INTRODUCTION
Traumatic brain injury (TBI) is a leading cause of mortality and morbidity in industrialized countries, often resulting in neurologic and motor impairment (1–4). These neurologic deficits, through clinical–pathologic correlation analysis, have driven research efforts to determine the anatomic and mechanistic sources. This has involved modeling of TBI, which has resulted in the extensive documentation of acute and delayed cell loss (5–14). Specific brain regions have received focused attention because of their clinical significance, vulnerability, and their proximity to modeled sites of injury (e.g., hippocampus, cortex, and thalamus). Nevertheless, clinical–pathologic correlation remains a complex task in the effort to develop neurologically meaningful interventions for TBI.

There are several reasons to expand the evaluation of TBI models to include an analysis of the cerebellum. First, neurologic deficits in TBI models include loss of motor control and coordination (15), qualities typically correlated to the cerebellum. In addition, recently, there has been increasing awareness of a greater and more integrated role of the cerebellum than previously appreciated in higher neurologic functions, independent of motor activity (16–18).

Accordingly, in the present study, we examined cellular changes in the cerebellum after a forebrain fluid percussion injury (FPI). We quantified spatial and temporal selective vulnerability of Purkinje neurons (PNs) after a range of FPI severities and report evidence of astrogliosis in regions of PN loss. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) and cleaved caspase-3 immunohistochemistry indicate that PN death does not occur by an apoptotic mechanism. Increased expression of calbindin-28k in the injured cerebellum also suggests that increased calcium buffering may be an adaptive mechanism in sparing surviving PNs.

MATERIALS AND METHODS
All surgical procedures were performed in accordance with guidelines established by the Animal Care Committee at St. Michael's Hospital.

Fluid Percussion Injury
The FPI model (Virginia Commonwealth University, Richmond, VA) has been extensively characterized in rat models of TBI (19–21). In brief, male Sprague Dawley rats (29–42 days old) were anesthetized with 2.0% to 2.5% halothane in compressed air. Temperature was maintained by a thermal heating blanket at 37°C. An extradural craniotomy
(approximately 2- to 3-mm diameter) was performed in the right lateral hemisphere, approximately 2 mm from the midline suture, midway between the bregma and lambda. A polyethylene tube was fixed to the opening with cyanoacrylate adhesive and dental acrylic, filled with 0.9% isotonic saline, and attached to the FPI device. For immunohistochemistry studies of PN loss, rats were subject to one of four grades of injury severity (1.5, 2.0, 2.5, and 3.0 atm of pressure injection), which were consistent with previously established levels corresponding to a range of mild to severe injury. Bone wax was used to close the hole in the skull and scalps sutured before recovery in a temperature-controlled chamber. Six injury groups were used for this study: naive, sham, 1-day, 3-day, 7-day, and 14-day postinjured groups.

Immunohistochemistry

Anesthetized rats were transcardially perfused with 0.9% isotonic saline followed by 4% paraformaldehyde. The cerebellum was postfixed overnight in 4% paraformaldehyde 0.5 M acetic acid solution before paraffin embedding. The cerebellar vermis was sectioned sagittally at 10-μm thickness. After deparaffinization of sections, endogenous peroxidase activity was quenched with 3% H₂O₂ in methanol for 20 minutes. Sagittal sections were permeabilized for 20 minutes at 37°C with 0.1% trypsin. Blocking of sections was performed in 0.1 M phosphate-buffered saline (PBS) containing 10% normal horse serum. Primary incubation of mouse anticalbindin-28k (Sigma, Oakville, ON) was performed at a dilution of 1:1000 in blocking solution overnight at 4°C. Secondary incubation with biotinylated horseradish peroxidase antibodies was performed for one hour at room temperature (RT) in blocking solution (1:1000 dilution). Amplification of secondary labeling was performed using an ABC kit (Vector Labs, Burlington, Ontario). Three successive washes in 0.1 M PBS were made between each step. Signal detection was performed with a DAB Chromagen substrate (Pierce, Rockford, IL) and hematoxylin (Sigma) was used for counterstaining. Negative controls were run simultaneously with the omission of primary antisera. Conventional hematoxylin and eosin (H&E) staining was performed on sham and injured sections to confirm that the absence of calbindin-expressing Purkinje cells was attributable to cell loss and not an atrophic response to injury.

