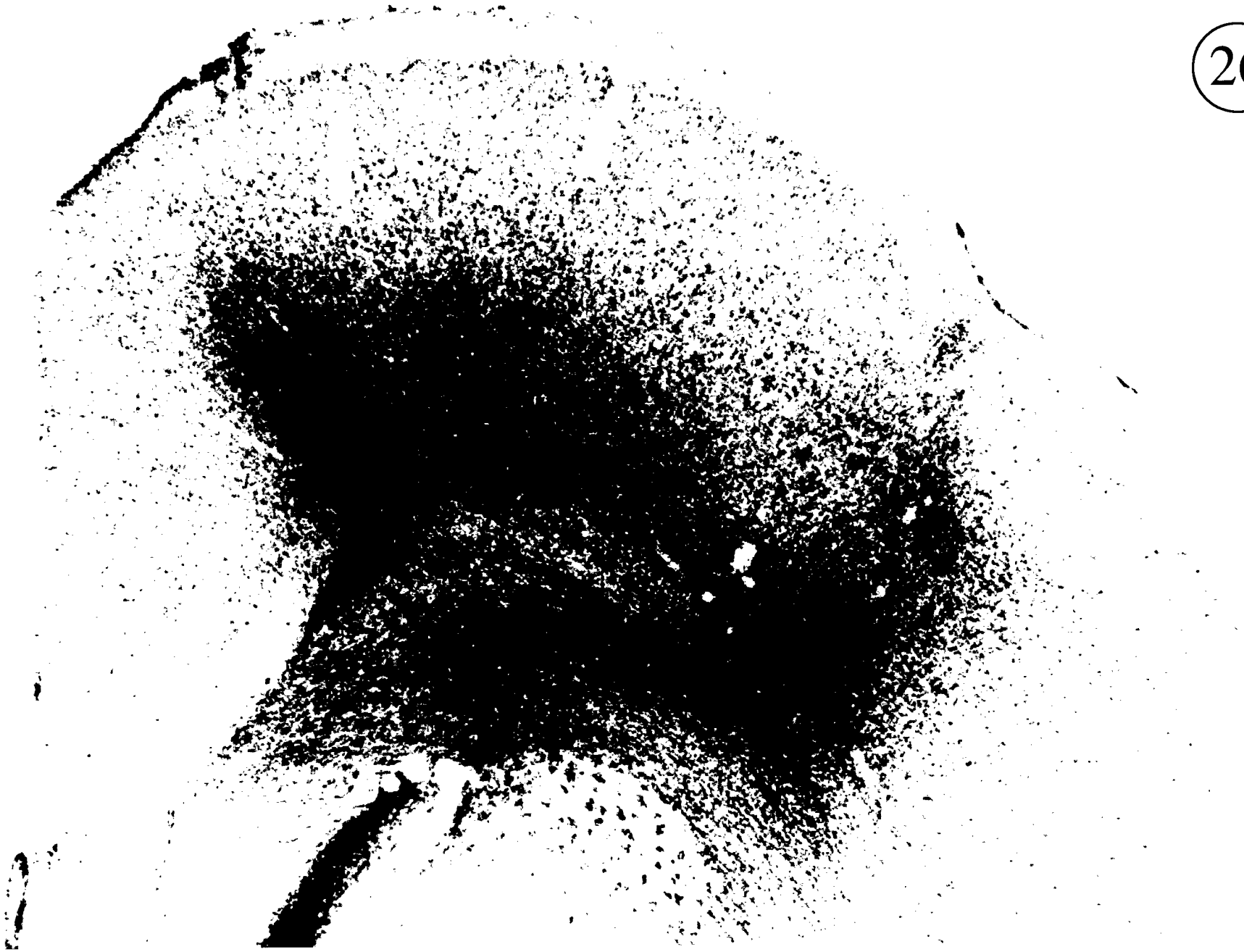


1 mm Inj C500 +2.0 8 Wks

1 mm Inj C502 +2.0 8 Wks



1 mm Inj C502 +2.0 8 Wks



Furthermore, axon damage under the piston face appears far greater than cell body damage in this area. Because of the severe dendrite degeneration, Figures 16,17,18, and the large amount of axon degeneration directly under and around surviving cortical neurons, perhaps, in effect, these cortical neurons have been disconnected from the rest of the brain and are functionless. Axon shearing in the cerebral mantle would explain the constant occurrence of a robust degeneration within sensorimotor fiber track systems, whether or not any cortex remained, Figure 4 and 25, and why fiber track degeneration after cortical impact injury wherein mantle is retained looks surprisingly like the fiber track degeneration seen after aspiration of the entire sensorimotor cortex; compare figures 22 and 24.

A considerable body of research exists concerning axonal damage following severe closed head injury but we are unaware of reports emphasizing the importance of axonal damage following focal brain injury. Whereas earlier considerations of diffuse axonal injury following closed head injury hypothesized axonal shearing at the time of injury, more recent evidence has shown that focal axonal separation does not occur at the time of injury but several hours later. With injury, axolemmal and neurofilament changes occur which obstruct axoplasmic flow. Axoplasmic flow builds up at the point of axolemmal or neurofilament damage and this build up leads to axonal rupture in the region of flow obstruction several hours after injury [23,24,25,26,27,28,29,30,31,32].

With this focal injury model we must address the same question. Does axonal damage under the site of piston impact occur immediately upon piston impact or does it occur after several hours' delay and by the same mechanisms proposed for diffuse axonal injury seen with closed head injury? Possibly two types of axonal injury may occur in our model: 1) immediate shearing of axons under the edges of the piston where the v-shaped wedge of severe brain damage is usually evident and 2) delayed axonal damage under the piston face where the cortical cells often appear intact. The preferential susceptibility of axons in closed, diffuse head injury has been well noted [14,23,33] and our model suggests that even with focal injury, axons appear more susceptible to damage than cell bodies. Thus, many of the mechanisms of injury and their treatment inherent in diffuse and focal brain injury merge.

Prior laboratory investigations have sometimes tested neuroprotective drugs with beneficial effects being measured by quantifying decreased cortical injury [10,11]. This

would appear to have little merit if a major problem with focal injury relates to underlying axonal damage. Clinically, several drug trials involving head injury have been undertaken but, unfortunately, all have failed [34]. Perhaps this is because drugs have not been specifically developed to prevent axonal damage from either diffuse or focal brain injury.

Behavioral data following right sensorimotor injury show that the rats in our experimental paradigm exhibit maximal left sided foot placing deficits during the first few days after injury. Flat beam behavior tests show that the rats make about an 85% recovery on their neurobehavioral scores over the subsequent 4 or 5 weeks, Figure 5, p. 13, [35]. Our fiber track degeneration studies, however, strongly suggest that most axons leading from the sensorimotor cortex have been destroyed even if viable sensorimotor neuron cell bodies persist. How substantial neurologic improvement occurs without a functionally connected sensorimotor cortex is mysterious. Neural plasticity must be a significant factor and indeed in adult rats after sensorimotor cortical ablation, dendritic sprouting in neurons of the contralateral sensorimotor cortex has been seen in conjunction with recovery [36,37,38].

This model of "focal" brain injury causes widespread cellular and axonal changes which extend far beyond the immediate area of injury. These findings may have relevance to human head injury if comparable effects also occur in man. For instance, extensive cell loss or cellular disconnections owing to axonal degeneration could add to expected functional impairments associated with a particular focal brain injury or explain unexpected neurological or neuropsychological changes seemingly unrelated to the known area of brain injury. The most salient finding of this study, the occurrence of widespread axonal damage following focal brain injury, has yet to be specifically addressed in the treatment of focal brain injury occurring in humans.

4 NEUROCHEMICAL STUDIES: A. TOTAL TISSUE AND EXTRACELLULAR FLUID AMINO ACIDS AND BIOGENIC AMINES FOLLOWING BRAIN INJURY

Introduction

Our fiber track degeneration studies show that following right sensorimotor cortical injury, widespread axonal degeneration occurs in cortical association and corticofugal axons descending to multiple brain stem nuclei and other regions. Despite this, however, the animals improved behaviorally in the weeks following injury. We sought to determine whether sensorimotor cortical injury results in either short or long lasting changes in either amino acid or biogenic amines in brain nuclei or brain areas related to the sensorimotor system. Amino acids and biogenic amines are believed to be neurotransmitters and knowledge of specific nuclear or brain area tissue levels of these substances after injury and during recovery might shed light on mechanisms involved in neural injury or post injury neurobehavioral recovery.

We hypothesized that with brain injury and recovery total tissue or extracellular fluid (ECF) concentrations of amino acids or biogenic amines would be altered in brain areas related to the sensorimotor system.

Methods

Rats intended for measurements of total tissue amino acids and biogenic amines received the standard brain injury and were then allowed to awaken from anesthesia. At 20 mins, 7, or 35 days after injury they were deeply anesthetized by pentobarbital (50 mg/kg ip) and sacrificed by rapid decapitation. We removed their brains within 1 minute and placed them in a -70^0 C freezer. Afterwards, their brains were thawed to -6^0 C in a freezing cryostat, sectioned, and the following areas sampled bilaterally: 1-injury site (sensorimotor cortex), 2-frontal, 3-parietal, and 4-cingulate cortices, 5-caudate nucleus, 6-thalamus, 7-red nucleus, 8-substantia nigra, 9-locus coerules, 10-hippocampus, 11-cerebellar folia and 12-deep cerebellar nuclei. Brain samples were prepared for the simultaneous HPLC processing of 12 amino acids plus GABA and the biogenic amines

epinephrine and norepinephrine. Of these only aspartate, glutamate, glutamine, serine, glycine, alanine, taurine and GABA occurred in high enough amounts to be reliably quantified.

Rats intended for in vivo microdialysis measurements of cortical amino acids in brain extracellular fluid had a right-sided craniectomy and a 3mm hole on the left side. Prior to injury a microdialysis probe with a 2mm membrane was inserted bilaterally into each cerebral cortex at the bregma level. Both microdialysis probes were then perfused with dialysate for 1 hour. Substrate equilibrium was established for the last 20 minutes. Following this the probes were removed and the rat injured. After injury the probes were immediately reinserted and perfusion continued for another 130 minutes. Dialysate issuing from the probe system was collected in 10 minute aliquots and analyzed by HPLC. One hundred and ninety minutes after injury each rat was deeply anesthetized with pentobarbital (50 mg/kg i.p.) and then perfused through the heart with 10% formalin.

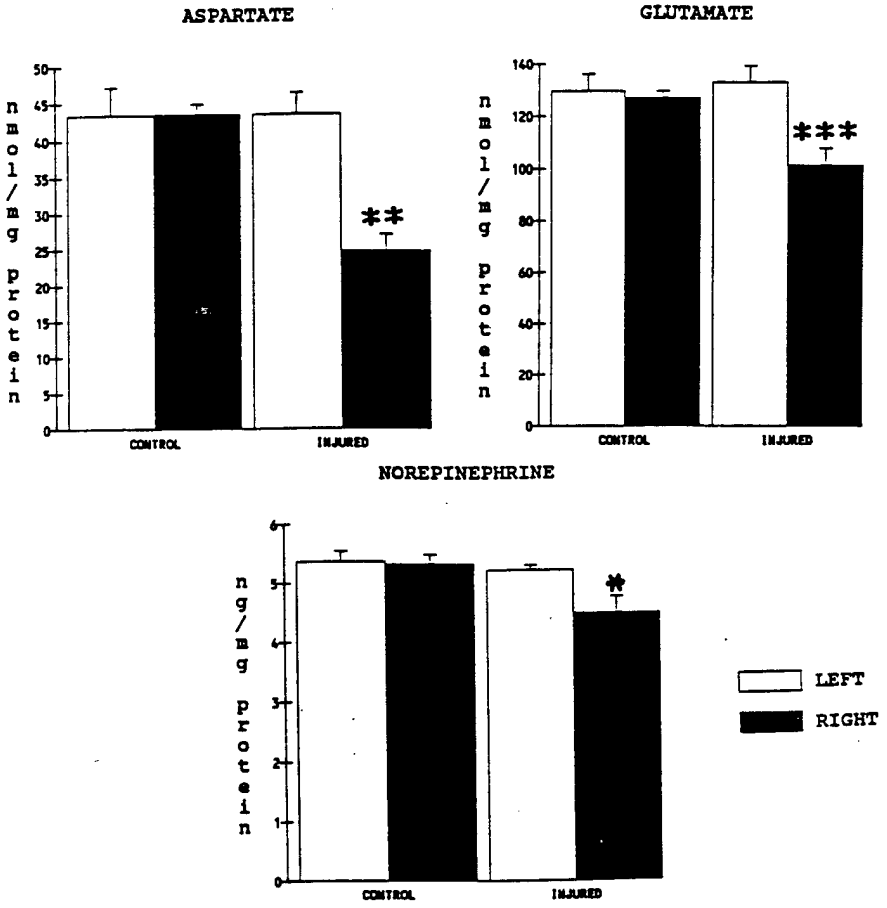
Data were analyzed by ANOVA using the experimental condition (injured vs non-injured rats) and side (left vs right) as main effects.

Results

1. Total Tissue Levels of Amino Acids and Biogenic Amines

Of the 12 areas measured bilaterally for total tissue amino acids, GABA, or biogenic amines, only the injury site plus the adjacent frontal and parietal cortices showed any significant changes. Total cortical aspartate, glutamate, and norepinephrine were decreased 20 minutes after injury, Figures 27,28,29, but were normal at 7 and 35 days, Figures 30 and 31.

Figure 27: Levels of Aspartate, Glutamate and Norepinephrine in the Sensorimotor Cortex 20 Minutes After Traumatic Brain Injury



Controls received only a scalp incision. Aspartate, glutamate and norepinephrine were decreased in only the area of impact compared to the same area on the left (uninjured) side, *-p<.05, **-p<.01, ***-p<.001; n-6 per group.

Figure 28: Levels of Aspartate, Glutamate and Norepinephrine in the Frontal Cortex 20 Minutes After Traumatic Brain Injury

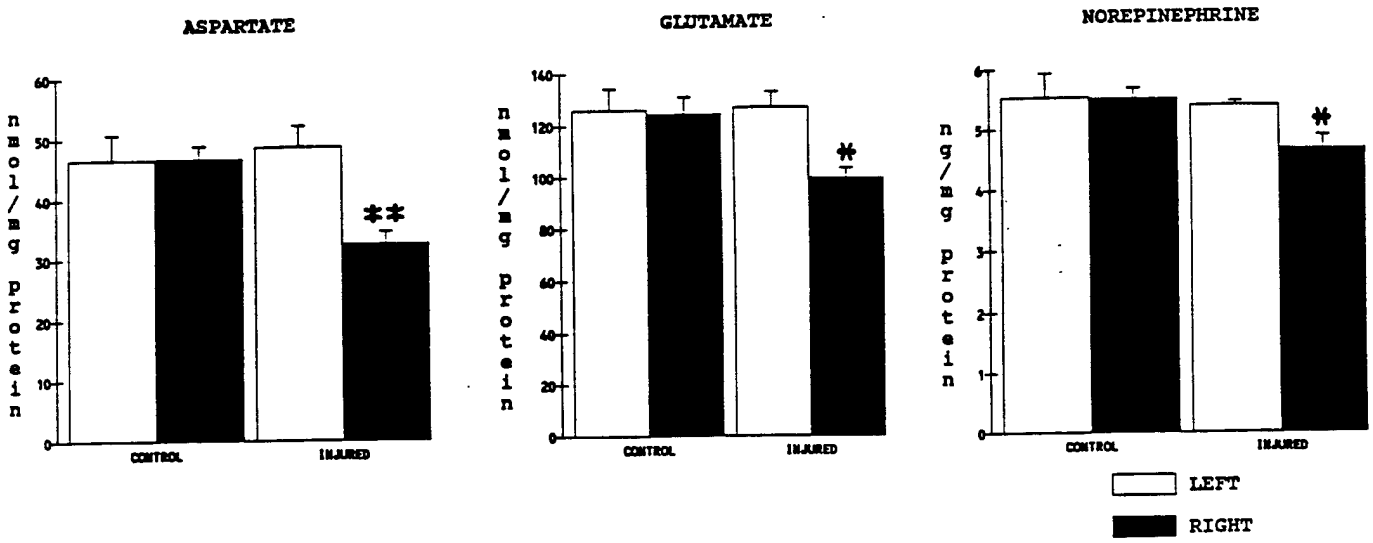
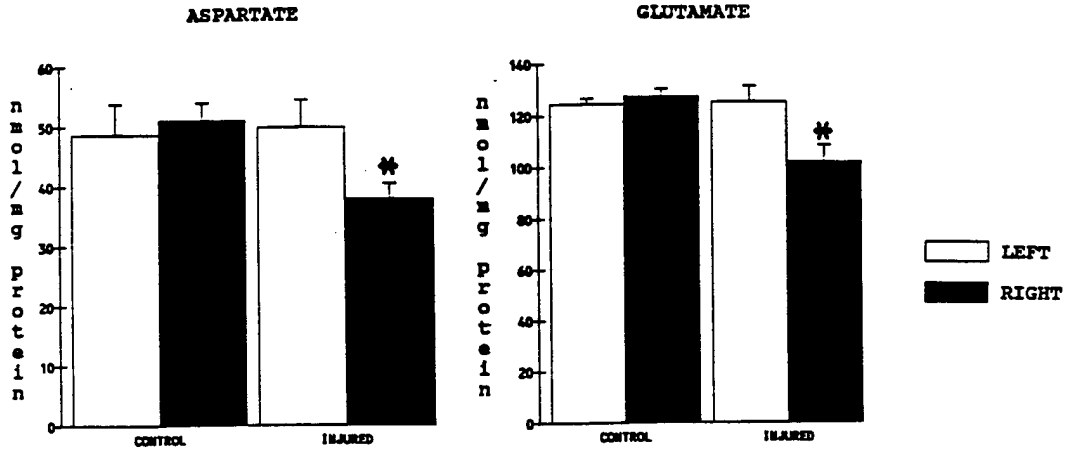


Figure 29: Levels of Aspartate and Glutamate in the Parietal Cortex 20 Minutes After Traumatic Brain Injury



Aspartate and glutamate were decreased in the right parietal cortices but not the left. *-p<.05, **-p<.01; n-6 per group. Norepinephrine was not decreased.

Figure 30: Aspartate, Glutamate and Norepinephrine Concentrations in the Sensorimotor Cortex 7 Days After Injury

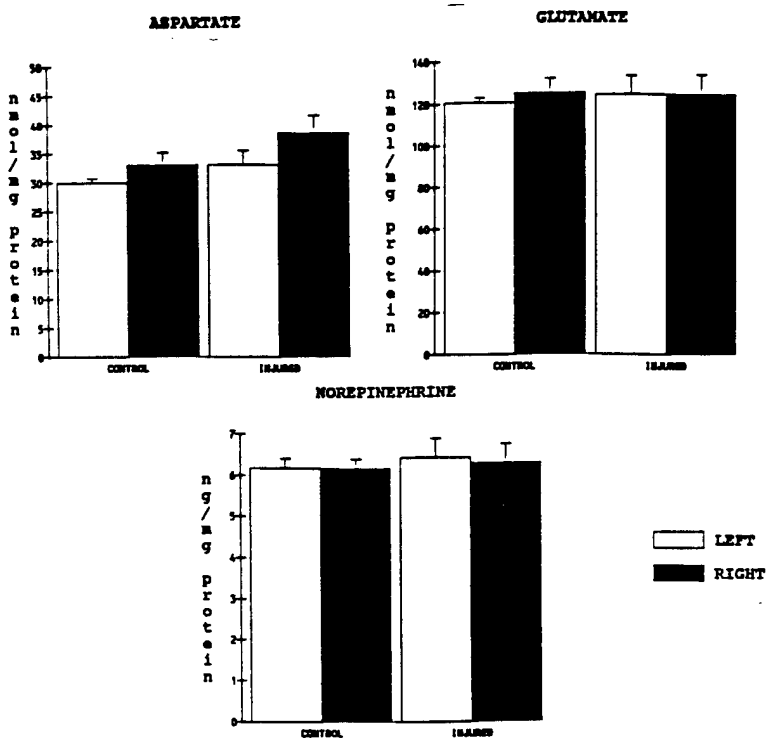
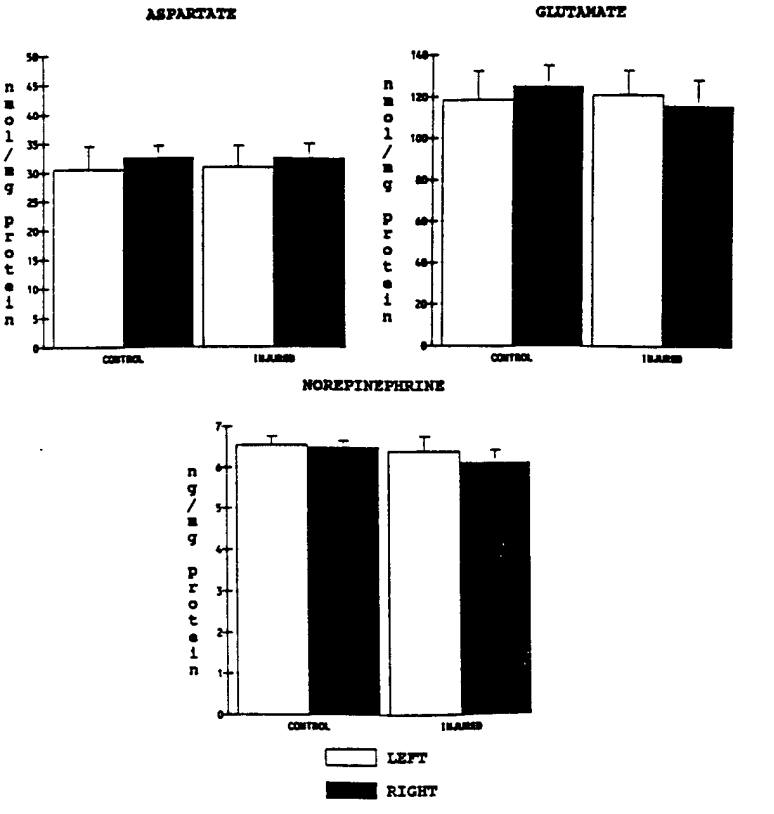


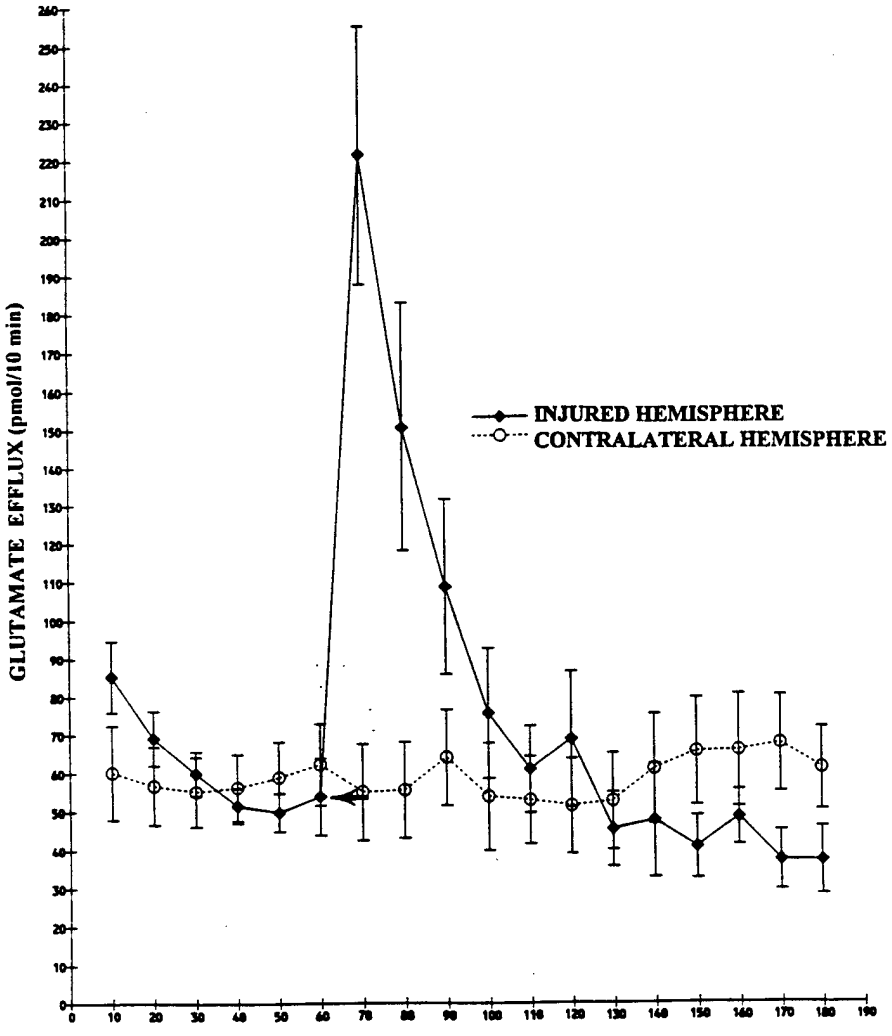
Figure 31: Aspartate, Glutamate and Norepinephrine Concentrations in the Sensorimotor Cortex 35 Days After Injury



2. Extracellular Fluid Measurements of Aspartate and Glutamate by in Vivo Microdialysis

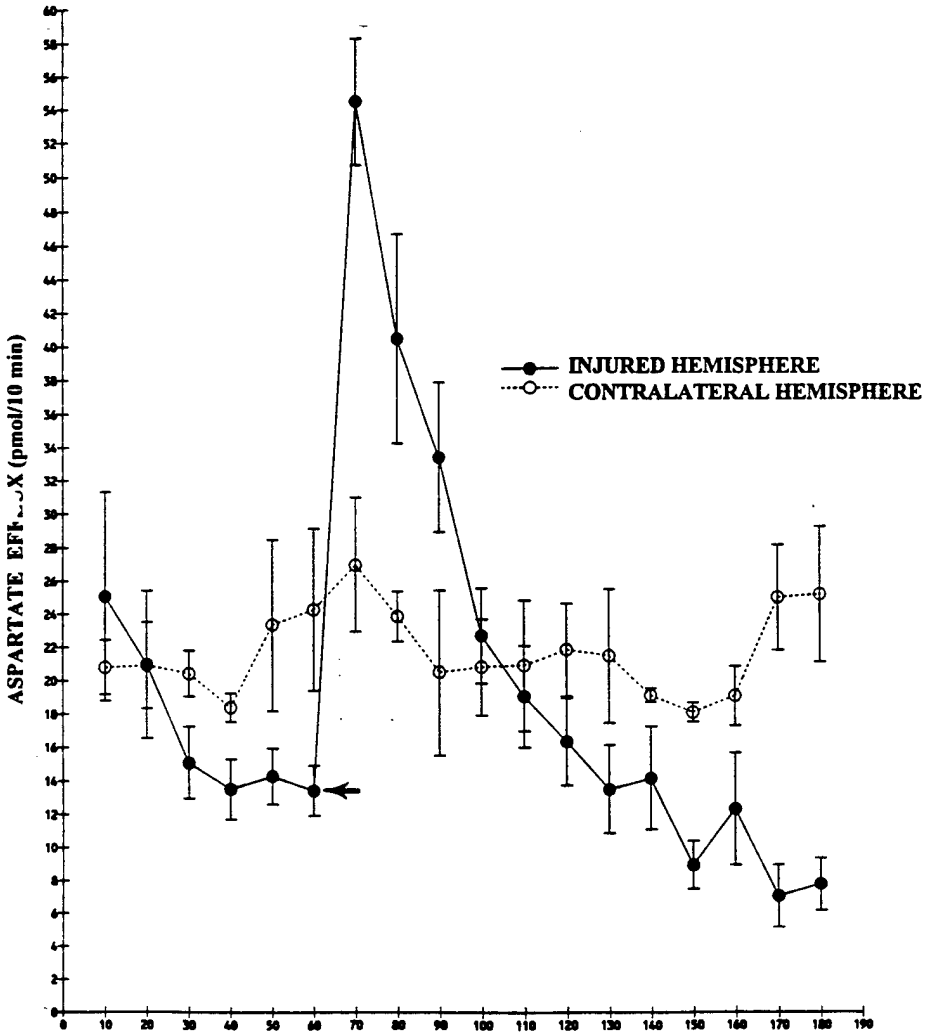
Aspartate and glutamate were markedly increased in the extracellular fluid at the injury site immediately after injury but returned to baseline levels ~40 mins later. ECF of the contralateral cortex did not show any posttraumatic increases in these excitatory amino acids, Figures 32, 33.

Figure 32: Extracellular Glutamate Following Injury



VALUES ARE MEAN ± SEM
ARROW DENOTES INJURY TIME (60 MIN).

Figure 33: Extracellular Aspartate Following Injury.



VALUES ARE MEAN ± SEM
ARROW DENOTES INJURY TIME (60 MIN).

The rapid return of normal concentrations of amino acids and biogenic amines at the injury site indicates that cells in this cortical region maintained or recovered important aspects of their metabolism if not function.

Discussion

The excitatory amino acids aspartate and glutamate are thought to play a pivotal role in cell injury and death after brain trauma by opening cell membrane calcium channels. The subsequent in rush of extracellular Ca^{2+} into the cell leads to impairment of intracellular second messengers systems, cell membrane injury, or cellular death [39,40]. Our microdialysis experiments indicate very high levels of both aspartate and glutamate in cortical extracellular fluid in the damaged area 20 minutes after injury, Figures 30,31. Total tissue concentrations of these amino acids plus epinephrine were decreased, however, Figures 27,28,29, suggesting that with injury, intracellular stores of these neurotransmitters are dissipated into the extracellular space and are then cleared from the area of injury over the next hour or so, probably by bulk ECF flow [41,42].

These short lived amino acid and biogenic amine changes occurred only in and around the area of injury and not in subcortical or other areas related to the sensorimotor system (e.g. contralateral homotypic sensorimotor cortex, basal ganglia, or cerebellum). Despite this, our histologic data show widespread, bilateral cortical chromatolysis lasting weeks after injury. Because aspartate and glutamate elevations occur only acutely around the injury area these excitatory amino acids are not likely to be directly responsible for these histologic changes which occur widely throughout the brain over a protracted period of time after injury.

Currently a hypothesis exists that sensorimotor deficits (diaschisis) are the result of depressed cerebral norepinephrine levels. Though we do not know exactly when norepinephrine levels became normal in our injury model, cortical norepinephrine concentrations had returned to normal at least by 7 days after injury, 2 weeks before sensorimotor deficits had maximally improved. Because return of cortical norepinephrine to normal levels did not coincide with maximal functional improvement it would appear that other factors must underlie the major portion of neurobehavioral recovery following brain injury.

The massive increase in aspartate and glutamate in the region of injury does not kill all cells in the area because our histologic studies show that considerable mantle often remains under the impact site, Figure 4, and that cell bodies from this mantle appear

histologically quite normal, Figure 14B. Furthermore, days to weeks after injury these cells exhibit normal levels of cortical amino acids and biogenic amines, Figures 32,33. Thus, some neurotransmittters, at least, continue to be made by normal appearing cells which lie directly under the piston impact. Despite the histologic and functional preservation of cell bodies under the piston impact site, these cells exhibit severe dendritic and axonal degeneration, Figures 15-18,26. Perhaps the massive extracellular glutamate/aspartate release consequent to injury plays a part in dendrite and axon destruction if not in cell body dissolution.

We have also documented posttraumatic gene and gene product changes in both the injured and uninjured hemispheres (see Section 9). Because no increases in either aspartate or glutamate were seen in the uninjured hemisphere after injury Figures 27,28 and 29 where robust posttraumatic gene changes occurred, it appears that such genetic changes occur by mechanisms not linked to excitatory amino acids. While it may be argued that gene changes occurring in the area of injury are epiphenomena associated with dead or dying brain, this cannot be said for the contralateral cortex which shows robost posttraumatic gene changes but appears histologically quite normal and has normal concentrations of cerebral cortical amino acids and biogenic amines. What triggers the posttraumatic gene changes particularly in the normal cortex remains unknown.

NEUROCHEMICAL STUDIES:

B. FREE FATTY ACIDS, DIACYLGLYCEROLS AND PHOSPHOLIPASE A_2 (PLA_2)

Introduction

A fatty acid molecule has two distinct regions: 1) a long hydrophobic, chemically unreactive carbon chain and a hydrophilic carboxylioc acid group which readily forms esters and amides. A fatty acid may be covalently linked to another molecule or may be free standing. If enzymes which can cleave fatty acids are activated (as by a brain insult) the normally covalently bonded fatty acids are released and become free. Palmitic (16:0), stearic (18:0), oleic (18:1), arachidonic (20:4), and docosahexaenoic (22:6) are the predominant free fatty acids found in brain tissue. Diacylglycerol (DAG) is a glycerol molecule with fatty acids covalently bonded in positions one and two. DAG levels are known to be increased by the action of phospholipase C (PLC) on membrane phospholipids.

Brain injury triggers a cascade of early cellular responses which then generates injury mediators that, in turn, activate secondary mechanisms of cellular and neuronal damage [43,44,45]. Severe perturbations in cellular metabolism, structure, and function lead to long-term neurologic deficits [44,45,46,47]. In our model, piston impact onto the brain shears axons and microvessels in the region of the right sensorimotor cortex, especially in brain under the edge of the piston. Plasma constituents including calcium leak into the damaged brain parenchyma [48,49]. In our model posttraumatic histopathological changes include neuronal alterations particularly affecting axons and dendrites, neuron cell loss, and astrogliosis. Neuronal and axonal alterations occur not only in the region of piston impact but also in brain areas not adjacent to the impact: contralateral hemisphere, brain stem, and cerebellum. Our histological studies have shown that both neuronal and axonal changes occurring after injury evolve throughout the brain over weeks (Section 3).

Prior research has implicated the rapid and massive accumulation of extracellular potassium and neurotransmitters (e.g. glutamate) after trauma in the initiation of

secondary cellular injury cascades [50,51,52,53,54]. Extracellular glutamate accumulation leads to neuronal calcium overload through postsynaptic N-methyl-D-aspartate (NMDA) receptor-gated calcium channels [55]. Mechanosensitive channels in the cell wall which are opened by shear forces from the mechanical insult may also contribute to calcium influx into neurons [48].

Intracellular neuronal calcium overload activates calcium-regulated enzymes such as phospholipases, proteases, and endonucleases. Activation of phospholipase A_2 and C (PLA_2 , PLC) signaling pathways leads to the release of free fatty acids (FFAs) and diacylglycerols (DAGs) early after brain injury [45,56,57]. PLA_2 activation also causes the accumulation of free arachidonic acid which, by itself, or via its further metabolism to eicosanoids along with the release of free radicals may affect membrane permeability or other cellular mechanisms which may impair neural function or lead to cell death [44,58,59,60]. Diacylglycerols, potent activators of protein kinase C (PKC), may also modulate other signaling pathways affecting the cellular responses to stimulation [61].

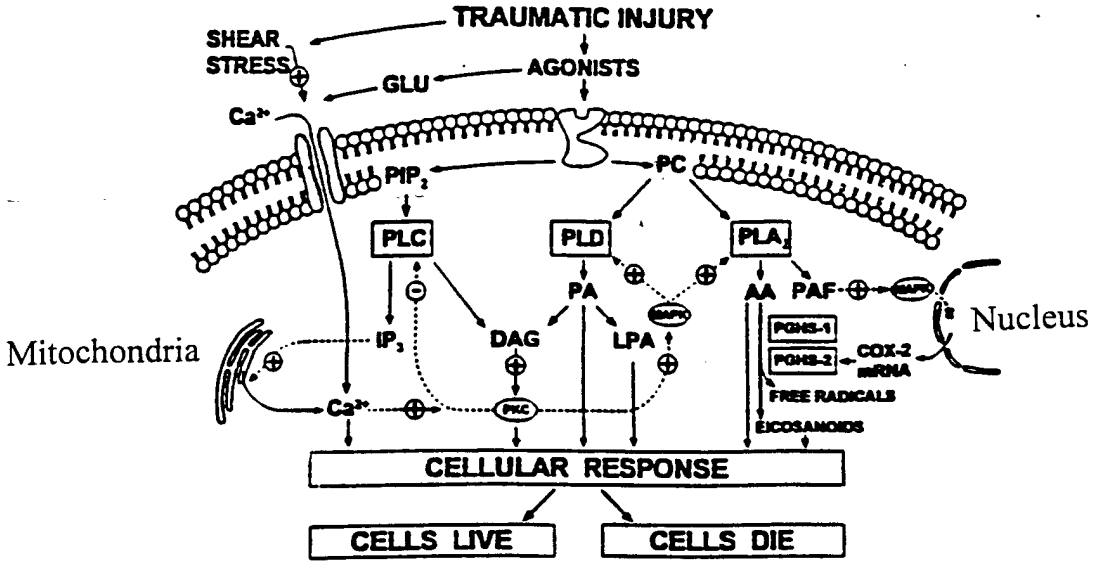


Figure 34: Overall scheme of important intracellular processes leading to free radical formation and cell recovery or cell death. COX= cyclooxygenase, PKC= protein kinase C, MAPK= mitogen activated protein kinase, GLU= glutamate, PL= phospholipase (C,D,A_2), PIP_2= phosphoinositol diphosphate, PC= phosphatydil choline, PA= phosphatylethanolamine, LPA= lysophosphatidic acid, AA= arachidonic acid, PAF= platelet activating factor, mRNA= messenger ribonucleic acid, PGHS prostaglandin synthase: 1 (constitutive) and 2 (inducible), IP_3= inositol triphosphate.

Prior investigators of brain injury have observed increases in FFAs, DAGs, and PLA_2 in the injured area or ipsilateral hippocampus within the first 24 hours after injury [62,63,57] and we have demonstrated free radical formation within minutes of impact injury to the cortex of anesthetized rats [64]. Our present studies on FFAs, DAGs and PLA_2 corroborate the findings of these earlier investigators for our early time points but we are unaware of experiments which have examined FFAs, DAGs and PLA_2, components of the "second messenger system" throughout the brain days and weeks after injury. We felt it important to measure FFAs, DAGs, and PLA_2 both early after injury, when perterbations would most likely be the result of destructive processes, and at later times when neural reconstructive and remodeling events would be occurring. Since we expected that major destructive processes would occur near the time of injury we assayed the damaged brain 30 mins, 24 hours, and 4 days after injury. Since behavioral recovery was complete at 35 days we also chose to measure FFAs, DAGs and PLA_2 then as well.

The central hypothesis we tested was that traumatic brain injury would be associated with both early and late changes in second messenger systems.

Method

All rats were anesthetized and received our standard brain injury. Rats for FFAs and DAGs studies were sacrificed 30 mins, 24 hours, 4 and 35 days after injury by head-focused microwave irradiation which immediately stops all enzymatic processes. Brains were immediately removed and eight different regions dissected out on a frozen plate: frontal right cortex (FRC) (area of injury), frontal left cortex (FLC), occipital right cortex (ORC), occipital left cortex (OLC), right hippocampus (RH), left hippocampus (LH), cerebellum (CER) and brain stem (BS). Lipid extracts were prepared from each brain area and total phosphorus measured [65]. Different lipid classes were separated by thin-layer chromatography using silica gel GHL plates (Analtech, Newark, DE) and developed in hexane:ether:acetic acid (40/60/1.3, by vol). FFAs and DAGs bands were scraped from plates, derivatized to their fatty acid methyl esters and analyzed by gas-liquid chromatography [65].

Rats for PLA_2 studies were similarly prepared and injured but were sacrificed by decapitation after light ether anesthesia at 30 mins, 24h, 4, 17, and 35 days after injury. Brains were immediately removed and the right and left cerebral cortices were dissected on a frozen plate. Cytosolic and microsomal fractions were prepared by differential centrifugation and PLA_2 measured by a procedure based on the release of radiolabeled arachidonic acid from labeled phosphatidylethanolamine [66].

Results

1. Free Fatty Acids

Tables 1 and 2 show the accumulation of total FFAs in eight different brain areas following cortical injury.

Table 1. Total Free Fatty Acid Content in Different Rat Brain Areas 30 min and One Day After Cortical Injury

	30 MIN		24 HOURS	
AREA	**SHAM**	**TRAUMA**	**SHAM**	**TRAUMA**
FRC	42.8 ± 5.1	71.4 ± 9.0*	35. 3 ± 2.6	134.0 ± 38.0*
FLC	36.1 ± 2.1	38.5 ± 2.3	2 8.9 ± 3. 3	30.2 ± 2.5
ORC	45.3 ± 3.7	62.1 ± 9.1	48.4 ± 5.4	63.3 ± 7.8
OLC	36.2 ± 2.3	36.3 ± 2.7	36.0 ± 3.1	38.9 ± 2.9
RH	46.8 ± 4.2	45.9 ± 4.3	61.7 ± 5.6	65.3 ± 5.8
LH	39.5 ± 2.4	37.7 ± 3.1	63.7 ± 6.7	54.7 ± 6.1
CER	33.4 ±1.4	33.5 ± 1.4	37.7± 3.6	34.6 ± 3.5
BS	38.5 ±0.9	36.0± 1.7	37.2±3.0	41.9 ± 4.5

Values are expressed as the mean ± SEM nmole of total FFA per mg lipid phosphorus from 9 to 10 individual determinations. FFA were quantified by GLC. Abbreviations are as follows: FRC, frontal right cortex (injured area); FLC, frontal left cortex; ORC, occipital right cortex; OLC, occipital left cortex; RH, right hippocampus; LH, left hippocampus; CER, cerebellum; BS, brain stem. Asterisk (*) denotes statistical significance with respect to sham ($p<0.05$, Student's t test).

Table 2. Total Free Fatty Acid Content in Different Rat Brain Areas After Cortical Injury.

	4 DAYS		35 DAYS	
AREA	**SHAM**	**TRAUMA**	**SHAM**	**TRAUMA**
FRC	26.4 ± 1.4	70.4 ± 12.2**	32.± 2.1	45.4 ± 3.6*
FLC	32.7 ± 2.1	50.9 ± 5.9*	31.6 ± 1.7	40.5 ± 2.3*
ORC	44.0 ± 4.1	91.8 ± 23.0	39.0 ± 3.1	46.1 ± 5.8
OLC	30.6 ± 2.2	41.8 ± 4.9	41.7 ± 2.3	41.2 ± 4.6
RH	40.4 ± 2.0	41.0 ± 3.6	51.9 ± 2.8	46.0 ± 5.2
LH	41.4 ± 8.5	44.6 ± 3.4	45.4 ± 5.7	56.9 ± 7.7
CER	33.4±4.3	36.7 ± 3.0	42.8 ± 4.6	50.0 ± 4.2
B S	40.3±3.9	37.8 ± 3.3	44.4 ± 3.7	41.7 ±3.6

Data are presented as mean ± SEM of 5-10 rats. FFAs were quantified by GLC. FRC, frontal right cortex (injured area); FLC, frontal left cortex; ORC, occipital right cortex; OLC, occipital left cortex; RH, right hippocampus; LH, left hippocampus; CER, cerebellum; BS, brain stem. Asterisk denotes values different from sham value (Student's t test, * $p<0.05$; ** $p<0.01$).

