Glibenclamide Reduces Hippocampal Injury and Preserves Rapid Spatial Learning in a Model of Traumatic Brain Injury

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Abstract
Cognitive disturbances after traumatic brain injury (TBI) are frequent, even when neuroimaging shows no overt hemorrhagic or other abnormality. Sulfonylurea receptor 1 (SUR1) plays a key role in various forms of CNS injury, but its role in hippocampal dysfunction after mild to moderate TBI is unknown. To assess the hypothesis that postinjury SUR1 upregulation in the hippocampus is associated with a later disturbance in learning, we studied a rat model of cortical impact TBI calibrated to avoid primary and secondary hemorrhage in the underlying hippocampus. The transcription factor, specificity protein 1, which regulates expression of SUR1 and caspase-3, was activated in the hippocampus 15 minutes after injury. Upregulation of SUR1 protein and of Abcc8 (which encodes SUR1) messenger RNA was evident by 6 hours. To assess the role of SUR1, injured rats were administered vehicle or a low dose of the specific sulfonylurea inhibitor glibenclamide for 1 week. At 2 weeks, the increase in activated caspase-3 in the hilus of glibenclamide-treated rats was half of that in vehicle-treated rats. Testing for rapid learning in a Morris water maze at 4 weeks showed significantly better performance in glibenclamide-treated rats; performance inversely correlated with Fluoro-Jade staining for degenerated neurons in the hilus. We conclude that glibenclamide may have long-term protective effects on the hippocampus after mild-to-moderate TBI.

Key Words: Caspase-3, Glibenclamide, Hippocampus, Morris water maze, Rapid spatial learning, Sfp1, Sulfonylurea receptor 1 (SUR1), Traumatic brain injury.

INTRODUCTION
Traumatic brain injury (TBI) affects 1.6 million Americans annually, with more than 5 million Americans currently living with disabilities resulting from TBI (1). Although physical impairments often improve with rehabilitation therapy, trauma-induced cognitive deficits are more persistent and often last many years (2). Patients with TBI report that disturbances in cognitive function have the greatest negative impact on their quality of life (3), with memory problems being the most severe and frequent symptom (4).

The hippocampus is crucial for certain types of memory (5, 6). Principal neurons of the hippocampus, called “place cells,” exhibit place-specific firing when rats explore a novel environment (7). Different hippocampal subfields along the transverse axis (dentate gyrus [DG], CA3, and CA1) make distinct computational contributions that are required specifically for rapid place learning (8–11) but not for incremental place learning (12–14). The rat cortical impact model of TBI reproduces many features of head injury in humans (15) and has been used to study pathophysiological sequelae in the hippocampus, including deficits in spatial learning and memory in Morris water maze (MWM) tasks (16–18).

Cognitive deficits after TBI in humans are often encountered in individuals with normal radiologic examinations or radiologic examinations in which the hippocampus and other structures integral to cognition and memory exhibit no overt abnormality. Fully 75% to 85% of patients with persistent postconcussive symptoms including neurobehavioral deficits have normal computed tomographic or magnetic resonance imaging at the time of injury (19, 20). In such cases, it is presumed that the kinetic energy absorbed by brain tissues, although not sufficient to cause primary or secondary hemorrhage, is still enough to incite a secondary injury response that results in neuronal dysfunction. The molecular mechanisms underlying neuronal dysfunction in the absence of hemorrhage or overt tissue destruction are poorly understood.

The sulfonylurea receptor 1 (SUR1)–regulated NCa-ATP channel has a key role in cellular and microvascular dysfunction after various types of CNS injuries (21). The channel is effectively blocked by low-dose glibenclamide, which confers significant improvements in edema, progressive secondary hemorrhage, tissue swelling, lesion size, inflammation, mortality, and neurologic function in rodent models of TBI, stroke, subarachnoid hemorrhage, and spinal cord injury (22–27). To date, however, no study has examined the role, if any, of the SUR1-regulated NCa-ATP channel in the cellular and functional abnormalities that occur in structures such as the hippocampus after a mild to moderate traumatic insult without primary or secondary hemorrhage.

Here, we used a rat model of cortical impact TBI specifically calibrated to avoid primary and secondary hemorrhage in the underlying hippocampus. Using this model, we assessed
i) the time-dependent upregulation of SUR1 protein and Abcc8 messenger RNA (mRNA) in the hippocampus underlying the site of cortical impact, ii) caspase activation and neuronal degeneration in the hippocampus at 2 and 4 weeks, respectively, and iii) incremental and rapid place learning at 2 and 4 weeks after injury. We report that SUR1 is transiently upregulated in the hippocampus despite the lack of overt hemorrhagic injury and that treatment with glibenclamide has long-term protective effects associated with significant preservation of rapid spatial learning.

MATERIALS AND METHODS

Subjects and Experimental Series

All experimental procedures were approved by the University of Maryland Institutional Animal Care and Use Committee. In accordance with “good laboratory practice,” an investigator blinded to treatment conducted all surgical procedures, behavioral tests, and quantitative analyses of the results.

