

# Upregulation of BDNF and NGF in Cervical Intervertebral Discs Exposed to Painful Whole-Body Vibration

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**Study Design.** *In vivo* study defining expression of the neurotrophins, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), in cervical intervertebral discs after painful whole-body vibration (WBV).

**Objective.** The goal of this study is to determine if BDNF and NGF are expressed in cervical discs after painful WBV in a rat model.

**Summary of Background Data.** WBV is a possible source of neck pain and has been implicated as increasing the risk for disc disorders. Typically, aneural regions of painful human lumbar discs exhibit hyperinnervation, suggesting nerve ingrowth as potentially contributing to disc degeneration and pain. BDNF and NGF are upregulated in painfully degenerate lumbar discs and hypothesized to contribute to this pathology.

**Methods.** Male Holtzman rats underwent 7 days of repeated WBV (15 Hz, 30 min/d) or sham exposures, followed by 7 days of rest. Cervical discs were collected for analysis of BDNF and NGF expression through RT-qPCR and Western blot analysis. Immunohistochemistry also evaluated their regional expression in the disc.

**Results.** Vibration significantly increases BDNF messenger ribonucleic acid (mRNA) levels ( $P = 0.036$ ), as well as total-NGF mRNA ( $P = 0.035$ ). Protein expression of both BDNF ( $P = 0.006$ ) and the 75-kDa NGF ( $P = 0.045$ ) increase by nearly 4- and 10-fold, respectively. Both BDNF mRNA ( $R^2 = 0.396$ ;  $P = 0.012$ ) and protein ( $R^2 = 0.280$ ;  $P = 0.035$ ) levels are significantly correlated with the degree of behavioral sensitivity (*i.e.*, pain) at day 14. Total-NGF mRNA

is also significantly correlated with the extent of behavioral sensitivity ( $R^2 = 0.276$ ;  $P = 0.044$ ). Both neurotrophins are most increased in the inner annulus fibrosus and nucleus pulposus.

**Conclusion.** The increases in BDNF and NGF in the cervical discs after painful vibration are observed in typically aneural regions of the disc, consistent with reports of its hyperinnervation. Yet, the induction of nerve ingrowth into the disc was not explicitly investigated. Neurotrophin expression also correlates with behavioral sensitivity, suggesting a role for both neurotrophins in the development of disc pain.

**Key words:** WBV, intervertebral disc, pain, neurotrophin, nerve growth factor, brain-derived neurotrophic factor.

**Level of Evidence:** N/A

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Whole-body vibration (WBV) is increasingly identified as a source of spinal pain. Epidemiological studies suggest that long-term WBV exposure increases risks for low back pain, spinal degeneration, and lumbar intervertebral disc (IVD) herniation.<sup>1-3</sup> Occupational exposure to WBV through frequent operation of vibrating vehicles is linked to higher incidences of neck pain.<sup>4-6</sup> For example, 33.5% of tractor drivers report neck pain at least once a week, with the frequency of episodes increasing with use.<sup>6</sup> Despite strong suggestions that WBV exposure is linked to chronic neck pain, the mechanism(s) involved remains unknown.

IVD damage and/or its degeneration are sources of pain, supported largely through work on the pathogenesis of chronic low back pain and disorders of the lumbar spine.<sup>7-9</sup> In healthy discs, the outer one-third of the annulus fibrosus is innervated; the remainder of the annulus fibrosus and the nucleus pulposus (NP) are both aneural and avascular.<sup>10</sup> However, in degenerate human discs, nerve ingrowth is also evident in the inner annulus fibrosus (IAF) and NP.<sup>11,12</sup> The extent of innervation is greatest at those spinal levels with pain,<sup>11</sup> and asymptomatic degenerate discs do not exhibit innervation.<sup>12</sup> Although hyperinnervation is evident in painful lumbar discs, it is unknown if the same develops in the cervical discs.

