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Injury severity determines Purkinje cell loss and microglial activation in the cerebellum after cortical contusion injury

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Abstract

Clinical evidence suggests that the cerebellum is damaged after traumatic brain injury (TBI) and experimental studies have validated these observations. We have previously shown cerebellar vulnerability, as demonstrated by Purkinje cell loss and microglial activation, after fluid percussion brain injury. In this study, we examine the effect of graded controlled cortical impact (CCI) injury on the cerebellum in the context of physiologic and anatomical parameters that have been shown by others to be sensitive to injury severity. Adult male rats received mild, moderate, or severe CCI and were euthanized 7 days later. We first validated the severity of the initial injury using physiologic criteria, including apnea and blood pressure, during the immediate postinjury period. Increasing injury severity was associated with an increased incidence of apnea and higher mortality. Severe injury also induced transient hypertension followed by hypotension, while lower grade injuries produced an immediate and sustained hypotension. We next evaluated the pattern of subcortical neuronal loss in response to graded injuries. There was significant neuronal loss in the ipsilateral cortex, hippocampal CA2/CA3, and laterodorsal thalamus that was injury severity-dependent and that paralleled microglial activation. Similarly, there was a distinctive pattern of Purkinje cell loss and microglial activation in the cerebellar vermis that varied with injury severity. Together, these findings emphasize the vulnerability of the cerebellum to TBI. That a selective pattern of Purkinje cell loss occurs regardless of the type of injury suggests a generalized response that is a likely determinant of recovery and a target for therapeutic intervention.
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Keywords: Traumatic brain injury; Controlled cortical impact; Neuronal loss; Cerebellum; Activated microglia; Purkinje cells

Introduction

Experimental traumatic brain injury (TBI) results in diffuse axonal injury, varying degrees of damage to the cortical mantle, and distinct patterns of subcortical neuronal loss (Morales et al., 2005). Diffuse axonal injury is a dominant feature after lateral fluid percussion (FP) injury (Thompson et al., 2005) whereas controlled cortical impact (CCI) is characterized by focal cortical damage and overt intraparenchymal hemorrhage (Dixon et al., 1991). Despite the unique features of each of these models, the temporal and regional patterns of subcortical neuronal loss are similar. This is perhaps best exemplified in the predictable pattern of neuronal cell loss in the hippocampus and thalamus (Baldwin et al., 1997; Chen et al., 2003; Colicos et al., 1996; Conti et al., 1998; Goodman et al., 1994; Hall et al., 2005; Hicks et al., 1996; Lowenstein et al., 1992; Newcomb et al., 1997; Scheff et al., 1997; Smith et al., 1995; Tang et al., 1997b), which likely contributes to functional deficits including cognitive impairment (Colicos et al., 1996; Scheff et al., 1997; Smith et al., 1995; Tang et al., 1997a). Such findings suggest that subcortical neuronal loss reflects a more generalized response to TBI.

We have previously reported Purkinje cell loss coincident with microglial activation in the cerebellum after mild FP injury (Fukuda et al., 1996; Mautes et al., 1996). Purkinje cell loss has since been replicated by others in a similar model of TBI and been shown to be dependent upon the magnitude of the initial injury (Park et al., 2006). These findings collectively emphasize the vulnerability of the cerebellum to TBI. What remains unclear is if cerebellar injury is unique to FP injury or in fact represents a more generalized response to TBI. We therefore examined cerebellar vulnerability after a graded CCI in the context of physiologic and anatomical parameters that have

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been shown by others to be sensitive to injury severity. We demonstrate that microglial activation in the injured hemisphere parallels neuronal cell loss in the cortex, hippocampus, and thalamus. Importantly, similar to FP injury, Purkinje cell loss and microglial activation are evident in the vermis of the cerebellum and vary in accordance with injury severity. Together, these findings in concert with previous reports (Fukuda et al., 1996; Mautes et al., 1996; Park et al., 2006) demonstrate that neurodegeneration in the cerebellum is a generalized response to TBI and as such, is a likely determinant of recovery and a target for therapeutic intervention.

