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A Model of Parasagittal Controlled Cortical Impact in the Mouse: Cognitive and Histopathologic Effects

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ABSTRACT

Controlled cortical impact (CCI), using a pneumatically driven impactor to produce traumatic brain injury, has been characterized previously in both the ferret and in the rat. In the present study, we applied this technique to establish and characterize the CCI model of brain injury in another species, the mouse, evaluating cognitive and histopathologic outcome. In anesthetized (sodium pentobarbital, 65 mg/kg) male C57BL mice, we performed sham treatment (no injury, $n = 12$) or CCI injury ($n = 12$) at a velocity of 5.7–6.2 m/sec and depth of 1 mm, using a 3-mm diameter rounded-tip impounder, positioned over the left parietotemporal cortex (parasagittal). At this level of injury, we observed highly significant deficits in memory retention of a Morris water maze task 2 days following injury ($p < 0.001$). Postmortem histopathologic analysis performed at 48 h following injury revealed substantial cortical tissue loss in the region of impact and selective hippocampal neuronal cell loss in the CA2, CA3, and CA3c regions, using Nissl staining. Analysis of degenerating neurons using modified Gallyas silver staining techniques demonstrated consistent ipsilateral injury of neurons in the cortex adjacent to the impact site and in the dentate gyrus of the ipsilateral hippocampus. Bilateral degeneration was observed at the gray matter-white matter interface along the corpus callosum. Glial fibrillary acidic protein (GFAP) immunohistochemistry revealed extensive reactive gliosis appearing diffusely through the bilateral cortices, hippocampi, and thalami at 48 h postinjury. Breakdown of the blood-brain barrier was demonstrated with antimouse IgG immunohistochemistry, revealing extravasation of endogenous IgG throughout the ipsilateral cortex, hippocampus, and thalamus. These results suggest that this new model of parasagittal CCI in the mouse mimics a number of well-established sequelae observed in previously characterized brain injury models using other rodent species. This mouse model may be a particularly useful experimental tool for comparing behavioral and histopathologic characteristics of traumatic brain injury in wild-type and genetically altered mice.

Key words: brain injury, cognition, controlled cortical impact, hippocampus

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INTRODUCTION

ALTHOUGH SEVERAL EXPERIMENTAL MODELS OF BRAIN INJURY have been characterized using various rodent species, the potential use of transgenic techniques in mice to isolate specific aspects of disease processes prompted us to develop a mouse model of traumatic brain injury (TBI) using the controlled cortical impact (CCI) technique. Experimental CCI employs a pneumatically driven impactor, which may precisely control deformation parameters and contact velocity. CCI was first characterized in the ferret (Lighthall, 1988) and has more recently been characterized in the rat, demonstrating many aspects of traumatic brain injury that are observed clinically, including cognitive dysfunction (Hamm et al., 1992), neurologic motor dysfunction, and histopathologic changes (Dixon et al., 1991; Hamm et al., 1992).

Cognitive dysfunction is recognized as one of the most common and tragic sequelae of TBI in humans, characterized by deficits in problem solving, learning, and memory (Levin, 1985; Parkin, 1984). However, little is known about potential anatomic substrates that may be responsible for posttraumatic cognitive dysfunction. Because of the clinical importance of the effects of brain trauma on cognition, posttraumatic alterations in learning and memory have been characterized in experimental models of TBI in the rat. Lyeth et al. (1990) were the first to demonstrate posttraumatic cognitive dysfunction using a vertex (midline) fluid-percussion (FP) brain injury in the rat, which induced an impairment in spatial learning (acquisition) of an eight-arm radial maze task. Subsequent studies using Morris water maze paradigms demonstrated that both vertex and parasagittal (lateral) FP brain injury in the rat induced learning and memory (retention) deficits (Gorman et al., 1993; Hamm et al., 1992; Pierce et al., 1993; Smith et al., 1991, 1994). In addition, spatial learning dysfunction of water maze tasks has been observed in rat brain injury models using weight-drop (Sutton et al., 1992) and CCI (Hamm et al., 1992) techniques.

Although our laboratory has extensively characterized the parasagittal FP brain injury model in the rat, we found in pilot studies that application of FP brain injury to the mouse was not optimal due to a much smaller, more fragile cranium and more porous and flexible cranial sutures when compared with larger rodents. We, therefore, chose to evaluate the CCI method of brain injury, which uses a rigid indentor to impact exposed brain at a controlled depth and velocity (Anderson, 1982; Dixon et al., 1991; Lighthall, 1988). The CCI technique was also chosen over a previously characterized weight-drop model of brain injury in the mouse (Hall, 1985) due to the enhanced ability of CCI to allow precise biomechanical control over the time course and amplitude of injury.