During the first 24 hours activation of lipolytic pathways was confined solely to the frontal right cortex (FRC) and 30 mins after injury total FFAs there were increased 1.7-fold over sham values. They reached a 3.8-fold increase by 24 hours. While total FFAs were uniformly increased in the injured FRC, individual responses varied. For instance, 24 hours after injury 6 of 10 rats had a moderate FFA increase (63 ± 9 nmole/mg lipid P vs sham value 35 ± 3) while 4 reached very high levels (240 ± 66). Such variability naturally produced large SEMs and affected statstical significance.

FFAs in the area of injury (FRC) remained elevated for the entire 5 week post injury recovery period but were much higher at earlier time points than at 35 days (3.8x vs 1.5x). Post injury FFA increases did not occur as widely throughout the brain as did DAG elevations.

Data on individual FFA elevations are in Appendix 3, pp 178-183.

2. Diacylglycerol

Tables 3 and 4 show the accumulation of total DAGs after cortical injury.

Table 3. Total DAG Content in Different Rat Brain Areas 30 Min and 24 Hours After Cortical Injury

	30 MIN		24 HOURS	
AREA	**SHAM**	**TRAUMA**	**SHAM**	**TRAUMA**
FRC	51.5 ± 3.9	91.4 ± 6.3 *	70.1 ± 6.4	111.0 ± 12.7*
FLC	69.8 ± 8.0	69.7 ± 6.9	52.8 ± 8.1	70.1 ± 9.3
ORC	57.2 ± 4.4	58.3 + 2.5	79.9 ± 8.3	93.1 ± 14.2
OLC	52.7 ± 6.8	46.5 ± 2.5	64.0 ± 6.5	57.9 ± 4.5
RH	48.0 ± 7.6	51.1 ± 4.2	95.0 ± 13.0	84.2 ± 8.0
LH	69.2 ± 4.8	51.6 ± 1.6	93.8 ± 17.5	88.3 ± 10.0
CER	40.6 ± 2.7	40.8 ± 4.4	42.9 ± 2.9	73.7 ± 10.4*
BS	24.9 ± 2.0	28.6 ± 2.7	38.8 ± 4.5	39.7 ± 5.7

Values are expressed as the mean ± SEM nmole per mg of lipid phosphorus from 9 to 10 individual determinations. DAG were quantified by GLC. Values significantly different from sham are indicated (Student's t test * $p<0.05$).

Table 4. Total Diacylglycerol Levels in Different Brain Areas After Cortical Injury.

	4 DAYS		35 DAYS	
AREA	**SHAM**	**TRAUMA**	**SHAM**	**TRAUMA****
FRC	37.9 ± 3.7	72.6 ± 7.7*	49.4 ± 3.7	118.8 ± 11.5**
FLC	51.7 ± 6.9	68.2 ± 5.2	46.2 ± 5.0	110.3 ± 8.3***
ORC	45.7 ± 6.0	54.0 ± 5.1	50.9 ± 7.5	114.1 ± 11.7*
OLC	56.5 ± 4.0	44.5 ± 5.3	49.8 ± 5.9	147.7 ± 18.0*
RH	51.1 ± 8.7	50.7 ± 5.6	69.1 ± 5.1	74.3 ± 7.1
LH	58.3 ± 4.8	48.0 ± 5.7	75.9 ± 8.9	69.5 ± 18.1
CER	31.6 ± 5.3	32.5 ± 2.8	70.4 ± 4.8	86.1 ±10.3
BS	38.1 ± 3.5	35.2 ± 4.9	46.2 ± 4.7	126.6 ± 15.1*

Data are presented as mean ± SEM of 5-10 rats. DAGs were quantified by GLC. Asterisk denotes value significantly different from sham value (Student's t test, * $p<0.01$; ** $p<0.001$; *** $p<0.0001$).

Total DAGs were increased 1.8 times in the damaged FRC 30 min after injury and remained at about this level (1.6 fold increase) at 24 hrs. A 1.7 fold increase also occurred in the cerebellum 24 hrs after injury (Table 4). We have hypothesized that the cerebellum was injured by being displaced against the inside of the occipital bone at the time of piston impact, Section 3 p 35.

Total DAGs demonstrated a continued elevation in the injured FRC throughout the entire 5 week post injury period but, interestingly, the increase in DAGs in this area was greatest at day 35, (2.4 fold increase). Unlike FFAs, the DAG elevations occurred widely throughout the brain, ~2.5 fold increases being noted at this late time in the left and right frontal lobes, both occipital lobes, and brain stem. Interestingly, the hippocampus of the 24 hour sham animals displayed higher levels of both FFAs and DAGs than the hippocampus of the 30 min sham controls (Tables 1 and 3), and these values were not significantly different from injured animals. This may indicate that surgical trauma has its own effects on the hippocampus. The reason for this is not clear.

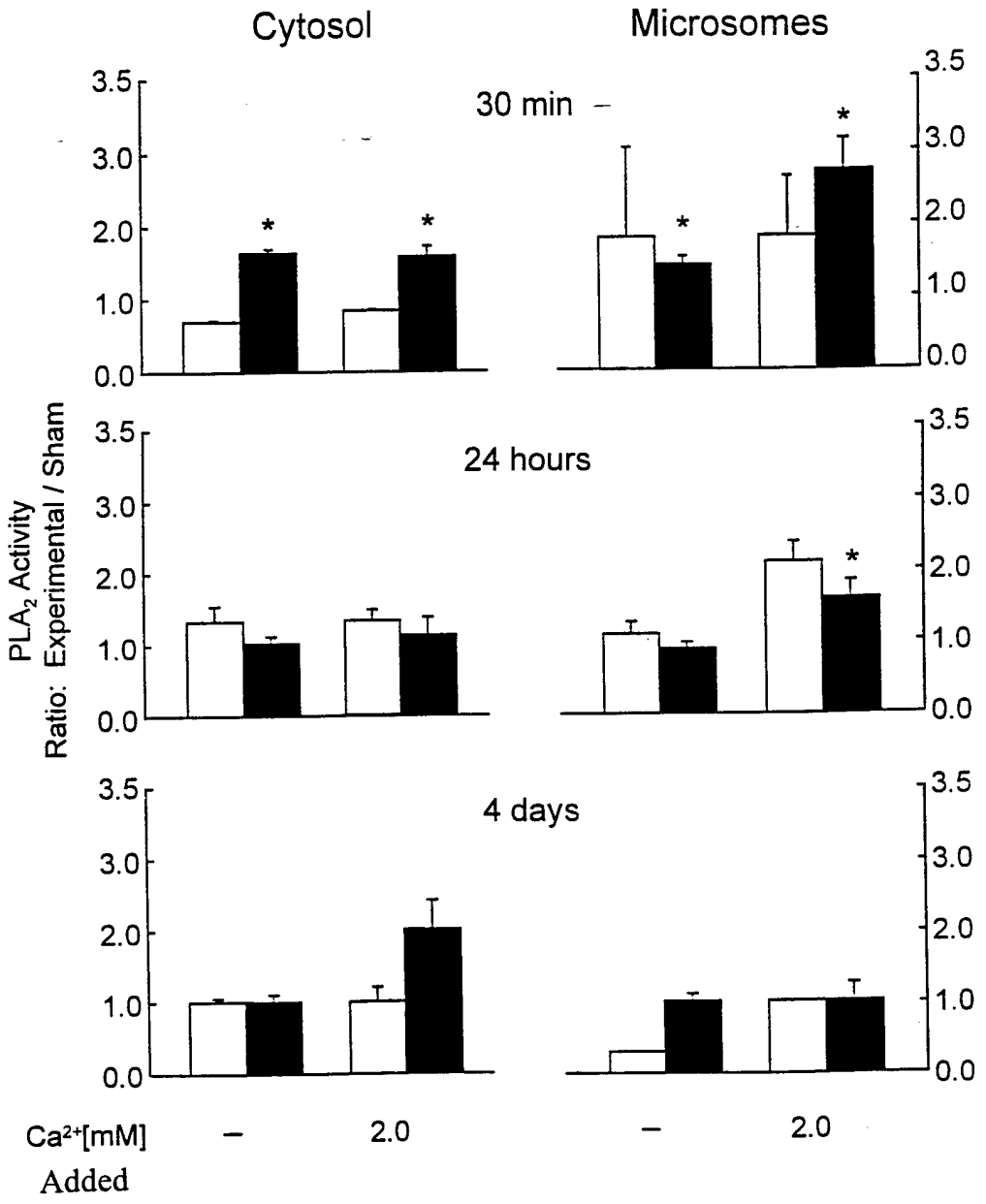
Data on individual DAGs will be found in Appendix 3 pp 178-183.

3. Phospholipase A_2 (PLA_2)

We also measured PLA_2 activity in cerebral cortical cytosolic and microsomal fractions at 30 mins, 24hrs, 4, 17 and 35 days after impact injury. Several forms of PLA_2 are present in neural tissue including the cytosolic calcium dependent ($cPLA_2$) and calcium indepent ($iPLA_2$) forms and a low molecular weight secretory PLA_2 ($sPLA_2$) [67,68,69] which is stored with neurotransmitters in synaptic vesicles and released upon stimulation [70]. This $sPLA_2$ may contribute, along with calcium-sensitive and arachidonoyl-specific $cPLA_2$, to the hydrolysis of arachidonoyl-phospholipids [71].

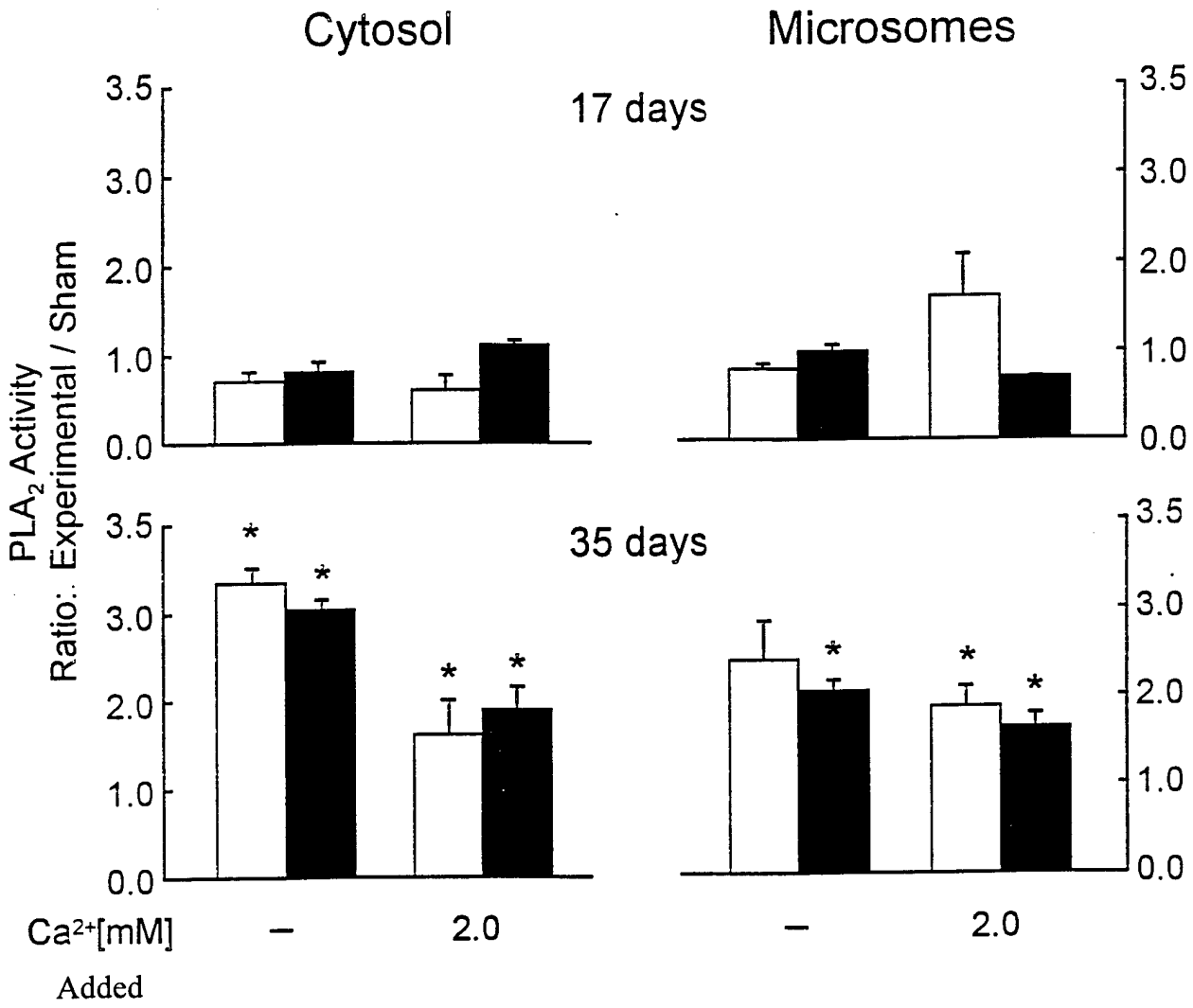
In these experiments we strove to measure calcium dependent and calcium independent forms of PLA_2 and so assays were performed with and without added calcium (2mM) in the incubation media. Data are presented in fold increase as compared to corresponding values in sham-operated rats run simultaneously in each individual experiment.

Figure 35 shows PLA_2 activity at 30 mins, 24 hrs and 4 days after injury in the injured and contralateral, uninjured cortex. Figure 36 shows PLA_2 activity at 17 and 35 days.



Early activation of PLA_2 after cortical impact injury. PLA_2 was measured (with and without added calcium) in cellular fractions of cerebral cortex at 30 min., 24 hours and 4 days after the insult. Values are the ratio of PLA_2 enzymatic activity between trauma and sham operated samples. Ratio is 1.0 when PLA_2 activity in experimental trauma sample was equal to sham value. Filled bars: ipsilateral cortex and empty bars: contralateral cortex. Asterisk (*) denotes values significantly different from sham control (Student's t test, $p<0.05$).

Figure 36



Sustained activation of PLA_2 after cortical impact injury. PLA_2 was measured (with and without added calcium) in cellular fractions of cerebral cortex at 30 min., 24 hours and 4 days after the insult. Values are the ratio of PLA_2 enzymatic activity between trauma and sham operated samples. Ratio is 1.0 when PLA_2 activity in experimental trauma sample was equal to sham value. Filled bars: ipsilateral cortex and empty bars: contralateral cortex. Asterisk (*) denotes values significantly different from sham controls (Student's t test, $p<0.05$).

Thirty minutes after the injury cytosolic PLA_2 activity was significantly increased about 50% in the injured cortex in media with no calcium added and also in media with 2 millimoles of calcium added. By 24 hours after injury PLA_2 was comparable to sham controls. Thirty minutes after injury microsomal PLA_2 from the damaged cortex showed a significantly higher 150% fold PLA_2 increase in the calcium rich media and at 24 hours this activity remained 50% increased. Most PLA_2 activity returned to sham levels 4 and 17 days after injury.

A late activation of PLA_2 occurred 35 days after the injury in both cytosolic and microsomal fractions (Figure 36). This activation was significantly higher (150% above sham values) in cytosolic fractions particularly in the calcium independent $iPLA_2$. This late $iPLA_2$ activation occurred in both the injured and contralateral uninjured cerebral cortex.

We tried to further characterize the PLA_2 activity by using two different PLA_2 inhibitors, MAFP (methyl arachidonyl fluorophosphate) a potent calcium-dependent PLA_2 inhibitor and BEL (E-6-(bromoethylene)-3-(1-naphthalenyl)-2 H-tetrahydropyran-2-one), an inhibitor for the calcium-independent isozyme. Each inhibitor, however, decreased PLA_2 activity about 80% in both cellular fractions and consequently was not helpful in further characterizing the cortical PLA_2 activity. A lack of specificity for PLA_2 inhibitions has been recently reported [72].

Discussion

As has been previously shown [56,57,62,63] traumatic brain injury triggers an acute activation of PLA_2 and phospholipid degradative pathways with the consequent accumulation of FFAs and DAGs not only in the damaged cortex but other brain areas as well. What has not been shown before is that these FFA, DAG, and PLA_2 increases last weeks beyond the traumatic event and occur widely throughout the brain.

Following right sensorimotor impact injury in our model the excitatory amino acids aspartate and glutamate increased only in the damaged cortex and for approproximately 1 hour after injury. The early increases in PLA_2, FFAs and DAGs

about the area of injury (right frontal) would be consistent with the concept of excitatory amino acids opening Ca^{2+} channels (or focal trauma causing a mechanical Ca^{2+} leak into the underlying cells) and activating calcium regulated enzymes including PLA_2, and PLC which leads to the release of FFAs and DAGs. Early time point elevations in PLA_2, FFAs and DAGs would be consistent with destructive cellular processes.

Brain trauma does not cause ischemia at least up to 90 minutes after injury [73] and vasogenic brain edema associated with injury does not become significant for more than 6 hours after the event [74]. Thus, the FFA and DAG changes seen within 30 minutes of injury would most likely represent phenomena associated with the injury itself. Molecular changes at 24 hours would be confounded by the effects of edema and thus will not be discussed in detail because the effects of injury become very difficult to sort out from the added effects of edema and possibly, late ischemia.

Though it is difficult to compare among laboratories because of the variability of control values, Table 5 suggests that the FFA increases following 30 minutes of ischemia are very much greater than 30 minutes after cortical brain trauma. This is particularly true for arachidonic acid (20:4).

Table 5: Increases in FFAs in Ipsilateral Cortex 30 Minutes After Insult (x Control Values)

	Rehncrona [75] (Ischemia)	Yoshida [76] (Ischemia)	Dhillon [62] (Trauma)	Homayoun◊ (Trauma)
16:0	5.6	6	1.7	-
18:0	11.6	18	2.5	1.7*
18:1	12.0	5.6	1.5	1.8*
20:4	>45	106	2.8	4*
22:6	>11	N/A	N/A	2.5*
Total	9.7	55	2.0	1.7

◊= present work
NA= not available
*= $p<0.05$ by Student's t test

Table 6 indicates that 30 minutes after either ischemia or trauma increases in total DAGs are fairly similar but 22:6 increases more after ischemia than after trauma (4x vs 2x).

Table 6: Increase in DAGs in Ipsilateral Cortex 30 Minutes After Insult (x Control Values)

	Yoshida [77] (Ischemia)	Homayoun◊ (Trauma)
16:0	2	1.4*
18:0	5.4	1.8*
18:1	3.1	2.0*
20:4	3.5	2.0*
22:6	14	2.1*
Total	3	1.8*

◊= present work
*= $p<0.05$ by Student's t test

The early mild increases in FFAs and DAGs observed with our 1mm impact injury suggests that with trauma, cell membrane damage is less severe than with ischemia. This possibly underlies the frequent preservation of cortical mantle and cells in the impact area. Perhaps the mild increases in FFAs and DAGs in our trauma model represent dendrite and axon damage more than cell body damage. This would be consistent with our histologic studies. The much lower elevations in FFAs after trauma also suggest that early after brain injury, ischemia is not a prominent event.

In our model total FFAs peaked at almost 4x normal 24 hours after injury but, surprisingly, were still significantly elevated (1.4x) 35 days after injury and not only in the injured right frontal lobe but also in the uninjured left frontal lobe as well, Table 2. As the uninjured left frontal lobe never exhibited a rise in excitatory amino acids, it appears that FFA increase there did not occur through the action of NMDA receptor regulated calcium channels.

Total DAGs were also elevated in the area of cortical injury 30 minutes after piston impact. They maintained a 1.6 fold increase in the injured right frontal cortex at

24 hours and 4 days after injury but by day 35 they had increased to 2.4x normal. These late DAG increases occurred not only in the damaged frontal lobe but widely through the brain in both cerebral hemispheres, cerebellum, and brain stem. Except for the damaged right frontal cortex these areas never exhibited posttraumatic aspartate or glutamate increases.

The occipital cortices, cerebellum and brain stem which had significant DAG elevations never showed any FFA increases Thus, posttraumatic DAG and FFA increases may occur independently of each other.

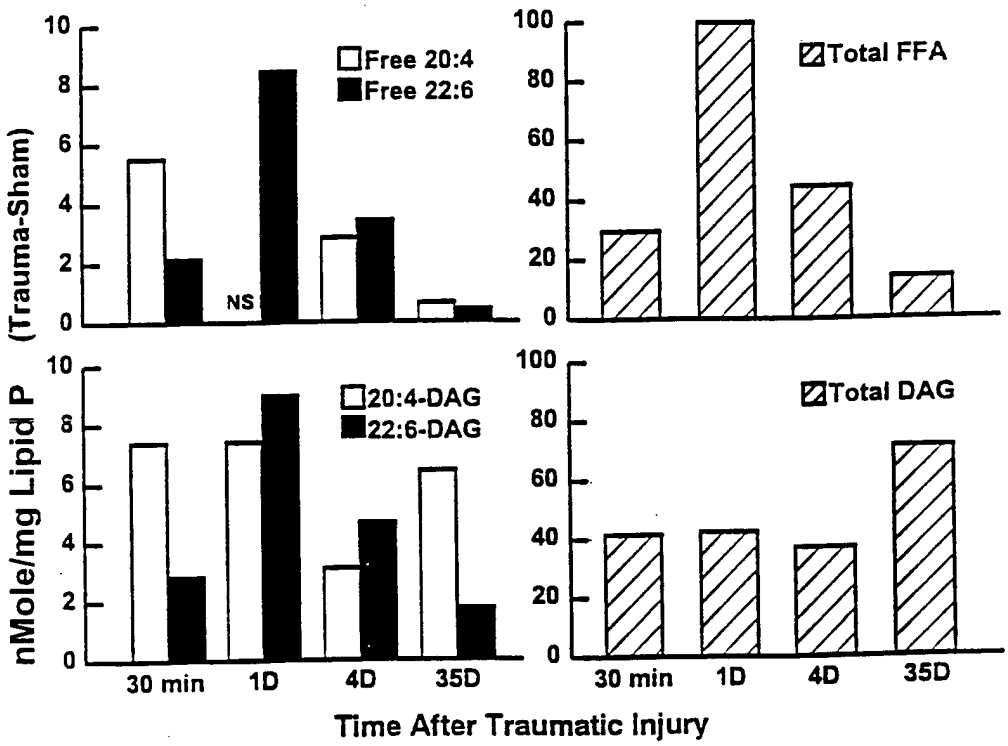
The DAG accumulation suggests that membrane phospholipid hydrolysis exceeds those metabolic pathways involved in the removal of DAGs such as their degradation via DAG lipase and/or their utilization for phospholipid resynthesis.

Within 30 minutes of injury both Ca-dependent and Ca-independent forms of PLA_2 were activated in the injured cortex. Cytosolic and microsomal PLA_2 activity then returned closer to control levels by days 4 and 17 but a second phase of PLA_2 stimulation was observed 35 days after injury in both the injured right cortex as well as the uninjured left cerebral cortex, (Figure 36). The calcium-independent isoenzyme was more pronounced.

The novel findings of these studies, the sustained elevations of PLA_2, FFAs and DAGs lasting over a month after brain injury, raise several interesting points. First, following cortical trauma the elevations in PLA_2, FFAs and DAGs occur in many regions and at many time points which never showed an increase in excitatory amino acids. Increases in these brain areas must occur by means other than glutamate→NMDA receptor→Ca^{2+} influx into cell. It may be argued that the long lasting elevations in PLA_2, FFA, and DAGs throughout the brain are just the play out of Wallerian degeneration processes which our fiber track degeneration studies show continue for at least 8 weeks after brain injury. Our histologic studies, however, strongly suggest that axonal degeneration peaks around one week after injury while DAG increases are highest 5 weeks after injury concomitant with a cytosolic and microsomal $iPLA_2$ resurgence. This late activation of $iPLA_2$ and DAG in many uninjured brain regions occurs when neurobehavioral recovery is maximal and may be a part of neuronal reparative or plasticity processes occurring throughout the brain. Moreover, PLA_2 has been involved

regeneration of peripheral nerves and noradrenergic axons [77,78].

Figure 37: Time Course of Most Pertinent FFAs and DAGs Findings



Accumulation of total FFA, DAG AA (20:4) and DHA (22:6) in the FRC at early and late times after cortical injury. Values represent the difference in nmole/mg lipid P between trauma and sham values that were statistically significantly different. Values for 4 and 35 days were recalculated from Homayoun et al., 1997.

Interestingly, cytosolic $iPLA_2$ is involved in the incorporation of arachidonic acid into membrane phospholipids [72]. This type of PLA_2 does not display selectivity for arachidonic acid phospholipids, and therefore generates 2-lysophospholipids that, in turn, can be reacylated to generate a new phospholipid. Arachidonic acid incorporation through this remodeling pathways is very active. Therefore high activity of $iPLA_2$ will favor the removal of free arachidonic acid and the remodeling of membrane arachidonic acid phospholipids. In fact, in our model the accumulation of free arachidonic acid showed the lowest increase by 35 days post-trauma concomitantly with the highest increase in $iPLA_2$ activity suggesting that arachidonic acid was indeed being incorporated into neural remodeling pathways at this time.

Table 7 shows how various neurochemical factors in the brain differ among brain insults: trauma, cryogenic injury or ischemia-seizures.

Table 7

	Ischemia-Seizures [45]	Cryogenic injury[79]	Traumatic Injury (Present Study)
PLA_2 - PLC	⊕	⊕	⊕
FFAs	↑↑	↑↑	↑
DAGs	↑	↑	↑
	FFA > DAG	FFA > DAG	FFA ≤ DAG
↑ FFA	TRANSIENT	SUSTAINED (48 hours)	SUSTAINED (35 days)
↑ DAG	TRANSIENT	SUSTAINED	SUSTAINED
ACYL GROUPS	↑ 18:0-20:4 (1:1)	↑ ALL, ↑ 22:6	↑ ALL, ↑ 22:6
CORTICAL AREAS			
IPSILATERAL INJURY SIDE	↑ FFA ↑ DAG	↑ FFA ↑ DAG	↑ FFA ↑ DAG
CONTRA-LATERAL	↑ FFA ↑ DAG	↑ DAG	↑ FFA ↑ DAG
HIPPOCAMPUS	⊕⊕⊕	N.A.	⊖

5 NEUROBEHAVIORAL STUDIES (DR. SOBLOSKY)

We selected the severity and cortical location of the brain lesion in our rat model to produce a reversible sensorimotor deficit. We felt that brain injury involving both fore-and hindlimb cortical areas would be most clinically useful because many brain injured people have a hemiparesis and because posttraumatic sensorimotor deficits can be quantified in the laboratory.

Careful observation of baseline sensorimotor ability and changes after injury is essential for obtaining meaningful results. Consistency in handling the rats was also important. Our full time research assistants (college graduates) had extended periods of handling our laboratory rats to ensure their familiarity with rat behavior in general and after injury. Two observers independently scored each rat's performance on the individual tests. Their scores were generally in close agreement. The wire grid test and horizontal ladder beam tests were videotaped. For each experiment approximately 12 rats were used in both experimental and control groups.

We intially used a battery of 4 already developed tests to evaluate both fore-and hindpaw function after injury. Four tests provided a more powerful demonstration of neurologic deficits than any single test and allowed insights concerning the improvement of function following brian injury impossible with a single test. These included:

1) The beam walking test wherein a rat crosses a thin horizontal beam placed above the floor. This test demonstrates both fore-and hindlimb deficits and evaluates the "style" by which the rats traverse the beam after injury. Toe misplacement on the beam, not usually exhibited before injury, accounts for most of the delay in neurobehavioral improvement after injury.

2) The pegged beam test in which a rat has to navigate around 4 upright pegs placed at intervals on a horizontal beam evaluates bilateral fore-and hindlimb sensorimotor function and coordination. Because the left forelimb of each experimental rat was impaired, the rat could not use this limb to compensate for any left hindlimb locomotor deficits. This increased the number of left hindlimb foot slips and showed hindlimb dysfunction at later times during recovery when the hindlimb dysfunction was

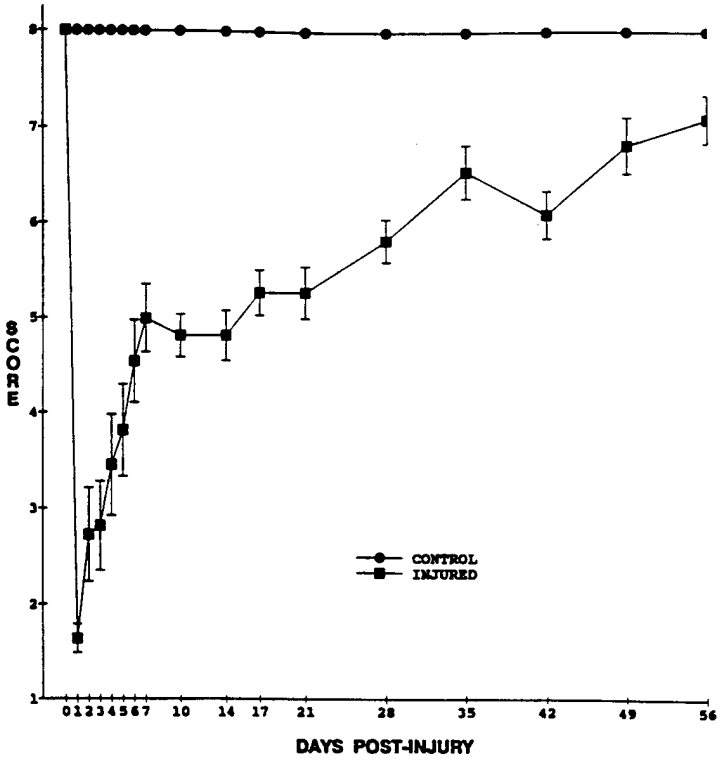
inapparent on the standard horizontal beam walking test. Increased foot slips were apparent up to 28 days after injury.

3) The wire grid test where left forepaw sensorimotor dysfunction was measured by left forepaw foot faults on a wire grid. Injured animals made left forepaw foot faults on this test for 28 days after injury.

4) The forepaw preference test measures how the cortical injury affects each rat's normal forepaw use for support, rearing, and landing. Forepaw use is recorded as a rat deports itself in a clear plastic box which allows the rat to rear up on its hindpaws. The rat does not require training or testing before the injury. Injured rats showed an initial extreme preference to use their right, uninjured, forepaw which diminished by 35 days after injury.

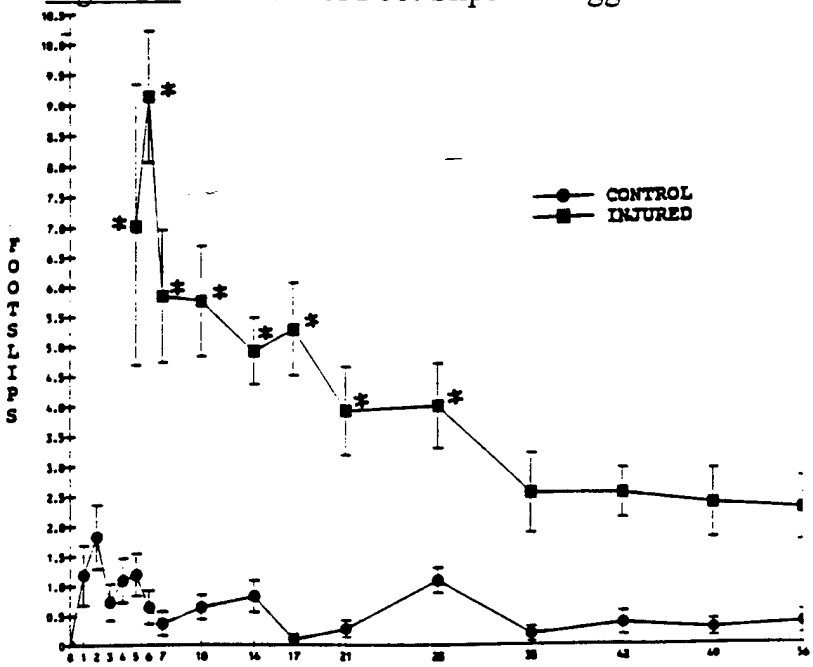
These tests showed excellent consistency in demonstrating neurological deficits, Figures 38,39,40,41.

Figure 38: Neurological Score on Beam Walking Ability After Brain Injury



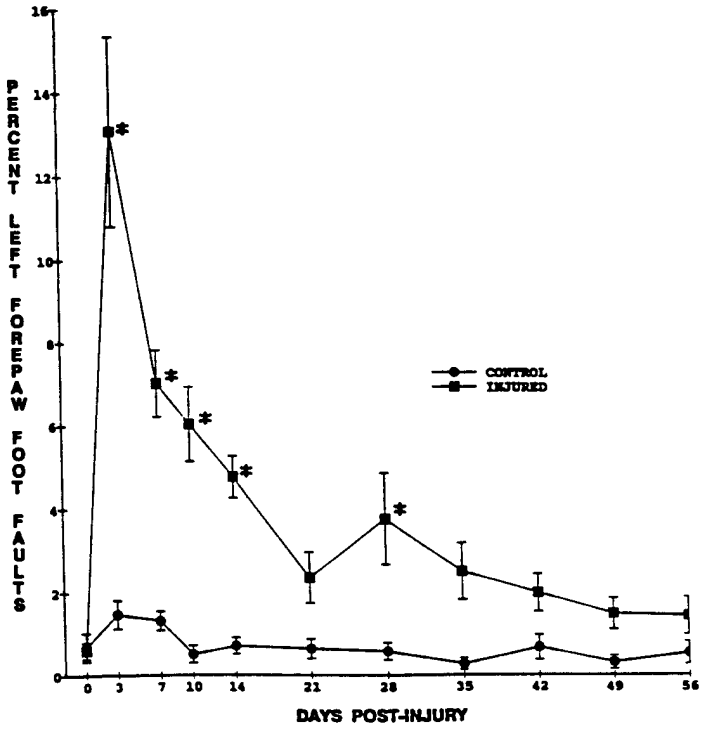
Data are $\bar{x}$ ± SEM: n=11 per group. Injured animals recovered rapidly during the first week and more slowly thereafter during the 56 day post injury observation ($p<.001$).

Figure 39: Number of Foot Slips on Pegged Beam



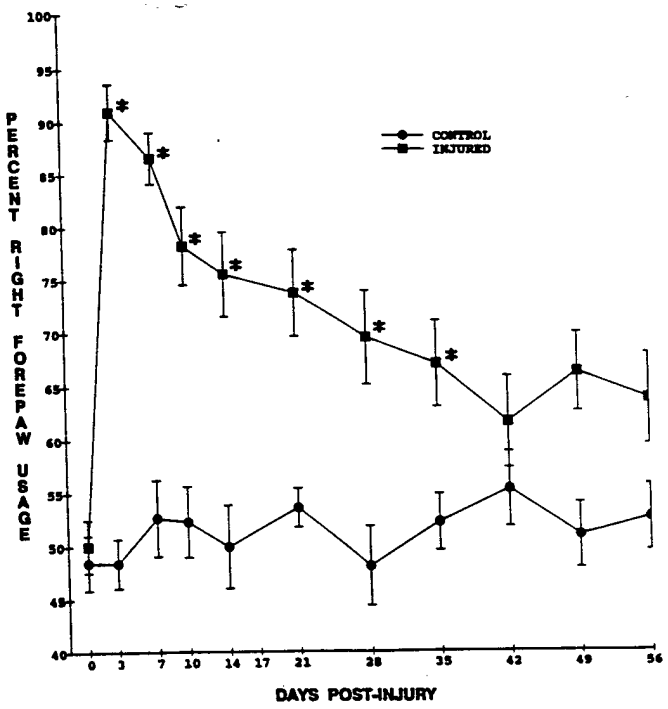
Data are $\bar{x}$ ± SEM: n=11 per group. *= p<.05 vs controls, Bonferroni test. Rapid recovery in first 7 days was followed by slower recovery and no statistical difference at 35 days.

Figure 40: Percentage of Left Forepaw Foot Faults After Injury



Data are $\bar{x}$ ± SEM: n=11 per group * = p <.05 vs controls. Rapid recovery in the first week was followed by a slower rate with no statistical difference at 35 days.

Figure 41: Paw Preference as Percentage of Right Forepaw Use



Data are $\bar{x}$ ± SEM: n=11 per group *= p <.05 vs controls. After initial rapid recovery in the first week, preference for uninjured paw decreased to no statistical difference at 42 days.

Using these tests we noted a longer time for hemiparesis improvement (28-35 days) than has been customarily noted when only a hindlimb deficit has been analyzed using a narrow horizontal beam where hindlimb paresis often improves to normal in 5-12 days [7,9,80,81,82].

While these above four tests or variants of them are used by many investigators worldwide to evaluate rat neurobehavior, they have drawbacks which we began to appreciate as we gained experience. For instance, foot misplacements on some tests may be difficult to observe in a rapidly moving rat. "Recovery" time determined by the narrow flat beam test depends on the scoring system used and one commonly used scheme encompasses overall postural form and style which are subjective determinants. This yields nonparametric data which cannot be used with more powerful parametric

statistical analyses.

To overcome such drawbacks Dr. Soblosky devised a horizontal ladder system where a rat's progress across a horizontal ladder can be videotaped and where both fore- and hindlimb deficits as well as foot or toe misplacements can be readily seen and quantified.

Each rat is videotaped crossing the horizontal ladder. The videotapes are played back and the rat's locomotion carefully quantified. Normal forelimb and hindlimb placements are depicted in Figure 42. From slow motion videotape playback we determine the number of forelimb footslips, (Figure 43), forelimb misplacements, (Figure 44), and hindlimb footslips, (Figure 45) each rat made during each crossing of the ladder beam.

FIG. 42

FIG. 43

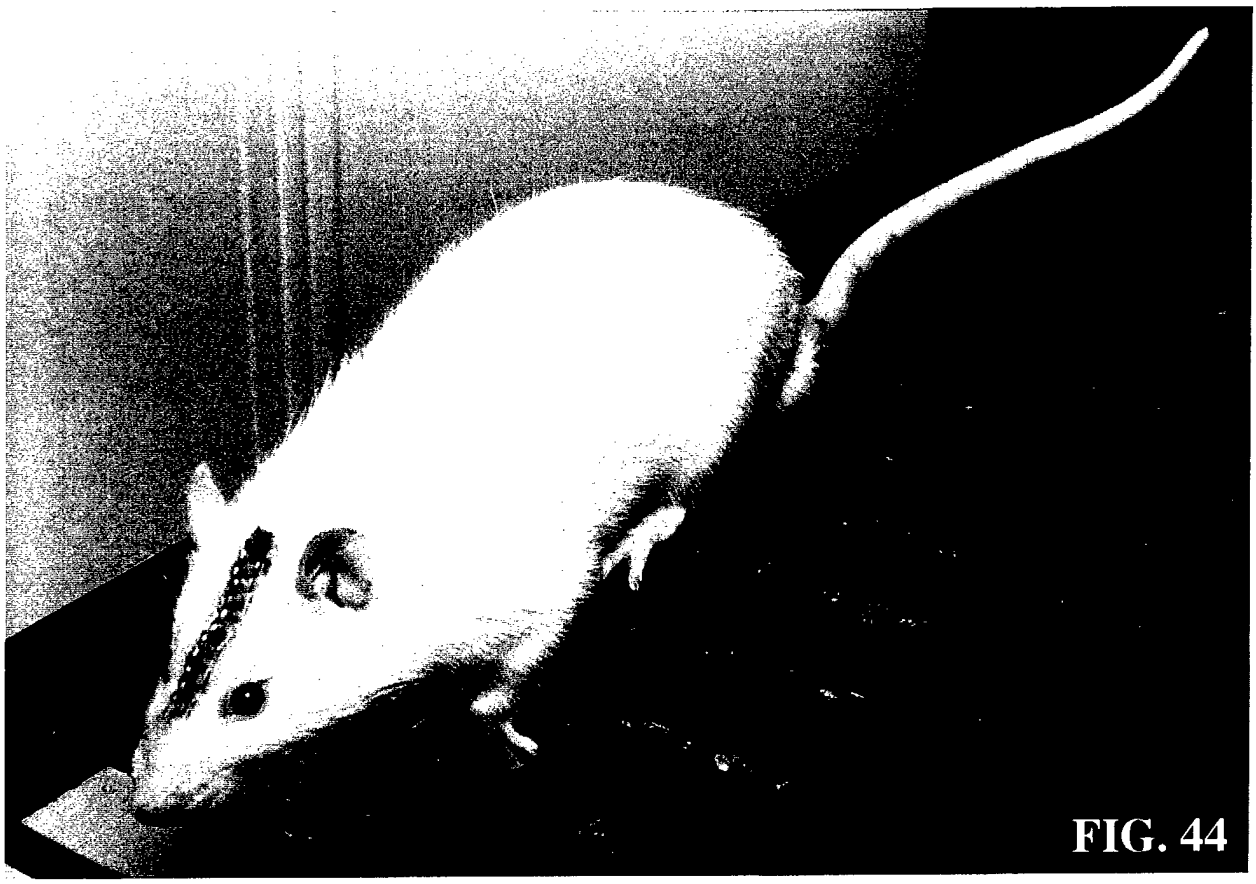


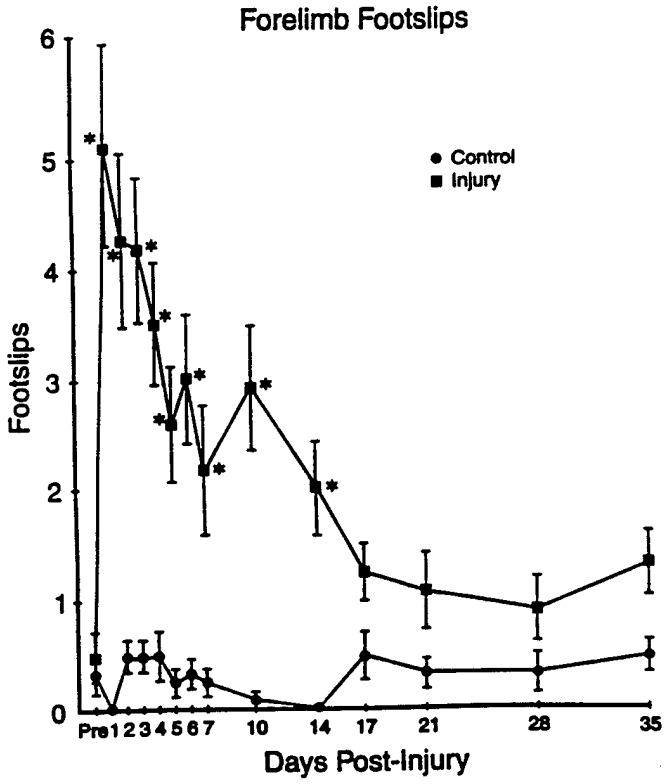
FIG. 44

FIG. 45

Results

The injured rats had more forelimb footslips than control rats until 17 days after injury (Figure 46) and more forelimb foot misplacements than controls for the entire 35 day test period (Figure 47). They made more hindlimb footslips than control rats up to 28 days after injury (Figure 48). Quantifying hindlimb misplacements was problematic for the injured rats because uninjured rats normally used various types of hindlimb placements on the ladder rungs.

