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Original Research

Neuronal survival of DRG neurons after neurite transection *in vitro* promotes by nerve growth factor and brain derived neurotrophic factor

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Abstract: Neurotrophic factors are growth factors that promote neuronal survival, regulate synaptic function and neurotransmitter release, and promote the plasticity and growth of axons in the peripheral and central nervous systems. This study focused on the roles of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in the survival of adult dorsal root ganglion (DRG) neurons following axotomy. To investigate this, we cultured adult mouse DRG neurons and administered NGF or BDNF to the culture medium at different doses before transection. After determining the optimal doses of NGF and BDNF, these factors were then applied in combination. Axotomy was performed using a precise laser beam and neuronal death was visualized through cell observer microscopy system, by adding propidium iodide to the culture medium. The results demonstrate that the optimal doses of NGF and BDNF for neuronal survival are 150 ng/mL and 50 ng/mL, respectively. The highest level of neuronal survival was observed in the cells treated with a combination of NGF and BDNF. In conclusion, NGF and BDNF have a positive effect, both individually and in combination, on the survival of DRG neurons following neurite transection.

Key words: Dorsal root ganglion; NGF; BDNF; Neuronal survival; Axonal injury.

Introduction

Neurotrophic factors, also known as neurotrophins, are growth factors that contribute to nerve regeneration and have triggered effects on neuronal survival. Neurotrophins are secretory peptides that play a critical role in the development of the nervous system and in plasticity in the adult (1, 2). Neurotrophins play important roles in protecting neural functions and repairing injuries by promoting neuronal survival, axonal growth and differentiation, synaptogenesis, and synaptic function (3-5). Much research has explored neurotrophins because of the role they play in supporting neuronal survival, regeneration, and the proliferation and maturation of certain types of neurons. Neurotrophins have therapeutic effects in mood disorders (6) and neurodegenerative diseases, including Parkinson's disease (7), Alzheimer's disease (8),and Huntington's disease (9). Neurotrophin family members include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and neurotrophin 4/5 (NT4/5). These factors bind to two different classes of transmembrane receptor proteins, Trks (tropomyosin receptor kinases) and the neurotrophin receptor p75. All neurotrophins bind to the p75 receptor with a low affinity while there are differences in the affinity for Trk receptors between neurotrophins; NGF binds to TrkA with a high affinity; BDNF and NT4/5 bind to TrkB with a high affinity; NT3 binds to TrkC with a high affinity (4, 10).

NGF was the first signaling protein to be identified. It was discovered by Rita Levi-Montalcini and Viktor Hamburger in the 1950s in mouse sarcoma cultures *in vitro* (11). NGF was found to promote the growth of sensory and sympathetic neurons in the chicken embryo (11). Subsequent studies have demonstrated that NGF plays an important role in the survival and maturation of developing neurons in the peripheral nervous system, in addition to the effects it has in the central nervous system. NGF is expressed in the brain, with the highest level in the hippocampus. In the CNS, NGF has a neuroprotective effect and can effect neural responses to injury in different cell types (12, 13). In addition to the nervous system, hematopoietic stem cells (14), neonatal cardiac myocytes (15), pancreatic cells (in the embryo and adult) (16, 17), and salivary glands (18) become sources of and targets for NGF. All of these findings indicate that NGF plays a very important role in the development of neurons as well as supports regeneration and survival after a lesion, most likely through the inhibition of apoptosis. A high expression of NGF in the nervous system and many other tissues does not only enhance tissue regeneration but also supports proliferation and functional recovery. After NGF, BDNF is the second best characterized member of the neurotrophin family that is expressed in the adult mammalian central nervous system, with a high level of expression observed in the hippocampus and cerebral cortex (19). The high level of expression of BDNF in the hippocampus (20) supports the hypothesis that this neurotrophin is involved in memory and learning. BDNF was first purified from the pig brain. This protein complex has been shown to promote the survival of cultured embryonic chicken spinal sensory neurons (21). BDNF has both neuroprotective and growth-promoting effects on different neural popu-

lations after injury. Additionally, BDNF can promote the regeneration and remyelination of injured axons (12) and plays an important role in neuronal survival and differentiation, as well as the outgrowth of neurons during development and in adulthood (6). Furthermore, a decrease in the expression of BDNF has been linked to mood disorders, including major depressive disorder and bipolar disorder (6), as well as several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and Huntington's disease (13).

