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Common Patterns of Bcl-2 Family Gene Expression in Two Traumatic Brain Injury Models

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Cell death/survival following traumatic brain injury (TBI) may be a result of alterations in the intracellular ratio of death and survival factors. Bcl-2 family genes mediate both cell survival and the initiation of cell death. Using lysate RNase protection assays, mRNA expression of the anti-cell death genes Bcl-2 and Bcl-x$_L$, and the pro-cell death gene Bax, was evaluated following experimental brain injuries in adult male Sprague-Dawley rats. Both the lateral fluid-percussion (LFP) and the lateral controlled cortical impact (LCI) models of TBI showed similar patterns of gene expression. Anti-cell death bcl-2 and bcl-x$_L$ mRNAs were attenuated early and tended to remain depressed for at least 3 days after injury in the cortex and hippocampus ipsilateral to injury. Pro-cell death bax mRNA was elevated in these areas, usually following the decrease in anti-cell death genes. These common patterns of gene expression suggest an important role for Bcl-2 genes in cell death and survival in the injured brain. Understanding the regulation of these genes may facilitate the development of new therapeutic strategies for a condition that currently has no proven pharmacologic treatments.

Keywords: Apoptosis; mRNA measurement; Cerebral cortex; Hippocampus; Traumatic brain injury; Animal models; Gene expression patterns

INTRODUCTION

Traumatic brain injury (TBI) in humans is a devastating condition that is a major cause of death and disability in the young, and increases the vulnerability of older individuals to neurodegenerative disease (Pentland *et al.*, 1986; Irving *et al.*, 1996; Graham *et al.*, 1999; Nemetz *et al.*, 1999; Akiyama *et al.*, 2000; Beschorner *et al.*, 2000; Lye and Shores, 2000; Jellinger *et al.*, 2001; Kay *et al.*, 2003; Liu *et al.*, 2003). TBI results in progressive neuronal loss in the cortex, hippocampus, cerebellum and thalamus (Adams *et al.*, 1985; Kotapka *et al.*, 1992; Ross *et al.*, 1993; Shiozaki *et al.*, 2001), a pattern that has been duplicated in various rodent models of TBI (Sutton, *et al.*, 1993; Dietrich *et al.*, 1994; Hicks *et al.*, 1996; Colicos *et al.*, 1997). Changes in cellular morphology indicative of neurodegeneration are evident almost immediately following the impact in the injured cortex and hippocampus (Dietrich *et al.*, 1994; Colicos *et al.*, 1996a; Hicks *et al.*, 1996). Electron microscopic analysis revealed that some of these degenerating neurons appear necrotic, with swollen mitochondria, vacuolated cytoplasm and pyknotic nuclei (Dietrich *et al.*, 1994), while others exhibit DNA fragmentation within shrunken cell bodies containing condensed chromatin and cellular structures characteristic of apoptosis (Rink *et al.*, 1995; Colicos and Dash, 1996b; Yakovlev *et al.*, 1997; Conti *et al.*, 1998; Fox *et al.*, 1998; Newcomb *et al.*, 1999). While the cellular mechanisms underlying regional cell death have yet to be elucidated, many neurochemical factors and proteins have been identified as potential candidates (Raghupathi *et al.*, 1995; Yakovlev and Faden, 1995; McIntosh *et al.*, 1998).

The Bcl-2 multigene superfamily include genes such as Bcl-2, Bcl-x$_L$ and Bak which mediate cell survival, and which participate in the inititiation of cell death (Bredesen, 1995). While Bcl-2 can protect cells from a variety of insults such as treatment with calcium ionophores, glutamate, free radicals and withdrawal of trophic factors (Reed, 1998), Bax has been suggested to be necessary for developmental cell death (Deckwerth *et al.*, 1996) and suppression of tumorigenesis (Yin *et al.*, 1997). Decreases in protein and/or mRNA for Bcl-2 and Bcl-x$_L$ and a concomitant increase in protein and/or mRNA levels for Bax in

injured neurons have been reported following both focal and global ischemia, nerve transection or administration of neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or kainic acid (Gillardon *et al.*, 1995; 1996a,b; Krajewski *et al.*, 1995; Chen *et al.*, 1996; Hassouna *et al.*, 1996). Conversely, an increase in Bcl-2 immunoreactivity was observed in neurons, glia and endothelial cells surrounding the infarcted cortex following focal ischemia (Chen *et al.*, 1995), while Bcl-2 and Bcl-x$_L$ levels were relatively unchanged in neurons that survived the ischemic insult (Gillardon *et al.*, 1996b; Honkaniemi *et al.*, 1996). Although these observations support the hypothesis that cell survival is dependent on the *ratio* of anti-apoptotic and pro-apoptotic Bcl-2 proteins (Korsmeyer, 1995), the specific mechanism(s) of action for either Bcl-2 or Bax following CNS injury is unclear.