Figure 46: Horizontal Ladder Test



Data are $\bar{x}$ ± SEM: n=11 per group: *= p <.05 versus controls, Tukey's test

Figure 47: Horizontal Ladder Test

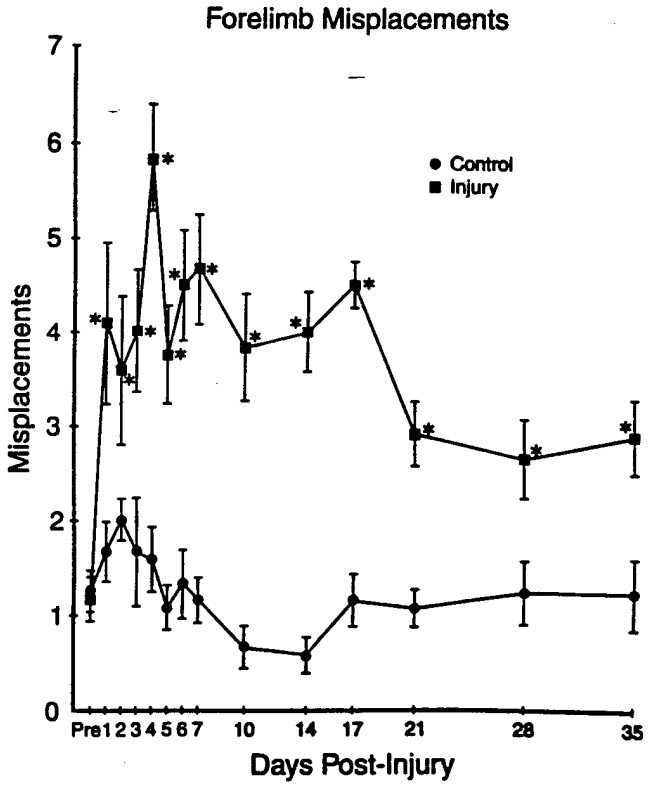
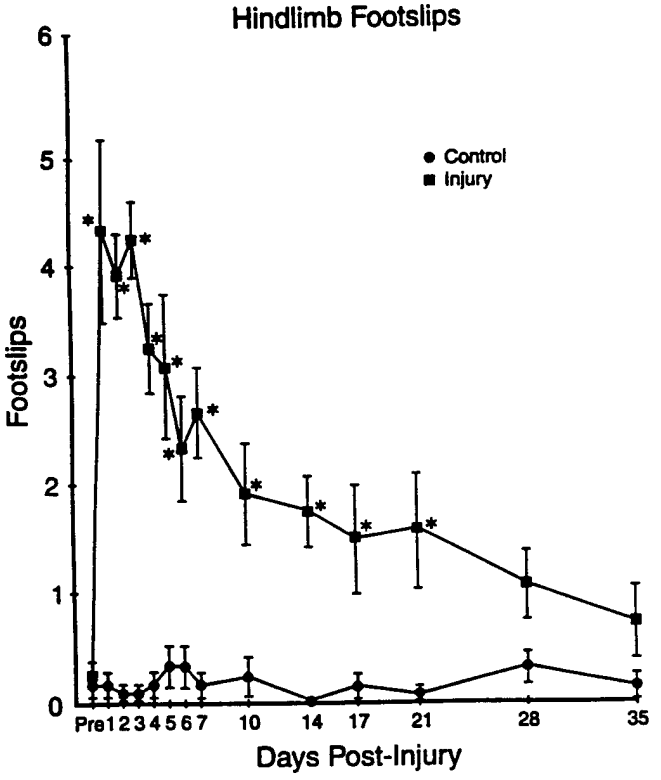


Figure 48: Horizontal Ladder Test



Discussion

Forelimb misplacement was the most sensitive aspect of the ladder beam, this abnormality being observed during the entire 35 day test period. Inability to accurately place the forelimb may have been a permanent deficit because there appears to be no late further improvement. By contrast, forepaw dysfunction appeared to resolve by day 28 when evaluated by the pegged beam or wire grid, Figures 39,40.

Any observed neural loss will be a direct function of the sensitivity of the evaluating test. The more sensitive the test the greater or more long lasting will be the apparent deficit. On the horizontal ladder beam each rat's performance is easy to record and quantitate; even subtle footslips and misplacements are convincing, easily seen, and unambiguous. The enhanced sensitivity of the ladder beam in depicting neural loss is useful because it shows a longer period of diminished behavioral function in which one could administer drugs to evaluate potentially therapeutic effects.

SPECIFIC ISSUES CONCERNING RECOVERY ADDRESSED BY OUR NEUROBEHAVIORAL TESTS

Rather than present our behavioral data (all of which have been published) in the usual background-methods-results-discussion format we will discuss the important concepts concerning the recovery from brain injury which have resulted from our behavioral testing.

1. THE RATS EXHIBIT A BIPHASIC IMPROVEMENT IN FUNCTION AFTER INJURY

When first tested after injury the rats had severe deficits which rapidly improved over the first 7 to 14 days. After this a slower rate of improvement occurred which lasted many weeks (Figures 5,38,39,40,41). Several interpretations of this biphasic improvement are possible. The early rapid improvement could reflect: 1) the resolution of the acute pathobiologic processes consequent to brain injury which potentiate actual neural injury consequent to the trauma [83,84,85]; 2) the rapid functional return of less

severely damaged cells around the periphery of the impact site or: 3) the rapid recruitment of other brain areas (as the caudate-putamen or globus pallidus) which normally participate in motor functions along with the cerebral cortex to take over some of the functions of the damaged cortex.

The later phase of improvement may reflect actual restoration of damaged cells and/or the further reorganization of ipsilateral and/or contralateral neural circuits. Actual neural changes in the contralateral homotypic sensorimotor cortex concomitant with behavioral (sensorimotor) changes have been described in adult rats [86,87,88]. The cerebellum has also been implicated in behavioral improvement following sensorimotor cortex injury. Norepinephrine infusions into the cerebellum contralateral to the injury have been shown to enhance recovery [80]. The cerebellum undergoes synaptogenesis as a result of motor learning, but not with random activity [89]. Possibly prolonged compensatory movements following brain injury induce synaptogenesis in the contralateral homotypic sensorimotor cortex, cerebellum or other brain areas and this new synaptogenesis is responsible for later stages of recovery.

While these various mechanisms are presently only theoretical possibilities the greatly differing recovery slopes early and later after brain injury, (see especially Figure 38), suggest that different processes account for early and late recovery. The concept is very important because drugs that might be effective early after injury (because they affect acute mechanisms) could be ineffective in the later phase of neural recovery and drugs enhancing late recovery processes might be totally ineffective if given before late recovery processes (e.g. enhance plasticity) became operative.

2. WHAT UNDERLIES NEUROBEHAVIORAL IMPROVEMENT AFTER BRAIN INJURY: RECOVERY OR COMPENSATION?

What actually underlies animal "recovery" is a vexatious issue [90-92]. The key problem involves actual neural recovery versus behavioral compensation or adaptation. True neural recovery would entail the complete return of pre-injury neural function and style in the affected limbs. Behavioral compensation or adaptation, however, does not necessarily require actual return of the lost neural function but occurs as a result of an altered (movement) style or strategy. Rose et al [93,94] contend that most studies

claiming to demonstrate environment enrichment-induced neural recovery are actually evaluating behavioral compensation. It may be difficult to ascertain the precise contribution of compensation/adaptation and true neural recovery in performance improvement following brain injury.

Several days following injury a rat places its hindlimb better on the surface of a horizontal flat beam but continued toe misplacement on the beam suggests that true neural recovery had not occurred. Furthermore, the pegged beam test shows a persistent hindlimb deficit (many footslips) in rats able to cross the horizontal flat beam without footslips. In other words the pegged beam unmasks deficits not apparent to horizontal beam testing.

Over time the rats significantly improve hindlimb placing even on the pegged beam but improvement on this beam always lags behind the non-pegged beam. Clearly, no transfer of compensatory movement from the non-pegged to the pegged beam occurred since the rats "recovered" hindlimb deficit as tested on the non-pegged beams reappeared on the pegged beam. If true neural recovery had occurred, whereby the brain had reestablished damaged neural circuits, over time one would expect the rat to navigate the pegged beam and horizontal ladder without footslips just as a normal rat.

Forelimb placements on the wire grid also improved after injury but the rats appeared to have altered their style or strategy in searching for the grid wires. They used quicker and shorter repetitive forelimb movements to search for a wire to engage. Over time forelimb placement continued to improve as the rats became more proficient in this compensative, searching strategy.

In the paw preference test using the unaffected forelimb for supporting and rearing activity is obviously compensation/adaptation.

Results from these tests lead us to conclude that a significant portion of any observed neurobehavioral improvement after brain injury occurs by compensation/adaptation mechanisms and not the resoration of truly normal movements which would indicate the restablishment of neural circuit integrity.

Compensation/adaptation processes themselves, however, may require new neural connections in undamaged brain areas which participate in and allow the compensation/adaptation. Pharmacologic agents which might aid compensatory adaptive processes would be of great benefit to the brain-injured and the test battery which Dr. Soblosky has implemented and devised provides an exquisite and unambiguous means to evaluate such drugs in the future.

3. DO TASK SPECIFIC TESTS ENHANCE POST INJURY NEUROBEHAVIORAL IMPROVEMENT OR DOES NEUROBEHAVIORAL IMPROVEMENT OCCUR AS READILY WITHOUT TASK SPECIFIC PRACTICE? **(IN OTHER WORDS DOES PHYSICAL THERAPY AID NEURAL IMPROVEMENT AFTER BRAIN INJURY?)**

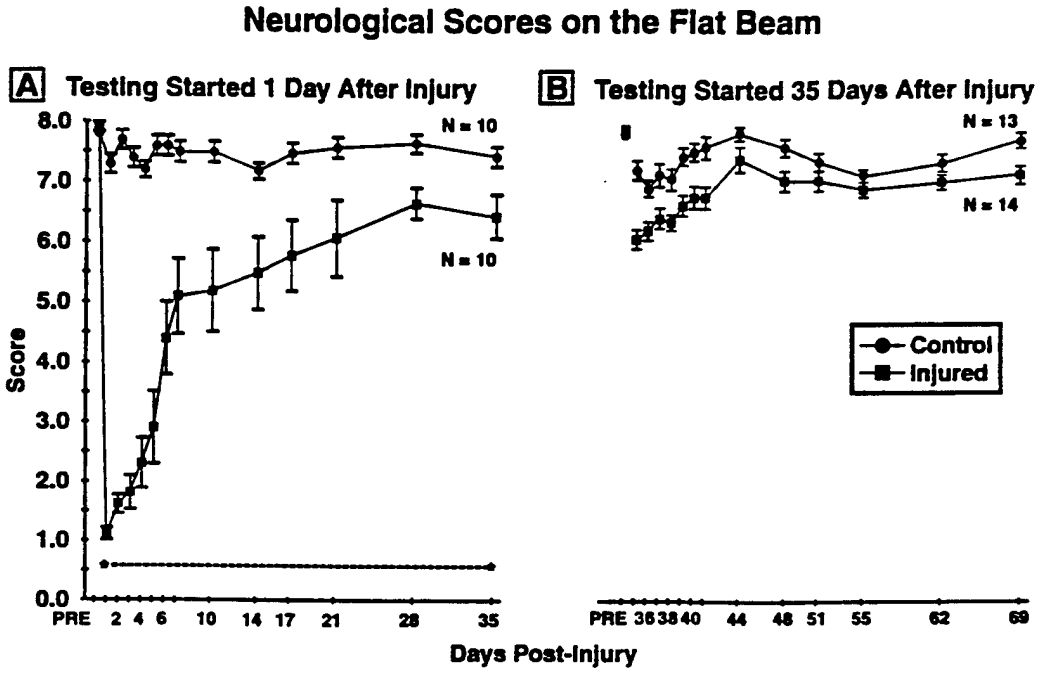
Several investigators have cautioned that after brain injury rapidity of neurobehavioral improvement documented by various tests may be a function of the testing frequency and that any observed improvement may be test-specific [95,96]. **In the laboratory setting specific testing to document post brain injury improvement may be considered the equivalent of physical rehabilitation and if practice of task-specific tests after injury improved post injury performance this would form a strong rationale for the use of physical rehabilitation after brain injury.**

Dr. Soblosky performed a set of experiments using the flat beam, pegged beam and paw preference test to determine if brain injured rats not subject to frequent task-specific testing would show neurobehavioral improvement as rapidly and as fully as brain injured rats which received frequent task specific testing right after their injury. If testing frequency were a factor in post injury improvement then the untested rats would appear perhaps only slighly recovered at 35 days post-injury because they had no practice on the motor tests. If testing frequency were not a factor, then the untested rats would show recovery even though they were never tested on specific motor tasks. This paradigm attempts to evaluate the efficacy of "rehabilitation" as a means of enhancing neural improvement after brain injury.

All 3 motor tests showed that the rats which were not tested until 35 days after injury performed as well by 37 days post injury as those which received frequent testing

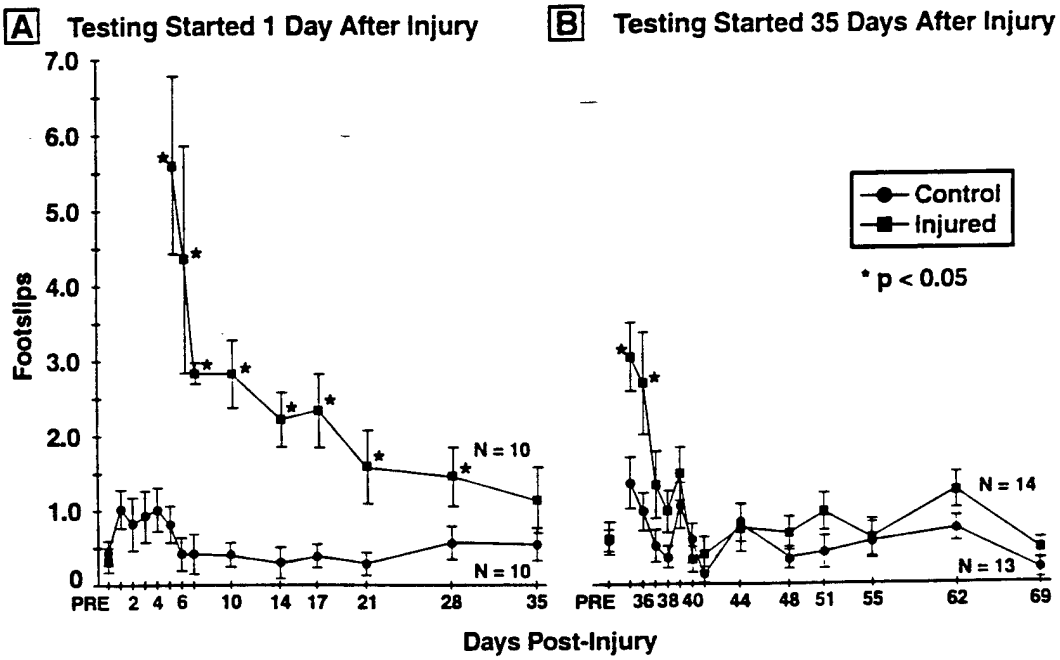
from right after injury through day 35, Figures 49,50,51.

Figure 49



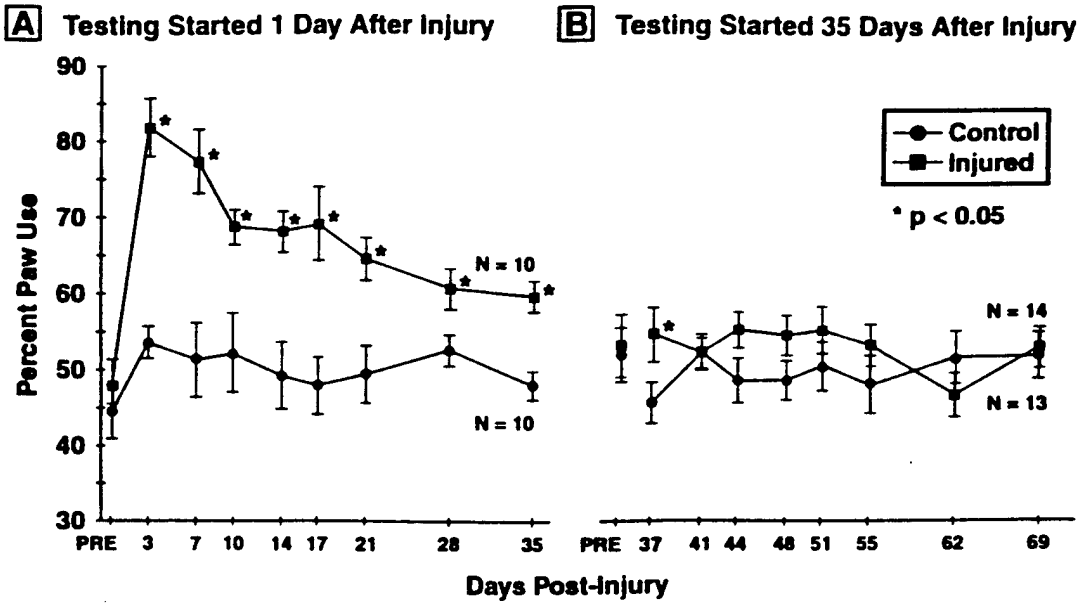
Animals not tested until 35 days after injury performed on their first test day (day 35) as well as rats tested 13 times over the first 35 post injury days, $\bar{x} \pm$ SEM. The non-parametric neurologic scores were evaluated by the Trapezoidal rule to calculate the areas under the curve. Resulting data were analyzed by the Kruskal Wallis test which indicated a significant difference among the four experimental groups. Specific comparisons were made using Dunn's procedure. This showed that injured rats that were tested beginning day 1 after injury performed worse than control rats for the entire 35 day test period ($Q4.823$, $p<0.01$). In contrast, injured rats not tested until 35 days after injury were not different than controls ($Q1.119$, $p>0.05$).

Figure 50



After the first 2 test days rats that were not tested until the 35th post injury day performed as well as rats tested right after surgery and subsequently tested 13 times, (ANOVA for repeated measures using a general linear model; specific comparisons by the Bonferonni test).

Figure 51



This test, not requiring practice, tracks adaptation occurring in the home cage. Rats not tested until 37 days after injury showed a right paw preference only for the first test day, (ANOVA plus Tukey's test).

These results strongly suggest that testing was not a factor in the rapidity or quality of the eventual neurobehavioral improvement which occurred following brain injury in rats. Once the neural mechanisms underlying post injury improvement were initiated, "task specific" practice allowed the relearning of specific motor movements but practice early after injury did not appear to enhance neurobehavioral improvement.

While it may be argued that just walking around the cage during the 35 days after injury and prior to formal testing allowed for a type of practice with the impaired extremities, it certainly was not task specific. Yet, after only one or two days of practice non tested, animals performed at fully improved levels. These results strongly suggest that after brain injury the underlying mechanisms responsible for neurobehavioral improvement, what ever they may be, proceed in at least a general way without the need for "task specific" practice. In other words the ability to reorganize the brain after injury appears internal and innate and does not rely on external task specific stimuli.

These results cause us to reappraise the whole concept of physical therapy or other rehabilitation efforts in regards to brain injury. If brain reorganization is innate and proceeds as well without task specific testing, what exactly does rehabilitation accomplish? In the case of hemiparesis, rehabilitation may keep the limbs supple and preserve muscle strength but one might question its role in any brain reorganization leading to post injury improvement in humans.

6 TESTING DRUGS TO IMPROVE RECOVERY AFTER BRAIN INJURY

1. DRUG MDL 28,170

Introduction

As mentioned in Section 4, one of the major causes of cell death after brain insults has been linked to the release of glutamate and other amino acids which, in turn, cause a massive calcium influx into cells. Calcium is thought to mediate a cytotoxic cascade via the activation of proteases, phospholipases, kinases and phosphatases. Cell damage resulting from calcium-mediated cytotoxicity may be the cause of behavioral deficits following brain injury. (See more extensive discussion, Section 4).

Calcium activated proteases (calpains) have been shown to be activated in traumatic brain injury, ischemia, hypoxia, and chemical electrolytic lesions [97,98,99,100,101]. The preferred substrates for calpains include cytoskeletal proteins (spectrin, MAP2, tubulin and neurofilament protein) as well as key intracellular enzymes (including protein kinase C and calcium/calmodulin dependent protein kinase II) [102]. Sustained calpain activation may cause the proteolysis of key cytoskeletal proteins causing cell death. Prolonged calpain activation has been described after experimental traumatic brain injury in regions that correlate with neural degeneration and cell death [97].

Because of the deleterious effects of calpains it was natural for investigators to try calpain inhibitors to limit brain damage. AMPA (glutamate agonist)-induced toxicity was reduced in cerebellar slices after calpain inhibition [103] and calpain inhibition after focal cerebral ischemia reduced infarction volume, edema and calcium-activated proteolysis [104,105]. Calpain inhibition has also been shown to attenuate motor and cognitive deficits after fluid percussion injury [106].

For our study we obtained a sample of MDL 28,170 from Hoechst Marion Roussel, Cincinnati, OH. MDL28,170 is a potent calpain inhibitor which has the advantage of being able to be administered peripherally. Most if not all other available calpain inhibitors are unable to cross the blood-brain barrier. Information regarding the dose and route of administration of MDL28,170 was kindly provided by Dr. Matthew Linnik of Hoechst Marion Rousell.

Methods

Each rat received our standard injury. MDL28,170 was dissolved in 10% ethanol and 90% PEG 300 in a concentration of 20 mg/ml. Immediately after injury each rat received a bolus dose of 20 mg/kg infused over 1.5-2 minutes. Each rat then received a 6 hour infusion at a dose of 3.34 mg/kg (Total dose = 40 mg/kg). Control rats received the appropriate amount of vehicle. There were two experimental groups: injury plus MDL28,170 (n=24) and injury plus vehicle, n=15.

We evaluated the effects of MDL28,170 by four tests: 1) beam traversing; 2) narrow pegged beam (footslips); 3) forelimb paw preference (forelimb asymmetry) ; 4) horizontal ladder.

Results

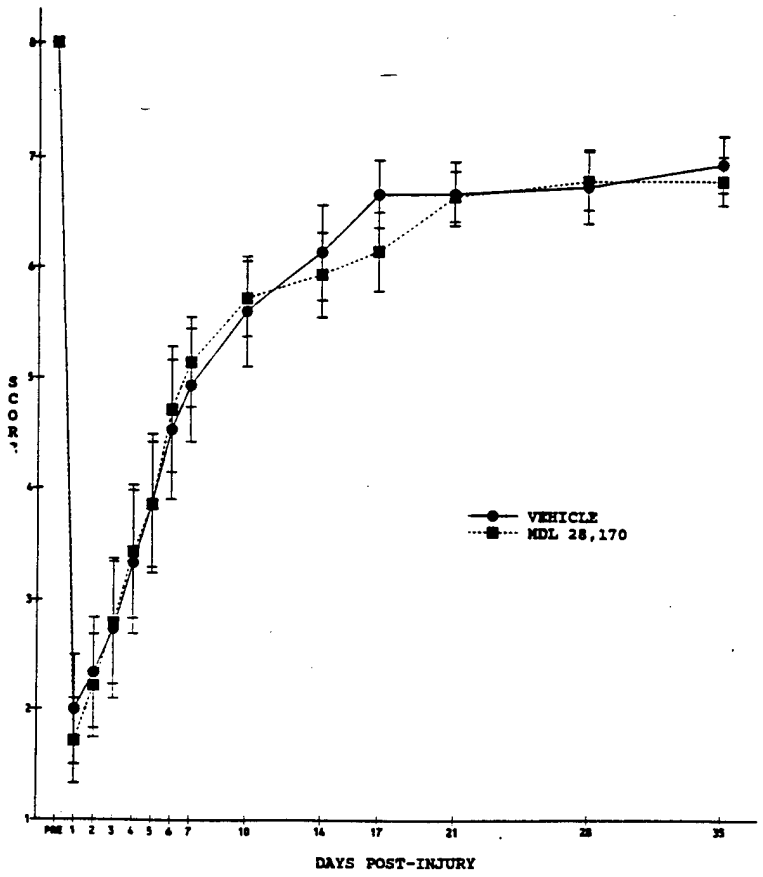
There were no significant differences between the injured drug-treated and injured vehicle-treated rats on any of our behavioral tests. In fact, results were remarkably similiar between the two groups, Figures 52 and 53.

Conclusions Concerning 28,170

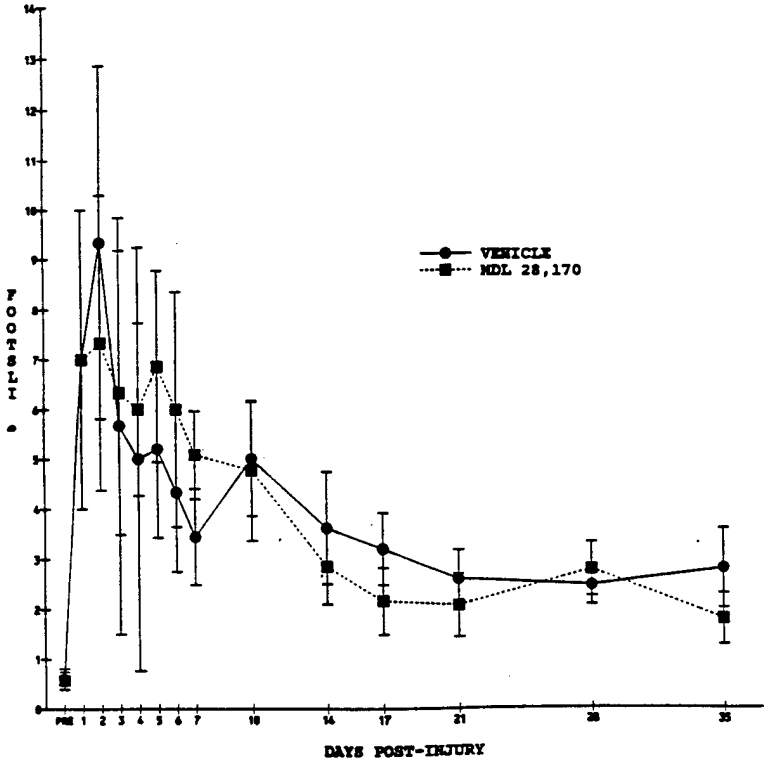
Our evaluation of MDL28,170 indicates that either calpain inhibition is not an effective treatment after cortical contusion injury or that the dose was not appropriate. Instructions on dose and administration, however, were obtained from the manufacturer. The dose we used was appropriate for ischemia studies, but perhaps the dose needs to be modified for traumatic brain injury. We did not evaluate alternate doses.

Figure 52: The Effect of the Calpain Inhibitor MDL28,170 on Recovery From Traumatic Brain Injury

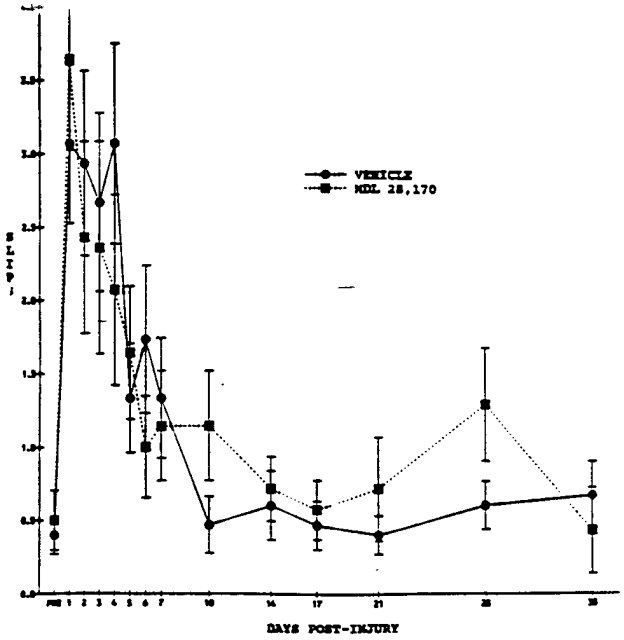
A. Flat Beam



B. Pegged Beam



A. Ladder Beam Forelimb Slips



B. Ladder Beam Forelimb Misplacements

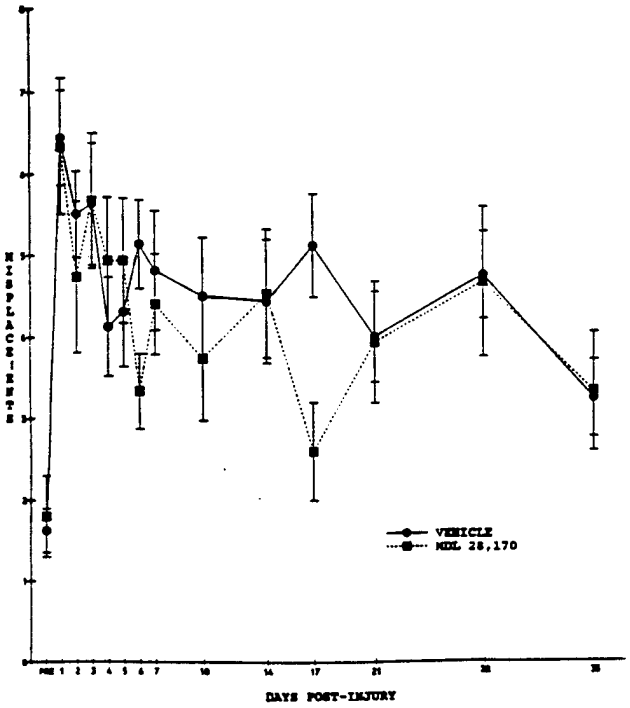
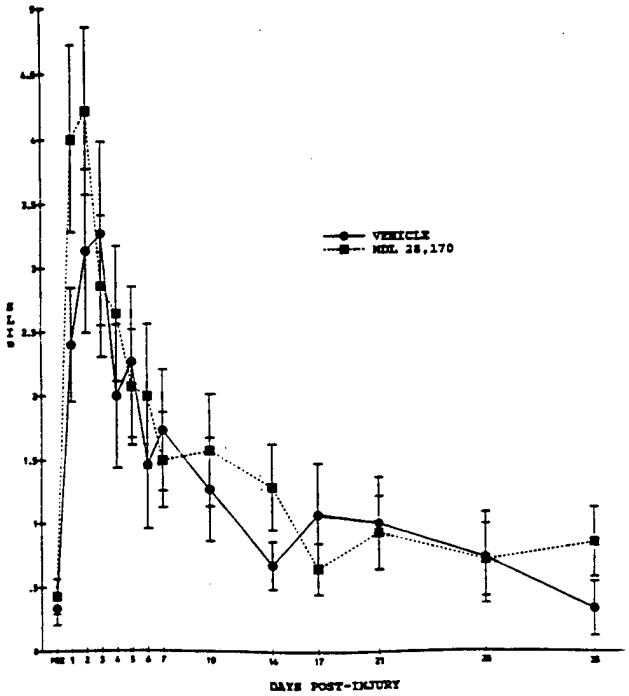


Figure 53

C. Ladder Beam Hindlimb Slips



2. DRUG BN52021

Introduction

Various neurotrauma models including our own (See Section 4A) have shown that neurotrauma causes the release of excess glutamate, increased calcium permeation into the cell through NMDA receptor-gated calcium channels, and activation of PLA_2. We have shown that PLA_2 activity is increased within 30 mins of injury in our model (see section 4B). PLA_2 activation leads to the generation of various membrane phospholipid-derived second messengers which increase eicosanoids and platelet activating factor, PAF (See Figure 34, p 52).

PAF is a bioactive lipid whose synthesis and release are increased in response to closed head injury, cerebral ischemia or seizures. PAF may contribute to neuronal damage after ischemia by decreasing regional cerebral blood flow, increasing blood-brain barrier permeability, causing vasoconstriction and by activating neutrophils. Administration of PAF antagonists has been shown to reduce both functional and histological damage caused by cerebral ischemia [107-110]. We therefore tested the PAF antagonist BN52021, to see whether this drug could improve post injury neural behavior.

Methods

All rats received our standard injury. BN52021 was obtained from BIOMOL (Plymouth Meeting, PA) and dissolved in 100% dimethylsulfoxide (DMSO). Each rat received two doses of either 10 mg/kg BN52021 in 100ul DMSO (i.p.). or 100ul of DMSO alone (i.p.) 30 mins prior to injury and two hours after injury. There were three experimental groups: injury plus BN52021, injury plus DMSO (vehicle) and no injury plus DMSO (vehicle).

Neurologic/Behavioral Evaluations

Post injury behavior was evaluated by: 1) beam traversing; 2) narrow pegged beam (footslips); 3) forelimb paw preference test (forelimb asymmetry); 4) ladder beam.

Statistics

Flat beam test non-parametric neurologic scores were evaluated using the trapezoidal rule to calculate the areas under the curves formed when each rat's score was plotted against time. The resulting data were then analyzed using the Kruskal Wallis test to determine differences in the animals' performance over time. Specific comparisons were made using Dunn's procedure.

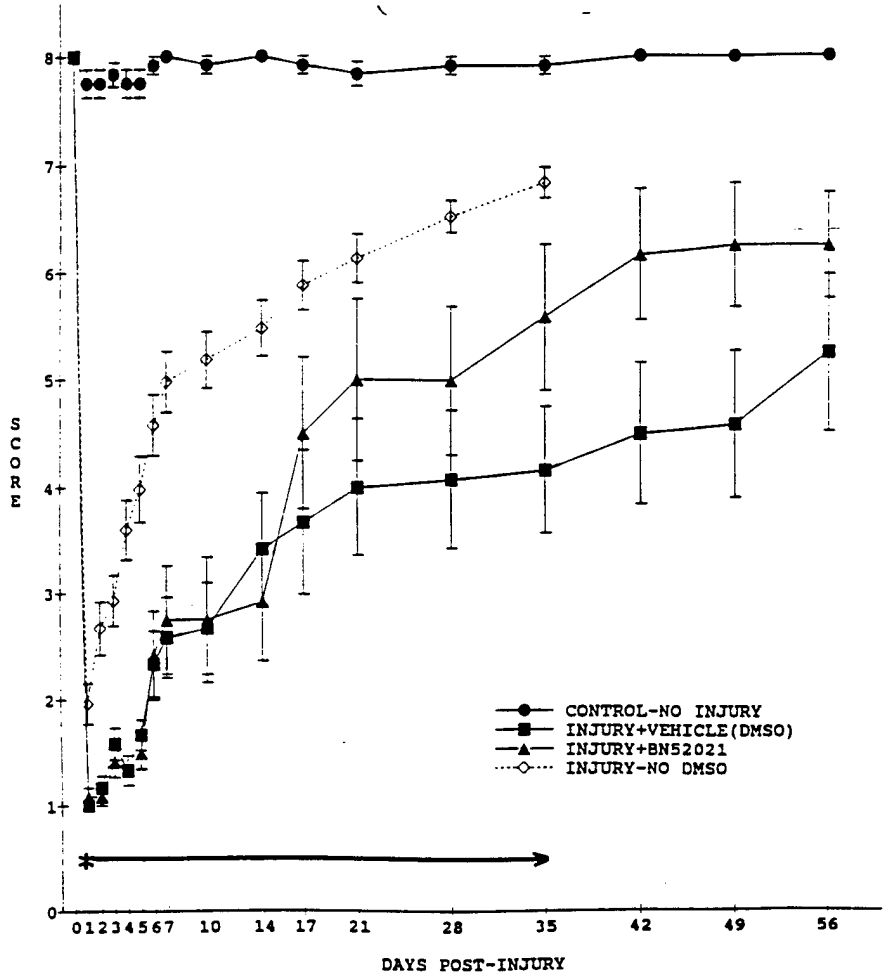
The pegged beam footslip data was incomplete at early time points for injured rats in which testing began one day post-injury. Most of these animals could not do the test until a later time. The data were analyzed by ANOVA using a General Linear Model. Individual comparisons were made using Tukey's test.

Data from the forelimb preferences test and ladder beam test were analyzed by ANOVA for repeated measures with specific comparisons made using Tukey's Test to compare the injured rats to controls at individual post-injury time points.

Results

Both the BN52021 in DMSO and the DMSO only treated groups had slower recovery rates and never reached the level of recovery attained by the injured only rats, Figure 54.

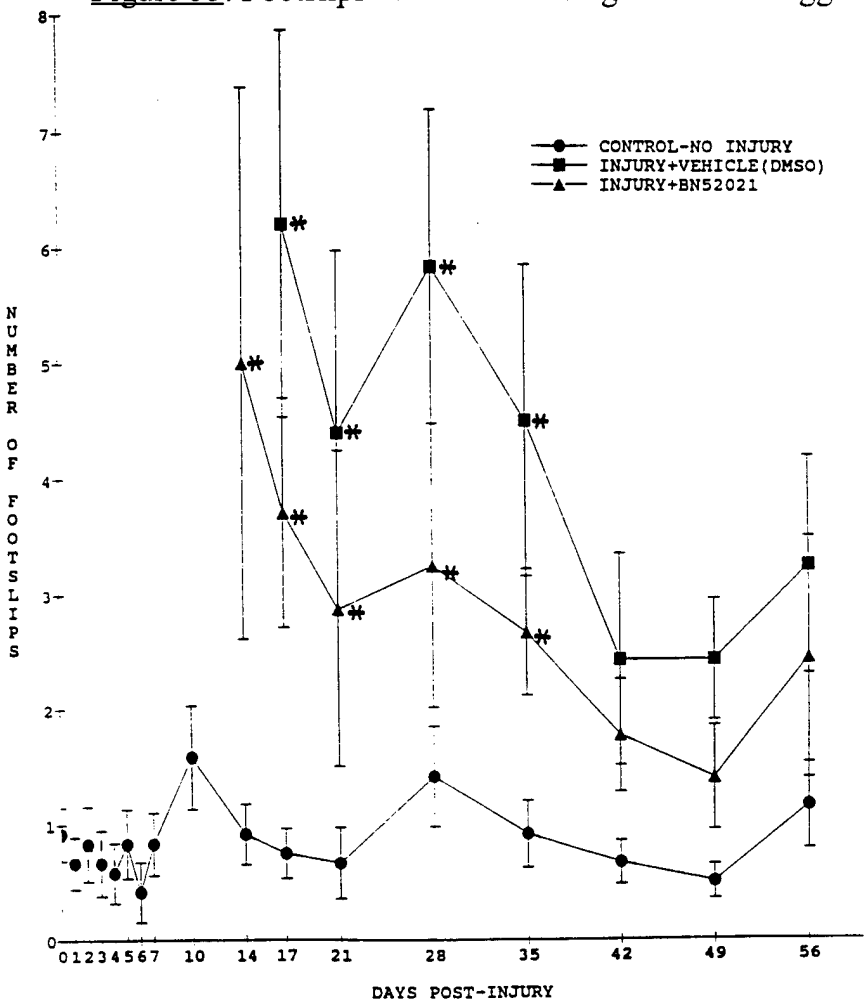
Figure 54: Beam Traversing



Kruskal Wallis analysis indicated a significant difference between the three groups but individual comparisons indicated that rats treated with BN52021 were no different than those receiving DMSO (Q=3.833, p.>05, Dunn's test).

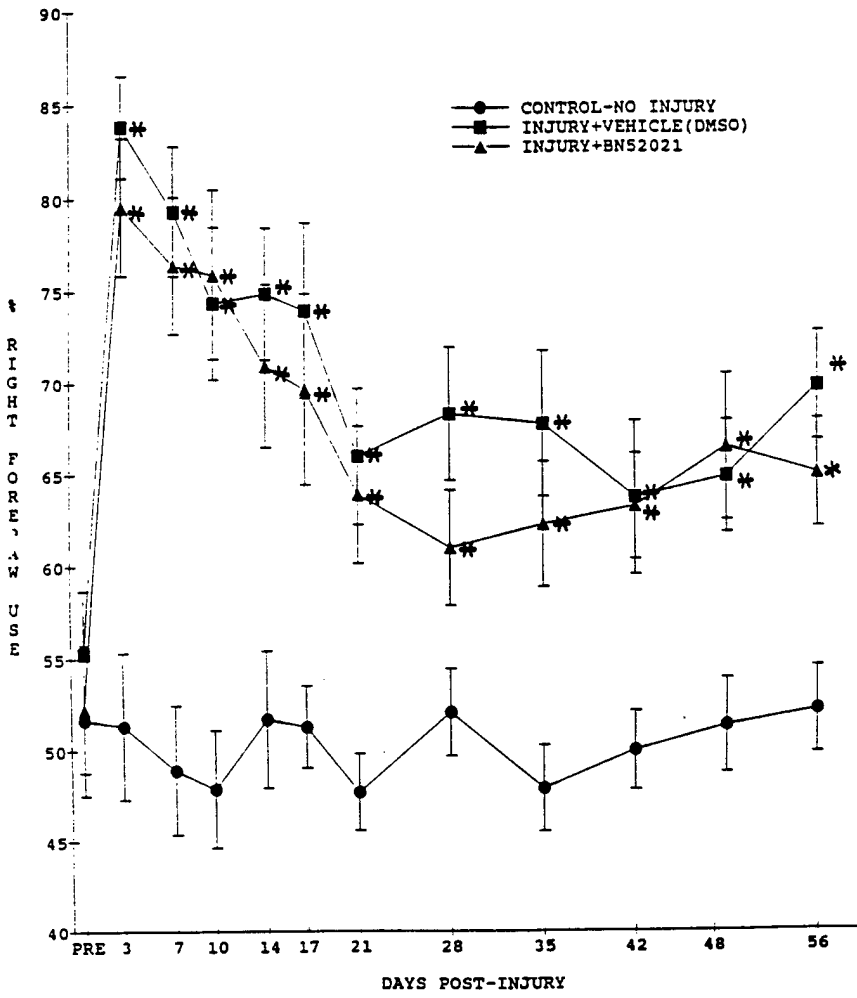
Injured rats treated with DMSO appeared to have a retarded improvement because our prior experimental data showed that injured rats appeared maximally improved by 35 days. Therefore we re-analyzed the data using data collected on 45 injured, but non DMSO-treated rats from 4 separate experiments. Re-analysis indicated a significant difference between the groups ($H=44.315$, $df=3$, $p<.001$) and individual comparisons demonstrated that injured rats receiving DMSO had a significantly impaired recovery compared to injured rats which did NOT receive DMSO ($Q=3.337$, $p<.05$, Dunn's test). **Thus, DMSO by itself was detrimental.**

Figure 55: Footslips While Traversing a Narrow Pegged Beam



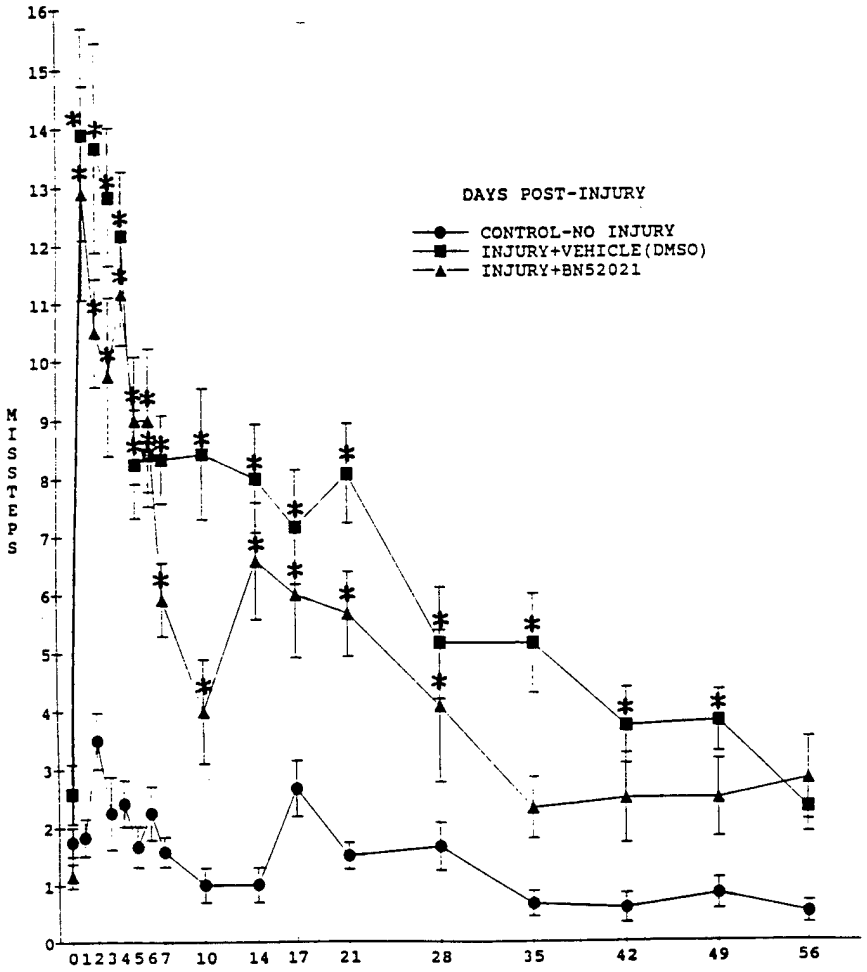
This test showed that rats treated with either BN52021 or DMSO alone were significantly differently from controls, but not from each other, up to day 35 ($p<.05$, Tukey's test). From day 42 on they were not different than controls. Thus, this test showed no improvement in injured rats given either BN52021 or DMSO.