Parvalbumin has been shown to be expressed in both Purkinje cells as well as basket and stellate cells of the molecular layer (22, 23). For double-label immunohistochemistry, parvalbumin was used as a marker to identify GABAergic interneurons of the molecular layer. A similar protocol was applied as described above with the omission of peroxide treatment. Both monoclonal antiparvalbumin and rabbit polyclonal anti-calbindin-28k (Sigma) (1:1000 for both antibodies) were incubated 1 hour at RT in 10% normal goat serum blocking solution. Alexa 488 goat antirabbit (1:1000) and Alexa 555 goat antimouse antibodies were used for visualization of labeled proteins. TO-PRO-3 (Molecular Probes) was used as a nuclear counterstain.

Double-label experiments for GFAP and calbindin-28k or cleaved caspase-3 (Chemicon, Temecula, CA) and calbindin-28k were performed as described here with the following changes: Microwave antigen retrieval was performed on tissue sections in 0.1 M sodium citrate solution for 20 minutes for sections labeled for GFAP. Monoclonal Cy3-conjugated anti-GFAP antibodies (Sigma) were used at a dilution of 1:1000 for 1 hour at RT. Trypsin permeabilization treatment was used for sections labeled for cleaved caspase-3 (1:500 dilution). Coronal sections from the injured forebrain were used as positive controls.

Fluorescent TUNEL method was performed according to the manufacturer’s protocol (Roche, Laval, Quebec). After the TUNEL procedure, sections were incubated overnight with monoclonal anticalbindin antibodies in 0.2% Triton-X, 10% normal goat serum at 4°C. Secondary Alexa 555 goat-antimouse antibodies were used for visualization of calbindin-28k expressing Purkinje cells. Hoechst was used as a nuclear counterstain.

Double-labeled fluorescent sections were captured on a BioRad Radiance 2100 confocal microscope using a 40× oil immersion objective. Image capture settings (i.e. laser power, aperture size, gain, and black level) were standardized for all images. A Z-series projection of eight to nine images at 1-μm step intervals was used for each image capture to ensure that observed changes were not the result of biases in section sampling.

Bright-field histologic sections used for Purkinje cell quantification were captured on an Olympus AX70 microscope using a 20× objective.

Purkinje Cell and Interneuron Quantification

Three to four nonadjacent vermis sections per animal (n = 4 animals per injury time point) were chosen for quantification. Three regions of the cerebellum were chosen for PN and interneuron quantification: (Fig. 1A, red boxes) region 1 (posterior cerebellum) corresponding to the gyrus of lobules VIII and IX; region 2 (middle cerebellum) gyrus of the horizontal fissure; and region 3 (anterior cerebellum) gyrus of lobules III and IV. Using SPOT capture software, a 1000-μm contour line was drawn along the PN layer on both sides of the fissure or gyrus for a total 2000-μm length sampling area. The number of PNs for each region was calculated as the average number of PNs counted between the four sections sampled for a specified region. Two criteria were used in cell counts: 1) the cell body was well defined (i.e. not blurred), and 2) had the general characteristic morphology of a Purkinje cell (i.e. round cell body approximately 25–30-μm diameter located between the granule cell and molecular layers).

Interneurons were quantified from equivalent regions sampled for PN counts. The total numbers of parvalbumin expressing interneurons in the molecular layer were quantified in a field with dimensions of 604 × 604 μm. Cell counts for PNs and interneurons were conducted in a blinded manner.

Western Blotting

Cerebellar vermis homogenates were obtained from sham, 1-, 3-, 7-, and 14-day postinjured rats. After decapsulation, the cerebellum was extracted in iced ACSF prepared fresh
daily (126 mM NaCl, 3 mM KCl, 1.4 mM KH2PO4, 2.4 mM CaCl2, 1.3 mM MgSO4, 26 mM NaHCO3, 20 mM glucose) and bubbled with carbogen mixture (95% O2, 5% CO2). The cerebellar vermis was collected whole or divided into three regions corresponding approximately to the regions used for immunohistochemistry quantification analysis. Tissue samples were homogenized in lysis buffer containing protease inhibitors (50 mM Tris-HCl, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin). Protein quantification was determined by the modified Lowry method (24). Samples were normalized for equal loading (40 μg/lane) and were electrophoresed on 7% SDS-PAGE gels and transferred overnight to nitrocellulose membranes.

