

EXPRESSION OF NEUROTROPHIC FACTORS AND THEIR RECEPTORS IN THE RAT SPINAL TRIGEMINAL NUCLEUS

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Abstract

The spinal trigeminal nucleus as part of the trigeminal sensory nuclear complex is associated with the transmission of discriminative tactile sensations, primarily pain, and temperature, from the orofacial region. It is divided into three parts – caudal, interpolar, and oral subnucleus which process pain and temperature stimulate. Our previous experiments have revealed the presence of certain neurotrophic factors such as the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) in this nucleus. These neurotrophins facilitate neuronal differentiation, survival, and plasticity by signalling through high-affinity transmembrane receptors. By using primary antibodies against NGF, BDNF, NT-3, and glial-derived neurotrophic factor (GDNF), and their corresponding receptors of the tyrosine receptor kinase (Trk) proto-oncogene family and GFR α 1, we found immunoreactive cells scattered along the whole length of the nucleus in rats in all three subnuclei. In particular, we observed that the majority of spinal trigeminal neurons were intensely immunostained for all the neurotrophic factors examined and that they were richly endowed with their Trk receptors. Our results also showed that NGF, NT-3, TrkC and GFR α 1 expression did not differ between different topographic regions of the nucleus, while BDNF, GDNF, and TrkA expression was highest in the SpVo, and TrkB expression was highest in the SpVi and SpVc. Given that these neurotrophic factors are involved in mechanisms of central sensitization in trigeminal nociceptive pathways, it can

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be inferred that neurotrophins may contribute to a better understanding of the fundamental mechanisms of orofacial pain.

Key words: spinal trigeminal nucleus, oral subnucleus, interpolar subnucleus, caudal subnucleus, neurotrophic factors, neurotrophic receptors

Introduction. The spinal trigeminal nucleus (SpV) is a sensory tract in the brain stem's lateral medulla. It is involved in the transmission of numerous sensory modalities from the ipsilateral side of the face, such as temperature, deep or crude touch, and pain. The spinal trigeminal nucleus further subdivides into the oral subnucleus (SpVo), interpolar subnucleus (SpVi), and caudal subnucleus (SpVc). Each nucleus serves its purpose. Inside the spinal trigeminal nucleus, the information is presented in the form of an analogy of onion leaves. The SpVc (in the upper cervical part of the spine and the lower medulla oblongata) represents the peripheral areas of the face (scalp, ears, and chin). The SpVi (in the upper medulla) represents the central areas (nose, cheeks, and lips), and sensory information from the mouth, teeth and pharyngeal cavity reaches the oral subnucleus [1]. The nucleus plays an important role in clinically significant conditions such as trigeminal neuralgia, chronic orofacial pain, and migraine attacks [2], where the functional and structural integrity of the nucleus depends on some neurotransmitters, trophic factors, and their receptors, acting on the neuronal population in an autocrine or paracrine manner [3].

The protein family of neurotrophic factors designated neurotrophins consists of the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) [4]. They promote neuronal differentiation, survival, and plasticity by signalling via both a low-affinity (pan)-neurotrophin receptor p75NTR and high-affinity transmembrane receptors, members of the tyrosine receptor kinase (Trk) proto-oncogene family. The latter include TrkA, TrkB, and TrkC, and exhibit a binding specificity. TrkA is a receptor for the NGF; TrkB is a receptor for the BDNF and NT-4, while TrkC is a receptor for NT-3. The glial cell line-derived neurotrophic factor (GDNF) is a member of the GDNF superfamily of ligands. Signalling by a cell surface-bound specific co-receptor alpha-1 (GFR α 1), it may be selectively committed to cells devoted to proprioceptive sensory transmission [5]. At present, there is convincing evidence that neurotrophic factors from the spinal trigeminal nucleus also play an important role in maintaining the structural and functional characteristics of the neurons [6].

To understand the importance of the neurotrophins in neurons of the SpV, we first examined the immunohistochemical localization of some neurotrophic factors and their corresponding receptors in adult rats and then compared their expression patterns in the different subnuclei.

Materials and methods. Adult (12-week-old; $n = 8$) Wistar rats of both sexes weighing 200–250 g were used in this study. The experiments were conducted

under the ethical guidelines of the EU Directive 2010/63/EU following a standard protocol established by the Bioethical Commission at the Institute of Neurobiology at the Bulgarian Academy of Sciences.

The rats were deeply anesthetized with Nembutal (50 mg/kg, i.p., Abbott) and transcardially perfused first with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the cervical part of the spinal cord and the brain were quickly removed, the caudal brainstem and upper spinal cord were dissected out and were then postfixed in the same fixative overnight at 4 °C, embedded in paraffin, and cut into 7 µm thick sections.