Figure 56: Forelimb Paw Preference Test (Forelimb Asymmetry)



Injured rats treated with either BN52021 or DMSO alone did not recover at any time point when compared to uninjured controls ($p<.05$, Tukey's test). Furthermore, injured rats treated with BN52021 were no different than those receiving DMSO (vehicle).

Figure 57: Ladder Beam



Rats treated with BN52021 showed a recovery after 35 days post-injury while rats treated with vehicle did not reach recovery until 56 days post injury ($p<0.5$) Tukey's test).

Conclusions Concerning BN52021

These tests are inconclusive as to the effectiveness of BN52021 in the treatment of traumatic brain injury because the vehicle, DMSO, recommended to us to dissolve the drug, appears to have deleterious effects on behavioral recovery. Although treatment with BN52021 appeared to enhance recovery, it never was statistically significant, presumably because the negative effects of DMSO could not be adequately counteracted by the drug.

When injured rats treated with either BN52021 or DMSO were tested on the flat beam (Figure 54) they appeared to have a retarded recovery compared to injured rats not receiving DMSO (We did not anticipate that DMSO would be deleterious, thus we did not include a second control for the vehicle and used historical data instead). Rats treated with BN52021 appeared to be recovering faster at later time points compared to DMSO-treated rats, but this was not significant. Their recovery was still depressed overall.

Performance on the pegged beam was also appeared to be negatively affected by DMSO because there were a number of rats which could not perform this test even after 56 days (4 treated with DMSO and 1 treated with BN52021). This is unprecedented for this test because injured rats can usually perform this test 14 to 17 days after injury. Rats treated with BN52021 appeared to be recovering faster compared to DMSO-treated rats, but the results were not statistically significant and the recovery was still depressed compared to untreated controls.

Data from the paw preference test, (Figure 56) indicated almost identical effects of both BN52021-treated and DMSO-treated rats. Data from the ladder beam test gave the best indication that BN52021 treatment may be efficacious because BN52021 treated rats appeared maximally improved by 35 days while it took 56 days from DMSO-treated rats to achieve such improvement, Figure 57. Unfortunately, this result is inconclusive. Subdural bleeding occurs with our model and DMSO may cause excessive intracranial bleeding retarding recovery. DMSO has also been noted to affect gene expression and our subsequent (non contracted) work indicates that genetic events play an important part in neural recovery (see Section 8). BN52021 should be reevaluated using another vehicle to dissolve the drug.

OVERALL CONCLUSIONS CONCERNING "NEUROPROTECTIVE" DRUGS BASED ON THIS RESEARCH PROJECT

The failure of these two drugs to improve post injury neurobehavior is perfectly consistent with the recently recognized failure of all drugs tried thus far, (~20) to improve outcome after head injury [34]. These persistent clinical failures are deeply disturbing to the brain trauma research community and to the pharmaceutical industry which has spent heavily to test potentially beneficial drugs. In view of the severe axon and dendrite damage which our present research has emphasized for the first time (Section 3), it is not surprising that drug therapy has failed. Furthermore, we know of no effort to develop drugs specifically designed to protect dendrites and axons after injury, the sites where, in our opinion, extremely significant damage occurs and where neuroprotection is vitally needed. The Prinicipal Investigator of this project does not think that "neuroprotection" will be a reality unless dendrites and axons are protected.

Drugs intended to improve outcome after brain injury could exert their effects either by saving tissue from damage (i.e. by neuroprotection) or by enhancing brain plasticity (i.e. by aiding and augmenting naturally occurring brain recovery mechanisms). Since our brain injured rats make a remarkable neurobehavioral recovery by themselves, often with severe sensorimotor mantle loss, (Figures 3 and 4) we feel that plasticity is the major factor in recovery and plasticity enhancement may be the most important effect of any future drugs to improve behavior after brain injury.

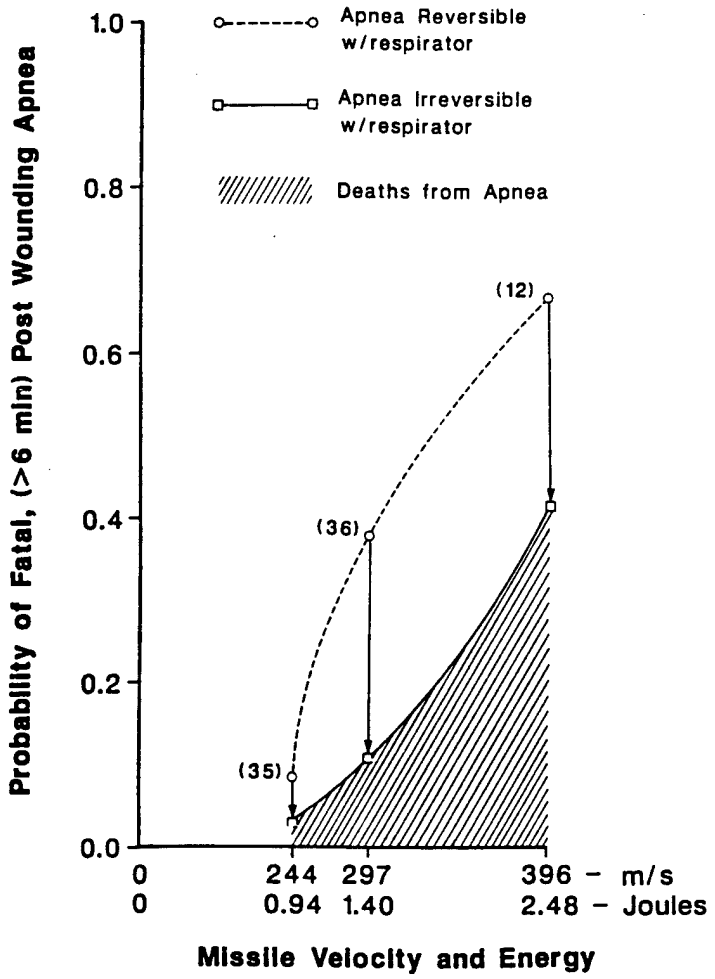
Using our model in the future, the effect of drugs on dendrite damage could be very accurately assessed by quantitative dendrite counts (Scholl method) and by dendritic spine counts. Axon damage could be assessed by deOlmos silver stains to note the intensity of axon degeneration after injury and then following drug treatment. These morphological markers could then be correlated with behavior. Similarly, post injury plasticity and the effects of drug treatment on plasticity in adjacent or contralateral homotypic brain could also be measured by quantitative dendrite and spine counts.

7 TRAUMATIC BRAIN INJURY AND APNEA

Introduction

Traumatic brain injury of sufficient force may be accompanied by cardiovascular and respiratory responses even when the trauma does not directly involve the brain stem. Lesser degrees of injury may stimulate the brain stem increasing respiration while more severe injury may depress function of the cardiorespiratory center causing prompt respiratory slowing or even apnea. If the duration of apnea is brief, no untoward results may occur; longer periods, however, might result in generalized hypoxia or may cause death [111-128]. Apnea has been reported after either blunt or penetrating brain injury, both clinically and experimentally [112-114, 120,121,126-131]. In our prior Army contract (DAMD17-86-C-6098) we examined the occurrence of apnea following an experimental missile wound to the right cerebral hemisphere of anesthetized cats.

Figure 58: The Probability of Apnea Following A Brain Missile Wound



Between 0.9 and 2.4 joules of missile energy deposit, the probability of an immediate fatal apnea was proportional to the missile energy deposit within the brain [113,131]. Immediate respiratory support will decrease the probability of apnea but the probability of fatal irreversible apnea (shaded area) rises with increasing missile energy deposit. Irreversible apnea was thought to represent structural damage to brain stem respiratory nuclei.

In the past other investigators have documented various brain stem lesions which could account for post injury apnea including punctate vascular lesions about the floor of the IV ventricle [117] as well as fiber track damage within the medulla [132,133].

Whereas punctate hemorrhages within the IV ventricle could possibly account for immediate apnea owing to their destructive effects upon respiratory neurons, axonal damage as usually described after brain injury may take hours to evolve [134-141] and might not, therefore, be the cause of immediate post injury apnea. Unfortunately, our research on apnea following brain missile wounding was terminated by the animal activists acting through Congress before we could investigate possible medullary structural lesions consequent to the right cerebral hemisphere wound.

Trauma research can no longer be done on cats or dogs owing to the animal activists. In proposing this present contract we had hoped that the rat would prove to be a good substitute for the cat in the study of posttraumatic apnea. Unfortunately, posttraumatic apnea is not as predictably produced in the rat as the cat so we do not feel that the rat model is appropriate for any future studies on apnea. Nevertheless, this model has provided important information which is presented below.

The purpose of this study was to examine the floor of the IV ventricle by scanning electron microscopy (SEM) in search of anatomic lesions in proximity to the medullary respiratory complex which could account for apnea occurring immediately following cortical injury.

Methods and Materials

Animals and Surgical Preparation

We used 26 male Sprague-Dawley rats (300 - 400 g) in this study. Animals were anesthetized with isoflurane (1.5%) with $N_20:0_2/5:1$. Six animals served as controls, the ependymal surfaces of the floors of their IV ventricles serving as normal specimens. The remaining 20 rats were the experimental animals. Twelve received a piston impact injury which depressed the dura 4 mm, 5 rats received a 2 mm dural depression, and 3 rats a 1 mm injury.

Within minutes of impact, each animal was even more deeply anesthetized with

sodium pentobarbital (Nembutal, 50 mg/kg. i.p.) and sacrificed via a thoracotomy and gravity perfusion fixation at a pressure of 120 mm Hg. An initial vascular pre-wash using O.1 M cacodylate buffer containing 0.1 M sucrose and 0.1 % procalne hydrochloride (pH 7.3-7-4) was followed by 3%, biological-grade, glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in O.1 M cacodylate buffer with O.lM sucrose(pH 7.3). Calvaria were then completely excised and skulls were severed and immersed overnight in 3% purified glutaraldehyde in 0.1M cacodylate buffer with O.lM sucrose (pH 7.3) at 4° C. Under 3.5x magnification we then removed the cerebellum and carefully dissected the brain stem from the skull base without distortion and immersed it in fresh 0.1M cacodylate buffer (pH 7.3). Afterwards, without touching the floor of the ventricle, the choroid plexus was removed from each brain using a dissecting microscope and microdissecting instruments. The brain stems were washed for an additional 24 hrs in multiple changes of 0.1M cacodylate buffer.

Scanning Electron Microscopy (SEM)

Each brain stem was postfixed with 2% OsO 0.1 M dehydrated by ethyl alcohol and critical point dried. The dried specimens were then mounted on aluminum stubs, coated in with a thin layer of gold-palladium, and examined in a JEOL T-300 scanning electron microscope at 20 kV (39-41).

Light Microscopy

Lesion sites in the floor of the IV ventricle were resected subsequent to glutaraldehyde fixation dehydrated and embedded in plastic for 200 μ thick sections cut on a Reichert-Jung (Ultracut E) ultramicrotome. Sections were mounted on glass slides and stained with aqueous, 1% toluidine blue.

Results

Control Animals (n=6)

Singh showed that the IV ventricle is rhomboidal in shape and divisible, via a median sulcus, into two symmetrical halves, (Figure 59), [142]. Each half of the ventricular floor is characterized by five ependymal zones which have distinct cell surface architectural features. The most caudal or inferior of these zones, Zone 1, lies adjacent to the median sulcus, rostral to the obex, and rostral yet continuous with the area postrema. The ependymal surface of this zone is characterized by a rather sparse, central tuft of cilia with numerous microvilli, Figure 60A. More laterally, the ependymal cells of Zone 1 are densely ciliated over the entire luminal surface, Figure 60B. Zone 2 lies lateral to Zone 1; its ependymal surface features fewer cilia and microvilli as well as pit-like holes, Figure 60C. Posttraumatic lesions found in this study were confined to Zones 1 and 2 so the histology of Zones 3,4 and 5 will not be disucssed.

Text for Figures 59, 60 pp 102, 103:

Figure 59: (p 102) SEM floor, caudal half of IV ventricle, control rat. Obex marked by *; median sulcus by white arrows; histologic zones numbered. (155x).

Figure 60: (p 103) Higher power SEM, Zones 1 and 2 of a control rat: A- Zone 1: cells have a sparce central tuft of cilia surrounded by numerous microvilli (2,365x); B- Zone 2 (medial): cilia are much denser (2,375x); C- Zone 2 (lateral): fewer cilia and microvilli (2,370x). Bar approximates 5μm.

Figure 59

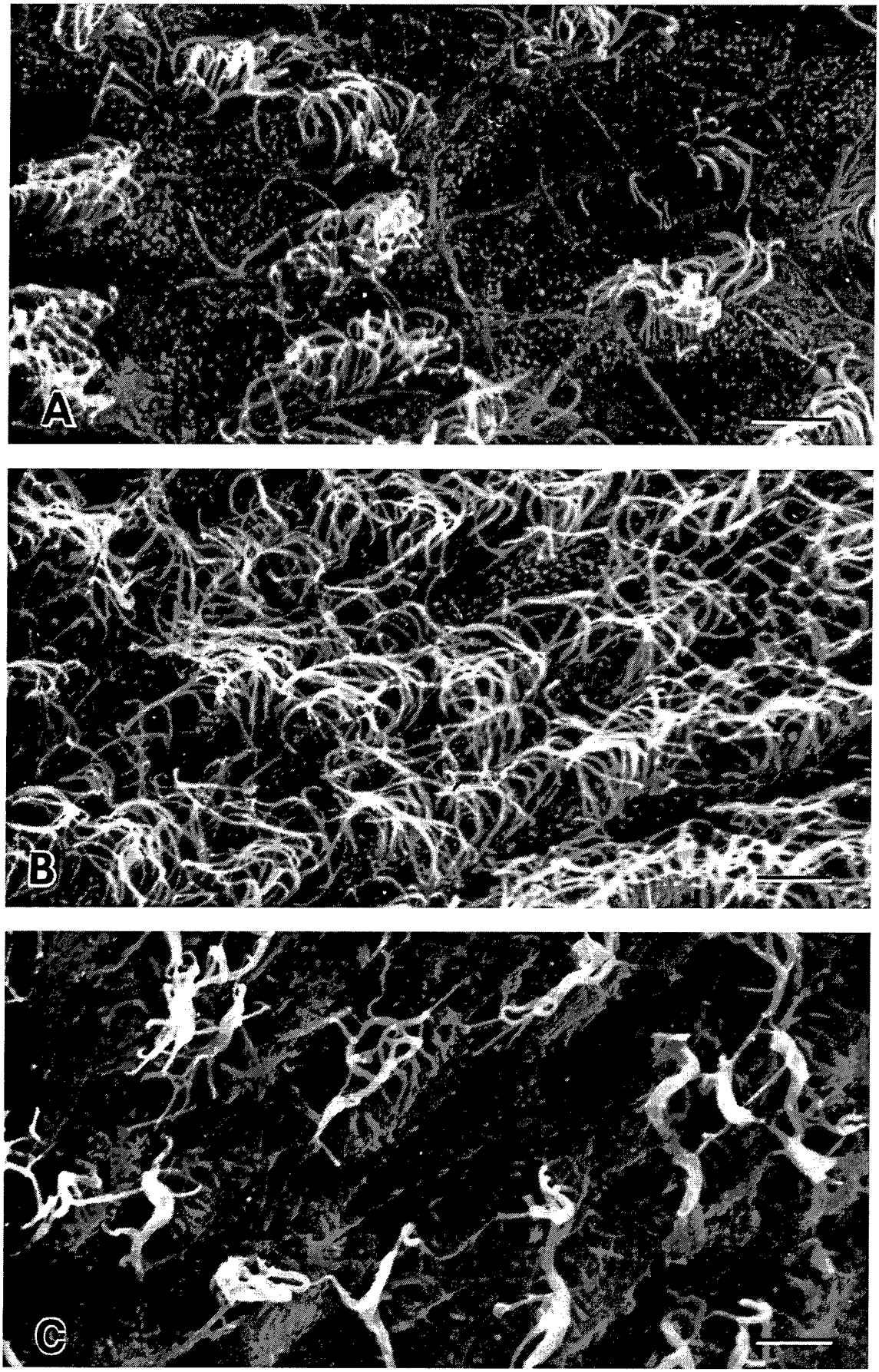


Figure 60

Brain Injured Animals (n=20)

1 mm and 2 mm Dural Impact Depths (n=3 and n=5)

Impacts depressing the dura 1 or 2 mm did not cause any significant respiratory changes and SEM of the IV ventricles from the animals in these two groups revealed no evidence of any disruption of the structural integrity of the ependymal floor. Occasional red blood cells were seen on the ventricular floor, (Figure 61).

Text for Figure 61, p 105:

Figure 61: This rat received a 2 mm cortical impact. No ventricular floor tears occurred in this or any of the 1 and 2 mm impacted animals though scattered red blood cells were generally evident on the IV ventricular floor. A- 2,370x; B- 3,480x. Bar approximates 5μm.

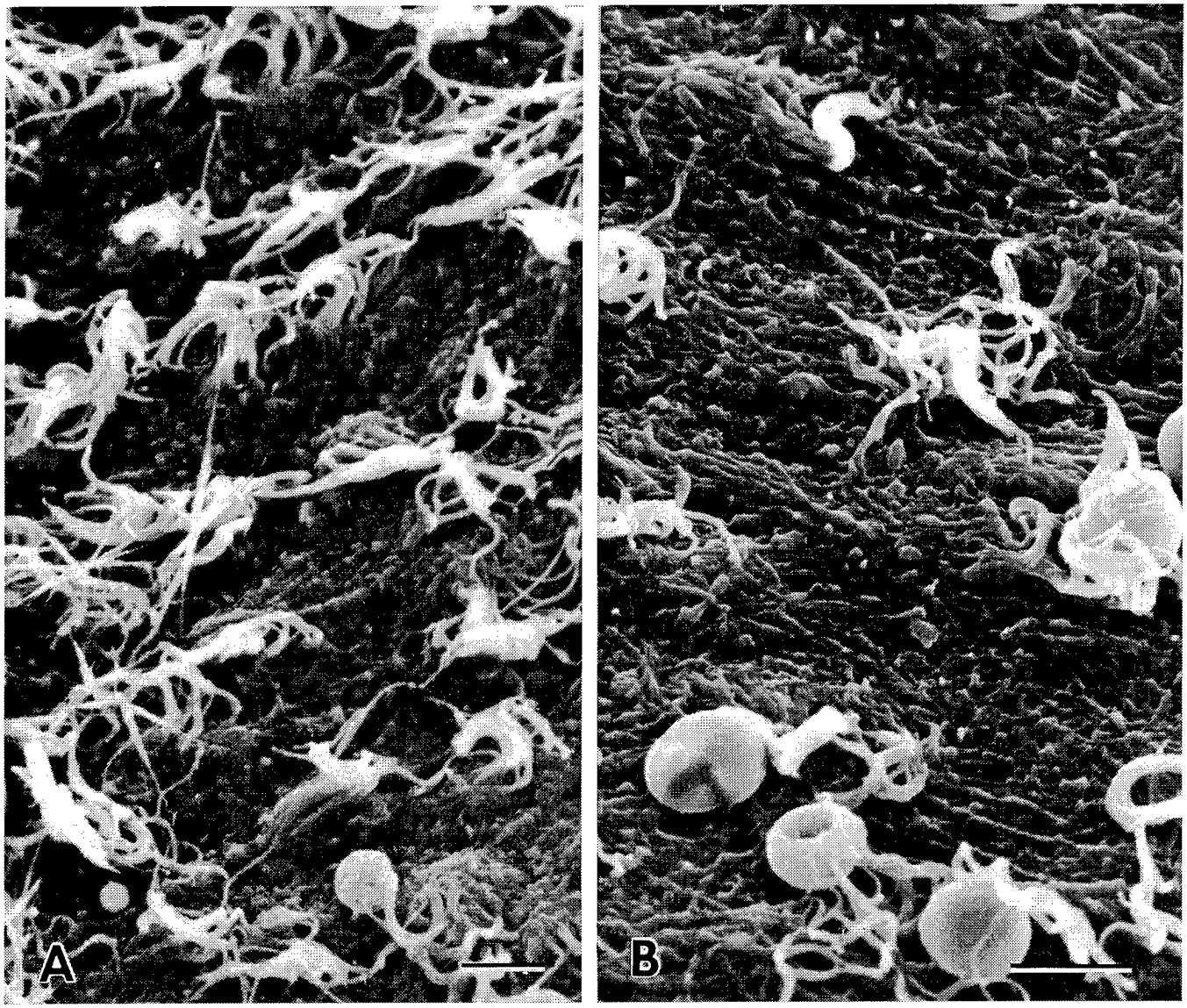


Figure 61

4 mm Dural Impact Depths (n=12)

Eight of the rats in this experimental group exhibited apnea. Four did not breathe for 15 seconds to 2 minutes after piston impact while respirations never resumed in four others. All 12 had subarachnoid hemorrhage extending to the base of the brain and encompassing the surface of the brain stem. No rat exhibited any intramedullary hemorrhage visible at 3.5X magnification. SEM of the IV ventricle floor of every rat showed one or more rostral to caudal, paramedian tears of the ependymal cell surface in Zones 1 and/or 2, (Figures 62,63) Five animals displayed multiple lacerations exposing the underlying medullary neuropil to the lumen of the IV ventricle while 7 displayed a single tear in either Zone I or 2. Light microscopy revealed these tears in the floor of the caudal IV ventricle to be oblique, medial to lateral lacerations into the subjacent medullary core with possible disruptions of interneuronal connections of adjacent reticular fields, (Figure 64). We microdissected the ventral brain stems of five of the 4 mm impacted animals and cleaned the surface of hemorrhage for examination with SEM. We found no evidence of tears on the ventral or lateral medulla, (Figure 65).

Text for Figures 62-65, pp 107-110:

Figure 62: (p 107) This rat did not resume respirations. (Picture is an oblique view, caudal medulla). A- Multiple tears are evident on the ventricular floor. Obex is just to right of asterisk. Tears are evident in lateral Zone 1 (large white arrow head) as well as Zone 2 (white arrow). Fissure marked by arrow courses towards area postrema (255x). B- Note jagged tear in ependymal cell surface membrane beneath cilia (7,250x) bar ~2μm. C-Tear in ependymal surface showing underlying neuropile (2,850x) bar ~5μm.

Figure 63: This rat did not resume respirations. A- A single paramedian tear (large white arrow) is evident on the IV ventricular floor. Small white arrow heads mark median sulcus. Asterisk marks caudal IV ventricle near obex, (120x). B-higher magnification of tear showing torn edges of ependymal surface (arrow) and exposed medullary neuropil, (800x).

Figure 64: This animal did not resume respirations. Section of caudal medulla showing floor of IV ventricle and oblique tear on floor through Zones 1 and 2. Open arrows: direction of median sulcus. Black arrow head: luminal surface; curved black arrows; course of fissure in medulla with interruption of intramedullary axons. A (50x) B (85x).

Figure 65: Ventral surface of brain stem showing no evidence of fissuring. Basilar artery indicated by asterisk. Cut nerve fibers on right (black arrow); (55x).

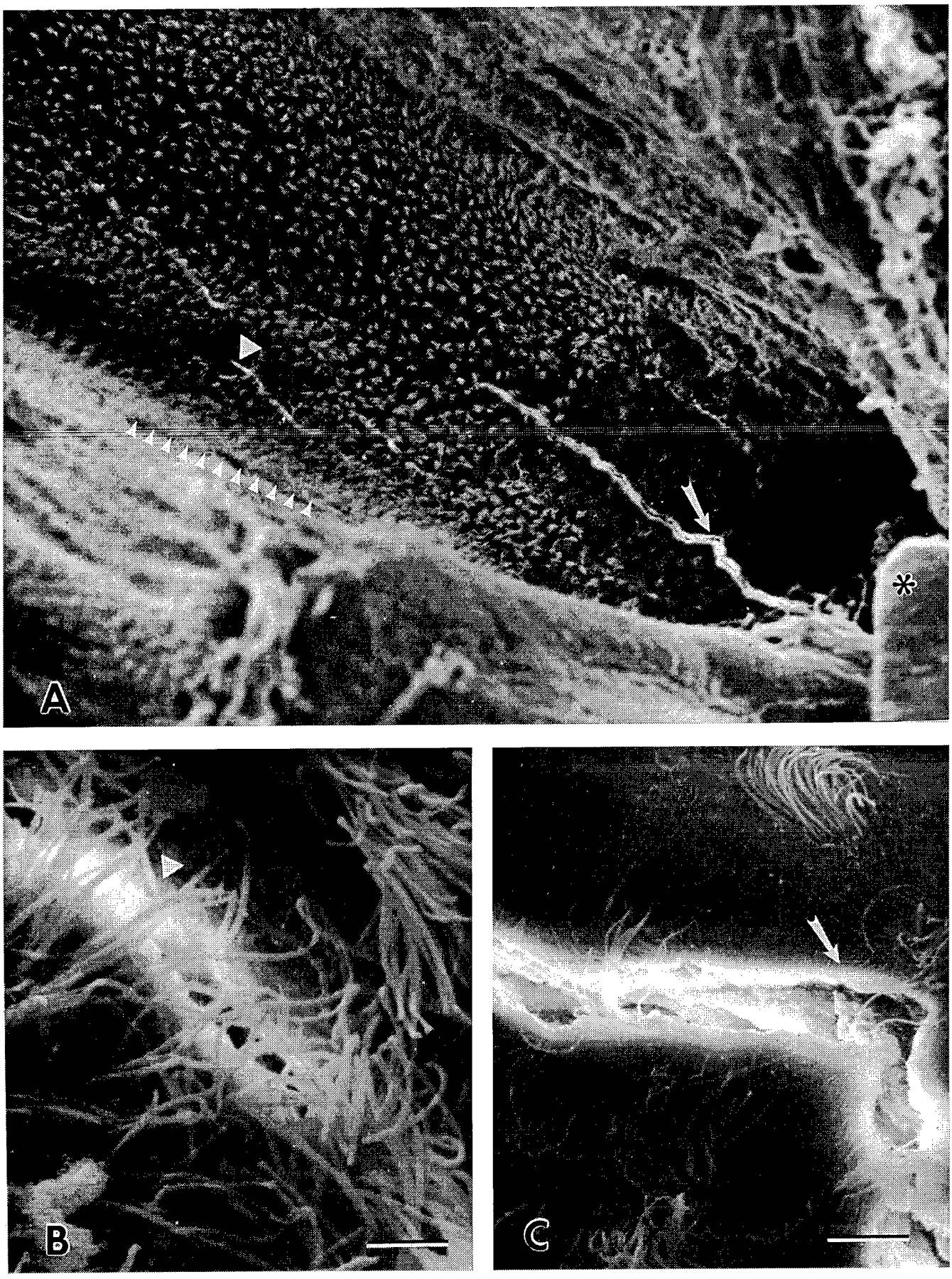


Figure 62

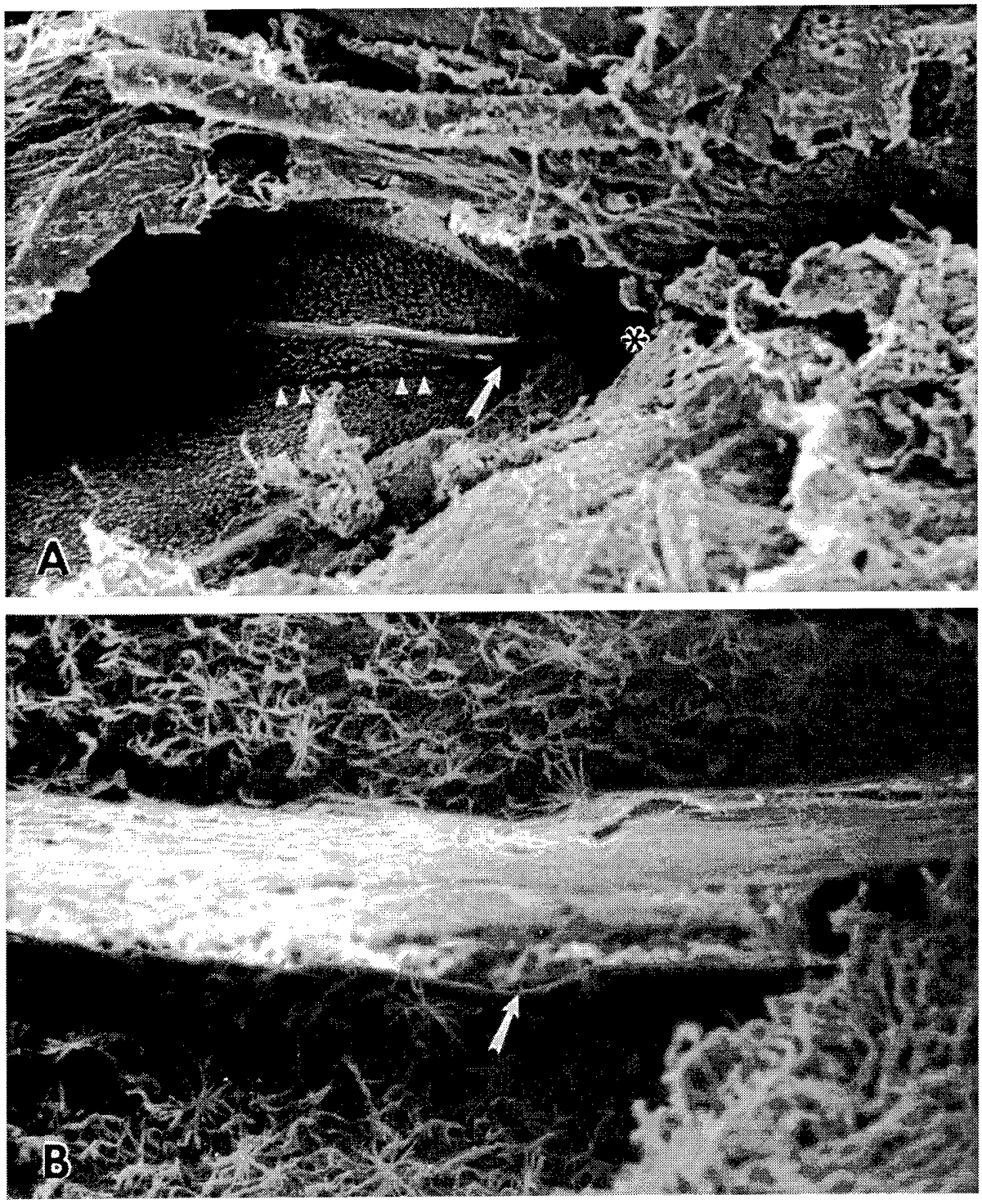


Figure 63

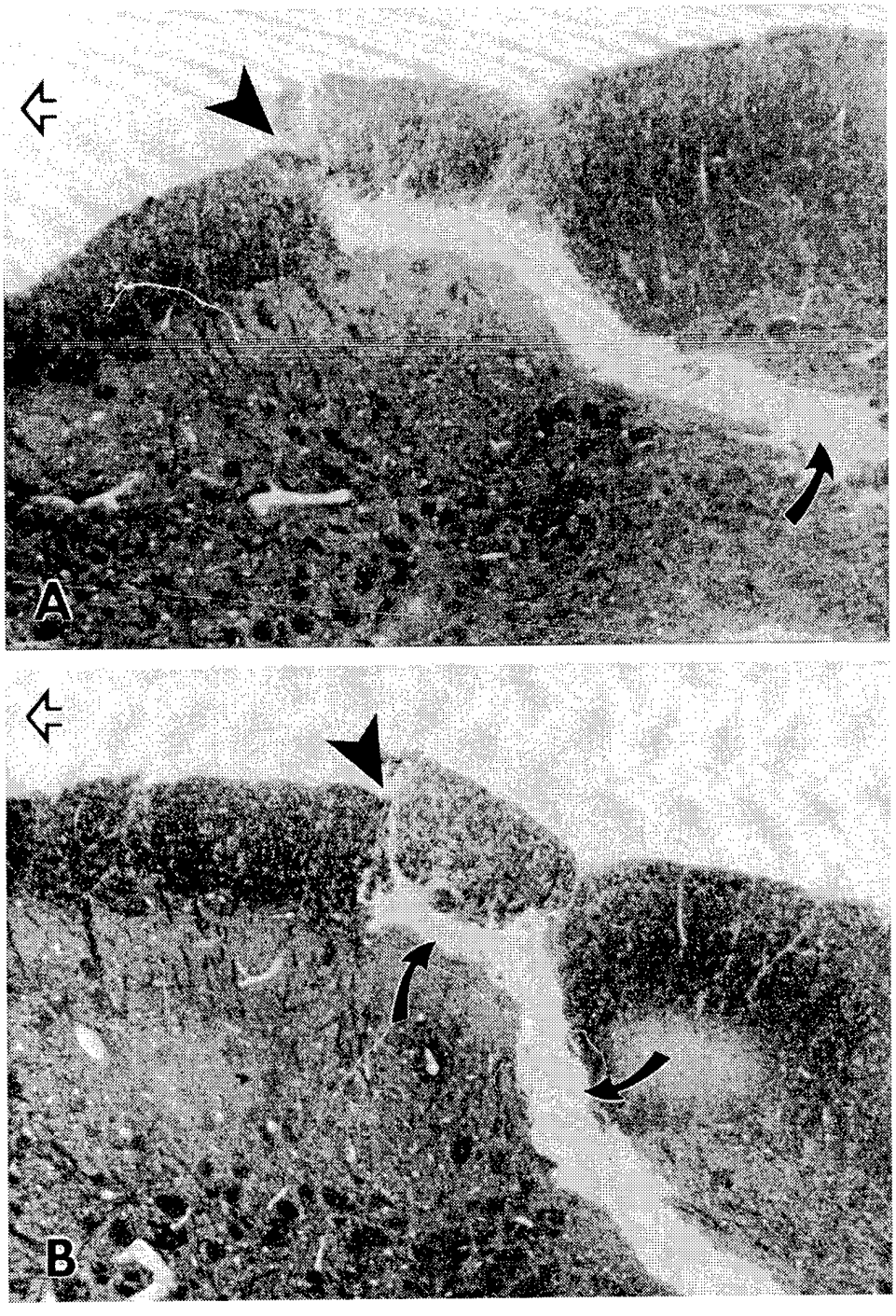


Figure 64

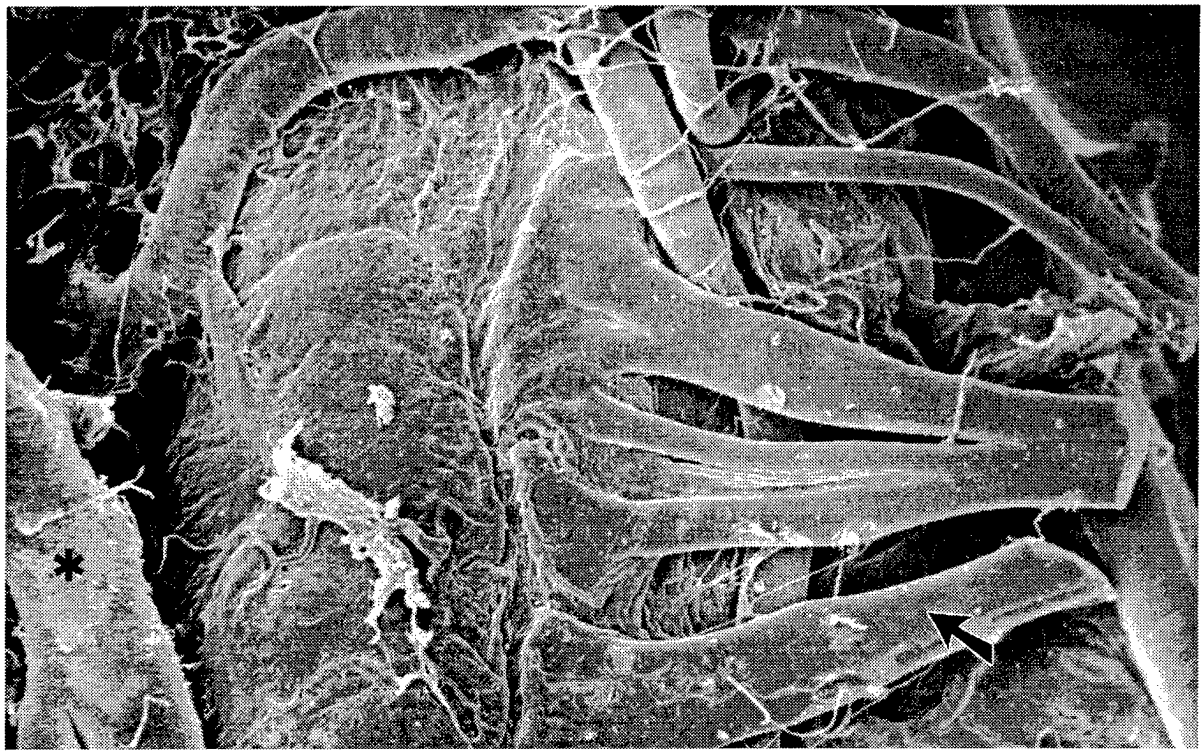


Figure 65

Discussion

The etiology of cardiorespiratory effects following traumatic brain injury has been studied for more than 100 years. For instance, Duret [117] noted the occurrence of petechial hemorrhages about the cerebral aqueduct and the floor of the IV ventricle with injuries which displaced the cerebral cortex. He proposed that injuries which deform the cranial vault and brain precipitated a forced flow of CSF through the cerebral aqueduct into the IV ventricle which impacted its floor and caused punctate hemorrhages on the ventricular floor as well as within the medulla. He noted that the force of such a surge could even tear the arachnoid about the foramen of Magendie. This hydrodynamic effect was referred to a "cerebral-spinal shock" and was associated with respiratory irregularities or apnea. Extensive white matter degeneration of nerve fibers as a result of shear stress and strain have been reported for both the cerebral hemisphere and brain stem as a result of head injury in humans [132]. An earlier study of experimental brain injury demonstrated a progressive chromatolysis of certain neurons 24 hrs post injury accompanied by varying amounts of axonal swelling especially in the caudal medulla and cervical spinal cord [133].

Bakay et al [129] studied the brain stem with electron microscopy following experimental brain injury and reported that within 1 hour of injury swollen mitochondria and hypertrophied extracellular spaces occurred in the medulla and in the upper spinal cord. Six hours after injury the myelin sheaths of axons had a "ruffled" appearance suggestive of a structural alterations. Subsequent studies of brain stem effects associated with head injury have been found to include macrohemorrhages, ischemic necrosis, and chromatolysis in reticular neurons of the medulla as well as scattered neuronal and axonal damage in other areas of the brain stem [114-116, 118-128, 143-145].

Earlier studies hypothesized that brain stem axonal damage occurred from immediate shear forces because of brain stem distortion following brain injury [114,120,121,127,128,132,143,146]. More recent studies, however, suggest instead that axoplasmic transport is impaired by neurolemmal infolding and neurofilament changes consequent to injury. Proximal axoplasmic flow buildup then bursts the axons proximal to the neurolemmal damage about 6 hours post injury [134-141, 147]. While it could be argued that neurolemmal or other axonal changes occurring with injury make the axon immediately dysfunctional, a process that takes 6 hours to evolve histologically does not readily explain a hallmark of severe head injury: immediate respiratory dysfunction or

apnea.

In the present study, animals were sacrificed within minutes of brain injury. Experimental animals receiving the 1 and 2 mm impacts did not manifest any respiratory abnormalities and exhibited no morphologic changes in the IV ventricle. Eight of 12 rats injured by a dural depression of 4 mm -impact depths demonstrated respiratory abnormalities including permanent apnea and all 12 exhibited tears of the IV ventricular floor near the obex. This suggests that a strong force impinged on the ependyma lining the floor of the caudal part of the ventricle. A surge of CSF out of the aqueduct onto the ventricular floor as Duret [117] proposed would not be an unreasonable supposition to account for such damage. The hydrodynamic effect of the injury appeared to be proportional to the severity of the cortical impact and depth of cortical depression in that no ventricular changes were evident when the sensorimotor dura was depressed 1 or 2 mm while all 12 of the animals whose dura was depressed 4 mm had defects in the floor of the IV ventricle. In the rat medulla, a dorsal group of respiratory neurons is located subjacent to the floor of the IV ventricle and would be susceptible to any of the lesions demonstrated in this study. Saether et al [148] reported that these neurons are largely (75%) inspiratory and are located within 2 mm of the opening of the central canal of the spinal cord at the obex. They lie from 0.5 mm to 2 mm below the surface of the ventricular floor [149,150] in Zones 1 and 2, where we have demonstrated posttraumatic ependymal tears. Mild interference with these inspiratory neurons could well cause respiratory irregularities while more severe disturbances or frank disruption of the neuron or their axons or dendrites could result in apnea.

