FINAL REPORT

The long-term effects of brief simulated LVVA loads on neurogenesis in cultured PC-12 cells

ABSTRACT

Objective: The cellular and molecular mechanisms for spinal manipulative therapy (SMT) are unclear. SMT is known to influence neurophysiological activities. However, a question remains: How these short transient changes of cellular electricity impact patients' long-term health? We hypothesize that SMT has a long-term neuroplasticity effect by promoting neurogenesis.

Methods: PC-12 neuronal precursor cells were briefly treated with simulated low velocity variable amplitude (LVVA) forces, followed by additional 3-day culturing in the presence or absence of nerve growth factor (NGF). Then cells were fixed for immuno-/or histochemical staining, photographed microscopically, and analyzed for mature neuronal features. Additional cell signaling studies were carried out in cell lysates by Western blotting analysis.

Results: As expected, NGF itself produced a remarkable increase of neurogenesis. With 36-second loading duration, only low-level LVVA loads produced a statistically significant promotion of neurogenesis in the absence of NGF ($P<0.05$, $N=6$), which became statistically insignificant under NGF co-incubation; further increased loading levels did not produce significant changes regardless of the presence or absence of NGF. However, when loading duration was increased to 60 seconds, mid-level LVVA produced a significant promotion of NGF-induced neurogenesis. Further cell signaling studies revealed that, at the 60-second loading duration, mid-level LVVA markedly increased NGF-stimulated phosphorylation of ERK 1/2 (extracellular signal–regulated kinases 1 and 2).

Conclusion: Our data indicate brief LVVA loads have long-term impacts on neurogenesis, which depends on the loading levels /durations and interactions with biological factors. Particularly, ERK signaling may be a key molecular cross-point between SMT mechanotransduction and long-term neuroplasticity.

INTRODUCTION

Chiropractic physicians have used SMT to deal with various health issues for over a hundred years. A growing body of literature supports the efficacy of SMT in the management of low back and neck pain.[1] It is well known that the manipulative forces used by chiropractic physicians influence neurophysiological activities: [2] While most investigators reported neuronal activity changes within seconds, a few reports indicated changes lasting up to 10 or 20 minutes after SMT in human volunteers.[2-7] However, it is not known how short transient changes in cellular electrical activity might impact the long-term health of patients.

Recent advancements in cell and molecular mechanobiology demonstrate that mechanical forces can modify cellular gene expression and cell differentiation processes. [8-10] In addition, mechanical stimulation has been shown to modulate neurogenesis in the spinal cord of adult rats.[11] This is quite significant, because it could implicate a neuroplastic mechanism by which mechanical forces may exert influence on neuronal morphology and structure. This notion has been further supported by a study showing that mechanical forces can directly influence neurite growth and neuronal differentiation in cell culture.[12] These advancements may implicate certain long-term mechanisms in chiropractic care, and thus provide us a unique opportunity to address this significant gap in our understanding of SMT.

In the present study, we hypothesize that SMT loads may promote neurogenesis as a part of its long-term beneficial mechanisms. To address this hypothesis, we utilized neuron cell cultures to apply mechanical forces that mimic one kind of SMT loads –low velocity variable amplitude (LVVA), and then examined the loading impacts on neurogenesis and associated molecular mechanisms.

METHODS

Materials: Reagents were obtained from the following sources: RPMI media from Cellgro (Herndon, VA), fetal bovine serum from Gibco BRL (Gaithersburg, MD), horse serum from Hyclone (Logan, UT), monoclonal antibodies against beta tubulin III antibody from Sigma-Aldrich (St. Louis, MO), polyclonal antibodies against total tubulin and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) from Santa Cruz Biotech. (Santa Cruz, CA), antibodies against phosphorylated or total ERK from Cell Signaling Tech (Beverly, MA), FITC-conjugated goat anti-mouse antibody from Jackson ImmunoResearch (West Grove, PA),NGF (nerve growth factor) from Sigma-Aldrich, PC-12 neuronal precursor cells from ATCC (Rockville, MD).

Cell culture: PC-12 cells were cultured as described previously by us and others.[13 - 14] Briefly, PC12 cells were plated at a density of 5 x10^4 cells in laminin-coated 6-well culture plates (FlexCell, Burlington, NC) in RPMI medium containing 5% fetal bovine serum, 10% horse serum, 2 mM L-glutamine, and penicillin (100 /ml)/streptomycin (100 mg/ml). After growing for 24 hrs, cells were changed into the differentiation media with reduced serum levels (0.5% fetal bovine serum, 1% horse serum) in the presence or absence of NGF(100 ng/ml).

Cellular loading: Six hours after changing into the differentiation media, cells were loaded with simulated LVVA forces generated by the FX-5000T tension system (FlexCell, Hillsborough, NC). The cellular loading lasted for 12 seconds each time, and repeated 3 to 5 times with a 10-second rest between the treatments (Fig. 1). Then cells were cultured further for 3 more days.

