Gene Expression Profiles of Neurotrophic Factors in Rat Cultured Spinal Cord Cells under Cyclic Tensile Stress

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Gene Expression Profiles of Neurotrophic Factors in Rat Cultured Spinal Cord Cells under Cyclic Tensile Stress

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Running head: Cyclic tensile stress to cultured spinal cord cells

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*** Structured Abstract (300 words)**

Study Design. An experimental study to investigate the *in vitro* gene expression of neurotrophic factors and receptors in cultured rat spinal cord cells subjected to cyclic mechanical stretch forces.

Objective. We evaluated *in vitro* expression of neurotrophic factors and receptors in cultured rat spinal cord cells under cyclic tensile stress.

Summary of Background Data. Application of compressive mechanical stress to the spinal cord results in various changes making it difficult to examine the expression of neurotrophic factors and their receptors. There are no *in vitro* studies that investigated the biological responses of cultured spinal cord cells to tensile stress.

Methods. Spinal cord cells were isolated for culture from 15-day Sprague-Dawley rat embryos. We used the FX3000® Flexercell Strain Unit to induce mechanical stress. We analyzed the effects of mechanical stress on cell morphology, mRNA expression levels of various neurotrophic factors and their immunoreactivities at 0, 2, 6, 12, 24 and 36 hours.

Results. Tensile stress for 6 hours resulted in reduction of spinal cord cells and loss of neurites. Cells that survived 24 hrs-stress showed swollen irregular-shaped soma, bleb formation, and fragmented neurites. The cell survival rate decreased while lactate dehydrogenase release increased significantly at 6 hours. There were significant increases in nerve growth factor, brain-derived neurotrophic factor, trkB, p75 neurotrophin receptor ($p75^{NTR}$), glial cell line-derived neurotrophic factor, and caspase-9 mRNA expressions during the early period after application of tensile stress.

Conclusion. Our results suggest survival of spinal cord neuronal cells under injurious tensile stress with increased synthesis and utilization of several neurotrophic factors and receptors, as well as expression of proteins related to cell apoptosis.

Mini-Abstract:

Exposure of cultured rat spinal cord cells to cyclic tensile stress resulted in increases in expressions of nerve growth factor, brain-derived neurotrophic factor, trkB, p75 neurotrophin receptor ($p75^{NTR}$), glial cell line-derived neurotrophic factor, and caspase-9 mRNAs at early time after tensile stress application.

Introduction

The spinal cord and neurons are always subjected to mechanical stress including tensile stresses, and during the spine movement, such stresses may be applied to the spinal cord in a very complex manner in association with subsequent symptomatic manifestation.[1,2] In addition, mechanical stress, a tensile stress, to the spinal cord may ultimately cause motoneuron dysfunction and axonal degeneration.[3,4] Longitudinal vertebral distraction and the physiological tension zone[5] of the spinal cord are closely correlated with each other when the spine is subjected to flexural position[6,7] and excessive kyphosis in the thoracic vertebrae.[8] Tensile stress applied to the spinal cord is potentially critical and linked to subsequent neuronal damage, but it is extremely difficult to estimate the isolated effect of tensile stress in the *in vivo* experimental setting.

Application of mechanical force on the spinal cord results in loss of function in some neurons while others show increased metabolic activity to resist injury. It is conceivable that mechanical stress of the spinal cord induces both neuronal survival and repair, or cell death. Recent studies of experimental spinal cord damage in animals examined the function as well as source and dynamics of induction of endogenous neurotrophic factors, including brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and their receptors,[9-15] that are essential for neuronal survival and repair as well as neurite outgrowth and arborization. However, in these experiments, excess mechanical stress could cause tissue or cell reactions such as activation of glial cells or invasion of foreign cells from the periphery. It could also induce various pathological events and release of inflammatory mediators that could

positively or negatively influence spinal cord function.[16] The complexity of the *in vivo* situation may result in a limited accessibility to the tissue of interest, preventing real-time and spatial measurement of biological or mechanical parameters.[17] Thus, to gain a better understanding of the neuronal response to spinal movement and physiological state, *in vitro* models of spinal cord stress could perhaps allow a better control of the extracellular environment, easy and perhaps repeated access to the cells, and help elucidate the mechanisms of response to mechanical stimuli.

To our knowledge, there are no studies that examined the biological and immunohistochemical responses of spinal cord cultured cells to mechanical tensile stress. Flexercell Strain Unit (FX3000®, Flexercell International, Hillsborough, NC) is a cell-stretching apparatus that allows application of cyclic tensile force to the cultured cells. The system has been used to elucidate the mechanism of mechanical signal in various types of cells.[18-20] The present study was designed to assess the effect of cyclic mechanical tensile force on the expression and synthesis of neurotrophic factors and their receptors in cultured spinal cord cells with the use of this equipment.