The neurotrophins, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), increase in painful

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degenerate human IVDs, and are thought to relate to its hyperinnervation.<sup>13,14</sup> Both neurotrophins are essential in neuronal development and maintenance, and modulation of pain.<sup>15</sup> NGF is a peripheral pain mediator, with increased release by dorsal root ganglia neurons in response to lumbar disc herniation.<sup>16</sup> Similarly, BDNF modulates nociceptive processing in the spinal dorsal horn in models of disc herniation.<sup>17</sup> Both BDNF and NGF are expressed in the annulus fibrosus and NP of nondegenerate human discs,<sup>13</sup> suggesting that neurotrophins in resident disc cells may promote nerve ingrowth. Furthermore, BDNF increases in severely degenerate discs relative to nondegenerate and moderately degenerate discs.<sup>13</sup> Despite their speculated role in disc degeneration and pain, no study has investigated the role of neurotrophins and innervation in discs after WBV in the context of neck pain.

We previously developed a rat model of repeated WBV exposure that produces cervical spinal compression and extension, and pain in the forepaw that is sustained for 7 days after the last WBV exposure.<sup>18</sup> We used that model to examine if repeated WBV alters expression of BDNF and NGF in the cervical discs at day 7 after WBV. Neurotrophin messenger RNA (mRNA) transcript and protein expression were quantified in the cervical discs using RT-qPCR and Western blot and correlated with the mechanical withdrawal thresholds on the day of harvest to examine if there is a relationship to behavioral sensitivity. Immunohistochemistry (IHC) localized both neurotrophins in the outer annulus fibrosus, IAF, and NP to determine if there are regional changes.

## MATERIALS AND METHODS

Procedures were approved by the University of Pennsylvania Institutional Animal Care and Use Committee and performed according to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.<sup>19</sup> Male Holtzman rats (250–275 g) (Harlan Sprague-Dawley Inc., Indianapolis, IN) were housed under Association for Assessment and Accreditation of Laboratory Animal Care-compliant conditions with a 12:12-hour light-dark cycle and free access to food and water.

Rats underwent either a repeated WBV or sham exposure, using published methods.<sup>18</sup> WBV was performed under isoflurane inhalation anesthesia (4% induction; 2.5% maintenance) for 30 minutes daily for 7 days in a prone position on a vibrating plate. The plate was cyclically vibrated along the long-axis of the rat's spine, with a peak-to-peak magnitude of 1.5 mm at 15 Hz and 0.55 g. A separate group of sham control rats received the same daily anesthesia but no vibration. All rats were allowed to rest for an additional 7 days after the final WBV. Behavioral hypersensitivity was measured in the forepaws prior to the start of the study (baseline) and daily until tissue harvest (day 14) by quantifying the mechanical withdrawal threshold.<sup>18,20,21</sup> Withdrawal thresholds were compared using a 1-way analysis of variance.

At day 14, cervical IVDs (C5–C8) were harvested to quantify BDNF and NGF mRNA levels using RT-qPCR. Rats (WBV  $n = 8$ ; sham  $n = 7$ ) were anesthetized with sodium pentobarbital (65 mg/kg) and transcardially perfused with

300 mL of ice-cold 0.1M phosphate-buffered saline (PBS; pH 7.4). Discs were dissected, snap frozen on dry ice, and stored at  $-80^{\circ}\text{C}$  before tissue processing. RNA was isolated with TRIzol (Life Technologies, Carlsbad, CA) or the Allprep kit (Qiagen, Germantown, MD) and cDNA was prepared using SuperScript III Reverse Transcriptase (Life Technologies). Cervical discs from naive unoperated rats ( $n = 2$ ) were included as controls. Power SYBR Green (Applied Biosystems, Carlsbad, CA) was used for RT-qPCR on the 7300 System (Applied Biosystems). Cycle conditions were  $50^{\circ}\text{C}$  for 2 minutes,  $95^{\circ}\text{C}$  for 10 minutes, followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 seconds and  $60^{\circ}\text{C}$  for 60 seconds, followed by melting curve analysis. Reactions were performed in duplicate, and primers confirmed to amplify a single band by 2% agarose gel electrophoresis. Efficiency was between 90% and 110%, and CyA was selected as the most stably expressed from a panel of housekeeping genes for relative quantification.<sup>21</sup> RT-qPCR was carried out using published BDNF primers<sup>22</sup> and primers specific to pro-NGF (forward 5'-CATCCACCCACCCAGTCTTC-3', reverse 5'-CACCTCCTTGCCCTTGATGT-3'), total-NGF (forward 5'-TACTGCACCACGACTCACAC-3', and reverse 5'-CCTGATGAACCTCCAGGCAG-3'). Quantification was performed using the comparative  $\Delta\Delta\text{Ct}$  method.<sup>23</sup>