The role of the Bcl-2 family proteins in TBI has been evaluated to a limited extent. In a model of combined TBI and hypoxemia, Clark and coworkers observed an upregulation of Bcl-2 in cortical neurons that survived the traumatic injury (Clark *et al.*, 1997), while increased Bax immunoreactivity was observed in apoptotic granule neurons in the dentate gyrus following controlled cortical impact injury (Kaya *et al.*, 1999). Direct evidence for a potential role of Bcl-2 in TBI neuropathology was based on the attenuation of post-traumatic cortical neurodegeneration in transgenic mice overexpressing human Bcl-2 (Raghupathi *et al.*, 1998; Nakamura *et al.*, 1999). We have recently reported that in the acute post-traumatic period, injured neurons exhibited a dramatic decrease in Bcl-2 immunoreactivity, which was followed at later times by an increase in Bax mRNA and protein (Raghupathi *et al.*, 2003). The initiation of delayed cell death requires new gene expression, so we hypothesized that certain bcl-2 homologs might be regulated at the mRNA level following TBI. Thus, we have measured changes in bcl-2 and bcl-x$_L$ (anti-apoptotic) as well as bax (pro-apoptotic) mRNA levels, in the cortex and hippocampus of rats injured in two distinct models of TBI. Similarities in gene modulation between the models may be an indication of more general genetic programs in the progression of secondary injuries following trauma.

MATERIALS AND METHODS

Animal Models
All protocols were approved by the appropriate Institutional Animal Care and Use Committee (IACUC). The relevance of the rat TBI models to clin-ical closed head trauma has been confirmed with respect to common findings in biochemical, histopathological and functional deficits (McIntosh *et al.*, 1989; Dixon *et al.*, 1991; Hamm *et al.*, 1992).

Fluid-Percussion Brain Injury
Lateral fluid percussion (LFP) is a model primarily of moderate diffuse brain injury. Adult male Sprague-Dawley rats (350-400 g, *n* = 24) were anesthetized with sodium pentobarbital (60mg/kg, i.p.), placed in a stereotactic frame and the scalp and temporal muscle were reflected. A hollow, female Luer-Lok fitting was rigidly fixed with dental cement to a 5.0 mm craniotomy centered between bregma and lambda sutures and over the left parietal cortex. The fluid-percussion injury device was connected to the animal via the Luer-Lok fitting and brain injury of moderate (2.3-2.6 atm) severity was produced in animals as previously described (McIntosh *et al.*, 1989). The device produces a pulse of 21-23 msec through the rapid injection of saline into the cranial cavity, resulting in brief deformation of brain tissue. Animals were sacrificed by decapitation at 1d (*n* = 6), 3d (*n* = 5) and 7d (*n* = 6). Sham-injured controls were surgically prepared but were not injured (*n* = 2 at each time point).

Controlled Cortical Impact Brain Injury
Lateral controlled cortical impact (LCI) is a model primarily of moderate focal brain injury. Adult male Sprague-Dawley rats (300-400 g, *n* = 31) were pre-anesthetized using 2% isoflurane, then given oxygen with 0.75% isoflurane through a fixed face mask on a stereotaxic platform. The cranium was exposed and a 6-mm craniectomy made laterally, midway between lambda and bregma, between the central suture and the left temporal ridge. The exposed dura was subjected to a 5 mm diameter piston impact, of 3.0 mm depth, 4 m/s velocity, and 100 ms duration. The incision was closed without replacement of the bone flap. Anesthesia was discontinued and recovery was assessed by return of pinna and corneal reflexes, and righting response. Animals were sacrificed by decapitation at 0.25d (~6h, *n* = 6), 1d (*n* = 6), 3d (*n* = 5) and 7d (*n* = 6) postinjury. Sham-injured controls were surgically prepared but not injured (*n* = 2 at each time point).

Tissue Preparation
Conscious animals were decapitated in a sharpened guillotine. For the LFP study, fresh cortex (inferolateral to the craniectomy site) and hippocampus (the entire vital structure) from each hemisphere were rapidly dissected on ice; tissue was immediately sonicated in 6M

guanidine thiocyanate, 0.13 M EDTA (6M GE) and stored at -80°C. For the LCI study, brains were rapidly dissected, frozen on powdered dry ice and stored at -80°C. Frozen brains were coronally cut into 300 μm sections in a cryostat microtome (IEC) at -8°C. Thick sections were rapidly thaw mounted on slides, refrozen on powdered dry ice, and stored at -80°C. Brain regions of interest were dissected from 300 μm frozen sections (at -10°C) using a 1000 μm micropunch cannula (Palkovits, 1973). Frozen tissue was immediately transferred to and sonicated in 40 μl of 6 M GE, and stored at -80°C (Strauss and Jacobowitz, 1993). Specimens for the LFP experiments were a mixture of parietal cortex or whole hippocampus, while those for the LCI experiments were microdissected from specific coronal levels anterior to the cortical impact site [Bregma -2.6 to -3.6 mm, according to the coordinates of Paxinos and Watson (Paxinos and Watson, 1986)]. Therefore, LFP specimens were uniformly more concentrated (though probably less homogeneous) than the LCI micropunch specimens (e.g., 571 ± 11 μg protein per assay in LFP cortex vs 230 ± 9 μg protein per assay in LCI cortex).