In research, the creation of damage in the nervous system of living animals is a common practice that involves sciatic, optic nerve and spinal cord transection and cortical wounding (22-25). These *in vivo* applications can explain some of the neuronal responses to neuritic injury. However, these applications can be inadequate in revealing some of the cellular changes and cell death that occur in injury. Additionally, these *in vivo* applications do not permit researchers to manipulate the cellular environment. In addition to *in vivo* injury models, there are several *in vitro* methods through which nervous tissue elements can be damaged in controlled culture conditions. Some of these *in vitro* models involve mechanical impact (26), using micro knives, scissors, blades, scalpels (27-32), glass capillaries (33), or water-jets (34). Another, easier, original, and more precise *in vitro* damage model is laser beam axotomy. This method was first used by Higgins and colleagues, who cultured a mixed population of cells from the embryonic mouse spinal cord, targeted the dendrite-like extensions originating from neurons, and damaged them using an UV laser (35-38). Cengiz and colleagues (2012) used a micro laser beam to cause damage in cultured dorsal root ganglion (DRG) cells *in vitro* (39). A laser was used to cause damage to the extended neurites without damaging other structures in the culture environment.

In the present study, we isolated the adult mouse DRG and cultured the neurons in high purity. We treated these cells with NGF, BDNF, and both two neurotrophins at different doses prior to making precise axotomy of the extended neurites with a micro laser beam. We investigated the effects of NGF and BDNF on neuronal survival in this *in vitro* model of injury.

Materials and Methods

Adult Balb-c mice, aged 6-12 weeks, were used in all of the experiments. Before removal of the DRGs, animals were anaesthetized by an I.P. injection of ketamine (100 mg/kg; Ketalar, Pfizer, İstanbul Turkey). Animals were sacrificed by cervical transection and L4-L5 DRGs were quickly and aseptically removed under a stereomicroscope. All of the attached nerves were trimmed from the DRGs in cold RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and the ganglions were then transferred to Neurobasal A medium (Invitrogen, Carlsbad, CA, USA), supplemented with 2% B27 (Invitrogen), containing: 2 nM Glutamax-I (Invitrogen), 100 units of penicillin, 100 mg streptomycin, 250 ng amphotericin B per ml (Sigma-Aldrich), and 100U/ml collagenase (Sigma-Aldrich). DRGs were incubated at 37 ^{0}C, in 5% CO_2, in an incubator for 40 min, then washed in Hank's buffered salt solution (Sigma-Aldrich) three times. The DRGs were then incubated with trypsin (1mg/mL; Sigma-Aldrich) in NBA-B27 for 15 min and triturated for about 15 min by gently and repeatedly pipetting through the tips of narrowing bores (from 2mm diameter down) and finally a 26-gauge injector needle. After trituration, DNAse (50µg/mL) (Sigma-Aldrich) was added to the cell suspension, followed by a 30 min incubation on a custom-made agitator that vibrated horizontally at 50 Hz. The neurons were then harvested in a pellet, by spinning the suspension at 120 g for 3 min, and resuspended in NBA-B27 supplemented with 10% fetal calf serum (Sigma-Aldrich) and 700 mg/mL trypsin inhibitor (Sigma-Aldrich). For selective isolation of the neurons, a gradient centrifuge technique was used. For this, the cell suspension was carefully pipetted on top of a three layer percol (Sigma-Aldrich) gradient (60, 35 and 10% from bottom to top) prepared with NBA-B27, centrifuged at 3000 *g* for 20 min and cooled down to 4 ^{0}C. The neurons that were collected from the 35% layer were washed with NBA-B27 and centrifuged once more at 120 *g* for 3 min. The supernatant was discarded and the pellet was re-suspended in NBA-B27. The cell suspension was seeded in 35 mm diameter glass-bottomed Petri dishes (WPI Inc., New Haven, CT, USA), which had been previously coated with poly-L-lysine (Sigma-Aldrich) (1.8µg/cm^2 2 h at RT) and laminin (Sigma-Aldrich) (40ng/mm^2, overnight at 37^{0}C). The preparations were kept in the incubator for 2 h, to let the neurons attach to the bottom of the dish, then filled with NBA-B27 and returned to the incubator (39). Forty-eight hours after incubation at different doses NGF, BDNF and both NGF and BDNF were added to the culture medium. The experiment was performed in nine experimental groups: control, 10 ng/mL NGF, 50 ng/mL NGF, 100 ng/mL NGF, 150 ng/mL NGF, 10 ng/mL BDNF 50 ng/mL BDNF, 100 ng/mL BDNF, and a combination of 150 ng/mL NGF and 50 ng/mL BDNF.