Because of the potential interrelationship between posttraumatic cognitive dysfunction and histopathologic changes, in the present study we investigated the effects of CCI in the mouse on memory function and performed a detailed and comprehensive histopathologic analysis of the brains of these animals. This histopathologic analysis included the evaluation of neuronal cell death (Nissl stain) and degeneration (silver degeneration stain), reactive gliosis [glial fibrillary acidic protein (GFAP) immunohistochemistry], and breakdown of the blood-brain barrier (extravasation of mouse IgG) following CCI injury.

MATERIALS AND METHODS

Morris Water Maze Training

C57BL male mice (25–32 g, $n = 24$) were trained in a Morris water maze (MWM) (Morris, 1984) to locate a stationary submerged invisible platform (0.5 cm below the surface) using external visible cues. The training and memory testing paradigm used in the present study has been described in detail previously for use in the rat (Smith et al., 1991, 1994). The water maze is a circular pool 1 m in diameter and 50 cm deep. The interior of the maze is painted white. The pool is filled with 21°C water to 24.5 cm in depth. A grid design of various derived zones is constructed with a computerized video system (Omnitech Videoscan) and is superimposed over the maze and viewed on a monitor. A plexiglass platform 24 cm tall (0.5 cm below the surface of the water) and 11.5 × 11.5 cm is placed into the tank so that the target zone (viewed on monitor) is completely within its borders. The maze is filled with nontoxic white paint rendering the maze opaque and obscuring the plateform from view. The essential feature of the MWM is that the animals can escape from the water onto the submerged platform.

For each training trial, the animals are placed randomly at four sites, 90° apart, along the tank's periphery. On the first day of training, each animal is given 1 min to find the platform (approximately one third of the animals do not find the platform on their first trial and must be placed on it once 1 min has elapsed). The animals are allowed to remain on the platform for 30 sec on their first trial and 15 sec on subsequent trials to spatially orient themselves to the external visual cues. Each animal receives a total of 10 trials on day 1 of training, and the latencies (time taken to find the platform) are recorded for each trial. On the second day of training, all animals are put through the identical training regimen of 10 trials, for a total of 20 trials over 2 days.

Controlled Cortical Impact

Two hours after the final training trial, the animals are anesthetized (sodium pentobarbital, 65 mg/kg i.p.). All

animals are then placed in a stereotaxic head holder, and ointment is applied to their eyes to protect vision during surgery. The top of the skull is exposed, and a 5-mm craniectomy is performed over the left parietotemporal cortex. *Care is taken not to disrupt the dura at this opening.* One hour after administration of anesthesia (near the end of the surgical plane of anesthesia), animals are subjected to either CCI ($n = 12$) or sham (no impact) treatment ($n = 12$). CCI is conducted using a beveled 3-mm flat-tip impounder at a velocity of 5.7–6 m/sec and at a depth of 1 mm. Animals remain in the stereotaxic head holder during CCI injury. The depth of injury (1 mm) is scaled according to the 2 mm depth used in the previously described rat CCI model (the dorsal-ventral diameter of a rat brain is typically 10 mm, whereas that of a C57 mouse is 5.5 mm). The chosen velocity (5.7–6 m/sec) is also based both on the previous studies performed in the rat model of CCI and on preliminary studies performed in the mouse, which demonstrated that a lower velocity of 4 m/sec ($n = 10$) did not produce consistently overt memory deficits (data not shown).

The CCI device, previously described in detail (Dixon et al., 1991), is a pneumatic cylinder wih a 5-cm stroke. The cylinder is rigidly mounted in a vertical position crossbar. The impactor tip may be manually adjusted to the desired depth. The upper rod is attached to a transducer core of a LVDT (Shaevitz Model 500 HR), which produces an analog signal recorded by a computer storage oscilloscope emulation computer program (R.C. Electronics) for analysis of time and displacement parameters of the impactor.

Postinjury Memory Test

At 2 days following injury, animals are tested for memory retention of the preinjury learned task in the MWM. The platform is removed from the MWM, and the animals are given 1 min to swim while a computer-video unit records their swimming patterns. Scores are assigned according to the time spent in each zone of a superimposed grid design, as previously described (Smith et al., 1991). During the tests, activity (swim) patterns and time spent in each zone are recorded using the Omnitech Videoscan zonal behavior program. A memory score is derived by assessing the animals' swimming behavior throughout different zones of the computerized grid design. Each zone is ranked in a weighted fashion according to its proximity to the platform site. These assigned numbers are multiplied by the number of seconds spent in the corresponding zone and totalled. Statistical analyses of memory scores are performed using the nonparametric Mann-Whitney U-test to examine relationships between the two groups ($p < 0.05$ is considered statistically significant).

