Neurobiology

Bcl-w Protects Hippocampus during Experimental Status Epilepticus

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Experimentally evoked seizures can activate the intrinsic mitochondrial cell death pathway, components of which are modulated in the hippocampus of patients with temporal lobe epilepsy. Bcl-2 family proteins are critical regulators of mitochondrial dysfunction, but their significance in this setting remains primarily untested. Presently, we investigated the mitochondrial pathway and role of anti-apoptotic Bcl-2 proteins using a mouse model of seizure-induced neuronal death. Status epilepticus was evoked in mice by intra-amygdala kainic acid, causing cytochrome *c* release, processing of caspases 9 and 7, and death of ipsilateral hippocampal pyramidal neurons. Seizures caused a rapid decline in hippocampal Bcl-w levels not seen for either Bcl-2 or Bcl-xl. To test whether endogenous Bcl-w was functionally significant for neuronal survival, we investigated hippocampal injury after seizures in Bcl-w-deficient mice. Seizures induced significantly more hippocampal CA3 neuronal loss and DNA fragmentation in Bcl-w-deficient mice compared with wild-type mice. Quantitative electroencephalography analysis also revealed that Bcl-w-deficient mice display a neurophysiological phenotype whereby there was earlier polyspike seizure onset. Finally, we detected higher levels of Bcl-w in hippocampus from temporal lobe epilepsy patients compared with autopsy controls. These data identify Bcl-w as an endogenous neuroprotectant that may

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Seizures, particularly when prolonged, are capable of causing neuronal loss within vulnerable brain structures such as the hippocampus. Delineating the mechanisms of such cell death may offer approaches to neuroprotection and perhaps anti-epileptogenesis.¹ The cell and molecular mechanisms of seizure-induced neuronal death originate in part from prolonged glutamate receptor activation resulting in elevations of intracellular sodium and calcium, culminating in activation of proteases, cell and organelle swelling, and necrotic cell death.^{2,3} However, cell and molecular hallmarks of apoptosis signaling have been reported in some experimental models of seizure-induced neuronal death and in temporal lobe material from patients with intractable epilepsy.^{4,5}

Apoptosis is orchestrated via either an intrinsic pathway that originates from within the cell or via extrinsic pathways mediated by activation of surface-expressed death receptors.⁶ Mitochondria are a critical point of apoptosis control on which a variety of cell death-promoting signals converge including raised intracellular calcium, free radicals, and Bcl-2 family proteins.⁴ Individual members of the Bcl-2 family can be classified as pro- or anti-apoptotic.⁶ All members of the family contain at least one of four Bcl-2 homology (BH) domains with two distinct proapoptotic subfamilies; the Bax/Bak subfamily and the BH3-only proteins. Killing by BH3-only proteins requires Bax or Bak,⁷ and mitochondrial permeabilization via Bax/Bak culminates in the release of cytochrome *c*. In the cytosol, cytochrome *c* promotes apoptosome forma-

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tion and activation of executioner caspases.⁸ On activation, caspases cleave critical structural and functional proteins,⁹ culminating in cell death.

In addition to regulating the intrinsic apoptotic pathway, several Bcl-2 proteins possess endogenous roles in intracellular processes, such as calcium signaling. In particular, Bcl-xl and Bak may directly influence neuronal excitability.^{10,11} Modulation of Bcl-2 proteins accordingly may influence neuronal function beyond life and death decisions.

Prolonged seizures in rats trigger cytochrome c release,¹² apoptosome formation,¹³ and executioner caspase-3 activation.^{12,14} This pathway may be instigated by BH3-only proteins, of which Bim plays a role *in vivo* and *in vitro* via a mechanism involving neutralization of Bcl-w.¹⁵ Bim and its transcriptional machinery are suppressed in hippocampi from patients with intractable epilepsy and after experimental brief protection-conferring seizures.¹⁵ Pharmacological tools for manipulating these pathways remain inadequate, however, because of cross-interference with necrosis-related signaling.¹⁶ We recently developed models in mice with which to exploit knockouts for the genes of interest.^{17,18} Presently, we examined the role of Bcl-2 family proteins after seizures in mice and report that anti-apoptotic Bcl-w has dual functions, abrogating seizure-induced neuronal death and polyspike seizure onset time, and find that Bcl-w is up-regulated in patients with intractable temporal lobe epilepsy.