Cell staining and microscopic imaging: At the end of each experiment, cells were washed twice in cold phosphate-buffered saline (PBS), fixed in 2% paraformaldehyde (in PBS) for 1 hr at room temperature with gentle rocking motion, and then stained with anti-beta tubulin III (a neuronal marker) antibodies and FITC-conjugated secondary antibodies or 0.01% coomassie brilliant blue G-250 (Bio-Rad) stain. Cellular microscopic images were taken by using an Infinity camera (Lumenera, Ottawa, Ontario) mounted on an EXI-310 microscope (ACCU-SCOPE, Commack, NY). Seven microscopic fields were randomly photographed for each well cultured without NGF, and 9 for each well cultured with NGF (as NGF caused a marked loss of cells).

Cell signaling studies and Western blotting: For cell signaling studies, cells were first seeded with normal growth media for 24 hrs, then withdrawn serum for 12 hrs, rested 30 mins after force loading, and finally challenged with NGF (100 ng/ml)for 15 mins before collecting cell lysates for further Western blotting analysis. Total cell lysate proteins (20 μg) were separated by routine SDS-PAGE and then transferred to PVDF membranes.[13] Specific proteins or their phosphorylation statuses were detected by using antibodies listed above with the assistance of an ECL kit (Peirce Biotechnology, Rockford, IL).

Neurogenesis assay: Microscopic images were downloaded into a computer and neurite bearing features were examined. A positive mature neuron was assigned for cells with at least one long neurite branch: The branch length should be twice greater than its own cell body.[14] Differentiated neurons and non-differentiated cells were both counted for each microscopic field. For each independent experiment, cell numbers were pooled together into 4 different conditions as mentioned above: the Control, Force only, NGF only, Force plus NGF. The neurogenesis rate was calculated for each group by dividing the total cell number with the neuron number in the same group. Then the fold increase of neurogenesis was calculated by dividing the neurogenesis rate of each specific condition against that of the Control in the same experiment. The fold increase number for the Control group in each independent experiment was calculated by dividing the

neurogenesis rate in that experiment against

the mean value of all Control groups at the same loading level. The fold increase numbers were then statistically analyzed. The investigator who counted cells was trained for cell morphology examination but not aware of the study hypothesis.

Data analysis: All data were analyzed using the unpaired *t* test in Excel program (Microsoft Windows 2010, Microsoft, Redmond, WA). $P < 5$ was considered statistically significant. The numbers were plotted in Fig. 4 and 5 with bars representing means $\pm$ SEM.

DISCUSSION

To our knowledge, this is the first attempt to study the long-term neuroplasticity effects of SMT, during which a potential novel mechanism is revealed and may help us in understanding the long-term benefits of chiropractic care.

The concept of "neuroplasticity" was first conceived by the founder of modern neuroscience – the famed Spanish scientist Santiago Ramón y Cajal (1852-1934), to describe physiological changes of adult brain structures. This concept has now been extended to cover both physiological and pathological changes in neuronal morphology, connectivity, neuronal death, neurogenesis, and neurochemistry.[21] In chiropractic research field, Boal and Gillette first proposed potential neuroplasticity mechanisms could be responsible for SMT benefits.[22] However, so far no concrete evidence has been reported, since neuronal electricity changes lasting milliseconds to minutes after SMT were too short to be classified as neuroplasticity. Thus, our study on the effects of simulated LVVA loads on neurogenesis may represent the first experimental evidence of neuroplasticity effects for SMT.

Our data suggest that the impact of simulated LVVA loads on neurogenesis is mild and also

complicated. One puzzle was that low-level LVVA promoted neurogenesis significantly in the absence of NGF (Fig. 3). This effect, however, disappeared with increased force levels or the addition of biological factors such as NGF (Fig. 3). We suspect that the increased forces would destabilize weak neurite growth cone,[23] thus stifle the potential promoting effects in a situation where neurogenic activities are already extremely low in the absence of NGF. Furthermore, when the powerful biological agent NGF was added into the culture, the huge increase of neurogenic activity drowned out the extremely low-rate effects of low-level LVVA, which could become a meaningless background noise in the face of strong NGF signaling storms. Interestingly, increasing the loading duration from 36 seconds to 60 seconds seems make a big difference. In that scenario, we observed a significant promoting effect on neurogenesis by mid-level LVVA, even in the presence of powerful NGF. Our 60-second duration closely mimics clinical practice where a single session of LVVA usually lasts from 70 to 80 seconds.[16] This may suggest a minimum loading duration is required to achieve a strong biological effect. Further cell signaling studies demonstrated that mid-level LVVA with longer loading duration remarkably potentiated NGF-stimulated phosphorylation of ERK1/2. ERK1/2 are major inter-mediators of NGF actions;[24-25] and ERK signaling pathway is important in neurogenesis, neurite growth, axon extension, and neuronal memory formation.[24-28] Thus LVVA forces target a major molecular pathway for neurotrophic actions. Interestingly, ERK is also known to mediate mechanotransduction,[29-30] which may be the reason it becomes a crossing point for SMT forces and NGF signalining (Fig. 5 & 6). Furthermore, ERK directly phosphorylates and activates transcription factors such as AP-1, Elk-1 and TLE1,[31-33] which in turn activate various gene expression programs. Therefore, it is highly possible that ERK, a key signaling molecule, could lead a potential SMT epigenetic regulatory pathway (Fig. 6); and this pathway may be responsible for SMT long-term benefits.