Following the memory test, the platform is placed back in the maze in a new location and made visible by raising it 1 cm above the surface of the water, with black tape placed around the exposed sites. The animals are given eight trials from random starting locations, and their latency to reach the platform is recorded.

Histopathologic Analysis

Following water maze evaluation, all animals are reanesthetized (48 h following injury) (100 mg/kg sodium pentobarbital) and perfused intracardially with 4% paraformaldehyde. Brains are removed and postfixed (12–15 h), transferred to 0.1 M phosphate buffer, pH 7.4, and subsequently submerged in 25% sucrose for cryoprotection. Before sectioning, the brains are quickly frozen in 2-methyl butane (−56°C). Serial cryostat sectioning of the brains is performed (10 μm) and alternate sections are stained to identify (1) cell loss determined by the absence of Nissl subtance using a 0.05% toluidine blue solution in acetate buffer, pH 4.4, (2) degenerating neurons using a modified Gallyas silver stain (sections are pretreated with a 4.5% sodium hydroxide-0.6% ammonium nitrate solution, impregnated with a 0.3% silver nitrate solution, washed in 0.012% ammonium nitrate-0.5% sodium carbonate solution in 95% ethanol, and developed in a 0.012% ammonium nitrate-0.05% citric acid solution in 95% ethanol with 0.55% formalin), (3) reactive gliosis, determined by an increase in GFAP immunoreactivity, and (4) blood-brain barrier breakdown, identified by extravasation of endogenous mouse IgG. Immunohistochemical analyses of GFAP and endogenous mouse IgG are performed as follows. Sections are treated with a 3% H_2O_2 solution and blocked in 5% normal goat serum. Primary antibody against either GFAP (Accurate, rabbit polyclonal, 1:100) or mouse IgG (Accurate, peroxidase-conjugated goat polyclonal, 1:1000) is applied, and sections are incubated overnight at 4°C. Labeling is visualized using diaminobenzidine (DAB) following incubation with either peroxidase-conjugated secondary antibody (Jackson, donkey antirabbit IgG, 1:1000) for GFAP labeling or streptavidin-peroxidase (Accurate, 1:1000) for IgG labeling. Sections labeled for GFAP are counterstained with hematoxylin following immunohistochemistry. For all histopathologic analyses, sections are qualitatively evaluated using standard light microscopy.

RESULTS

Effects of CCI on Cognition in the Mouse

Two days after CCI in the mouse, we observed a profound and highly significant ($p < 0.001$) deficit of mem-

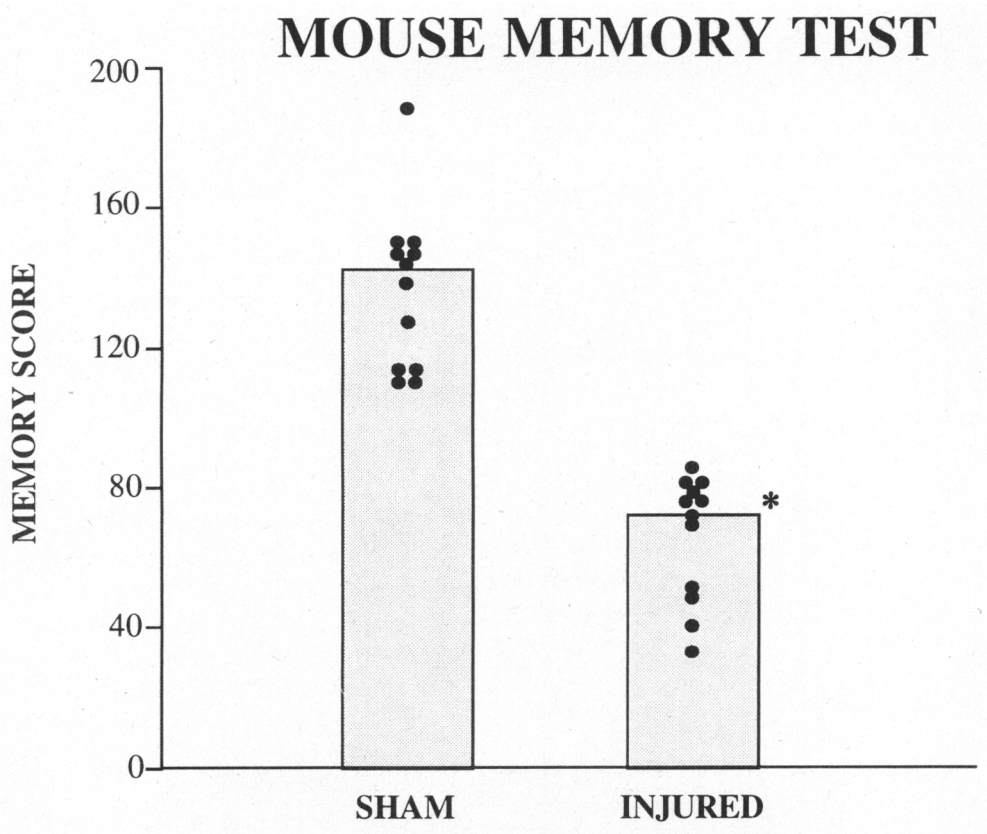

FIG. 1. Posttraumatic memory score at 48 h after parasagittal CCI injury. Bars represent median memory scores of sham and brain-injured mice. Dots represent individual memory scores. *$p < 0.001$, compared with sham animals.

