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Dynamic Mechanical Stretch of Organotypic Brain Slice Cultures Induces Differential Genomic Expression: Relationship to Mechanical Parameters

Although the material properties of biological tissues are reasonably well established, recent studies have suggested that the biological response of brain tissue and its constituent cells may also be viscoelastic and sensitive to both the magnitude and rate of a mechanical stimulus. Given the potential involvement of changes in gene expression in the pathogenic sequelae after head trauma, we analyzed the expression of 22 genes related to cell death and survival and found that a number of these genes were differentially regulated after mechanical stretch of an organotypic brain slice culture. Twenty-four hours after stretch, the expression of BDNF, NGF, and TrkA was significantly increased, whereas that of bcl-2, CREB, and GAD_{65} was significantly decreased (MANOVA followed by ANOVA, $p<0.05$). Expression of CREB and GAD_{65} was negatively correlated with strain, whereas expression of APP695 was negatively correlated with strain rate (all $p<0.05$). This study demonstrates that a subset of genes involved in cell death and survival are differentially regulated after dynamic stretch in vitro and that the expression of specific genes is correlated with mechanical parameters of that stretch.
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Keywords: Genomic Expression, In Vitro Model, Traumatic Brain Injury, Stretch Injury, cDNA Array

Introduction

Recently, several *in vitro* models have been introduced to study the cellular and molecular aspects of the response of cells and tissues of the central nervous system (CNS) to mechanical injury [1]. *In vitro* systems offer several advantages over *in vivo*, whole-animal models for studying mechanical injury to the nervous system, including the precise specification of loading parameters (strain and strain rate), control of the extracellular environment (temperature, ion concentration, partial pressure of gases), easy and repeated access to the cells, and simplified administration of pharmacological compounds. By precisely controlling loading conditions, the quantitative relationship between prescribed injury severity and injury response can be examined.

Brain tissue, like most other biological materials, is a nonlinear, viscoelastic material [2]. Increasing evidence suggests that the biological response of single living cells mimics the mechanical behavior of the tissue, i.e., the response of the cell is strongly influenced by the magnitude and rate of the mechanical stimulus. For example, the resting potential of the giant squid axon was transiently depolarized in response to a uniaxial strain, and as the rate of loading increased, the time required for recovery also increased [3]. Similarly, a post-stretch increase in intracellular calcium concentration ($[Ca^{++}]_i$) in neuronal-like cells has been shown to be dependent on both strain and strain rate of the stretch [4–6]. These mechanically dependent changes are not unique to neurons. Cultured astrocytes exhibited similar post-stretch Ca^{++} dynamics [7]. Cell injury, as measured by lactate dehydrogenase (LDH) activity or nucleic acid staining using cell impermeable dyes, has been shown to be dependent on strain and strain rate, as well [5,6,8–10]. Taken together, these data suggest that some functional responses of CNS-derived cells to stretch appear to mimic the viscoelastic properties of the tissue.

Although it can occur in less than a second, the mechanical event that causes injury initiates an extended cascade, which may develop over minutes to hours or over a much longer period. For example, a transient disruption of Ca^{++} homeostasis may be an early event in a series of aberrant signaling cascades that ultimately leads to cell dysfunction or death. One extended consequence of this molecular cascade may be changes in gene expression that are necessary for cell recovery or cell death. Increases in expression for several immediate early genes (IEG) including *c-fos*, *c-jun*, junB, and zif/268 [11–14] have been documented in various *in vivo* models of TBI, suggesting that many other genes are potentially regulated after trauma as well. Indeed, the differential, post-traumatic expression of several genes has been explored *in vivo*, including brain-derived neurotrophic factor (BDNF) [15,16], nerve growth factor (NGF) [17,18], caspase-1, caspase-3 [19], tumor necrosis factor α (TNF-α) [20], interleukin 1β (IL-1β) [21], and bcl-2 [22,23].

In this study, we hypothesized that: (1) Mechanical stretch of an organotypic brain slice culture would cause the differential regulation of certain genes, and (2) that this differential regulation would be a graded response dependent on mechanical parameters of that stretch. An organotypic brain slice culture preparation was chosen to maintain the complex three-dimensional architecture and connectivity between heterogeneous cell populations to mimic as closely as possible the *in vivo* situation both mechanically and biologically. Twenty-four hours after *in vitro* stretch, the expression of several genes was differentially regulated, and the expression of a subset of genes was correlated with stretch parameters. These data are the first to correlate changes in gene expres-

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sion in brain tissue with a precisely controlled and measured mechanical injury and offer further insight into the molecular consequences of traumatic brain injury.