Long-Evans rats (250–300 g; Harlan, Indianapolis, IN) (n = 59) were used in 4 experimental series as follows. In series 1, 24 rats underwent cortical impact and were killed at 24 hours (n = 6), 12 hours (n = 6), 6 hours (n = 6), 3 hours (n = 3), or 15 minutes (n = 3) after injury; 6 other rats underwent sham operation and were killed at 24 hours (n = 3) or 6 hours (n = 3) after craniectomy. These brains were used for gross pathologic, histochemical, immunohistochemical, and quantitative reverse transcription–polymerase chain reaction (RT-PCR) assessments. In series 2, 10 rats underwent cortical impact, received vehicle (n = 5) or glibenclamide (n = 5), and were maintained for 2 weeks after injury; 3 other rats underwent sham operation and were killed at 2 weeks. These brains were used for histochemical and immunohistochemical assessments. In series 3, 10 rats underwent cortical impact, received vehicle (n = 5) or glibenclamide (n = 5), and were tested in the MWM before killing 4 weeks after injury; 3 other rats underwent sham operation and were tested in the MWM before killing at 4 weeks after surgery. These brains were used for histochemical and immunohistochemical assessment. In series 4, 3 uninjured rats were killed, and their brains were used for histochemistry and immunohistochemistry.

Cortical Impact Injury

Anesthetized (ketamine 60 mg/kg and xylazine 7.5 mg/kg intraperitoneally) rats breathed air spontaneously. The head was fixed in a stereotaxic frame (Stoelting Co, Wood Dale, IL). Body temperature was maintained at 37°C using an isothermal heating pad (Deltaphase; Braintree Scientific, Inc, Braintree, MA). Using aseptic techniques, an 8-mm craniectomy in the left parietal bone was made midway between bregma and lambda and abutting the sagittal suture and left lateral ridge. Care was taken to avoid thermal and mechanical injury to the dura mater.

Impact injury was induced using a modified Feeney device (28), as previously described (22), with some modifications. The footplate was replaced with a 4.7-mm Teflon sphere, and the depth of cortical depression was not limited.

The vertical guide tube (inside diameter, 6 mm), angled +10 degrees relative to the stereotaxic frame in the transverse plane, and the spherical impactor were gently positioned on the dura at −3.6 mm anteroposterior and +2.6 mm mediolateral to the bregma. Before activating the impactor, the stereotaxic frame with the animal and vertical guide tube was rotated −10 degrees so that the guide tube was orthogonal to the table surface. The impactor was activated by dropping a 10-g mass 3 cm down the shaft of the guide tube onto the Teflon sphere (impact velocity of 0.77 m/sec). The mass was removed within 3 seconds of impact. The dura was not lacerated by this procedure. The craniectomy was left open, and the scalp was closed.

Treatment

A stock solution of glibenclamide (Sigma, St Louis, MO) was prepared by placing 25 mg into 10 ml of dimethyl sulfoxide; solutions for treatment were made by placing 200 μl of stock solution into 2.3 ml of unbuffered normal saline and clarifying the solution with a few microliters of 0.1N NaOH to achieve a pH of ~8.5. For rats in series 2 and 3, within 10 minutes of impact injury, a loading dose of glibenclamide (10 μg/kg intraperitoneally) or vehicle was given and a mini osmotic pump (Alzet 2001, 1.0 μm/hr; Alzet Corp, Cupertino, CA) was implanted subcutaneously to deliver 200 ng/hr of glibenclamide or vehicle for 7 days. This treatment yields plasma concentrations ~5 ng/ml (J. M. Simard, unpublished observation) and has a minimal effect on serum glucose (23, 26, 29).

Quantitative RT-PCR

To measure mRNA abundance of Abce8, Kcnj11, which encode SUR1, Kir6.1, and Kir6.2, respectively, total RNA from control and injured rat hippocampi were extracted using TRIzol (Invitrogen, Carlsbad, CA). RNA was quantified by assessing optical density at 260 and 280 nm. To avoid contamination from genomic DNA, the RNA was treated with DNase I (Invitrogen). Total RNA (1 μg) from each sample was reverse-transcribed using SuperScript III kit (Invitrogen) with random hexanucleotides and analyzed by real-time PCR using ABI PRISM 7300 Sequence Detector System (Applied Biosystems, Foster City, CA). To normalize samples, S18 mRNA abundance was also measured. The primer sequences used were as follows: 5′-CTGCGAGTACTCAACACAAAC-3′ (forward) and 5′-AACCAGTCGGGATCTGTAT-3′ (reverse) for S18; 5′-GAGTGCGAATCTCGCCT-3′ (forward) and 5′-CCTTTGAGACTGGAACCAGC-3′ (reverse) for Abce8; 5′-AGCTGCGCTTCTGCTCCTCA-3′ (forward) and 5′-CCCCTCAACACCAATGGTACT-3′ (reverse) for Kcnj11; and 5′-CCGAGCGGCCCACACAGAACATC-3′ (forward) and 5′-CCGAGCGGCCCACACAGAACATG-3′ (reverse) for Kcnj11.

