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TRAUMATIC INJURY INDUCES DIFFERENTIAL EXPRESSION OF CELL DEATH GENES IN ORGANOTYPIC BRAIN SLICE CULTURES DETERMINED BY COMPLEMENTARY DNA ARRAY HYBRIDIZATION

B. MORRISON III,* J. H. EBERWINE,† D. F. MEANEY* and T. K. MCINTOSH*‡†§

Departments of *Bioengineering, †Neurosurgery, and ‡Pharmacology, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

Abstract—The expression of a large panel of selected genes hypothesized to play a central role in post-traumatic cell death was shown to be differentially altered in response to a precisely controlled, mechanical injury applied to an organotypic slice culture of the rat brain. Within 48 h of injury, the expression of nerve growth factor messenger RNA was significantly increased whereas the levels of bcl-2, α-subunit of calcium/calmodulin-dependent protein kinase II, cAMP response element binding protein, 65,000 mol. wt isoform of glutamate decarboxylase, 1β isoform of protein kinase C, and ubiquitin messenger RNA were significantly decreased. Because the expression levels of a number of other messenger RNAs such as the neuron-specific amyloid precursor protein, β_2 microglobulin, bax, bcl_{xl}, brain-derived neurotrophic factor, cyclooxygenase-2, interleukin-1β, interleukin-6, tumor necrosis factor-α, receptor tyrosine kinase A, and receptor tyrosine kinase B were unaffected, these selective changes may represent components of an active and directed response of the brain initiated by mechanical trauma.

Interpretation of these co-ordinated alterations suggests that mechanical injury to the central nervous system may lead to disruption of calcium homeostasis resulting in altered gene expression, an impairment of intracellular cascades responsible for trophic factor signaling, and initiation of apoptosis via multiple pathways. An understanding of these transcriptional changes may contribute to the development of novel therapeutic strategies to enhance beneficial and blunt detrimental, endogenous, post-injury response mechanisms. © 2000 IBRO. Published by Elsevier Science Ltd.

Key words: genomic expression, *in vitro* model, traumatic brain injury, organotypic culture, stretch injury, cDNA array.

The primary mechanical event associated with traumatic injury to the CNS initiates a cascade of molecular and cellular events which include changes in gene expression, culminating in cell dysfunction and/or death. Although the initial injury occurs in less than 1 s, the post-traumatic sequelae may take hours or days to develop, thereby providing an opportunity to therapeutically attenuate detrimental or augment beneficial endogenous responses in an attempt to limit resultant cell death. A detailed analysis of these specific molecular events may aid in the rational development of novel therapies for the brain-injured patient. Many genes which have been reported to be up-regulated following experimental models of traumatic brain injury (TBI), such as *c-fos*, *c-jun*, junB or zif/268,[24,54,58,69] are transcription factors that control the expression of other genes, suggesting that the expression of a large number of genes, including those involved in cell death or survival, may be affected by trauma.

In this regard, expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), necessary for neuronal and other cell survival, has been shown to be increased after controlled cortical impact (CCI) injury in the rat.[13,26,71,73] Alterations in the expression and activity of cell death genes such as caspase-1 and caspase-3 have also been documented after CCI.[70] Morphological evidence of apoptotic cell death has been demonstrated in various models of TBI[8,9,57,70] suggesting that regulation of apoptotic pathways may contribute significantly to the post-traumatic sequelae. Genes associated with inflammatory mediators such as the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) have been implicated in both apoptotic and necrotic cell death pathways, and their expression has been shown to be up-regulated after fluid percussion injury (FPI) in the rat.[18,19] Collectively, these studies suggest that the brain's response to trauma may be an active process encompassing energy-dependent mechanisms such as changes in gene expression.

The expression of a large panel of genes associated with cell death or survival pathways and hypothesized to be involved in the post-traumatic sequelae was evaluated in the present study. Mechanical injury of brain tissue may result in shearing of various neuronal connections resulting in the loss of afferent inputs to certain cells which in turn could lead to cell death due to trophic factor withdrawal. Therefore, expression of NGF and BDNF as well as their receptors (TrkA and TrkB, respectively) was analysed in this study. Loss of afferent input and trophic support could, in turn, lead to cell death via apoptotic mechanisms. Alternatively, mechanical injury itself could affect the ratio of anti-apoptotic to pro-apoptotic proteins and initiate apoptosis.[35] Therefore, expression of several pro- and anti-apoptotic members of the bcl-2 family (bcl-2, bax, and bcl_{xl}) was examined.

Changes in intracellular Ca^{2+} homeostasis subsequent to

§To whom correspondence should be addressed at: 3320 Smith Walk, Hayden Hall Room 105C, Philadelphia, PA 19104, U.S.A. Tel.: +1-215-573-2871; fax: +1-215-573-3808.