Since our data suggest that SMT forces may promote neurotrophic actions, potential clinical applications of SMT in nerve injuries and neurodegenerative conditions may warrant further investigation. In situations such as spinal injuries, healers want to promote neurite growth for axon repairing to overcome local intrinsic inhibitory factors.[34] While in neurodegenerative conditions, whether is due to aging, [35] subluxation,[36] or other specific issues, [37] loss of neurons is a paramount impairment to those patients. There have been tremendous efforts in biomedical research field to save those neurons or promote adult neurogenesis to replace those dying ones.[38-42] Thus, our study may hint a potential application of natural external forces such as SMT for promoting axon extension and neurogenesis in some of those conditions.

In addition, our data are in agreement with reports that mechanic forces were able to promote adult neurogenesis in the spinal cord and to enhance neuronal differentiation in cell culture.[11-12] As mentioned above in the Introduction section, mechanical stimulation has been shown to modulate neurogenesis in the spinal cord of adult rats,[11] which was one of reports that initially inspired our current work. In the rat study, a single touch (lasting only a few seconds) promoted the neurogenesis of inhibitory neurons in the spinal cord.[11] Thus, LVVA, a force with much longer loading duration than a simple touch could produce an even stronger promoting effect on the neurogenesis of inhibitory neurons in spinal cord. If this is true, it could be the underlying mechanism for LVVA and other SMT forces in the management of neck and back pain. Clearly further experimenting is required to test this intriguing hypothesis.

REFERENCES

1. Bronfort G, Haas M, Evans RL, Bouter LM. Efficacy of spinal manipulation and mobilization for low back pain and neck pain: a systematic review and best evidence synthesis. *Spine J*. 2004;4:335-56.
2. Pickar JG. Neurophysiological effects of spinal manipulation. *Spine J*. 2002;2:357-71.
3. Terrett ACJ, Vernon HT. Manipulation and pain tolerance: a controlled study of the effect of spinal manipulation on paraspinal cutaneous pain tolerance levels. *Am J Phys Med*. 1984;63:217-25.
4. Glover JR. Back pain and hyperaesthesia. *Lancet.* 1960;1(7135):1165-9.
5. Dishman JD, Dougherty PE, Burke JR. Evaluation of the effect of postural perturbation on motoneuronal activity following various methods of lumbar spinal manipulation. *Spine J*. 2005;5(6):650-9.
6. Haavik-Taylor H, Murphy B. Cervical spine manipulation alters sensorimotor integration: a somatosensory evoked potential study. *Clin Neurophysiol*. 2007;118(2):391-402.
7. Dishman JD, Weber KA 2nd, Corbin RL, Burke JR. Understanding inhibitory mechanisms of lumbar spinal manipulation using H-reflex and F-wave responses: a methodological approach. *J Neurosci Methods.* 2012;210(2):169-77.
8. Yang Y, Beqaj S, Ariel I, Kemp P, Schuger L. Stretch-induced alternative splicing of serum response factor promotes bronchial myogenesis and is defective in lung hypoplasia. *J Clin Invest*. 2000;106(11):1321-30.
9. Shivashankar GV. Mechanosignaling to the cell nucleus and gene regulation. *Annu Rev Biophys*. 2011;40:361–378.
10. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell.* 2006;126:677–689.
11. Shechter R, Baruch K, Schwartz M, Rolls A. Touch gives new life: mechanosensation modulates spinal cord adult neurogenesis. *Mol Psychiatry.2011*;16(3):342-52.
12. Chang YJ, Tsai CJ, Tseng FG, Chen TJ, Wang TW. Micropatterned stretching system for the investigation of mechanical tension on neural stem cells behavior. *Nanomedicine.* 2013;9(3):345-55.
13. Zhang, L, Jope, RS. Oxidative stress differentially modulates growth factor-induced phosphorylation of ERK, p38 and CREB in PC12 cells. *Neurobiology of Aging.* 1999;20:271.
14. Wade A, Thomas C, Kalmar B, Terenzio M, Garin J, Greensmith L, Schiavo G. Activated leukocyte cell adhesion molecule modulates neurotrophin signaling. *J Neurochem.* 2012;121(4):575-86.