Discs were also analyzed to quantify protein levels by Western blot after WBV ( $n = 8$ ) and sham ( $n = 8$ ). Samples were homogenized in lysis buffer (150mM NaCl, 50mM Tris-Cl, pH 8.0, 1mM EDTA, 1% Triton X-100) with protease and phosphatase inhibitors (Sigma, St. Louis, MO); protein was measured by BCA assay (Thermo Scientific, Waltham, MA). Equal amounts of protein were loaded on gradient gels and transferred to PVDF membranes. Membranes were incubated overnight at  $4^{\circ}\text{C}$  with mouse anti-GAPDH (1:1000; Cell Signaling, Danvers, MA), rabbit anti-NGF (1:200; Santa Cruz Biotechnology, Dallas, TX), and rabbit anti-BDNF (1:200; Abcam, Carlsbad, CA) and then incubated for 2 hours with goat anti-rabbit IRDye 800 (1:15,000; Li-Cor, Lincoln, NE). Membranes were imaged using the Odyssey Infrared Imaging System and quantified using the Odyssey Application Software (Li-Cor). Expression was normalized to GAPDH, which served as a loading control for each sample. Normalized BDNF and NGF mRNA and protein levels were each compared between groups using separate Student  $t$  tests. Separate linear regressions evaluated if the percent reduction in withdrawal thresholds from baseline correlated with transcript or protein levels for each of BDNF and NGF; significance was determined by separate analyses of variance.

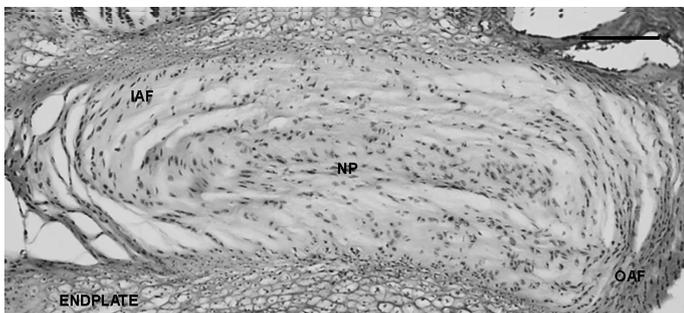
Spatial localization of neurotrophin expression was analyzed using immunohistochemistry from separate group of rats (WBV:  $n = 4$ ; sham:  $n = 3$ ). Rats were anesthetized on day 14 with sodium pentobarbital (65 mg/kg) and perfused with 300 mL of PBS followed by 250 mL of 4% paraformaldehyde in PBS and postfixed in 4% paraformaldehyde overnight at  $4^{\circ}\text{C}$ . Spinal columns were harvested *en bloc* and stored in 30% sucrose in PBS at  $4^{\circ}\text{C}$  and then decalcified using 10% EDTA (pH 7.2–7.4) for 2 weeks. Samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA) and serially sectioned (20  $\mu\text{m}$ ) at the sagittal midline and thaw-mounted onto slides at room temperature. Endogenous

peroxidase activity was quenched with 0.3% hydrogen peroxide in 0.01M PBS. Antigen retrieval was performed by incubating slides in DeCal Antigen Retrieval (BioGenex, Fremont, CA) solution for 30 minutes. Slides were washed, blocked with normal horse serum (Vector, Burlingame, CA) for 90 minutes, and incubated in primary antibody against either NGF or BDNF (1:500, Santa Cruz Biotechnology) overnight at 4°C. After washing, sections were incubated with biotinylated donkey-anti-rabbit secondary antibody (1:1000; Vector) for 30 minutes and developed using 3,3-diaminobenzidine (Vector). Slides were imaged using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