ory retention of the water maze spatial task (Fig. 1). There was no overlap in memory scores between the injured and sham groups, which had median scores of 72 and 143, respectively. Both injured and sham animals exhibited the same swim speed during the memory test, with no observable impairment of swimming ability. In addition, following the memory test, injured and sham mice were able to navigate to a visible platform with equal proficiency over eight trials, demonstrated as a mean latency of 6 sec for each group. Representative swim patterns of sham and injured mice during the memory test are shown in Figure 2.

Histopathologic Effects of CCI in the Mouse

Gross observation of coronal brain sections revealed an extensive cortical lesion 2 days following injury, which can be best visualized in the endogenous IgG-stained sections shown in Figure 3. The cortical lesion, characterized as a total loss of tissue, extended from the center of the impact site (Fig. 3B) rostrally to the level of the striatum, where the lateral ventricles widen, and caudally to the level of the pontine nuclei. At its maximum depth, this cortical tissue loss reached the subcortical white matter. This pattern of gross tissue loss was consistent in all injured animals. Breakdown of the blood-brain barrier, as shown by substantial immunohistochemical labeling of extravasated mouse IgG, was observed throughout the rostral-caudal extent of the brain (Fig. 3). The most concentrated IgG staining was found in cortex adjacent to the lesion and in the ipsilateral subcortical white matter. Less intense but easily discernible labeling of mouse IgG was also observed in the ipsilateral hippocampus and dorsolateral thalamus.

In addition to the gross cortical tissue loss and blood-brain barrier breakdown, Nissl staining revealed selective neuronal loss in the ipsilateral dorsal hippocampus following CCI brain injury (Fig. 4A,B,C,D). Overt neuronal cell loss was observed consistently in areas CA3 and CA3c. Some cases also showed neuronal loss in area CA2 (Fig. 4B, arrow 1). In these same regions, some remaining neurons appeared pyknotic (darkly stained and shrunken).

Marked gliosis was also observed following injury, demonstrated by increased GFAP immunoreactivity (Fig. 4F). Gliosis was seen throughout the ipsilateral cortex