Materials and Methods

Organotypic Brain Slice Cultures. Cultures were grown in custom-built wells, which have been described previously [24]. In brief, the wells consisted of a 60-mm-dia stainless steel well with an 18-mm-dia hole in the center. A Sylgard membrane 380 μm (0.015 in.) thick (Specialty Manufacturing, Saginaw, MI) 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) and 200 μg/ml poly-L-lysine (Sigma, St. Louis, MO), in sterile, deionized water in an incubator (Fisher Scientific, Pittsburgh, PA), with 5 percent CO_2 at 37°C. The wells were then rinsed sequentially with 2 ml of sterile, deionized water and Neurobasal media (Gibco).

The brain of a four-day-old Sprague-Dawley rat pup was aseptically removed (routinely within 2 minutes after decapitation) and sectioned coronally at 350 μm with a McIlwain Tissue Chopper (Brinkmann Instruments, Westbury, NY). The brain was transferred to ice-cold Gey's salt solution (Gibco) with 6.5 percent glucose, and the slices carefully separated with a finely polished spatula. Slices were individually transferred to an assembled and coated well and allowed to attach before the addition of 1.5 ml of Neurobasal media supplemented with 6.5 percent glucose and B-27 Supplement (Gibco) and then transferred to a rocker (Elmeco Engineering, Rockville, MD). The cultures were fed twice weekly with 1.5 ml of Neurobasal media supplemented with 6.5 percent glucose and B-27 Supplement and were maintained *in vitro* for 18 days before stretch. Cultures were generated from a total of 5 different litters and 43 pups, of which 20 were male.

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 forth in the Guide for the Care and Use of Laboratory Animals, U.S. Department of Health and Human Services, Publication Number 85-23, 1985.

Histology. After 18 days *in vitro*, uninjured cultures were fixed in 10 percent neutral buffered formalin (Sigma) for 30 minutes and then processed in an automated tissue processor (Shandon Hypercenter XP, Shandon Scientific Instruments, Cheshire, UK). The tissue was embedded in paraffin and sectioned on a rotary microtome (Leica, Malvern, PA) at 6 μm in either a coronal or transverse plane and mounted on slides before being dewaxed and re-hydrated. To examine the distribution of cells throughout the thickness of the cultured tissue, sections were cut in the transverse plane through the cultures and stained with hematoxylin and eosin. To confirm the presence of different cell types normally found *in vivo* within the cultures, immunohistology was performed for cell-type specific markers. Coronal sections processed for immunohistology were blocked with 2 percent normal horse serum (NHS, Hyclone, VWR Scientific Products, West Chester, PA) in 1×phosphate buffered saline (PBS, 100 mM phosphate, 0.9 percent NaCl, pH 7.4) and then stained for three different antigens: glial fibrillary acidic protein (GFAP) to identify astrocytes, neurofilament (NF-M) to identify neurons, and cyclic nucleotide phosphatase (CNP) to identify oligodendrocytes. The antibody to GFAP (G-9269, Sigma, St. Louis, MO) was a rabbit polyclonal antibody used at 1:1000. The antibody to NF-M (RMO 44) [25] was a gift from Dr. John Q. Trojanowski (Department of Pathology, University of Pennsylvania, Philadelphia, PA) and was a mouse monoclonal used at full strength. The antibody to CNP was also a mouse monoclonal (Clonetech, Palo Alto, CA) and used at 1:1000. Primary antibodies were individually applied to the tissue and incubated for 24 hours at 4°C. After rinsing in 1×PBS, the appropriate secondary antibody (biotin conjugated anti mouse IgG for both the RMO 44 and CNP or biotin conjugated anti rabbit IgG for GFAP, both from Jackson Immuno Research Laboratories, West Grove, PA) was applied for 2 hours in 2 percent NHS in 1×PBS. After rinsing in 1×PBS, the tertiary antibody (streptavidin conjugated horseradish peroxidase, Jackson Immuno Research Laboratories) was applied for 2 hours in 2 percent NHS in 1×PBS. Negative controls were performed without primary antibodies. Visualization was accomplished by incubating the tissue in 0.05 percent diaminobenzadine in 1×PBS, 1 mM imidizole, and 0.03 percent H_2O_2 for 10 minutes followed by a thorough rinsing. Tissue sections were then dehydrated, coverslipped, and photographed on a Leitz microscope (Leica, Allendale, NJ) with a digital camera (SoundVision, Farmingham, MA).