Materials and Methods

Experimental Seizure Model

All animal procedures were approved by the Legacy Institutional Animal Care and Use Committee, the Research Ethics Committee of the Royal College of Surgeons in Ireland, and the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Studies were performed according to previously described methods.^{17,18} C57BL/6 mice were obtained from commercial sources (Charles River, Wilmington, MA; or Harlan, Bicester, UK). BALB/c wild-type and littermate Bcl-w-deficient mice were provided by Dr. G. Macgregor (Emory University, Atlanta, GA) and bred in-house. Adult male mice (20 to 25 g) underwent seizures induced by unilateral stereotaxic microinjection of kainic acid (KA) into the basolateral amygdala nucleus.¹⁹ Briefly, mice were anesthetized (isoflurane), maintained normothermic via a heat pad (Harvard Instruments, Holliston, MA), and a femoral vein was catheterized, and then animals were placed in a mouse-adapted stereotaxic frame (Kopf Instruments, Tujunga, CA). Mice were affixed with three recording electrodes (Plastics One, Inc., Roanoke, VA) and a 26-gauge steel guide cannula over the dura using dental cement (Plastics One Inc.). Anesthesia was discontinued, electroencephalography (EEG) recordings were commenced, and then a 31-gauge internal cannula (Plastics One Inc.) was inserted into the lumen of the guide to inject KA [0.3 μ g in

0.2 μ l of vehicle; phosphate-buffered saline (PBS), pH adjusted to 7.4] into the amygdala. Nonseizure control animals underwent the same surgical procedure but received intra-amygdala vehicle injection. The EEG was monitored until intravenous lorazepam (6 mg/kg) administration at 40 minutes and then recorded for up to 30 minutes thereafter. Mice were euthanized 0.5, 1, 4, 8, 24, or 72 hours after anticonvulsant, and brains were microdissected on ice for immunoblotting or flash-frozen whole for immunohistochemistry as previously described.^{15,18} Brains from additional naïve (noninstrumented) wild-type and Bcl-w-deficient mice were used for the examination of hippocampal and amygdala neuroanatomy and gene expression.

Induction of Brief Seizures in Vivo

Additional C57BL/6 mice were subject to brief seizures delivered via a Woodbury & Davenport electroshock apparatus as previously described.¹⁵ Maximal electroshock seizures (MESs) were delivered via orbital (corneal) electrodes, adapted for use in mice, using stimuli of 25 V, 500 mA, and 0.2-second duration. In mice, this paradigm induces a tonic-clonic convulsion that lasts ~15 to 30 seconds. Mice were subject to one, three, or nine MESs throughout a period of 1 to 3 days and euthanized 24 hours after the last MES. Control mice were handled and placed in contact with the electrodes without current.

Bcl-w-Deficient Mice Genotyping

Bcl-w-deficient mice were originally generated using a retroviral gene-trap system in which lacZ (β -galactosidase) replaced *bcl-w*.²⁰ Mice have a BALB/c background and had been backcrossed for more than eight generations. Mice of either gender were used, and the endogenous or disrupted gene was detected by polymerase chain reaction analysis of tail DNA extracts using the primer sequences as described previously.²⁰

Human Brain Samples

This study was approved by the Legacy Health System Institutional Review Board, and informed consent was obtained from all patients. Data for controls and epilepsy patients have previously been published.¹⁵ In brief, epilepsy patients ($n = 10$) were between 15 to 57 years of age, included seven females and three males, and were referred for surgical resection of the left or right temporal lobe because each had previously been determined to have medically intractable epilepsy with a history of recurring seizures. The resected hippocampus was flash-frozen in liquid nitrogen and stored at -70°C until use. Coronal slabs of ~1-mm thickness were prepared for biochemical analysis. Human control tissue ($n = 6$) was obtained from the Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD. These people were between 13 to 53 years of age, included five males and one female, and did not have neurological disease. These specimens were also fresh-frozen, *en bloc* hippocampi.

Western Blotting

Western blotting was performed as previously described.¹⁵ Protein concentration was determined using Bradford reagent spectrophotometrically at A⁵⁹⁵ nm, and 50- μ g samples were boiled in gel-loading buffer and separated on 12 to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) and then incubated with antibodies against the following: α -tubulin, actin, Bax, and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); Bcl-w, Bim, and manganese superoxide dismutase (MnSOD) (Stressgen, Victoria, BC, Canada); Bcl-xl and cytochrome c (BD Transduction Laboratories, Lexington, KY); cleaved caspase-3, caspase-7, and caspase-9 (Cell Signaling Technology, Beverly, MA); and GluR5-7 (Chemicon, Temecula, CA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) followed by chemiluminescence detection (NEN Life Science Products, Boston, MA), and then exposed to Kodak Biomax film (Kodak, Rochester, NY).

Subcellular Fractionation

Mouse hippocampal tissue was fractionated to obtain the mitochondria according to previous methods.²¹ In brief, samples (pools of four hippocampi) were homogenized in a mannitol/sucrose buffer containing a protease inhibitor cocktail and then centrifuged twice at 1200 $\times g$ for 10 minutes. The postnuclear supernatant was then centrifuged twice at 10,000 $\times g$ for 15 minutes, and the resulting mitochondrial pellet was resuspended in a sucrose buffer and purified through a Percoll bilayer by centrifugation at 41,000 $\times g$ for 30 minutes.