Molecular probes

The bcl-2 riboprobe was from SalI linearized pGEM-5Zf+ (Promega) containing a bcl-2 PCR fragment (639 bp) generated from rat brain cDNA (using primers at 247-259 (forward) and 885-863 (reverse) with respect to the initiation codon, Genbank accession # L14680). The bax riboprobe was from pGEM-5Zf+ (Promega) containing a bax PCR fragment (370 bp) generated from rat brain cDNA (using primers at 190-212 (forward) and 559-537 (reverse) with respect to the initiation codon, Genbank accession # U49729) and subcloned in the NcoI and SalI site of the plasmid. The bcl-x_L riboprobe was from SaII linearized pGEM-5Zf+ (Promega) containing a bcl-x PCR fragment (589 bp) generated from rat brain cDNA (using primers at 21-42 (forward) and 606-628 (reverse with respect to the initiation codon, GenBank accession# X82537). The sequence spans the regions across which alternate splicing occurs for the short (pro-apoptotic) isoform (Shiralwa et al., 1996), yielding full length fragments only for bcl-x_L. The PCR fragments were synthesized using high fidelity PCR (Boehringer-Mannheim) and rat brain cDNA (Clontech), sequenced, and sequences were confirmed using a BLAST search in the GENBANK database. The cyclophilin cDNA (CYC, a "housekeeping" gene, has a constant level of expression under most circumstances) was subcloned into the pGEM-4Z vector with the PstI/NcoI 309 bp fragment

of rat cyclophilin cDNA (-8 to 300, (Danielson *et al.*, 1988)). Clones were also confirmed by restriction enzyme analyses. RNA probes were synthesized from linearized, purified cDNA templates, using T7 RNA polymerase in the presence of ^{32}P-labeled ribonucleoside triphosphates (specific activity $\sim$5 x 10^5 dpm/ng). Full-length antisense transcripts were purified by acrylamide-urea gel electrophoresis, autoradiography (10s), excision from the gel and elution in 0.5 M ammonium acetate (pH 6.3), 1 mM EDTA, 0.2% sodium dodecylsulfate (SDS).

RNase Protection Assays (RPA)

To measure mRNA levels we used a lysate RNase protection assay, and direct counting of radionuclide activity in the protected fragments. The lysate-RPA was carried out as described (Strauss and Jacobowitz, 1993). Briefly, an excess of ^{32}P-labeled antisense riboprobes was hybridized directly to the target mRNA in the cell or tissue lysates at 37°C overnight. A small portion of each RPA sample was set aside for a protein determination (Bradford microassay, BioRad), and the total sample protein was calculated for normalization purposes. Double-stranded RNA complexes were protected from RNase degradation, purified away from background contaminants by organic extractions, ethanol precipitation, and native polyacrylamide gel electrophoresis. Gel pieces containing full sized protected fragments were excised from the dried gel using the autoradiogram as a guide. Background was determined using specimens hybridized on dry ice, but otherwise treated the same as experimental specimens. Scintillation counting was used to detect radionuclide decay (dpm). Only dpm > 2 standard errors of the mean above background values was considered quantifiable. The signal (dpm - background) was converted to moles (by the probe specific activity) or to grams of mRNA (based on the length of the endogenous mRNA). An internal control probe (cyclophilin) was included with each assay for normalization.

Statistics

The mRNA results presented are mean ± standard error. To conclude that a target mRNA had changed, we required that changes be consistent (extent and direction) by both methods of normalization, and statistically significant by at least one method. Analysis of variance was performed with *post hoc* Dunnett test for sham comparisons, or paired Student's *t*-test for left *vs* right side comparisons. A p <0.05 was required to reject the null hypothesis that the group means were equivalent (p<0.1 for one-sided *t*-test).

RESULTS

Lysate RNase protection assays were used to measure brain bcl-2, bcl-x$_L$, and bax mRNA levels. Radionuclide decay from the protected target mRNA:riboprobe hybrids yielded absolute amounts of mRNA in the sample. Values reported in Tables I, II, and III were grams of target mRNA normalized to total protein in the sample (fg/μg protein). Relative mRNA levels presented in FIGs. 1, 2, and 3 were moles of target mRNA normalized to moles of cyclophilin mRNA (mole %) and further normalized to sham control levels.