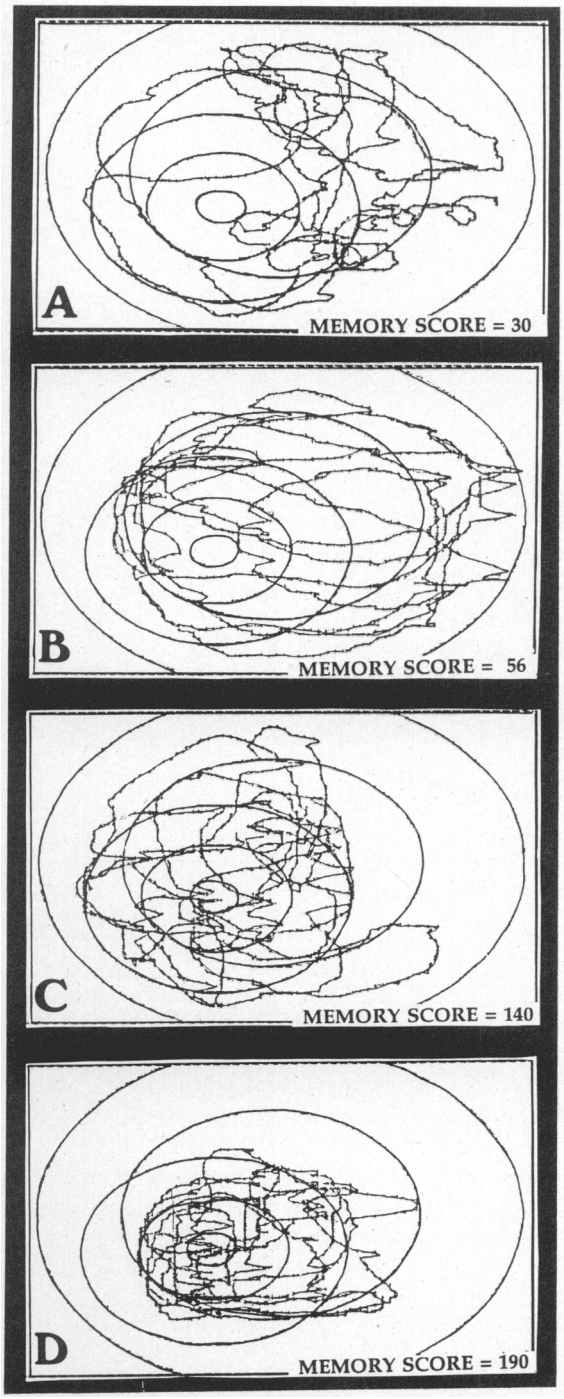

FIG. 2. Representative swim patterns of mice during the memory test. Platform-seeking behavior of CCI injured mice is shown in **A** and **B,** and that of non-injured mice is shown in **C** and **D.**

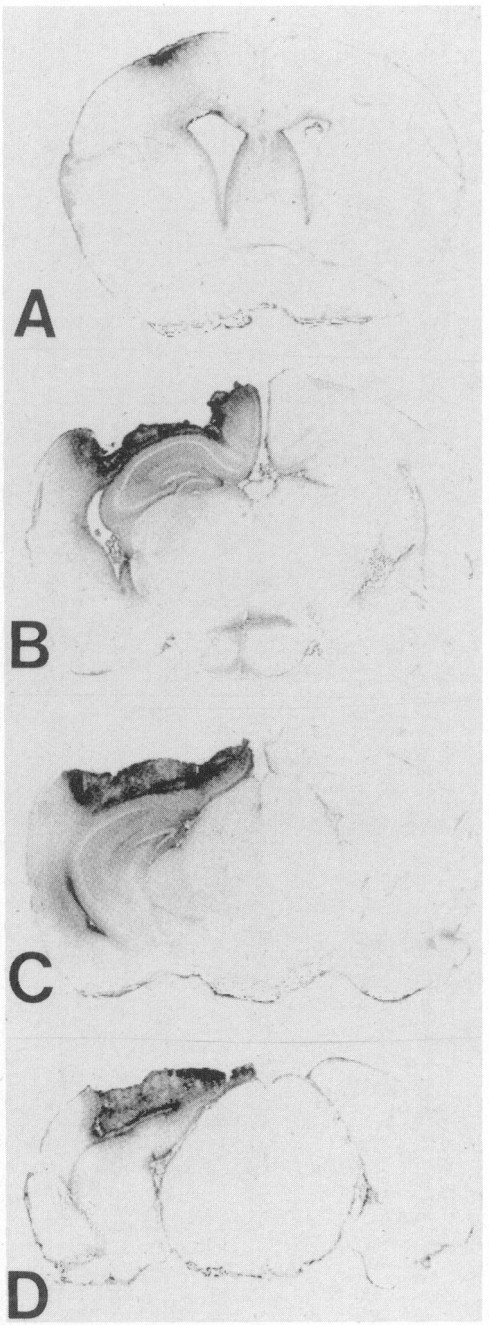

FIG. 3. Coronal brain sections, immunostained with antibodies against mouse IgG 48 h following CCI injury. Note the dark staining outlining a gross loss of tissue in the left parietotemporal cortex (**B** and **C**). Also note that the mouse IgG immunostaining extends the entire rostral (**A**) to caudal (**D**) extent of the brain and extends into the left hippocampus and dorsolateral thalamus (**B** and **C**).