Immunohistochemistry

Cryosections were immunolabeled using standard techniques. After permeabilization (0.2% Triton X-100 in PBS) and blocking (2% donkey or 5% goat serum; D-9663; Sigma) for 1 hour, the sections were incubated with primary antibodies: goat anti-SUR1 (1:200; SC-5789; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), mouse anti-Kir6.1 (1:100; SC-33627; Santa Cruz), mouse anti-Kir6.2 (1:100; SC-33628; Santa Cruz), mouse anti-Abcc8 (1:100; MAB46454; R&D Systems, Minneapolis, MN), and mouse anti-Abce8 (1:100; AB55029; Merck Millipore, Billerica, MA). After primary incubation, the sections were washed with PBS and incubated with secondary antibodies in PBS for 1 hour: donkey anti-goat (1:200; sc-2160; Santa Cruz) and donkey anti-mouse (1:200; sc-2020; Santa Cruz). After washing, the sections were mounted with ProLong Antifade Mountant with DAPI (Invitrogen, Carlsbad, CA) and imaged using a Zeiss LSM 800 confocal microscope (Carl Zeiss, Inc, Thornwood, NY). Immunoreactivity was quantified with ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD).
Biotechnology, Santa Cruz, CA), mouse anti-NeuN (1:100; Clone A60; Chemicon, Temecula, CA), rabbit anti–nuclear factor κB p65 (1:200; SC-372; Santa Cruz Biotechnology), rabbit anti–specificity protein 1 (Sp1, 1:200; SC-59; Santa Cruz Biotechnology), or rabbit anticleaved caspase-3 (1:100; Asp175; Cell Signaling Technologies, Beverly, MA). Fluorescent-labeled, species-appropriate secondary antibodies (Invitrogen) were used for visualization. Omission of primary antibody and competition with antigenic peptides, when available, were used as negative controls. The sections were coverslipped with polar mounting medium containing antifade reagent and the nuclear dye, 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), and were examined using epifluorescence microscopy (Nikon Eclipse 90i, Nikon Instruments Inc., Melville, NY).

**Histochemistry**

For routine histology, cryosections were stained with hematoxylin and eosin and examined using light microscopy. Fluoro-Jade C (FJC) histofluorescent staining was carried out as described (30). Briefly, cryosections were first immersed in a solution containing 1% NaOH in 80% EtOH for 5 minutes. Sections were then rinsed for 2 minutes each in 70% EtOH and distilled water before being oxidized for 10 minutes in 0.06% KMnO4 solution. After a rinse in distilled water, sections were stained in 0.0001% FJC (Chemicon) and 0.0001% DAPI (Sigma) solution for 10 minutes. Finally, slides were washed 3× with distilled water for 1 minute and dried on a slide warmer at 50°C for 30 minutes. After clearing in xylene and coverslipping with nonpolar mounting medium (Cytoseal XYL; Richard-Allan Scientific, Kalamazoo, MI), sections were examined with an epifluorescence microscope under a fluorescein isothiocyanate filter for FJC signal, with care being taken to limit the time of exposure to reduce the likelihood of photobleaching.

**Quantitative Immunohistochemistry**

Unbiased measurements of signal intensity within regions of interest (ROIs) were obtained using NIS-Elements AR software (Nikon Instruments, Melville, NY) from sections immunolabeled in a single batch, as described (25, 31). All ROI images for a given signal were captured using uniform parameters of magnification, area, exposure, and gain. Segmentation analysis was performed by computing a histogram of pixel intensity for a particular ROI. For SUR1 and FJC, specific labeling was defined as pixels with signal intensity greater than 2× that of background (SUR1) or 1.5× that of background (FJC), and the area occupied by pixels with specific labeling was used to determine the percent area with specific labeling (% ROI). For cleaved caspase-3, the number of DAPI+ nuclei with specific labeling in the granule or pyramidal cell layer of the ROI was manually counted with the use of Adobe Photoshop software (Adobe Systems, San Jose, CA) and a cell counter for precision. Manual cell counting was carried out for nuclear-localized Sp1 in the cortex, corpus callosum, hilus of the hippocampal DG, CA3 of the hippocampus, and dorsal thalamus ipsilateral to the cortical impact.

Regions of interest were located as follows: for the SUR1 signal, the entire ipsilateral hippocampus with the alveus forming the inferolateral border; for the FJC signal, the hilus of the hippocampus; for nuclear-localized cleaved caspase-3 cell counts, 2 noncontiguous 411.8 × 329.4-μm² areas, the first of which contains the suprapyramidal and infrapyramidal limbs of the hippocampal granule cell layer in the corner of the field and the second contains the hippocampal CA3b pyramidal cell layer in the corner of the field; and for nuclear-localized Sp1 cell counts, 4 noncontiguous 411.8 × 329.4-μm² areas, the first of which contains the suprapyramidal and infrapyramidal limbs of the hippocampal granule cell layer in the corner of the field, the second contains the dorsal thalamus directly below the lateral end of the suprapyramidal limb of the hippocampal granule cell layer, the third contains the hippocampal CA3b pyramidal cell layer in the corner of the field, and the fourth contains the corpus callosum directly above the hippocampal CA3b subfield.

**FIGURE 1.** Cortical impact model spares the hippocampus from primary and secondary hemorrhage. (A, B) Surface view and perfusion-fixed coronal section (A) and hematoxylin and eosin-stained cryosection (B) obtained 24 hours after impact injury. The contusion involves the cortex and corpus callosum but spares the underlying hippocampus from hemorrhagic injury. (C) Surface view and perfusion-fixed coronal section obtained at 4 weeks after impact showing the lesion confined to cortex. Images shown are representative of 5 to 6 rats at each time point.
Morris Water Maze

Rats were maintained in the animal facility under standard laboratory conditions (lights on at 0600 and off at 1800), housed in pairs or triplets, and had access to food and water ad libitum after surgical procedures and throughout MWM testing. All MWM trial blocks were conducted at the same time each day.