Immunoprecipitation

For immunoprecipitation studies,¹⁵ hippocampal protein samples (0.5 mg) were first incubated overnight at 4°C with 5 μ g of anti-Bcl-w and then incubated with protein A/G agarose beads (Santa Cruz Biotechnology) for 2 hours at 4°C. The protein-bead complex was collected by centrifugation and boiled, and samples were subject to Western blotting with anti-Bim. Nonspecific binding was assessed by omission of the primary (immunoprecipitation) antibody, and whole cell lysates were run concurrently to confirm molecular weights.

Analysis of Amygdala KA Receptor Expression

To determine whether KA receptor (GluR5-7) levels were equivalent between wild-type and Bcl-w-deficient mice, bilateral tissue cores (~ 1 mm wide by 2 mm deep) of the amygdala nuclei (including the basolateral amygdala) were extracted from a selection of naïve wild-type or knockout mice. Tissue samples were then processed for Western blotting as described above.

Histopathology, Immunohistochemistry, and DNA Fragmentation Analysis

Coronal mouse brain sections (12 μ m) were air-dried and postfixed in 100% methanol (Bcl-w immunostaining) or 10% formalin (histopathology and NeuN immunostaining) for 30 minutes, followed by washes in PBS. For histopathology, sections were stained with Cresyl violet. For immunohistochemistry, sections were first blocked with 5% goat serum and then incubated overnight at 4°C with antibodies against Bcl-w (Stressgen) or NeuN (Chemicon). Sections were washed in PBS and then incubated for 2 hours at room temperature in a 1:1000 dilution of goat anti-mouse or 1:500 dilution of goat anti-rabbit Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR). After additional washes sections were counterstained with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA), to assess nuclear morphology. Counts of NeuN-stained hippocampal CA1 and CA3 and amygdala neurons from naïve wild-type and Bcl-w-deficient mice were the mean of five $\times 60$ lens fields from two adjacent sections at the level of bregma -1.7 mm.¹⁹ Analysis of cells exhibiting DNA fragmentation was performed using a fluorescein-based terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique (Promega, Madison, WI) as previously described.¹⁸ Images were visualized using a Hamamatsu Orca 285 camera (Hamamatsu Corporation, Bridgewater, NJ) attached to a Nikon 2000s epifluorescence microscope (Melville, NY) under Ex/Em wavelengths of 330 to 380/420 nm (blue), 472/520 nm (green), and 540 to 580/600 to 660 nm (red).

Production of Bcl-wTAT and Electrophysiology

TAT-conjugated proteins were produced according to previously described techniques.²² cDNAs encoding the human *bcl-w* (99% identical to mouse sequence) or green fluorescent protein (GFP; control) open reading frame were inserted into the *NcoI* and *EcoRI* sites of the pTAT-HA vector (a gift from S. Dowdy, Washington University School of Medicine, St. Louis, MO), amplified, purified, and subjected to sequence analysis as described by Vocero-Akbani and colleagues.²³ The pTAT-HA plasmids were transformed into high-expressing BL21 (DE3)LysS (Novagen, Madison, WI) bacteria and amplified, and expression of the recombinant protein analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recombinant proteins were denatured with 8 mol/L urea buffer, purified on a Ni-NTA column (Qiagen, Chatsworth, CA), and eluted. Purified proteins were applied to G-25 Sephadex desalting columns equilibrated in phosphate buffer to remove urea. Eluted fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fractions containing the TAT fusion proteins frozen in 10% glycerol and stored at -80°C until use. TAT-proteins were applied to mouse cortical neuron cultures (10 μ mol/L) 4 hours before recordings. Patch-clamp recordings of γ -amino butyric acid (GABA)-mediated currents were performed accord-

Figure 1. Seizure-induced hippocampal injury in the mouse: cytochrome *c* release and processing of caspase-9 and -7. Photomicrographs show representative Nissl-stained contralateral (**A**) and ipsilateral (**B**) mouse hippocampi 24 hours after seizures. Note the loss of CA3 neurons (**arrows**) in **B**, and some CA1 injury. **C–H**: TUNEL staining of the ipsilateral hippocampus. **C** and **D**: CA3 (**C**) and CA1 (**D**) subfields at 24 hours. **E–H**: Images of individual TUNEL-stained dying CA3 and CA1 neurons within the hippocampus. **I**: Western blot detection of cytochrome *c* in subcellular fractions ($n = 4$ hippocampi per lane) 1 hour and 4 hours after seizure termination in mouse brain. Strong cytochrome *c* expression was present in mitochondrial fractions (mito) in both contralateral and ipsilateral fractions. Seizures triggered cytochrome *c* release into the cytoplasmic (cyto) fraction of the ipsilateral but not contralateral hippocampus at 4 hours. **J**: Representative Western blot ($n = 2$ per lane) showing expression and cleavage of caspase-9 and caspase-7 after seizures in the mouse hippocampus. Note emergence of cleaved (~ 39 kd) caspase-9 at ~ 4 hours and emergence of cleaved caspase-7 at ~ 4 to 8 hours. Tubulin is shown as a loading control. Time is shown in hours after termination of seizures. Western blot data are representative of three independent experiments. Molecular masses in kd are shown to the left of the blots. Scale bar = $30\ \mu\text{m}$.