E-mail address: mcintosh@seas.upenn.edu (T. K. McIntosh)

Abbreviations: APP, amyloid precursor protein; aRNA, antisense amplified RNA; BDNF, brain-derived neurotrophic factor; CCI, controlled cortical impact; $CKII_{\alpha}$, α-subunit of calcium/calmodulin-dependent protein kinase II; CREB, cAMP response element binding protein; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetate; FPI, fluid percussion injury; GAD_{65}, 65,000 mol. wt isoform of glutamate decarboxylase; IL, interleukin; NGF, nerve growth factor; P, postnatal day; $PKC_{1\beta}$, 1β isoform of protein kinase C; RNH, reverse northern hybridization; SDS, sodium dodecyl sulfate; SSC, standard saline citrate buffer; SSPE, standard saline phosphate EDTA buffer; TBI, traumatic brain injury; TNF, tumor necrosis factor; Trk, receptor tyrosine kinase.

glutamate release have been hypothesized to play a major role in post-traumatic damage and cell death caused by excitotoxicity.[43] Although changes in intracellular Ca^{2+} cannot be measured directly by measuring changes in gene expression, the functional consequences of a disruption of Ca^{2+} homeostasis may be inferred by assessing changes in the expression of various proteins involved in Ca^{2+} signaling such as $CKII_{\alpha}$ (α-subunit of calcium/calmodulin-dependent protein kinase II), $PKC_{1\beta}$ (1β isoform of protein kinase C) and CREB (cAMP response element binding protein) which were analysed in the present study. The major inhibitory neurotransmitter of the brain, GABA, could potentially prevent the excitotoxic effects of widespread glutamate release.[55] Although GABA concentrations cannot be measured by changes in gene expression, changes in expression of genes involved in GABA synthesis may reflect changes in GABA signaling. Therefore, the expression of GAD_{65} (65,000 mol. wt isoform of glutamate decarboxylase), an enzyme which synthesizes GABA from glutamate, was measured after injury.

Changes in cytokine expression have been documented in both animal models of brain trauma as well as in brain-injured patients.[63] Cytokines are involved in the inflammatory response and may play a role in the infiltration of the brain by systemic, inflammatory cells which in turn cause damage to the CNS.[31] Therefore, the expression of three cytokines, IL-1β, IL-6, and TNF-α, was analysed after trauma.

A histological hallmark of diffuse axonal injury is intra-axonal accumulation of the amyloid precursor protein (APP).[52] Expression of the neuron-specific isoform of APP (APP695) was analysed to determine whether it was differentially regulated after trauma.[34] Ubiquitin is a protein which tags other intracellular proteins for degradation. After ischemia and trauma, ubiquitin is hypothesized to be part of the cellular stress response involved in the breakdown of damaged cellular components and was included in this study to measure the transcriptional activity of the stress response.[39,47]

Although several *in vivo* models of TBI appear well suited for the study of the clinically relevant response of the whole animal,[53] the complexity of the *in vivo* situation prevents free access to the tissue, thereby interfering with spatial or real-time measurements of mechanical or biological parameters. Several *in vitro* models of mechanical trauma have recently been developed[5,17,48] and possess specific advantages over *in vivo* models including the precise control of the extracellular environment, easy access to the tissue, and the isolation of specific cellular and molecular events from potentially confounding factors found *in vivo*.

The present study utilized a novel, highly reproducible, *in vitro* model of mechanical brain injury which affords unique control over the amount and rate of mechanical insult applied to the culture. An organotypic culture preparation was chosen so as to maintain the complex three-dimensional architecture and local connectivity between heterogeneous cell populations to mimic as closely as possible the *in vivo* situation both mechanically and biologically. In conjunction with a sophisticated cDNA-array based technique (reverse northern hybridization, RNH),[16] the present study is the first to characterize the differential, transcriptional response of specific genes (18 in total) associated with cell death or survival pathways in organotypic brain slice cultures subjected to mechanical injury.

EXPERIMENTAL PROCEDURES

Organotypic brain slice cultures

An organotypic brain slice culture preparation was chosen for these studies so as to maintain the complex three-dimensional architecture between heterogeneous cell populations as well as the local connectivity between them, although long-distance fiber tracts may not have been maintained. Cultures were grown in custom-built wells which have been described previously.[48] In brief, the wells consisted of a 60 mm diameter stainless steel well with an 18 mm diameter hole in the center. A sheet of Sylgard membrane 0.015 inch thick (Specialty Manufacturing, Saginaw, MI, U.S.A.) was stretched across the bottom of the well and held in place with an O-ring. After autoclaving, the wells were coated overnight with 500 μl of a coating solution, 50 μg/ml of laminin (Gibco, Grand-Island, NY, U.S.A.) and 200 μg/ml poly-L-lysine (Sigma, St Louis, MO, U.S.A.), in sterile, deionized water in an incubator (Fisher Scientific, Pittsburgh, PA, U.S.A.), with 5% CO_2 at 37°C. The wells were then rinsed sequentially with 2 ml of sterile, deionized water and Neurobasal media (Gibco).

A total of 44 slice cultures was utilized for these experiments and was harvested from a total of 19 pups from three separate litters. In summary, the brain of a four-day-old Sprague–Dawley rat pup was aseptically removed, transferred to the stage of a McIlwain Tissue Chopper (Brinkmann Instruments, Westbury, NY, U.S.A.) and sectioned coronally at 350 μm. The brain was then transferred to ice-cold Gey's salt solution (Gibco) supplemented with 6.5% glucose, at which time the slices were carefully separated with a finely polished spatula. The slices were transferred to an assembled and coated well and allowed to attach before 1.5 ml of Neurobasal medium supplemented with 6.5% glucose and B-27 Supplement (Gibco) was added to the well which was placed on a rocker (Elmeco Engineering, Rockville, MD, U.S.A.). The cultures were fed twice a week with 1.5 ml of Neurobasal media supplemented with 6.5% glucose and B-27 supplement and were maintained *in vitro* for 18 days before injury.