The maze consisted of a circular pool (1.45 m diameter, 0.6 m high) with a black interior filled with water (20 ± 1°C) to a depth of 0.53 m. White Tempera nontoxic paint powder was used to render the water opaque. A clear Plexiglass platform (11 cm in diameter) was submerged 2 cm below the water surface (invisible to the rat) at a distance of 30 cm from the maze periphery during learning trials. The platform was flagged and raised 1 cm above the water surface (visible to the rat) during vision and motor testing trials. The testing room had several extramaze cues (tables, shelf, wall markings, etc). The investigator, dressed in blue scrubs, stood in the same location during test trials after releasing the rat into the maze with its face toward the pool wall. A computerized tracking system (ANY-Maze; Stoelting Co) was used to record animal movements during probe trials and to calculate the average swim speed during vision and motor testing trials.

At 2 weeks after injury or sham injury, rats were evaluated for performance in incremental place learning (32). Starting on postinjury day 14, rats were trained to locate the submerged platform in the northwest (NW) quadrant of the pool, based on extramaze cues. Rats underwent 4 trials per day for 5 consecutive days, with an intertrial interval of 30 minutes. Rats started a trial once from each of the 4 possible start locations (north [N], east [E], south [S], and west [W]), separated by 90 degrees around the maze periphery. The order of the starting location was randomized. Each trial lasted until the rat climbed onto the platform or until 120 seconds had elapsed, whichever occurred first. Rats that failed to locate the escape platform within the allotted time were manually guided to it. All animals remained on the platform for 30 seconds before being placed in a heated drying cage between trials. The rat’s latency to reach the platform was recorded for each of the 20 trials, and the average for each day was calculated as a measure of incremental place learning ability.

A memory probe trial for incremental learning ability, with the platform removed from the pool, was run on postinjury day 19. Rats were released from the south start point for the 60-second probe trial. The percent time spent in the NW quadrant was measured only during the first 30 seconds of the trial because animals with good hippocampal function abandon their search in the correct quadrant after this period if no escape platform exists (33–35).

Vision and motor abilities were tested on postinjury days 20 and 21 with 4 trials per day. The visible platform was located at the same location (NW), as during incremental learning trials. The rats started a trial once from each of the 4 possible start locations. Rats were allowed up to 120 seconds to find the platform, 30 seconds to remain on the platform, and 30 minutes in a heated cage between trials. The animal’s escape latency to the visible platform was measured for each of the 8 trials and averaged for each day. The average swim speeds for the 8 trials were calculated using the tracking software.

At 4 weeks after injury or sham operation, the same rats underwent an adapted version of the reversal and rapid place learning tests (32, 36). On postinjury day 28, rats were given 1 trial (released from N start site) to learn a new location of the submerged platform in the southeast (SE) quadrant. As before, the animals were allowed up to 120 seconds to find the hidden platform and 30 seconds to remain on the platform. The platform was then removed from the pool, and after a 30-minute intertrial interval in a heated cage, they underwent a memory probe trial for 60 seconds (released from E start site). The percent time spent in the SE quadrant during the first 30 seconds of the probe was measured for each rat.

**Statistical Analysis**

Data are reported as mean ± SEM. Student t-test, analysis of variance (ANOVA), or repeated-measures ANOVA were used for group comparison, as appropriate, with Bonferroni post hoc comparisons. A difference between groups was deemed significant if p < 0.05. Correlation was determined using a test for linear regression and Pearson product-moment correlation coefficient.

**RESULTS**

**Impact Injury to the Cortex and Hippocampus**

The impact used to induce trauma resulted in a hemorrhagic contusion in the cortex but left the underlying hippocampus seemingly uninjured, with no apparent tissue disruption or primary or secondary hemorrhage (Fig. 1A). Histologic examination of coronal sections through the epicenter at various times after the impact (15 minutes and 3, 6, 12 and 24 hours) revealed hemorrhagic lesions involving the cortical mantel and corpus callosum (Fig. 1B) but no hemorrhagic injury in the hippocampus. At 2 and 4 weeks after the impact, cavitory lesions restricted to the cortex were apparent

**FIGURE 2.** Upregulation of sulfonylurea receptor 1 (SUR1) protein and Abcc8 mRNA in the hippocampus. (A–D) Low-magnification (A, B) and corresponding high-magnification (C, D) images of the hippocampus in sham-injured rat (A, C) and 12 hours after impact injury (B, D), showing a visible increase in SUR1 expression (white or red) with injury; nuclei labeled with DAPI (blue). The images shown are representative of 3 sham and 6 injured rats at 12 hours. (E) Quantitative immunohistochemistry showing the change in SUR1 expression in the hippocampal region of interest (ROI), obtained at the times indicated after impact; 3–6 rats per time point; *p < 0.05; **p < 0.01. (F) Quantitative RT-PCR of hippocampus showing a change in mRNA for Abcc8 but not for Kcnj8 or Kcnj11 6 hours after impact; data are normalized to S18; 3 rats/group. (G) Cocommunolabeling of the hippocampus for SUR1 and NeuN, as indicated, 12 hours after impact. A superimposed image demonstrates neuronal expression of SUR1. Images are representative of 6 rats at 12 hours.
but with no overt morphologic disturbance of hippocampi (Fig. 1C).