15. Barrett GL. The p75 neurotrophin receptor and neuronal apoptosis. *Prog Neurobiol.* 2000;61(2):205-29.

16. Gudavalli MR, Cox JM. Real-time force feedback during flexion-distraction procedure for low back pain: a pilot study. *J Can Chiropr Assoc.* 2014;58(2):193-200.

17. Lamour G, Eftekhari-Bafrooei A, Borguet E, Souès S, Hamraoui A. Neuronal adhesion and differentiation driven by nanoscale surface free-energy gradients. *Biomaterials*. 2010;31(14):3762-71.

18. Shioda N, Han F, Fukunaga K. Role of Akt and ERK signaling in the neurogenesis following brain ischemia. *Int Rev Neurobiol.* 2009;85:375-87.

19. Ye Y, Zhao Z, Xu H, Zhang X, Su X, Yang Y, Yu X, He X. Activation of sphingosine 1- phosphate receptor 1 enhances hippocampus neurogenesis in a rat model of traumatic brain injury: an involvement of MEK/Erk signaling Pathway. *Neural Plast.* 2016;2016:8072156.

20. Pillat MM, Lameu C, Trujillo CA, Glaser T, Cappellari AR, Negraes PD, Battastini AM, Schwindt TT, Muotri AR, Ulrich H. Bradykinin promotes neuron-generating division of neural progenitor cells through ERK activation. *J Cell Sci.* 2016;129(18):3437-48.

21. Fuchs E, Flügge G. Adult neuroplasticity: more than 40 years of research. *Neural Plast.* 2014;541870.

22. Boal RW, Gillette RG. Central neuronal plasticity, low back pain and spinal manipulative therapy. *J Manipulative Physiol Ther.* 2004;27(5):314-26.

23. Betz T, Koch D, Lu YB, Franze K, Käs JA. Growth cones as soft and weak force generators. *Proc Natl Acad Sci USA.* 2011;108(33):13420-5.

24. Vaudry D, Stork PJ, Lazarovici P, Eiden LE. Signaling pathways for PC12 cell differentiation: making the right connections. *Science.* 2002;296(5573):1648-9.

25. Tabakman R, Jiang H, Shahar I, Arien-Zakay H, Levine RA, Lazarovici P. Neuroprotection by NGF in the PC12 in vitro OGD model: involvement of mitogen-activated protein kinases and gene expression. *Ann N Y Acad Sci.* 2005;1053:84-96.

26. Shioda N, Han F, Fukunaga K. Role of Akt and ERK signaling in the neurogenesis following brain ischemia. *Int Rev Neurobiol.* 2009;85:375-87.

27. Ye Y, Zhao Z, Xu H, Zhang X, Su X, Yang Y, Yu X, He X. Activation of sphingosine 1-phosphate receptor 1 enhances hippocampus neurogenesis in a rat model of traumatic brain injury: an involvement of MEK/Erk signaling Pathway. *Neural Plast.* 2016;2016:8072156.

28. Pillat MM, Lameu C, Trujillo CA, Glaser T, Cappellari AR, Negraes PD, Battastini AM, Schwindt TT, Muotri AR, Ulrich H. Bradykinin promotes neuron-generating division of neural progenitor cells through ERK activation. *J Cell Sci.* 2016;129(18):3437-48.

29. Hatanaka K, Ito K, Shindo T, Kagaya Y, Ogata T, Eguchi K, Kurosawa R, Shimokawa H. Molecular mechanisms of the angiogenic effects of low-energy shock wave therapy: roles of mechanotransduction. *Am J Physiol Cell Physiol.* 2016;311(3):C378-85.

30. Li S, Zhang H, Li S, Yang Y, Huo B, Zhang D. Connexin 43 and ERK regulate tension-induced signal transduction in human periodontal ligament fibroblasts. *J Orthop Res.* 2015;33(7):1008-14.

31. Cohen-Armon M, Visochek L, Rozensal D, Kalal A, Geistrikh I, Klein R, Bendetz-Nezer S, Yao Z, Seger R. DNA-independent PARP-1 activation by phosphorylated ERK2 increases Elk1 activity: a link to histone acetylation. *Mol Cell.* 2007;25(2):297-308.