ing to previously described methods.^{24,25} Patch electrodes had a final diameter of 1 to 2 μm and resistance between 3 to 5 $\text{mol/L}\Omega$. Membrane potentials were recorded in current-clamp mode using Axopatch 1-D amplifiers (Axon Instruments, Foster City, CA). Data were filtered at 2 kHz and digitized on-line using Digidata 1320A DAC units (Axon Instruments) with acquisition by pClamp software (version 8.0; Axon Instruments). All electrophysiological experiments were done at room temperature (22 to 24°C). A multibarrel perfusion system (SF-77B; Warner Instrument Co., Hamden, CT) was used to achieve a rapid exchange of GABA (5 $\mu\text{mol/L}$).

Data Analysis

Data are presented as mean $\pm$ SEM. Protein bands from Western blots were semiquantified using gel-scanning integrated optical density software (AlphaEaseFC, version 4.0; Bioquant, Nashville, TN) and were corrected to the intensity reading for the control sample in each independent experiment. Data were analyzed using analysis of variance with post hoc Fisher's PLSD test or for two group comparisons Student's *t*-test (StatView software; SAS Institute, Inc., Cary, NC). For the human data analysis, a Huber-White robust variance estimates was undertaken. Additional specialized statistical testing

was undertaken for EEG analysis where specified in the results (see Supplemental Table 1 at <http://ajp.amjpathol.org>). Significance was accepted at $P < 0.05$.

Results

Hippocampal Damage after Seizures in Mice Is Associated with Activation of the Intrinsic Mitochondrial Pathway

Status epilepticus (40 minutes) evoked in C57BL/6 mice by intra-amygdala KA resulted in unilateral hippocampal neuronal death (Figure 1B). Twenty-four hours after seizure termination, extensive DNA fragmentation was notable within ipsilateral CA3 neurons (Figure 1C) with smaller numbers of CA1 neurons (Figure 1D) also degenerated. Higher magnification images of TUNEL-positive cells are shown in Figure 1, E–H. Damage was not found contralaterally (Figure 1A), and no TUNEL-positive cells were detected in the ipsilateral hippocampus of mice that received intracerebral vehicle injection (data not shown).

We next examined cytochrome *c* release as a key marker of intrinsic pathway induction and mitochondrial dysfunction. Cytochrome *c* was exclusively present within the mitochondrial fraction at both 1 hour and 4

hours within contralateral hippocampus (Figure 1I). In contrast, cytochrome c release was detected within the ipsilateral hippocampus at 4 hours (but not 1 hour) as evidenced by its presence within the cytoplasmic fraction (Figure 1I).

Caspase-9, which is activated via apoptosome formation downstream of cytochrome c release, was constitutively expressed in mouse hippocampus (Figure 1J). Cleaved caspase-9 could be detected ~4 hours after seizure termination with fragment levels peaking at 72 hours (Figure 1J). Quantification of the cleaved caspase-9 levels revealed a statistically significant difference between the cleavage products evident at 72 hours compared with controls ($P < 0.01$) and to 0.5 and 4 hours ($P < 0.05$). Activated caspase-9 is capable of cleaving executioner caspases such as caspase-3 and -7. Although cleaved caspase-3 is detected in this model,¹⁷ caspase-7 has not been investigated. Western blotting detected procaspase-7 at low levels in control mouse hippocampus (Figure 1J). Cleaved caspase-7 could be detected ~4 hours after seizure termination through 72 hours (Figure 1J). Again quantification of the levels of cleaved caspase-7 revealed a statistically significant difference between 24 hours and control ($P < 0.05$) and between 72 hours and control, 0.5, 4, and 8 hours ($P < 0.01$).

Selective Regulation of Bcl-w during Seizures

Because Bcl-2 family proteins regulate events upstream of mitochondrial dysfunction in the intrinsic pathway, we next examined expression of key members in our model. Bax, Bcl-xl, and Bcl-2 were all constitutively expressed in mouse hippocampus, but levels were primarily unchanged during seizure-induced neuronal death (Figure 2A). Remarkably, seizures induced a rapid decline in anti-apoptotic Bcl-w levels, which began at 0.5 and peaked 4 hours ($P < 0.05$) after seizures, followed subsequently by recovery of expression at later time points up to 24 hours (Figure 2, A and B). A tendency to lower Bcl-w levels at 72 hours was noted but was variable and did not reach statistical significance.

To evaluate more completely the responsiveness of Bcl-w to seizures, we subjected mice to one or more brief, nondamaging electroshock seizures, which we and others have shown to be associated with generating a tolerant and/or protected state.^{15,26} Western blotting revealed a dose-related increase in hippocampal Bcl-w levels but not Bcl-xl (Figure 2C) or Bcl-2 (data not shown).