Mechanical Stretch Device. The device allowed for the independent specification of critical mechanical parameters of stretch including maximum substrate strain, strain rate, relaxation rate, and duration [24]. At the time of injury, the culture well was bolted to a heated brass plate; together they formed a vacuum chamber. Cultures were stretched by deformation of the substrate on which they had adhered by the application of a vacuum to the underside of the membrane as depicted in Figs. 1 and 2(*A*). This displacement generated a non-equal biaxial strain over the radial extent of the membrane. Membrane strain was not directly measured in this study; however, a mathematical model of the membrane deformation has been previously presented and validated, which allowed for the calculation of the complete strain field from the displacement of the center of the membrane [24]. Therefore, a laser displacement transducer (Omron Electronics, Schaumberg, IL) was employed to measure the dynamic displacement of the center of the membrane so that peak membrane strain could be calculated. Strain rate was calculated from the strain data. Representative data traces from an injury are shown in Fig. 2(*B*). Cultures were removed individually from the incubator and attached to the device while temperature was maintained at 37°C. To avoid potential fluid shear forces, the medium was removed from the well immediately prior to stretch, and it was replaced immediately following stretch.

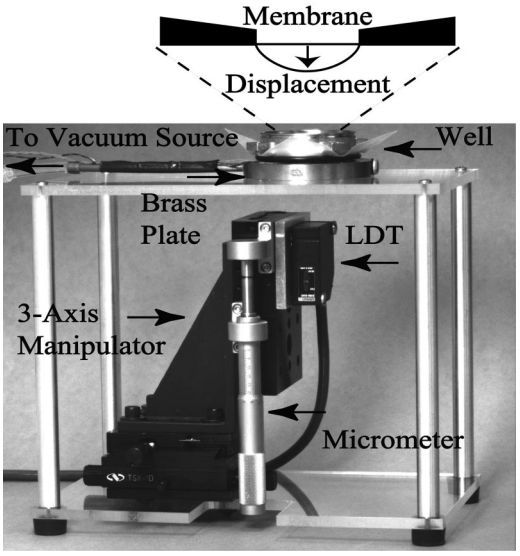

Fig. 1 An annotated picture of the Mechanical Stretch Device demonstrates its major components. A laser displacement transducer (LDT), mounted on a three-axis manipulator, measures the dynamic displacement of the membrane, allowing for calculation of the peak membrane strain. The organotypic brain slice cultures are grown in custom-built wells on a silicone membrane that is deformed by an applied vacuum as is depicted in the exploded view illustration.

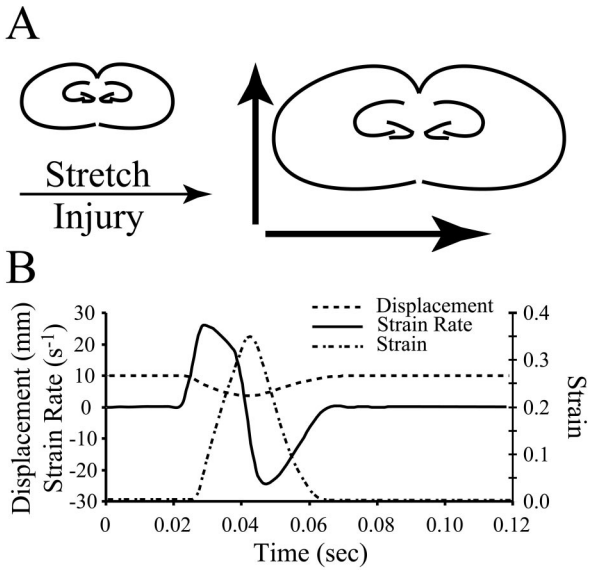

Fig. 2 (*A*) A schematic representation of the deformation of an organotypic brain slice culture during the dynamic stretch. (*B*) Representative data traces including displacement, strain, and strain rate acquired from the laser displacement transducer during the dynamic displacement of the culture substrate, i.e., the silicone membrane. This particular event was terminated in approximately 40 ms with a strain of approximately 0.35 and a strain rate of approximately 25 s^{-1}.