■Materials and Methods

Cell Cultures. Primary cultures were established using the method described previously.[21,22] In brief, spinal cords of Sprague-Dawley rat embryos were dissected out at post-coital day 15 and stripped of the dorsal root ganglia and the meninges. Dissected tissues were rinsed with cold Ca^{+2}- and Mg^{+2}-free Hanks balanced salt solution (HBSS) supplemented with 4 g/L glucose, and incubated at 37°C for 20 minutes with 0.03% (w/v) trypsin solution in HBSS with mild shaking. They were

transferred into HBSS containing 0.1% (w/v) soybean trypsin inhibitor (Sigma, St. Louis, MO) and 0.2% (w/v) bovine serum albumin (BSA), and triturated very mildly. The cell suspension was filtered through nylon mesh (70 μm, Cell Strainer; Becton Dickinson, Bedford, MA). The culture medium consisted of 75 mL Leibovitz's L-15 medium supplemented with glucose (4 g/L), 1.0 mL N2 supplement, 15 mL 0.15 M sodium bicarbonate, 10 mL heat-inactivated horse serum, 1 mL of 100 mM L-cysteine and 1 mL penicillin G 10^4 U/mL and neutralized with CO_2. After centrifugation at 400 x *g* for 15 minutes at 4°C, precipitated cells were gently re-suspended in a fresh culture medium and plated at a density of 4.0 x 10^5 cells/well onto a 6-well culture plate with a flexible-polystyrene bottom coated with type IV collagen (BioFlex® Baseplate, Flexercell International).

The experiment was carried out in the Orthopaedic Spinal Cord Laboratory of Fukui University. The experimental protocol strictly followed the Ethics Review Committee Guidelines for Animal Experimentation of our University Medical Faculty.

Application of Tensile Stress to Cultured Spinal Cord Cells. The cell stretching device used in this study was Flexercell FX3000® (Flexercell International). The device consists of a computer-controlled vacuum unit (Figure 1**A**), a culture plate with a flexible-polystyrene well-bottom coated with type IV collagen (BioFlex® Baseplate) (Figure 1**B**), and another culture well plate with a non-deformable culture well bottom constructed of the same material. The culture plates consisted of 6-well (35 mm diameter) flexible-bottomed culture plate with a hydrophilic surface. Application of vacuum provides a hemispherically downward deforming force to the flexible bottom,

resulting in a non-homogenous strain profile with a maximum at the periphery and a geometric decline toward zero at the center of the culture well bottom. The cells were placed in the culture well plates at an average density of 4.0 x 10^5 cells/well. For these experiments, spinal cord cells in the culture were subjected to cycles of 1 second of a maximum 12% elongation (vacuum level, 10.3 kPa). The flexible-bottomed culture plates including the control plates were then placed on the vacuum baseplate in the incubator (37ºC in 5% CO_2). After three days of cell seeding, cells were subjected to cyclic stretch stress for 48 hours. Repeated examinations by phase microscopy (IX70, Olympus, Tokyo, Japan) showed that the cells remained attached to the substratum during elongation. The cells were observed morphologically following application of tensile stress and various assays, quantification of mRNA expression of neurotrophic factors, and immunoreactivity were conducted at 0, 2, 6, 12, 24 and 36 hours after the application of tensile stress.

Assessment of Cell Survival and Cell Damage. To determine possible cellular damage due to tensile stress, cell survival was examined by manual cell counting and by measurement of lactate dehydrogenase (LDH) release at 0, 2, 6, 12, 24, 36 hours after the application of tensile stress. Calcein-acetoxymethyl ester (calcein AM: 3.00 μM) was used to identify living cells (Live/Dead Assay, Molecular Probes, Eugene, Oregon). Calcein-AM is a membrane-permeable dye that is cleaved by intracellular esterase to produce an impermeant green-wavelength fluorophore in living cells.[23,24] The culture medium was removed and the cells were then washed twice with PBS, and stained for 75 minutes at 32ºC. The numbers of attached living (green) cells in at

least 6 high-power fields (each containing at least 100 cells) were counted using fluoromicroscopy (IX70, Olympus) and a color image analyzer (MacSCOPE, Mitani, Fukui, Japan) in more than three wells for each time point. The cell survival rate (%) was calculated relative to the cell number at 0-hour. Because spinal cord cells do not proliferate, the cell counts were almost uniform and at a density from 3.3 x 10^5 to 4.8 x 10^5 cells/well during 3 days after dissemination on Bioflex® Baseplate in the absence of tensile stress.

LDH, a stable enzyme present in the cytoplasm of all cells, is rapidly released into the culture medium following damage to the plasma membrane. The culture medium was sampled at the aforementioned time points after mechanical stimulation and analyzed using the CytoTox-ONE™ kit (Promega, Madison, WI). This assay has been used to discriminate between apoptotic and necrotic cell death.[25,26] Following incubation of the cells with the reagent, each reaction was stopped by stop solution provided with the kit to prevent further generation of fluorescent product. LDH release was assessed using a chemiluminescence imaging analyzer (IS-8000-OH, Alpha Innotech, San Leandro, CA) in more than three wells for each time point. LDH release was almost uniform in the absence of stress during 2 days after dissemination on Bioflex® Baseplate. LDH release rate (%) following mechanical stress was calculated per volume, and expressed relative to that at 0 hour.

Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR). The gene expressions of nerve growth factor (NGF), BDNF, NT-3, NT-4/5, trkA, trkB, trkC, p75 neurotrophin receptor ($p75^{NTR}$), glial cell line-derived neurotrophic factor

(GDNF), GDNF family receptor (GFR)-α1, caspase-3, and caspase-9 were examined by real-time PCR at each time point after mechanical stimulation. Briefly, the cultured cells on each well were disrupted in a lysis buffer containing β-mercaptoethanol and total RNA was purified using RNeasy® Mini Kit (Qiagen, Valencia, CA) and treated with DNase I (Takara Biomedicals, Kyoto, Japan). Reverse transcription was performed using 1 μg of total RNA, AMV reverse transcriptase XL (Takara Biomedicals, Ohtsu, Japan) and random primer. Real-time PCR was performed on PRISM 7000 (ABI) using 1 μl of the synthesized cDNA and SYBR Green PCR master mix (Applied Biosystems, Foster, CA). Table 1 lists the primer sequences used in this study. The target genes were amplified and analyzed in triplicate using ABI Prism 7000 SDS Software (Applied Biosystems). The expression levels of target genes were revised with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at each time, and the relative expression of target genes were calculated relative to that at 0 our.

Immunohistochemical Analysis. Neuronal survival under cyclic tensile stress was determined by manual cell counting of NeuN-labeled cells using a fluorescence microscope. A round flexible bottom well (35 mm in diameter) was divided into six sectors; the cutting bottom attached cultured cells was used as a section during the immunocytochemical procedure. After application of tensile stress, the cultured spinal cord cells were washed twice with PBS and fixed in 2% paraformaldehyde for 15 minutes, then incubated at room temperature for 10 minutes in BlockAce (UK-B25, SnowBrand, Tokyo) to prevent nonspecific reactions. Cells were processed for

immuno-cytochemical detection of NeuN by incubation with mouse antibody to NeuN (1:400; Chemicon International, Temecula, CA) for 20 hours at 4ºC, followed by incubation with goat anti-mouse Alexa Flour® 488/fluorescein-conjugated antibody (1: 400; Molecular Probes, Eugene, OR) for 1 hour at room temperature. Sections were counterstained with nuclear marker DAPI (Abbott Molecular, Des Plaines, IL). The immunostained cells were visualized under a fluorescence microscope (AX80, Olympus) with U-MNIBA cube (BP460-490 nm excitation and BA515-550 nm emission) and U-MWU cube (BP330-385 nm excitation and BA420 nm emission). DAPI-labeled and double-labeled cells in at least 6 high-power fields (magnification, x 100), each containing at least 10 cells, were counted in more than three wells for each time point. The ratio of DAPI-labeled cell count and NeuN-labeled cell count was calculated automatically, which represented neuronal survival rate (%), at each time using a color image analyzer (Mitani).

For identification of immunoreactivity for neurotrophic factors and their receptors in neurons, following incubation with mouse NeuN antibody and goat anti-mouse Alexa 488, the cells were incubated with rabbit antibody to NGF (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), or with rabbit antibody to BDNF (Chemicon International) or with rabbit antibody to $p75^{NTR}$ (1:400; Santa Cruz Biotechnology) or rabbit antibody to GDNF (1:400; Santa Cruz Biotechnology). Cells were subsequently incubated with goat anti-rabbit antibody Alexa Flour® 594/fluorescein-conjugated antibody (1: 400; Molecular Probes) and counted using a confocal microscope equipped with a 15-mWatt crypton argon laser (model TCS SP2, Leica Instruments,

Nusslosh, Germany). The 488- and 543-nm lines of an argon/helium-neon laser were used for fluorescence excitation.

Statistical Analysis. All values are expressed as mean±SEM. Differences between values at particular time and those of the corresponding control were tested by one-way ANOVA and Tukey post hoc test. A *P* value less than 0.05 denoted the presence of a statistically significant difference. The above tests were conducted using SPSS software version 11.0(SPSS, Chicago, IL).

■Results

Effects of Cyclic Tensile Stress on Cell Morphology and Cell Survival

Under phase contrast microscopy, the control spinal cord cells (no application of tensile stress) had smooth oval-shaped cell soma (Figure 2**A**). Application of tensile stress for ≥6 hours decreased the number of spinal cord cells and resulted in loss of neurites, compared to baseline findings (Figure 2**B**). Application of tensile stress for 24 hours resulted in swelling of the remaining cells with irregularly shaped cell soma and appearance of several blebs within the cells, together with fragmentation of some neurites (Figure 2**C**).

The cell survival rate as assessed by manual counting of the number of living cell decreased in a time-dependent manner under cyclic tensile stress, from 74±22% at 2 hours to 53±16% at 6 hours, 48±11% at 12 hours, 40±12% at 24 hours, and 38±7% at 36 hours (Figure 3**A**). The decrease in the number of living cells became significant after 6 hours. On the other hand, LDH release in the culture medium was increased at

6 hours, reached a plateau over the subsequent 12 hours (Figure 3**B**). The time course of changes in LDH release correlated with the morphological changes seen in mechanically-stressed cells.