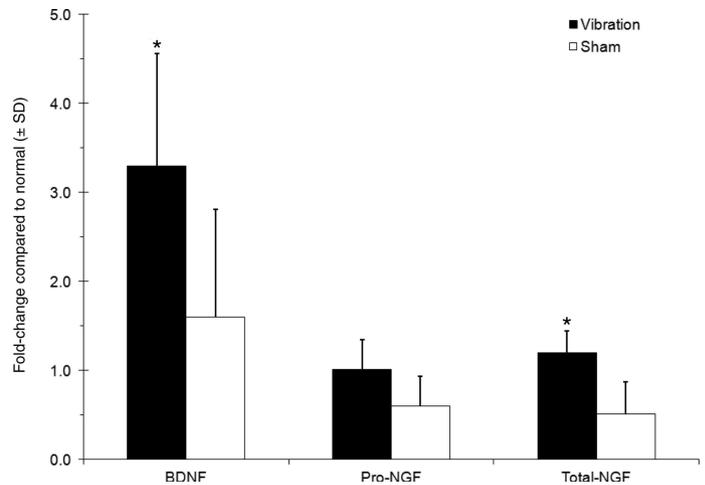
At least 2 representative images of each disc were obtained at 20× magnification. Images were analyzed using semi-quantitative measures to grade the extent of NGF and BDNF expression in different regions of the disc: the outer annulus fibrosus (OAF), IAF and NP (Figure 1). Each region was identified through landmarks in the extracellular matrix, endplate and NP; the OAF and IAF were distinguished using cell morphology,<sup>24</sup> and graded by 2 evaluators blinded to the study. Each region of the disc was separately graded using an adapted scoring technique previously used to grade human discs.<sup>25</sup> Scoring used a 4-point scale, based on the distribution of immunopositive cells: no staining (–), mild staining (±), moderate staining (+), and robust staining (++) . Scores from each observer were averaged by region in each disc. Because the scoring data were not normally distributed a nonparametric Mann-Whitney *U* test determined significance between groups. Significance between regions within the same group was determined using a nonparametric Wilcoxon signed rank test.

## RESULTS

WBV induced behavioral sensitivity that was sustained throughout the exposure and rest periods. The mechanical withdrawal thresholds for the forepaw were significantly lower after WBV than sham exposures ( $P = 0.018$ ). Both BDNF and NGF mRNA were detected in all groups (Figure 2). BDNF levels were greater than normal in both groups, with a nearly double and significant increase in mRNA after vibration over sham ( $P = 0.036$ ) (Figure 2). No differences were detected for the prodomain of NGF mRNA. Yet, total-NGF mRNA had significantly elevated after vibration over sham ( $P = 0.035$ ).

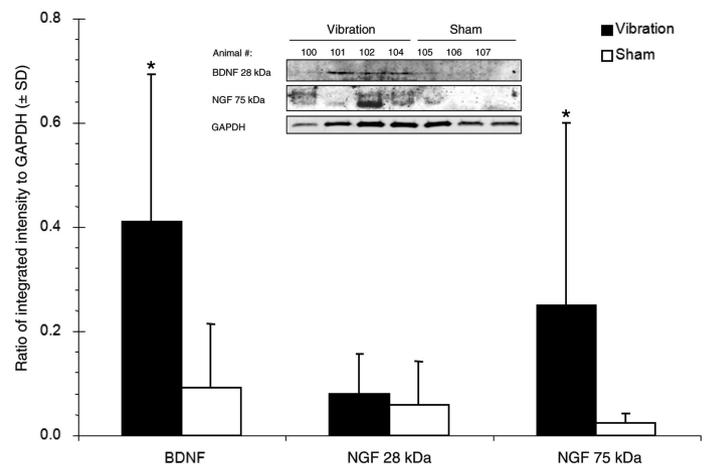


**Figure 1.** Hematoxylin and eosin stain schematic of cervical disc in the sagittal plane showing the IAF, OAF, NP, and endplate regions of the IVD. Scale bar is 100  $\mu$ m. IAF indicates inner annulus fibrosus; OAF, outer annulus fibrosus; NP, nucleus pulposus; IVD, intervertebral disc.