Animal procedures used in these studies were fully approved by the University of Pennsylvania's Institutional Animal Care and Use Committee and we carefully adhered to the animal welfare guidelines set out in the Guide for the Care and Use of Laboratory Animals, U.S. Department of Health and Human Services, Publication Number 85-23, 1985. This work utilized a novel *in vitro* model of TBI which reproduced many aspects of the *in vivo* post-traumatic sequelae and offers an alternative to *in vivo* techniques.

Injury

Tissue deformation is considered a proximal cause to the primary nerve and vascular injuries that occur in TBI.[1,22] Using techniques to compare the estimated pattern of tissue deformation to the *in vivo* injury distribution, it has been proposed that deformations between 0.10 and 0.50 with strain rates in the order of 10–50/s were necessary to cause primary mechanical tissue damage.[41,44] The device utilized in the current study was capable of accurately and reproducibly generating deformations within the range associated with *in vivo* damage and allowed for the independent specification of critical mechanical parameters of injury including maximum strain, strain rate, relaxation rate and duration.[48] This injury was produced by deformation of the silicone membrane on which the cultures were grown. Injury level or strain was calculated from the displacement of the center of the membrane as measured by a laser displacement transducer (Omron Electronics, Schaumberg, IL, U.S.A.), and strain rate was calculated from strain. Injury severity was in the mild to moderate range: strain was below 0.40 and strain rate below 30/s.

A total of 28 cultures was individually injured under aseptic conditions at the following parameters (mean $\pm$ S.D.): strain $= 0.18 \pm 0.09$, strain rate $= 9.7 \pm 7.1$/s and relaxation rate $= 13.2 \pm 10.3$/s, which encompass a range of strain and strain rate values believed to be responsible for *in vivo* TBI.[40] Furthermore, this wide range of values was deliberately chosen to minimize the chance of missing a post-traumatic response. An additional 16 cultures was subjected to sham injuries which consisted of placing the culture on the injury device without engaging the device. After injury, cultures were returned to the incubator until harvested at 6, 24 and 48 h after injury. These time points were chosen to focus on specific effector genes downstream of immediate-early genes and to explore the genomic sequelae of mechanical CNS injury which may be amenable to future, therapeutic, clinical intervention.

Table 1. Clone information

Insert	Size (bp)	Species	Accession	Sequence
Apoptosis				
bax	370	Rat	U49729	190–559
bcl-2	639	Rat	L14680	247–885
bcl-x_l	589	Rat	S76513	40–628
Ubiquitin	2224	*Manduca*	X53524	4–231
Ca^{2+} signaling				
$CKII_\alpha$	1510	Rat	J02942	1–1510
CREB	1125	Rat	X14788	1–1125
$PKC_{1\beta}$	2481	Rat	M19007	45–2524
Cytokines				
IL-1β	908	Rat	M98820	53–960
IL-6	911	Rat	M26744	1–911
TNF-α	708	Rat	X66539	1–708
Trophic signaling				
NGF	1682	Rat	M36589	1–1682
BDNF	1169	Rat	M61178	1–1169
TrkA	451	Rat	M85214	836–1286
TrkB	481	Rat	M55291	1337–1817
Others				
APP695	2822	Human	X06989	33–2854
COX-2	4154	Rat	S67722	1–4154
GAD_{65}	1966	Rat	M72422	1–1966
β_2-microglobulin	328	Rat	Y00441	277–604

These plasmid clones were utilized for this study. Pertinent information for each clone is given including the size of the insert, species and Genbank accession number. The column labeled "Sequence" indicates which nucleotides of the corresponding Genbank sequence are present in the plasmid. The ubiquitin clone consisted of nine tandem repeats of the ubiquitin monomer. Clone identity was confirmed by sequencing and/or restriction digestion.