Effects of Cyclic Tensile Stress on Neuronal Survival

Among DAPI-labeled cells, 71% cells were positive for NeuN before the application of tensile stress 3 days after plating. Neuronal survival rate decreased in a time-dependent manner when cells were under cyclic tensile stress, from 71±10% at 0 hour (before application of tensile stress), to 40±15% at 2 hours of application, 22±9% at 6 hours, 18±7% at 12 hours, 12±4% at 24 hours, and 10±3% at 36 hours, compared to non-stress cells at each time (Figure 4).

Effects of Cyclic Tensile Stress on mRNA Expression Levels

Time-dependent mRNA expression of NGF, BDNF, NT-3, NT-4/5, trkA, trkB, trkC, $p75^{NTR}$, GDNF, GFRα-1, caspase-3, and caspase-9, was examined in neuronal-rich cultures subjected to mechanical stress (Figure 5). The mRNA expression levels of NGF, BDNF, and GDNF significantly increased at an early period of mechanical stress, while NT-3 and NT-4/5 mRNA levels remained the same throughout the application of the cyclical force. NGF and GDNF mRNA levels started to increase 2 hours after the application of stress, reached at peak levels at 6 or 12 hours and gradually declined until 36 hours. However, changes in BDNF mRNA level were relatively small, though significant; i.e., an increase was detected first at 6 hours and persisted until 24 hours after mechanical stress. The mRNA expression levels of trkB

and $p75^{NTR}$, but not those of trkA, trkC, GFR-α1, were significantly up-regulated, compared to the control. The $p75^{NTR}$ mRNA level started to rise somewhat later; 6 hours after stress application, attained maximum level at 12 hours after stress, and gradually decreased until 36 hours. The mRNA expression level of caspase-9 was significantly increased at 2 or 6 hours and gradually declined until 24 hours; while that of caspase-3 increased in the early period after stimulation, but then decreased subsequently and no difference with the control was noted at later time intervals.

Effects of Cyclic Tensile Stress on NGF, BDNF, $p75^{NTR}$, and GDNF Expression in Spinal Cord Neurons

While NeuN-labeled cells decreased gradually in number in a time-dependent manner, NGF, BDNF, $p75^{NTR}$, and GDNF immunoreactivities increased soon after the application of tensile stress on primary spinal cord cells. Immunoreactivities to NGF, BDNF, $p75^{NTR}$ and GDNF were increased at 6 hours under cyclic tensile stress and co-localized with the majority of the diminishing NeuN-positive cells (Figure 6). These results demonstrate that surviving neurons subjected to mechanical stress synthesized some neurotrophins and their receptors.

■Discussion

Several researchers have used *in vitro* models of mechanical trauma to the central nervous system, but studies on neuronal cells appeared to be limited to the usage of immortalized cell lines, such as NG108-15 [27,28] and PC12 [29,30]. These studies indicated that mechanical stretch disrupted ionic homeostasis, [27] increased cell membrane

permeability,[29] and disrupted membrane integrity followed by neuronal loss or release of LDH. Pfister *et al*[28] used glioma cell line and reported that stretch-injury resulted in overexpression of Bcl-2 family (NG108-15) followed by neuronal cell death. However, the use of neuronal cell lines as *in vitro* injury model is associated with one major disadvantage. Although some are derived from neuronal cells, these cell lines consist of immortalized or cancerous cells with the ability to divide uncontrollably, suggesting that their pattern of gene and/or protein expression may be significantly different from the finally differentiated, functioning neurons. Hence, although limited to the immature or developing spinal cord, we used primary cultured cells and the method requires no treatment and retains the biomechanical and molecular fidelity of spinal cord cells *in vivo*.

Previous studies showed that subjecting the cells to relatively high non-physiological strain induced axonal injury and neuronal cell death.[27-29] These data of physiological strain on neuronal cells also varied probably due to the use of different equipments. However, recent studies examining the effect of *in vitro* mechanical stress on neuronal cells indicated that mild neurotrauma induces secondary mechanisms that ultimately lead to differentiation of neurons in mixed cortical cultures,[31] and that the effect of micro-texture on neurite outgrowth is more prominent under low than high mechanical stress.[32] Based on the early studies, we selected the tensile stress most appropriate to our culture system in a series of preliminary experiments. However, our results showed morphological changes of vacuole formation within the cell soma and shrinkage of neurite arborization and release of LDH during the time course of tensile stress application. Unexpectedly, our results

also indicated a decrease in neuronal survival rate from 71% at 0 hour to 12% at 36 hours, even under lower physiological mechanical condition. In primary cultures, isolated neurons may be more susceptible to mechanical damage than astrocytes or other cells.[17] Further studies are warranted on the condition of culture cells (time, concentration, and others), material coating of the well bottom during stretch injury necessary to maintain a higher survival rate of spinal cord neurons.