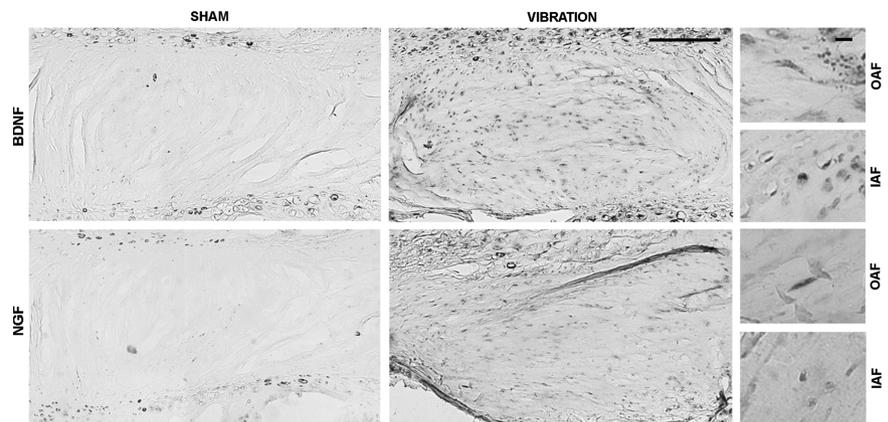
**Figure 2.** Transcript levels of BDNF and NGF are upregulated in the cervical disc after WBV. BDNF and total-NGF remain significantly ( $*P < 0.036$ ) higher in vibrated discs compared with shams. BDNF indicates brain-derived neurotrophic factor; NGF, nerve growth factor; WBV, whole-body vibration.

Protein levels for both neurotrophins exhibited similar trends as mRNA levels, with increases after WBV (Figure 3). BDNF protein increased significantly ( $P = 0.006$ ) more than 4-fold after WBV over sham. Two forms of NGF protein were detected: one at 28 kDa and one at 75 kDa (Figure 3). The 28-kDa form was not different between groups; but, 75-kDa NGF was more than 10-fold greater after WBV than sham ( $P = 0.045$ ). Increased neurotrophin expression was also confirmed by immunopositive staining in the IVDs after vibration (Figure 4; Table 1). Immunoreactivity was detected in fibroblast-like cells in the OAF and rounded, chondrocyte-like cells in the NP and IAF for both neurotrophins (Figure 4). BDNF expression after WBV was present in all samples, but exhibited variable expression, ranging from mild to robust.



**Figure 3.** Expression of BDNF and NGF protein as measured by Western blot and normalized to GAPDH levels. After WBV, BDNF and the 75-kDa isoform of NGF are each significantly upregulated ( $*P < 0.045$ ) compared with levels in the sham discs. The inset shows representative bands labeled for both neurotrophins and GAPDH. BDNF indicates brain-derived neurotrophic factor; NGF, nerve growth factor; WBV, whole-body vibration.

**Figure 4.** Increased BDNF and NGF labeling is evident in discs after WBV compared with sham discs. Both neurotrophins are expressed in rounded cells of the OAF and fibroblast-like cells of the IAF and NP. Scale bars are 200 μm in full panel and 100 μm in the panels on the right. BDNF indicates brain-derived neurotrophic factor; NGF, nerve growth factor; WBV, whole-body vibration; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; NP, nucleus pulposus.



BDNF in the sham discs was generally absent or only mild (Figure 4; Table 1). NGF exhibited a similar trend, with 2 samples with no expression and only mild expression in a single control rat. NGF labeling was evident in all samples that underwent vibration, with either moderate or robust expression (Figure 4; Table 1). Overall, expression of both BDNF and NGF had significantly increased in the discs after vibration ( $P = 0.003$  for BDNF;  $P = 0.001$  for NGF).

Expression of both neurotrophins had increased in all regions of the disc after WBV (Figure 5). The distribution of BDNF and NGF positive labeling varied within the regions of discs exposed to WBV (Figure 5), with greater labeling for both neurotrophins in the NP and IAF than the OAF (Table 1). BDNF expression was greatest in the IAF and NP, and significantly increased in the IAF ( $P = 0.003$ ) and the NP ( $P = 0.002$ ) over expression in the OAF (Figure 5). However, there was no difference in BDNF between the IAF and the NP

(Table 1). NGF labeling was also greatest in the NP and IAF; but, this increase was only significant in the NP compared with the OAF ( $P = 0.007$ ). There were no regional differences detected in the sham discs.