A total of 41 cultures were each stretched once for the gene expression analysis under aseptic conditions over a continuum of levels at the following parameters ($\pm$SD): $0.0 \leqslant$ strain $\leqslant 0.47$, mean 0.16 ± 0.09; $0 \leqslant$ strain rate $\leqslant 22\ s^{-1}$, mean $6.0\ s^{-1} \pm 6.1\ s^{-1}$; and $0 \leqslant$ relaxation rate $\leqslant 21.3\ s^{-1}$, mean $6.2\ s^{-1} \pm 6.2\ s^{-1}$. This wide range of values was deliberately chosen to analyze the correlation between stretch parameters and post-stretch gene expression. For the gene expression studies, a total of seven cultures were subjected to sham stretch stimuli that consisted of placing the culture on the stretch device without firing the device. After stretch or sham stretch, the well was returned to the incubator until cultures were harvested 24 hours after stretch.

A second set of cultures was used to measure cellular response as determined by lactate dehydrogenase (LDH) release. A total of six cultures was injured at the following stretch parameters: strain $= 0.40 \pm 0.08$, strain rate $= 20.22 \pm 4.18\ s^{-1}$, and relaxation rate $= 28.39 \pm 6.79\ s^{-1}$. A total of five cultures, different from those used as controls for the gene expression data, was subjected to sham stretch. Media samples (10 μl) were taken at 6, 24, and 48 hours after stretch, and the level of LDH release was measured as described below.

Reverse Northern Hybridization. At 24 hours after stretch or sham stretch, cultures were individually homogenized in 1 ml of RNA-STAT (Tel-Test ''B,'' Inc., Friendswood, TX) and total RNA was isolated per the manufacturer's instructions. The final pellet 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 [26–31]. In the current study, amplified and radioactively labeled RNA from a single sample was hybridized to a single array. 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) and a specific poly$(dT)_{24}$-T7 primer at 37°C for 1 hour (20 μl reaction volume, 50 mM Tris, 120 mM KCl, 10 mM dithiothreitol (DTT), 6 mM $MgCl_2$, 250 μM dNTPs, pH 8.3, 20U RNAsin (Promega, Madison, WI)). 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 hours (Boehringer Mannheim, Indianapolis, IN) 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 minutes (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) at 37°C for 4 hours (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 3000Ci/mmol (NEN Life Science Products, Boston, MA), pH 7.5). This aRNA was used to probe a slot blot.

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

The membranes were removed from their bottles and washed sequentially at 50°C for 1 hour each in 2×SSPE, 0.1 percent SDS, and then 0.1×SSPE, 1 percent SDS. Membranes were then wrapped in plastic wrap and apposed to a PhosphorImager cassette (Molecular Dynamics, Sunnyvale, CA) for 48 hours 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). The bax, bcl-2, and bcl-x_l clones were gifts from K. I. Strauss (Temple University, Philadelphia, PA). The protein kinase $C_{1\beta}$ ($PKC_{1\beta}$) clone was a gift from E. Slosberg (Columbia University, New York, NY). The β2 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, Conshohocken, PA). The cAMP response element binding protein (CREB) clone was a gift from M. R. Montminy (Salk Institute, La Jolla, CA). The ubiquitin clone was a gift from L. M. Schwartz (University of Massachusetts, Amherst, MA). The TrkA, TrkB, NGF, BDNF, cyclooxygenase 2, α subunit of calcium/calmodulin dependent kinase II ($CKII_\alpha$), and GAD_{65} clones were gifts from J. H. Eberwine (University of Pennsylvania, Philadelphia, PA). The GFAP clone was a gift from A. Brooks-Kayal (Children's Hospital of Philadelphia, Philadelphia, PA). The cyclophilin clone was purchased from ATCC (Bethesda, MD) and is dbEST #295733 from the I.M.A.G.E. consortium as was the β-actin clone dbEST #292226 and the GAPDH clone dbEST #295897. Specific sequence information regarding each clone used in this study is given in Table 1. The pUC18 plasmid (Clonetech, Palo Alto, CA) was chosen as a negative control and was used to account for any nonspecific hybridization signal.

Quantification of Gene Expression. The level of expression of a particular gene on a given blot was determined as follows with the aid of the Analytical Imaging Station software (Imaging Research, Ontario, Canada). From the digitized image, for a single

Table 1 Specific information regarding each clone utilized in this study, including the size of the insert, the species, and the Genbank accession number. The last column indicates that bases of the Genbank sequence were in the plasmid insert.