Neurotrophins are required for neuronal survival and influence neurite elongation during development.[33] The expression of NGF family (NGF, BDNF, NT-3, and NT-4/5) and their receptors (trkA, trkB, and trkC) under mechanical compression may be essential for maintaining cell survival mechanism as well as prevention of cell death. We have been also keen to know the capacity of mechanically injured spinal cord to restore its function.[3,4,13,14,34-36] In the present study, NGF, BDNF, trkB mRNA expression levels increased significantly at an early time period following application of tensile stress, but NT-3, NT-4/5, trkA, trkC mRNA levels showed no significant elevation throughout the experiment. Expression of NGF, BDNF, and trkB genes is dependent on neuronal activity, and changes in intracellular Ca^{2+} homeostasis activate these signaling pathways.[37,38] Disruption of intracellular Ca^{2+} homeostasis after mechanical tensile stress[27] may induce overexpression of NGF, BDNF, and trkB mRNAs for survival and prevention of cell death. Our results showed that NGF and GDNF gene expression levels were similar to those reported previously.[15,39] While it has been shown that GDNF is a neurotrophic factor for motoneurons and central nervous system neurons,[40,41] it is also expressed in glial cells especially in activated microglia/macrophages of injured neural tissue.[42] Although neuronal-rich culture cells

(71% of NeuN-positive cells) were used in our experiment, other cells including glial cells, with the exception of neurons, could affect our results because this culture has a heterogeneous cell population. NT-3 is also known to play a role in neurite elongation,[13,34] thus in the present study, cultured neuronal cells required NGF and BDNF as well as GDNF but not NT-3 or, presumably, NT-4/5 peptides.

Apoptotic and necrotic cell death after mechanical injury occurs through several and different pathways in neuronal tissue. The $p75^{NTR}$ is a neurotrophin receptor that can bind to all neurotrophins at equal affinity in most cells but it binds with a higher affinity to the proform NGF in neurons.[43] While the function of $p75^{NTR}$ remains elusive, it is known to promote cell survival either in association with tyrosine kinase receptors or by itself.[44] Paradoxically, its activation has also been shown to promote apoptotic cell death in neurons and oligodendrocytes, and $p75^{NTR}$-induced cell death follows the intrinsic apoptotic death pathway with release of cytochrome C from the mitochondria and activation of caspase-9.[45] Our preliminary results on $p75^{NTR}$ and caspase-9 gene expression profiles are in agreement with these reports. On the other hand, caspase-3 increased after stimulation but the difference from the control was not significant. One possible explanation for this finding is that since caspase-3 is activated by both the intrinsic and extrinsic pathways, it is possible that upregulation of the intrinsic pathway may reduce apoptosis through the extrinsic pathway. Further studies are warranted to examine the roles of $p75^{NTR}$ and caspase mRNAs or proteins in the apoptotic pathways activated by tensile stress in the spinal cord. At this stage, however, there is virtually no information on the roles of $p75^{NTR}$ in spinal cord cells under tensile stress.

In conclusion, our results suggest that spinal cord neuronal cells survive by increasing the synthesis and utilization of several neurotrophic factors and their receptors under injurious tensile stress. Changes in the expression of these genes at an early period after mechanical stress suggest the readiness of spinal cord neurons to undergo apoptosis or necrosis. A close examination of the effects of mechanical stress on spinal cord cells *in vitro* may be the key to elucidating the adaptation of spinal cord to mechanical tensile stress.

*** Key Points (3-5 main points of the article)**

Key Points:

- We investigated the *in vitro* effects of cyclic tensile stress on cultured spinal cord cells, especially the expressions of neurotrophins and their receptor genes.
- Increased expression levels of NGF, BDNF, trkB, $p75^{NTR}$, GDNF, and caspase-9 were evident at 6 hours after application of tensile stress.
- Cyclic tensile stress increased mRNA expression and immunoreactivities of several neurotrophic factors and their receptors, and induced morphologically-confirmed necrotic cell death and increase in LDH release.

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Tables

Table 1. Sequences of Primers used for real-time PCR.

Target Protein	Forward Primer	Reverse Primer	PCR Product Size (bp)	Sequence Accession No.
NGF	5'-CCATGTGGTTCCTGATCCTGTTC-3'	5'-TCCAACAACCCGAGACTGGAC-3'	83	NM031523
BDNF	5'-TCCTGATAGTTCTGTCCATTCAGCA-3'	5'-GCCATTCATTCAGGCTTCCA-3'	93	NM012513
NT-3	5'-CATGTCGACGTCCCTGGAAATAG-3'	5'-TGGACATCACCTTGTTCACCTGTAA-3'	82	NM031073
NT-4/5	5'-GAGGTGGAGGTGCTGTTGAC-3'	5'-TCCCACTCAGGAGCCAGAA-3'	150	NM013184
trk A	5'-CAAGATGCTGGTGGCTGTCAA-3'	5'-AGCAGCTCTGCCTCACGATG-3'	81	NM021589
trk B	5'-CCTTGACCGATCTGGCTTCTGTA-3'	5'-TAGTTGTGGTGGGCAAACTGGA-3'	107	NM012731
trk C	5'-CATGAAGCATGGAGACCTGAACA-3'	5'-ACCATGCCGGAGGCTATCTG-3'	147	NM019248
$p75^{N1R}$	5'-AGGGCTGGTCCATTGGTCTATTC-3'	5'-TTAAGGGCCGTGTTGGCTTC-3'	132	NM012610
GDNF	5'-CCGGACGGGACTCTAAGATGA-3'	5'-GTCAGGATAATCTTCGGGCATATTG-3'	194	NM019139
GFRα-1	5'-GGGACGCTTTGGTGTCTGAA-3'	5'-CCAGGTACACTTGGATGTTGGATG-3'	132	NM012959
caspase-3	5'-GCAGCAGCCTCAAATTGTTGACTA-3'	5'-TGCTCCGGCTCAAACCATC-3'	144	NM012922
caspase-9	5'-CTGAGCCAGATGCTGTCCCATA-3'	5'-CCAAGGTCTCGATGTACCAGGAA-3'	168	NM031632
GAPDH	5'-GGCACAGTCAAGGCTGAGAATG-3'	5'-ATGGTGGTGAAGACGCCAGTA-3'	143	NM017008