The samples of the SpV were then deparaffinized with xylene and ethanol and processed for avidin-biotin-horseradish peroxidase complex immunohistochemistry. All sections were passed through the immunohistochemical procedures simultaneously to avoid variations in staining intensity. The endogenous peroxidase was blocked with 1.2% hydrogen peroxide in absolute methanol, followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) for up to 30 min. After washing in PBS, the sections were preincubated for 1 hour at room temperature in 5% normal goat serum. Between the separate steps, the sections were rinsed with PBS/Triton X-100. Subsequently, they were incubated in a humid chamber overnight at 4 °C with the following primary antibodies provided by Santa Cruz Biotechnology: rabbit anti-NGF (sc-548), rabbit anti-BDNF (sc-546), goat anti-NT-3 (sc-13380), mouse anti-GDNF (sc-13147), rabbit anti-TrkA (sc-118), rabbit anti-TrkB (sc-8316), rabbit anti-TrkC (sc-117), and rabbit anti-GFR α 1 (sc-10716) in working dilution 1:500. After rinsing in PBS, the sections were incubated for 1 h at room temperature with complementary secondary antibodies: biotinylated goat anti-rabbit IgG (Dianova, Hamburg, Germany), rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA, USA), horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:250, respectively. Following rinsing, the ABC complex (Vectastain Elite Kit; Vector Laboratories) was applied and colour development with diaminobenzidine as a chromogen was done for 3–5 min.

After immunostaining, the slides were viewed and photographed with Olympus VS120-L100 Virtual Slide System equipped with VS-ASW 2.9 Image Acquisition software.

Twenty-six images taken from all three parts of the spinal trigeminal nucleus of five rats provided an adequate sample size. The grayscale intensity (range 0–255: black = 0, white = 255), corresponding to the neurotrophic factors and their corresponding receptors immunostaining, was defined and the area of reaction was extracted. Each grayscale image was binarized and represented the analyzed reaction and was further used for grayscale measurements using open-source Fiji ImageJ software. To illustrate the distribution of the data, we have displayed them as box-and-whisker plots. The central line in each box plot represents the median, whereas the lower and upper halves of the box are the 25th and 75th

percentiles, respectively. The whiskers on the box are each outlier for the group.

Statistical data analysis was performed by SigmaStat® 11.0 software (Systat Software Inc., San Jose, CA, USA). Experimental data were evaluated by Student's *t*-test or Mann–Whitney *U* test, to parametric and nonparametric data, respectively. Differences were considered statistically significant if *p*-values were < 0.05.

Results. Using descriptive statistical analysis, we were able to demonstrate and compare the expression levels of the neurotrophic factors and their receptors in the three subnuclei of the SpV. The data showed that NGF was evenly expressed by the neurons in the nucleus with no statistically significant difference (Fig. 1, 3A). Increased immunoreactivity for TrkA was observed in SpVo compared to the SpVi subnucleus (median, 173.290 vs. 187.110, **p* = 0.038) and SpVc subnucleus (median, 173.290 vs. 189.690, ****p* ≤ 0.001) (Fig. 2, 3B). Mann–Whitney *U* test revealed slightly stronger expression of TrkA in neurons of SpVi subnucleus according to SpVc (median, 187.110 vs. 189.690, °*p* = 0.025) (Fig. 3B).