**Upregulation of SUR1 in Hippocampus After Impact Injury**

Although there was no overt hemorrhagic injury in the hippocampus, the impact to the overlying cortex still deposited enough kinetic energy in the hippocampus to elicit an injury response. There was time-dependent upregulation of SUR1 protein in the hippocampus (Figs. 2, A–E). Immediately after the impact, and at 3 hours later, SUR1-specific immunolabeling in the hippocampus was the same as that in sham-operated animals (Figs. 2A, C), but at 6 hours after the impact, SUR1 expression was more prominent in the stratum moleculare and hilus of the hippocampal DG. By 12 hours, SUR1 expression had spread to the strata oriens, radiatum, and lacunosum/moleculare of the hippocampus proper (Figs. 2B, D). At 24 hours after the impact, SUR1-specific labeling in the hippocampus had returned to levels seen at 6 hours (Fig. 2E).

Upregulation of SUR1 protein was associated with upregulation of Abcc8 mRNA, which encodes SUR1 (Fig. 2F). Quantitative RT-PCR measurements of mRNA for Abcc8 showed a 2-fold increase at 6 hours, whereas mRNAs for Kcnj8 and Kcnj11, which encode Kir6.1 and Kir6.2, respectively, were unchanged. This pattern of transcription was shown previously to be associated with de novo upregulation of SUR1-regulated NCa-ATP channels and not of KATP channels (23). Double-labeling experiments indicated that SUR1 upregulation in the hippocampus occurred primarily in NeuN+ neurons (Fig. 2G).

**Activation of Sp1 in the Hippocampus After Impact Injury**

De novo upregulation of Abcc8 mRNA and SUR1 protein implies the involvement of 1 or more transcription factors. The transcription factors that have been linked to SUR1 upregulation in CNS injury include specific protein 1 (Sp1) and nuclear factor kB (NF-kB) (23, 25). Of these, NF-kB is activated by mechanical stimuli (37, 38), but to our knowledge, Sp1 has not been linked to mechanotransduction.

Nuclear localization of NF-kB (p65) was prominent in the hemorrhagic penumbral region of the cortical contusion (Fig. 3A), the location where we previously established the role of SUR1 in progressive secondary hemorrhage (22). In nonhemorrhagic tissues beyond the penumbra (i.e. the parapenumbra), including the hippocampus, NF-kB signaling was absent (Fig. 3B).

By contrast, immunolabeling for Sp1 showed prominent nuclear localization in regions beyond those with p65 nuclear localization, thereby distinguishing parapenumbra from penumbra. In the parietal cortex, both the penumbra, identified by p65 nuclear localization, and the parapenumbra showed extensive labeling for Sp1 (Fig. 3C). Specific protein 1 labeling was quite prominent in hippocampal areas in which p65 was absent (Fig. 3D).

Cells that exhibited robust nuclear localization of Sp1 also showed strong expression of SUR1 (Fig. 3E), consistent with the role of Sp1 in SUR1 transcription (23, 39). Indeed, they appeared to be temporally related. Specific protein 1 activation was observed as early as 15 minutes after impact and remained elevated for 3 hours preceding SUR1 upregulation (Fig. 3F). Both Sp1 and SUR1 peaked at 12 hours, after which both declined. We did not explore the molecular mechanism responsible for activation of Sp1 in the hippocampus by the mechanical stimulus associated with the impact injury but the temporal relationship between Sp1 activation and SUR1 upregulation, combined with the well-established role of Sp1 in transcriptional regulation of SUR1, is consistent with the involvement of Sp1 in the injury response in the hippocampus.

**Blockage of SUR1 With Glibenclamide After Impact Injury**

We next examined the effect of pharmacological block of SUR1 using the highly specific, highly potent sulfonylurea inhibitor, glibenclamide (40). Previous work on a similar TBI model showed that glibenclamide effectively blocks progressive secondary hemorrhage in the penumbra (22), but an effect on neuronal function in the parapenumbra has not previously been investigated. For these experiments, injured rats were administered a single loading dose followed by constant infusion of either vehicle or low-dose glibenclamide for 1 week. The dose of glibenclamide used has been shown repeatedly to be too low to have any appreciable effect on serum glucose (23, 26, 29).

In these experiments, we used activated caspase-3 in hippocampal neurons as an outcome measure for 2 reasons. First, like SUR1, caspase-3 is transcriptionally regulated by Sp1 (41), suggesting that levels of the enzyme might be elevated after impact injury. Second, neuronal apoptosis associated with activation of caspases plays an important role in TBI, both in humans and in various animal models (42). We quantified the number of hippocampal place cells with nuclear localization of cleaved caspase-3 in the vehicle-treated control group versus the glibenclamide-treated group.