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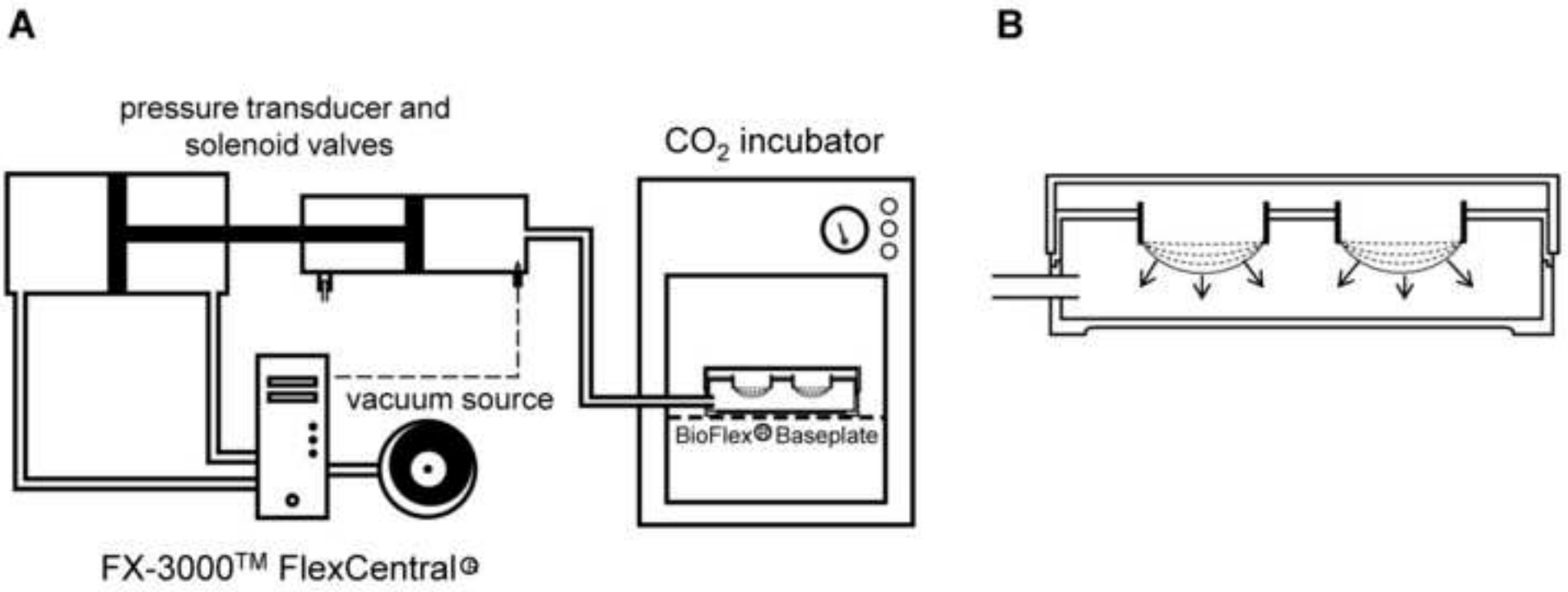