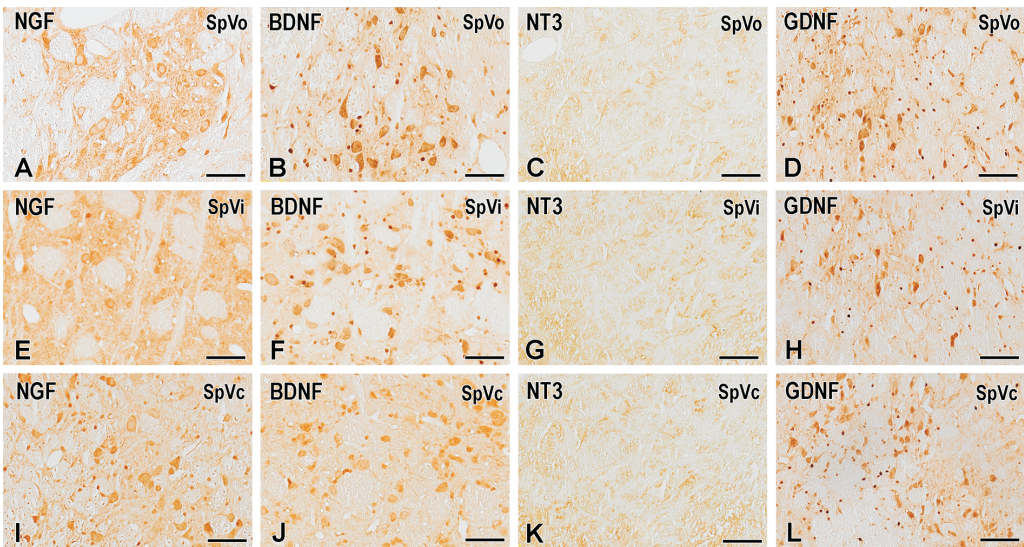


Fig. 1. Immunihistochemical demonstration of neurotrophic factors in the three subnuclei of the spinal trigeminal nucleus. NGF expression in the oral subnucleus (A), interpolar subnucleus (E), and caudal subnucleus (I). BDNF expression in the oral subnucleus (B), interpolar subnucleus (F), and caudal subnucleus (J). NT-3 expression in the oral subnucleus (C), interpolar subnucleus (G), and caudal subnucleus (K). GDNF expression in the oral subnucleus (D), interpolar subnucleus (H), and caudal subnucleus (L). Scale bars: 50 µm

For the BDNF expression levels, the comparison between the three subnuclei of SpV revealed that they have different expression levels for the neurotrophic factor (Fig. 1). Turns out that BDNF had the highest expression in the neurons of the SpVo subnucleus, followed by the ones in the SpVc (median, 148.869 vs. 156.240, **p* = 0.041 when compared to SpVo; median 156.240 vs. 165.068

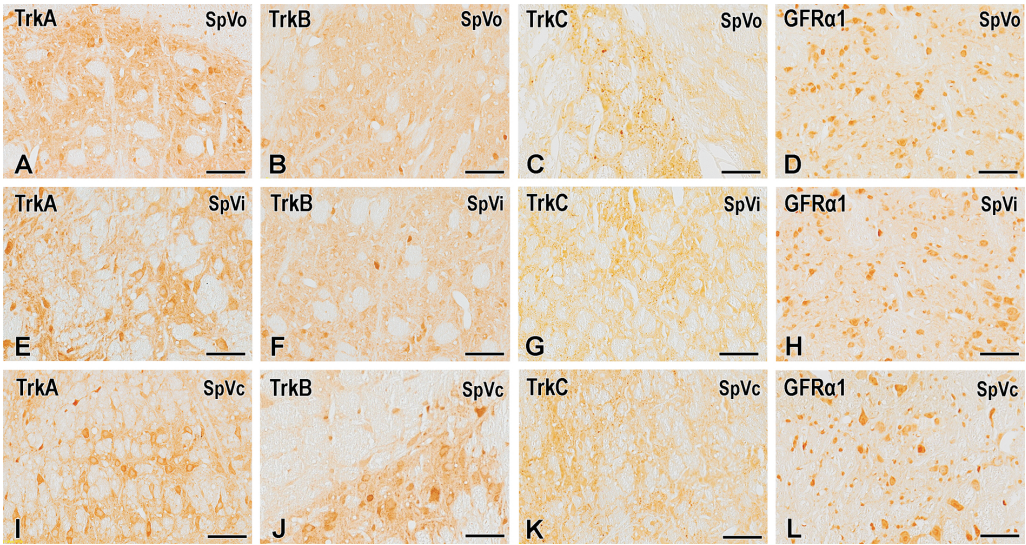


Fig. 2. Immunohistochemical demonstration of neurotrophic receptors in the three subnuclei of the spinal trigeminal nucleus. TrkA expression in the oral subnucleus (A), interpolar subnucleus (E), and caudal subnucleus (I). TrkB expression in the oral subnucleus (B), interpolar subnucleus (F), and caudal subnucleus (J). TrkC expression in the oral subnucleus (C), interpolar subnucleus (G), and caudal subnucleus (K). GFR α 1 expression in the oral subnucleus (D), interpolar subnucleus (H), and caudal subnucleus (L). Scale bars: 50 μ m

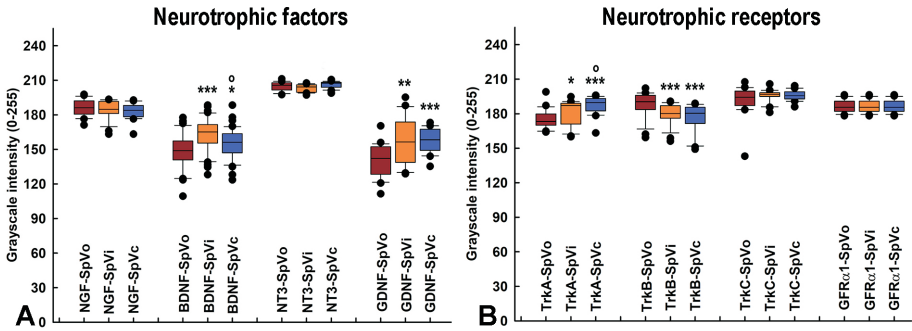


Fig. 3. Statistical comparison of the immunostaining intensity for neurotrophic factors (A) and their corresponding receptors (B) in the three subnuclei of the spinal trigeminal nucleus (SpV). Morphometric data are presented as box plots (median, 25–75%), where the boxes represent the second and third quartiles and the line within the box represents the median, and lines outside the box represent the spread of values.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (vs. SpVo); ° $p < 0.05$ (vs. SpVi)

