Loss of forebrain cholinergic neurons following fluid-percussion injury: implications for cognitive impairment in closed head injury

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Disturbances in memory, concentration, and problem solving are common after even mild to moderate traumatic brain injury. Because these functions are mediated in part by forebrain cholinergic and catecholaminergic innervation, in this study the authors sought to determine if experimental concussive injury produces detectable morphological damage to these systems.

Fluid-percussion head injury, sufficient to cause a 13- to 14-minute loss of righting reflex, was produced in rats that had been anesthetized with halothane. Injury was delivered either at midline or 2 mm off midline and compared with appropriate sham-injured controls. After 11 to 15 days, the rat brains were stained in serial sections for choline acetyltransferase, tyrosine hydroxylase, dopamine β-hydroxylase, acetylcholinesterase, and nicotinamide adenine dinucleotide phosphate diaphorase. Cell counts were determined for the entire population of ventrobasal forebrain cholinergic cells. Midline injury produced a bilateral loss of cholinergic neurons averaging 36% in area Ch1 (medial septal nucleus), 45% in Ch2 (nucleus of the diagonal band of Broca), and 41% in Ch4 (nucleus basalis of Meynart), (p ≤ 0.05). Lateralized injury resulted in cholinergic neuron loss of similar magnitude ipsilaterally (p ≤ 0.05), but a smaller contralateral loss of between 11% and 28%. No loss of neurons was detected in the pontomesencephalic cholinergic groups Ch5 and Ch6. There was no visible effect of head injury on forebrain dopaminergic or noradrenergic innervation.

A significant and apparently selective loss of ventrobasal forebrain cholinergic neurons following brief concussive injury in rats is demonstrated in this study. This type of injury is known to produce significant disturbance in cognitive tasks linked to neocortical and hippocampal cholinergic function. It remains to be determined how this neuron loss occurs, whether it can be prevented with neuroprotective agents, how it affects innervation in target tissues, and whether it occurs in human victims of traumatic brain injury.

KEY WORDS • fluid-percussion injury • traumatic brain injury • acetylcholine • catecholamine • nucleus basalis • septal nucleus • rat

Over 500,000 people in the United States sustain traumatic brain injuries each year; the vast majority are between the ages of 11 and 40 years. Although 360,000 of these injuries are mild (Glasgow Coma Scale (GCS)19 13–15), over 70,000 persons die from head injury annually, and another 70,000 individuals survive after suffering moderate (GCS 9–12) or severe (GCS 3–8) brain injury. Apart from focal deficits attributable to injury of specific sensory and motor pathways, head injury is associated with pervasive disturbances in consciousness and cognitive function. A significant percentage of survivors of even mild head injury report disabling alterations in memory, concentration, problem solving, and motivation. These problems are nearly ubiquitous in survivors of more severe injury. Rimel, et al., 39 have reported that, as a result of these nonspecific problems, 69% of patients with moderate injury and 34% with mild injury are unable to resume preinjury employment at 3 months postinjury. Although such deficits tend to diminish over time, permanent disability may persist in 10% of cases of mild injury and 66% of cases of moderate injury.

The neuronal basis for this “postconcussive state” is presently unknown. The stereotypical nature of these nonspecific problems suggests that the brain mechanisms subserving these functions are particularly vulnerable to disruption by trauma. Considerable experimental and pharmacological evidence has accumulated over the past two decades that associates a number of specific neurochemical systems in the brain with the kinds of functions that tend to be disrupted in the postconcussive state. In particular, cholinergic neurons within the ventrobasal forebrain are essential for memory and cognitive function, and central noradrenergic and dopaminergic systems participate in selective attention and motivation.
Cholinergic neurons in concussive head injury

The goal of this study was to determine whether mild to moderate fluid-percussion injury produces any discernible injury to central cholinergic or catecholaminergic systems in experimental animals. Immunocytochemical and histochemical techniques were used to visualize these neuronal systems. Injured animals were compared to animals in a sham-injured control group and also to those in a group in which the fluid-percussion injury was largely confined to one hemisphere.

Materials and Methods

Animal Preparation

Adult male Sprague-Dawley rats, weighing 275 to 325 g, were used in this study. All experiments were performed in compliance with animal use standards established by the U.S. Public Health Service and were approved and monitored by the animal care committee of the University of Washington. Animals were anesthetized during all procedures.