32. Yao W, Oh YT, Deng J, Yue P, Deng L, Huang H, Zhou W, Sun SY. Expression of Death Receptor 4 Is Positively Regulated by MEK/ERK/AP-1 Signaling and Suppressed upon MEK Inhibition. *J Biol Chem.* 2016;291(41):21694-21702.
33. Zahavi T, Maimon A, Kushnir T, Lange R, Berger E, Kornspan D, Grossman R, Anzi S, Shaulian E, Karni R, Nechushtan H, Paroush Z. Ras-Erk signaling induces phosphorylation of human TLE1 and downregulates its repressor function. *Oncogene.* 2017.
34. Hulsebosch CE. Recent advances in pathophysiology and treatment of spinal cord injury. *Adv Physiol Educ.* 2002;26(1-4):238-55.
35. Chow HM, Herrup K. Genomic integrity and the ageing brain. *Nat Rev Neurosci.* 2015;16(11):672-84.
36. He X, Dishman V. Spinal motor neuronal degeneration after knee joint immobilization in the guinea pig. *J Manipulative Physiol Ther.* 2010;33(5):328-37.
37. Torriglia A, Jaadane I, Lebon C. Mechanisms of cell death in neurodegenerative and retinal diseases: common pathway? *Curr Opin Neurol.* 2016;29(1):55-60.
38. Verity NC, Mallucci GR. Rescuing neurons in prion disease. *Biochem J.* 2011;433(1):19-29.
39. Gould TW, Oppenheim RW. Motor neuron trophic factors: therapeutic use in ALS? *Brain Res Rev.* 2011;67(1-2):1-39.
40. Bikbova G, Oshitari T, Baba T, Yamamoto S. Neurotrophic factors for retinal ganglion cell neuropathy - with a special reference to diabetic neuropathy in the retina. *Curr Diabetes Rev.* 2014;10(3):166-76.
41. Xilouri M, Stefanis L. Chaperone mediated autophagy to the rescue: A new-fangled target for the treatment of neurodegenerative diseases. *Mol Cell Neurosci.* 2015;66(Pt A):29-36.
42. Valdés P, Schneider BL. Gene Therapy: A Promising Approach for Neuroprotection in Parkinson's Disease? *Front Neuroanat.* 2016;10:123.
43. Ishii T, Warabi E, Siow RC, Mann GE. Sequestosome1/p62: a regulator of redox-sensitive voltage-activated potassium channels, arterial remodeling, inflammation, and neurite outgrowth. *Free Radic Biol Med.* 2013;65:102-16.
44. Suter DM, Miller KE. The emerging role of forces in axonal elongation. *Prog Neurobiol.* 2011;94(2):91-101.
45. Nakamura T, Aoki K, Matsuda M. FRET imaging and in silico simulation: analysis of the signaling network of nerve growth factor-induced neuritogenesis. *Brain Cell Biol.* 2008;36(1-4):19-30.

FIGURE LEGENDS

Fig. 1. The FX-5000T tension system. The system is controlled by a FX5K^{TM} Tension FlexLink machine (located at the right corner of the upper panel) with the assistance of FlexSoft FX-5000 software preloaded in a computer. The machine is linked with a computer (upper panel), a vacuum machine (not shown), and a cell loading station (lower panel).

Fig. 2. Oscillating waveforms generated by different loads of simulated LVVA forces. Three different loading ranges were tested: low (up to -42 kPa), middle (up to -68 kPa), and high (up to -91 kPa). Examples of actual waveforms from experiments are shown for each loading range.

Fig. 3. NGF induced neurogenesis in PC-12 cells: PC-12 cells were cultured in differentiated media with 100 ng/ml NGF for 3 days. Then cells were fixed and immunocytochemical stained with primary anti- β-tubulin III antibodies and FITC-conjugated secondary antibodies. Note several well-differentiated neurons with long, fine developed neurite branches. Cell lysates from parallel cell cultures were analyzed by Western blotting (20 μg protein for each sample). The

right panel shows that the expression of β-tubulin III was markedly increased by 3-day NGF treatment as comparing to that of control protein GAPDH (glyceraldehyde 3-phosphate dehydrogenase). C: control; N: NGF treatment.

Fig. 4. Effects of simulated LVVA forces (36s) on neurogenesis. Various levels of simulated LVVA force were loaded on cells for 36 seconds, then cultured in the presence or absence of NGF for additional 3 days. Then, neurogenesis assay was performed as described in the text. Fold increase numbers against the control group are plotted for each loading levels with a 36-second duration and different culture conditions (means $\pm$ SEM, n = 6). * $P < 0.05$, ** $P < 0.01$ compared to the control group.

Fig. 5. Prolonged mid-level LVVA promoted neurogenesis via potentiating signaling. Mid-level LVVA with a 60-second loading duration was focused here. Similar neurogenesis assay was performed as described above. Fold increase numbers against the average value (0.28%) of control groups were calculated and plotted in the upper panel (means $\pm$ SEM, n = 4). * $P < 0.05$ compared with NGF treatment alone. Parallel cell cultures were set up for cell signaling studies as described in the text. Twenty 20 μg proteins for each sample were run for Western blotting analysis. Representative immunoblots are shown for phospho-ERK (p-ERK) and total tubulin expression in the lower panel. Note mid-level LVVA loading (force) markedly increased NGF-stimulated phosphorylation of ERK.