The piston impact injury model we used is not a closed head injury but does somewhat resemble a missile injury model in that both produce a brief overpressure and sudden brain displacement. The resultant ventricular compression could cause a sudden, forceful flow of CSF through the cerebral aqueduct.

It could also be argued that supratentorial overpressures from missile wounding as well as the 4 mm dural depression are both capable of displacing the entire brain caudally and that any axonal neurofilament injury or shearing observed could be the result solely of such brain stem displacement. Despite the difficulty of examining the ventral and lateral sides of the medulla owing to hemorrhage in the subarachnoid space, these surfaces were examined and never appeared to be cracked or torn as was the floor of the

IV ventricle. If in severely injured rats, two-thirds of which demonstrated post injury respiratory dysfunction, brain damage occurred solely from a rostral - caudal displacement of the medulla, one might expect to have evidence of the surface damage from such stress to be more evenly distributed over the surface of brain stem. In our study, however, the floor of the IV ventricle appeared preferentially damaged with the lesions localized in sites about the obex and the area postrema just dorsal to the respiratory complex. This zonal damage on the floor of the ventricular floor may well reflect increased CSF flow or a transmitted pressure surge. The confinement of damage to Zones 1 and 2 may be a function of the geometry of the anatomical region making it the area of maximal destruction. The present findings do not discount posttraumatic neurofilament damage or axonal shearing within the brain stem. In fact, our light microscopy confirms the possibility of such an occurrence.

This study demonstrates that with severe brain injury, tears occur in the floor of the IV ventricle near respiratory nuclei which may well explain not only the occurrence of immediate posttraumatic apnea but why it may be irreversible in some instances. These studies do not duplicate a human closed head injury but do suggest that it may be worthwhile to evaluate the IV ventricular floor near the obex in people who die abruptly following a closed head injury. The occurrence of frank disruptions of respiratory nuclei would mean that respirations could not be restored by cardiopulmonary resuscitation in some brain trauma victims including those wounded by missiles.

8 THE EFFECT OF HYPOXIA AND HEMORRHAGE UPON RECOVERY FROM BRAIN INJURY

Clinically, if hypoxia or hypotension follow brain injury, subsequent mortality and morbidity increase 2 to 4 times [151-155]. Despite the importance of these so-called "secondary insults" for those with brain trauma, very little laboratory research has been done to ascertain the exact pathophysiological mechanisms which hypoxia and hypotension provoke after brain injury so their effects can be ameliorated.

Experimentally, hemorrhagic hypotension following brain injury produces a prolonged reduction of cerebral oxygen delivery despite normalization of systemic oxygenation [156]. Bilateral carotid artery occlusion leading to cerebral ischemia in rats normally produces no hippocampal cell loss. If such occlusion is coupled with systemic hypotension significant hippocampal cell loss ensues [157].

Since the soldier is exposed to extreme conditions in combat where hypoxia or hypotension may complicate brain injury, we thought it important to begin systemic research on these problems. We secured 3 years of concomitant funding from the Joe W and Dorothy Dorsett Brown Foundation of New Orleans to begin this work. These additional, related experiments, carried out jointly with this present DAMD17-93-C3008 contract, were done by a Brown Foundation-funded technician. The Brown Foundation has provided an additional $200,000 worth of research for this model of brain injury to help the soldier in combat.

Though we were successful in developing both the brain injury-hypoxia and the more demanding brain injury-hypotension models, we eventually concentrated on the brain injury plus hypotension model because Bellamy has determined that hemorrhage is "the greatest threat to life on the battlefield" causing about 50% of all deaths in combat [2].

Methods

1. Brain Injury Plus Hypoxia

Anesthetized rats received the standard brain injury. Immediately thereafter they were subject to a lower oxygen tension by reducing their inspired oxygen for 20 minutes.

After this, normal oxygenation was restored and the animals allowed to awaken. In initial experiments the rats' PaO_2 was lowered to 45 mmHg by adjusting the inspired gas mixtures and kept at this level for 20 minutes. This injury-hypoxia combination caused subsequent behavior scores to be suggestively reduced. Therefore, another group of animals was tested where the PaO_2 was lowered to 23 mmHg for 20 minutes after injury. This injury-hypoxia combination caused a significant decrement in subsequent behavior testing.

In these experiments we used 6 groups of rats with 12 rats in each group:

Controls
Injured only
Hypoxia only (PaO_2 45 mmHg)
Hypoxia only (PaO_2 23 mmHg)
Injured + hypoxia (PaO_2 45 mmHg)
Injured + hypoxia (PaO_2 23 mmHg)

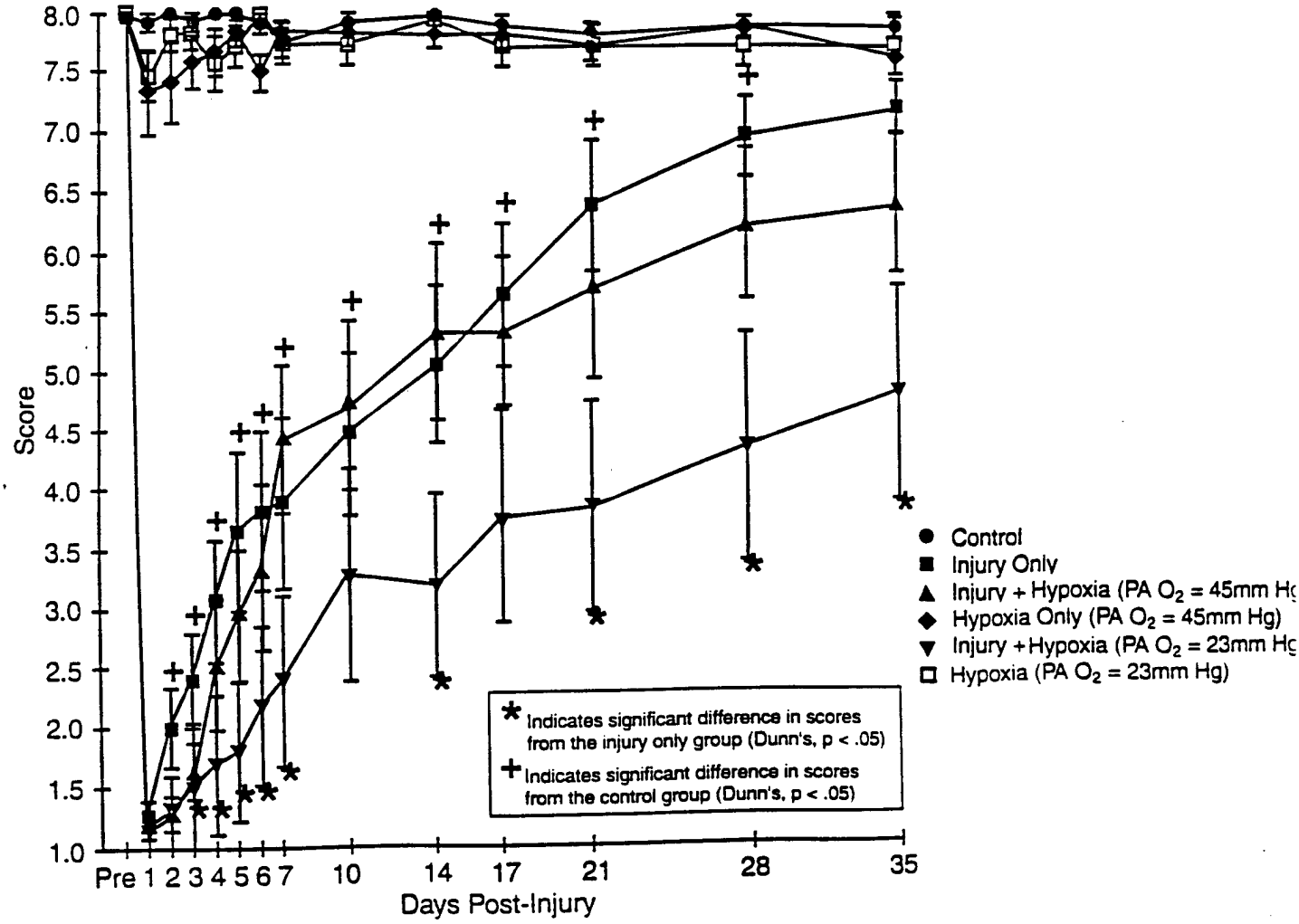
We measured behavior after injury-hypoxia by the flat beam, pegged beam, wire grid, and paw preference tests.

Statistical analysis was accomplished by using ANOVA plus the Tukey test to evaluate performance on the pegged beam, wire grid, and paw preference tests. Flat beam performance was measured by the Kruskal-Wallis one way analysis of variance by ranks with post hoc comparisons by Dunn's test.

Results

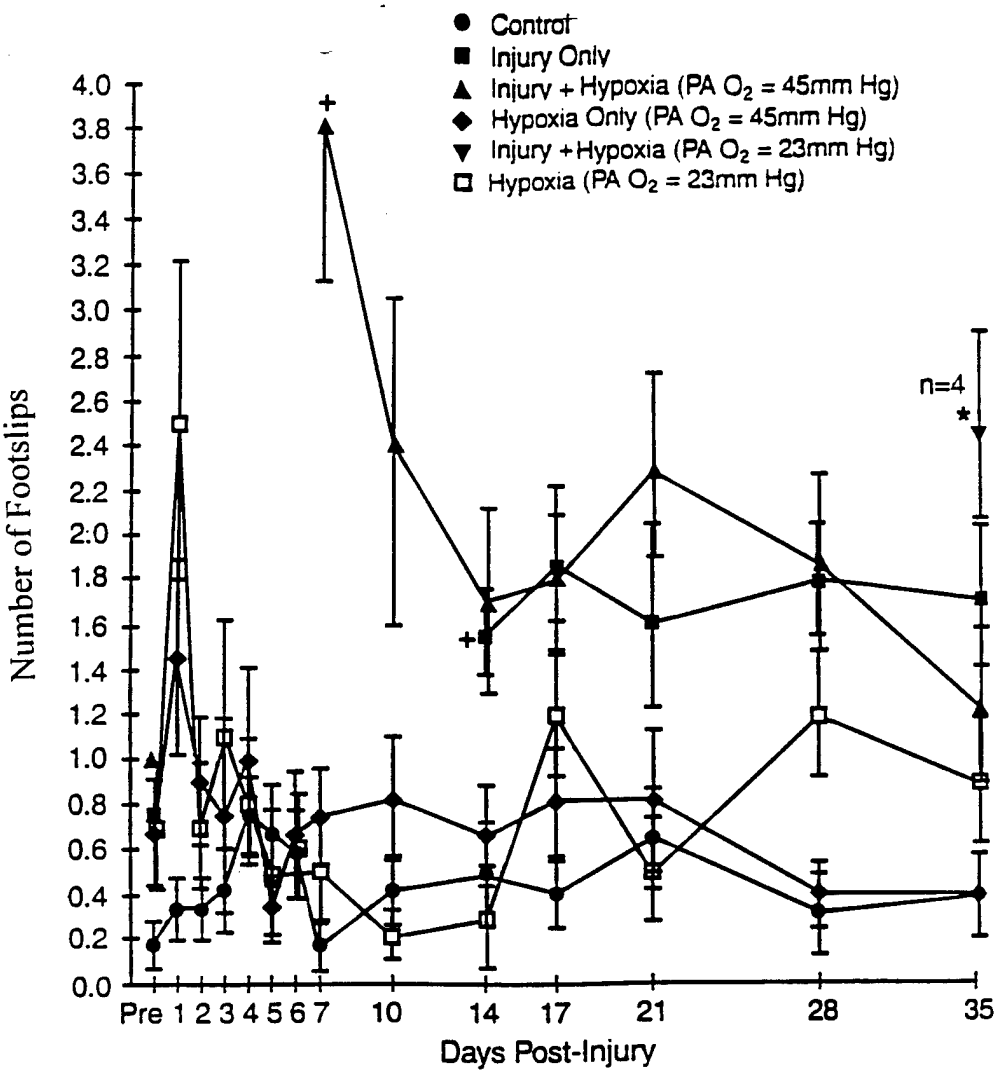
1. Brain injury plus hypoxia

Figure 66: The Flat Beam Test



Flat beam: Hypoxia alone which reduced PaO_2 to either 45 or 23 mmHg for 20 minutes produced no behavioral effects. As expected, normoxic brain injured rats (■) had significantly reduced behavioral scores. Brain injured rats which had their PaO_2s lowered to 45 mmHg (▲) performed essentially as normoxyc brain-injured rats. If brain injury was followed by hypoxia such that the PaO_2 was 23 mmHg for 20 minutes (▼), behavioral scores were significantly lowered for the entire 35 day observation period.

Figure 67: The Pegged Beam Test

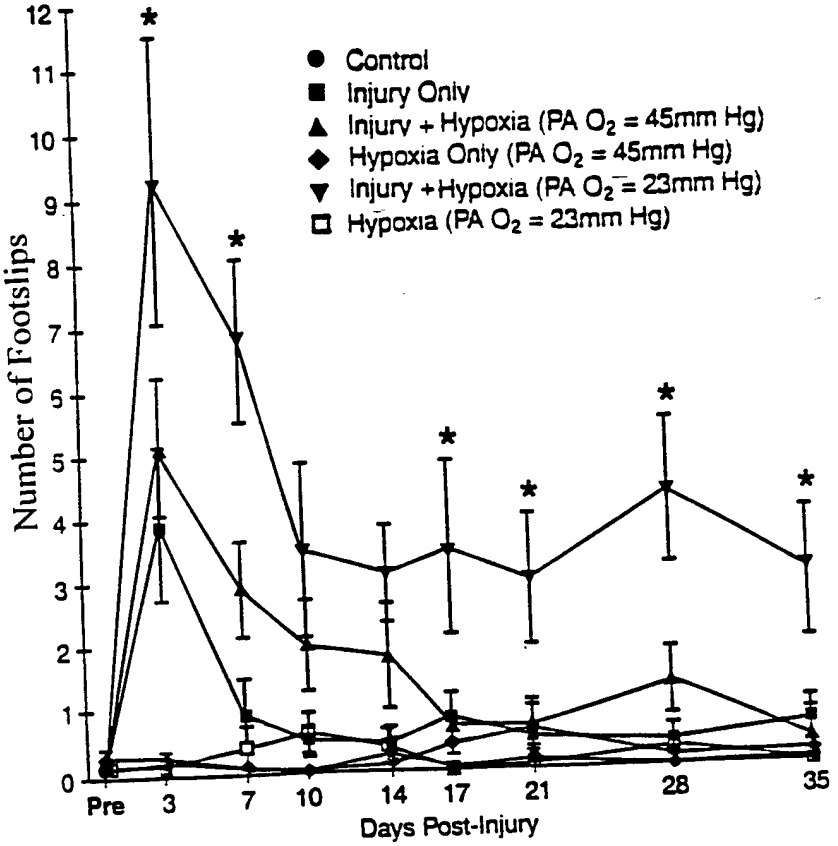


* Indicates significant difference in start day compared to the injury only group (Tukey, $p < .05$)

+ Indicates significant difference in start day compared to the control group (Tukey, $p < .05$)

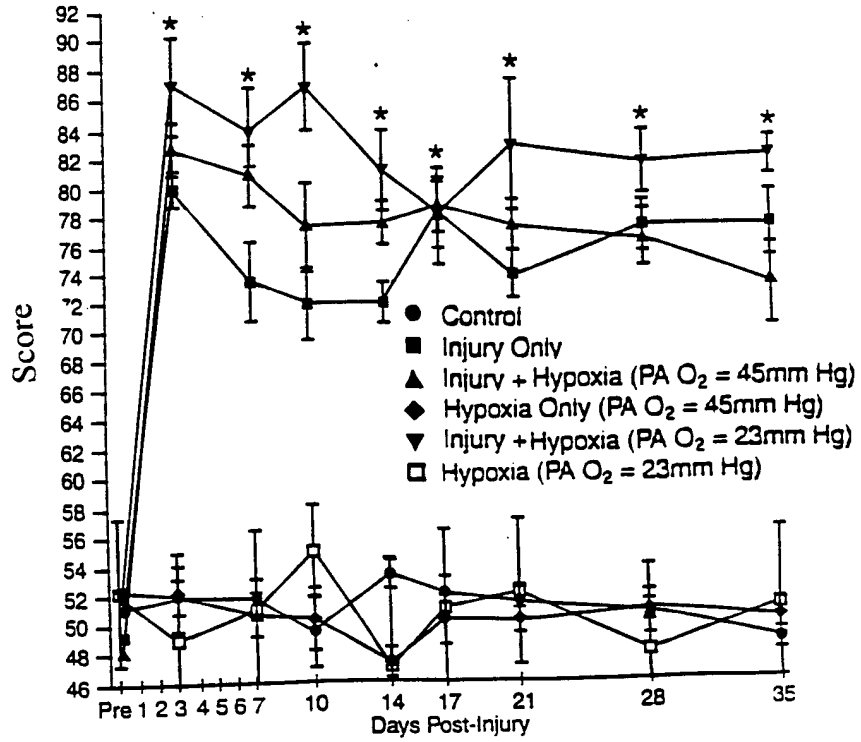
Pegged Beam: Hypoxia alone (PaO_2 either 45 or 23 mmHg for 20 minutes) caused no behavioral deficits. Reducing PaO_2 to 45 mmHg for 20 min (▲) after brain injury did not affect performance; in fact these animals could perform the pegged beam test sooner than the injured, normoxyc rats. Hypoxia which reduced the PaO_2 to 23 mmHg after injury (▼) severely and significantly impaired the rats' ability to begin negotiating the pegged beam. Only 4 of 12 rats could complete this task by day 35. Though these 4 rats made more footslips than the normoxyc injured rats at this time their increased number of errors was not significantly increased owing to the small number of testable rats in the experimental group (n=4).

Figure 68: The Wire Grid Test



Wire grid: Reduction of the PaO_2 to 23 mmHg for 20 min after injury caused the rats to make 6 to 7 times the number of footslips as normoxyc, brain injured rats. (*= significant differences in scores compared to injury only group, Tukey, $p<.05$)

Figure 69: The Paw Preference Test



Paw Preference Test: Hypoxia did not produce a deleterious effect on paw preference beyond that of injury alone because injury alone maximized use of the unaffected paw. (*= significant difference in scores between injured and uninjured groups, Tukey, $p<.05$)

2. Brain Injury Plus Hemorrhagic Hypotension

Preliminary Tests

Since post injury behavioral testing on these rats was essential, we could not measure blood pressure via femoral artery cannulation for any experimental rats because limb ischemia might result which would interfere with subsequent testing. Tail cuff blood pressures were consistent with femoral arterial recordings at normal blood pressures but erroneous at low blood pressures. To circumvent this problem we measured systemic arterial blood pressure in more than 20 rats subject to exsanguination and determined that when 5-6 ml of blood were withdrawn the systemic blood pressure invariably fell to between 40 and 50 mmHg. We felt confident, therefore, that removing this amount of blood in our experimental animals would produce systolic blood pressures below 50 mmHg in virtually all rats even though we did not directly measure this.

In initial experiments blood pressure was reduced by bleeding to 40-50 mmHg systolic for 10 minutes after brain injury. This shed blood was then reinfused. Subsequent behavior testing scores were reduced but not significantly so. Consequently, another group of rats was subject to 20 minutes of hemorrhagic hypotension. After this interval the shed blood was reinfused. This injury-hemorhagic hypotension combination produced significant reductions in subsequent neurobehavioral test scores.

Method

In these experiments we also used 6 groups of rats, 12 rats per group.

1. Control
2. Injury only
3. Hypotension 10 min
4. Hypotension 20 min
5. Injury + 10 min hypotension
6. Injury + 20 min hypotension

For these experiments each rat was anesthetized and had a jugular vein cannula

inserted. Control rats had a craniectomy. After this their scalp was closed and the jugular vein cannula tied off and the neck incision closed before allowing the rat to awaken. Rats only made hypotensive were treated as above but had 5-6 ml of blood withdrawn from the jugular vein cannula. The shed blood was kept warm in a heparinized syringe for either 10 or 20 minutes and then reinfused. Afterwards the jugular vein cannula was removed, all skin incisions closed and the animals allowed to awaken. Rats subject to brain injury plus hemorrhagic hypotension underwent the usual surgical preparation (craniectomy) and had a jugular vein catheter placed for blood withdrawal. Immediately after injury 5-6 ml of blood were withdrawn via the jugular vein cannula into a warmed, heparinized syringe. This simulated a soldier getting a brain wound plus hemorrhagic shock from another wound. After either 10 or 20 minutes of hemorrhagic shock the warmed, shed blood was reinfused, the jugular catheter was tied off, all wounds were closed and the rats allowed to awaken.

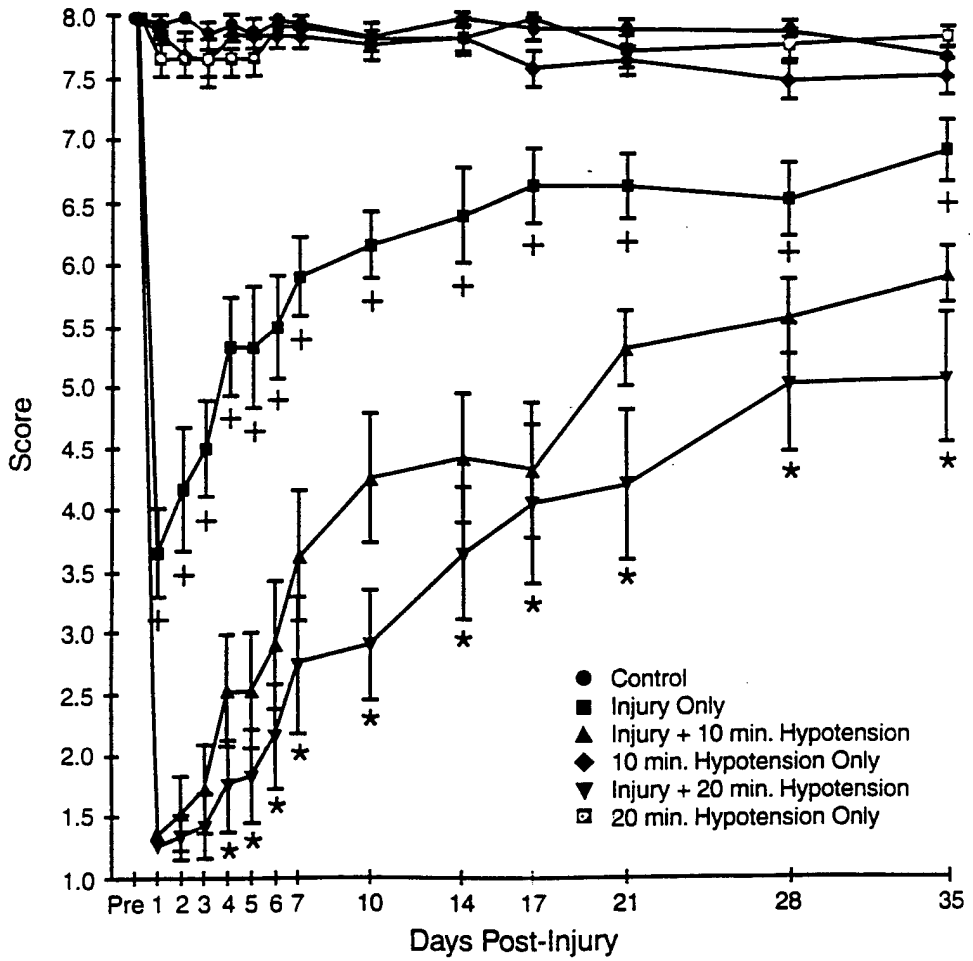
Sensorimotor function was subsequently tested in all groups of rats by the flat beam, pegged beam, wire grid and paw preference tests.

Histological correlates were ascertained by Dr. Hector LeBlanc, LSU neuropathologist, and fiber track degeneration stains were done by Neuroscience Associates, Knoxville TN (see Section 3 for tissue handling details).

Results

Hypotension alone for either 10 or 20 minutes did not affect performance on any test.

Figure 70: Flat Beam Test

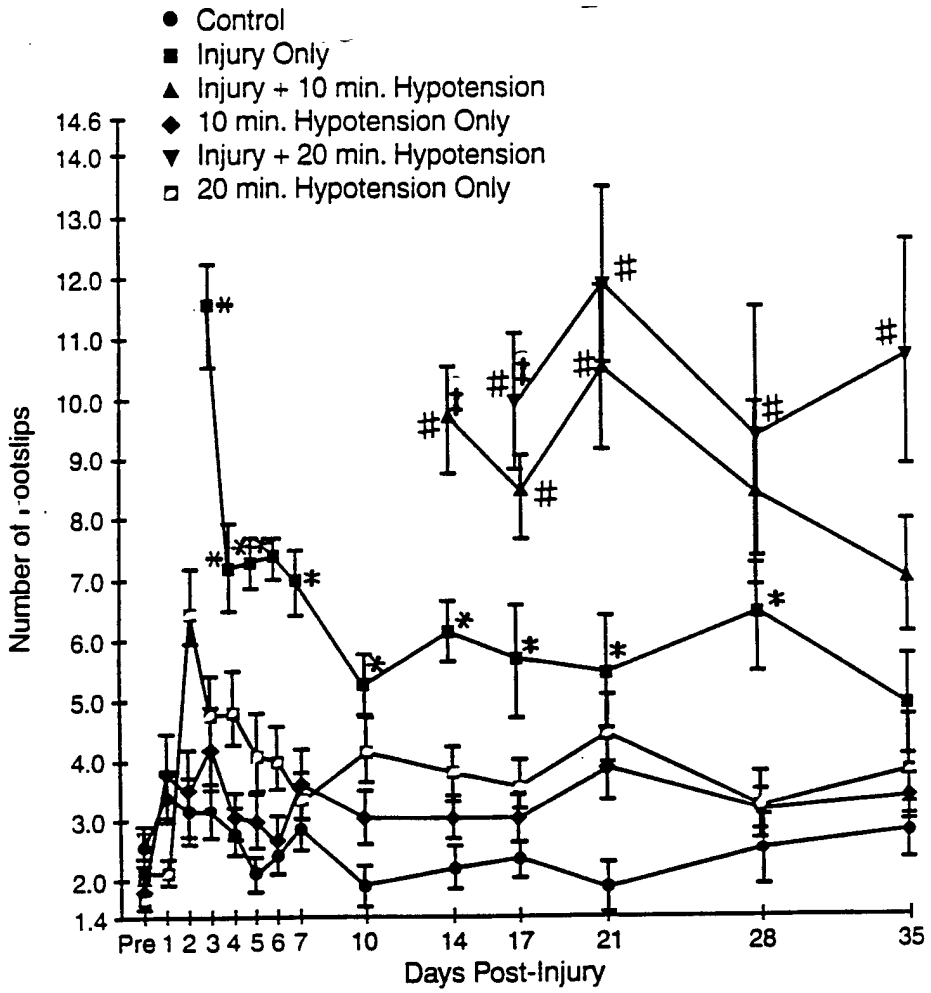


*Significant differences in scores as compared to injury only group (Dunn's, $p<.05$)

+Significant differences in scores as compared to control group (Dunn's , $p<.05$)

Flat Beam: Whereas the performance of brain injured rats made hypotensive for 10 minutes immediately after injury was suggestively lowered, it was not statistically worse than normotensive, brain injured rats. If brain injury was followed by 20 minutes of hypotension, however, flat beam scores were significantly lowered.

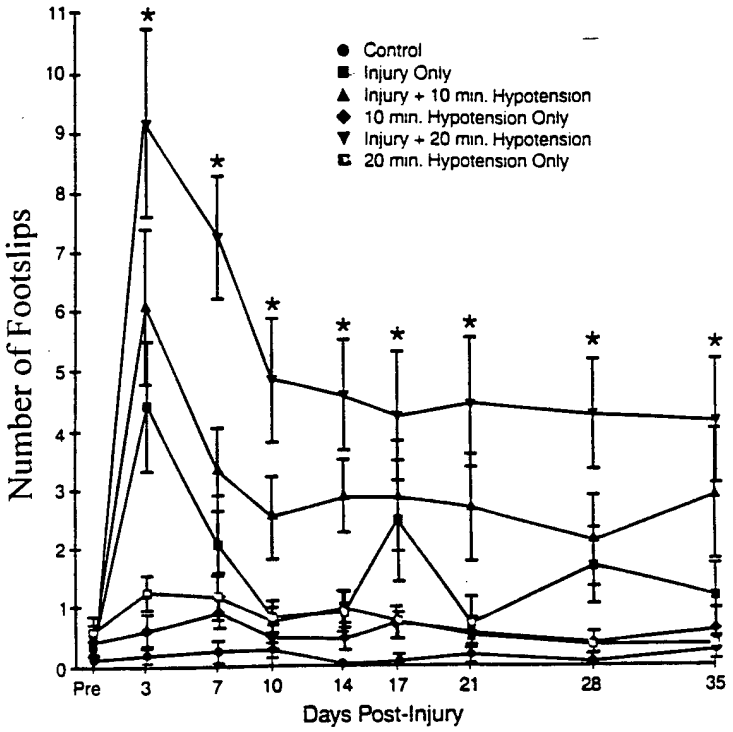
Figure 71: Pegged Beam test



*-indicates significant differences from the control group (Tukey, $p<.05$).
#-indicates significant differences from the injury only group (Tukey, $p<.05$).
‡-indicates significant differences in test start day compared to injury only group (the first day the animal could complete the test) (Tukey, $p<.05$).

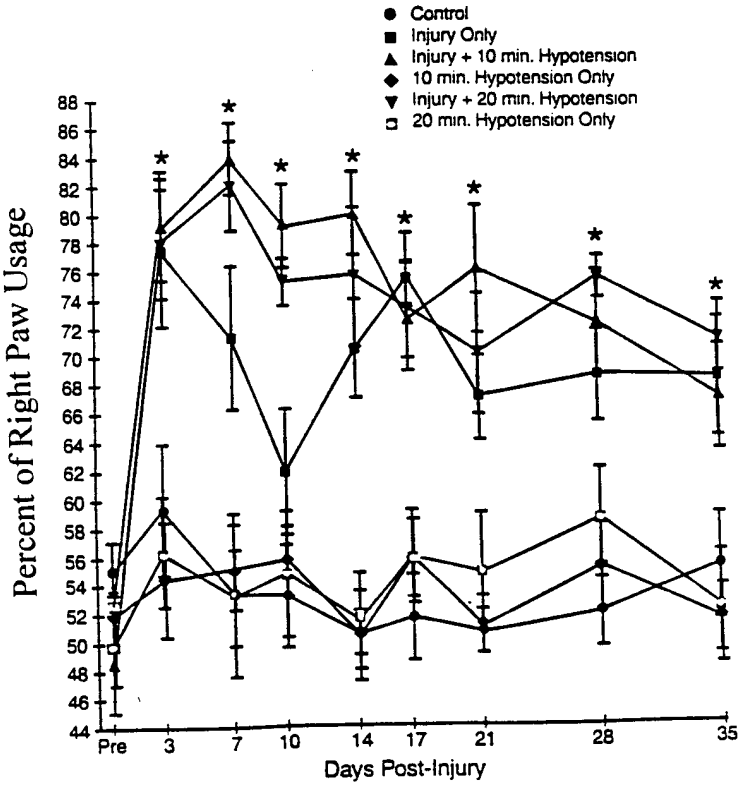
Pegged Beam: Animals subject to either 10 or 20 minutes of hypotension after brain injury were significantly delayed in their ability to perform this test. Normotensive injured animals could navigate the pegged beam on day 3 while animals made hypotensive for 10 minutes could begin this test only on day 14; those hypotensive for 20 minutes could begin on day 17. By day 35 rats made hypotensive for 10 minutes after injury made about 50% more footslips than injured, normotensive rats (not significant). At 35 days, rats which were hypotensive for 20 minutes made more than twice as many footslips as the injured normotensive rats, a significant difference. Furthermore, there was no evidence that the number of footslips was decreasing over time in this group.

Figure 72: Wire Grid Test



Wire grid:
Rats which sustained a 20 minute hypotensive episode always made significantly more footslips than normotensive injured rats. (*=significant differences in scores as compared to injury only group, Tukey, $p<.05$)

Figure 73: Paw Preference Test



Paw Preference Test:
As in the hypoxia study, hypotension did not add to the percent right paw use because injury alone caused maximal shift to the right paw. (*= significant differences between brain and injured groups as compared to non-brain-injured groups, Tukey, $p<.05$)

Histologic Correlates of Brain Injury Plus Hemorrhagic Hypotension

Evaluation of H and E stained brains from 6 rats subject to injury alone or injury plus hemorrhagic hypotension at 24 hours and 5 weeks after this trauma failed to reveal any consistent cellular findings that could be construed as being caused by the added hypotension.

DeOlmos fiber track degeneration studies of 4 injured-hypotensive rats are presented in Figures 74,75,76 and should be compared to such studies in brain injured, normotensive rats, Figure 4 and Appendix 1. Mantle loss appears variable in either injured-normotensive or injured-hypotensive rats. While there is a suggestion that mantle loss was greater in the injured-hypotensive rats, in some of these rats it was quite well preserved. Conversely, some normotensive injured rats also had severe mantle loss. The pattern and intensity of degeneration of sensorimotor projection and corticofugal fibers appear very similar between the brain injury normotensive and hypotensive groups.

Discussion

Neither histologic nor axon degeneration studies provide a ready explanation why the secondary insult of hypotension so adversely affected recovery from brain trauma which our neurobehavioral tests so definitively demonstrated. Hypotension following brain injury did not appear to increase the extent of tissue damage nor the extent of degeneration within corticoprojection or corticofugal sensorimotor fibers. As stated in section 6 we feel that post injury plasticity mechanisms are the major factors in neural recovery after brain injury. Since hypotension following brain injury does not appear to increase the extent of damage **we hypothesize that the secondary insult of hypotension (and hypoxia) following brain trauma impairs recovery by affecting brain plasticity mechanisms which underlie normal post injury adaptation/recovery.**

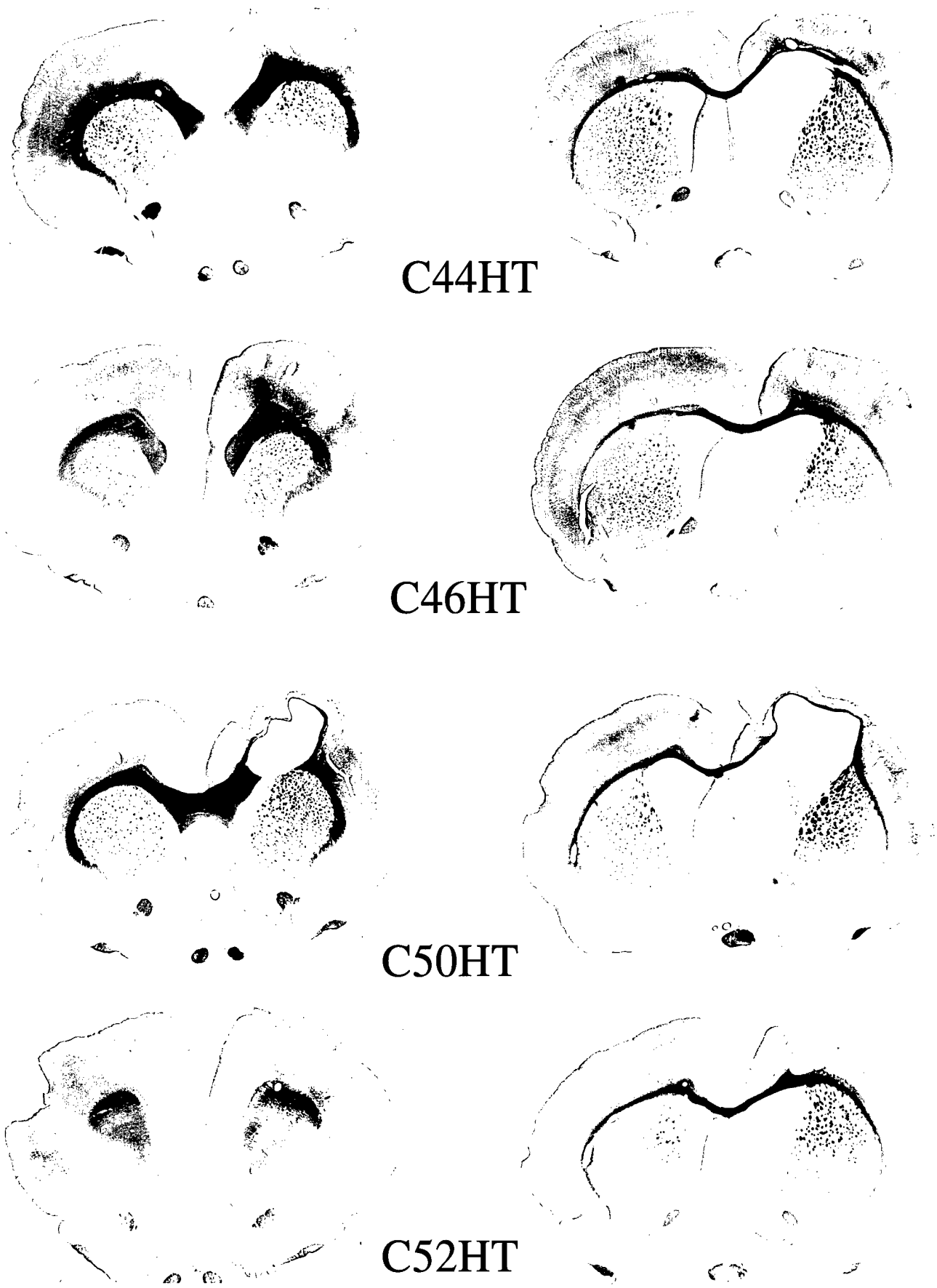
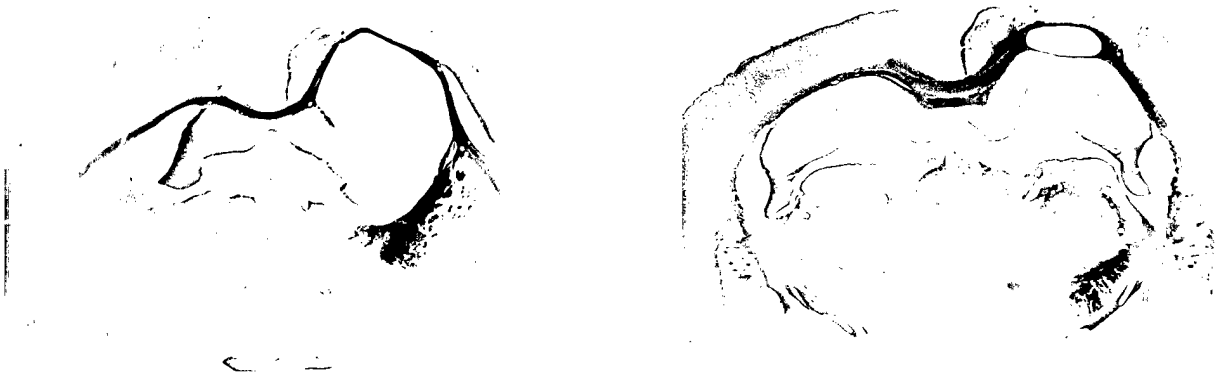


Figure 74

-2.0 mm

-5.0 mm



C44HT

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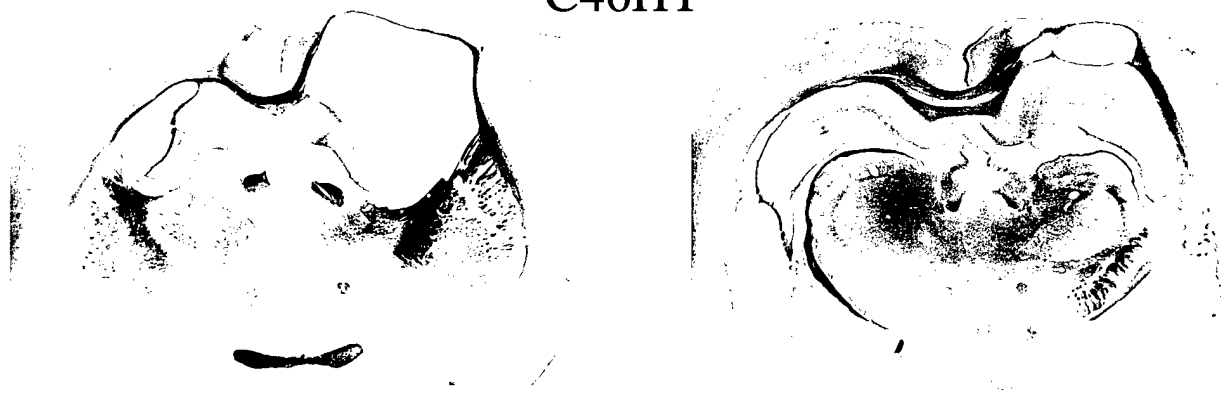

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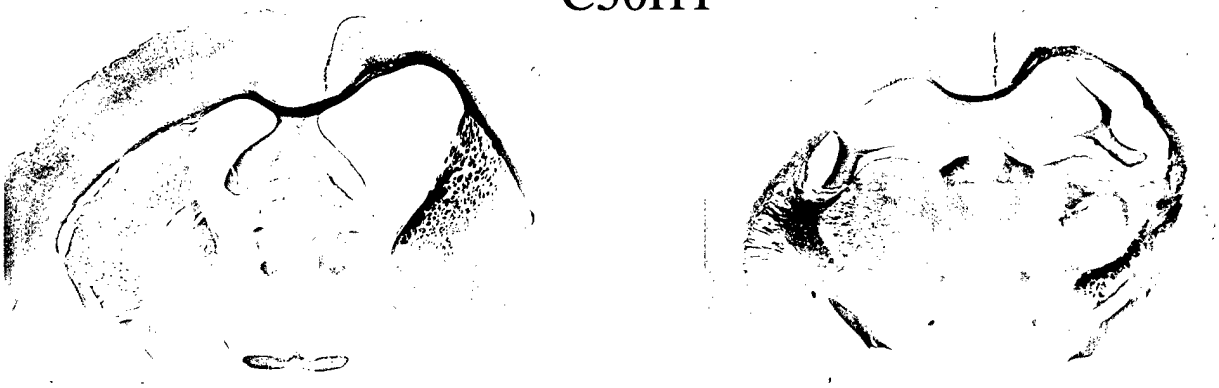

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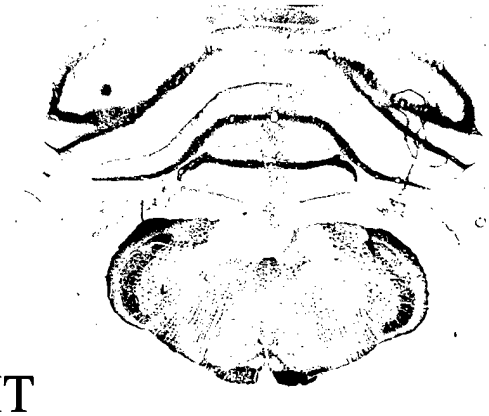
Figure 75

-8.0 mm

-12.0 mm



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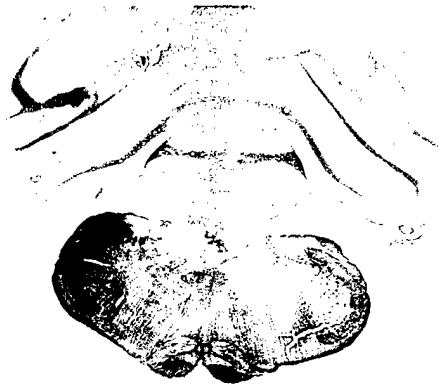

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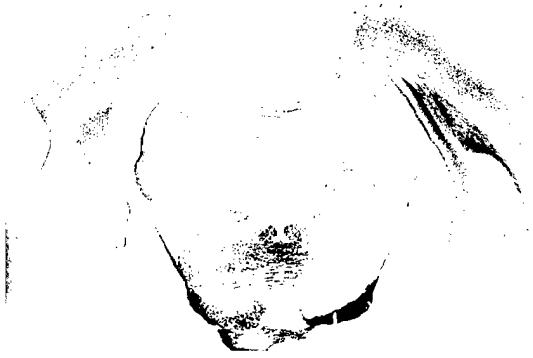

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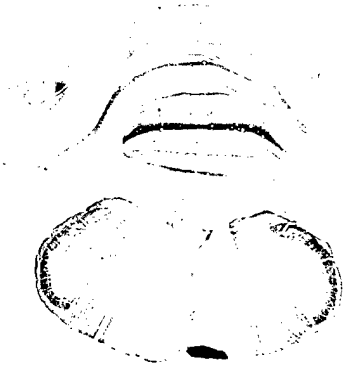


C52HT

Figure 76

9 BRAIN INJURY AND CEREBRAL CORTICAL GENE EXPRESSION

Background

Since we could find no obvious histological reasons for the decreased performance of rats which sustained a bout of hypotension after their brain injury, we sought help from molecular biology to see whether changes in gene expression might provide an explanation for the deletious effect of hemorrhagic hypotension.