Microlaser Beam Axotomy

Within 6-8 h of incubation, the cultured DRG neurons began to regenerate axons that were long enough for a transection to be performed (Figure 1). After 48 h, suitable neurons were selected using a computer-controlled inverted microscope with a stage incubator that provided physiological conditions (Cell Observer; Zeiss, Oberkochen Germany) (40). Propidium iodide (7.5µM; Sigma) was added to the medium to enable the selection of viable cells for axotomy. The cell coordinates were recorded when a viable cell was identified. Before axotomy, the axons to be transfected were marked on the electronic images and the exact points of transection, at 200 µm distance from the perikaryon, were marked on the pointouts.

Axon transection of the selected neurons was per-

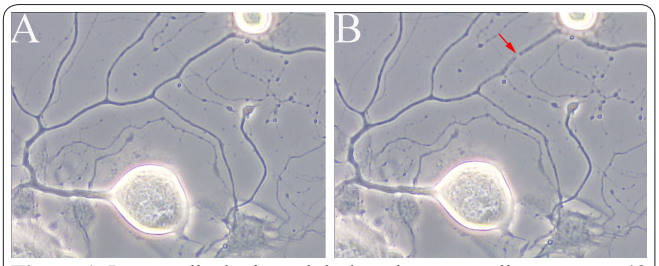

Figure 1. Images displaying adult dorsal root ganglion neurons 48 h after incubation; (A) before and (B) after laser beam axotomy.

formed using a laser microdissection system (PALM Microbeam, Zeiss, Germany) that was equipped with UV laser units that operated at 337 nm and produced 1-30 pulses per second. Axotomies were performed using a 63X dry phase contrast objective (LD Archoplan N.A. 0.75, Zeiss Germany). The transections were precise and formed a gap in the axon. During the axotomy, the cell cultures were removed from the incubator for no longer than 30 min to avoid any change in the pH of the medium (39, 41).

The preparations were transferred to a computer-controlled inverted microscope where phase-contrast and red fluorescence images of axotomize neurons were taken using a dry 20X objective. Images were obtained at several timepoints after the axotomy; immediately after the axotomy, 24 h and 48 h after axotomy.

Statistical Analyses

The statistical significance of the differences between the results from the experimental groups were evaluated through one way ANOVA. P values < 0.05 were considered to be statistically significant. All experiments were repeated five times.

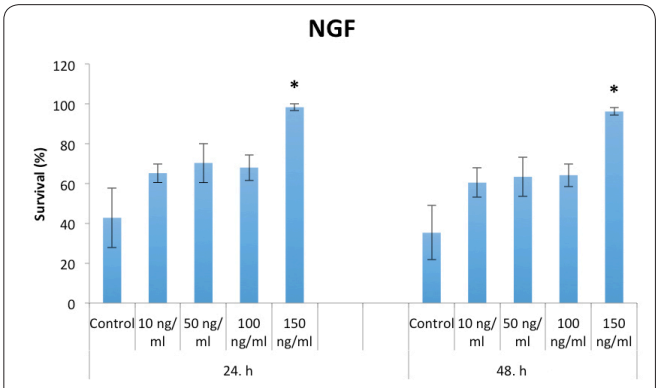

Figure 2. Neuronal survival of adult dorsal root ganglion neurons after axotomy following exposure to nerve growth factor (NGF). Cells were plated 24 and 48 h after axotomy. Results are expressed as means ± SEM. *p < 0.05.