Luer-lock injury cannulas were surgically affixed over a cranial burr hole using methyl methacrylate cement on animals anesthetized with ketamine cyclazine 1 to 2 days prior to injury. Cannulas were positioned either centrally (3 mm anterior to the lambdoid suture at midline) or parasagittally (2 mm posterior and lateral to the bregma). On the day of injury, the animals were anesthetized with halothane, intubated endotracheally, ventilated with 1.75% halothane in air to maintain PCO2 within a normal range, and kept on a warming pad. Injury or sham injury was administered using a fluid-percussion device, which in the case of injury delivered a 3.2-atm pressure pulse over an 8-msec period.42 Immediately before the moment of injury (10–15 seconds) the halothane supply was terminated. Under these conditions, noninjured animals would awaken within 3 to 4 minutes. Care was taken to equalize anesthetic conditions in both injury and sham groups. Following injury, note was made of the times required for the return of the following reflex functions: spontaneous respiration, pupil size and reactivity, corneal blink, pinna reflex, hindpaw pinch withdrawal, righting reflex, and sniffing. Animals were then kept overnight under a heat lamp and weighed daily to monitor hydration. Intraperitoneal injections of 20 cc dextrose 5% in lactated Ringer solution were given daily for 4 to 5 days until the injured groups were able to maintain adequate oral fluid intake.

Histochemical Studies

The animals were killed between 11 and 15 days postinjury by injection of methohexital–pentobarbital, and their bodies were serially perfused with saline, 1% paraformaldehyde, and 4% paraformaldehyde–0.05% glutaraldehyde. After removal, the brains were immersed for 24 hours in the same fixative and then infiltrated with 30% sucrose in phosphate-buffered saline. Brains were sectioned at 40-μm intervals in the coronal plane, collecting multiple series of every sixth section. One of each series was then used for immunocytochemical staining for choline acetyltransferase (CAT) with monoclonal No. 770 990 (1:2000 final dilution); 8 One series was stained for nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase using the method of Scherer-Singler,41 which was modified by the inclusion of magnesium.49 Another series was stained for acetylcholinesterase (AChE) using the method described by Mesulam and colleagues.31,40 Immunocytochemical staining was performed on free-floating sections using the sensitive avidin–biotin complex method44 and peroxidase was developed using the diaminobenzidine–glucose oxidase technique with nickel ammonium sulfate intensification.43 Choline acetyltransferase–stained sections were additionally enhanced with ammoniacal silver.24 Additional brain tissue was serially sectioned in the sagittal plane and stained for TOH and DBH as described above to visualize the catecholamine fiber bundles running longitudinally through the mesencephalon and diencephalon. Tissue sections were stained with toluidine blue to identify nonspecific neuron loss.

Quantitative Analysis of Cholinergic Neurons. Cell counts of cholinergic neurons stained for CAT in the forebrain and NADPH diaphorase in the brainstem were obtained from the 1:6 series of coronal sections using the nomenclature of Mesulam and coworkers45 to identify individual cholinergic nuclei. In the forebrain, the histological appearance of the fornix, anterior commissure, and sepal region was used to select corresponding sections from each animal. Counts were combined from two sections containing the Ch1 and Ch2 groups and from three sections containing the Ch4 group (Fig. 1). In the brainstem the Ch5 and Ch6 groups were counted over their entire extent. Each section was counted at least twice until intercount differences were less than five neurons.

Statistical Study

All quantitative data were analyzed by a one-way analysis of variance and by the Student-Newman-Keuls’ test, which was used to identify individual differences between sides and groups. Significance was determined at the level of p < 0.05.

Supplies

For the immunocytochemical studies, monoclonal No. 770 990 from Boehringer Mannheim, Indianapolis, Indiana, was used to stain for CAT and polyclonal No. TE101 TH and No. TE103, each available from Eugene Tech International, Allendale, New Jersey for TOH and DBH, respectively. The avidin–biotin complex method was achieved using Vectastain, provided by Vector Laboratories, Burlingame, California.