The ability of brain injured individuals to improve at least somewhat after their injury suggests that posttraumatic changes occur whereby the damaged central nervous system undergoes cellular rearrangement, makes new connections, and functionally restructures the remaining cellular elements. This is known as plasticity [158]. Following neural injury, rapid changes in the levels of expression of some genes suggest that early molecular events underlie the ultimate fate of a damaged cell: survival and the beginning of neuroplasticity or death [158-161]. Gene expression encompasses all processes from transcription (the conversion this genetic information from DNA into RNA) to translation (the conversion of this RNA to effector proteins). Differentiating transcriptional from translational activity is important because a change in DNA coding in response to a stimulus (transcription) , may not be translated into action at the extranuclear cellular level (i.e. protein production may not have occurred). Synthesized effector proteins participate in specific cellular responses such as growth of axons and establishing synaptic connections so it can be seen that the final result of gene expression, specific protein production, underlies functional recovery.

Starting, stopping, and regulating protein synthesis as well as the synthesis and degradation of RNA are important factors controlled by genes particularly the so-called immediate early genes such as *c-fos, c-jun and egr-1*. An orderly sequencing of brain neuron gene expression is critical for maintenance of normal neuron function including the processing of afferent, sensory information into motor behavior. Following injury, new genes may be expressed and recovery of function depends on patterns of expression of a number of genes regulated over time: first for an immediate response to the injury and then to rebuild new neuronal connections [159,162,163]. The first response to injury is carried out by the protein products of immediate-early genes (IEGs), such as *c-fos* [164]. Neural activity, seizures, hypoxia, and mechanical damage may lead to a rapid and sustained rise in the expression of immediate early genes which control the expression of a wide variety of subsequent genes. The protein products of these genes include neurotrophic factors, neuropeptides, and structural genes involved in neuronal

regrowth and synaptogenesis.

The IEGs *c-fos, c-jun, and egr-1* are particularly important to measure since this is the starting point of the injury response. *C-fos and c-jun* control the important transcription factor AP-1 [161,165,166]. *Egr-1* (formerly zif 268) reflects vascular damage and activation of excitatory amino acid receptors and may control basic fibroblast growth factor (FGF-2) as well as platelet derived growth factor- beta (PDGFB) [167,168]. Increasing evidence points to *egr-1* in brain structural plasticity, perhaps by participating in the regulation of synapsin II associated with synaptic vesicles. Since *egr-1* is also increased by activation of membrane receptors linked to the mitogen activated kinase (MAPK) pathway, this gene may be expressed to signal the production of neurotrophins.

Neurotrophins, particularly the trk family, are receptors important for the regulation of growth and deafferentation in normal and damaged neurons [168,169]. Trk-A and trk-B encode a tyrosine kinase that binds to brain derived neurotrophic factor as well as neurotrophin-3 (NT-3). Stimulation of these receptors is associated with cell survival, neuron outgrowth with axonal growth, and reformation of synaptic connections, both important factors in recovery in the first few days after impact injury.

With the help of Mr. Steven Zeiller, a 3rd year medical student, and Dr. Roger Beuerman, a molecular biologist, we studied simultaneously several cortical brain genes in injured normotensive rats and injured hypotensive rats whose systolic blood pressure was lowered to ~45 mmHg for 20 minutes after brain injury.

We studied the immediate early genes (IEGS), *fos, fos 2B, jun, egr-1,* as well as nerve growth factor (NGF), trk B- a neurotropin receptor, tumor necrosis factor (TNF), acidophilic fibroblast growth factor (AFGF) and platelet derived growth factor B receptor (PDGFBR). Each of these genes has been implicated in the cellular response to injury.

Method

We used 4 groups of animals (3 rats per group) in these experiments.

1. Controls
2. Uninjured hypotensive (exsanguination to SBP ~45 mmHg for 20 min followed by blood reinfusion)
3. Injured normotensive
4. Injured hypotensive (exsanguination SBP ~45 mmHg for 20 min followed by blood reinfusion)

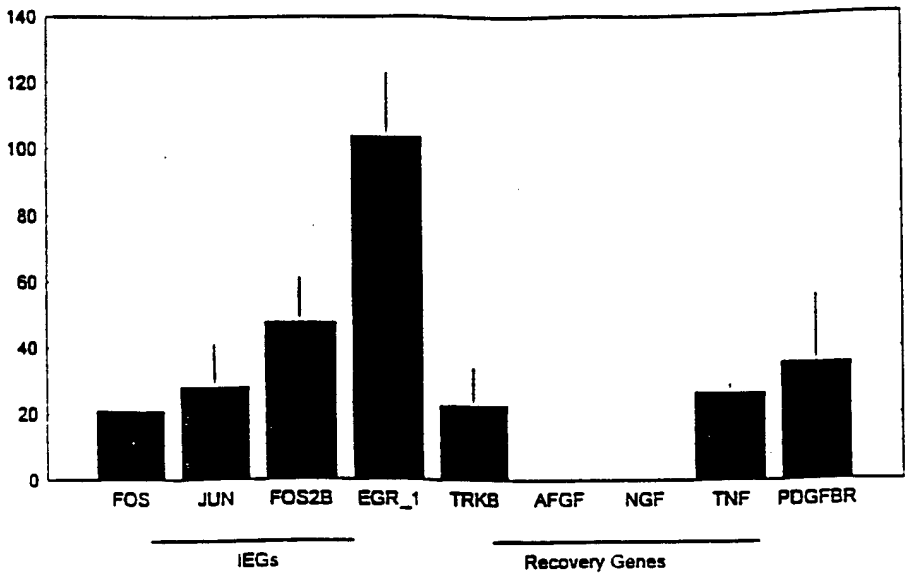
After surgical preparation and specific manipulation according to their assigned group, the rats were allowed to awaken and then were sacrificed (i.p. pentobarbital 50 mg/kg) at 120 minutes. Their brains were quickly removed, sectioned, and placed in liquid nitrogen. The right (injured) and left (uninjured) cerebral cortices were then analyzed for *fos, fosB, jun, egr-1*, NGF, trkB, AFGF, TNF and PDGFBR.

Results

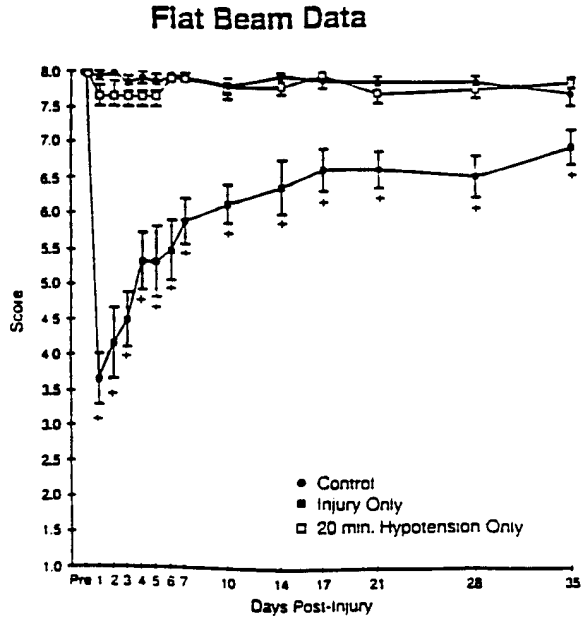
The cortex of normotensive controls expressed only *egr-1,* an early immediate gene. Hemorrhagic hypotension alone was not associated with any brain gene changes.

1. Cortical gene changes following brain injury in normotensive rats.

Figure 77: A- Brain gene changes occurring in the cerebral cortex on the injured side 120 minutes after injury in normotensive rats. B- Behavioral improvement after injury associated with this post injury gene pattern.



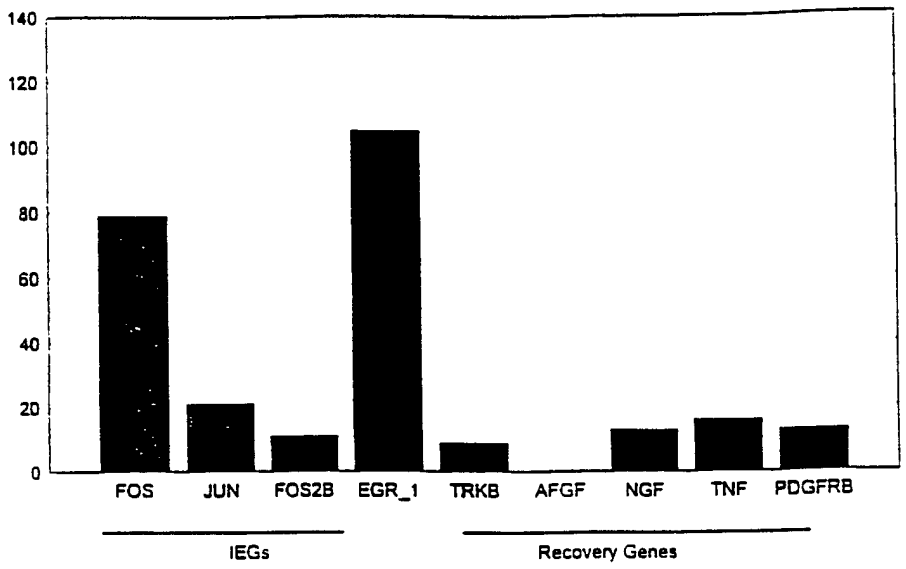
A



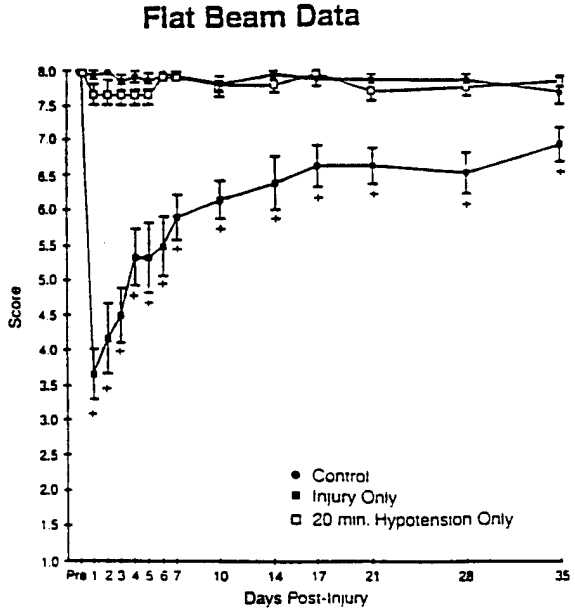
B

Following injury in normotensive rats, at the brain injury site elevations occurred in *fos, fos 2B, jun, egr-1*, trkB, TNF and PDGFBR with *egr-1* showing the largest increase. The normal post injury flat beam neurobehavioral improvement pattern discussed in section 5 is shown. B. Significance – $p<0.05$ Dunn's test.

Figure 78: A- Brain gene changes occurring in the cerebral cortex on the uninjured side 120 minutes after injury in normotensive rats. B- Behavioral improvement associated with this post injury gene pattern.



A



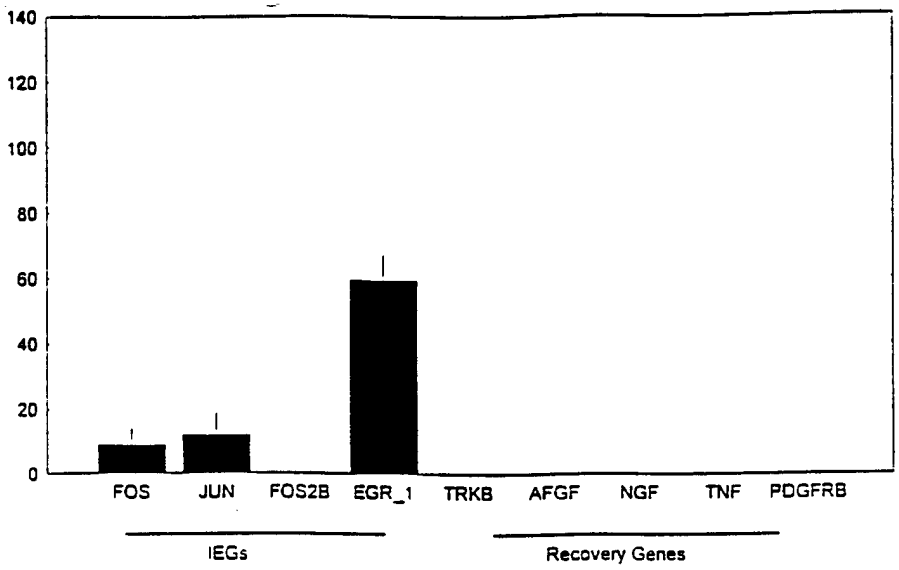
B

In the contralateral (uninjured) cortex *fos* and *egr-1* showed the largest increases, but *jun, fos 2B*, trk-B, NGF and PDGFRB were also expressed. This posttraumatic pattern of gene expression in the contralateral, uninjured hemisphere is associated with a normal post injury recovery pattern. B. Significance -$p<0.05$ Dunn's test.

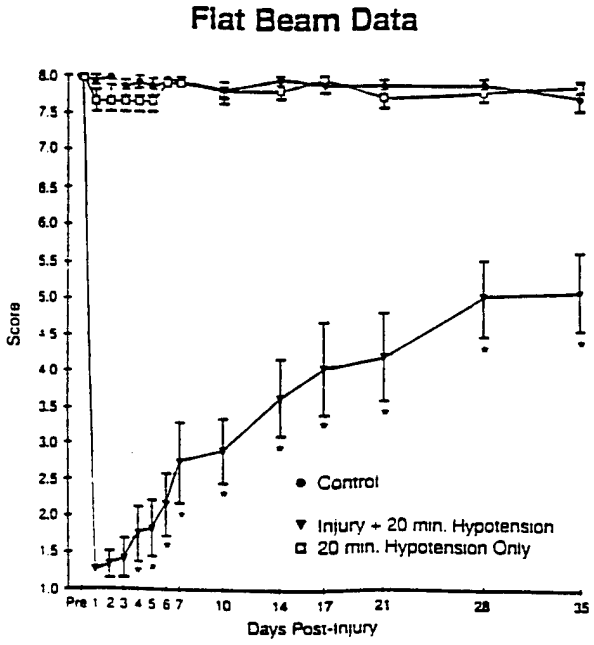
2. Cortical gene changes following brain injury plus hemorrhagic hypotension

If brain injury is followed by hemorrhagic hypotension (SBP~45 mmHg for 20 minutes followed by shed blood reinfusion) all gene expression is down regulated, not only at the brain injury site but contralaterally as well, Figures 79A, 80A. In Section 8 we have shown that recovery is significantly decreased if brain injury is followed by 20 min of hemorrhagic hypotension (SBP ~45 mmHg), and Figures 79B and 80B show the impaired recovery pattern associated the severe down regulation of many brain genes.

Figure 79: A- Brain gene changes occurring in the cerebral cortex on the injured side 120 minutes after injury in hypotensive rats. B- Impaired behavioral improvement after injury associated with hypotension and this post injury gene pattern.



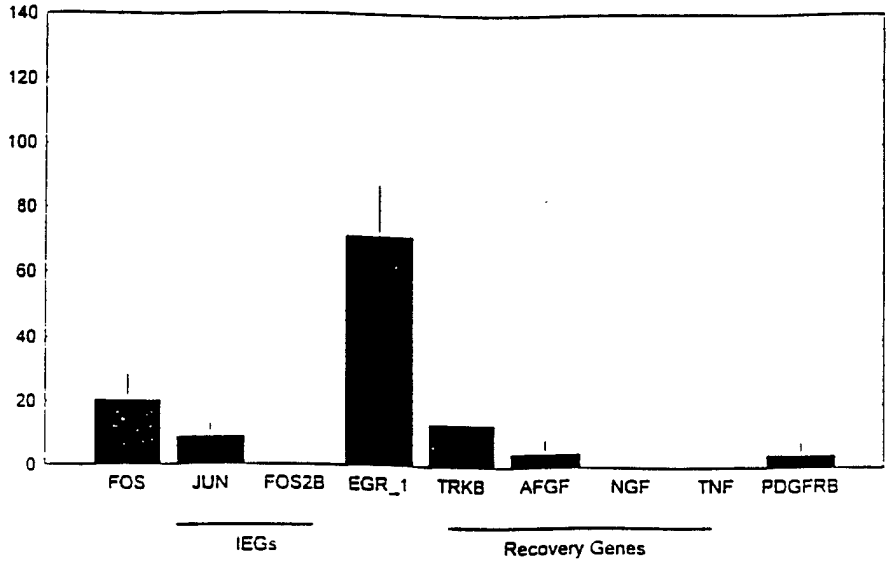
A



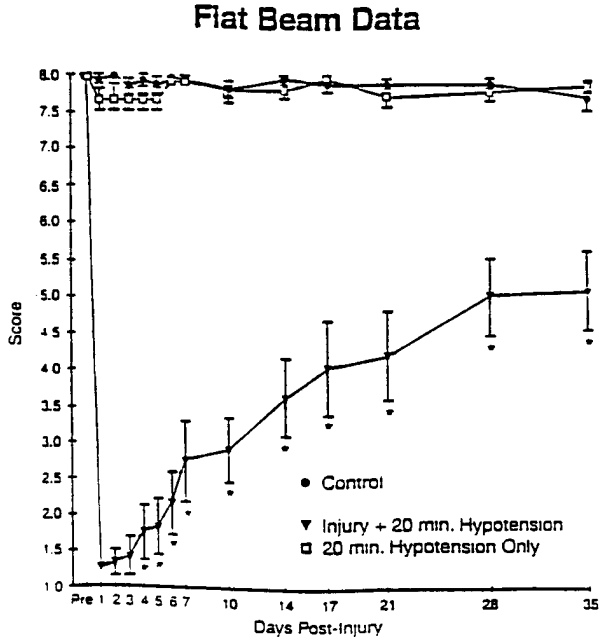
B

Following injury in hypotensive rats, at the brain injury site the expression of all IEGs was down regulated and *fos2B* was abolished; the expression of "recovery genes" was totally abolished. The impaired post injury flat beam neurobehavioral pattern associated with brain injury plus hemorrhagic hypotension (Section 8) and this gene pattern is shown, significance *-$p<0.05$ Dunn's test.

Figure 80: A- Brain gene changes occurring in the cerebral cortex on the uninjured side 120 minutes after injury in hypotensive rats. B- Impaired behavioral improvement after injury associated with hypotension and this post injury gene pattern.



A



B

Following brain injury and hemorrhagic hypotension IEGs in the contralateral cerebral cortex were also down regulated. *fos2B* expression was abolished as were the expressions of "recovery genes" NGF and TNF. The impaired post injury flat beam neurobehavioral pattern associated with brain injury plus hemorrhagic hypotension (Section 8) and this gene pattern is shown, significance *-$p<0.05$ Dunn's test.

Discussion

To our knowledge this is the first time this type of experiment has been done. Using the "gene screen" technique we have demonstrated a wide variety of gene expression changes after brain trauma. We grouped the examined genes into 2 categories: 1) the immediate early genes and 2) genes that are usually thought to be affected after the IEGs. These would include trk-B, AFGF, NGF, TNF and PDGFRB. To help us understand the multiple gene changes which we have shown in these "down stream" genes, we are tentatively calling these genes "recovery genes" because their expression is clearly associated with a normal post injury recovery pattern (Figures 77A,B, 78A,B) and their down regulation or absence is associated with impaired recovery, Figures 79A,B, 80A,B.

While brain injury has long been known to affect brain genes [164], the novel finding from these studies is that if brain injury is followed by hemorrhagic hypotension, both IEG and recovery gene expression in the injured cortex and contralateral uninjured cortex are down regulated or abolished. Abolition or severe down regulation of genes normally expressed after injury is associated with significantly impaired recovery: down regulated genes= down regulated recovery. We believe that this is the first time such an association has been demonstrated.

Normally, following injury the expression of many cortical genes is upregulated even in the impacted cerebral cortex, Figure 77. It may be argued that the abolition of gene expression in the injured cortex in the hypotensive rats occurs because the cells are severely damaged and dying owing to the effects of the injury plus hypotension. Such gene findings then would have little meaning. Perusal of Figures 74,75, and 76, however, show that often the mantle is often preserved at the injury site in injured hypotensive animals. Thus, all cells at the injury site do not die. Furthermore, gene expression is down regulated or abolished in the left, uninjured cortex if injury is followed by hemorrhagic hypotension and the contralateral mantle is invariably preserved in brain injured hypotensive rats, Figures 74,75,76. We infer, therefore, that the down regulation of gene expression that occurs if hemorrhagic hypotension follows brain injury represents much more than just dying brain. Most importantly, the down regulation of IEGs and recovery genes may well underlie the impaired recovery associated with brain injury and hypotension (and brain injury-hypoxia as well). Subsequent diminished production of various neurotrophins, which are known to help preserve neurons, perhaps prevents normally occurring plasticity mechanisms which underlie normal recovery.

We hypothesize that down regulation of IEGs and "recovery genes" underlies the decreased recovery seen if hypotension (or hypoxia) follows brain injury. Based on these genetic findings the principal investigator feels that future brain trauma research directed at the gene level will be necessary to develop more effective treatments to truly benefit those with brain injury. The expression of specific brain genes following brain injury underlies normal plasticity mechanisms which allows a modicum of improvement after injury to occur. True neuroprotection will preserve or enhance the expression of specific genes required to maintain neuronal structure and function.

While the brain injury-hypotension model was developed primarily to gain insight into mechanisms underlying the deleterious effects of hypotension upon recovery following brain injury, our results have transcended this aim. We have provided a way to down regulate brain genes after injury and have shown for the first time that gene down regulation within hours of injury is associated with long term behavioral deficits. Possibly these down regulated genes affect plasticity mechanisms. Further research might indicate which specific down regulated genes cause the impaired recovery. If the down regulation of such genes could be prevented or their expression augmented possibly impaired recovery could be prevented. Conversely, augmentation of specific genes which are normally expressed after brain injury might improve recovery after brain injury. Clearly in the future gene manipulation after brain injury (or stroke) will provide a fertile field for improving impaired neurons and associated clinical recovery.

10 SUMMARY OF SIGNIFICANT FINDINGS

1. THE MULTIDISCIPLINE APPROACH ITSELF- one of the most important findings of this contract is the demonstration of the power of the multidiscipline approach. For instance, because our posttraumatic gene studies are linked to behavior it is quite likely that these gene findings really do affect recovery and are clinically relevent. Similarly, the occurrence of free fatty acids (FFAs), diacylglycerols (DAGs), and phospholipase A_2 (PLA_2) elevations weeks after injury when the animals are showing post injury behavioral improvement suggests that at late post injury time points these molecules are active in recovery processes. The persistance of histologic (cells, dendrites, axons) damage after brain injury in the face of neurobehavioral improvement strongly suggests that plasticity mechanisms in uninjured brain areas must be sought as the source of this post injury improvement. **SIGNIFICANCE: the multidisciplinary approach to the study of brain injury provides insights far in excess of those obtained through any single discipline. Accordingly, the multidiscipline approach is more cost effective because research findings in a particular area must immediately integrate with findings in all other areas. Findings from a single discipline cannot stand in isolation but must consistently fit into the overall picture. This approach minimizes tangential research and allows more pertinent questions to be asked and answered sooner. This will speed up the biologic treatment of brain injury.**

2. HISTOLOGICAL STUDIES- A. Changes in neuronal morphology occurring up to 2 hours after injury are not typical of ischemia. B. We have shown for the first time that cells directly under an area of cortical injury are often strikingly well preserved, appear to produce certain metabolites normally, but have severe axon and dendrite damage. Though the cell bodies may be preserved and metabolize normally, they, nevertheless, may be functionless because their afferents and efferents have degenerated. **SIGNIFICANCE: these findings of severe axon and dendrite destruction around an area of focal brain injury may fundamentally alter the way brain injury is perceived. A major goal for future brain injury neuroprotection research must involve preservation of axons and dendrites. This has not heretofore been emphasized.**

3. NEUROCHEMICAL STUDIES- The early (30 min) free fatty acids (FFAs) and diacylglycerols (DAGs) increases which occur after brain injury are not as intense as

early after ischemia. **SIGNIFICANCE: this also suggests that ischemia is not a major event, within 30 minutes of focal brain trauma.** Aspartate and glutamate changes in the brain following injury occur acutely, briefly, and only about the injury site. Any changes in FFAs, DAGs and phospholipase (PLA_2) which occur at a distance from the injury site or which occur days or weeks later are unlikely to have been initiated by the action of extracellular glutamate or aspartate increases acting upon NMDA receptors and calcium channels. **SIGNIFICANCE: classical glutamate-NMDA receptors and calcium channel interactions do not explain many of the FFA, DAG, and PLA_2 changes we have demonstrated. Other mechanisms to explain elevations of these molecules occurring distant to the injury site or weeks after injury will have to be found.** The early increases in FFAs, DAGs and PLA_2 associated with brain injury (which we have also demonstrated) are well known and would be related to destructive mechanisms associated with cellular injury. We have shown for the first time that FFAs, DAGs, and PLA_2 levels are also highly elevated widely throughout the brain weeks after injury. **SIGNIFICANCE: the late increases in FFAs, DAGs and PLA_2 , heretofore unknown, are probably related to constructive brain plasticity mechanisms and post injury neuronal remodelling.**

4. BEHAVIORAL STUDIES- Behavioral testing has been the lynch pin which has allowed us to relate all observed effects from other disciplines to the animals' actual post injury clinical status. Our behavioral tests reveal a biphasic improvement after injury indicating the interaction of early and late recovery processes. **SIGNIFICANCE: the early rapid improvement probably represents resolution of acute injury phenomena. The later, less rapid but longer lasting improvement probably represents the effect of brain plasticity mechanisms.** The normal improvement in performance after injury which rats exhibit probably represents adaptation and compensation rather than true neural recovery. Improvement of neurobehavioral function after injury proceeds as well without task specific practice as with it. Thus, practice does not appear necessary for the internal process of neural reworking to achieve post-injury neurobehavioral improvement. **SIGNIFICANCE: these latter experiments, if they can be extrapolated to humans, suggests that physical therapy probably does not help reorganize brain neural networks though it may keep limbs limber and with good muscle mass while intrinsic brain recovery mechanisms proceed.**

5 THE ADVERSE EFFECT OF HYPOXIA AND HYPOTENSION UPON RECOVERY- We have been able to model the clinically known deleterious effects of hypoxia and hemorrhagic hypotension on neural recovery occurring after brain injury. Reducing systolic blood pressure to ~45 mmHg for 20 minutes by bleeding after brain

injury (with subsequent reinfusion of the shed blood) severely retarded the extent of neurobehavioral recovery in our rats. This decreased performance is not explained by H and E or silver staining histological studies. **SIGNIFICANCE: our studies strongly suggest that hemorrhagic hypotension following brain injury exerts its deleterious effects not by increasing the extent of damage but by decreasing brain plasticity mechanisms needed for normal neural recovery. Future research should document posttraumatic plasticity mechanisms, how they are degraded by hypoxia or hypotension and how they can be enhanced.**

6. GENE CHANGES AFTER BRAIN INJURY- We have demonstrated that the expression of specific immediate early genes (IEGs) and "recovery genes" is normally upregulated widely throughout the brain following focal brain trauma. Hemorrhagic shock following brain injury concomitantly abolishes or severely reduces the expression of IEGs as well as "recovery genes", those genes associated with neurotrophin production. This down regulation of IEGs and "recovery genes" may underlie the observed impaired recovery which we have demonstrated when hypotension follows brain injury. **SIGNIFICANCE: a normal gene response to injury must underlie normal brain plasticity mechanisms inherent in post injury neural improvement. Hemorrhagic hypotension, by down regulating or abolishing the normally occurring gene response after injury, may severely curtail brain plasticity mechanisms and, hence, neural recovery. Methods of enhancing important genes inherent to neural recovery after trauma should be devised. Their effect on brain plasticity and neural behavior should be determined.**

7. POST TRAUMATIC APNEA- Immediate apnea is often seen with brain injury and may actually be the cause of death. Using scanning EM we have identified posttraumatic fissures on the floor of apparently normal IV ventricles in the region of the respiratory nuclei which may account for posttraumatic apnea. **SIGNIFICANCE: to our knowledge this is in the first demonstration of such findings in apparently normal IV ventricles which provide an anatomical reason for post traumatic apnea and the occurrence of permanent post traumatic respiratory arrest.**

11 DIRECTIONS FOR FUTURE RESEARCH

We have spent 5 years developing a brain trauma model in normotensive rats for the Army linking histological, neurochemical, genetic, and behavioral changes consequent to a standard amount of brain trauma. Our major findings are presented in this report.

The genetic response to brain trauma may underlie much of the damage resulting from focal injury and undoubtedly holds the key to normal or impaired recovery. The number of genes actually involved in brain injury and subsequent normal recovery should be further elucidated. Our brain injury-hypotension model shows that the genetic response to brain injury can be manipulated with subsequent behavioral changes. Methods to increase or decrease specific gene expression instrumental in brain recovery processes should be sought because the ability to manipulate neuronal genes may be a requisite for improving post injury recovery.

The time course of the severe axon and dendrite damage associated with focal brain injury should be thoroughly investigated so that any possible window of opportunity for their treatment can be delineated. Treatments to ameliorate both axon and dendrite damage should be sought. Treatments could include hypothermia, drugs, and specific genetic manipulations.

Future research should involve a multidiscipline approach involving molecular biology (genetics), histology, and behavior. For instance, it should be ascertained whether specific genes can be upregulated following brain injury and whether this upregulation will improve recovery. If histological studies reveal that dendrite or axon damage can be prevented this, too, should be coupled to behavioral tests to ascertain whether such histologic improvements result in clinical improvement. Constantly coupling such studies with behavior will ensure that any genetic or histological findings are relevent to neural recovery and are not epiphenomena.

Continued development and utilization of this model by the Army linking genetic and histologic studies with behavior will put Army brain trauma research in the forefront of brain trauma research. This will help the Army better care for soldiers, not only preserving the fighting strength but also optimally aiding those who can no longer serve.

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APPENDIX 1

Coronal Sections of Control and Injured Rat Brains
From + 2.0 mm to -12.0 mm Relative to Bregma (0.0 mm on p 12)

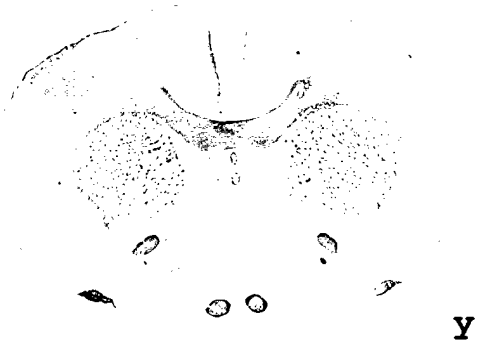
+2.0 mm

47

Control

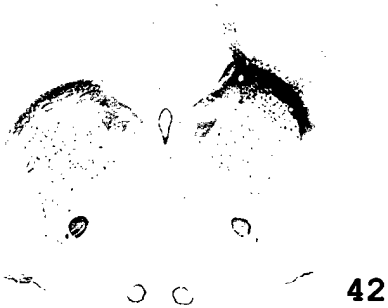
53

Control



y

Control



42

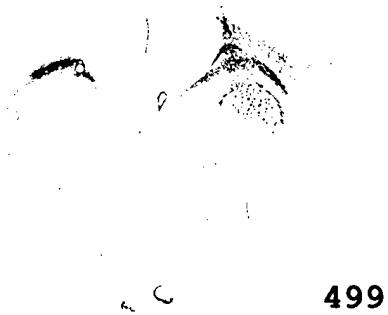
Injured - 8 Wks

51

Injured - 8 Wks

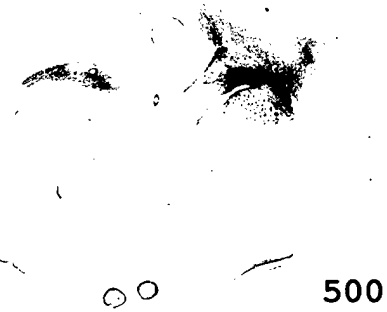
54

Injured - 8 Wks



499

Injured - 8 Wks



500

Injured - 8 Wks

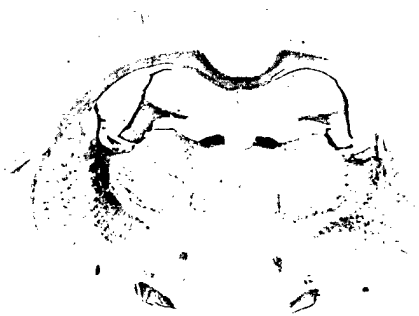
501

Injured - 8 Wks

-2.0 mm

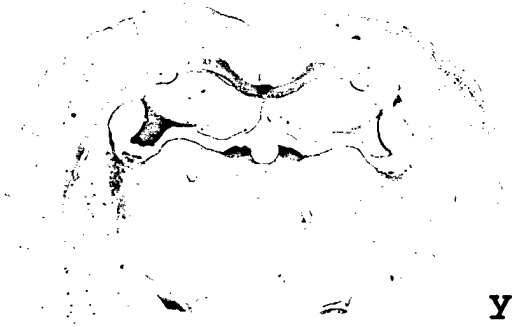
47

Control



53

Control

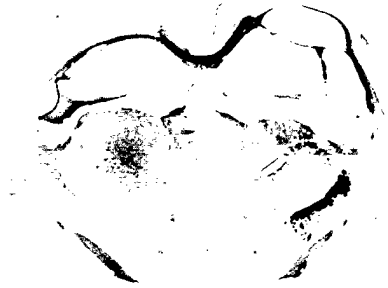


y

Control

42

Injured - 8 Wks.



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54

Injured - 8 Wks.

499

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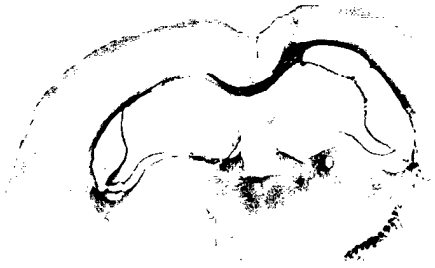

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501

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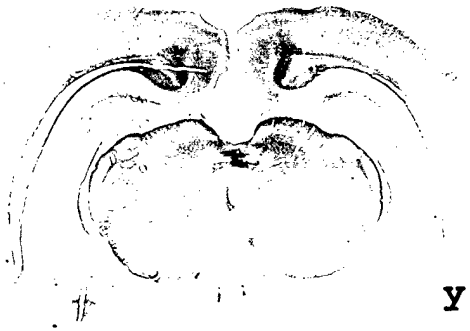
-5.0 mm

47

Control

53

Control



y

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42

Injured - 8 Wks.

51

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54

Injured - 8 Wks.

499

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500

Injured - 8 Wks.

501

Injured - 8 Wks.

-8.0 mm

47

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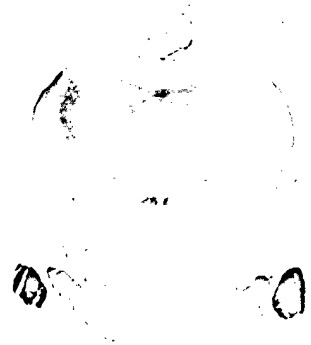

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Control

y

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42

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51

Injured - 8 Wks.

54

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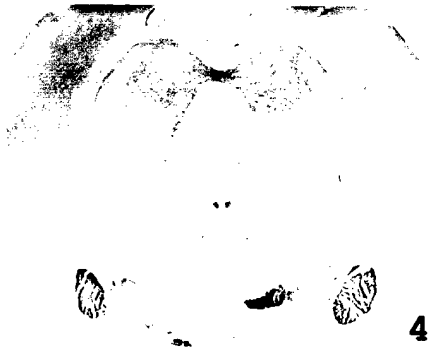

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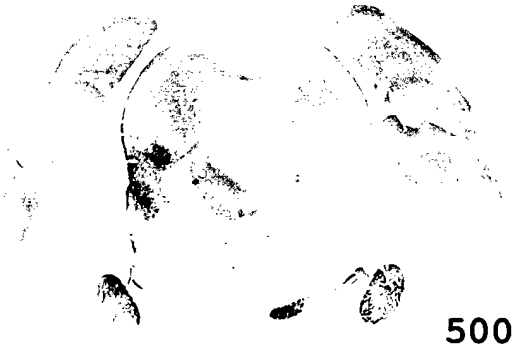

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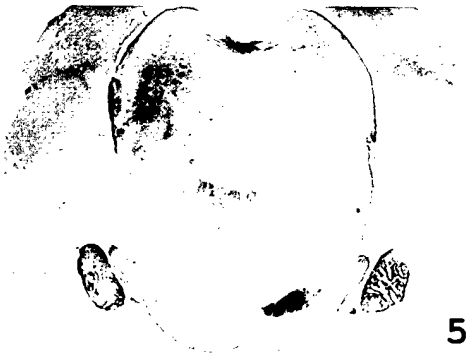

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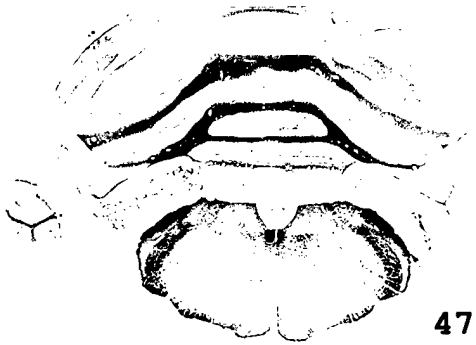
-12.0 mm



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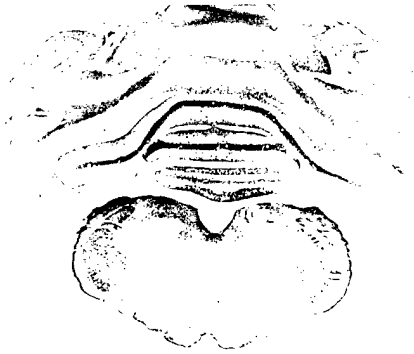

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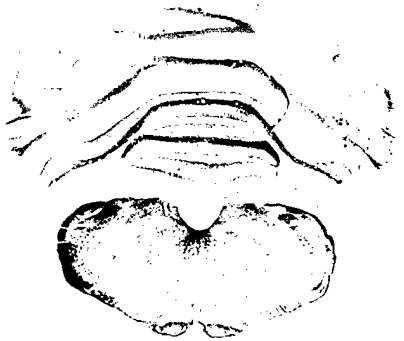

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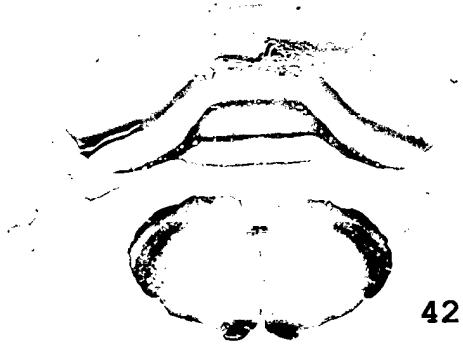

42

Injured - 8 Wks

51

Injured - 8 Wks

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Injured - 8 Wks

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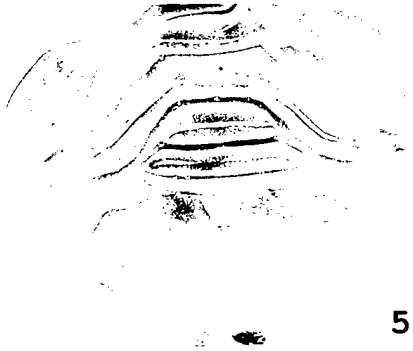
Injured - 8 Wks



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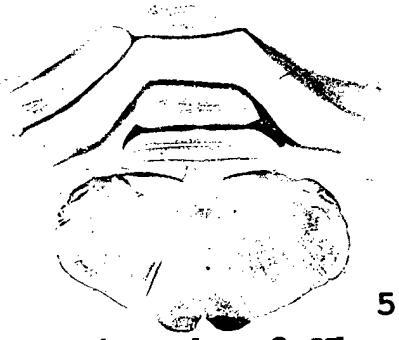

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Injured - 8 Wks

APPENDIX 2
HISTOPATHOLOGIC STUDIES
(Read by Dr. James Nelson, Neuropathologist, LSU)

Immediately after injury

There is an area of pallor associated with fresh, subarachnoid and multifocal parenchymal hemorrhage involving the anterior four-fifths of the dorsal cerebral convexity. In cross-section, the anterior and posterior thirds of this lesion are triangular in outline, while the middle third is rectangular. Focal parenchymal hemorrhages are present throughout the extent of the lesion. The area of pallor extends through the cortex into the subcortical white matter. Occasional foci of hemorrhage are present in the white matter. Neurons within and immediately adjacent to the area of pallor often have shrunken, faintly eosinophilic cell bodies with scalloped borders and deeply staining nuclei with an effaced chromatin pattern. These neuronal changes are accompanied by small numbers of enlarged astrocytic nuclei which rarely form pairs. Blood vessels, other than those associated with the hemorrhage, are patent with intact, flattened endothelium. There is no cellular infiltrate. Scanty, fresh subarachnoid hemorrhage is present over the contralateral superior and medial neocortex. Scattered neurons with morphologic changes similar to those in the traumatized hemisphere are evident in the cortex beneath the subarachnoid hemorrhage. Sections of brain stem and cerebellum are unremarkable.