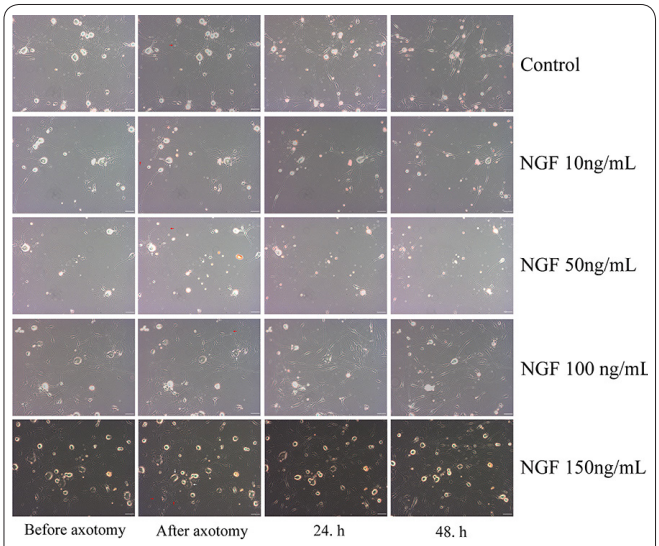

Figure 3. Nerve survival after axotomy following exposure to nerve growth factor (NGF; 10, 50, 100, and 150 ng/mL). Image indicates where the axon was cut, 200 µm away from the cell body (red arrow). Adult dorsal root ganglion neurons were stained with propidium iodide at 24 and 48 h after laser beam axotomy. Scale bars, 50 µm.

Results

We tended to observe higher means in all of the percentage viability values in the groups exposed to NGF than in the control group (Figure 2 and 3.). However, this increase was only significant at the dose of 150 ng/mL NGF (24 h, $P < 0.05$). When compared with the survival observed in the control group, cells that were exposed to NGF at different doses displayed increased survival at the 48 h timepoint. We determined that only the 150 ng/mL dose of NGF produced a significant increase in cell survival (Figure 2). Propidium iodide staining analysis showed that ability of dorsal root ganglion neurons survival in the experimental group than the control group at 24 and 48 h after laser beam axotomy (Figure 3).

Compared with the viability observed in the control group at 24 h following axotomy, it was determined that exposure to BDNF at 50 ng/mL and 10 ng/mL doses tended to increase viability, while BDNF at 100 ng/mL tended to decrease viability, compared to the viability observed in the control group. The difference in the percentage viability was only significant in the group exposed to 50 ng/mL BDNF. Although the viability tended to decrease in the group exposed to 100 ng/mL BDNF, the viability was not significantly different to that observed in the control group (Figure 4 and 5). At the 48 h timepoint after axotomy, an increase in cell viability was observed at doses of 10 ng/mL and 50 ng/mL BDNF, when compared with the viability observed

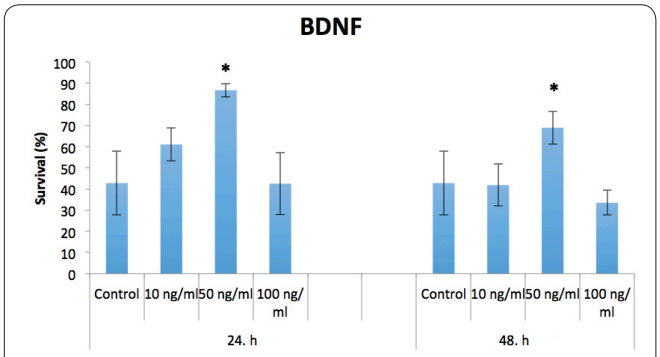

Figure 4. Neuronal survival of adult dorsal root ganglion neurons after axotomy following exposure to brain derived neurotrophic factor (BDNF). Cells were plated 24 and 48 h after axotomy. Results are expressed as means ± SEM. *p < 0.05.

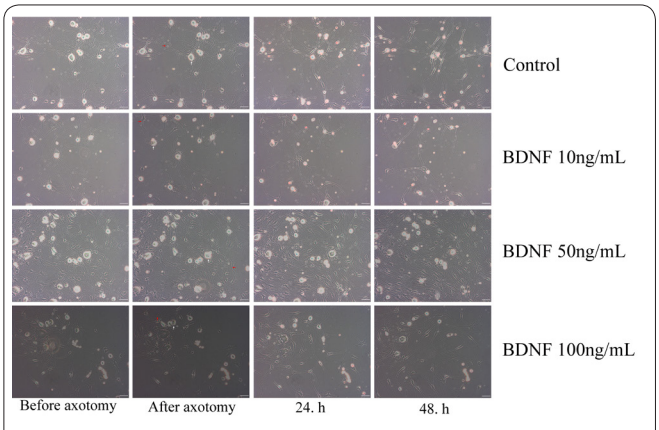

Figure 5. Nerve survival after axotomy following exposure to brain derived neurotrophic factor (BDNF; 10, 50, and 100 ng/mL). Image indicates where the axon was cut, 200 µm away from the cell body (red arrow). Adult dorsal root ganglion neurons were stained with propidium iodide at 24 and 48 h after laser beam axotomy. Scale bars, 50 µm.

in the control group. Conversely, at the 48 h timepoint after axotomy, a trend towards a decrease in viability was observed in the cells exposed to BDNF at 100 ng/mL. The cell viability was only significantly different to that observed in the control group in the group that was exposed to 50 ng/mL BDNF.