Figure 2. Expression of Bcl-2 family members after seizures in the mouse. **A:** Representative Western blots ($n = 2$ hippocampi per lane) showing Bax, Bcl-2, Bcl-xl, and Bcl-w expression after seizures in the mouse hippocampus. Note the reduction in Bcl-w levels at 0.5 and 4 hours after seizures. Tubulin is shown below as a loading control. Time is shown in hours after termination of seizures 40 minutes after intra-amygdala KA injection. Data are representative of three independent experiments. **B:** Graph showing quantification of Bcl-w levels after seizures. * $P < 0.05$ compared with control. **C:** Western blot ($n = 3$ hippocampi per lane) showing increasing Bcl-w expression in proportion to the number of brief nondamaging seizures in the mouse hippocampus. Mice were subjected to one, three, or nine MESs throughout a period of 1 to 3 days and euthanized 24 hours after the last MES. Western blots of Bcl-xl and tubulin are shown as controls. **D:** Representative Western blots ($n = 4$ hippocampi per lane) of Bcl-w immunoprecipitation and Western detection of Bim from control hippocampus and hippocampi 1 hour and 4 hours after seizure termination. Control immunoprecipitation (no antibody incubation) is shown on left of panel. IgG Western blot is shown as loading control. Molecular masses in kd are shown to the left of the blots.

Figure 3. Localization of Bcl-w in control and seizure-damaged mouse hippocampus. **A:** Immunohistochemical co-localization of NeuN (green) with Bcl-w (red) in CA3 neurons of control mouse hippocampus. **B:** Overlay showing immunohistochemical co-localization of Bcl-w with NeuN in control CA2 cells in which three neurons to the left coexpress Bcl-w; the arrowhead indicates a rare Bcl-w-positive cell not expressing NeuN. **C:** Co-localization of Bcl-w with TUNEL-positive CA3 cells 24 hours after seizures. Note punctuate, mitochondrial-like localization of Bcl-w in degenerated neurons. **D:** Western blot ($n = 4$ hippocampi per lane) showing increasing Bcl-w levels in the mitochondrial fraction of seizure-damaged mouse hippocampal tissue. Manganese superoxide dismutase (MnSOD) is shown as a loading control. Time is shown in hours after lorazepam administration. Data are representative of two independent experiments. Scale bars: 8 μ m (A); 4 μ m (B); 6 μ m (C).

Seizure-Induced Redistribution of Bcl-w

Although reported in rat,²⁷ the cellular distribution of Bcl-w protein in mouse hippocampus, and its response to seizure injury, has not been described. Accordingly, we performed fluorescence microscopy experiments using antibodies against Bcl-w on control and seizure-damaged mouse hippocampus (Figure 3, A–C). Bcl-w was constitutively present within adult mouse hippocampus and almost exclusively neuronal (Figure 3A). Extensive Bcl-w immunostaining was apparent within NeuN-positive pyramidal cell bodies of CA3 (Figure 3A) and CA2 (Figure 3B). Additional staining was present within CA1 and granule neurons of the dentate gyrus, with occasional staining of microvessel-like structures and glia (data not shown). The distribution and appearance of Bcl-w was noticeably altered in seizure-damaged ipsilateral hippocampus at 24 hours (Figure 3C). Here, Bcl-w appeared in a punctuate distribution consistent with mitochondrial localization within seizure-damaged TUNEL-positive CA3 (Figure 3C) and CA1 (not shown) neurons. To verify the specificity of the Bcl-w antibody, Bcl-w-

deficient hippocampal tissue was incubated with this antibody, and no Bcl-w immunoreactivity was detected (data not shown).

To support these microscopy observations, we examined Bcl-w levels in the mitochondrial compartment after subcellular fractionation. The mitochondrial fraction of control hippocampus contained a small amount of Bcl-w. In contrast, Bcl-w levels were elevated in fractions from seizure-damaged hippocampus at 0.5, 4, and 8 hours (Figure 3D).

Bcl-w Binds Bim during Seizures in Mouse Hippocampus

Bcl-w is targeted by proapoptotic BH3-only protein Bim during seizure-induced neuronal death in the rat.¹⁵ On binding Bim, Bcl-w is inactivated and integrated into mitochondrial membranes.²⁸ To investigate whether Bcl-w interacts with Bim in the mouse, Bcl-w was immunoprecipitated from control and seizure hippocampus and lysates immunoblotted for the presence of Bim. In

control brain, Bcl-w was not associated with Bim. However, 1 hour and 4 hours after seizures, we detected increased binding of Bim to Bcl-w (Figure 2D).