Materials and methods

Animals

Adult male Sprague-Dawley rats ($n=45$, 340–400 g, Simonsen, Gilroy, CA) were housed in pairs before and after surgery in a temperature-regulated room with a 12-h light/dark cycle. All animals were provided food and water *ad libitum*. All procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

CCI

Rats were anesthetized with 3.6% chloral hydrate (10 ml/kg, intraperitoneal injection). Once anesthetized, a polyethylene catheter (PE50) was inserted into the left femoral artery to monitor blood pressure and obtain samples for blood gas analysis. The skull was then exposed with a left coronal scalp incision and reflection of the underlying soft tissues. A circular craniectomy, 7 mm in diameter, was made in the left parietal bone between bregma and lambda with the medial edge of the craniectomy 1 mm lateral to midline. The animal was then positioned on a stereotaxic frame and subjected to CCI. Injury was produced with a convex impactor tip 6 mm in diameter that was oriented perpendicular to the surface of the brain. The impact was set to a velocity of 4 m/s with a sustained depression of 150 ms. Severity of injury was determined by adjusting the depth to which the impactor tip deformed the surface of the brain. Animals were assigned to four groups based on depth of deformation: 0 mm (sham controls, $n=4$), 1.5 mm (mild injury, $n=10$), 2.0 mm (moderate injury, $n=13$), and 2.7 mm (severe injury, $n=18$). Sham controls underwent identical surgical procedures but were not injured. After injury or sham surgery, the scalp was closed and each animal was given 10 ml of saline subcutaneously. Body temperature was monitored with a rectal probe throughout the procedure and maintained at 36.5–37.5°C with a water-circulating heating pad. Blood for blood gas analysis was collected before and 5 min postinjury. Blood pressure recordings were taken prior to injury and at regular intervals up to 2 min postinjury.

All surviving animals (0.0 mm: $n=4$; 1.5 mm: $n=9$; 2.0 mm: $n=12$; 2.7 mm: $n=10$) were euthanized at 7 days postinjury. Each animal was perfused through the aorta with 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at a rate of 100 ml/min. Brains were removed and postfixed with the same fixative for 4 h. Coronal sections of the cortex and cerebellum were cut 40 μm in thickness using a sliding microtome (Micron, Waldorf, Germany) or a vibratome (Technical Products International Inc., St. Louis, MO). Sections were collected from the forebrain (Bregma −1.8 mm to Bregma −4.3 mm), which included the impact site, and from the cerebellum (Bregma −11.3 mm to −14.3 mm, Paxinos, 1995).

Immunocytochemistry

In the forebrain, neuronal cell counts were determined in sections immunostained with a mouse monoclonal antibody to vertebrate neuron-specific protein (NeuN, Chemicon, Temecula, CA; Mullen et al., 1992; Wolf et al., 1996). A mouse monoclonal antibody to the complement C3b receptor (OX-42, Serotec, Kidlington, OX, England) was used to evaluate microglial activation. All tissue sections were washed twice in 0.05 M phosphate-buffered saline, pH 7.4, for 5 min and then incubated in 1% H_2O_2 for 10 min to quench any endogenous peroxidase activity. Sections were then incubated in 2% sheep serum/0.2% Triton-X 100/0.1% bovine serum albumin (SS/TX/BSA) for 5 min followed by a 20-min incubation in 10% SS/TX/BSA. Incubation with primary antibodies (NeuN, 1:500; OX-42, 1:1000) was done overnight at 4 °C in 2% SS/TX/BSA. Sections were then washed in phosphate-buffered saline (PBS, 5 min ×3), after which they were incubated with a biotinylated horse anti-mouse secondary antibody in 2% SS/TX/BSA for 1 h. Secondary antibodies were visualized using avidin–biotin–horseradish peroxidase complex (Vectastain ABC kit, 1:100 in PBS, Vector Labs, Burlingame, CA) for 30 min followed by a PBS wash (5 min ×3), 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.02% H_2O_2 for 20 min, and a final PBS wash (5 min ×3).

Cerebellar sections were examined for Purkinje cell loss using anti-PEP-19 (courtesy of Dr. James Morgan, St. Jude's Children's Research Hospital), a rabbit polyclonal antibody that recognizes a developmentally regulated neuropeptide in Purkinje cells (Ziai et al., 1988, 1986). Rabbit anti-calbindin D-28 polyclonal antibody (calbindin, Chemicom) was also used for the detection of Purkinje cells. Cerebellar sections were stained for either PEP-19 (1:4000) or calbindin (1:1000) as described above with the exception that goat serum was used instead of sheep serum. In addition, cerebellar sections were stained with OX-42 as described above to examine microglial activation.

After staining, all sections were mounted on Superfrost/Plus glass slides (Fisher), air dried overnight, dehydrated in graded alcohols, cleared in Hemo-De (Fisher), and coverslipped in DePeX (Biomedical Specialties, Santa Monica, CA).

Morphometric analysis

All quantification was conducted by an observer who was blinded to the experimental conditions. Three adjacent sections

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