Thirty minutes after injury

Small numbers of monouclear cells are accumulating in the subarachnoid hemorrhage over the traumatized cortex. Increased numbers of enlarged, paired astrocytic nuclei and multifocal collections of vacuoles involving the cortical neuropil are evident within the traumatic cortical lesion. A few structures resembling apoptotic bodies are in the neuronal layers of the cortex. Otherwise, the leptomeningeal and parenchymal changes and the extent of the traumatic lesion are similar to their counterparts seen immediately following trauma. The changes in the contralateral hemisphere are also similar to those seen immediately after the cortical impact.

Two hours after injury

The overall size of the lesion is smaller than those observed immediately after and 30 minutes after injury. The posterior border of the lesion is at the level of the mid thalamus.

The number of mononuclear cells within the subarachnoid hemorrhage is increased in comparison to the 30' lesions. These cells are beginning to infiltrate the parenchyma. The astrocytic reaction is less extensive than at 30'. There are occasional small foci of apparent neuronal loss within the borders of the traumatic lesion. Otherwise, the leptomeningeal and parenchymal changes are similar in both hemispheres to their counterparts at 30'.

Four hours after injury

Fresh subarachnoid hemorrhage, zones of parenchymal pallor, and foci of fresh parenchymal hemorrhage involve the dorsal convexity of one cerebral hemisphere extending from the frontal pole to the level of the midbrain. A small number of erythrocytes are present in the subarachnoid space over the dorsal convexity of the contralateral hemisphere. In the anterior portion of the severely affected hemisphere there are two wedge-shaped areas of parenchymal pallor separated by morphologically normal parenchyma. Posteriorly, the zone of pallor extends uninterrupted across the dorsal cerebrum. The pale staining lesions are most extensive in the cortex, however, the underlying white matter is also significantly affected. The histologic changes in the cortex comprise eosinophilic neuronal necrosis, proliferation of astrocytes, occasional apoptotic cells, subpial infiltration of small numbers of neutrophils, and foci of fresh petechial hemorrhage. These alterations are most extensive within the pale staining areas, however, small numbers of necrotic neurons can be seen interspersed among morphologically intact neurons in adjacent areas of cortex which stain normally. Vacuoles, small cavities, apparent loss of myelin, focal hemorrhages, and a few apoptotic cells are evident in the white matter contiguous with the cortical lesions. The brain stem and cerebellum are unremarkable.

Twenty-four hours after injury

There is a well defined area of acute, cerebral cortical necrosis, fresh hemorrhage, and spongiform change involving the dorsal convexity of the traumatized hemisphere. The area of necrosis is roughly wedge shaped with the base along the cortical surface and the apex within the parenchyma pointing in a ventromedial direction. In the more posterior sections the lesion is separated into the lateral and medial zones by morphologically normal cortex. Fresh hemorrhage is present chiefly in the molecular layer. Other less frequent or extensive foci of hemorrhage involve the subarachnoid space and the central portions of the lesion. A single focus of hemorrhage is located in the corpus callosum ventral to the cortical lesion. Within the affected cortex there is neuronal necrosis, apoptosis of adjacent oligodendroglia, endothelial proliferation, and small numbers of

perivascular neutrophils. The latter are also present in leptomeningeal blood vessels and subarachnoid space. Anteriorly, neuronal necrosis extends through the outer one half to two thirds of the cortex. Posteriorly, neuronal necrosis extends to the junction of cortex and white matter.

Forty-eight hours after injury

The shape, extent, and cortical location of the lesions at 24 and 28 hours are similar. The histologic features of the lesion at 48 hours represent progression of changes evident at 24 hours. There is advanced neuronal necrosis with loss of nuclear definition, vascular proliferation, subarachnoid and subpial mononuclear cell infiltration with occasional phagocyte formation. There are increased numbers of astrocyte nuclei in the cortex adjacent to the lesion. Parenchymal and subarachnoid hemorrhage are present within and are overlying some portions of the necrotic lesion. Subpial and subarachnoid hemorrhages are present.

Seventy-two hours after injury

In addition to focal subpial and parenchymal hemorrhage, there is focal replacement of parenchyma in the molecular layer with collections of phagocytes. Rarely, a phagocyte may contain pigment resembling hemosiderin. Mononuclear cells and phagocytes are present in subarachnoid and perivascular spaces. In some sections the number of astrocyte nuclei may be increased.

Five days after injury

Well defined areas of cortex have been replaced by collections of phagocytes, thin walled blood vessels, and a few foci of hemorrhage. Some of the blood vessels adjacent to the lesion are proliferating. Small numbers of phagocytes and mononuclear cells are present in the subarachnoid space. The anterior two thirds of the lesion consist of two foci with the histologic features just described separated by cerebral cortex with increased numbers of astrocyte nuclei. Posteriorly, the lesions are separated by necrotic tissue. In most sections the lesions are confined to the cortex. Occasionally, however, the subcortical white matter is involved.

Seven days after injury

The cerebral hemispheres are asymmetrical with the traumatized hemisphere being smaller than the non-traumatized hemisphere, A well-defined parenchymal defect is present in the traumatized hemisphere involving the cerebral cortex and white matter over

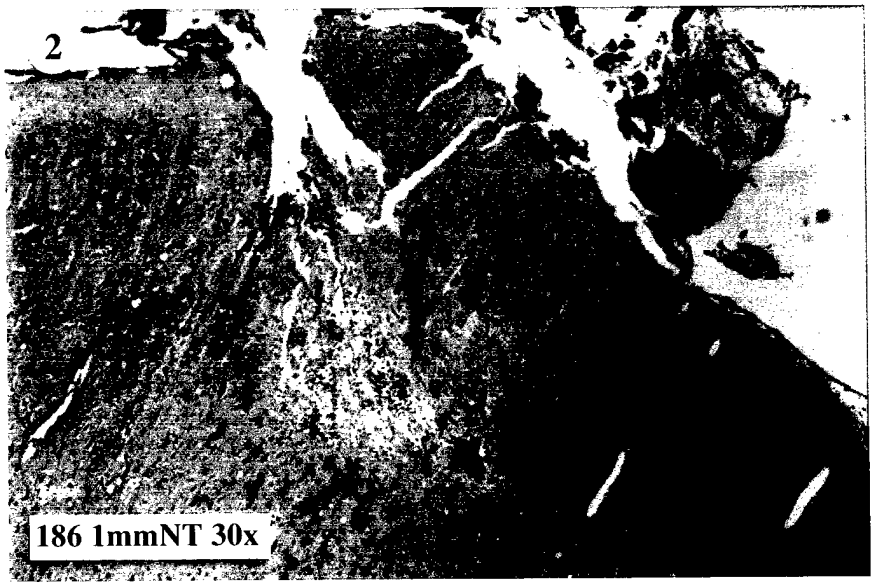
the dorsal cerebral convexity. The lesion extends from near the frontal pole anteriorly to the level of the aqueduct posteriorly. In rat 327, the anterior third of the lesion consists of dorsal medial and lateral parenchymal defects separated by cerebral cortex in which astrocytes and a few proliferating blood vessels are evident. The parenchymal defect extends through the cortex into the underlying white matter. In some sections there is a cavitary defect in the white matter with gliovascular trabeculae and macrophages which appear to be unconnected with the cortical defect. The cortical defect is covered or partially filled with a fibrovascular membrane continuous with the leptomeninges and the connective tissue investments of the parenchymal blood vessels. Macrophages, focal hemorrhage, fragments of coagulated brain and foci of calcification are present within the membrane. A thin rim of astrocytes, a few preserved neurons or myelinated axons, macrophages and occasional proliferating blood vessels form the border of the defect within the parenchyma. Outside this rim, the parenchyma is morphologically intact. The lateral ventricle adjacent to the parenchymal defect is dilated. Increased numbers of astrocytes are present in the contralateral cortical molecular layer along the dorsomedial surface of the hemisphere. Sections of brain stem and cerebellum are unremarkable.

Four weeks after injury

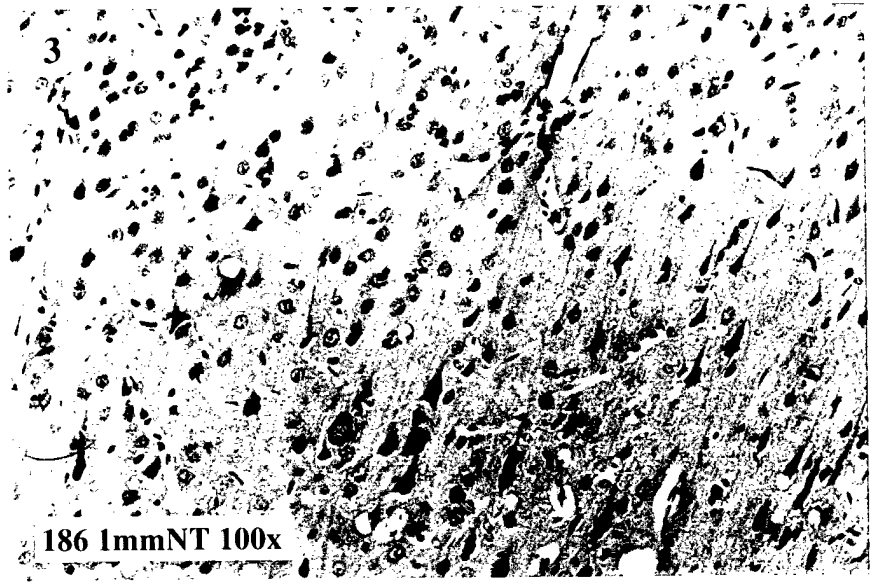
Parenchymal defects similar in location, extent, and configuration to those described at one week are present in the traumatized cerebral hemisphere which is smaller than its non-traumatized counterpart. The lateral ventricle adjacent to the parenchymal defects is dilated. Erythrocytes are no longer evident and the number of macrophages is reduced in the parenchymal defect. Similarly, the parenchymal border of the defect appears thinner with fewer macrophages in comparison with the border at one week. Increased numbers of astrocytes are evident in the cortical molecular layer along the dorsomedial surface of the contralateral hemisphere. The brain stem and cerebellum are unremarkable.

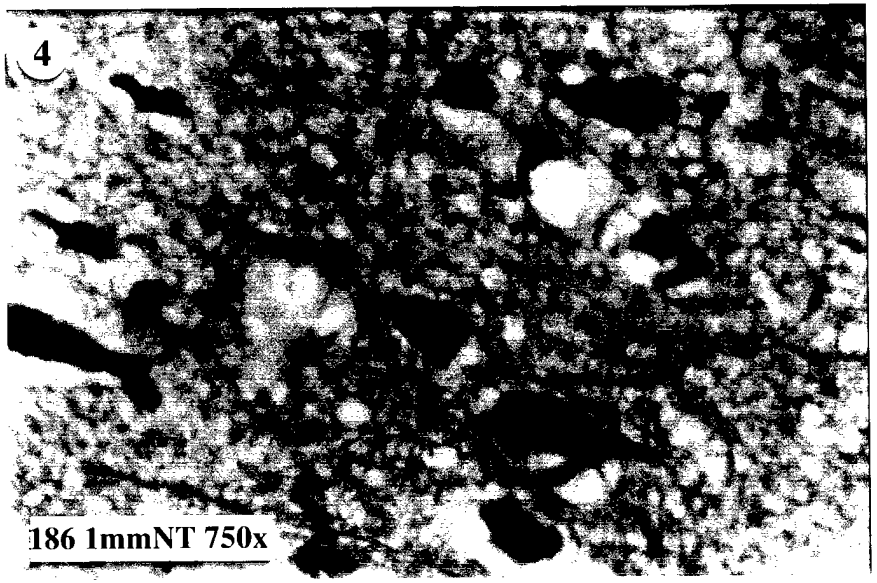
Eight weeks after injury

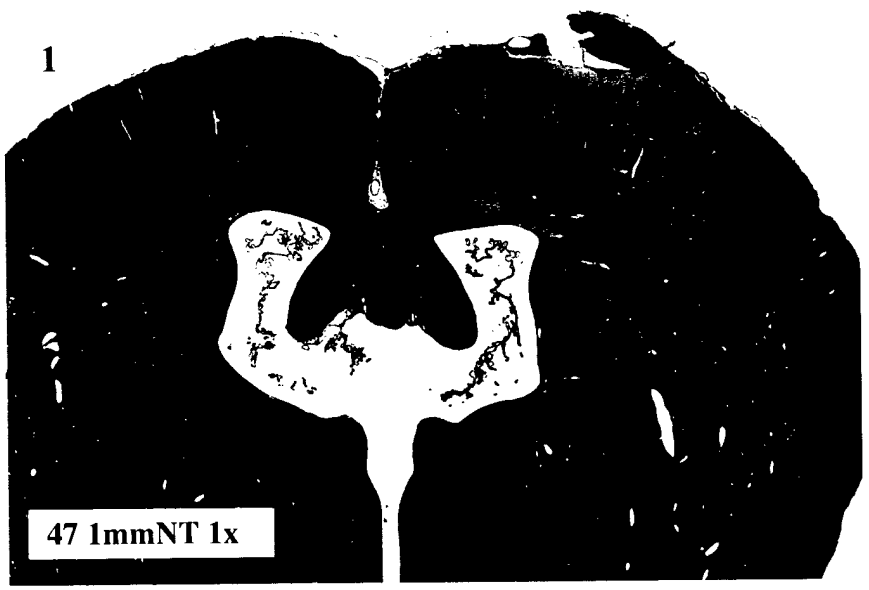
There are no significant differences in the form, location and histologic characteristics of the cerebral lesions at eight weeks in comparison with those at four weeks.
Unilateral corticospinal tract atrophy (including the pyramid) is present in the brain stem. The cerebellum is unremarkable.

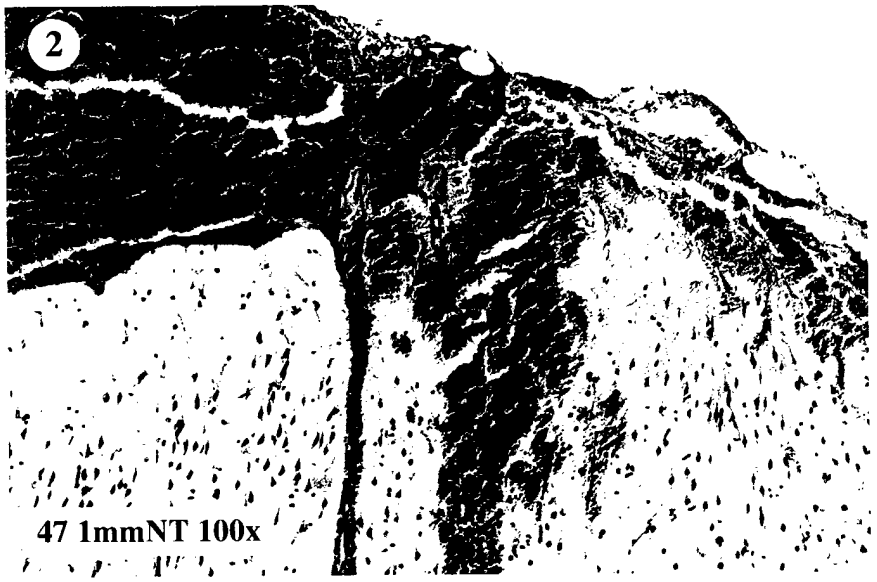


Immediately After Injury

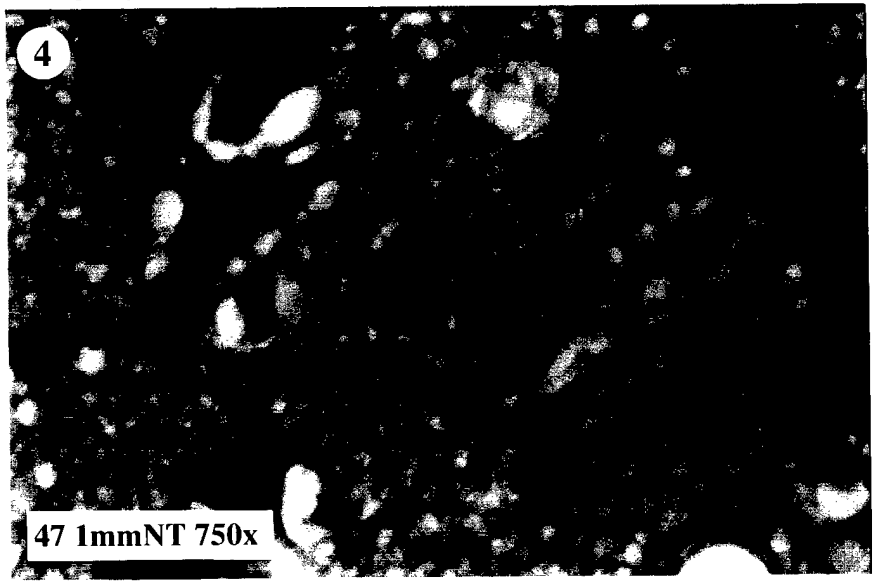


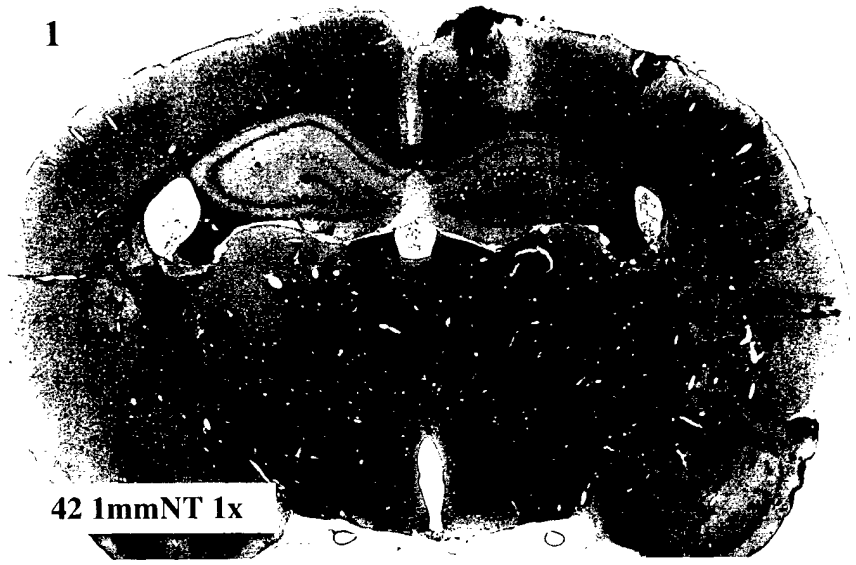


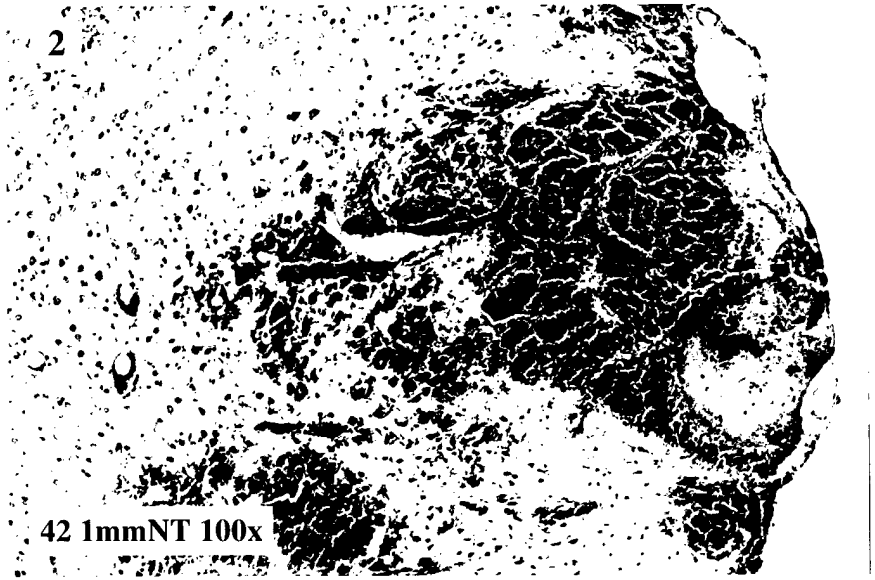




30 min. After Injury







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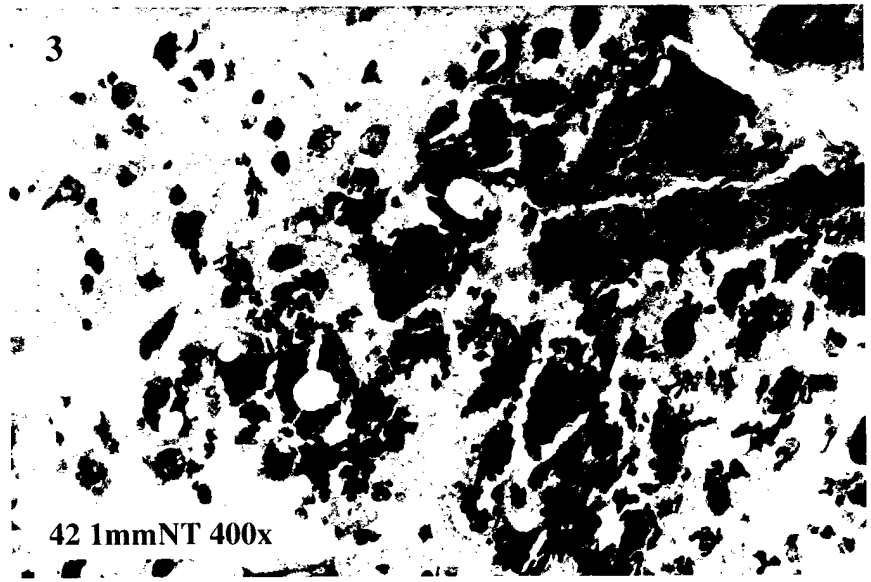

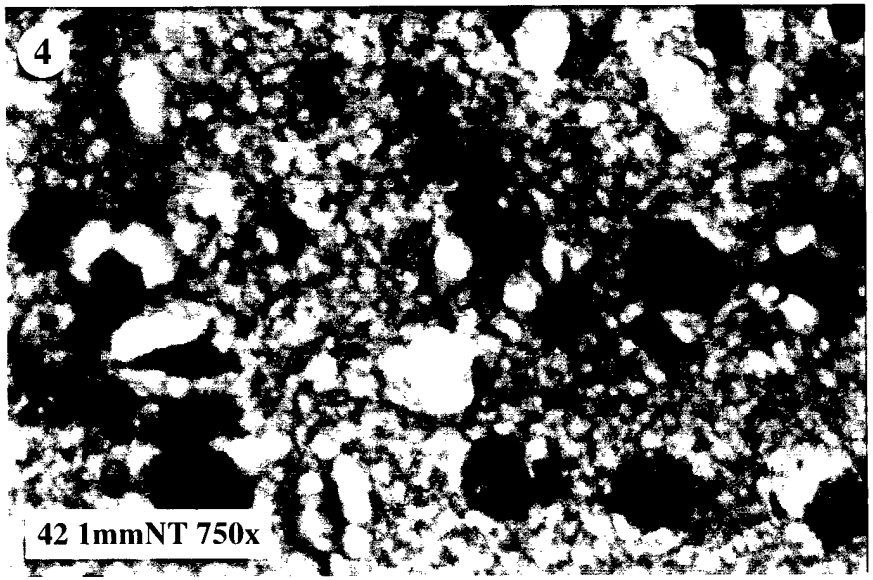

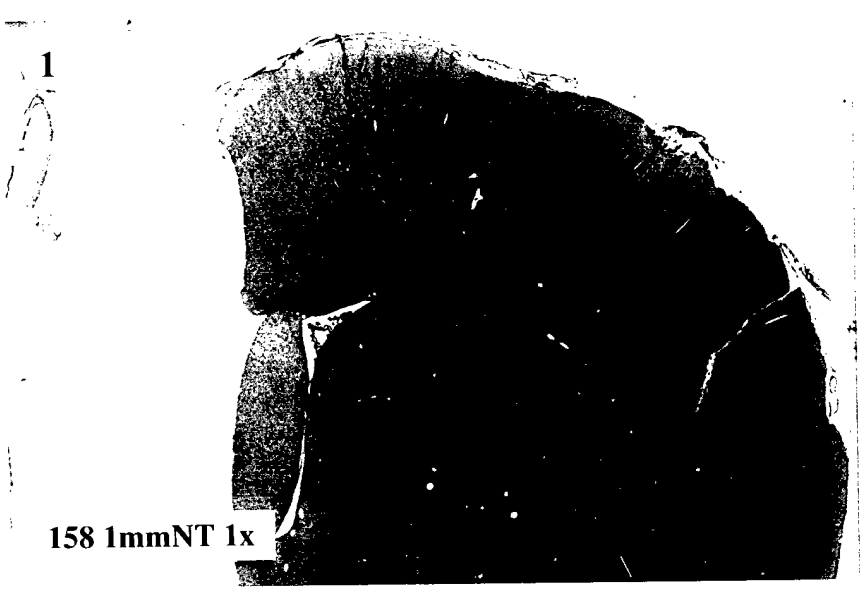

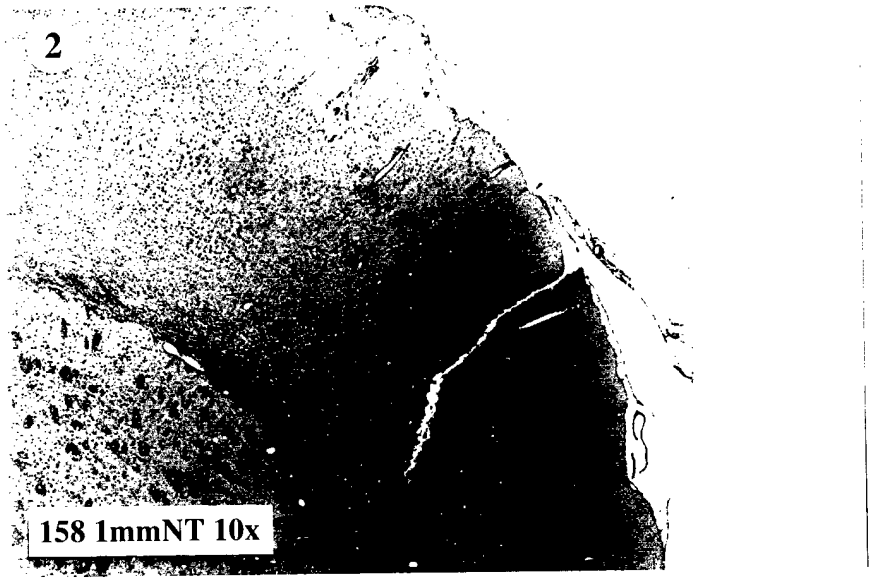

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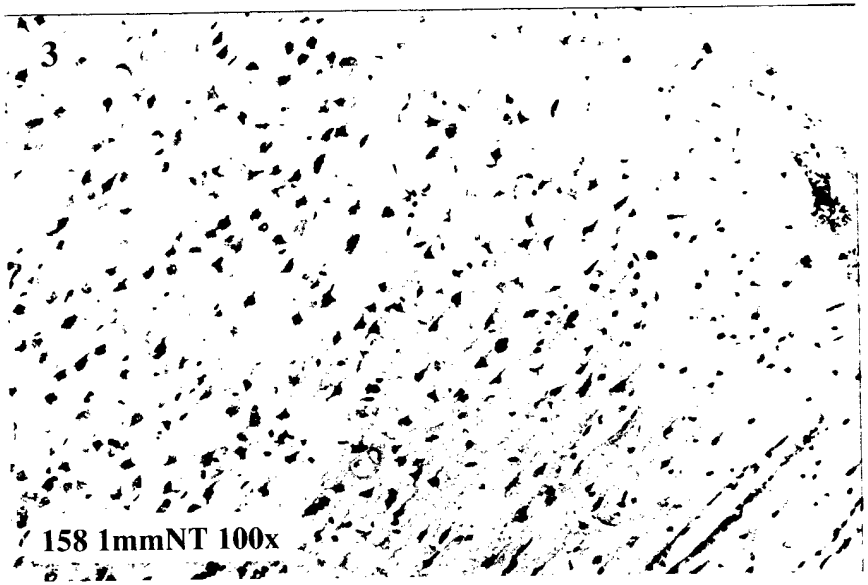

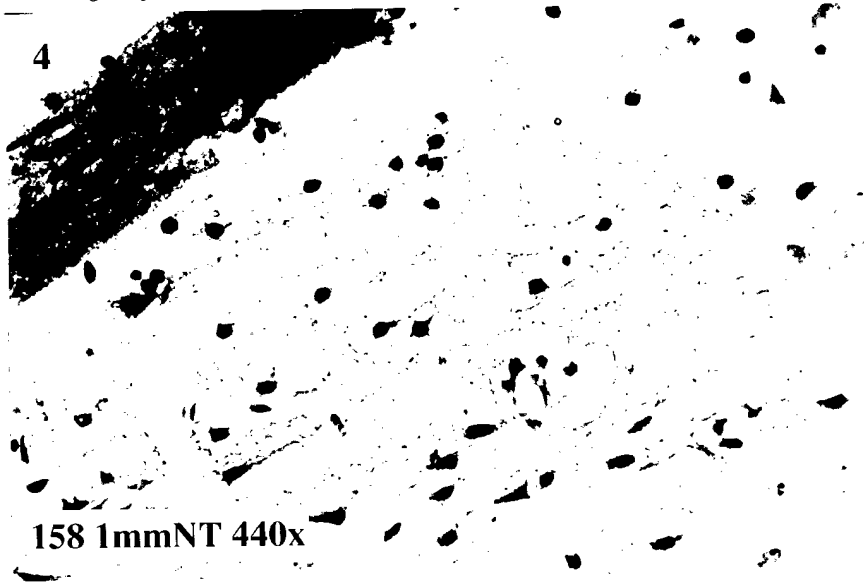

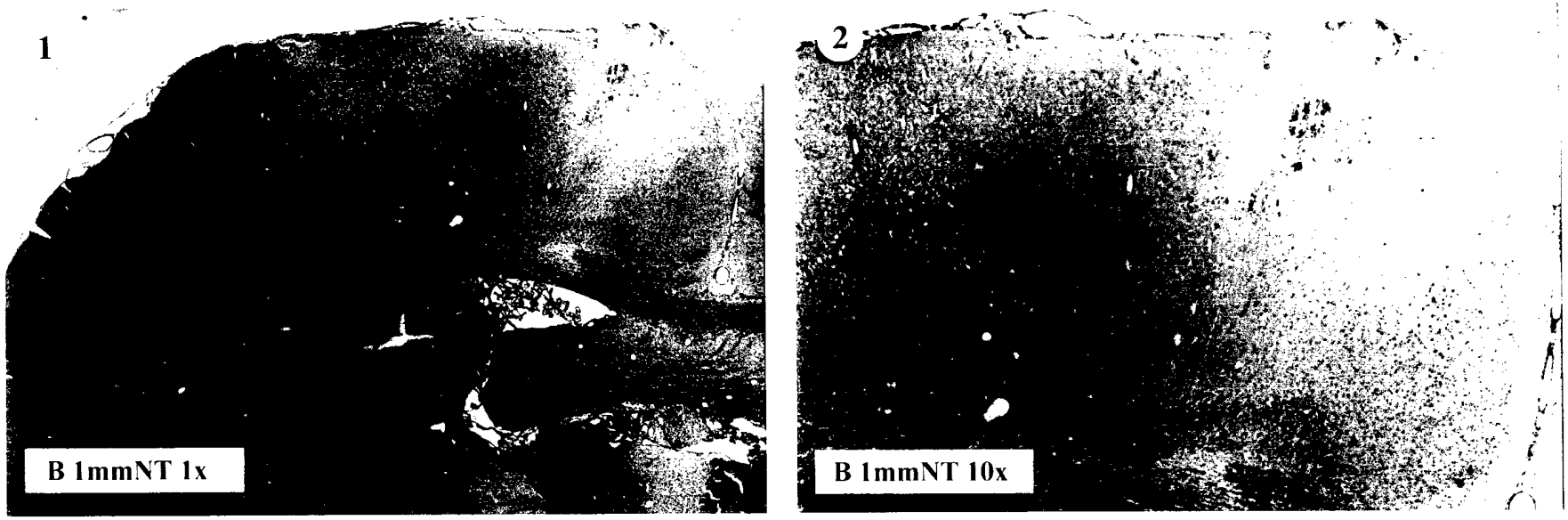

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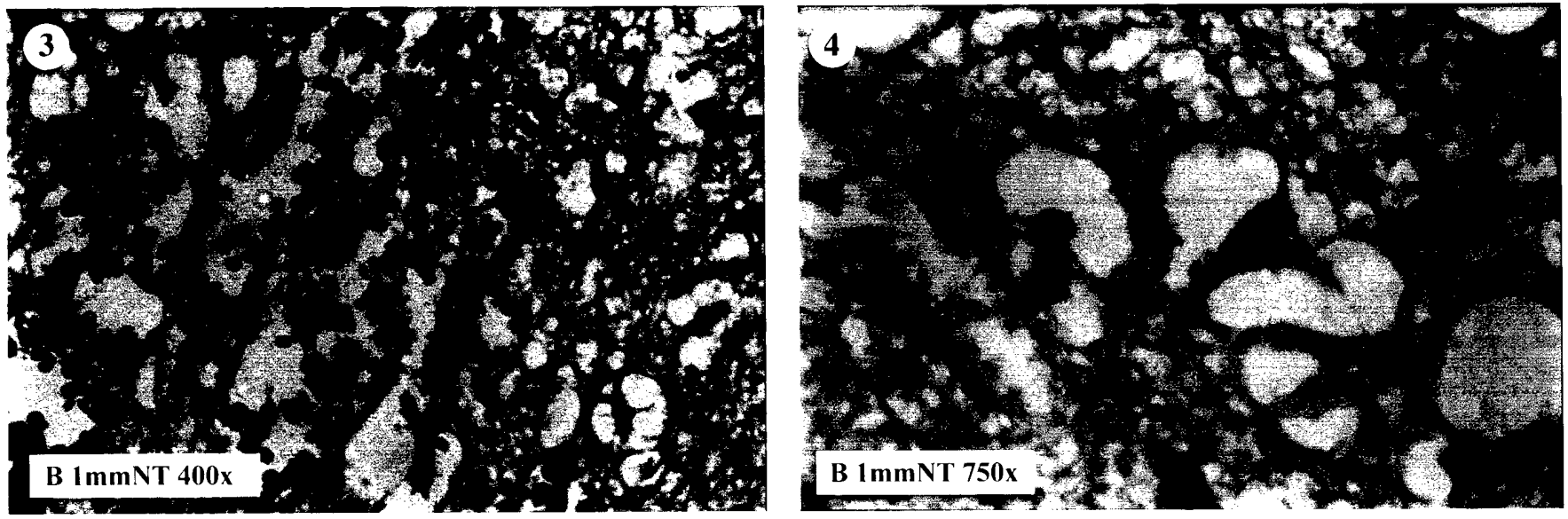
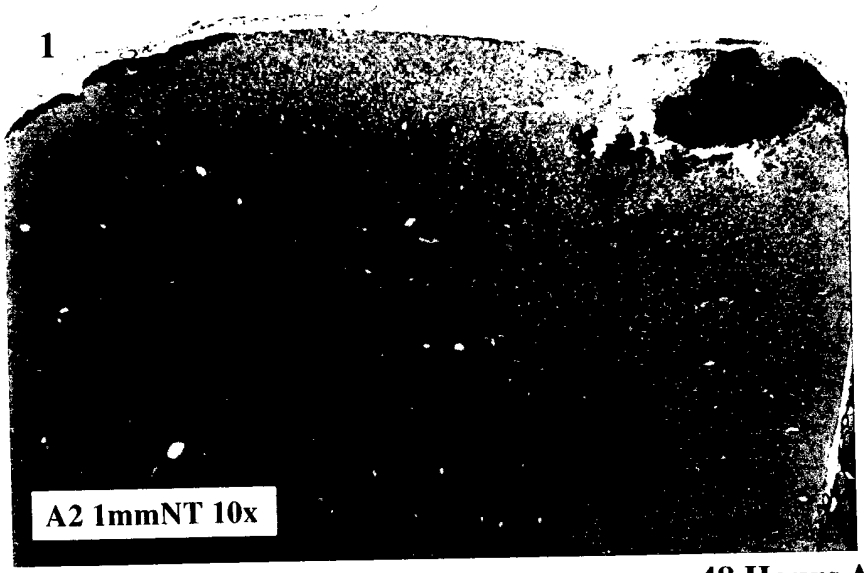
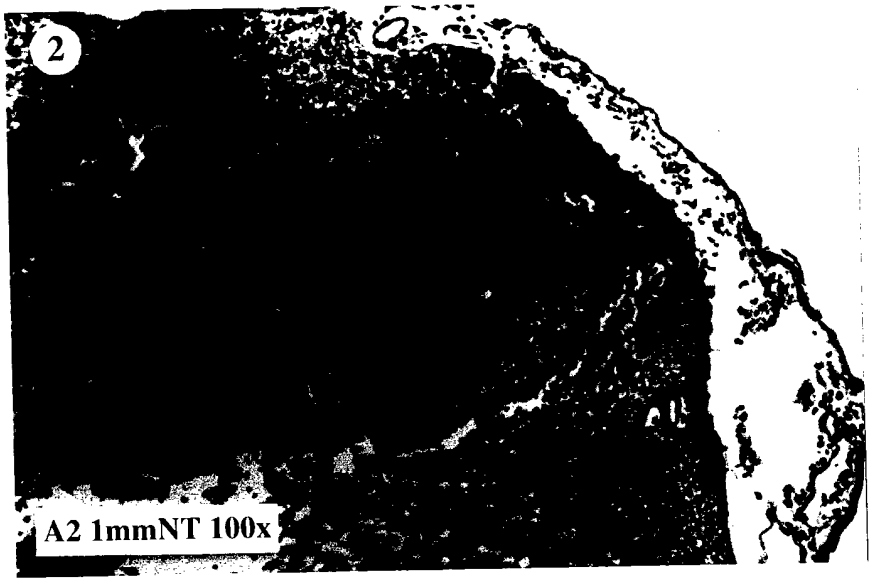
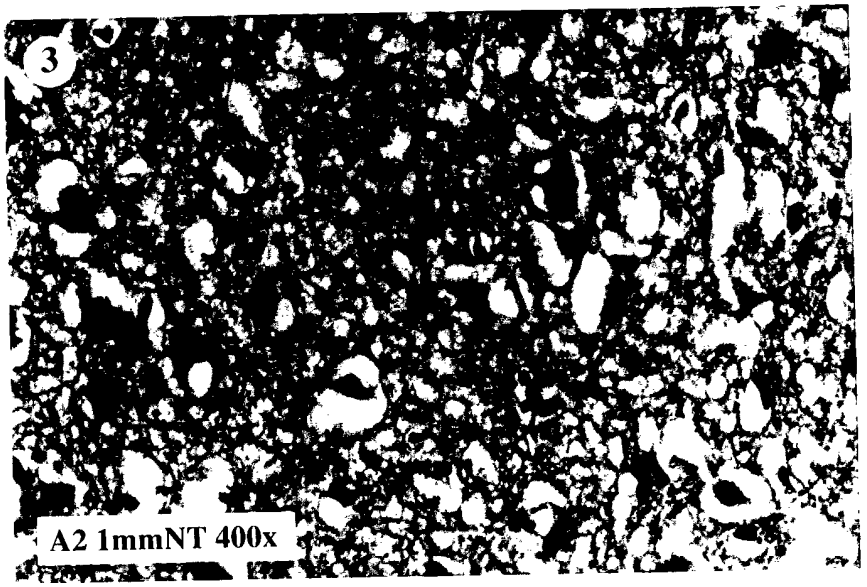
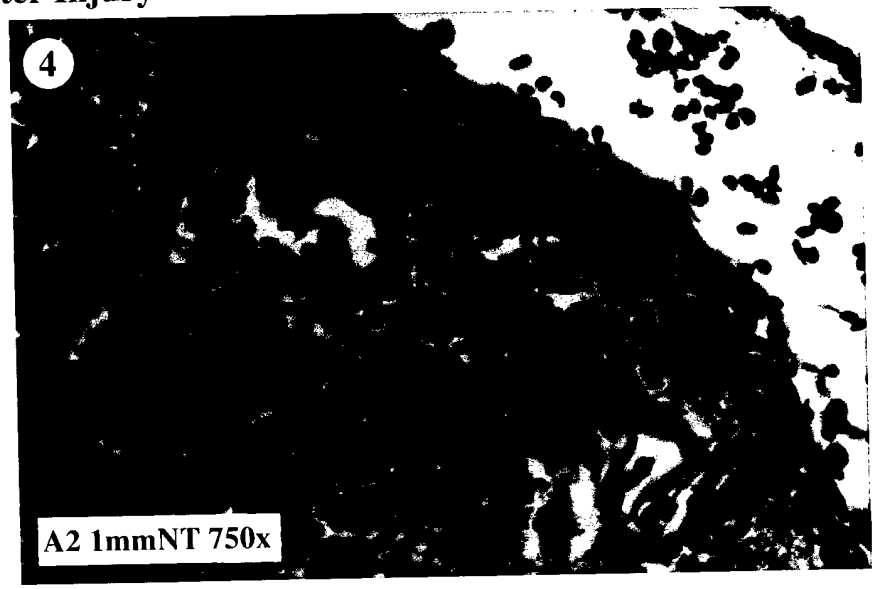