Exacerbated Seizure Damage in Bcl-w-Deficient Mice

To investigate the functional significance of Bcl-w during seizure-induced neuronal death, we undertook experiments using mice lacking *bcl-w*.^{20,29} These mice were raised on a BALB/c background,²⁰ a strain that displays a somewhat more damage-resistant phenotype to C57BL/6, whereby seizure-induced hippocampal lesions are confined to CA3a.¹⁸ Analysis of hippocampal and amygdala neuron counts, levels of cell death regulatory genes, and glutamate receptor expression determined Bcl-w-deficient mice displayed no differences that could adversely affect subsequent experiments (Supplemental Figure 1, see <http://ajp.amjpathol.org>).

Next, wild-type and Bcl-w-deficient mice were subjected to status epilepticus. Twenty-four hours after seizure termination, histopathology revealed wild-type mice exhibited ~30% reduction in hippocampal CA3 neuron number compared with vehicle-injected nonseizure controls (Figure 4, A and B). In contrast, seizures in Bcl-w-deficient mice caused ~65% CA3 neuron loss compared with nonseizure controls and significantly more neuronal loss than in seizure-affected wild-type littermates (Figure 4, A and B). Staining hippocampal sections for DNA fragmentation revealed seizures triggered significant TUNEL staining in wild-type mice compared with nonseizure controls (Figure 4, C and D). Bcl-w-deficient seizure mice exhibited significantly higher TUNEL counts than wild-type seizure mice (Figure 4, C and D).

Many CNS diseases display sexual dimorphism in treatment responses or neuropathological variability. Male gender is associated with enhanced vulnerability to excitotoxicity³⁰ and hemispheric volume loss is greater in male temporal lobe epilepsy (TLE) patients.³¹ Because we used Bcl-w-deficient mice of either sex in our study we examined our histology data for differences arising from gender. Subgroup analysis of histopathology data revealed no statistical differences in either CA3 neuron loss or TUNEL counts between seizure-damaged male ($n = 4$) and female ($n = 5$) Bcl-w-deficient mice (data not shown).

Neurophysiological Phenotype in Bcl-w-Deficient Mice

We next undertook quantitative analysis of cortically recorded seizure EEG between wild-type and Bcl-w-deficient mice. First signs of seizure activity (low-amplitude, high-frequency EEG; Figure 5A) emerged 201 ± 41 seconds ($n = 9$) after KA injection in Bcl-w-deficient mice, which was not significantly different ($P = 0.6$) to onset in wild-type mice (242 ± 57 seconds, $n = 10$). Polyspike paroxysmal seizure bursts (Figure 5A) had the same characteristics of amplitude, frequency, and mean dura-

Figure 4. Exacerbated hippocampal injury in Bcl-w-deficient mice after seizures. **A:** Graph showing hippocampal CA3 neuron counts in control and seizure wild-type (+/+) mice and seizure-damaged Bcl-w-deficient (-/-) mice ($n = 7$ to 9 per group). Note significantly lower CA3 numbers in Bcl-w-deficient seizure mice compared with seizure wild-type and nonseizure wild-type controls. **B:** Representative low-power field views of Cresyl violet-stained hippocampal sections from wild-type and Bcl-w-deficient mice 24 hours after seizures. **Arrows** indicate sites of cell loss. **C:** Graph showing hippocampal CA3 TUNEL counts in control and seizure wild-type mice and seizure-damaged Bcl-w-deficient mice ($n = 7$ to 9 per group). Note significantly higher CA3 TUNEL counts in Bcl-w-deficient mice compared with wild-type after seizures. **D:** Representative low-power field views of TUNEL-stained hippocampal sections from wild-type and Bcl-w-deficient mice 24 hours after seizures. Scale bar = 70 μ m.

tion between wild-type and Bcl-w-deficient mice (Figure 5, B and C). However, the delay from KA injection to first polyspike paroxysmal seizure burst was significantly shorter in Bcl-w-deficient mice compared with wild types (Figure 5D). This had the secondary effect that the total duration of all polyspike paroxysmal seizure bursts within the 40-minute experimental period between KA injection and anti-convulsant administration was significantly longer in Bcl-w-deficient mice compared with wild types (Figure 5E), and the total number of polyspike paroxysmal seizure bursts was greater in Bcl-w-deficient than wild types (Figure 5F). Gender subgroup analysis of Bcl-w-deficient mice EEG data revealed no differences for any EEG parameter between male and female mice (data not shown).