Reverse northern hybridization

At 6 ($n=8$), 24 ($n=9$) and 48 h ($n=11$) post-injury, cultures (approximately 10 mg of tissue) were individually homogenized in 1 ml of RNA-STAT (Tel-Test "B", Friendswood, TX, U.S.A.) and total RNA was isolated as per the manufacturer's instructions. The final pellet (approximately 10 μg of total RNA) was resuspended in 10 μl of RNAse-free water. The RNH has been described in detail elsewhere and has been shown to be a quantitative method to measure simultaneously the abundance of multiple species of mRNA from a limited supply of RNA, as little as from a single cell (0.1 pg RNA).[7,10,16,37,65] This method differs from other cDNA array techniques which hybridize two fluorescent samples of reverse-transcribed cDNA to a single array.[14,59] In the current study, amplified and radioactively labeled RNA from a single sample was hybridized to a single array or slot blot. Internal controls, described below, were utilized to allow for comparisons between separate arrays or samples. The mRNA from the experimental preparation was reverse transcribed into a first strand of cDNA using 20 U AMV reverse transcriptase (Seikagaku America, Rockville, MD, U.S.A.) and a specific poly$(dT)_{24}$-T7 primer at 37°C for 1 h [20 μl reaction volume, 50 mM Tris, 120 mM KCl, 10 mM dithiothreitol (DTT), 6 mM $MgCl_2$, 250 μM dNTPs, pH 8.3, 20 U RNAsin (Promega, Madison, WI, U.S.A.)]. The single-stranded cDNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated and resuspended. A second strand of cDNA was then created from the first strand with a combination of 1 U T4 and 2 U Klenow polymerases at 14°C for 4 h (Boehringer Mannheim, Indianapolis, IN, U.S.A.) via hairpin self priming (50 μl volume, 100 mM Tris, 10 mM DTT, 10 mM $MgCl_2$, 250 μM dNTPs, pH 7.4). The hairpin was digested with 18 U S1 Nuclease (Boehringer Mannheim) at 37°C for 7 min [450 μl volume, 50 mM NaCl, 50 mM NaOac, 1 mM $Zn(SO_4)_2$, pH 4.6]. The double-stranded cDNA was used as a template to generate antisense, amplified RNA (aRNA) using 1000 U T7 RNA polymerase (Epicentre Technologies, Madison, WI, U.S.A.) at 37°C for 4 h [25 μl volume, 40 mM Tris, 5 mM DTT, 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 250 μM ATP, 250 μM GTP, 250 μM UTP, 17.5 μM CTP, 0.37 μM [^{32}P]CTP 3000 Ci/mmol (NEN Life Science Products, Boston, MA, U.S.A.), pH 7.5]. This aRNA was used to probe a slot blot.

To determine its complexity, 2×10^6 c.p.m. of the aRNA was used to probe a northern blot of total RNA from the brain of a 22-day-old rat. Total RNA (10 μg) was electrophoresed through a 1% agarose/3% formaldehyde gel, transferred to a nylon membrane, hybridized for 16 h and washed at high stringency. An adjacent lane on the membrane was stained with Methylene Blue so that the 18S and 28S ribosomal bands could be used as markers.

To generate the slot blots, linearized plasmids, containing sequences of known genes, were denature at room temperature for 15 min [2 μg/100 μl in 0.1× standard saline citrate buffer (SSC) and 0.3 N NaOH] and neutralized with an equal volume of 20× SSC before applying 1 μg of each to nylon membranes (Hybond Nylon, Amersham, U.K.) with the aid of a slot blot manifold (Millipore, Bedford, MA, U.S.A.). The membranes were pre-hybridized at 42°C in pre-hybridization mix [50% formamide, 6× standard saline phosphate EDTA buffer (SSPE), 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 100 μg/ml Sheared DNA] which was replaced with fresh mix before the aRNA was allowed to hybridize for 72 h at 42°C.

The membranes were removed from their bottles and washed sequentially at 50°C for 1 h each in 2× SSPE, 0.1% SDS, and then 0.1× SSPE, 1% SDS. Membranes were then wrapped in plastic wrap and apposed to a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for 48 h before digitizing on a Storm plate reader (Molecular Dynamics).

Clones

The plasmid clones utilized in these experiments were obtained from a number of different sources. The neuron-specific amyloid precursor protein (APP695) clone was a gift from J.Q. Trojanowski (University of Pennsylvania, Philadelphia, PA, U.S.A.). The bax, bcl-2 and bcl-x_l clones were gifts from K.I. Strauss (Temple University, Philadelphia, PA, U.S.A.). The $PKC_{1\beta}$ clone was a gift from E. Slosberg (Columbia University, New York, NY, U.S.A.). The β_2-microglobulin clone was a gift from M. Kress (Genetique Moleculaire et Integration des Fonctions Cellulaires, France) and was used as a housekeeping gene and a positive control. IL-1β, IL-6 and TNF-α clones were gifts from G. Z. Feuerstein (SmithKline Beecham Pharmaceuticals, Conshohochen, PA, U.S.A.). The CREB clone was a gift from M. R. Montminy (Salk Institute, La Jolla, CA, U.S.A.). The ubiquitin clone was a gift from L. M. Schwartz (University of Massachusetts, Amherst, MA, U.S.A.). The pUC18 plasmid was chosen as a negative control and was used to account for any non-specific hybridization signal. Specific information pertaining to each plasmid is presented in Table 1 including insert size, species, Genbank accession number and relevant sequence portion.

Quantification of gene expression

The level of expression of a particular gene in a given sample on an individual blot or array was determined as follows with the aid of the Analytical Imaging Station software (Imaging Research, Ontario, Canada). From the digitized image, the expression of a particular gene for a single sample was determined by summing the value of each pixel in the image corresponding to that particular slot. The expression level was corrected for background by subtracting the level of expression given by a cDNA slot of plasmid without an insert (pUC). To make comparisons between individual blots and therefore between samples, the level of expression of a particular gene was then divided by the average level of expression of all the genes on a given slot blot yielding the relative expression of each gene with respect to the average expression of all genes for that sample.[7]

Statistical analysis

The relative gene expression data were first logarithmically transformed to preserve a normal distribution. Control groups at all three time-points were pooled and a one-way ANOVA was performed followed by LSD post hoc comparisons for a particular gene between the various time-points. Results were assumed to be significant if $P<0.05$.