![FIGURE 3. Impact-induced activation of specificity protein 1 (Sp1) in the parapenumbra. (A–D) The hemorrhagic penumbra of the parietal cortex (A, C) and the underlying nonhemorrhagic parapenumbral hippocampus (B, D) immunolabeled for p65 (A, B) and for Sp1 (C, D) 12 hours after impact, showing nuclear localization (pink) of both transcription factors in the penumbra but only Sp1 in the parapenumbra; nuclei are labeled with DAPI (blue). Images are representative of 6 injured rats at 12 hours. (E) Immunolabeling of the hippocampus for Sp1 and sulfonylurea receptor 1 (SUR1), as indicated, 12 hours after impact. Superimposed image demonstrates nuclear localization of Sp1 in SUR1+ cells. Nuclei are labeled with DAPI. Images are representative of 6 rats at 12 hours. (F) The number of hippocampal nuclei with localization of Sp1 at the times indicated after impact (left-sided ordinate); 3 to 6 rats per time point; **p < 0.01. Also shown are data from Figure 2E on SUR1 expression at the same times (right-sided ordinate).](http://jnen.oxfordjournals.org/Download)
At 2 weeks after injury, cleaved caspase-3 immunolabeling was prominent in numerous ipsilateral hippocampal place cells in the granule cell layer of the DG and in the pyramidal cell layer of CA3 (Figs. 4, A–E). The vehicle-treated control group had significantly more cleaved caspase-3+ place cells in these layers than the glibenclamide-treated group, with glibenclamide treatment being associated with a 7-fold and a 4-fold reduction in activated caspase-3 labeling in the DG and CA3, respectively (Fig. 4E).

At 2 weeks after injury, the contralateral hippocampus also showed cleaved caspase-3-specific immunolabelling, albeit at lower levels compared with that of the ipsilateral hippocampus. As in the ipsilateral hippocampus, glibenclamide treatment was associated with less cleaved caspase-3 labeling in the contralateral hippocampus, although the reduction was only statistically significant in the CA3 subfield (vehicle = 21 ± 3 in DG ROI, 51 ± 11 in CA3 ROI; glibenclamide = 15 ± 6 in DG ROI, 17 ± 5 in CA3 ROI, p < 0.03 for CA3 ROI).

Cognitive Performance in the MWM After Impact Injury

Two other groups of rats were subjected to impact injury and were administered either vehicle or glibenclamide for 1 week, as above. They were tested for MWM performance starting at day 14 after impact injury with the goal of assessing incremental place learning, which is not wholly dependent on the hippocampus (12–14), versus rapid place learning, which requires intact hippocampal function (8–11).

Analysis of incremental place learning trials revealed a significant day-to-day effect, demonstrating an overall improvement in escape latency to the hidden platform over time (Fig. 5A). No significant difference between vehicle and glibenclamide groups was found on any day of testing (2-way repeated-measures ANOVA with Bonferroni comparisons; p = 0.39 on day 1 and p = 1 on subsequent days; Fig. 5A). The memory probe trial assessing retention of incremental place learning also did not reveal significant group differences in search preference for the correct quadrant during the first 30 seconds of the probe, with all 3 groups spending significantly more time in the correct quadrant than expected by chance (sham-operated = 66 ± 9%, vehicle = 60% ± 5%, glibenclamide = 55% ± 1% time in the NW quadrant). Platform-seeking behavior was not affected by motivational, motor, or vision disparities between groups because average swim speed (sham-operated = 0.218 ± 0.068, vehicle = 0.217 ± 0.026, glibenclamide = 0.211 ± 0.012 m/sec) and escape latencies during visible platform trials were similar for all the groups (Fig. 5A).

After a 1-week rest period, the rats were retested in the MWM under an adapted reversal and rapid place learning paradigm on day 28 after impact injury. After 1 learning trial
for a “reversed” platform location (moved to the SE quadrant after having been in the NW quadrant for 28 trials), rats underwent a memory probe to assess their retention of this position. Notably, the sham-operated and glibenclamide-treated groups exhibited similarly high search preferences for the new SE quadrant after 1 learning trial. In contrast, the vehicle control group spent no more time in the correct quadrant than expected by chance (sham-operated = 47% ± 3%, vehicle = 23% ± 4%, glibenclamide = 41% ± 3% time in the SE quadrant; Fig. 5B). The swim paths during this memory probe revealed quadrant-focused search patterns by all 3 groups, but the vehicle control rats continued to focus their search in the old NW quadrant, in contrast to sham-operated and glibenclamide-treated animals (Figs. 5C, D). This lack of “pliancy” in untreated brain-injured animals to efficiently switch search strategies is a hallmark of hippocampal damage (43). A similar long-term outcome has been reported in a more severe fluid percussion model of TBI (36).

**Fluoro-Jade C Hippocampal Neuronal Degeneration**

Fluoro-Jade C is a putative marker for neuronal degeneration and delineates patterns of neurodegeneration that are identical to silver stains and its predecessor dyes (30). Increasing severity of traumatic injury is associated with an increasing number of FJC+ neurons in the rat hippocampus (44). We quantified FJC histofluorescent staining of hilar cell bodies and processes at 1 month after impact injury in the same vehicle- and glibenclamide-treated rats that were assessed for MWM performance.

One month after impact injury, there was prominent FJC staining of neuronal processes and, to a lesser extent, cell bodies in the ipsilateral hippocampal hilus (Figs. 6, A–F). The vehicle-treated control group had significantly more FJC+ neurons in the ipsilateral hilus than the glibenclamide-treated group (Fig. 6G). Glibenclamide treatment was associated with a 3-fold reduction in FJC+ hilar cell death in the ipsilateral hippocampus (p < 0.05).