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^{++} 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
		Housekeeping		
β2 microglobulin	328	Rat	Y00441	277-604
β-actin	1143	Rat	H31602	Partial
Cyclophilin A	685	Rat	H35109	Partial
GAPDH	494	Rat	H35273	Partial
		Others		
APP695	2822	Human	X06989	33-2854
COX-2	4154	Rat	S67722	1-4154
GAD_{65}	1966	Rat	M72422	1-1966
GFAP	646	Rat	U03700	485-1130

sample, the expression of a particular gene 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 samples, the level of expression of a particular gene was divided by the average level of expression of all 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 [27]. Typically a single housekeeping gene, which is assumed to be expressed at a constant level regardless of experimental condition, is utilized to normalize the expression of all other genes. However, after injury, even traditional housekeeping genes may be differentially regulated. The alternate normalization scheme used for these experiments was chosen for two reasons. First, normalizing to an internal control allowed for comparisons to be made between samples regardless of the amplification efficiency in the aRNA amplification procedure. Second, normalizing to the average expression of all genes was less sensitive to either directed or random fluctuations in the expression of any single gene.

LDH Release. To assess the degree of plasma membrane permeability caused by mechanical stretch, the level of LDH, an intracellular enzyme, was measured in the culture media. Samples of media (10 μl) were taken 6, 24, and 48 hours after stretch and assayed for levels of LDH according to the manufacturer's instructions (Procedure 500, Sigma). In brief, media samples were incubated at 37°C for 30 minutes with NADH and pyruvate. A color reagent was then added to the samples and incubated at room temperature for 20 minutes, at which time the color was developed by the addition of 0.4N NaOH. The absorbance of the resulting color product was measured on a spectrophotometer (Perkin Elmer, Norwalk, CT) at 464 nm, and LDH values were calculated from a standard curve. LDH values were then normalized to the total releasable LDH values obtained after addition of Triton-X 100 to the culture media at a final concentration of 0.2 percent for 2 hours.

Statistical Analyses. All statistical analyses was performed on Statistica (Statsoft, Tulsa, OK). LDH release data were analyzed first by a one-way ANOVA (Control versus Injured) to determine the overall effect of stretch. A series of *post hoc* Scheffe tests were then performed to determine at which time points stretch caused LDH release.

To determine which genes were differentially regulated after *in vitro* stretch, a one-way MANOVA (Sham versus Stretch) was first performed on the data to determine the overall effect of stretch. To determine which specific genes were affected, *post hoc* comparisons for each dependent variable were performed. It was unclear what, if any, steps were necessary to control the experiment-wise error rate, α, which was initially set at $\alpha = 0.05$, since a total of 22 different *post hoc* tests would be performed, one for each gene. Applying a Bonferroni adjustment would have set $\alpha = 0.05/23 \sim 0.002$, which seemed overly conservative. A study by Hummel and Sligo examined the effect of group size and number of variables on the experiment-wise error rate when either ANOVA or MANOVA was applied to multivariate data [32]. Their analysis demonstrated that if one applies only ANOVA tests to multivariate data, the experiment-wise error rate can be greatly inflated (as high as $\alpha = 0.20$). However, if ANOVA tests were performed after a significant MANOVA analysis, the experiment-wise error rate was slightly more conservative than that specified. Both the Bonferroni and the Hummel and Sligo methods were employed for data interpretation.

To examine the possibility that gene expression was correlated with mechanical stretch parameters, a multiple regression was performed for each gene on strain and strain rate. A point was considered to be an outlier if its Mahalanobis distance was greater than 12.23, which is appropriate for a data set with two predictors (strain and strain rate) and approximately 50 observations [33]. In a similar manner, data points with a Cook's distance close to 1 were considered outliers. Any outliers were eliminated from the analysis.

Results

Stained transverse sections demonstrated that the cultured tissue remained several cell layers thick (approximately 275 μm) with cells distributed throughout the thickness (Fig. 3(*A*)). The presence of astrocytes, neurons, and oligodendrocytes within the organotypic brain slice cultures was confirmed by specific immunohistological staining for GFAP (Fig. 3(*B*)), NF-M (Fig. 3(*C*)), and CNP (Fig. 3(*D*)), respectively. No staining was observed in negative control sections. Astrocytes demonstrated a typical star-shaped morphology with short thick processes. Neurons demonstrated large nuclei and proximal portions of processes and were found in anatomically preserved structures such as the hippocampus. Oligodendrocytes were observed throughout the tissue, as well as within white matter tracts such as the alveus, which were maintained in these cultures.