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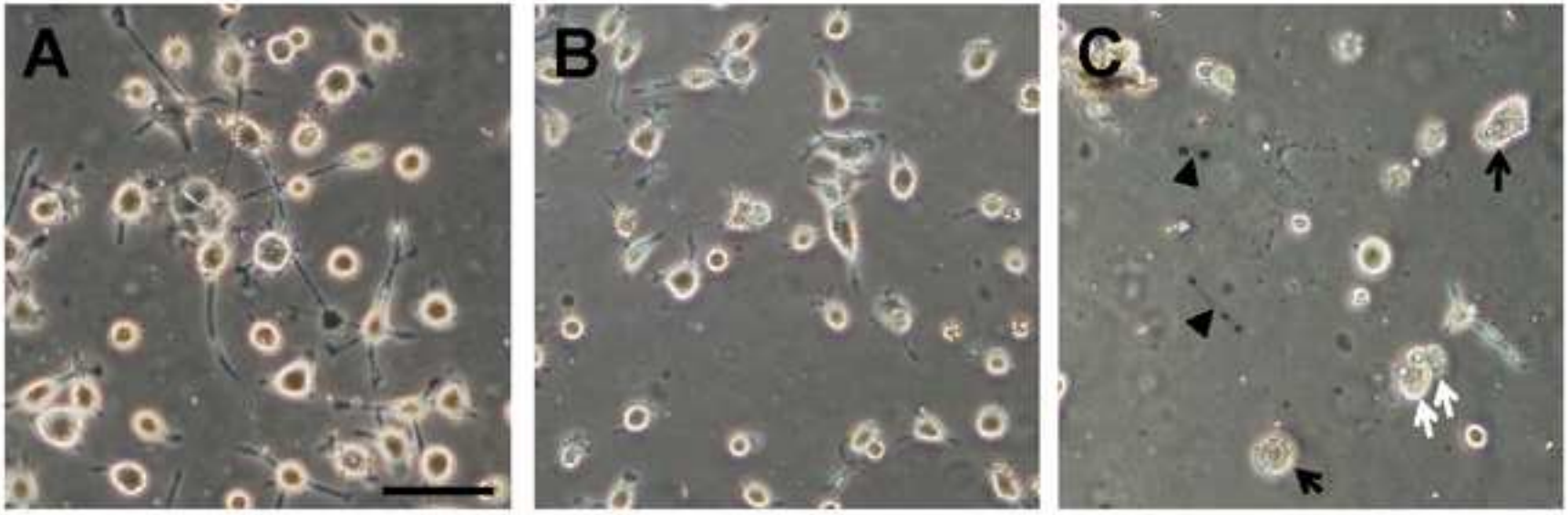

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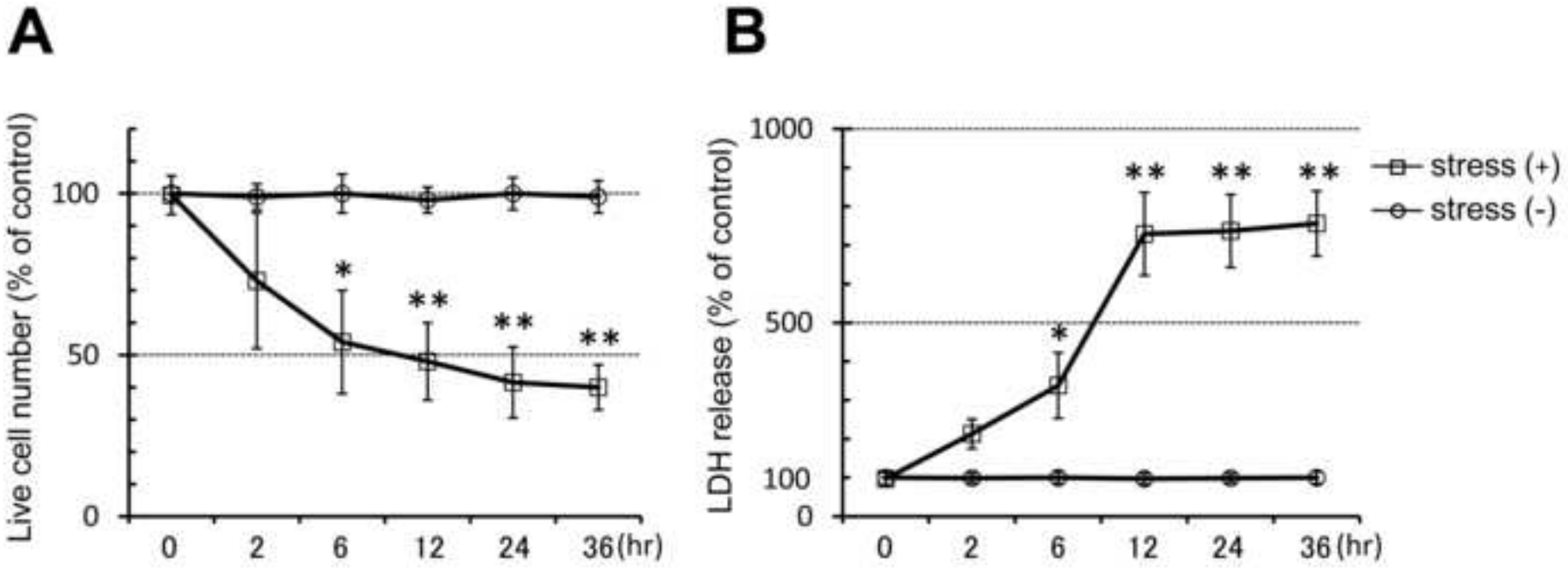