The contralateral hippocampus also showed signs of delayed neuronal cell degeneration, similar to that observed with activated caspase-3 at 2 weeks after injury. As with cleaved caspase-3 labeling, the contralateral hippocampus exhibited less FJC staining than the ipsilateral hippocampus and the vehicle control group had greater FJC staining of hilar neurons than glibenclamide-treated rats, but within the SEM (not shown). Sham-operated and naive animals displayed virtually no FJC staining in ipsilateral or contralateral hippocampi.

**Correlation Between Cognitive Performance and Hilar Neuron Degeneration**

Rats with higher FJC staining in the hippocampal hilus had poorer performance during the memory probe for rapid place learning. Memory impairment was highly correlated with the number of FJC+, degenerating neurons in the hilus of the ipsilateral DG (Pearson product-moment correlation coefficient, r = −0.74, R² = 0.5419; Fig. 6H). This result corroborates previous findings of a similarly strong correlation (45–47). In addition, this analysis showed that, on a plot of memory performance versus ipsilateral hilar neurodegeneration, glibenclamide-treated rats clustered much closer to the sham-operated rats than to the vehicle control rats (Fig. 6H), reaffirming the protective effect of glibenclamide.

**DISCUSSION**

Severe impact trauma delivers kinetic energy to the brain that dissipates with distance and induces primary hemorrhage at the epicenter, progressive secondary hemorrhage in the surrounding penumbra (22), and nonhemorrhagic secondary injury in regions outside the hemorrhagic penumbra, that is, in the parapenumbra. Mild-to-moderate impact trauma may lack the hemorrhagic components and thus may be restricted to nonhemorrhagic secondary injury, arguably one of the least well-understood aspects of TBI. In humans, the most frequent form of TBI is closed head injury without hemorrhagic contusion. Despite seemingly normal radiologic imaging, many of these patients experience a wide variety of neurocognitive deficits, often lumped under monikers such as “postconcussive syndrome” and “posttraumatic stress disorder,” either of which may persist for years (19, 20).

The model of TBI that we studied here was calibrated so that the hippocampus would lie outside the hemorrhagic penumbra but would still be vulnerable to a nonhemorrhagic secondary injury response. We found that 2 well-known transcription factors undergo early activation and nuclear translocation in a manner that effectively distinguished the hemorrhagic penumbra from the nonhemorrhagic parapenumbra. Both NF-κB and Sp1 were identified in the penumbra; in the parapenumbra, however, NF-κB was absent, whereas Sp1 was prominent. Previous work identified an important role for NF-κB in TBI (48), where it may be

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**FIGURE 6.** Glibenclamide reduces Fluoro-Jade C in the hippocampus 4 weeks after impact. (A–D) Low-magnification images of hippocampi from naive (A), sham-injured (B), vehicle-treated injured (Veh) (C), and glibenclamide-treated injured (Glib) (D) rats labeled with Fluoro-Jade C at 4 weeks after injury. There is absence of labeling in controls (A, B) and visibly less labeling with glibenclamide (D) versus the vehicle-treated rat (C). (E, F) High-power views of Fluoro-Jade C labeling in vehicle-treated rats at 4 weeks after impact injury showing prominent labeling in principal cells (E) and in the hilus (F); nuclei are labeled with DAPI (blue). Images are representative of 3 rats/group for naive and sham and 5 rats/group for vehicle and glibenclamide. (G) Quantitative evaluation of Fluoro-Jade labeling in the hippocampal region of interest (ROI), in the 4 groups of rats indicated; *p < 0.05. (H) Plot of the percent time spent in the correct Morris water maze quadrant (data from Fig. 5B) versus quantified Fluoro-Jade labeling in sham-injured rats and in injured rats treated with vehicle or glibenclamide. The best-fit regression line is also shown.
involved in penumbral capillaries responsible for progressive secondary hemorrhage (22). To our knowledge, this is the first report to implicate Sp1 in TBI. Activation of Sp1 was evident in the parapenumbra as early as 15 minutes after the injury, a time that rivaled observations with “immediate early genes” such as c-fos, c-jun, and others after more severe TBI (49, 50). Such a rapid response was not expected for this transcription factor, which is typically considered to serve merely a “housekeeping” role. Nuclear factor κB is well known to be activated by mechanical stimuli, especially in microvessels (37, 38), but to our knowledge, this is the first report to suggest that Sp1 may also be rapidly activated by a mechanical stimulus.

The importance of Sp1 lies in its critical role in transcriptional regulation of caspase-3 (41) and SUR1 (23, 39). Caspase-3 is an end-executioner of apoptosis and has long been linked with TBI (42). More recent work has identified the SUR1-regulated NC\textsubscript{C\textsc{a}-ATP} channel as serving the crucial role of end-executioner in oncotic (necrotic) cell death (23). Transcriptional regulation of these 2 molecular entities is undoubtedly complicated, but to have both of these mechanisms of cell death under partial control of a single transcription factor argues strongly for the importance of Sp1 activation after impact injury to the brain.

In the penumbra, a dominant response to injury involves upregulation of SUR1 in the microvascular endothelium, which is associated with progressive secondary hemorrhage (22). In the parapenumbra, where Sp1 showed early activation, an important response to injury included upregulation of SUR1 in neurons of the hippocampus. Upregulation of SUR1 in the hippocampus occurred within 6 hours of injury, somewhat later than the 3 hours that we previously reported for a more severe contusive force (22). When expressed in neurons alone, SUR1 leads to cell demise, as indicated by caspase-3 activation at 2 weeks and FJC staining at 4 weeks, both of which were significantly ameliorated by inhibiting SUR1 with glibenclamide. Upregulation of SUR1 was previously associated with oncotic cell swelling, oncotic (necrotic) cell death (23, 51) and caspase-3 activation (25, 51), but in those cases, the inciting insult was ischemia or subarachnoid hemorrhage not a nonhemorrhagic impact trauma.