The anti-apoptotic bcl-2 and bcl-x$_L$ genes were downregulated in both the LFP and LCI models of TBI. Temporal profiles in the injured cortex showed 75-80%

depletion of bcl-2 mRNA early on, with the LCI levels returning to normal by 1 day (FIG. 1, top, solid squares), and the LFP bcl-2 levels remaining significantly depressed for at least 3 days postinjury (FIG. 1, top, solid circles). In the contralateral cortex, LFP showed significant decreases in bcl-2 mRNA, while LCI did not (FIG. 1, top, stripes). In the hippocampus, low levels of bcl-2 mRNA were observed in the sham animals, and while detectable, postinjury bcl-2 levels were below the level of quantitation. Using non-parametric analysis, bcl-2 levels were significantly decreased in the injured hippocampus from 1 to 7 days postinjury (data not shown). In the LCI model, bcl-2 was undetectable in micropunches of hippocampus, probably due to the low amount of tissue sampled (53 ± 3 μg protein per assay from the CA1-CA3 regions, *vs* 619 ± 25 μg protein per assay in the LFP whole organ homogenates).
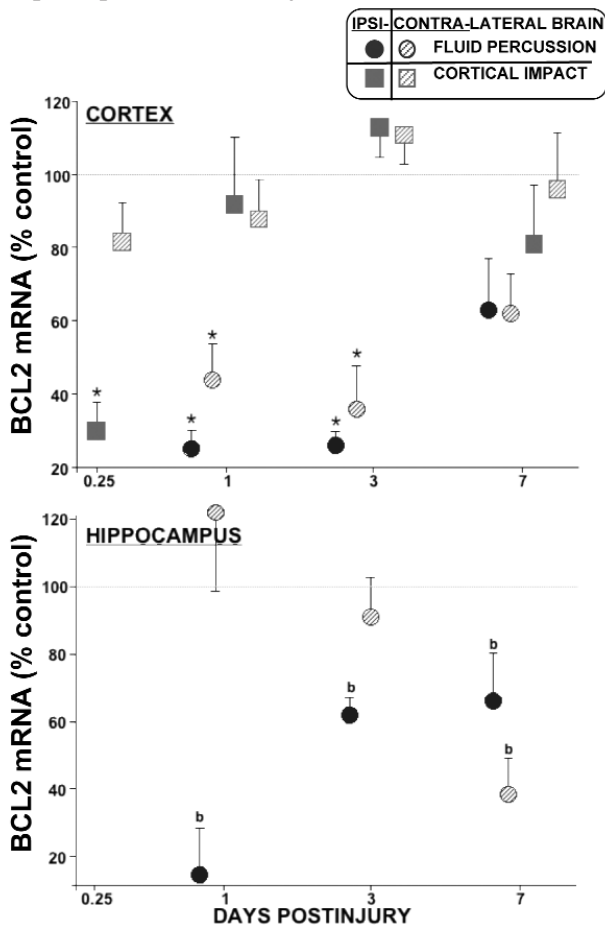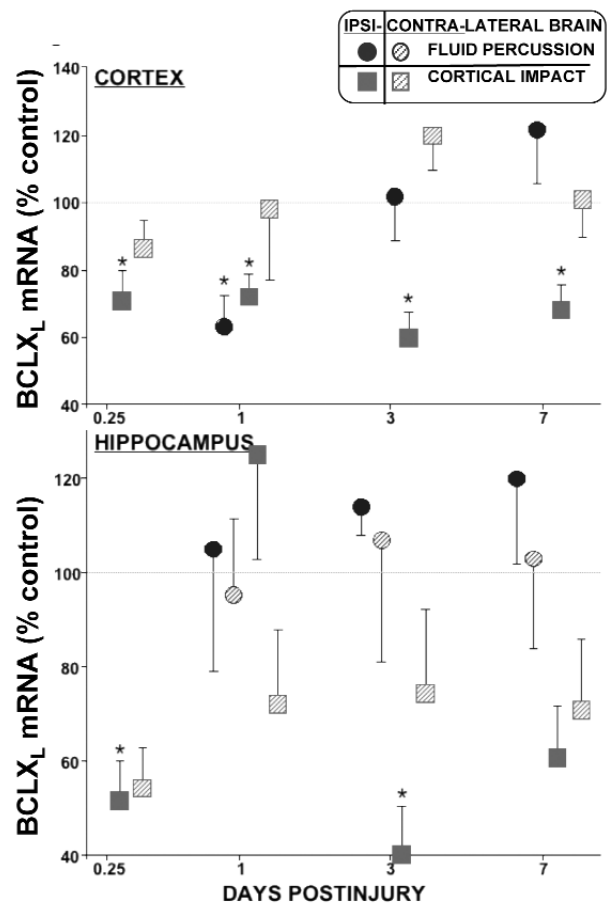

FIGURE 1 Relative levels of bcl-2 mRNA in cortex and hippocampus after traumatic brain injury. In the injured cortex (top), bcl-2 mRNA decreased 70-80% at 0.25 d after LCI (solid squares) and from 1 to 3 d after LFP (solid circles) TBI. Contralateral cortex showed no changes (LCI, striped squares), or 50-60% decreases in bcl-2 mRNA from 1 to 3 d postinjury (LFP, striped circles). Hippocampal bcl-2 levels (bottom) were constitutively below (LCI, not shown) or dropped below the limit of quantitation (b) after LFP TBI. Relative mRNA levels (mean ± SEM) are the mole percent of cyclophilin mRNA, normalized to sham controls. Ipsilateral: solids, Contralateral: stripes. *p <0.05 *vs*. shams, ANOVA, Dunnett.