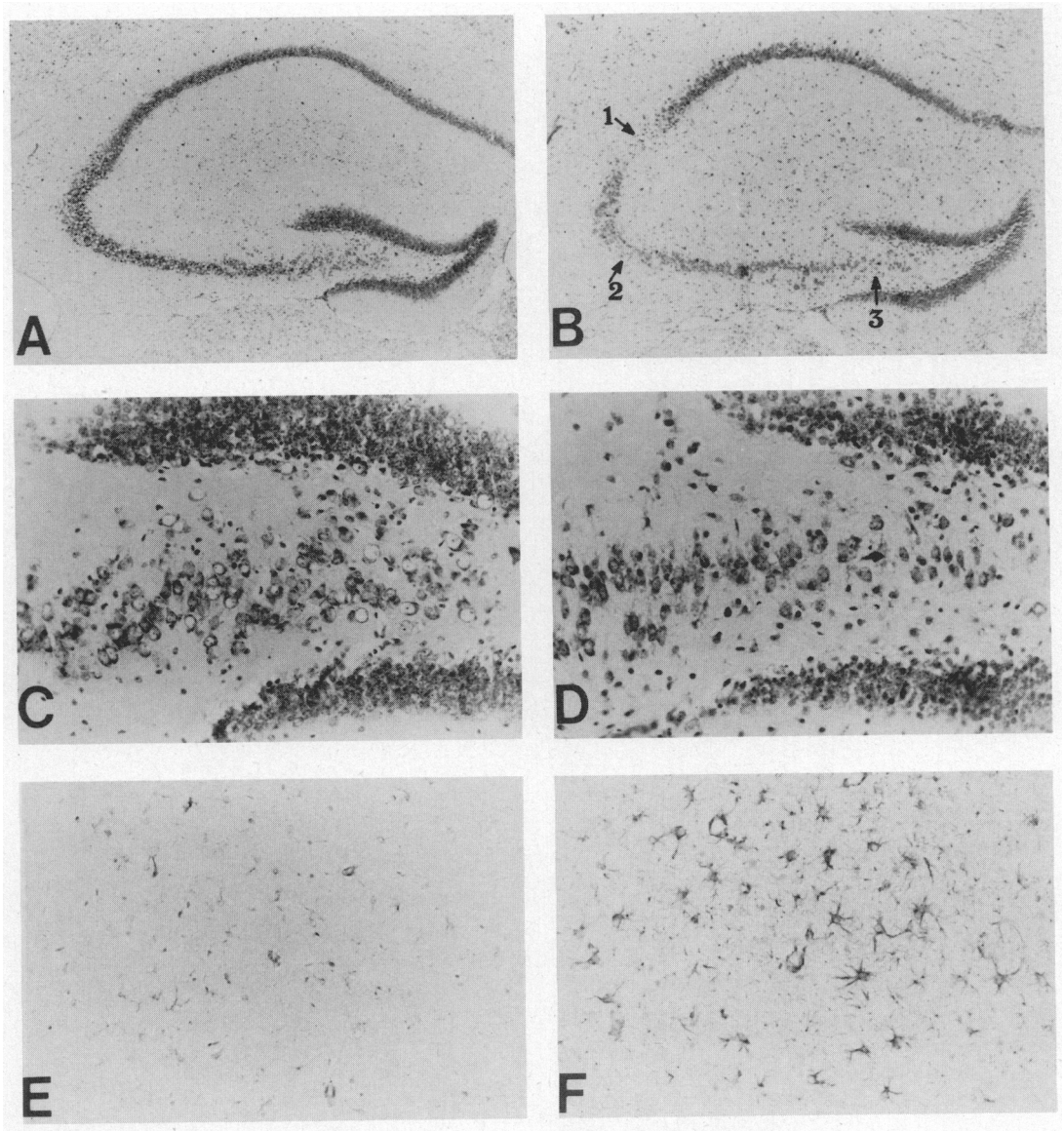

FIG. 4. Representative photomicrographs of coronal mouse brain sections of the left hippocampi of non-injured **(A, C, E)** and CCI-injured **(B, D, F)** animals. Nissl-stained sections **(A** and **B)** demonstrate loss of neurons in the hippocampus of the injured animal **(B)** in the CA2 *(arrow 1),* CA3 *(arrow 2),* and CA3c *(arrow 3)* regions, compared with a non-injured animal **(A).** Higher magnification of these sections in the region of the dentate gyrus further demonstrates the posttraumatic loss of neurons in the CA3c region and the appearance of shrunken, pyknotic neurons **(D)** compared with the non-injured animal **(C).** Gliosis, determined by an increase in GFAP, is demonstrated in the region of the hippocampal fissure of an injured animal **(F),** compared with a non-injured animal **(E).** Counterstained with hematoxylin.

and hippocampus. Although hippocampal neuronal cell loss and degeneration appeared to be restricted to regions directly beneath the center of the impact, increased GFAP immunoreactivity extended throughout the rostral-caudal extent of the hippocampus.

Silver degeneration staining of neurons was most strongly observed in the ipsilateral dentate gyrus, in cortical neurons immediately adjacent to the cortical tissue loss, and bilaterally along the gray matter-white matter interface of the corpus callosum at 2 days following injury (Fig. 5). Although within the hippocampus no overt cell loss was detected in the dentate gyrus, this was the only region where neurons were consistently argyrophilic. In contrast, we observed marked neuronal cell loss in the CA3 region, with virtually no labeling for degenerative changes in the surviving neurons at 2 days fol-