Neurotrophin responses correlated with behavioral sensitivity, although weakly. Both BDNF and total-NGF mRNA levels were significantly correlated ( $p_{BDNF} = 0.012$ ;  $p_{NGF} = 0.044$ ) with the percent reduction in withdrawal thresholds (Figure 6), though both were weak ( $R^2 = 0.396$  BDNF;  $R^2 = 0.276$  total-NGF). Neither form of NGF showed any relationship to behavioral sensitivity; BDNF protein was significantly correlated ( $R^2 = 0.280$ ;  $P = 0.035$ ) with behavioral sensitivity.

**DISCUSSION**

Painful WBV is associated with upregulation of both BDNF and NGF, especially in the inner regions of the cervical disc, even after a period of rest after the last exposure (Figures 2–5). These neurotrophins also increase in painful and degenerate lumbar IVDs and have been associated with innervation of typically aneural regions of the disc.<sup>9,13</sup> Despite such reports, this is the first study to note changes in these neurotrophins associated with pain due to WBV. Although the cervical spine undergoes significant mechanical deformation in this painful WBV model,<sup>18</sup> the physiological mechanisms responsible for pain onset and/or maintenance are not defined. Increased disc innervation has been reported in ovine models of accelerated degeneration due to annular lesions or tears and in humans with painful IVD.<sup>12,26,27</sup> The known role of BDNF and NGF in promoting nerve growth in the disc,<sup>9,13</sup> together with the significant correlations between their expression and pain in our model (Figure 6), support their potential role in regulating disc pain from WBV.

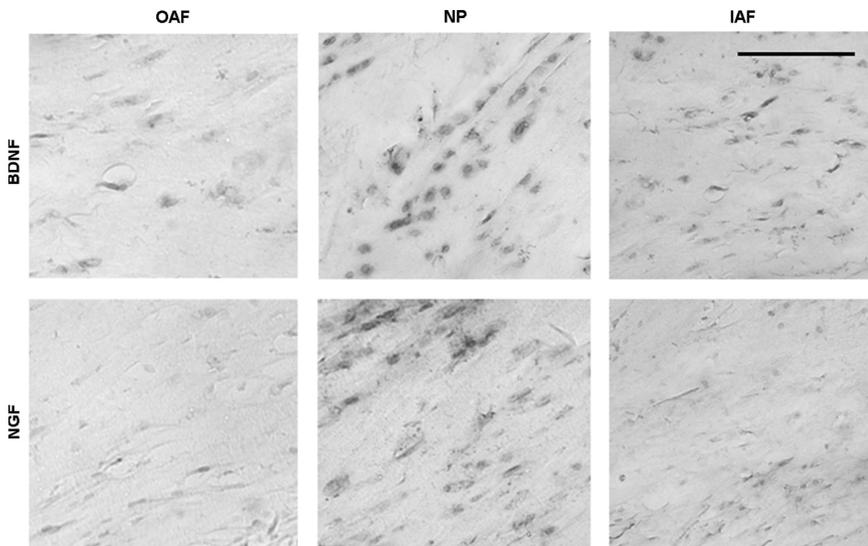
Both BDNF transcript and protein levels are increased in discs exposed to WBV and are significantly correlated with increased behavioral sensitivity at day 14 (Figures 2, 3, and 6), suggesting a role for BDNF in the pathomechanisms governing cervical neck pain. In addition to regulating the growth and survival of neural cells, BDNF modulates nociception *via* its anterograde transport from the DRG and release from primary afferents, increasing neuronal hyperexcitability in the spinal dorsal horn.<sup>15,28–32</sup> In fact, sequestering spinal BDNF using its TrkB receptor attenuates pain after spinal joint injury.<sup>33</sup>

**TABLE 1. Scoring on NGF and BDNF Labeling in the OAF, IAF, and NP**

Rat ID	BDNF			NGF		
	OAF	IAF	NP	OAF	IAF	NP
WBV						
20	±	±	+	+	+	++
21	+	–	±	+	+	+
23	+	++	++	++	++	++
24	++	++	++	+	++	++
Sham						
32	–	–	–	±	±	±
33	–	±	N/A	–	–	–
34	±	+	±	–	–	–

– denotes no staining; ±, mild staining; +, moderate staining; ++, robust staining.