Sulfonylurea receptor 1 forms the regulatory subunit of 2 very different ion channels: the potassium-selective, hyperpolarizing, SUR1-regulated K\textsubscript{ATP} channel and the sodium-conducting, depolarizing, SUR1-regulated NC\textsubscript{C\textsc{a}-ATP} channel. The difference between the 2 resides in their pore-forming subunits, known to be Kir6.2 for the hippocampal K\textsubscript{ATP} channel (52), and hypothesized to be TRPM4 for the NC\textsubscript{C\textsc{a}-ATP} channel (53). In the context of ATP depletion, opening of K\textsubscript{ATP} channels has been associated with protection of neurons, albeit to a limited extent and for a limited duration (54). By contrast, opening of NC\textsubscript{C\textsc{a}-ATP} channels has been associated with oncotic (necrotic) cell death (23) and activation of caspase-3 (25). In this study, we did not identify which pore-forming subunit accompanied SUR1 upregulation in the hippocampal parapenumbra after impact injury. However, we showed that the pore-forming subunits of K\textsubscript{ATP} channels are not upregulated. Moreover, the significant beneficial effects of glibenclamide in reducing activated caspase-3 at 2 weeks and FJC labeling at 4 weeks, along with the preservation of rapid spatial learning at 4 weeks, argue in favor of involvement of the SUR1-regulated NC\textsubscript{C\textsc{a}-ATP} channel.

**Rapid Place Learning Performance**

Damage to the hippocampus impairs performance in spatial memory tasks (55). All 3 groups of rats (sham, vehicle, and glibenclamide) were able to learn the location of the hidden platform from distant visual cues by the third day of learning on postinjury at day 16. They continued to undergo 2 more days of learning before being tested for memory retention during a probe trial on postinjury day 19 because a difference in probe trial performance has been shown to be a more accurate measure of cognitive function than results obtained in acquisition learning trials (56). Unlike other investigators who have noted impairments in memory acquisition during 5 days of place learning (16, 57–62) or in memory retention after incremental learning (16) in rats with focal cortical contusion, we did not observe any significant group difference in MWM performance during or after incremental place learning. This preserved ability for incremental place learning is likely due to our utilization of a milder injury and, we believe, reflects a clinically relevant feature of our model. Humans with hippocampal lesions may also exhibit accurate place memory when incremental learning is possible (63). Given enough trials over several days, stable place information can be acquired and consolidated by the neocortex alone, even with total hippocampal lesions (12–14).

While incremental learning was possible for all rats, only sham-operated and glibenclamide-treated rats exhibited memory retention for a new hidden platform location after a single learning trial at 4 weeks after injury. The ability for one-trial rapid learning is a hippocampus-specific cognitive function requiring place information to traverse the full trisynaptic pathway (entorhinal cortex → DG → CA3 → CA1 → entorhinal cortex) (11). In addition, the feed-forward pathway from the entorhinal cortex to the DG and on to CA3 is needed for pattern separation (10). The increased FJC+ neurodegeneration in the DG and caspase-3 activation in the DG and CA3 after impact injury in vehicle-administered rats may explain their inability for rapid place learning and pattern separation, as demonstrated by their continuing to search for the hidden platform in the old position acquired during incremental learning. Our results closely follow those obtained by Bast et al (32), in which only shams and rats with an intact intermediate hippocampus showed significant preference for the correct quadrant after rapid learning. Our results are also consistent with reports of rats with hippocampal damage being unable to switch search strategies efficiently after a shift in platform position (36, 43).

We found significantly greater FJC-stained neuronal processes and cell bodies in the hilus of the DG in vehicle-administered rats compared with sham-operated or glibenclamide-treated rats. There was also a strong inverse correlation between the number of FJC+ degenerating neurons in the hilus of the DG at 4 weeks after impact injury and performance in the MWM after rapid learning. This was similar to...
findings of a significant correlation between hilar cell loss and memory impairment in models with focal cortical contusion (58) and fluid percussion injury (45). Injury to the hilus is of importance because the hilus receives information from the entorhinal cortex via the perforant pathway, and it contains interneurons responsible for inhibition of DG granule cell neurons. Hyperexcitability of the granule cell neurons due to hilar cell loss may disrupt the flow of place information in the trisynaptic pathway necessary for rapid learning and may even cause certain forms of trauma-induced temporal lobe epilepsy (46, 64). Similar findings of neurodegeneration in the hilus have been reported 2 weeks after focal cortical contusion (58) and in fluid percussion models at 2 days (45), 1 week (46), and 1 year (65) after injury.

In conclusions, the present study demonstrates for the first time that administration of low-dose nonhypoglycemic glibenclamide significantly improves long-term histologic outcome and preserves cognitive performance in a clinically relevant rodent model of TBI. Whereas previous work indicated that glibenclamide may have an important therapeutic benefit in severe TBI with hemorrhagic contusion (22), the present study suggests that glibenclamide may also be useful in mild-to-moderate TBI associated with cognitive dysfunction.

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