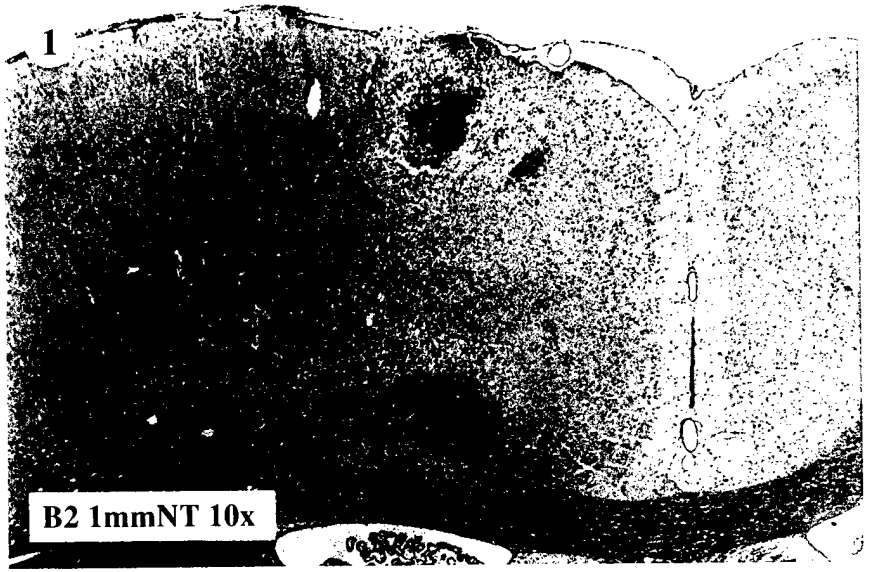


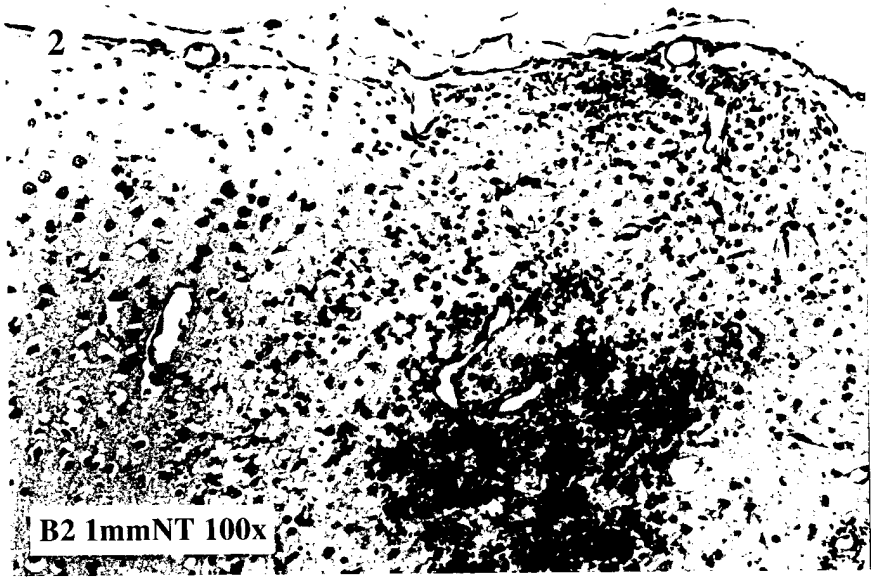


48 Hours After Injury









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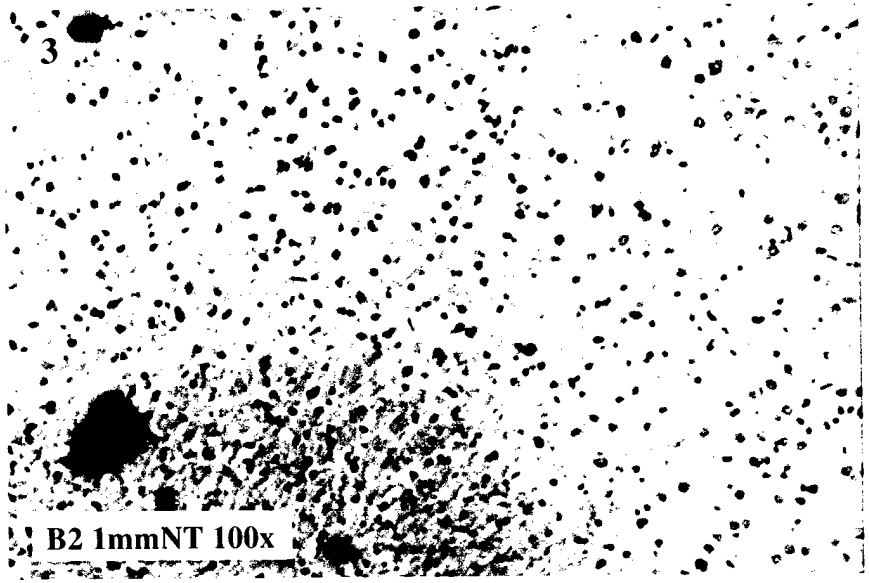
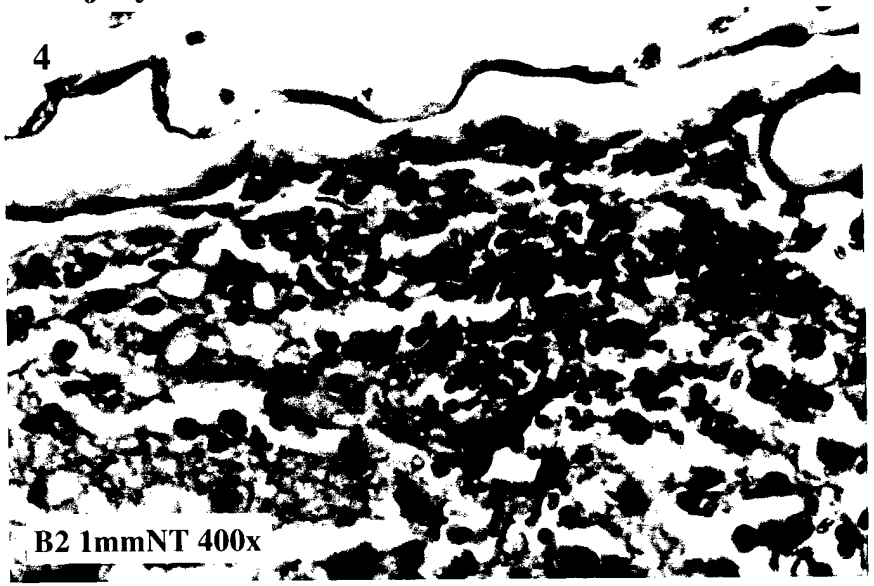
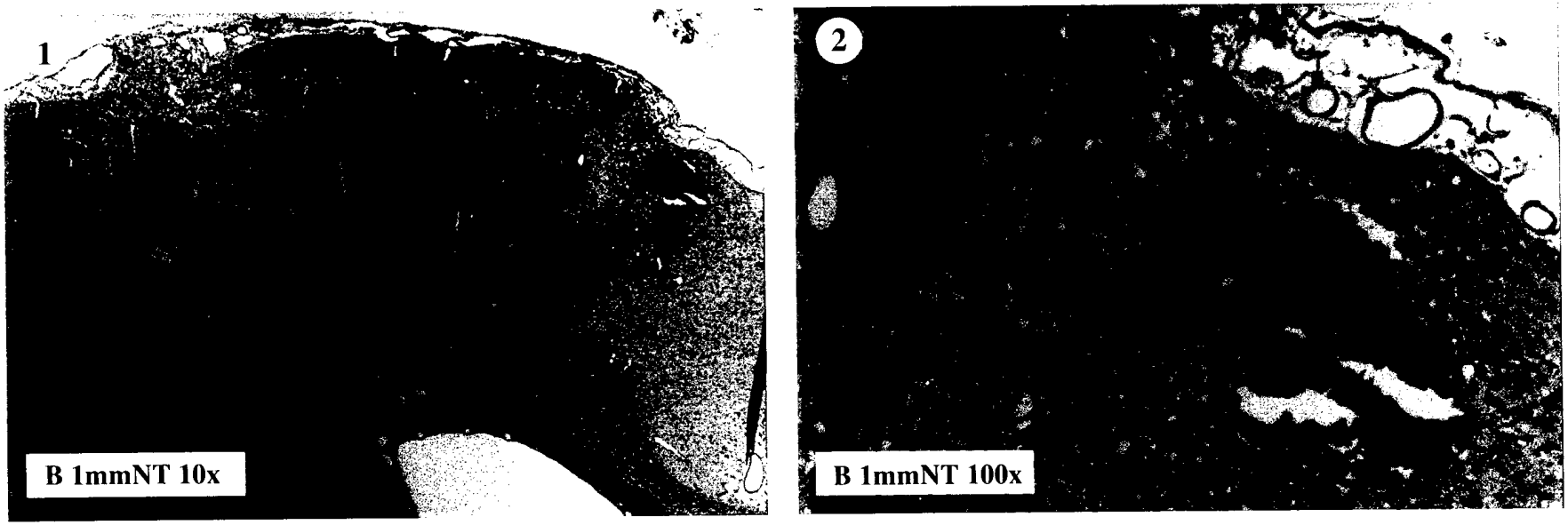
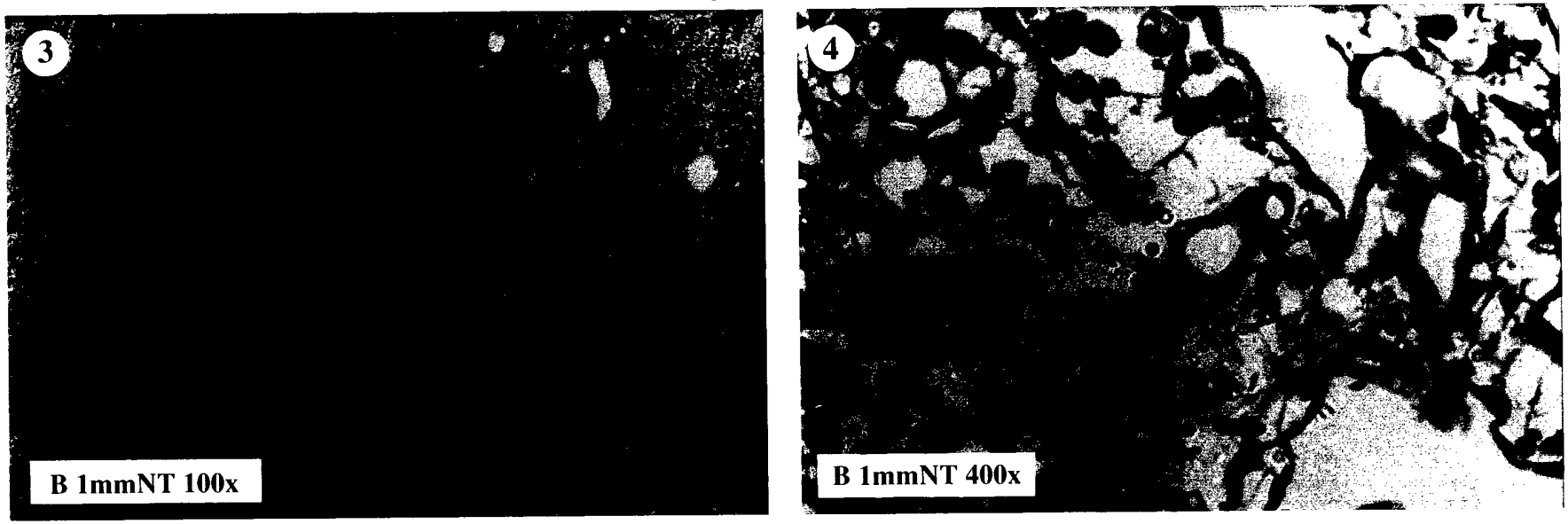
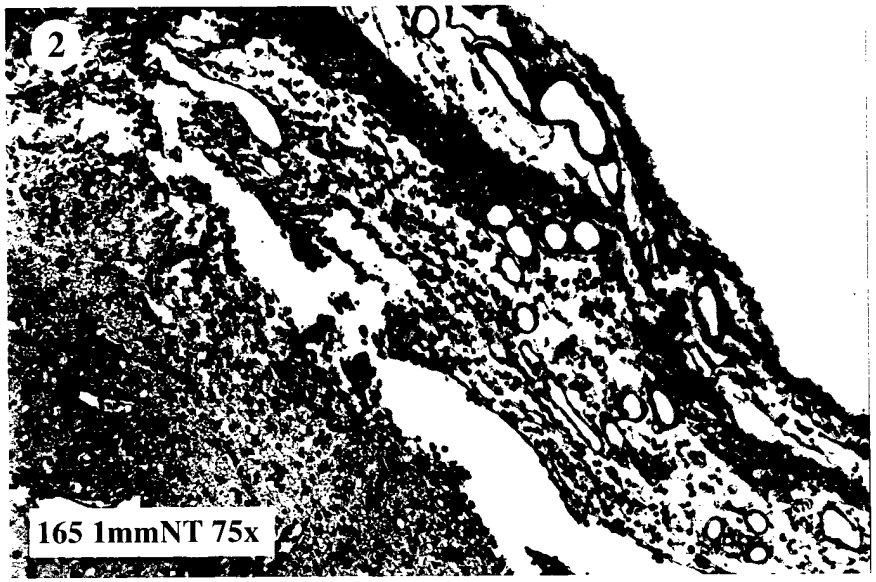




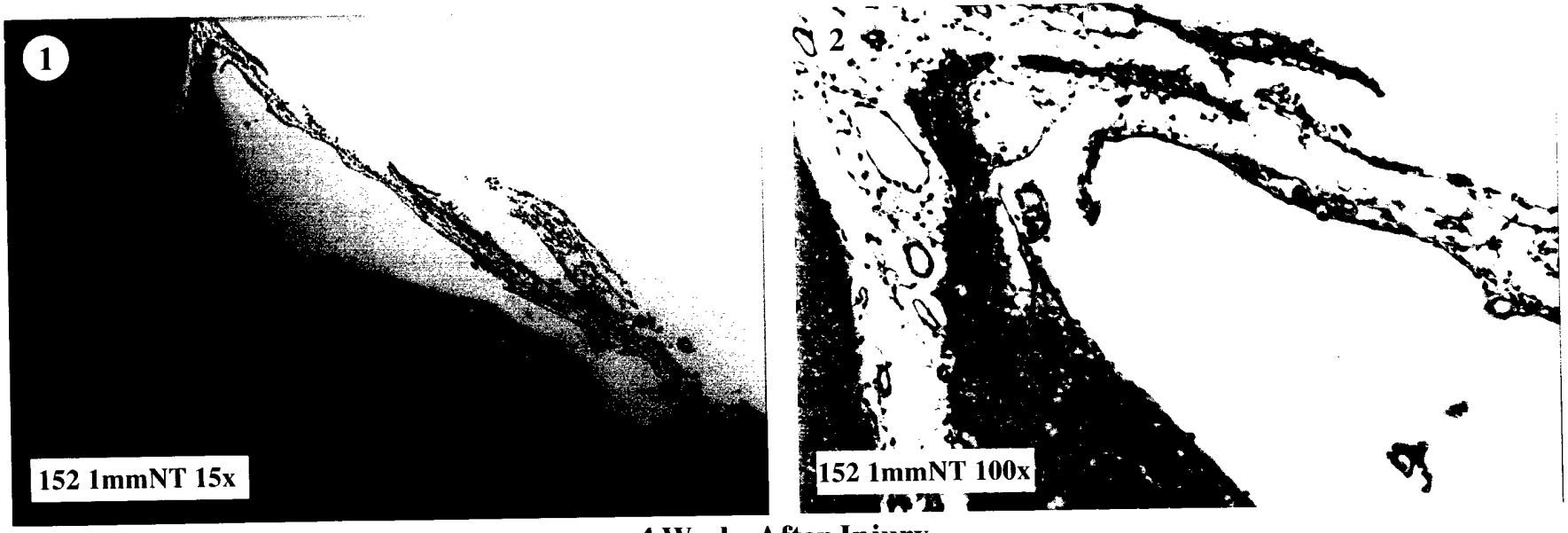


5 Days After Injury

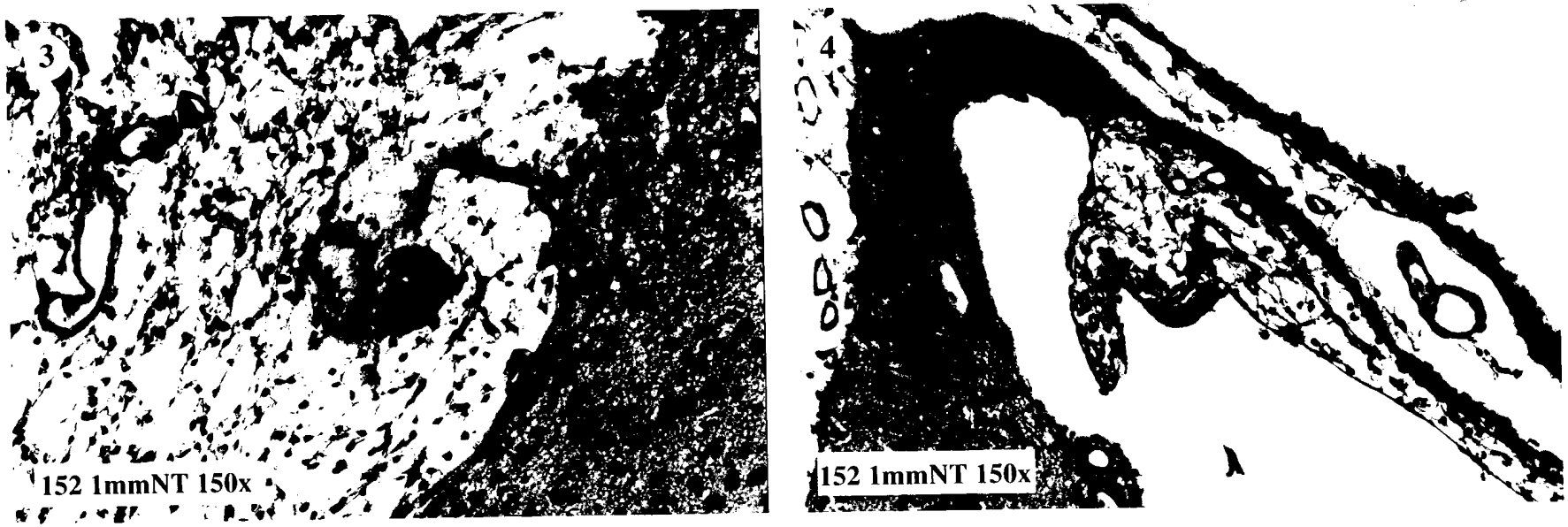




1 Week After Injury



4 Weeks After Injury



APPENDIX 3
DETAILS OF NEUROCHEMICAL STUDIES

Individual FFAs

Thirty minutes after injury in the damaged right frontal cortex 20:4 and 22:6 showed the highest relative increases, (4 and 2.6 fold respectively). Stearic acid (18:0) and oleic acid (18:1) showed 1.7 and 1.8 fold increases while palmitic acid (16:0) remained unchanged, Figure 1. By 24 hrs all FFAs in the FRC showed a tendency to higher values but, because of large variations among individual rats, differences were not significant except for 22:6 and 18:0 (7- and 4-fold increase above sham values), Figure 2. The only other brain area to show a significant change was the ORC where 22:6 increased (sham 1.2 ± 0.1; trauma: 2.6 ± 0.8, $p<0.03$), Figure 3.

Many FFAs remained significantly increased in the injured FRC at 4 days when compared to sham-operated rats with docosahexaenoic acid (22.6), stearic acid (18:0) and arachidonic acid (20:4) displaying the greatest elevations (4.4,3.1, and 2.8-fold). In the contralateral frontal lobe (FLC) all fatty acids tended to increase compared to sham but only 22:6 achieved significance (1.8 fold). In the ORC 22:6 and 20:4 displayed significantly higher values than sham (2.3-fold increase each), while no changes were detected in the OLC nor in right and left hippocampus, cerebellum, and brain stem, Figure 4. Thirty-five days after injury, free 20:4, 18:1, and 18:0 remained increased about 1.5 fold in the right frontal cortex while 20:4 and 18:1 showed a similar elevation in the FLC, (Figure 5). Free 20:4 was also increased 1.5x in ORC but no significant individual FFA changes were observed in any other brain areas.

The individual FFA changes are summarized in Table 3.

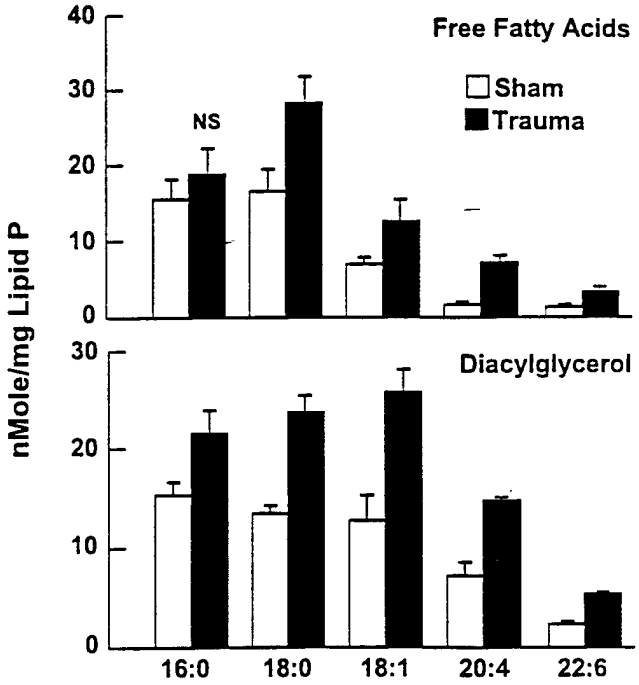
Individual Diacylglycerols

Thirty minutes after injury all DAG groups in the injured FRC tended to be increased 1.4 to 2.0 fold above sham values. At 24 hours all DAG acyl groups continued to trend higher with 18:1 DAG reaching a significant 2x inrease.

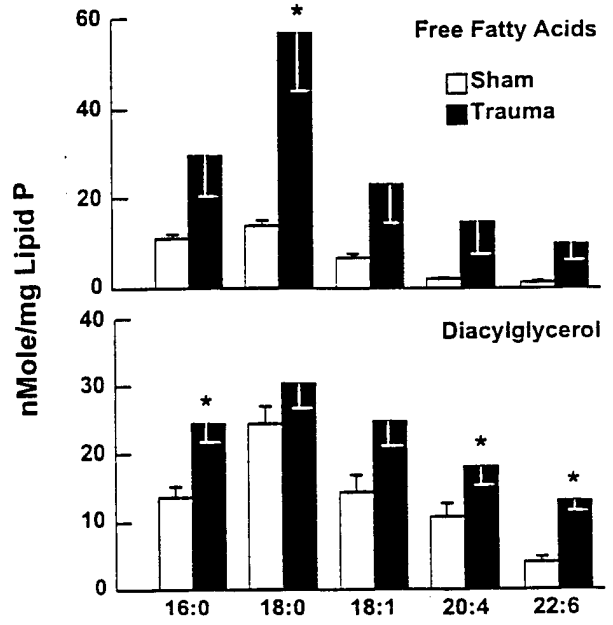
Four days after trauma all fatty acids in the injured FRC, except for palmitic acid (16:0), were increased above sham. The highest was 22:6 (3.3-fold) followed by 18:0

(2.9-fold) and 20:4 (1.8-fold). In the frontal left cortex (FLC) only 18:0 and 22:6 (1.6-fold) were significantly increased.

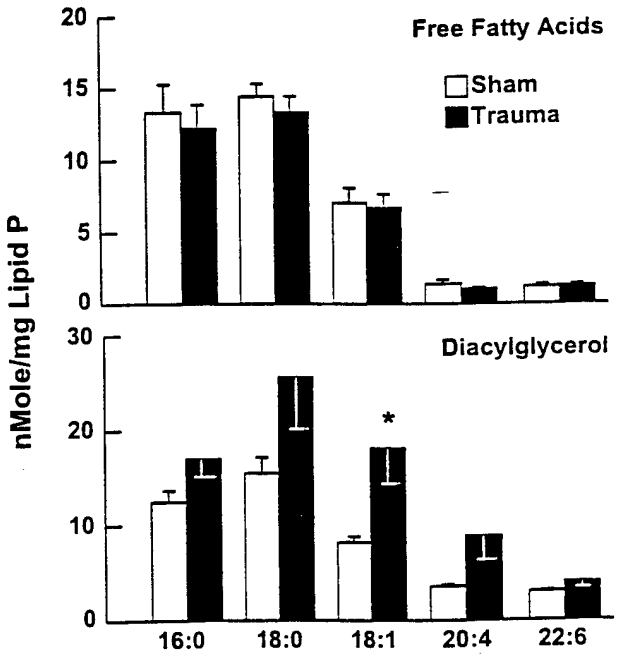
By day 35 after injury all DAGs-acyl groups were highly increased in many brain areas: the FRC, FLC, ORC and OLC, cerebellum and brain stem(Figure 6). At this time all 4 cortical areas exhibited 3 to 5 fold increases in 18:1 and 20:4-DAGs while other acyl groups were increased by 2-fold in the cortex. In the brain stem high accumulation of 20:4-DAGs (5-fold), followed by 18:1 DAGs (3.8-fold), and 18:0 DAGs (2.6 fold) occurred while in cerebellum 18:1 DAG was significantly increased (Figure 7).



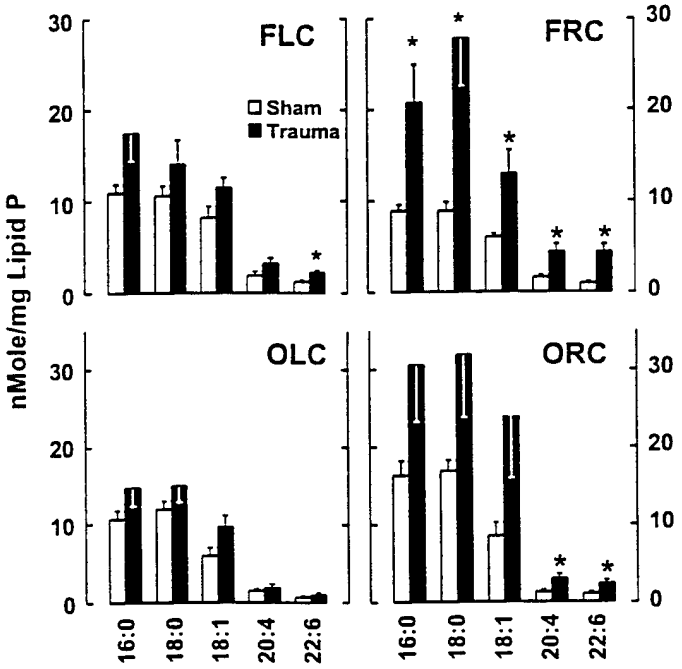
AP 3-Figure 1. Accumulation of FFA and DAG in the frontal right cortex 30 minutes after cortical impact injury. Mean ± SEM are shown. All experimental values were significantly different from sham except for free 16:0 (NS). Other details as in Table 1 legend.



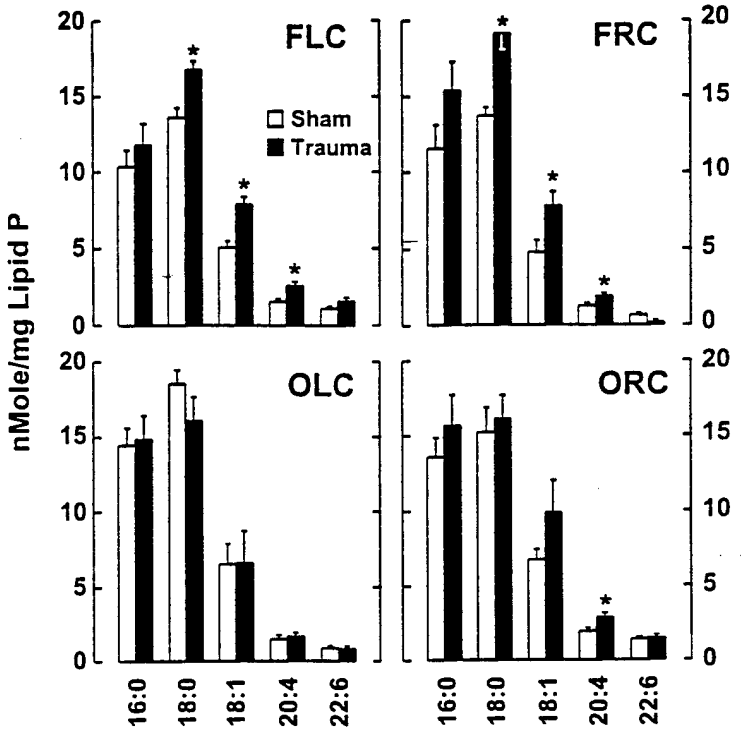
AP3-FIGURE 2. Accumulation of FFA and DAG in the frontal right cortex one day after cortical impact injury. Mean ± SEM are shown. Asterisks indicate values significantly different from sham values.



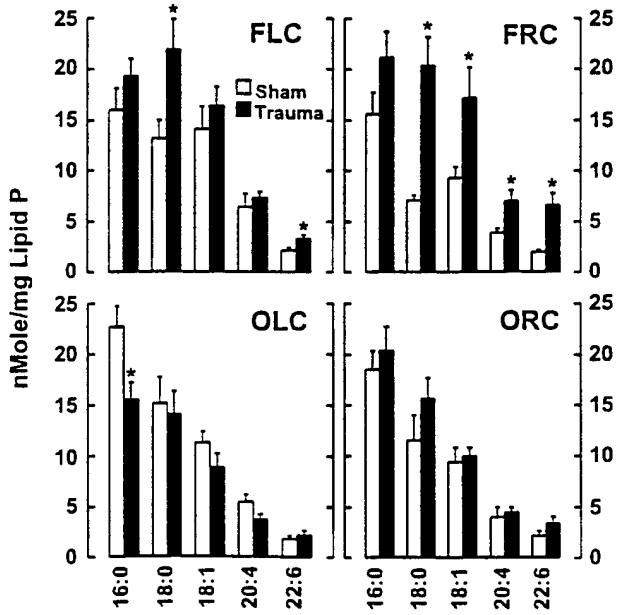
AP3-FIGURE 3. Accumulation of FFA and DAG in the cerebellum 1 day after controlled cortical impact injury. Details as in Figure 1 legend.



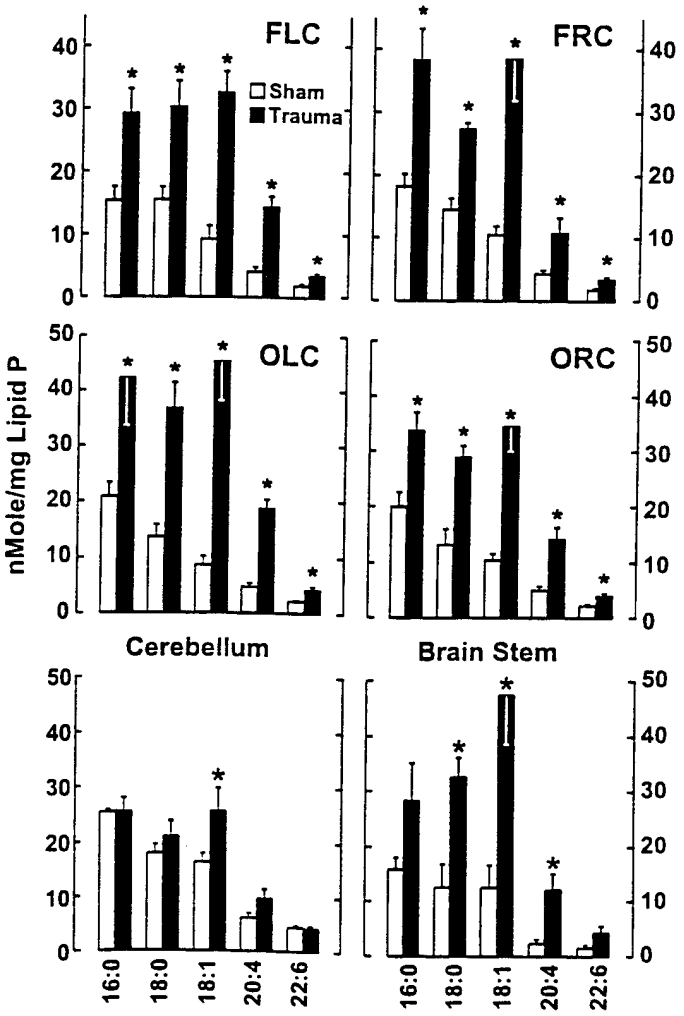
AP3-FIGURE 4. Accumulation of FFAs in rat brain cortical areas 4 days after controlled cortical impact injury. Rats were injured in the frontal right sensorimotor cortex (FRC). Mean ± SEM values are shown. Asterisks denote values significantly different from sham (Student's t test, $p<0.05$). Other details as in Table 1 footnotes.



AP3-FIGURE 5. Accumulation of FFAs in cortical areas 35 days after controlled cortical impact injury. Asterisks denote significant differences (Student's test, $p<0.05$)



AP3-FIGURE 6. DAG-acyl group levels in rat brain cortical areas 4 days after controlled cortical impact injury. Asterisks denote significant differences. (Student's t test, $p<0.05$)



AP3-FIGURE 7. DAG-acyl group levels in rat brain cortical areas, cerebellum, and brain stem 35 days after controlled cortical impact injury. Asterisks denote significant differences at $p<0.05$ (Student's t test).

APPENDIX 4

PUBLICATIONS AND ABSTRACTS RESULTING FROM THIS RESEARCH

A. Publications in Refereed Journals

1. Matthews MA, Carey ME, Soblosky JS, Davidson JF,Tabor SL: Focal brain injury and its effects on cerebral mantle neurons and fiber tracts. Brain Research 794: 1-18, 1998

2. Soblosky JS, Colgin LL, Parrish CM, Davidson JF, Carey ME: A procedure for the sample preparation and handling for the simultaneous determination of amino acids, monoamines and metabolites from microdissected brain regions of the rat. J. Chromatography, 1998 (In Press).

3. Soblosky JS, Colgin LL, Chorney-Lane D, Davidson JF, Carey ME: Ladder Beam and camera video recording system for evaluating forelimb and hindlimb deficits after sensorimotor cortex injury in rats. Journal of Neuroscience Methods 78: 75-83, 1997.

4. Soblosky JS, Colgin LL, Chorney-Lane D, Davidson JF, Carey ME: Some functional recovery and behavioral sparing occurs independent of task-specific practice after injury to the rat's sensorimotor cortex. Behavioural Brain Research 89: 51-59, 1997.

5. Homayoun P, Rodriguez de Turco EB, Parkins NE, Lane DC, Soblosky JS,. Carey ME, Bazan NG: Delayed phospholipid degradation in rat brain after traumatic brain injury. J. Neurochem 69: 199-205, 1997.

6. Soblosky JS, Tabor SL, Matthews MA, Davidson JF, Chorney DA, Carey ME: Reference memory and allocentric spatial localization deficits after unilateral cortical brain injury in the rat. Behavioural Brain Research 80: 185-194, 1996.

7. Soblosky JS, Matthews MA, Davidson JF, Tabor SL, Carey ME: Traumatic brain injury of the forelimb and hindlimb sensorimotor areas in the rat: physiological, histological and behavioral correlates. Behavioural Brain Research 79: 79-92, 1996.

B. Publications Pending

1. Sarphie TG, Carey ME, Matthews MA, Soblosky JS, Davidson JF, Tabor SL: Scanning electron microscopy of the brainstem ventricular area in rats subjected to impact injury of the parietal cortex. (Submitted to J. Neurosurgery).

2. Homayoun P, et al: Early free fatty acid and diacylglycerol changes in rat brain after traumatic brain injury (Submitted to J. Neurochemistry).

3. Soblosky JS, Parrish CL, Colgin LL, Davidson JF, Carey ME: Functional Recovery after sensorimotor cortex injury generalizes to similar but harder tasks without needing task-specific practice (Submitted to Behavioural Brain Research).

4. Homayoun P, et al: Phospholipase A2 changes after traumatic brain injury (Submitted to J. Neurochemistry).

5. Zeiller S, Awasthi D, Carey ME, Beuermen RW, Nguyen D, Soblosky JS, Colgin LL:. Effects of cortical impact injury plus hypotension on gene expression.

6. Mervis RF, Soblosky JS, Carey ME: Dendrite changes following traumatic brain injury; qualitative and quantitative studies.

7. Lane DC, Soblosky JS, Carey ME: Hemorrhagic hypotension delays recovery from experimental traumatic brain injury.

C. Meeting Abstracts

1. Soblosky JS, Parrish, C.L., Colgin LL, Davidson JF, Carey ME: Functional recovery after sensorimotor cortex injury generalizes to similar but harder tasks without needing task-specific practice. (To be presented at Society for Neuroscience Annual Meeting, 1998. Los Angeles, CA.)

2. Mervis RF, Soblosky JS, Carey ME : Cortical damage following traumatic brain injury in the rat: a morphological assessment of Golgi-impregnated neurons. J. Neurotrauma 14(10): 764, 1997.

3. Soblosky JS, Colgin LL, Chorney-Lane D, Davidson JF, Carey ME: Ladder beam and camera video recording system for evaluating forelimb and hindlimb deficits after sensorimotor cortex injury in rats. Soc. Neurosci. Abstr. 23, 270, 1997.

4. Zeiller S, Awasthi D, Carey ME, Beuermen RW, Nguyen D, Soblosky JS, Colgin LL: Effects of cortical impact injury plus hypotension on gene expression. Soc. Neurosci. Abstr. 23, 1122, 1997.

5. Chorney-Lane DA, Soblosky JS, Colgin LL, Davidson JF, Carey ME: Effects of hypotension and hypoxia on traumatic brain injury. Soc. Neurosci. Abstr. 22, 1904, 1996.

6. Soblosky JS, Colgin LL, Chorney, D.A., Davidson JF, Carey ME: Recovery of function after traumatic brain injury (TBI): Is ultimate recovery dependent upon

testing frequency? Soc. Neurosci. Abstr. 22, 1180, 1996.

7. Homayoun P, Rodriguez de Turco EB, Soblosky J, Carey M., Bazan, N: Effects of experimental traumatic brain injury on regional levels of free fatty acid and diacylglycerol. J.Neurochemistry 66: Suppl. 1, S12B, 1996.

8. Matthews MA, Soblosky JS, Davidson JF, Carey ME: Scanning electron microscopy of the brain stem ventricular area in traumatically injured rats. J. Neurotrauma 12(5): 991, 1995.

9. Soblosky JS, Matthews MA, Chorney DA, Davidson JF, Colgin LL, Carey ME: Pattern of axonal degeneration in the brain after traumatic brain injury (TBI) of rat sensorimotor cortex. Soc. Neurosci. Abst. 21, 1003, 1995.

10. Chorney DA. Soblosky JS, Colgin LL, Davidson JF, Carey ME: Effects of secondary insult on traumatic brain injury. Soc. Neurosci. Abst. 21, 1731, 1995.

11. Soblosky JS, Tabor SL, Davidson JF, Carey ME: Traumatic brain injury: Effect of long term and short memory changes as evaluated in an 8 arm radial maze. J. Neurotrauma 12(1): 143, 1994.

12. Soblosky JS, Davidson JF, Tabor SL, Carey ME: Traumatic brain injury of rat hind and forepaw sensory/motor areas. Soc. Neurosci. Abst. 20, 194, 1994.

APPENDIX 5

SUMMARY OF WORK CONTRACTED, WORK PERFORMED AND EXTRA NON-CONTRACTED WORK PERFORMED FOR THE BENEFIT OF THE U.S. ARMY UNDER CURRENT CONTRACT DAMD17-93-C-3008

Contracted	Year to be Done	Status
Develop, characterize rat head injury model of piston impact to right sensorimotor cortex including physiological effects, standardize anesthesia.	01	Done *
Histologic evaluation of head trauma immediately after injury to 8 weeks after injury by light microscopy using H&E staining for general morphology.	01-02	Done *
Electron Microscopy *c-fos* immunohistochemistry.	01-02	Done
Evaluate apneic response after brain injury. Electron microscopy of floor of IV ventricle	02-03	Study terminated because the rat is not a good model for posttraumatic apnea studies. No drug studies done to reverse post-traumatic apnea nor brain stem microdialysis done because of this. Significant anatomic lesions identified on IV ventricular floor. #

* = papers published

= papers submitted for publication

Contracted	Year to be Done	Status
Regional effects of acute trauma on excitatory amino acids and biogenic amines in cortex and brain stem at our standard injury level.	02	Done
Evaluate the acute effects of head injury on synaptic phospholipase A_2 (PLA_2).	02	Done #
Evaluate membrane inositol lipid: diacylglycerol and $1P_3$ turnover in membrane phospholipids.	02	Not Done
Evaluate cortical excitatory amino acids and catecholamines up to two hours after injury.	03	Done
Evaluate effects of brain injury on membrane free fatty acids and diacylglycerol	02	
4 to 35 days after injury		Done #
30' to 4 days.		Done *
Time course of platelet-activating factor (PAF) following brain trauma.	03	Effort abandoned because assay said to be reliable by kit manufacturer was found to be unreliable by Dr. Bazan's lab.

* = papers published

= papers submitted for publication

Contracted	Year to be Done	Status
Evaluate long term effects of brain trauma on neurological behavioral parameters up to 14 days after brain injury	04	Done up to 8 weeks *
a) motor testing		Done
b) memory testing.		Done
Evaluation of excitatory amino acids and catecholamines in recovering animals.	04	Done
Acute effects of head injury on platelet activating factor (PAF).	05	Abandoned owing to unavailability of reliable PAF assay
Effects of drugs including a PAF antagonist on enhancing neurological recovery following brain injury	05	
MDL 28, 170 (Calpain Inhibitor)		Done
BN 52021 (PAF antagonist).		Done

Non Contracted Additional Research for Army at No Additional Cost

1) The adverse effect of systemic arterial hypotension upon neural recovery following brain injury (Brown Foundation grant).	Done
2) The adverse effect of hypoxia upon neural recovery following brain injury (Brown Foundation grant).	Done
3) The effect of task specific testing on neural recovery.	Done *

* = papers published

= papers submitted for publication

Non Contracted Additional Research (cont)	Status	
4) Development of a horizontal ladder to more accurately evaluate posttraumatic locomotion defects.	Done	*
5) Silver stains to determine axonal degeneration.	Done	*
6) GFAP stains of injured neurons.	Done	
7) Descriptive and quantitative evaluations of dendrites after injury using Golgi stains.	Being Done	
8) Quantitative analysis of immediate early genes and "recovery genes" following brain injury alone and following brain injury plus systemic arterial hypotension.	Done	
9) Evaluate regional cytochrome oxidase and NADPH after brain injury (Brown Foundation grant).	Being Done	

* = papers published

= papers submitted for publication

APPENDIX 6

PERSONNEL ENGAGED IN THIS RESEARCH

Principal Investigator:	Michael E. Carey, MD
Senior Research Associate:	Joseph Soblosky, PhD (Core Lab)
Core Laboratory Personnel:	June Davidson, BA Laura Colgin, BA Diane Lane, BA (Brown Foundation) Christine Parish, BA (Brown Foundation)
Neurochemistry (Dr. Bazan):	Parvin Homayoun, PhD Elena Rodriguez de Turco, PhD Nilda Parkins, MS
Histology: (Dr. Matthews)*	T.G. Sarphie, PhD Olga Isaeva
Molecular Biology (Dr. Beuerman):	Steven Zeiller, BS Doan Nguyen, PhD

* deceased