BDNF indicates brain-derived neurotrophic factor; NGF, nerve growth factor; WBV, whole-body vibration; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; NP, nucleus pulposus; N/A, not available.

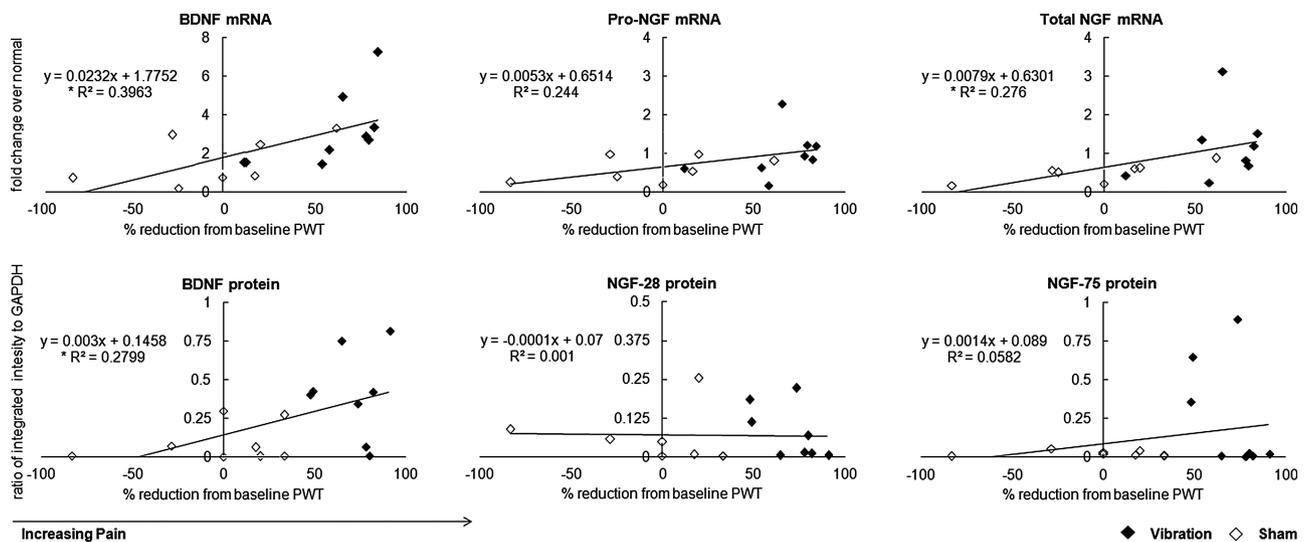


**Figure 5.** Positive labeling for both neurotrophins is evident in all regions of the disc. BDNF labeling in the IAF and NP is greater than in the OAF. NGF expression is greater in the NP than the OAF. BDNF indicates brain-derived neurotrophic factor; NGF, nerve growth factor; IAF, inner annulus fibrosus; OAF, outer annulus fibrosus; NP, nucleus pulposus.

Orita *et al*<sup>34</sup> also showed that intradiscal inhibition of BDNF in a puncture model decreases expression of the neuropeptide, calcitonin gene-related peptide, in the neurons that innervate the disc. Because BDNF expression in the disc increases with degenerative grade, BDNF signaling has been implicated in discogenic pain.<sup>13,35</sup> The findings of our study add further support to such hypothesis, particularly related to WBV.