The earlier onset of polyspike seizures in Bcl-w-deficient mice raises the possibility that the exacerbated hippocampal injury was a consequence of the mice experiencing additional seizure time, rather than damage

Figure 5. Altered polyspike seizure onset in Bcl-w-deficient mice and effects of Bcl-w on GABA currents. **A:** Representative EEG traces of baseline, initial change to ictal EEG, and a polyspike paroxysmal seizure burst from a wild-type (+/+) mouse. **B:** Representative traces showing polyspike paroxysmal seizure bursts from a wild-type and a Bcl-w-deficient (-/-) mouse. Note similarity of burst pattern and duration. **C-F:** Quantitative analysis of polyspike seizure EEG in wild-type ($n = 10$) and Bcl-w-deficient ($n = 9$) mice show mean duration of individual polyspike bursts are similar (**C**), but time between KA injection and onset of first polyspike paroxysmal seizure burst (**D**), total polyspike paroxysmal seizure (PS) duration within experiment (**E**), and total seizure burst number within experiment (**F**) all differ between wild-type and Bcl-w-deficient mice. * $P < 0.05$; ** $P < 0.01$ compared with control. **G:** Representative patch clamp trace showing fast inhibitory current induced by application of GABA. **H:** Representative patch clamp trace showing enhanced GABA inhibitory current in a neuron treated for 4 hours with Bcl-wTAT. **I:** Graph quantifying GABA-induced currents in control ($n = 10$) neurons or neurons treated with Bcl-wTAT ($n = 12$). **J:** Correcting Bcl-wTAT effects for cell capacitance did not alter its GABA-promoting effects. Scale bar = 1.5 seconds.

vulnerability attributable to loss of a neuroprotective gene. To assess this possibility, we ran statistical analyses on group seizure duration and damage relationships between wild-type and Bcl-w-deficient mice (Supplemental Table 1, see <http://ajp.amjpathol.org>). These analyses showed that the additional seizure time in Bcl-w-deficient mice was not sufficient to account for the exacerbated cell death.

Bcl-w Overexpression Augments GABA Currents

We next explored the cause of the neurophysiological phenotype displayed by mice lacking *bcl-w*. Among several potential mechanisms, we investigated whether Bcl-w could influence GABA signaling, because GABA is the main inhibitory neurotransmitter in the CNS, and dis-

ruption of GABA signaling triggers seizures. To test this, we transiently overexpressed Bcl-w in mouse primary neurons by conjugation to the protein transduction domain of the HIV TAT protein.²³ This technique has been used previously to deliver Bcl-2 family proteins and larger proteins into neurons *in vitro* and *in vivo* and modulate seizure-induced neuronal death.^{14,22,32,33} Whole cell patch-clamp recordings confirmed GABA application activated a fast inward current in mouse neurons (Figure 5G). Surprisingly, GABA currents were potentiated in cultures exposed to Bcl-wTAT (Figure 5, H and I). Normalizing current amplitudes for cell capacitance to reduce effects of differences in cell volume did not alter the observed Bcl-w effect (Figure 5J). Furthermore, the Bcl-w effect was not related to nonspecific actions of the TAT protein transduction domain because treatment of cultures with GFP-TAT protein ($n = 10$) did not significantly affect GABA-mediated currents (data not shown).

Selective Alteration of Bcl-w Levels in Human TLE Brain

Last, we hypothesized that levels of Bcl-w, like those of Bim,¹⁵ may be altered in the brains of patients with TLE. To test this we analyzed Bcl-w levels in patient and autopsy control hippocampal samples from this original study. Bcl-w levels in whole cell lysates from TLE samples were 1.7-fold higher than controls ($P < 0.05$) (Figure 6, A and B). In contrast, there were no significant differences in levels of either Bcl-x or Bax between these groups (Figure 6A).

Discussion

The major findings of the present study are that i) prolonged focal limbic seizures in mice activate the molecular machinery of apoptosis in hippocampus; ii) Bcl-w is an endogenous inhibitor of seizure-induced neuronal death in mice, and its absence leads to a neurophysiological phenotype characterized by earlier polyspike seizure onset; and iii) Bcl-w levels are raised in hippocampi from patients with intractable seizures. Bcl-w or its targets might be manipulated to mitigate neuronal injury after acute prolonged seizures and influence seizure initiation and their propensity for damage in chronic intractable epilepsy.

Despite pharmacological evidence that targeting apoptosis-signaling pathways blocks seizure-induced hippocampal injury, doubts remain as to their functional significance with findings between laboratories contradictory, even within the same model.^{34,35} The mouse data here complement evidence from rat models that an intrinsic cell death pathway is activated by seizures, which features early cytochrome c release and caspase processing. We detected processing of caspase-7 in mouse hippocampus that does not occur after seizures in rats³⁶ but is present in resected hippocampus from patients with intractable seizures.³⁷ Because this mouse model also reproduces CA1 injury seen in clinical TLE material

Figure 6. Altered expression of Bcl-w in human TLE brain. **A:** Western blots ($n = 1$ per lane) showing hippocampal expression of Bcl-w, Bcl-xl, and Bax in control and TLE patient whole cell lysates. Note significantly higher levels of Bcl-w in TLE samples compared with control. **B:** Quantification of Bcl-w levels in TLE samples compared with autopsy controls. * $P < 0.05$ compared with control.

but not in rat using the same approach,^{21,38} the mouse may be a better model from which to extrapolate findings on seizure-induced apoptosis signaling to patients.