All cultures remained attached to the membranes at all times including after stretch, indicating that there was no slippage between the culture and the membrane. Because each culture was well-adhered to the membrane, we assumed that each culture experienced the same strain history as the membrane during stretch. Although the strain field was not equi-biaxial over the radius of the membrane, maximum center strain was used as a measure of injury level. In addition, only cultures that were centered on the membrane were injured.

In vitro, stretch of organotypic brain slice cultures resulted in the significant release of LDH into the media as compared to control cultures at all time points after stretch (6 h, $p < 0.05$; 24 h, $p < 0.01$; 48 h, $p < 0.001$) as shown in Fig. 4. This significant increase in LDH release caused by injury was superimposed on a lower basal release of LDH in both control and injured cultures. Such LDH release over time is normally observed in primary cultures of CNS tissue [34,35].

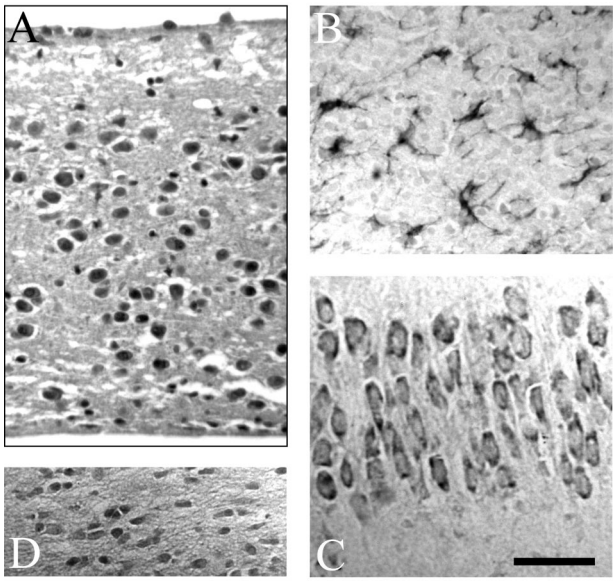

Fig. 3 Photomicrographs of organotypic brain slice cultures after 18 days *in vitro* demonstrating the presence of various cell types. (*A*) A transverse section cut through the culture and stained with H & E demonstrates that the cultures remain several cell layers thick (approximately 275 μm) with cells dispersed throughout the thickness. The lower surface was adhered to the membrane and the upper surface was bathed in media. All other sections shown (B–D) were cut in a plane parallel to the membrane. (*B*) Cells stained for GFAP demonstrated a typical astrocytic morphology of a starlike shape with short, thick processes. (*C*) Neurons stained for NF-M throughout the cultures as well as in anatomically defined structures such as the CA3 region of the hippocampus. Neurons in this region demonstrated the typical morphology of pyramidal neurons with large nuclei and apical processes. (*D*) Oligodendrocytes stained for CNP and were concentrated within white matter tracts such as the alveus. These cells were typically smaller than other cells and closely apposed to adjacent cells in a linear fashion. Scale bar represents 50 μm in all panels.

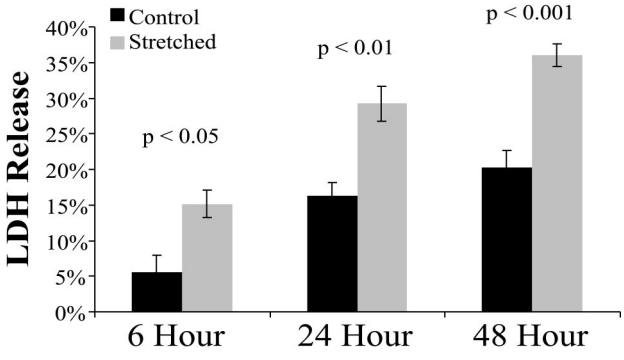

Fig. 4 LDH release was measured at 6, 24, and 48 hours after stretch or sham stretch and calculated as a percentage of total releasable LDH. At each time point, stretched cultures released significantly more LDH than did control cultures, indicating that dynamic substrate strain resulted in membrane damage. Both control and stretched cultures demonstrated a basal level of LDH release that is normally observed in primary cultures [34,35]. For the control group $n=5$, and for the stretched group $n=6$. Values are presented as mean ±SEM.