The cell viability at both the 24 and 48 h timepoints after axotomy was significantly higher in the group that was exposed to a combination of NGF and BDNF than in the control group. The cell viability observed in this group was not significantly different to the viability observed in the group that was exposed to 150 ng/mL NGF alone or the group that was exposed to 50 ng/mL BDNF alone. However, there was a higher level of cell viability in the group exposed to NGF and BDNF in combination than in the control group (Figure 6 and 7.).

Discussion

In this study, we aimed to search the effect of NGF and BDNF on neuronal survival after laser beam axotomy in a controllable and manipulated environment in vitro. The roles of neurotrophins in modulating the neuronal survival and function of neurons have affected our research.. There are many evidence for effects of trophic support of neurotrophins and it has been shown in both in vivo and in vitro experiments by exogeneous applications. Also neurotrophin withdrawal in sensory and sympathetic neurons has been searched and resulted loss cells through programmed cell death mechanisms (42). The main tasks of neurotrophins are the promotion of neuronal survival, proliferation, differentiation, myelination, axonal growth, and synaptic plasticity. Neurotrophins are known to contribute to axonal regeneration, in addition to all of these effects. The expression of neurotrophins increases in the distal part of the injured nerves, returning to normal levels following axonal regeneration and target innervation. This suggests that neurotrophins are directly or indirectly influenced by regeneration (4, 43). These factors increase their activation at certain times in the nerve cell cycle, as well as an increase in activation and levels of the damaged cells.

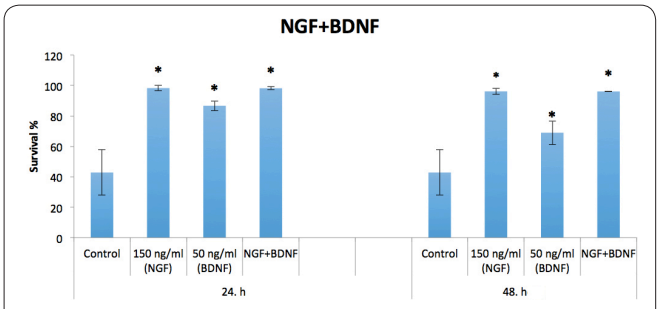

Figure 6. Neuronal survival of adult dorsal root ganglion neurons after axotomy following exposure to nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), or a combination of NGF and BDNF. Cells were plated 24 and 48 h after axotomy. Results are expressed as means ± SEM. *$p < 0.05$.

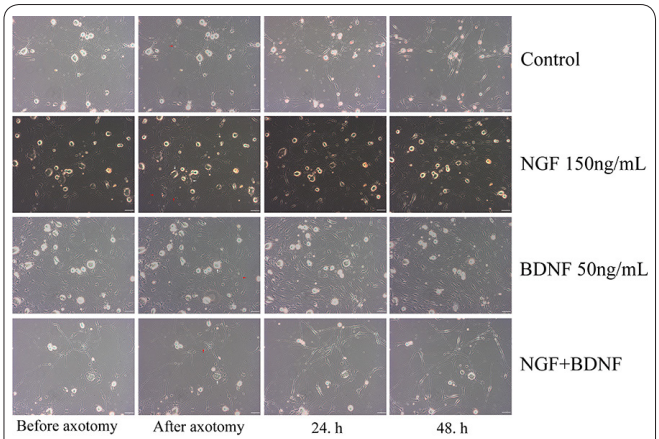

Figure 7. Nerve survival after axotomy following exposure to nerve growth factor (NGF; 150 ng/L), brain-derived neurotrophic factor (BDNF; 50 ng/L) or a combination of BDNF and NGF. Image indicates where the axon was cut, 200 µm away from the cell body (red arrow). Adult dorsal root ganglion neurons were stained with propidium iodide at 24 and 48 h after laser beam axotomy. Scale bars, 50 µm.