FIGURE 2 Relative Levels of bcl-xL mRNA in cortex and hippocampus decreased early on in both models of TBI. In the injured cortex (top), bcl-x$_L$ decreased 30-40% in both models. In the hippocampus (bottom), bcl-xL mRNA decreased in a biphasic manner after LCI (solid squares), while high variation after LFP (circles) obscured possible changes. Relative mRNA levels (mean ± SEM) are the mole percent of cyclophilin mRNA, normalized to sham controls. Ipsilateral: solids, Contralateral: stripes. *p <0.05 *vs*. shams, ANOVA, Dunnett.

Levels of bcl-x_L mRNA in the injured cortex also decreased (~30%) early on in both LFP and LCI models of TBI (FIG. 2). While the LFP levels returned to normal by 3 days, in the LCI injured cortex bcl-x_L mRNA remained depressed for at least 7 days postinjury (FIG. 2, solid squares). Hippocampal bcl-x_L mRNA levels decreased 40-60% in the LCI model, but not at all in the LFP model. Measurements of bcl-x_L mRNA in the hippocampus showed large errors in most groups in both models, indicating the likelihood of some ongoing dynamic process (rather than a problem with sample collection). This was further supported by the biphasic decreases observed in the LCI model at 0.25 and 3 days, but not 1 day postinjury (FIG. 2). There were no significant differences in contralateral hippocampal bcl-x_L mRNA levels. In the LFP model, bcl-x_L mRNA levels in the ipsilateral diencephalon decreased (~50%, $p < 0.05$, Dunnett) at 1d postinjury; there were no changes at other times or contralateral to injury (data not shown). Apparent decreases contralat-

**Table I   Bcl-2 mRNA Levels Decrease After Traumatic Brain Injury**

BRAIN REGION	MODEL	SHAMS	0.25	TIME POSTINJURY (days) 1	3	7
CORTEX				bcl-2 mRNA (fg/µg protein) [a]		
IPSILATERAL	LFP	0.87 ± 0.17	-	0.35 ± 0.09*	0.29 ± 0.10*	0.50 ± 0.09
	LCI	1.08 ± 0.24	0.33 ± 0.08*	1.00 ± 0.20	1.22 ± 0.11	0.88 ± 0.17
CONTRALATERAL	LFP	0.80 ± 0.14	-	0.22 ± 0.04*	0.22 ± 0.04*	0.54 ± 0.12
	LCI[£]	1.41 ± 0.22[^]	1.16 ± 0.17[#]	1.12 ± 0.14	1.08 ± 0.16	0.98 ± 0.18
HIPPOCAMPUS						
IPSILATERAL	LFP	0.36 ± 0.08	-	0.05 ± 0.05[b]	0.22 ± 0.02[b]	0.24 ± 0.07[b]
	LCI	-[b]	-	-	-	-
CONTRALATERAL	LFP[£]	0.54 ± 0.06[#]	-	0.67 ± 0.13[#]	0.49 ± 0.09[#]	0.20 ± 0.07[b]
	LCI	-[b]	-	-	-	-

[a]Messenger RNA levels (mean ± SEM) determined by RNase protection assay (see Methods). Sham values from multiple time points showed no differences and were combined for each anatomical area. Lateral fluid percussion (LFP) or lateral cortical impact (LCI) injury was performed as described in Methods. [b]Below the limit of quantitation, "-" not done (hippocampal bcl-2 mRNA levels in the LCI specimens were below the limit of quantitation). *$p < 0.05$, ANOVA, Dunnett vs SHAMS. [#]$p < 0.05$, paired t-test, contralateral vs ipsilateral ([^]$0.05 < p < 0.10$); [£]$p < 0.05$, paired t-test, contralateral vs ipsilateral over time groups combined.