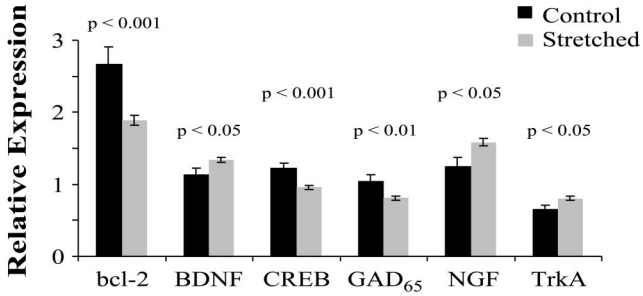

Fig. 5 Stretch of organotypic brain slice cultures affected the expression of several genes. Twenty-four hours after stretch the expression of bcl-2 ($p<0.001$), CREB ($p<0.001$), and GAD_{65} ($p<0.01$) was significantly decreased, whereas that of BDNF ($p<0.02$), NGF ($p<0.02$), and TrkA ($p<0.05$) was significantly increased over expression in control cultures. For the control group $n=7$, and for the stretched group $n=41$. Values are presented as mean ±SEM.

A MANOVA analysis of post-stretch gene expression demonstrated a significant overall effect of stretch on gene expression ($p<0.05$). A subsequent ANOVA analysis demonstrated that the following genes were differentially regulated after stretch: bcl-2 ($p<0.001$), BDNF ($p<0.02$), CREB ($p<0.001$), GAD_{65}($p<0.01$), NGF ($p<0.02$), and TrkA ($p<0.05$) as shown in Fig. 5. Although the remaining genes analyzed were expressed at levels quantifiable by the RNH technique, none was differentially regulated after stretch including APP695, β_2 microglobulin, β-actin, bax, bcl_{xl}, $CKII_{\alpha}$, cyclophilin, cyclooxygenase-2, GAPDH, GFAP, IL-1β, IL-6, $PKC_{1\beta}$, TNF-α, TrkB, and ubiquitin.

To determine whether the expression of any gene was correlated with stretch parameters, a multiple regression analysis was performed for each gene on strain and strain rate. No data points were considered to be outliers as determined by their Mahalanobis or Cook's distances. A significant multiple regression was found for APP695, CREB, and GAD_{65} as shown in Table 2. Standardized regression coefficients (regression coefficients after variables were adjusted to a mean of 0 and a variance of 1) were calculated for the genes that demonstrated significant correlation with stretch parameters (APP695, CREB, and GAD_{65}) to determine the loading on strain and strain rate as shown in Table 2 in the column labeled β. The Pearson correlation coefficient and significance level for the multiple and simple regression analyses are also given in Table 2.

Table 2 Results of the multiple regression and simple regression analyses of gene expression and mechanical parameters are tabulated for those genes that demonstrated a significant multiple regression. The loading of this relationship on Strain and Strain Rate is given by the standardized regression coefficients in the column labeled β. App695 was negatively correlated with strain rate, whereas CREB and GAD_{65} were negatively correlated with strain. As indicated by the correlation coefficients (R^2), these correlations were not particularly strong. No data points were determined to be outliers by either their Mahalanobis or Cook's distances.

Gene	Regression	β	R^2	$p<$
APP695	Multiple		0.17	0.01
	Strain	0.061	0.01	0.70
	Strain Rate	**−0.430**	**0.17**	**0.01**
CREB	Multiple		0.17	0.01
	Strain	**−0.440**	**0.15**	**0.01**
	Strain Rate	0.125	0.00	0.40
GAD_{65}	Multiple		0.11	0.05
	Strain	**−0.343**	**0.05**	**0.02**
	Strain Rate	0.269	0.02	0.07

Discussion

This study is the first to correlate quantitative changes in gene expression in brain tissue with biomechanical parameters associated with a mechanical stretch. Twenty-four hours after a single mechanical stimulus of organotypic brain slice cultures, mRNA expression for several genes was differentially regulated, including an increase of BDNF, NGF, and TrkA message and a concomitant decrease of bcl-2, CREB, and GAD_{65} message. The level of gene expression of APP695, CREB, and GAD_{65} was significantly correlated with stretch parameters. APP695 expression was negatively correlated with strain rate while CREB and GAD_{65} expression was negatively correlated with strain. However, the multiple regression could account for no more than 17 percent of the variability in the expression data, indicating that the relationship between gene expression and stretch parameters was not particularly strong,

The organotypic brain slice culture preparation utilized for these studies offers several advantages over dissociated cell cultures. Typically, *in vitro* preparations consist of dissociated cells of homogenous or heterogeneous populations. During the harvest procedure, the cells are mechanically or enzymatically dissociated, thereby severing connections between cells and disrupting any three-dimensional architecture or spatial arrangement of cells. An organotypic brain slice culture preparation presents an attractive alternative that maintains the morphological organization of the tissue as well as the local interconnections between cell types in a three-dimensional matrix several cell layers thick. This heterogeneous population of neurons, astrocytes, and oligodendrocytes allows for realistic interactions between different cell types, and our data demonstrate that mechanical stretch of organotypic brain slice cultures results in cell injury as measured by LDH release. By maintaining this high level of biological complexity in the culture, this tissue preparation in conjunction with the stretch device [24] may represent an *in vitro* system that reproduces the *in vivo* situation of TBI both mechanically as well as biologically.