The studies of neurotrophin withdrawal started with the discovery of NGF (44). NGF supports different neuron populations to maintain their phenotypic and functional characteristics. NGF continues to play a role as a vital factor after the postnatal period. NGF is produced and released by tissues due to peripheral neuropathy, held by specific receptors expressed at the nerve terminal and transport retrogradely to soma (45, 46). NGF is necessary for the development of neurons in the peripheral nervous system, the protection of cell phenotype, and the maintenance of the functional integrity of cholinergic neurons in the central nervous system. Amino acid and mRNA sequence analysis of this neurotrophin have been performed, revealing NGF to be a very well conserved molecule, showing a very high level of homology between different species (46). In studies that were conducted on TrkA receptor knock out mice with the NGF gene and high expression level of NGF, a large amount of pain and temperature-sensitivity was lost in the small DRG neurons as well as some sympathetic neurons (47, 48). These findings support the hypothesis that NGF protects immature DRG neurons and sympathetic neurons from cell death after axotomy (49, 50). In parallel with these previous studies, we observed that exposure to NGF stimulated the regeneration and neuronal survival of cultured DRG neurons following axotomy.

BDNF played pivotal role in the regulation of neuronal modification and survival in the nervous system (51). It has been reported that the absence of BDNF genes or the TrkB receptors, to which BDNF binds with a high affinity, leads to the elimination of medium sized DRG neurons (52). Animals that have damaged BDNF genes were viable until the eighth postnatal day, while severe central and peripheral anomalies were observed in mice with deficient TrkB receptors, and these animals did not survive more than 24 h after birth (53). Another study indicated that BDNF rescues the axotomized neurons of neonatal mice and rats and decreases the mortality of retinal ganglion neurons that naturally occurs during development (54, 55). Previous studies show that BDNF has neuronal protective effects against injury in vivo and in vitro. In the present study, we found that application of BDNF resulted in an increase in neuronal survival rates after axotomy *in vitro*.

To date, studies have supported a trophic role of NGF and the other factors that are released from the peripheral and central nervous system to the adult DRG neurons. Application of NGF to the sciatic nerve after axotomy demonstrated that, compared with the results

from an untreated control group, NGF protects L4-L6 DRG neurons from death (56, 57). In this field, the most common experimental model is the local application of NGF to the proximal part of the interrupted peripheral nerve. These studies demonstrate the active role that NGF plays in the protection of neuronal vitality following axotomy (58). In this study, *in vitro* DRG neuron cultures were exposed to different doses of NGF and BDNF, alone or in combination, and axonal injury was performed with a very sensitive laser axotomy model. Using this method, the direct effects of these neurotrophins on cells are clearly demonstrated.

The most common the *in vivo* damage models are sciatic, optic, or spinal cord transections and cortical injuries. Although these *in vivo* models help to explain the responses to neuronal damage, they are not enough when they used alone. However, *in vitro* methods are more effective at investigating the molecular and cellular responses and mechanisms involved in nerve injuries. There are many *in vitro* injury models that can be applied in controllable culture media; including mechanical damage caused by materials such as microblades, scissors, and glass capillaries (26, 59). In this study a laser beam, used as the injury model. This is a new and unique method that is easier and more effective than other models and enables more precise damage to be elicited. This method, which has been used systematically in the neurite transections, was performed for the first time by Higgins and colleagues (35).

As a result of this study, it was determined that the most effective dose of NGF to promote cell viability was 150 ng/mL while the most effective dose of BDNF was 50 ng/mL. Exposure to NGF increased survival at a dose of 100 ng/mL, though this was not found to be the most effective dose. Conversely, exposure to BDNF at a dose of 100 ng/mL decreased survival. For this reason, the dose of BDNF was not increased while the dose of NGF was increased. After the optimal doses were determined, cells were exposed to these factors in combination. Because this study was performed entirely *in vitro*, it was possible to intervene directly in the cell from the outside, and neurotrophins could be added in varying concentrations. In addition, the use of the laser beam axotomy method, which provides a highly effective and sensitive axotomy, also provided value to this study.

In conclusion, the effects of neurotrophins on neuronal survival after *in vitro* axotomy are demonstrated for the first time in this study. A detailed analysis was performed by determining the survival and death of neurons after axotomy using a time lapse microscopy system that provided detailed observations. The results of this analysis indicate that exogenous BDNF and NGF application enhances neuronal survival. These findings may provide a basis for further studies on neuronal death mechanisms following axotomy.

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Disclosure statement

No potential conflict of interest was reported by the author.

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