Despite reports of expressional regulation of anti-apoptotic Bcl-2 family proteins by seizures,^{39,40} their functional significance remains primarily untested. Experiments here show that in mice, as in rats using the same model,^{21,38,39} seizures have modest or no effect on hippocampal levels of Bcl-2 or Bcl-xl. Rather, seizures cause several changes to Bcl-w levels and localization, including its up-regulation after brief electroshock seizures that are known to confer tolerance against seizure damage.²⁶ We also find higher Bcl-w levels in the hippocampus of patients with intractable TLE; the first clear evidence of anti-apoptotic Bcl-2 family gene regulation in this brain region in patients. These mouse and human Bcl-w data exactly mirror in the opposite way changes to Bim after seizures in rats and in patients' hippocampi.¹⁵ Accepting the caveats of our control human material, up-regulation of Bcl-w, like the down-regulation of Bim, could influence seizure-mediated neuronal injury.

Binding of Bim to Bcl-w triggers membrane integration and inactivation of its anti-apoptotic properties.²⁸ We find in mice, as in rats,¹⁵ that Bim binds Bcl-w after seizures. This may explain the initial drop seen in Bcl-w levels in mouse whole cell lysates after damaging seizures, with

Bim driving Bcl-w's integration into mitochondrial membranes and inactivation. Indeed, our microscopy and fractionation experiments show Bcl-w protein within hippocampal neurons transitions to a mitochondrial localization, a response also observed after focal ischemia.⁴¹

By using mice in which *bcl-w* was genetically deleted, we have demonstrated endogenous levels of Bcl-w protect neurons against seizure damage. Bcl-w-deficient mice exhibited markedly exacerbated hippocampal injury after seizures. These data complement in the reverse manner evidence that Bcl-w overexpression protects against excitotoxic insults.⁴² They also imply that although Bcl-w is redundant during brain development, in contrast to Bcl-2 and Bcl-xl,^{43,44} its absence is likely not compensated by these genes during seizure-induced neuronal death. Bcl-w's higher endogenous levels in adult rodent brain²⁷ may contribute to its importance in situations in which cell death begins rapidly, such as after seizures. Bcl-w's significance may also reside in its capacity to block calcium- as well as Bax-mediated dysfunction of brain mitochondria.⁴¹ Anti-apoptotic Bcl-2 family proteins have similar affinities for proapoptotic Bcl-2 family proteins,⁴⁵ and other anti-apoptotic Bcl-2 family proteins have been shown to regulate intracellular calcium homeostasis.⁴⁶ However, interaction partners seem to differ for each anti-apoptotic Bcl-2 family protein in hippocampus,^{15,38} whereas differences in subcellular localization or defects in transcriptional responses could also explain the nonredundancy of Bcl-w.

Unexpectedly, our study reveals a second potential mode of action by which Bcl-w could modulate seizure-induced hippocampal injury, reducing seizure duration by countering initiation of the synchronous, polyspike seizure bursts during status epilepticus. The finding initially raised the question of the extent to which such an effect might contribute to extended damage in these mice. Damage is a function of duration in most seizure models,¹ but extending seizure times from 15 to 90 minutes has quite small effects on hippocampal damage in our mouse model.¹⁷ Critically, our statistical analysis of the relationship between seizure duration and damage establishes the additional polyspike activity experienced by Bcl-w-deficient mice (<4 minutes) would not be enough to have resulted in the observed damage difference from wild-type mice. Thus, Bcl-w's neuroprotective properties are a function of its anti-apoptotic actions, with perhaps a secondary indirect benefit resulting from its effects on polyspike seizure onset.

To account for the observed neurophysiological phenotype in Bcl-w-deficient mice, we explored GABAergic signaling, long been implicated in seizure initiation, and the development and termination of status epilepticus. Although exploratory, the electrophysiology experiments undertaken here demonstrate Bcl-w can potentiate inhibitory GABA currents. Absence of Bcl-w might therefore lead to impairment of some aspect of GABAergic function, although extensive further studies including analyses of endogenous Bcl-w targets are required to build on these data. Interestingly, neurophysiological phenotypes have previously been reported for Bcl-2 family proteins,^{10,11} which result from endogenous functions of the

proteins, for example in regulating neuronal mitochondrial calcium dynamics. Of additional note, GABA-B receptors interact with 14-3-3 proteins,⁴⁷ and Bcl-w co-immunoprecipitates with 14-3-3 from rodent hippocampus (D.H. and C.K.S., unpublished observation).

In conclusion, our study provides evidence that Bcl-w has potent protective properties against seizure-induced neuronal death. Two functionally distinct modes of action may contribute to neuronal survival after seizures, and the raised Bcl-w levels in patient brain may contribute to the epileptic hippocampus' endogenous capacity to reduce its damage vulnerability and perhaps proclivity to seizure propagation. Therapeutic strategies targeting this gene might be beneficial for the protection of brain during status epilepticus or in intractable epilepsy.

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