Utilizing other non-CNS cell types and different loading conditions, the expression of several genes has been shown to be correlated with parameters of a mechanical stimulus. For example, *c-fos* expression in vascular smooth muscle cells was correlated with the degree of uniaxial stretch (0–20 percent) applied to the culture substrate [36]. The expression of transforming growth factor $\beta 1$ in mesangial cells demonstrated a similar correlation with biaxial substrate strain from 5 to 20 percent [37]. In an isolated mesenteric artery preparation, expression of *c-myc* was correlated with wall stress as opposed to strain [38]. Others have found that gene expression can be differentially regulated depending on the level of mechanical stretch, although an attempt to correlate expression with stretch was not made. For example, tissue plasminogen activator has been shown to be up-regulated with 10 percent but not 6 percent stretch in endothelial cells [39]. In a similar manner, a shear stress of 36 dynes/cm^2 but not of 15 dynes/cm^2 in endothelial cells increased the expression of basic fibroblast growth factor [40], and a shear stress of 25 dynes/cm^2 increased expression of C-type natriuretic peptide over 4 dynes/cm^2 [41] in endothelial cells. Although gene expression appears to be regulated by mechanical factors in cells that are normally exposed to a mechanically active environment (e.g., the circulatory system), the ability to respond to mechanical stimuli may be a universal property of all tissues, including brain. However, in contrast to our hypothesis, this regulation may not be highly correlated with the mechanical stimulus in a tissue that is normally protected, in this case, by a rigid skull. Alternately, although upstream events in the mechanotransduction cascade, such as changes in $[Ca^{++}]_i$, may be correlated with stretch parameters, downstream events such as changes in gene expression at 24 hours post-stretch may be more of an all-or-none phenomenon acting as a molecular switch.

One possible consequence of mechanical injury of the brain could be the aberrant activation of the molecular pathways responsible for mechanotransduction. This activation could, in turn, be responsible for a portion of the secondary cellular dysfunction and damage associated with TBI such as apoptotic cell death [42]. For example, both bcl-2 and NGF are involved in cell survival. Increases in NGF expression may represent an endogenous response of the injured tissue to protect itself by attempting to supplement trophic support. However, NGF's trophic actions are mediated in part by the transcription factor CREB. After mechanical stretch to our organotypic brain slice cultures, we observed that the expression of CREB is decreased, suggesting that the intracellular pathways responsible for NGF's actions could be impaired. Although NGF may be present in sufficient concentrations to sustain healthy cells, increased NGF expression may not be sufficient to sustain injured cells that may die by apoptosis due, in part, to trophic factor withdrawal. Cells may also initiate the apoptotic cascade by additional mechanisms. The fate of a cell has been hypothesized to be dependent on the ratio of cell-life to cell-death proteins such as bcl-2 and bax, respectively [43]. Decreases in bcl-2 expression with concomitant maintenance of bax expression could shift the intracellular ratio of life and death proteins in favor of initiation of apoptosis and cell death. By unraveling the molecular consequences of TBI, new therapies could be rationally designed for the head-injured patient.

Changes in gene expression are likely to be a universal response of biological tissues or cells to mechanical stimuli. Studies in other cell types normally exposed to mechanical stimuli have demonstrated that the expression of certain genes is correlated with parameters of a mechanical stimulus. In this study, we have demonstrated that specific genes are differentially regulated in cultured brain tissue after a single mechanical stimulus, and that the post-traumatic expression of some of these genes was weakly dependent on the biomechanical parameters of that stretch. These data are the first to correlate changes in gene expression in brain tissue with a precisely controlled and measured mechanical injury and offer further insight into the molecular consequences of traumatic brain injury. However, these downstream, delayed changes (24 hours) may be components of an all-or-none molecular switch as opposed to the more highly correlated, early cellular responses such as changes in $[Ca^{++}]_i$ or immediate early gene expression.

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