RESULTS

Figure 1 demonstrates the complexity of the aRNA product which was used to probe a northern blot of rat brain total

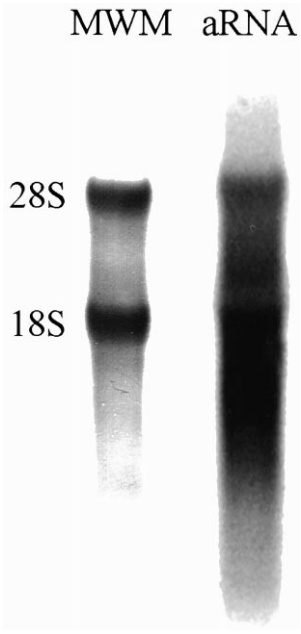

Fig. 1. A northern blot of rat brain total RNA was probed with a sample of aRNA used for these studies. The complexity of the aRNA was demonstrated by hybridization to a wide range of sequences in the total RNA indicating that the aRNA was representative of the mRNA found in the cultured tissue. An adjacent lane was stained with Methylene Blue to provide molecular weight markers as indicated by the ribosomal RNA bands.

RNA. The aRNA hybridized to a wide range of sequences, indicating that it was representative of the RNA found in the tissue. Note that the aRNA does not preferentially hybridize to the 18S and 28S ribosomal bands, although these bands typically account for a majority of the total RNA indicating that the amplification procedure was specific for mRNA.[65]

Figure 2 shows a representative RNH blot demonstrating low background and high, specific hybridization signal to the immobilized cDNA. Analysis of gene expression at various time-points after *in vitro* mechanical injury revealed that several genes including bcl-2, $CKII_{\alpha}$, CREB, GAD_{65}, NGF, $PKC_{1\beta}$ and ubiquitin were differentially expressed. The earliest time-point examined in this study was 6 h post-injury so as to focus the analysis on effector genes downstream of immediate-early genes and to explore the genomic consequences of TBI which may be amenable to future, therapeutic intervention. The expression of several genes followed a common pattern after injury in which expression was close to control values at 6 h post injury but significantly decreased at 24 h and returned to control values by 48 h post-injury. Specifically, expression of bcl-2 decreased at 24 h after injury compared with control ($P < 0.005$), 6 h ($P < 0.01$) or 48 h ($P < 0.01$) and returned to baseline at 48 h (Fig. 3A). Expression of $PKC_{1\beta}$ decreased at 24 h compared with control ($P < 0.05$) and 6 h ($P < 0.05$) after injury and remained slightly depressed (Fig. 3B). Expression of ubiquitin decreased at 24 h compared with control ($P < 0.005$), 6 h ($P < 0.05$) and 48 h ($P < 0.005$) post-injury and returned to baseline at 48 h (Fig. 3C). Expression of GAD_{65} decreased at 24 h compared with control ($P < 0.05$) and 48 h ($P < 0.01$) and returned to baseline at 48 h (Fig. 3D).

The remaining genes that were regulated after trauma followed different temporal profiles. Expression of $CKII_{\alpha}$ decreased at 6 h ($P < 0.05$) and 24 h ($P < 0.05$) after injury compared with 48 h which, although slightly elevated, was no different to control levels (Fig. 3F). CREB expression decreased at 6 h ($P < 0.05$) compared with 48 h which was slightly higher than control levels (Fig. 3G). NGF expression increased at 6 h compared with control ($P < 0.005$) and 48 h ($P < 0.005$). In addition, at 24 h after injury, NGF expression was increased compared with control ($P < 0.05$) and 48 h ($P < 0.01$) levels (Fig. 3H). The expression of all other genes analysed was not significantly altered at any time-point after injury, including APP695, β_2-microglobulin, bax (Fig. 3E), bcl_{xl}, BDNF, cyclooxygenase-2, IL-1β, IL-6, TNF-α, TrkA and TrkB (data not shown). It is important to note that these genes were expressed at levels which were quantifiable by the RNH procedure and that the lack of post-injury regulation was not due to a limitation of the technique or lack of sensitivity.

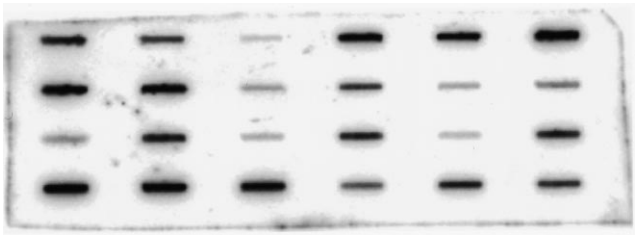

Fig. 2. A representative slot blot displaying relative expression levels of specific genes. The slot blot consists of linearized cDNA of specific genes in the following pattern: (left to right) row 1: TrkA, TrkB, c-jun (not analysed), bax, bcl_{xl}, bcl-2; row 2: NGF, BDNF, cyclophilin A (not analysed), APP695, β-actin (not analysed), ubiquitin; row 3: glyceraldehyde 6-phosphodehydrogenase (not analysed), cyclooxygenase 2, growth-associated protein 43 (not analysed), β_2-microglobulin, pUC, $PKC_{1\beta}$; row 4: IL-1β, IL-6, TNF-α, $CKII_{\alpha}$, CREB, GAD_{65}. Genes not analysed as indicated above were below the limits of detection by RNH with the particular plasmids used in this study. All other genes analysed were readily detectable, including those which did not show post-injury regulation. Each slot blot was hybridized to a single sample of aRNA. Each slot is 7 mm long.