Table II   Bcl-X_L mRNA Levels Decrease After Lateral Traumatic Brain Injury

BRAIN REGION	MODEL	SHAMS	0.25	TIME POSTINJURY (days) 1	3	7
CORTEX				bcl-x_L mRNA (fg/µg protein) [a]		
IPSILATERAL	LFP	5.69 ± 0.57	-	3.80 ± 0.51*	5.75 ± 0.72	6.90 ± 0.91
	LCI	17.5 ± 1.3	12.4 ± 1.6*	12.6 ± 1.2*	10.5 ± 1.4*	12.0 ± 1.3*
CONTRALATERAL	LFP	-	-	-	-	-
	LCI[£]	13.5 ± 1.6	11.7 ± 1.2	13.2 ± 2.8[#]	16.1 ± 1.3[#]	13.6 ± 1.5
HIPPOCAMPUS						
IPSILATERAL	LFP	3.31 ± 0.56	-	3.48 ± 0.86	3.77 ± 0.20	4.00 ± 0.61
	LCI	14.4 ± 1.8	7.43 ± 1.24*	18.0 ± 3.2	5.79 ± 1.47*	8.76 ± 1.59
CONTRALATERAL	LFP	3.98 ± 0.87	-	3.79 ± 0.65	4.25 ± 1.04	4.11 ± 0.77
	LCI	13.2 ± 1.1	7.13 ± 1.14	9.50 ± 2.07	9.78 ± 2.35[^]	9.32 ± 1.99

[a]Messenger RNA levels (mean ± SEM) determined by RNase protection assay (see Methods). Sham values from multiple time points showed no differences and were combined for each anatomical area. Lateral fluid percussion (LFP) or lateral cortical impact (LCI) injury was performed as described in Methods. "-" not done. *$p < 0.05$, ANOVA, Dunnett vs SHAMS. [#]$p < 0.05$, paired t-test, contralateral vs ipsilateral ([^]$0.05 < p < 0.10$); [£]$p < 0.05$, paired t-test, contralateral vs ipsilateral over time groups combined.
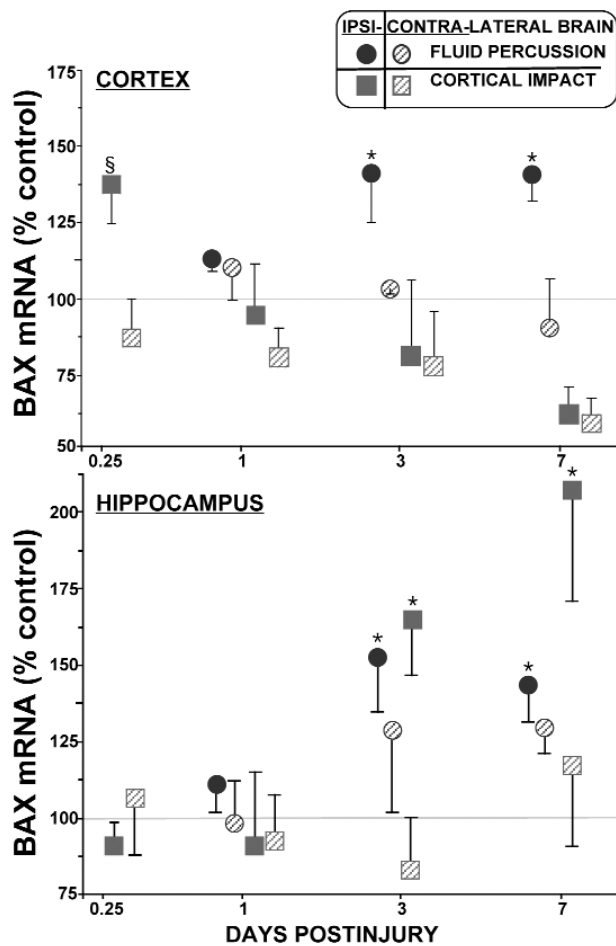
eral to injury (Table II) were not statistically significant by either means of normalization.

Pro-apoptotic bax mRNA levels increased in both TBI models (FIG. 3). Injured cortex showed ~40% increases in bax mRNA at 0.25 days in the LCI model, and at 3 and 7 days in the LFP model. In the injured hippocampus, bax mRNA increased by 50-100% at 3 and 7 days postinjury in both models (FIG. 2, bottom). In the LFP model, bax mRNA levels decreased in the ipsilateral diencephalon (~60%, $p < 0.05$, Dunnett) only at 1d postinjury (data not shown). No other changes were observed contralaterally, or in ventral cortex (distant from the injury site), in either model.

Despite differences in injury phenotype (LFP is somewhat diffuse *vs*. LCI which is more focal) and sample collection (fresh macrodissection *vs* frozen microdissection) between the models, common patterns of bcl-2 family gene expression were observed

FIGURE 3   Relative Levels of bax mRNA in cortex and hippocampus after traumatic brain injury. Levels of bax mRNA are increased in both models of traumatic brain injury. In the injured cortex (top), bax mRNA increased ~40% by 0.25 d after LCI (solid squares), and from 3 to 7 d after LFP (solid circles). In the hippocampus (bottom), bax levels increased in both TBI models from 3 to 7 d after injury by ~50% (LFP, solid circles), up to ~200% (LCI, solid squares). Relative mRNA levels (mean ± SEM) are the mole percent of cyclophilin mRNA, normalized to sham controls. Ipsilateral: solids, Contralateral: stripes. *$p < 0.05$ *vs* shams, ANOVA, Dunnett (§$p < 0.05$ by Fisher PLSD).