Fig. 6. A potential epigenetic pathway for SMT long-term neuroplasticity. Neurotrophic factors such as NGF can stimulate the phosphorylation of ERK, which can be potentiated by SMT forces. Activated ERK enters into nuclei to phosphorylate numerous transcription factor. The activated transcription factors will be able to bind DNA sites and then regulate gene transcription. This leads to long-term neuroplasticity such as generating new neurons or neurites.

Fig. 1. The FX-SOOOT tension system.

Fig. 2. Oscillating waveforms generated by different loads of simulated LVVA forces.

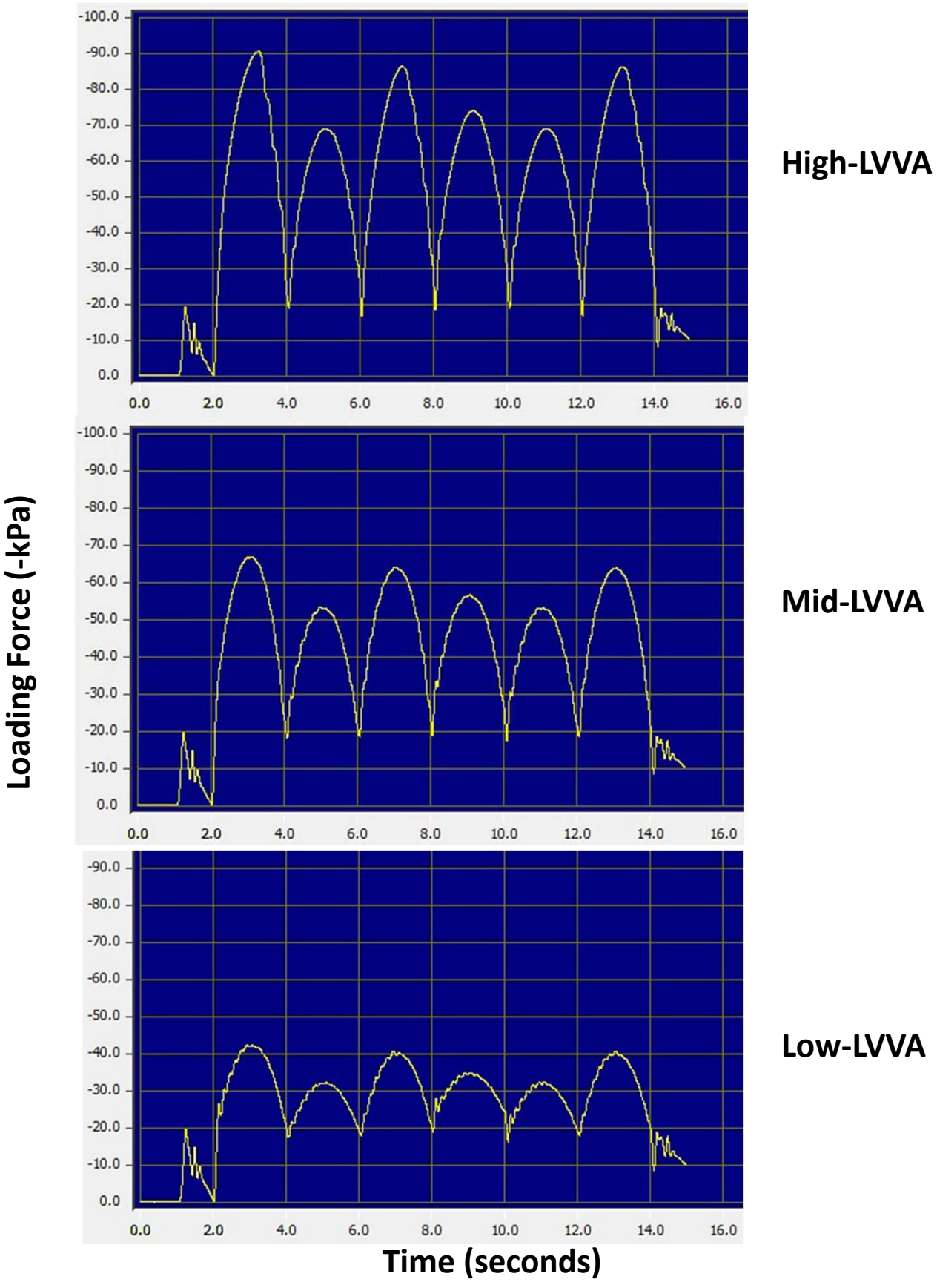
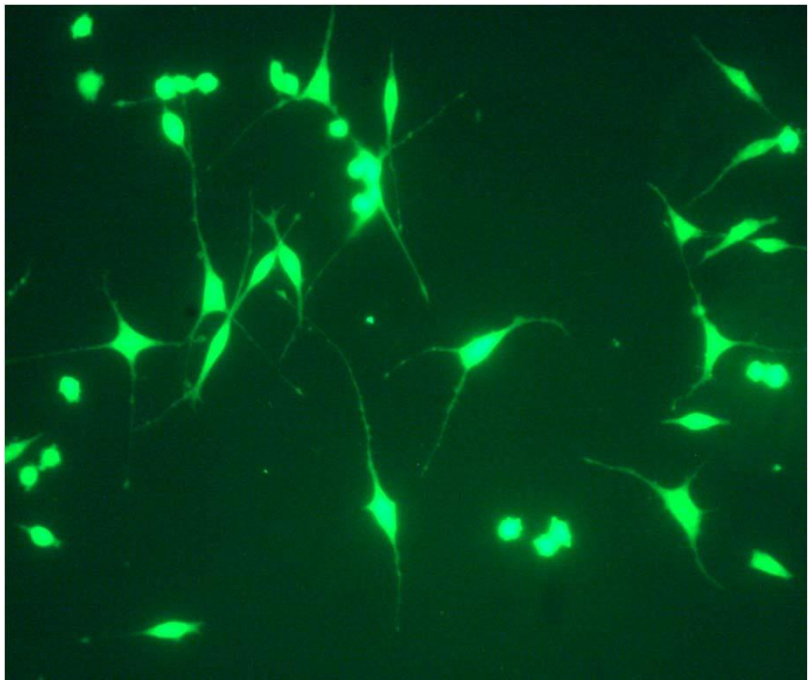
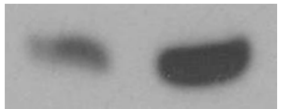
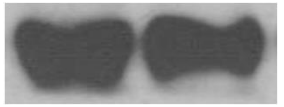