when compared to SpVi, ° $p = 0.018$), and the SpVi expression (median, 148.869 vs. 165.068, *** $p < 0.001$) in last place (Fig. 3A). TrkB as a receptor for BDNF showed different expression levels in the three subnuclei as well; the one in the caudal subnucleus being the highest and the expression in the oral being the lowest

(Fig. 2). Statistical difference was observed between the oral and the interpolar subnuclei (median, 190.430 vs. 180.246, $***p \leq 0.001$) and between the oral and caudal subnuclei (median, 190.430 vs. 180.420, $***p \leq 0.001$) (Fig. 3B).

NT-3 expression (Fig. 1) was the lowest out of the set of neurotrophic factors scrutinized in the study along with the expression levels of its TrkC receptor (Fig. 2) with no statistical difference in the three subnuclei whatsoever (Fig. 3A, B).

On the contrary, GDNF expression was abundant in the neurons of the three subnuclei of the spinal trigeminal nucleus (Fig. 1). The highest and statistically significant expression was observed in the oral subnucleus compared to the interpolar (median, 142.250 vs. 156.490, $**p = 0.003$) and caudal (median, 142.250 vs. 158.418, $***p < 0.001$). GFR α 1 receptor immunostaining intensity did not differ in all three examined subnuclei of the spinal trigeminal nucleus (Fig. 2, 3B).

Discussion. The present study provides immunohistochemical evidence that the three different parts of the spinal trigeminal nucleus release trophic factors, which helps to explain some mechanisms underlying the development of chronic conditions related to nociception. Our results show that the three subnuclei highly express neurotrophic factors of the NGF, BDNF, NT-3, and GDNF family and their corresponding receptors. Here we demonstrate the occurrence of NGF, BDNF, NT-3, and GDNF proteins in the vast majority of neurons which are also endowed with their cognate receptors TrkA, TrkB, TrkC, and GFR α 1.

This is the first report on difference in the expression levels of the neurotrophic factors and their receptors which have been compared in the three parts of the spinal trigeminal nucleus of the rat. The differences in terms of expression between the three subnuclei have never been studied before. We now know that the BDNF and GDNF along with their receptors take a great deal of the set of neurotrophic factors investigated in this study; and, on the contrary, the NT-3 and its TrkC receptor seem not that well expressed by the neurons in the spinal trigeminal nucleus. There is strong evidence that some neurotrophic factors, such as BDNF and GDNF, working together are important regulators for the development of sensory cholinergic neurons in the central nervous system, the latter leading sensory impulses to higher areas of the central nervous system [7]. Previous studies have shown that many growth and trophic factors are found in mammalian cell populations of the spinal trigeminal nucleus [8].

Our studies confirm previous findings and show that NGF and its corresponding TrkA receptor, which binds with high affinity to NGF, are expressed primarily by neurons in the spinal trigeminal nucleus [9]. Even though there is evidence that neurons do not depend on NGF and BDNF for survival and thus the growth requirements are different for these cells; nevertheless, the increase in the chance of survival depends on the activation of protein kinase B [10]. In the present study, we additionally visualized NGF and TrkA immunoreactivity in rat spinal trigeminal nucleus cells. Our results are consistent with the hypothesis that the increased expression of molecules in the spinal trigeminal nucleus, which regulates

its postnatal growth, may affect the way pain is perceived at the central level.

In conclusion, our study suggests that the expression of neurotrophic factors in the three subnuclei of the spinal trigeminal nucleus varies. In particular, the majority of neurons contain NGF, BDNF, NT-3, GDNF, and the corresponding receptors, and their expression levels differ significantly in the different parts of the nucleus. It is also likely that the cellular populations in the nucleus may exert, via the entire cocktail of neurotrophins present in them, trophic and/or regulatory control on the adjacent cells in the spinal trigeminal nucleus suggesting a possible autocrine and paracrine role. Last but not least, lessons from the expression of neurotrophins may be applied when understanding better their involvement in the genesis and/or temporal evolution of conditions affecting the sensory pathways for pain from different regions of the head.

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