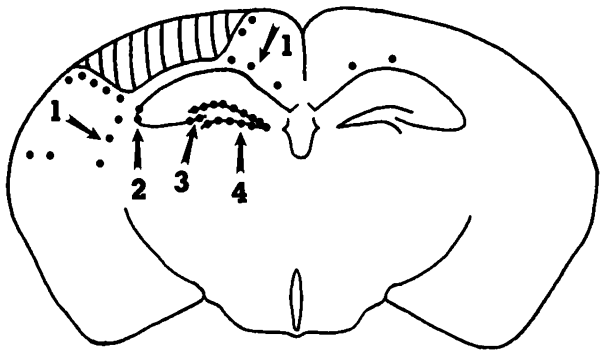

FIG. 5. Schematic representation of a coronal section of a CCI-injured mouse brain 48 h following injury, stained with a silver degeneration stain. Each dot represents a minimum of five positively stained neuronal cell bodies. Hatched region identifies fragmented or lost tissue at impact site. *Note:* Degeneration of neurons at gray matter-white matter interface along the corpus callosum *(arrow 1),* in the ipsilateral hippocampus in the CA2-CA3 region *(arrow 2),* in the CA3c region *(arrow 3),* and in the dentate gyrus *(arrow 4).*

lowing injury. Although the CA1 region of the left hippocampus was directly beneath the site of impact, the neurons in this region appeared to be spared from neuronal cell loss or degeneration.

It is important to note that there were no deaths resulting from CCI injury, and all animals were awake and ambulatory by 1 h after injury.

DISCUSSION

The present study demonstrates that parasagittal CCI brain injury in the mouse induces profound spatial memory dysfunction (retrograde amnesia), observed 2 days following injury. Surprisingly, C57 mice learn the MWM task with a level of proficiency similar to that of Sprague-Dawley rats, demonstrating an equivalent swim speed (Smith et al., 1991), even though they are approximately one-fifteenth the size. Furthermore, the swim patterns and memory scores obtained during the memory test of noninjured mice were almost identical to those previously observed in noninjured rats. In association with the posttraumatic memory deficits, we observed gross cortical tissue loss, hippocampal neuronal cell loss and neuronal degeneration, reactive gliosis, and extensive breakdown of the blood-brain barrier 2 days following injury. The neuronal cell loss in the hippocampus was observed primarily in the ipsilateral CA2, CA3, and CA3c regions and appeared to be selective, since adjacent regions of hippocampus (such as CA1) demonstrated no overt decrease in neuronal number. However, this observation may be limited, since hippocampal damage may continue beyond the 48 h postinjury time point of evaluation.

In preliminary studies, parasagittal CCI injury of lower severity (3.5–4.6 m/sec) in mice did not produce consistently robust memory deficits or histopathologic damage (data not shown). At the injury velocity of approximately 6 m/sec, significant memory deficits were observed in association with cortical tissue loss and neuronal cell loss in the CA2, CA3, and CA3c regions of the hippocampus. This pattern of cortical and hippocampal damage is similar in extent and distribution to the previously characterized hippocampal cell loss following parasagittal FP brain injury in the rat at moderate levels of severity (Cortez et al., 1989; Hicks et al., 1993; Smith et al., 1993). However, following vertex CCI in the rat, only intraparenchymal hemorrhage, but no overt neuronal cell loss, could be observed in the hippocampus (Dixon et al., 1991). In the present study, although the dura remained overtly intact following a 6 m/sec injury, the loss of tissue in the parietotemporal cortex resembles ablation or penetrating injury, with almost total loss of tissue in the region of impact. In contrast, vertex CCI in the rat (6 m/sec) produces much less profound cavitating lesions and necrotic changes in the medial cortex underlying the impact site (Dixon et al., 1991). Dixon et al. (1991) also observed that vertex CCI in the rat induced axonal injury (retraction balls-terminal clubbing) throughout the brain (not assessed in the present study).

Since the new model of parasagittal CCI in the mouse was scaled to the rat CCI model used by Dixon et al. (1991), the disparity in histopathologic damage between the mouse and the rat may have important biomechanical implications. Both models used comparable CCI velocity (6 m/sec) at a depth of approximately 20% the dorsal-ventral diameter (1 mm for the mouse, 2 mm for the rat) and similarly scaled impounder diameters (3 mm for the mouse, 10 mm for the rat). Differences in histopathology between the rat and mouse following CCI may reflect differences in the dynamics of injury, possibly due to (1) differing positions of the impact site (vertex for rat versus parasagittal for mouse), (2) differences in the actual tissue compliance or cytoarchitecture between mouse and rat brain, (3) differences in the shaping of the impounder tip, or (4) differences in volume displacement. In a recently described model of *parasagittal* CCI in the rat (Sutton et al., 1993), greater cortical loss was observed compared with the vertex CCI in the rat (Dixon et al., 1991), suggesting that the location of the impact site does play an important role in the severity or extent of injury or both.