Results

Acute Neurological Effects of Injury

A summary of the acute neurological effects of fluid-percussion injury in the three experimental groups used for the quantitative histochemical analysis is presented in Table 1. In the sham-injured group, anesthesia alone caused a brief loss of brainstem reflexes, which returned to normal within 4 minutes. The effect of fluid-percussion injury was similar in the central cannula and parasagittal cannula groups. The corneal, pinna, and paw-pinch reflexes were lost for a period of 8.5 to 12 minutes. The right-
ing reflex returned between 13 and 14.5 minutes, and sniffing behavior, indicating that an animal was awake enough to explore the environment, returned between 19 and 20 minutes. All of the animals remained alert and interactive in the postinjury period; however, the injured animals showed a marked decrease in both spontaneous activity and feeding behavior, which lasted up to 7 or 8 days. At Day 5 body weights in these animals fell to a nadir averaging 82% of preinjury weight. Two animals, not included in the data provided in Table 1, died between Days 3 and 5, for an overall mortality rate of 20% in the injured groups. None of the animals displayed any focal neurological deficits such as limb weakness or dilated pupils.

At the level of injury used here, fluid percussion did not result in any significant visible damage to the brain parenchyma. At most, slight superficial hemorrhage, confined to the subpial border, was visible in several brains immediately beneath the injury cannula site. Most of the injured brains did show evidence of thin smears of subdural–subarachnoid hemorrhage around the mesencephalic and prepontine cisterns, but there was no evidence of cavitation, necrosis, or deep cerebral hemorrhage in any of the brain tissue, and no evidence of diffuse cerebral swelling or herniation.

Effects of Injury on Cholinergic Histochemistry

Fluid-percussion injury resulted in a significant change in the morphology of cholinergic neurons in the ventrobasal forebrain at a survival time of 11 to 15 days. As compared to neurons from control animals (Fig. 2), those from injured animals were smaller and had fewer and shorter neuritic processes, especially in the Ch4 group (Fig. 3). The intensity of staining did not visibly differ between injured and control groups, and there were no stained remnants of degenerated neurons present. Cell counts obtained from the Ch1, Ch2, and Ch4 regions demonstrated a significant decrease in ventrobasal forebrain cholinergic neurons following fluid-percussion injury (Fig. 4 upper). In the central cannula group, cholinergic neurons were decreased bilaterally an average of 36%, 45%, and 41% in the Ch1, Ch2, and Ch4 groups, respectively. In the parasagittal cannula group, cholinergic neuron dropout was significantly greater on the side ipsilateral to the injury and markedly less on the contralateral side, except in the septal nucleus (Ch1) in which neuronal dropout was equivalent on both sides.

In the brainstem, both CAT immunocytochemistry and NADPH diaphorase histochemistry stained identical populations of neurons. There were no differences in the morphological appearance or in the number of these neurons (Fig. 4 lower) between the control and injured groups.

Because the CAT antiserum used in this study does not adequately stain the axons and nerve terminals of central cholinergic neurons, it is generally not possible to use this material to correlate the reduction in cholinergic neurons with loss of innervation within terminal fields. However, the basolateral and posterolateral amygdalar nuclei did stain densely for CAT, and in many of the injured brains there was a decrease in the intensity of this staining (Fig. 5). Acetylcholinesterase histochemistry demonstrat-

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Injury (atm pressure pulse)</th>
<th>Apnea</th>
<th>Corneal Blink</th>
<th>Pinna Pinch</th>
<th>Pinch Withdrawal</th>
<th>Righting Reflex</th>
<th>Sniffing</th>
</tr>
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<tbody>
<tr>
<td>control (5 animals)</td>
<td>0 ± 0</td>
<td>0.0 ± 0</td>
<td>1.6 ± 0.43</td>
<td>2.3 ± 0.58</td>
<td>1.8 ± 0.38</td>
<td>3.8 ± 0.64</td>
<td>3.1 ± 0.72</td>
</tr>
<tr>
<td>central cannula (4 animals)</td>
<td>3.23 ± 0.05†</td>
<td>0.63 ± 0.6</td>
<td>11.8 ± 3.42†</td>
<td>8.6 ± 2.32†</td>
<td>9.8 ± 2.52†</td>
<td>13.1 ± 2.42†</td>
<td>19 ± 7.35†</td>
</tr>
<tr>
<td>parasagittal cannula (4 animals)</td>
<td>3.20 ± 0.02†</td>
<td>0.75 ± 0.48</td>
<td>10.6 ± 2.25†</td>
<td>9.9 ± 0.51†</td>
<td>9.8 ± 1.02†</td>
<td>14.4 ± 2.11†</td>
<td>20 ± 4.22†</td>
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* Data are expressed as mean ± standard error of the mean.  † Difference is significant from control group (p ≤ 0.05).