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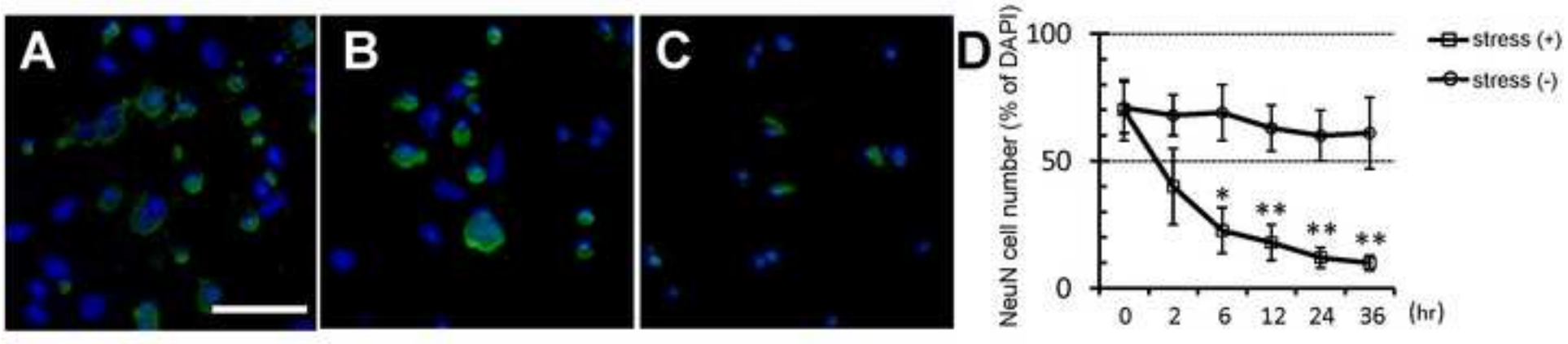

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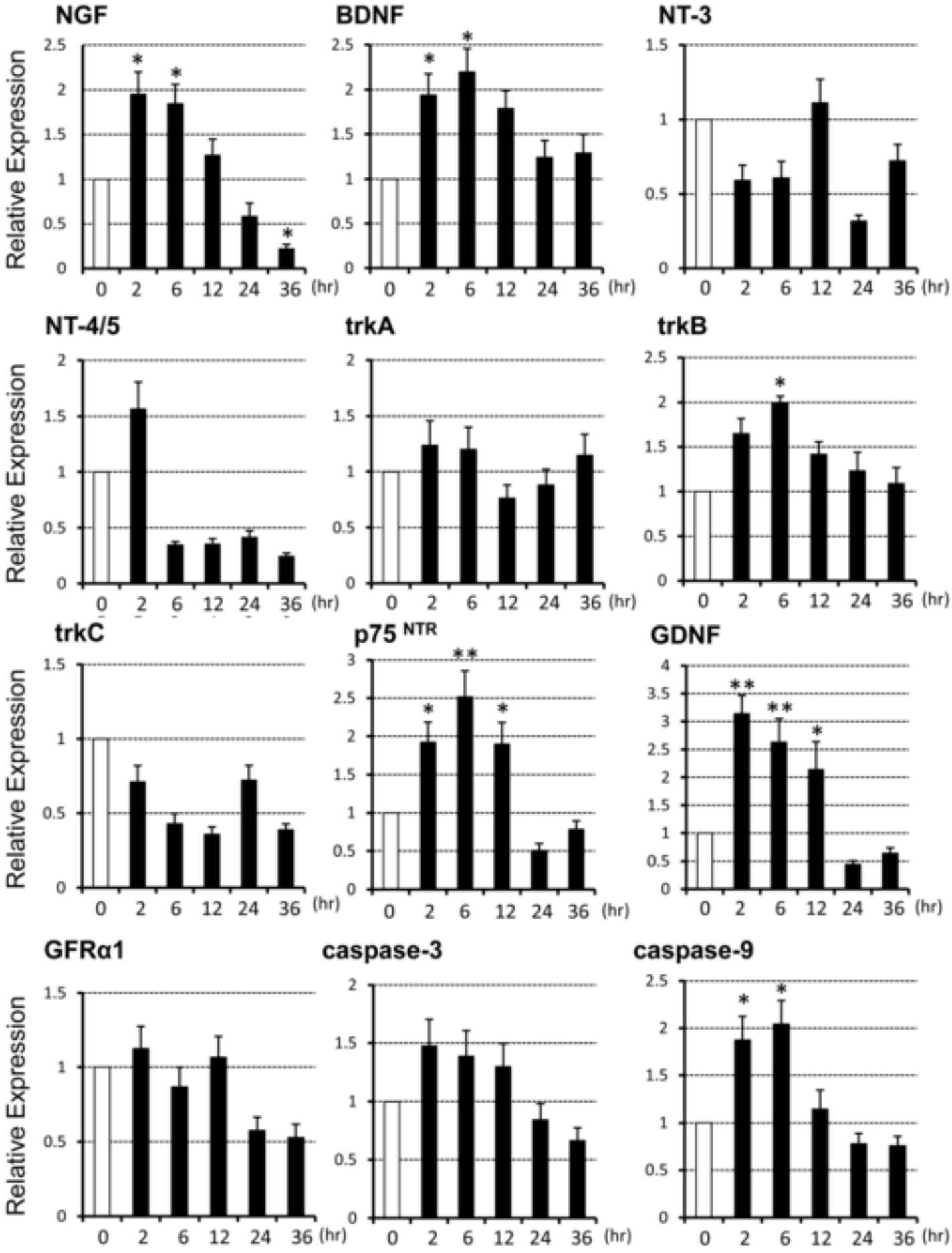

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