Although gene expression for total-NGF significantly increases after painful WBV, the pro-NGF gene does not (Figure 2). Yet, total-NGF accounts for *both* the pro-NGF and mature transcripts of NGF. Pro-NGF (~30 kDa), a precursor of NGF, is active in several inflammatory conditions.<sup>36,37</sup> Intraplantar injection of pro-NGF, which binds the p75<sup>NTR</sup> NGF receptor, can induce hyperalgesia<sup>38</sup>; pro-NGF levels do not correlate with pain after WBV (Figure 6). Because only total-NGF levels correlate with behavioral

sensitivity, the mature transcript may be crucial in the development of disc pain. Furthermore, pro-NGF protein increases only slightly after WBV, whereas the high molecular weight 75-kDa NGF species exhibits a robust expression (10-fold) (Figure 3). Although this higher molecular weight species has not been previously reported in the disc, it is prevalent in extracerebral blood vessels and the external carotid artery.<sup>39</sup> Freemont *et al*<sup>9</sup> reported microvascular blood vessels expressing NGF adjacent to ingrown nerves in painful human lumbar IVDs. Regardless, increases in both of the NGF isoforms indicate its active production after WBV, despite not being directly related to pain. NGF in all regions of nondegenerate discs,<sup>13,40</sup> together with evidence in discs after WBV in our study (Figure 4; Table 1), indicate that NGF may support disc health and may be increased in response to pain or injury.



**Figure 6.** Correlations of BDNF and NGF mRNA and protein levels with percent reduction in PWT at day 14. BDNF ( $P = 0.012$ ) and total-NGF ( $P = 0.044$ ) mRNA are significantly correlated with greater decreases in PWT. Protein levels for both neurotrophins are weakly correlated with PWT, with only BDNF being significant ( $P = 0.035$ ). BDNF indicates brain-derived neurotrophic factor; NGF, nerve growth factor; mRNA, messenger RNA; PWT, paw withdrawal threshold.

Although both BDNF and 75-kDa NGF increase after WBV, there is a high degree of variability (Figure 3). This variability may be due to fact that tissue processing techniques for RT-qPCR and Western blot assay the entire disc and regional analyses were not performed, as with the IHC (Figures 4, 5). In fact, regional analysis of both neurotrophins indicates that there is an overall difference and regionally (Figures 4, 5; Table 1). The upregulation in the inner disc regions after WBV is also observed in patients with disc pain,<sup>9,13</sup> suggesting that localization of these neurotrophins in the nucleus may induce subsequent innervation of the disc.

Increased expression of NGF in the inner regions of the disc is seen exclusively in patients with painful discs and hypothesized to occur simultaneously with hyper-innervation, suggesting that nerve fibers only grew into discs with local production of NGF.<sup>9</sup> NGF production by human degenerate NP cells has been reported to promote nerve ingrowth *in vitro*.<sup>41</sup> In our study, NGF is greatest in the NP (Figure 5; Table 1), suggesting that repeated painful WBV may induce its production. Recently, BDNF has been purported to promote nerve ingrowth into degenerate disc tissue.<sup>13,35</sup> Both neurite outgrowth and the percent of neurite expressing cells decrease with anti-BDNF treatment in cultured degenerate human NP cells.<sup>35</sup> Because BDNF expression is greater in the IAF and NP than the OAF (Figure 5; Table 1), BDNF may “also” promote innervation into the disc. However, this study did not investigate whether such subsequent innervation was induced; additional studies are needed to evaluate if there is indeed a causal relationship between vibration, expression of these neurotrophins in the disc, and pain.

This is the first study demonstrating that painful WBV is not only associated with increased neurotrophins in the cervical disc, but those increases are in the disc regions where similar upregulation is observed in painful lumbar IVDs in humans. Moreover, BDNF transcript and protein, and NGF transcript levels correlate with the extent of behavioral sensitivity. This study provides additional support for these neurotrophins as having roles in disc pain, possibly *via* regulating nerve growth. Although additional studies are needed to define the specific temporal relationships between neurotrophins, nerve growth, and pain, these findings provide encouraging data supporting the notion that neck pain from WBV may result from direct biochemical changes in the disc.

## ➤ Key Points

- ❑ WBV induces upregulation of transcript and protein expression of the neurotrophins BDNF and NGF.
- ❑ BDNF expression in cervical discs after painful vibration is increased in the IAF and NP more than in the OAF. NGF expression is significantly upregulated only in the NP.
- ❑ Upregulation of neurotrophins in the inner annulus fibrosus and NP may contribute to WBV-induced neck pain.

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