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**FIG. 2.** Photomicrographs of brain sections from sham-injured control animals showing staining for choline acetyltransferase. Monoclonal No. 770 990. A: Overview of cells in Ch1 and Ch2 cholinergic cell groups. Original magnification × 400. B: Detail of Ch1 neurons bilaterally. Note the fine, vertically oriented axonal fibers expressing marker. Original magnification × 100. C: Overview of cholinergic neurons in region Ch4 on the right. Midline is noted by the arrow. Original magnification × 100. D: Detail of Ch4 neurons. Note the large size of the neurons and their long bipolar or multipolar neuritic processes. Original magnification × 100.
ed a fine reticular network of nerve terminals with distinct lamination in the cerebral cortex and hippocampus, as well as distinct patterns of innervation within subcortical nuclei. Injury did not produce any qualitative alteration in the pattern of AChE staining; however, a number of brains did show decreased intensity within the basolateral and posterolateral amygdalar nuclei.

**Effect of Injury on Catecholamine Histochemistry**

The TOH and DBH stains provided excellent visualization of catecholamine and noradrenergic neurons, as well as their axon bundles and terminal ramifications. Visual observation revealed no apparent differences between the control and injured groups with respect to the number or morphology of neurons in any of the various catecholamine nuclei extending from the medulla to the rostral diencephalon. Because of their extremely large number, it has not been possible to analyze these neurons quantitatively in this material. A number of major catecholamine axon bundle systems were specifically examined, including the dorsal noradrenergic bundle, central tegmental tract, median forebrain bundle, and nigrostriatal bundle; no evidence of axonal injury or dropout was apparent in the injured brains. Furthermore, there was no apparent decrease in terminal field density in a large number of areas, including cerebral cortex, hippocampus, prefrontal cortex, cingulate gyrus, septal region, ventrobasal forebrain, thalamus (including the anteroventral nucleus and habenular nuclei), striatum olfactory tubercle, and nucleus accumbens. Even areas of cortex and hippocampus closest to the injury cannula showed no visible decrease in catecholamine innervation.

**Discussion**

**Histopathology of Head Injury**

Current understanding of the pathophysiology of traumatic brain injury attributes much of the neural damage to diffuse axonal injury, typified acutely by widespread axonal retraction balls, especially within white matter tracks. As the work of Adams and colleagues has shown, the severity of neurological injury and its clinical prognosis correlates with the extent of diffuse axonal injury.

Up to the present time no attempt has been made to address how specific neurochemical systems are affected by diffuse axonal injury or to determine whether any neuronal systems are more vulnerable to this type of injury than others. The observation that severe and moderate
multiple sites within the brainstem and especially to the thalamus.32,50

This study demonstrates a significant reduction in stainable septal and ventrobasal forebrain cholinergic neurons in the subacute period following mild to moderate head injury in rodents. This effect was seen in cases of both central and lateral injury, although lateral injury resulted mainly in an ipsilateral reduction of neurons. Nonspecific staining of neurons in tissue sections stained with toluidine blue did not reveal any evidence of injury or cell loss in the ventrobasal forebrain or septal regions. Thus we infer a selectivity in the cholinergic injury. The results of this study also confirm and greatly extend the preliminary findings of Leonard and coworkers26 who noted an injury-related decrease in septal cholinergic neurons with staining to either CAT or nerve growth factor receptor. Although the present study suggests a loss of cholinergic staining in the amygdala, further work will be required to quantify this and the extent of any changes in other target sites for cholinergic nerve terminals.

Functional Implications

Ventricular forebrain and septal cholinergic neurons have been strongly linked with memory and learning behavior. Lesions in these sites markedly disturb learning of spatial memory tasks in rodents;17,33 Alzheimer’s disease is associated with a loss of these neurons.5,10,35 Traumatic brain injury in rodents has been noted to impair spatial learning as measured in the Morris water maze.14,44 Recent epidemiological analysis has shown that a history of concussive head injury in the past is a significant risk factor for the development of Alzheimer’s dementia.12,34 These observations are in accord with the present study, which demonstrates a selective loss of cholinergic neurons following traumatic brain injury. Cholinergic neurons in the septum and ventrobasal forebrain would appear to have a heightened vulnerability to trauma, and this susceptibility may underlie, in part, the phenomenon of memory loss and cognitive dysfunction in the postconcussive period.

The mechanism that accounts for the susceptibility of cholinergic neurons to trauma is unknown. One possibility is that trauma disrupts the complex mechanism of nerve growth factor production and transport necessary to sustain these neurons.13 Alternatively, these neurons may be susceptible to the excitotoxic milieu of potassium and glutamate, which is known to occur following fluid-perfusion injury.14,20,23 Elucidating the mechanism of injury may permit the development of specific strategies to preserve and restore these neurons.