Table III   Bax mRNA Levels Increase After Traumatic Brain Injury

BRAIN REGION	MODEL	SHAMS	TIME POSTINJURY (days)			
			0.25	1	3	7
CORTEX			bcl-x$_L$ mRNA (fg/µg protein) [a]			
IPSILATERAL	LFP	5.27 ± 0.51	-	6.67 ± 0.49	7.45 ± 0.85*	7.44 ± 0.46*
	LCI	2.04 ± 0.25	2.81 ± 0.32[§]	1.92 ± 0.35	1.57 ± 0.38	1.28 ± 0.17
CONTRALATERAL	LFP[£]	4.39 ± 0.42	-	5.76 ± 0.67[#]	5.55 ± 0.14[^]	3.21 ± 0.44[#]
	LCI[£]	2.91 ± 0.56[^]	2.40 ± 0.38	2.35 ± 0.29	2.43 ± 0.65[^]	1.71 ± 0.26
HIPPOCAMPUS						
IPSILATERAL	LFP	1.68 ± 0.27	-	1.86 ± 0.17	2.59 ± 0.30*	2.40 ± 0.20*
	LCI	3.45 ± 0.86	2.74 ± 0.27	2.76 ± 0.80	5.69 ± 0.62*	7.25 ± 1.35*
CONTRALATERAL	LFP	1.69 ± 0.20	-	1.62 ± 0.25	2.18 ± 0.57	2.13 ± 0.12
	LCI	5.28 ± 0.71	5.65 ± 1.06[#]	4.86 ± 0.91	4.38 ± 0.95[#]	6.18 ± 1.43

[a]Messenger RNA levels (mean ± SEM) determined by RNase protection assay (see Methods). Sham controls from multiple time points showed no differences and were combined for each anatomical area. Lateral fluid percussion (LFP) or lateral cortical impact (LCI) injury was performed as described in Methods. "-" not done. *$p < 0.05$, ANOVA, Dunnett *vs* shams; §$p < 0.05$, ANOVA, Fisher PLSD *vs* shams. #$p < 0.05$, paired *t*-test, contralateral *vs* ipsilateral ([^]$0.05 < p < 0.10$); £$p < 0.05$, paired *t*-test, contralateral *vs* ipsilateral over time groups combined.

following TBI (Table IV). All of these comparisons were between injured brain areas at various postinjury time points and the corresponding sham brain area (combined for all time points). There were also many cases of differences in gene expression between ipsilateral and contralateral regions (Tables I, II and III). These differences did not always correspond to injury-related changes. For example, in LCI animals bcl-2, bcl-x_L and bax mRNA levels were all significantly higher in the contralateral cortex. In LFP animals, levels of bcl-2 and bax mRNAs were higher in the contralateral cortex and hippocampus, respectively. Left-right differences in gene expression in certain sham groups were most likely the effect of surgical manipulations, however, it is possible these findings indicate lateralization (no naïve animals were examined in these studies to test this). Nevertheless, when normalized to the proper control group (*i.e.*, comparable brain area from the sham group, FIGs. 1-3), the temporal and neuroanatomical changes in gene expression described above were independent of these left-right differences.

DISCUSSION

The present study quantifies the common patterns of bcl-2, bcl-x_L and bax gene expression in two rat models of TBI. An early decrease (6-24 h) in bcl-2 and bcl-x_L mRNA levels is followed by a delayed phase (3-7d) increase in bax mRNA levels. This bipartite/biphasic pattern (decline in anti-apoptotic activity followed by augmented pro-apoptotic activity) was seen in ipsilateral cortical and subcortical (hippocampus) structures proximal to the injury site, but not in distal areas (ventral cortex or contralateral parietal cortex, contralateral hippocampus, diencephalon, brain stem, cerebellum).

In the cortex, bcl-2 and bcl-x_L mRNA levels decreased early, with one or both of these complementary genes diminished for at least 3 days postinjury

(and out through 7 days in the LCI model). In the hippocampus, bcl-2 and bcl-x_L mRNA levels also decreased early on. In fact, hippocampal bcl-2 levels were undetectable in the LCI model, and became unmeasurable following injury in the LFP model. Large variances were evident in hippocampal bcl-x_L mRNA levels in both models over the observation period. In the LCI model, bcl-x_L mRNA levels appeared to decrease 50-60% at 6 hours and 3 days, but not at 1 day postinjury.