Blocking of membranes was performed in 5% nonfat milk blocking solution. Immunoblotting for calbindin-28k expression (1:3000) was performed overnight at 4°C in blocking solution. Rabbit anti-ERK1, 2 (1:30,000) (Sigma) was used as a loading control in densitometry analysis of calbindin expression. Secondary antibody incubation was performed for one hour with HRP conjugated goat antimouse (1:3000) and goat antirabbit (1:3000) secondary antibodies. Washes in three changes of TBST were performed between incubations. Bands were visualized using a chemiluminescent ECL kit (Perkin Elmer, Boston, MA) and exposure to x-ray film. All immunoblotting experiments were repeated in triplicate (n = 3 per injury time point for whole and regional protein analysis). Densitometry analysis of calbindin expression after injury was performed using Gel-Pro Analyzer software (Media Cybernetics, San Diego, CA). Sham levels of expression were assumed to be 100%, whereas changes in protein expression were indicated as percent changes from sham levels.

Statistical Analysis
SigmaStat statistical software (SPSS Inc., Chicago, IL) was used for analysis of Purkinje cell quantification. A one-way analysis of variance (ANOVA) was used to examine trends in cell loss in each injury severity group. A 3-way ANOVA was used to examine trends between time versus injury versus region. Post hoc analysis for pairwise comparisons and interactions was performed using the Student-Newman-Keuls method. Statistical significance was assumed at a p value < 0.05.
RESULTS

Purkinje Cell Quantification

Purkinje cells in sham animals were observed at regularly spaced intervals between the molecular and granule cell layers (Fig. 1B). Cell bodies were round and clearly defined and dendritic arborizations were visible. After FPI, acute PN loss at one day postinjury was evident in region 1 of the cerebellum (Fig. 1C) at all injury severities. After injury, abnormal PN morphology was observed in some cells that had diffuse calbindin staining and poorly defined soma (Fig. 1D, E).

One-way ANOVA in region 1 indicated a significant difference (p < 0.001) in PNs between time points for all levels of injury severity. Post hoc analysis indicated that region 1 was highly susceptible to early cell loss at 1 day postinjury after 2.0, 2.5, and 3.0 atm injuries (p = 0.014, p = 0.006, and p = 0.001, respectively) (Fig. 2A). The 1.5-atm injury group reached a significant decline in PNs at 7 days postinjury (p = 0.007). Surviving PNs from region 1 did not vary significantly over the 14-day time course with the exception of the 1.5-atm injury group. This mild injury group had a significant decline in PNs between 1 day and 14 days postinjury (p = 0.021).

Analysis of surviving PNs in the middle and anterior regions of the cerebellum (regions 2 and 3, respectively) indicated a spatial and temporal gradient of PN loss after lateral FPI. Regions 2 and 3 were less susceptible to acute PN loss in comparison to the posterior cerebellum. In region 2, PNs did not decline significantly until 14 days postinjury (all injury levels). The highest injury severity group had a significant decline in PNs at 7 days postinjury (p = 0.003) (Fig. 2B). Region 3 was the least susceptible cerebellar region. Delayed PN loss was observed at 7 days postinjury at

FIGURE 2. (A) Region 1 exhibited the greatest number of Purkinje cell loss at 1 day postinjury. The asterisk indicates value were significant compared with controls. (B, C) There were significant declines in Purkinje neurons in regions 2 and 3 but only at higher injury severities and at later time periods. This temporal and spatial relationship was supported by 3-way analysis of variance indicating a significant interaction between regional susceptibility and survival time (p < 0.0001). Dose-response relationship was supported by significant differences in injury severity allowing for effects of differences in survival time and region Purkinje neuron loss (p = 0.004).
3.0-atm injury and at 14 days in the 2.0-atm injury group 
\((p = 0.034, p = 0.02)\).