Fig. 3. NGF induced neurogenesis in PC-12 cells





β-tubulin III



GAPDH

C N

Fig. 4. Effects of simulated LVVA forces (36s) on neurogenesis

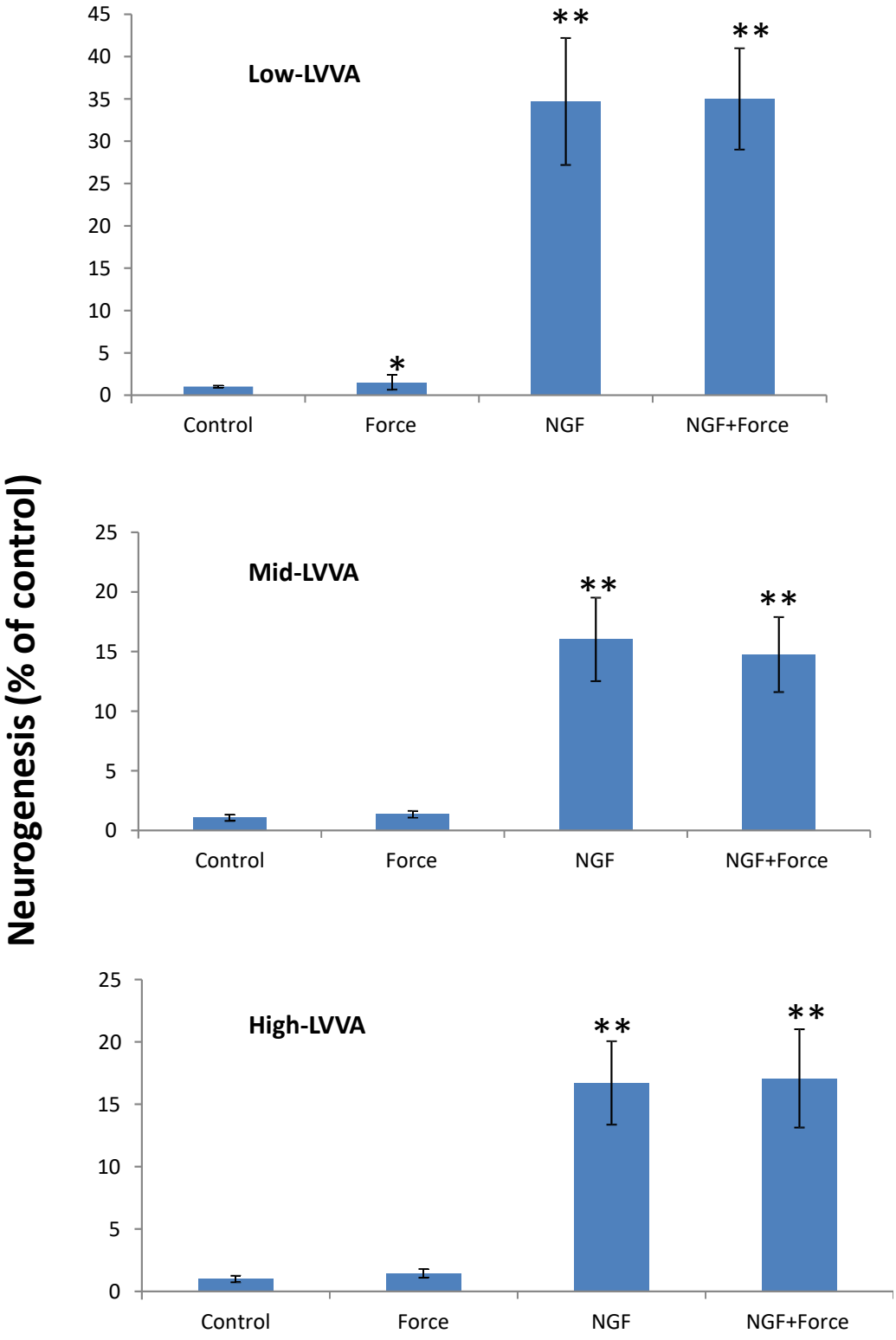
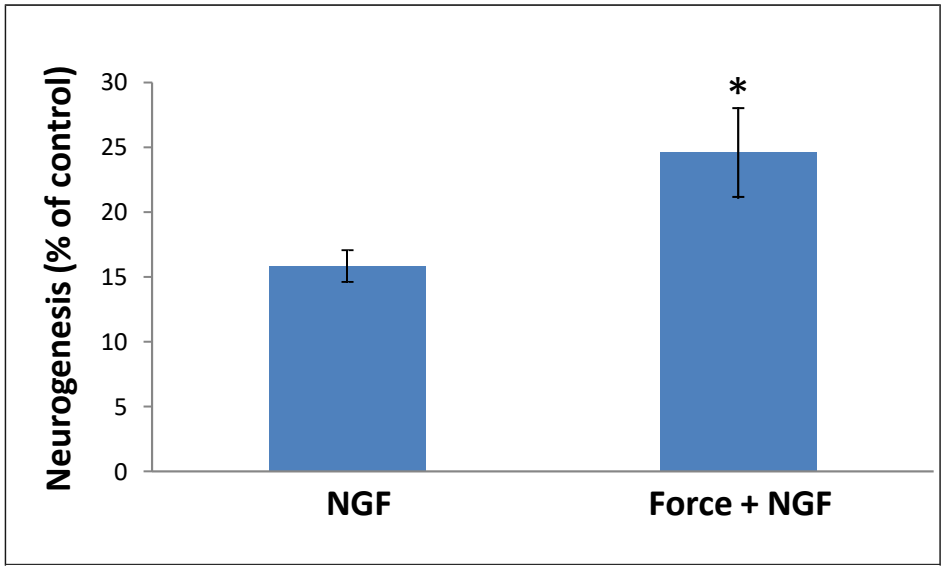