DISCUSSION

In vitro, mechanical injury to organotypic cultures of rat brain selectively altered the expression of several classes of genes hypothesized to play a role in the post-traumatic sequelae of brain injury including those involved in apoptosis (bcl-2 and ubiquitin), intracellular Ca^{2+} signaling ($CKII_{\alpha}$, CREB, $PKC_{1\beta}$), trophic factor signaling (NGF) and intercellular signaling (GAD_{65}). The expression of both $CKII_{\alpha}$ and CREB was significantly decreased 6 h post-injury, the earliest time-point examined in this study, and returned to control levels by 48 h. In contrast, the expression of NGF was significantly increased at 6 and 24 h, returning to baseline 48 h after injury. The remaining differentially regulated genes, bcl-2, GAD_{65}, $PKC_{1\beta}$ and ubiquitin, demonstrated a common pattern of significantly depressed expression at 24 h which recovered by 48 h. These results suggest that, after mechanical trauma, the brain mounts an active response which includes the transcriptional regulation of a number of cell death or survival-related genes, either protective or detrimental which include increases as well as decreases, arguing against a generalized transcriptional impairment after injury.

A major strength of the current study was the simultaneous measurement of expression of a large number of genes, allowing for the interpretation of these changes in a co-ordinated,

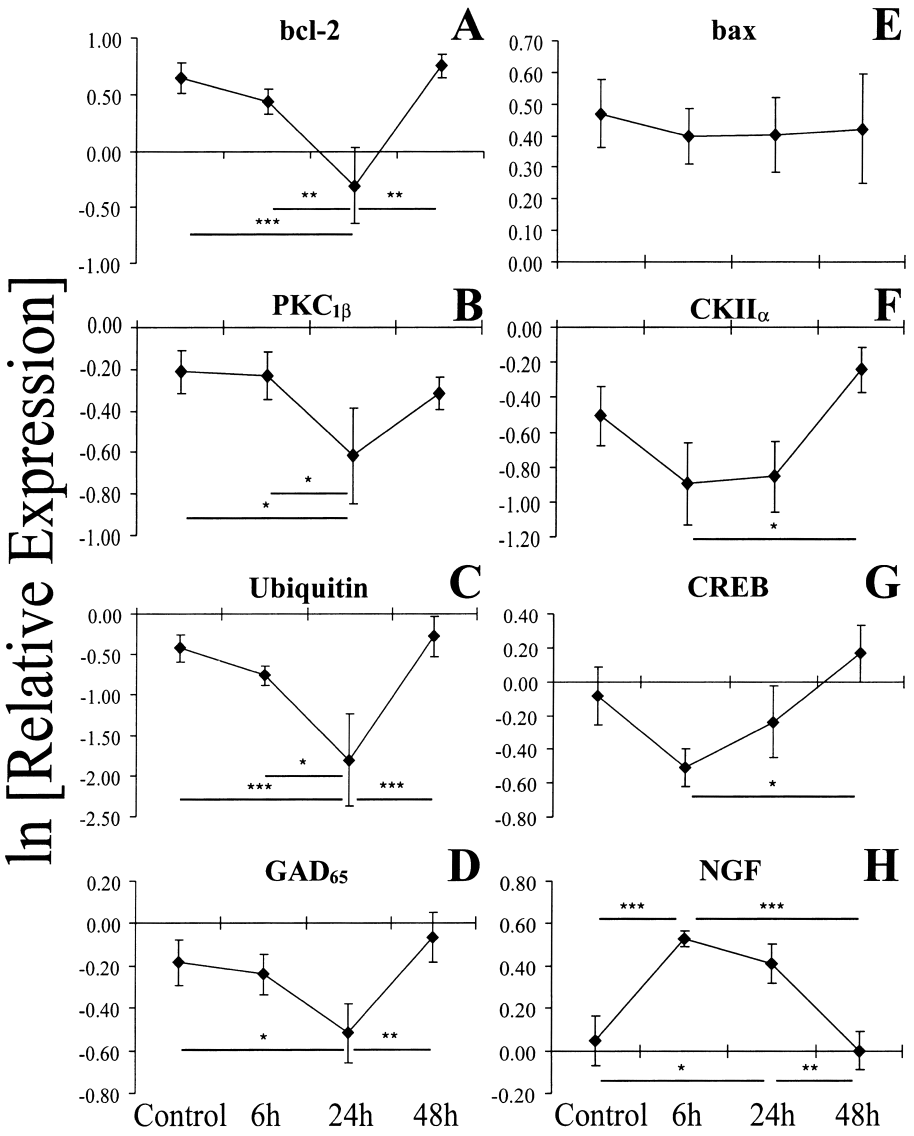

Fig. 3. Temporal profile of changes in gene expression in organotypic brain slice cultures after a single mechanical injury. Data is plotted as the logarithmic transformation of the ratio of the hybridization signal of a particular gene to the average hybridization signal of all genes on a particular blot. Several genes demonstrated a common temporal expression pattern post-injury (A–D). Several other temporal profiles of expression were observed including no regulation (E) and a post-injury increase (H). Error bars denote S.E.M. Significance is noted as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.005$. Genes which were not differentially regulated included APP695, β_2 microglobulin, bax (E), bcl_{xl}, BDNF, cyclooxygenase-2, IL-1β, IL-6, TNF-α, TrkA, and TrkB. The failure to demonstrate post-traumatic regulation was not due to a low level of expression below the sensitivity limit of the RNH assay but because their expression levels, which were readily detectable by RNH, were not statistically different at any time-point after injury. The groups consisted of the following numbers of cultures, respectively: Control, 16; 6 h, 8; 24 h, 9; 48 h, 11.