Three-way ANOVA indicated a significant difference in 
injury severity after allowing for effects of differences 
in survival time and region assessed (\(p = 0.004\)). Post hoc analysis indicated significant differences between 1.5-atm and 2.5-atm as well as 2.0- and 3.0-atm injury groups (\(p = 0.029\) both comparisons), thus indicating a dose–response relationship between PN cell death and injury severity. A spatial and temporal relationship of cell loss was indicated by the interaction between survival time and regional response to injury. PN counts were significantly different at 1 day in region 1 versus region 2 (\(p < 0.001\)) and region 1 versus region 3 (\(p < 0.001\)) but not between regions 2 and 3.

**Protein Expression of Calcium-Binding Proteins**

We hypothesized that alterations in calbindin expression after injury could contribute to delayed PN loss as result of impaired calcium-buffering capacity. Densitometry analysis of Western blot data from whole cerebellum vermis sections indicated no change in expression over the course of injury despite immunohistochemistry data indicating a loss of PNs (Fig. 3A). However, given that calbindin is highly expressed in Purkinje cells, variations in total calbindin expression may have been masked as a result of the low sensitivity in detecting relatively small changes in total expression. As such, we examined region-specific changes in calbindin expression. Western blot analysis indicated a decline in calbindin expression at one day postinjury in regions 1 and 3. However, by 14 days postinjury, expression of calbindin recovered to near sham levels for region 1. Furthermore, densitometry analysis in region 2 indicated an increase in calbindin expression at 7 and 14 days postinjury above sham levels of expression (Fig. 3B). Region 3 also exhibited a moderate increase in expression levels at 3 and 7 days postinjury.

Expression of parvalbumin calcium-binding protein was also examined after injury. Although parvalbumin is expressed in stellate and basket cells of the molecular layer, it has also been shown to be expressed in Purkinje cells (22, 23). There was no change in parvalbumin expression over the course of the injury (data not shown).

**Cerebellar Astrogliosis**

Astrogliosis is a characteristic pathologic occurrence in injured central nervous system tissue (25, 26). We examined cerebellar sections for the presence of astrogliosis at 14 days postinjury. Astrocyte processes in the molecular layer were arranged in stripes perpendicular to the granule cell layer (Fig. 4A). In injured sections, there was an increase in GFAP immunoreactivity in regions where PN loss occurred (Fig. 4B). GFAP expression did not appear to change in the granule cell layer or white matter tracts.

**Sparing of Parvalbumin-Expressing Interneurons**

Survival of interneurons of the molecular layer was examined by double-labeling sections for parvalbumin and calbindin. Both calcium-binding proteins colocalized to Purkinje cell bodies in both injured and sham tissue. Interneurons were present in both sham and one-day postinjury sections despite the loss of PNs (Fig. 4C, D). At 14 days postinjury, despite the obvious loss of PNs, interneurons were spared (Fig. 4E). Quantification of interneurons indicated no significant difference in cell numbers within regions or between groups in sham and 14-day postinjury animals (mean

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**FIGURE 3.** (A) Western blot from whole vermis homogenate. There was no change in calbindin expression over the course of injury despite the decrease in the number of Purkinje neurons, suggesting a possible increase in expression in surviving Purkinje neurons. (B) Densitometry analysis from Western blots from specific regions of the cerebellar vermis indicate an initial decrease in calbindin expression in regions 1 and 3. Expression levels in region 1 are comparable with sham levels of expression at 14 days postinjury, suggesting an increased level of expression in surviving Purkinje cells. Region 2 had a 1.5-fold higher level of calbindin expression at 7 and 14 days postinjury.

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sham counts: 148 ± 20 standard deviation [SD]; mean 14-day counts: 143 ± 23 SD; p = 0.484).