Fig. 5 Prolonged mid-level LVVA promoted neurogenesis via potentiating ERK signaling



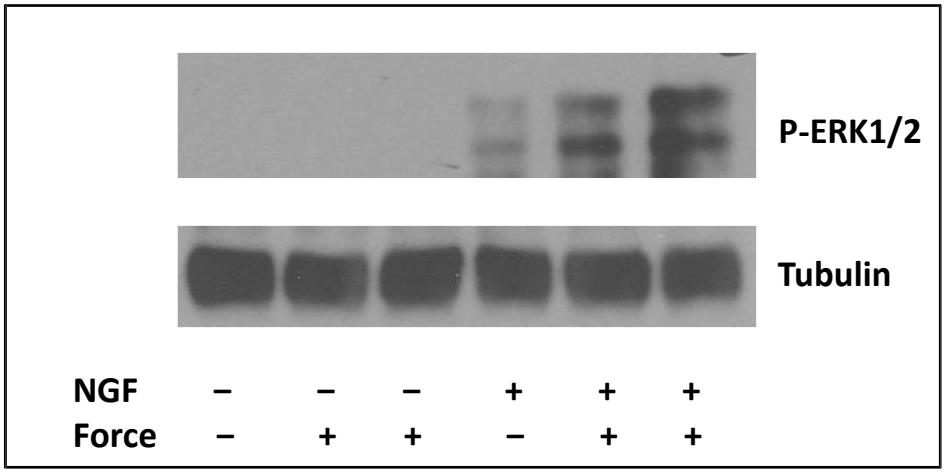


Fig. 6. A potential epigenetic pathway for SMT long-term neuroplasticity