In contrast to cells in the forebrain, cholinergic neurons in the pons and mesencephalon (Groups Ch5 and Ch6) did not show any change in number with the head injury paradigm used here. Functionally these neuron groups seem to influence strongly the reticular activating system, and extensive data exists that links cholinergic function in the vicinity of the tegmental nuclei with changes in consciousness.16,21,23 Head injury transiently increases metabolism and cholinergic turnover in this area,15,16,22,40,51 and cholinergic antagonists attenuate the severity of behavioral suppression from traumatic brain injury in rodents.27,28 and humans.38 The failure of the present study to

Central Cholinergic Neurons

Anatomically, central cholinergic projection neurons are situated in two major sites within the brain. The forebrain site consists of neurons within the medial septal nucleus (Group Ch1), nucleus of the diagonal band of Broca (Groups Ch2 and Ch3), and nucleus basalis of Meynart (Group Ch4). The Ch1 and Ch2 neurons account for the major cholinergic projection to the hippocampus via the fimbria-fornix, whereas the Ch4 neurons provide innervation to the cortical mantle and amygdala.3,10,32 The Ch5 and Ch6 neuronal groups consist of cells in the pontomesencephalic reticular formation and laterodorsal tegmental gray, respectively. These neurons project to

traumatic brain injury is associated with fairly stereotypical alterations in consciousness, cognitive function, and behavior suggests that one or more of the neuronal systems subserving these functions has a heightened vulnerability to the pathophysiological mechanisms triggered by head trauma. This hypothesis of selective vulnerability of key brain systems is further supported by the significant frequency with which mild head injury produces disturbances in memory, concentration, problem solving, and initiative, even in the absence of any gross pathological or radiographic changes. The goal of this study was to begin to define how specific central cholinergic and catecholaminergic systems are affected in a rodent model of mild to moderate diffuse traumatic brain injury.

FIG. 5. Photomicrographs of choline acetyltransferase staining in the lateral amygdaloid nucleus (arrow). The control brain tissue (A) shows dense cholinergic terminal staining throughout the nucleus, which is markedly diminished following fluid-percussion injury (B). Staining medial to the lateral amygdaloid nucleus is nonspecific (asterisk). Monoclonal No. 770 990, original magnification × 25.
find any quantitative change in the number of these neurons following fluid-percussion injury indicates that any alteration in their status may be preferentially metabolic.

Central Catecholamine Neurons

A number of central catecholamine projections have important roles in behavioral activation, reward, alertness, and focused attention, including the mesocortical and mesolimbic dopamine neurons, and the noradrenergic neurons of the locus ceruleus. Pharmacological agonists of these transmitters may facilitate cognitive recovery in patients after head injury, and antagonists such as haloperidol appear to retard functional improvement. One may thus anticipate functional impairment of these projections following traumatic brain injury. The immunocytochemical techniques used in this study were sensitive enough to demonstrate the full anatomical extent of the central dopaminergic and noradrenergic projections, although we have no histological means at present to quantify these projections. Qualitatively, however, we detected no apparent damage to these systems after a moderate level of fluid-percussion injury. As in the case of pontomesencephalic cholinergic neurons, any injury-related damage to these catecholamine projections may be functional rather than anatomical, or anatomical changes may require a higher level of injury. Quantification of catecholamine levels and turnover needs to be done in these animals.

Conclusions

With the availability of a humane and inexpensive model of concussive head injury, our understanding of the neurochemistry and neuroanatomy of traumatic brain injury will become increasingly sophisticated. Examining the brain at the level of discrete neuronal systems will be necessary to understand the generalized deficits these patients have and also to understand the physiology and neurochemistry of coma. Neuroscience research in the last 15 years has clearly established that many neuronal systems in the brain, including the forebrain cholinergic and the central catecholaminergic projections, are capable of a functional response in response to lesions. We should thus anticipate that such mechanisms may contribute to the process of recovery after head injury. This opens vast new avenues for clinical intervention into this devastating problem: 1) to interrupt the cascade of events producing secondary injury to neuronal systems; 2) to facilitate repair and recovery mechanisms; and 3) to compensate specifically for the effects of neuronal loss. The demonstrations outlined in this paper of a selective loss of cholinergic neurons in a major forebrain system essential for normal memory and cognitive function is a first step forward in this process.

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