**Apoptosis After Injury**

Calbindin colocalization with TUNEL or cleaved caspase-3 labeling addressed the possibility of an apoptotic mechanism to account for PN loss. TUNEL/cleaved caspase-3 was assessed in at least four sections per time period in each injury group. There was relatively little TUNEL observed despite previous histologic PN quantification that indicated delayed cell loss. At one day postinjury, limited TUNEL in the granule cell layer was observed in region 1; however, there was no colocalization with calbindin expression (Fig. 4F). A general finding at all injury severities was the presence of TUNEL in the granule cell layer (Fig. 4G) with the exception of the 1.5-atm injury group, which did not
display TUNEL at any time period. There was no indication of TUNEL at any time period beyond 1 day postinjury. Immunolabeling for caspase-3 was negative in all sections examined with the exception of positive control forebrain tissue sections (data not shown).

**DISCUSSION**

The pathophysiological consequences of TBI in rats has most frequently been attributed with motor impairment and memory/learning deficits associated with injury to the sensorimotor cortex or hippocampal regions, respectively (4, 15, 27–34). However, functional deficits such as impairment of motor coordination after TBI are findings that have been associated with cerebellar function. Despite this correlation, there exists little data on the pathophysiological changes that occur in the cerebellum after forebrain TBI. More recent studies have expanded the traditional notion of cerebellar function to include both motor and higher-order cognitive processing independent of motor function (18, 35–37). This new appreciation of cerebellar function suggests that cerebellar injury after TBI could play an important role in the progression of neurologic and motor impairments that have not yet been addressed. As such, we examined the susceptibility of the cerebellum to forebrain TBI and determined that this structure, remote from the area of impact, exhibits significant reductions in neuronal cell population and alterations in calcium-binding protein expression.

The graded levels of injury severity demonstrated a dose–response relationship to PN death after forebrain injury. There was a significant difference between injury groups having allowed for differences in survival time and regional susceptibility. Furthermore, a time–region effect was observed, which indicated a temporal and spatial progression of selective PN loss. For example, the posterior region of the cerebellum was susceptible to acute losses in PNs at one day postinjury, whereas regions 2 and 3 displayed delayed loss of PNs at higher grades of injury severity. Thus, PN susceptibility to forebrain trauma includes both acute and delayed mechanisms.

Two previous studies have demonstrated delayed Purkinje cell loss after midline and lateral FPI (38, 39); however, a distinction in regional assessment of the cerebellum’s susceptibility was not made and the acute cell loss accounting for the majority of PN loss was not described. The loss of PNs at one day postinjury in the posterior region suggests that initiating mechanism is primary in nature, possibly as a result of the contre-coup effect. Interestingly, there was no statistical significance in post hoc analysis of injury severity in the posterior cerebellum. This may be indicative of a high susceptibility of PNs to mechanical forces. Although contre-coup injuries are generally associated with the contralateral side of impact, the exact mechanism of these injuries is not well understood but likely involves deceleration and inertial forces causing the brain to impact on a stationary skull (40). It is possible that such phenomenon occurs in the cerebellum after forebrain FPI. Radial tissue stress and strain from the point of impact may cause the cerebellum to displace at a sufficient velocity to induce traumatic impact against the occipital region of the skull. The relevance of this model to human cases presents different modeling dynamics given the differing anatomic location of the cerebellum in humans and rodents. However, pathologic changes in the cerebellum in clinical cases of TBI have noted diffuse axonal injury, cerebellar atrophy, and various degenerative changes as indicated by magnetic resonance imaging, which suggests that this indirect cerebellar injury model in rats is relevant to human studies (41–43). Furthermore, patients with cerebellar injuries demonstrate impairment in learning tasks and complex nonmotor processing, which indicates a close relationship between cerebrocerebellar communication (44). Thus, a compartmentalized approach to TBI studies, although helpful in focusing on region-specific mechanisms, may not adequately convey the range of complexity of TBI pathophysiology, which likely affects regions of the brain both close and distant from the area of impact.