cellular context which was not artificially limited to one or two genes. Such a global approach explores the dynamic genomic response to trauma and generates a molecular fingerprint of the post-traumatic sequelae. Existing literature demonstrates that after trauma changes in mRNA correlate with changes in protein expression and, therefore, changes documented in the current study will probably reflect changes at the protein level as well. For example, up-regulation of message and protein are correlated after brain injury for NGF,[13,23] glial fibrillary acidic protein,[49] S100β,[30] basic fibroblast growth factor,[33,75] TNF-α, IL-6, IL-1β,[18,19,31,60,63,67] c-fos, c-jun, junB and zif-286.[32,54,72] An understanding of these transcriptional changes may suggest future therapeutic strategies for brain-injured patients specifically targeted at augmenting beneficial or blunting detrimental, endogenous, post-injury response mechanisms with the ultimate goal of minimizing the resultant progression of damage.

Although the primary mechanism of cell death after various CNS injuries including ischemia, spinal cord injury and TBI is believed to be necrotic, a portion of cells throughout the injured tissue undergoes apoptosis.[9,11,38] Bcl-2, a protein with anti-apoptotic activity, is expressed in neurons which survive different types of injury *in vivo* such as ischemia[6] and CCI.[8] In the present study, bcl-2 mRNA expression was significantly decreased at 24 h post-injury, returning to baseline levels by 48 h. Interestingly, bax mRNA levels were not altered after injury, suggesting that the cellular ratio of bax:bcl-2 protein may have increased, thereby favoring initiation of apoptosis which has been shown to occur after TBI in the rat.[9,57] Ubiquitin plays a vital role in the targeted degradation of damaged proteins and is also essential for the cellular stress-response

and consequent cell survival.[42] After forebrain ischemia in the gerbil, ubiquitin immunoreactivity initially disappears but reappears in the CA3 and dentate granule cells, but not in the CA1 which is destined to die.[47] An earlier study found similar regional changes after forebrain ischemia in the rat, leading the authors to suggest that the stress response was severely depressed in cells destined to die.[39] The transient decrease in ubiquitin mRNA demonstrated in the present study at 24 h may be indicative of a dysfunctional stress response which, with the coincident decrease in bcl-2 mRNA, may be indicative of delayed cell death in response to a mechanical trauma. These acute changes in cell death genes support the time-course of *in vivo* apoptosis after TBI which is multiphasic, with peaks at 24 h and one week in the cortex, 48 h in the hippocampus and two weeks in the thalamus.[9,57] The results presented herein predict the acute initiation of apoptosis at 24 and 48 h. However, because the latest time-point studied was 48 h, a delayed decrease in bcl-2 occurring in the cortex at one week indicative of a delayed phase of apoptosis may have been missed.

The loss of afferent inputs and trophic support due to mechanical injury can also result in cell death and, as a protective mechanism, the brain may attempt to augment such trophic support. An increase in NGF protein and mRNA has been reported in models of TBI in the rat[13] and in human TBI patients,[51] and similar changes were seen in the present study as mRNA levels of NGF were significantly elevated at 6 and 24 h after stretch injury. Additional studies in a FPI model in the rat have demonstrated the therapeutic efficacy of NGF infusion to reduce post-traumatic cholinergic cell loss, apoptosis and cognitive deficits,[15,61] suggesting that an endogenous response of the brain can be enhanced by exogenous manipulation to attenuate post-traumatic consequences. The combination of an *in vitro* model of TBI and sophisticated molecular techniques could prove useful in the search for novel therapeutic strategies.

Disruption of intracellular Ca^{2+} homeostasis after traumatic injury is hypothesized to be responsible for cell damage and/or death.[43] Pathological activation of pathways involved in Ca^{2+} signaling may result in down-regulation of these systems as part of an endogenous protective response. In the present study, mRNA for three proteins involved in Ca^{2+} signaling, $CKII_{\alpha}$, CREB and $PKC_{1\beta}$, were transiently decreased 24 h after injury. CKII activity as well as mRNA levels have been shown to be decreased at 24 h and recovered by 48 h after ischemic injury in the gerbil.[28,29] In the present study, we demonstrated a similar temporal pattern of $CKII_{\alpha}$ gene expression following mechanical trauma which decreased at 6 and 24 h and recovered at 48 h. In mechanically responsive cells such as endothelial cells, PKC is believed to be involved in the transduction of mechanical stimuli into intracellular signaling cascades which can ultimately affect gene expression via protein kinase A-dependent phosphorylation of CREB and changes in intracellular Ca^{2+}.[4,46,64,66] PKC activity has been shown to be increased after FPI in the rat at 1 and 3 h after injury, suggesting that similar mechanotransduction pathways may also be present in CNS cells.[62,74] We have demonstrated here a post-traumatic decrease in levels of $PKC_{1\beta}$ mRNA at 24 h which may represent another aspect of an endogenous, neuroprotective response to pathological Ca^{2+} signaling and enzyme activation. After FPI in the rat, CREB phosphorylation increased, as did induction of *c-fos* which contains an activator protein-1 promoter, suggesting that Ca^{2+} signaling pathways were activated.[12] In the same study, protein levels of CREB remained unaltered at 1 h but were decreased 3 h post-injury. In the present study, after *in vitro* stretch injury, mRNA levels of CREB were decreased at 6 h post-injury compared with slightly elevated levels at 48 h. This decrease in CREB mRNA may be part of a protective, negative feedback mechanism.