Previously, Hamm et al. (1992) demonstrated that vertex CCI in the rat produced severe and long-term deficits

in learning (acquisition) ability. In the present study, we observed profound deficits in spatial memory retention following parasagittal CCI in mice, demonstrated by a decrease in time spent in the escape platform zone (with the platform removed). The severity of this memory loss or retrograde amnesia is very similar to that observed in the rat model of parasagittal FP brain injury (Smith et al., 1993, 1994) and may reflect an important, clinically relevant component of this TBI model. Further studies will determine the temporal course of this cognitive deficit.

Although the hippocampus has been shown to be selectively involved in spatial learning and memory (Morris et al., 1982; Scoville and Milner, 1957), in the clinical setting, damage specifically to the hippocampus has yet to be identified as playing a major role in the development of human posttraumatic cognitive dysfunction. In experimental lesion studies, damage to either the hippocampus, parietal cortex, amygdala, thalamus, or cerebellum has been shown to impair acquisition performance (learning) (Crowne et al., 1989; DiMattia and Kesner, 1988). However, impairment of memory retention appears to be dependent on *bilateral* hippocampal damage, which may be exacerbated with damage to other structures (Jucker et al., 1990; Kametani and Kesner, 1989). Taken together, these data support the hypothesis that although learning or acquisition may be dependent on several important brain structures, the hippocampus may be the primary structure involved in initial memory storage and processing. In the present study, profound memory deficits were observed following CCI injury, although only unilateral hippocampal loss and degeneration were identified consistently. However, this observation may not be inconsistent with the hypothesis of the dependence of memory retention with hippocampal integrity. The vertex CCI and the vertex FP models of brain injury in the rat both produce a posttraumatic learning dysfunction without overt cell loss in the hippocampus (Dixon et al., 1991; Lyeth et al., 1990). Nevertheless, in both of these models, a loss of microtubule-associated protein (MAP)2 in the hippocampus has been observed (Taft et al., 1992, 1993), suggesting that substructural cytoskeletal damage has occurred in this region in the absence of overt cell loss. In contrast, following parasagittal FP brain injury in the rat, both memory dysfunction and learning dysfunction have been observed in association with bilateral hippocampal cell loss, and a correlation has been observed between the severity of memory dysfunction and the extent of selective loss of hippocampal neurons (Hicks et al., 1993). Similar to vertex CCI and vertex FP brain injury, parasagittal FP injury also induces a decrease of MAP2 in hippocampal regions not associated with cell loss (Smith et al., 1993; Hicks et al., 1994). These results suggest that posttraumatic cognitive deficits may result from either overt or covert damage of key brain structures.

Selective posttraumatic loss of hippocampal neurons observed in the present and previous studies (Cortez et al., 1989; Kotapka et al., 1991) may be related to a marked posttraumatic release of excitatory amino acid (EAA) neurotransmitters into the extracellular space (Choi, 1988; Faden et al., 1989; Katayama et al., 1990; Nilsson et al., 1990; Palmer et al., 1993). The hippocampus, which contains the highest concentration of EAA receptors (Monaghan and Cotman, 1986), may be selectively vulnerable to toxic effects from a high extracellular concentration of EAAs. Therefore, the preponderance of posttraumatic cognitive deficits observed both clinically and experimentally may be partially explained by the selective vulnerability of hippocampal neurons to EAA toxicity following brain injury.

In the present study, we observed damage to the left parietotemporal cortex (tissue loss and degeneration) and ventrolateral thalamus (blood-brain barrier disruption and gliosis) following brain injury in the mouse, which encompassed regions involved in motor function and visual recognition. However, as with pilot studies, injured animals swam at the same velocity as noninjured animals, with no apparent neurologic motor deficits affecting the swimming task (e.g., circling behavior). In addition, injured animals swam to a visible platform as well as noninjured animals, suggesting that visual impairment or posttraumatic changes in motivation did not contribute to the observed posttraumatic memory dysfunction.

The regional posttraumatic breakdown of the blood-brain barrier and gliosis observed in the present study may play an important role in a secondary injury cascade. Serum albumin has been suggested to greatly potentiate EAA toxicity (Eimerl and Schramm, 1991; Menzies et al., 1993). Serum also contains free EAAs at much greater concentrations than found in brain extracellular space. Moreover, breakdown of the blood-brain barrier may allow the passage of macrophages and lymphocytes into the brain, potentially initiating inflammatory and autodestructive processes. The gliosis observed following CCI in the mouse may reflect these potential initial inflammatory events and may play a role in perpetuating inflammatory responses to brain injury, such as the proposed link between the posttraumatic gliosis and an increase in the gene expression and production of cytokines, observed in other models of experimental brain injury (Fan et al., 1994; Taupin et al., 1993).

The posttraumatic memory dysfunction in association with neuronal damage and degeneration, reactive gliosis, and IgG extravasation observed in the present study sug-

gests that the mouse model of CCI brain injury may be a particularly useful tool for performing studies of TBI in genetically altered animals.

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