The relative lack of TUNEL and activated caspase-3 and their failure to colocalize with calbindin indicated that PN loss in this indirect trauma model did not occur by apoptosis. It is possible that apoptosis was involved in PN loss at time periods earlier than those assessed. However, such an early initiation of apoptosis would not account for the delayed cell loss observed at 14 days postinjury. It is possible, however, that a second apoptotic-initiating event that was not assessed in our time points was responsible for the delayed cell loss observed at the interval between 7 and 14 days postinjury. Given the high calcium-buffering capacity of PNs conferred by parvalbumin and calbindin, there may be mechanisms other than disruption of calcium homeostasis that induced delayed cell death. For example, microglia were observed in perpendicular stripes in regions of Purkinje cell loss in a previous study examining PN loss after indirect trauma (39). The authors hypothesized that given the close anatomic proximity between microglia and PNs, injury may have been the result of microglial activation. Regardless, the precise mechanism of delayed PN loss remains unknown.

Changes in calbindin expression after injury suggested neuroprotective mechanisms involved in sparing surviving PNs. Although the largest decline in Purkinje neurons in region 1 was observed at 14 days postinjury, calbindin expression was comparable to sham levels of expression. Given that there were approximately 30% fewer PNs in this region, the normalized calbindin levels, which were comparable to sham levels, indicates increased expression of calbindin in surviving cells. Furthermore, there was an increase in calbindin expression approximately 1.5-fold higher in region 2 at 7 and 14 days postinjury. This increase also correlates with notably less PN loss in region 2 than region 1. However, it is difficult to discern whether cell survival in this region was the result of its distance from the point of secondary impact (e.g., contre-coup) or from increased calcium-buffering capacity. Nevertheless, the increase in calbindin expression is likely to alter PN responses to synaptic input, thus reflecting some plasticity changes at work. Studies have demonstrated that calbindin and parvalbumin play a significant role in modulating Ca2+ transients after synaptic stimulation (45–47). How these
changes affect synaptic transmission of the cerebellum after injury remains to be determined.

Reactive astrocytosis after central nervous system injury is a phenomenon that is not well understood. There is conflicting evidence supporting neuroprotective and regenerative roles for astrocytes as well as data suggesting that astrogliosis is an impediment to axonal regrowth and contributes to neurotoxicity (for reviews see [25, 26, 48]). After experimental TBI, global neuronal depolarization and spreading depression are associated with increases in extracellular K+ (49, 50). Under conditions of high extracellular K+, astrocytes have been shown to release glutamate through reversal of electrogenic glutamate transporters (51, 52). PNs have been shown to be selectively vulnerable to AMPA/KA receptor-mediated injury (53, 54). Reversal of glutamate transport in cerebellar astrocytes after FPI is a possible mechanism contributing to excitotoxic injury in PNs. This may account for mechanisms of delayed PN death that were not identified by TUNEL. Further investigation may elucidate the relationship between astrogliosis and mechanisms of delayed Purkinje cell loss given the close anatomic proximity of molecular layer astrocytes to Purkinje cell dendritic arborizations.

A greater appreciation for the complexity of TBI pathophysiology can be gleaned from the susceptibility of the cerebellum after forebrain injury. Although the behavioral outcome as a result of these pathophysiological changes is not known, the literature provides some evidence to suggest that cerebellar dysfunction contributes to TBI neuropathology. Deficiencies in cued learning responses in Morris water maze testing indicate that some of these deficits are associated with extrahippocampal damage (57). The possibility of cerebellar contribution to neurologic deficits after TBI are strengthened by data from hemicerebellar-ectomized animals, which exhibit deficits in cued learning responses that are similar to those observed in TBI models (18, 36). Furthermore, there is an increasing database of knowledge that demonstrates changes in regions both proximal and distal from the location of trauma in animals and clinical cases of TBI (41, 58–61).

CONCLUSIONS

An increasing number of studies have begun to demonstrate the full spectrum of injury to brain structures after TBI. The vulnerability of the cerebellum to forebrain injury has implications for how TBI should be assessed in behavioral studies and emphasizes the complexity of the pathophysiological response. As a structure involved in motor coordination, as well as higher-order cognitive functions, the integrated role of the cerebellum in brain functions requires a reevaluation of clinical-pathologic correlation analysis in TBI.

Furthermore, not only would an analysis of the cerebellum expand our understanding of modeled TBI with respect to morphology and mechanism, but it also provides the opportunity to study the dimensions of injury remote from the site of impact and delayed from its time of occurrence.

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