An alternative interpretation of the co-ordinated changes in gene expression documented in this study suggests that cells may be preparing to undergo apoptosis within the first 48 h after injury. Inhibition of PKC by staurosporine can induce apoptosis,[50] whereas activation can prevent apoptosis in neurons and glia.[36] Ubiquitin is essential for the targeted degradation of short lived, pro-apoptotic proteins, since inhibition of ubiquinating enzymes can induce apoptosis.[45] Under these conditions, bcl-2 can block apoptosis;[50] however, in the present study, expression of bcl-2 was decreased together with expression of $PKC_{1\beta}$ and ubiquitin. While the expression of pro-apoptotic proteins such as bax was maintained, a decrease in anti-apoptotic proteins may alter the ratio of cell death to cell survival genes and, according to the "apopstat" theory, initiate apoptosis after injury.[35]

The maintenance of BDNF and NGF mRNA expression after injury as well as that of their receptors, TrkB and TrkA, is suggestive of a protective mechanism to prevent cell death after trauma. Certain cellular activities of trophic factors are known to be mediated by intracellular kinases, such as ERK, p38, and CKIV, which ultimately phosphorylate CREB.[20,56,68] However, the reduction in mRNA coding for CREB observed following injury suggests that there may be no substrate to phosphorylate. Therefore, after trauma, although levels of trophic factors and their receptors may be maintained, the intracellular signaling cascade responsible for their trophic actions may be down-regulated or impaired. Taken as a whole, the changes in gene expression at 6 and 24 h may indicate a readiness for cells to undergo apoptosis, as has been shown *in vivo*.[9,57] The mechanism(s) underlying the normalization of gene expression by 48 h are currently unknown. However, if the mechanism(s) could be elucidated and enhanced, post-injury cell death and dysfunction could be ameliorated.

A small subset of genes, including the cytokines IL-1β, IL-6 and TNF-α, showed no change in expression after *in vitro* injury, but have been shown to be post-traumatically altered in *in vivo* models of needle lesion and FPI in the rat.[18,19,31,63] In the current study no changes were seen in mRNA levels for IL-1β, IL-6 or TNF-α. Cytokine expression is normally undetectable *in vivo* in the brain under control conditions, but is up-regulated and released by neurons when cultured.[21] In a similar manner, cytokines were expressed at high levels in the cultures utilized for these studies (data not shown), suggesting that cytokine expression may have been maximally expressed prior to injury and may not have been capable of further induction. Alternatively, the increase in cytokine production after *in vivo* brain injury may, in part, be due to infiltrating immunocompetent cells or humoral factors entering the brain owing to blood–brain barrier breakdown. Because of inherent limitations of *in vitro* models and a lack of blood–brain barrier, our model system may not be suitable for the study of the post-traumatic inflammatory response.

A post-traumatic regulation of BDNF and its receptor TrkB

was not observed in the present study, in contrast to *in vivo* findings. In the fluid percussion model of TBI in the rat, the peak of post-injury induction of BDNF *in vivo* occurs at 3 h, is returning to control levels as early as 6 h, and is decreased by 72 h post-injury in the cortex.[25–27] Data from the current study indicated a non-significant increase in expression of BDNF at 6 h after injury (data not shown), suggesting that a significant increase in BDNF expression may have been missed at our earliest time-point. A second possible explanation for a lack of regulation of TrkB and BDNF mRNA is due to the age of the pups [postnatal day (P)4] utilized for this study. *In vivo*, the dentate gyrus does not develop until between P5 and P10.[2,3] Because the greatest increase *in vivo* of both TrkB and BDNF mRNA occurred in the dentate gyrus, it may not be surprising that neither BDNF nor TrkB was up-regulated in the current system.

This study utilized a clinically relevant *in vitro* model of injury to the CNS to examine the differential and co-ordinated changes in expression of a large panel of selected genes which are thought to play a central role in the post-traumatic sequelae and resultant cell death. Because the expression levels of several other genes, readily detectable by RNH, were not altered, we conclude that the observed changes in gene expression are components of an active and directed tissue response initiated specifically by trauma. The simultaneous measurement of these co-ordinated changes is a powerful tool with which to formulate and test sophisticated hypotheses concerning alterations in cellular physiology after mechanical injury. In addition, this approach may be useful for predicting which differentially regulated genes should be analysed in more detail at the protein level. An increased understanding of the post-traumatic sequelae may suggest novel therapeutic approaches to enhance beneficial and limit detrimental endogenous responses to trauma.

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