The level of bax mRNA expression was consistently elevated by 40-100% at 3 and 7 days postinjury in the cortex (LFP only) and hippocampus (both models). Early elevation of bax mRNA was observed in the cortex (LCI) at 6 hours postinjury. Thus, bax showed more acute changes in LCI cortex, with more extended changes in LFP, consistent with the temporal course of cavitation in LCI *vs* LFP. Bax expression in the hippocampus was similar, consistent with the temporal course of delayed cell death in this region.

Clearly, in both models the pro-apoptotic bax gene was up-regulated in hippocampus, a region distal to the injury site. However, variation in mRNA levels was greater for the LCI experiments. This was most likely due to the method of specimen collection. Anatomical variability between rats leads to subtle differences in injury localization, changes in gene expression and subsequent cellular responses. Despite these variations, apoptosis-related bcl-2 family gene expression measured in these animals showed fairly consistent patterns that reflect moderate injury to the somatosensory cortex and underlying neocortex.

Differences in absolute mRNA levels between the models might also be attributable to the method of collection. Homogenization of gross brain regions (LFP specimens) would tend to dilute changes in the target mRNA among less affected tissue, while the micropunched regions (LCI samples) were chosen using the *in*

Table IV Patterns of Bcl-2 gene expression in LFP and LCI models of traumatic brain injury[a]				
Ipsilateral Brain Region	**Model**	**Bcl-2 mRNA**	**Bcl-x_L mRNA**	**Bax mRNA**
Parietal Cortex	LFP	⇓1d→3d	⇓1d only	⇑3d→7d
	LCI	⇓6h only	⇓6h→7d	⇑6h only
Hippocampus	LFP	⇓1d→7d	No change§	⇑3d→7d
	LCI	below LOQ	Biphasic§: ⇓6h and ⇓3d	⇑3d→7d

[a]Summary of results presented in Tables I-III, Figures 1-3. LFP: lateral fluid percussion TBI; LCI: lateral cortical impact TBI; ⇓ decrease; ⇑ increase; LOQ: limit of quantitation; §high variances may indicate a dynamic process.

situ hybridization histochemistry analyses (Raghupathi *et al.*, 2003) as a guide. In the case of bcl-2 in the hippocampus, the low level of expression favored detection in the LFP samples which were more concentrated (tissue per unit volume). Decreased mRNA levels in either model could reflect cell loss, however cyclophilin and protein levels, also measured in these studies did not change significantly with time or injury.

The Bcl-2 gene family members participate in apoptosis, either tipping the balance toward cell survival (bcl-2, bcl-x_L, etc.) or toward programmed cell death (bax, bcl-xS, etc.). After experimental brain injuries, delayed apoptotic cell death has been observed in the cortex, hippocampus and other brain areas. Neuronal apoptosis predominates in the cortex and hippocampus, with astrocytic and monocytic apoptosis being scarce or absent (Chen *et al.*, 1997; Conti *et al.*, 1998; Wennersten *et al.*, 2003). In the present study, the regional and temporal changes in Bcl-2 family gene expression suggest an association with delayed cell death following lateral fluid-percussion (LFP) and lateral cortical impact (LCI) TBI. The results are consistent with the "apostat" mechanism of cell death, which suggests that a shift in the cellular ratio of cell death activator (Bax, Bad, Bcl-xS, c-Jun N-terminal kinase, etc.) and suppressor (Bcl-2, Bcl-x_L, extracellular signal regulated kinase, etc.) proteins regulates the fate of a cell (Oltvai *et al.*, 1993; Xia *et al.*, 1995). Thus, cell (neuronal) survival may be compromised due to decreased expression of Bcl-2, and a concomitant increase in Bax levels.

Delayed cell death following TBI can occur due to the activation of multiple pathways which include the differential expression of the Bcl-2 family of genes. Overexpression of bcl-2 *in vivo* leads to neuroprotection following axotomy (de Bilbao and Dubois-Dauphin, 1996); and mice overexpressing human bcl-2 in neurons have a markedly reduced cortical contusion following experimental brain injury (Raghupathi *et al.*, 1998; Nakamura *et al.*, 1999). It is tempting to speculate that the acute disappearance of bcl-2 and the overexpression of bax in the injured brain exacerbates neuronal vulnerability to trauma (Raghupathi *et al.*, 2003).

Alternatively, the parallel findings in different brain injury models of time- and brain region-specific decreases in apoptosis suppressor genes, with subsequent increases in apoptosis promoter genes support the existence of a conserved pattern of apoptosis-related gene expression. The evolution of this biphasic pattern may represent a culling process to remove irreparably damaged cells from the injured brain. An initial phase of reduced anti-apoptotic activity may allow cell death promoters to kill damaged cells. Subsequently, cells that survive but are not fully functional (for example, cells that cannot repair DNA or produce adequate survival factors) may be eliminated by the wave of increased bax expression. Therapeutic strategies to modulate the expression of these genes may confer resistance to trauma-induced delayed cell death, but careful timing and targeting of these treatments will be necessary to attenuate functional deficits after TBI.

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