

NITRIC OXIDE SYNTHASE IMMUNOREACTIVITY IN THE RAT SPINAL TRIGEMINAL NUCLEUS

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Abstract

The spinal trigeminal nucleus (SpV) is involved in the transmission of nociceptive information from the orofacial region. This study investigates nitric oxide synthase (NOS) immunohistochemical staining and conducts statistical data analysis within the three subnuclei of the SpV in rats. Our immunohistochemistry study revealed that the SpV has a high expression of nNOS, with immunostaining mostly seen in the perikarya and nerve processes of some neurons. Furthermore, we found that glial cells in the nucleus surrounding blood vessels display a significant nNOS immunohistochemical staining. NOS-immunopositive neurons had small cell bodies. Strong staining is also evident in their processes. NOS-immunoreactive neuronal processes encircled a significant amount of blood vessels. There were statistically significant variations in the nNOS expression between the SpV subnuclei. In particular, the caudal subnucleus exhibited the greatest expression, followed by the interpolar subnucleus, and the oral subnucleus showed the lowest expression. Remarkably, there was no discernible variation in the expression of nNOS in the interpolar and oral regions of the SpV. We suggest that NO can act as a neurotransmitter in increasing nociceptive stimuli in the SpV.

Key words: spinal trigeminal nucleus, subnuclei, nitric oxide synthase (NOS), immunohistochemistry, rat

Introduction. Nitric oxide (NO) is an unusual endogenous signalling gas molecule that is involved in a variety of processes. There are three different

isoforms of its synthesizing enzyme, nitric oxide synthase (NOS), which are designated neuronal nNOS (or NOS I), inducible iNOS (or NOS II), and endothelial eNOS (or NOS III). Neuronal, nNOS is expressed constitutively in certain neurons of the brain. In addition to brain tissue, nNOS has been identified by immunohistochemistry in various other tissues and organs, such as the spinal cord, sympathetic ganglia, and adrenal glands, in epithelial cells of various organs, in the macula densa of the kidney, in the cells of the islets of Langerhans in the pancreas, and vascular smooth muscle [1]. Certainly, several earlier findings have shown widespread expression of nNOS-immunoreactivity in trigeminal ganglia in mammals [2] as well as in birds [3], and reptiles [4]. The small- and medium-sized neuronal cell bodies constitute the majority of those that produce NOS [5]. The neuronal isoform of NOS has been demonstrated along the length of the spinal cord meaning that nNOS-containing neurons may be involved in preganglionic sympathetic and parasympathetic, somatosensory, visceral sensory, and maybe motor pathways in spinal cord neurotransmission [6]. Particularly, the presence of nNOS in the superficial layers of the dorsal horn of the spinal cord suggests that NO has functions in sensory and pain processing [7]. There is evidence that NO has a key role in hyperalgesia and sensitization of sensory neurons [8]. The primary afferent neurons of the trigeminal system in the cat produce NOS both peripherally in their nerve fibres and their perikarya. Indeed, NOS-immunopositive neurons have been observed in the cat mesencephalic trigeminal nucleus and the trigeminal ganglion [5]. Expression of NOS isoforms has been investigated in parts of the SpV, particularly its caudal subdivision during acute and chronic arthritis showing stable levels of nNOS immunoreactivity and mRNA, but an increase in the activity of the region [9]. The latter experiment has supported the antinociceptive of nNOS. Having collected these data, this study aims to investigate the presence of the nNOS in the SpV by performing NOS immunohistochemistry and subsequent statistical analysis within its three subnuclei, the oral (SpVo), inter-polar (SpVi), and caudal (SpVc).

Material and methods. We used six adult male Wistar rats with a weight range of 200–250 g in this study. The research procedures adhered strictly to the ethical guidelines outlined in the EU Directive 2010/63/EU, safeguarding the welfare of animals used for scientific purposes. The protocol was established following the standards set by the Bioethical Commission of Biomedical Research at the Institute of Neurobiology of the Bulgarian Academy of Sciences. Rigorous efforts were made to minimize the number of animals used in this study, mitigate any discomfort, and avoid the pain experienced by laboratory animals. Rats were deeply anesthetized with Nembutal (50 mg/kg, i.p., Abbott) and then they underwent transcardial perfusion with 0.05 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Subsequently, the cervical segment of the spinal cord and the brain were quickly dissected out and postfixed overnight at 4 °C in the same fixative. The tissue samples were

processed following fixation, including washing, dehydration, paraffin embedding, and sectioning into 7 μm thick sections. The first section of each series was routinely stained with hematoxylin and eosin to assess the nuclear morphology, while the remaining sections were designated for immunohistochemical analysis.

The sections through the SpV underwent deparaffinization and were subjected to ABC (avidin-biotin horseradish peroxidase complex) immunohistochemistry to analyze the nitrergic profile of neurons in the nucleus. To ensure uniform staining intensity, all sections underwent immunohistochemical procedures simultaneously in the same slide tray. Initially, endogenous peroxidase activity was blocked using 1.2% hydrogen peroxide in absolute methanol, followed by antigen retrieval in 10 mM citrate buffer (pH 6.0) for up to 30 min in a microwave oven. After washing in PBS, sections were preincubated for 60 min at room temperature in 5% normal goat serum to minimize nonspecific staining. Between steps, sections were rinsed with cold PBS/Triton X-100. Subsequently, sections were incubated overnight at 4 °C with a monoclonal mouse primary antibody against nNOS (BD Biosciences, 1:100 dilution) in a humid chamber. Following PBS rinses, sections were incubated for 2 h at room temperature with a complementary secondary antibody, biotinylated horse anti-mouse IgG (Vector Labs) at a dilution of 1:250. After additional rinsing, the ABC complex (Vectastain Elite Kit; Vector) was applied, followed by colour development with diaminobenzidine as a chromogen for 3–5 min.

To ascertain antibody specificity, both positive and negative controls were conducted. Negative controls involved omitting the specific primary antibody and replacing it with PBS or non-immune serum at an equivalent dilution, resulting in the absence of specific immunostaining.

Following immunostaining, slides were examined and captured using an Olympus VS120-L100 Virtual Slide System equipped with VS-ASW 2.9 Image Acquisition software. Before image acquisition, the system underwent meticulous calibration to ensure accurate image capture. This calibration included standardizing the light source, including colour filters, and camera settings for consistency across all images. Additionally, adjustments for brightness, contrast, colour, and sharpness were meticulously calibrated. The resulting digital images were saved in TIF format for further analysis.

Thirteen images captured from each segment of the SpV constituted a sufficiently robust sample size. These immunostained images were transferred to a laptop equipped with the open-source image analysis platform Fiji [10] for subsequent analysis of the NOS expression. Within each subnucleus, three randomly selected fields with consistent staining were identified for analysis. To perform morphometric analysis, the micrographs of immunostained sections were initially converted to grayscale images. Grayscale intensity, indicative of NOS immunostaining, was determined, and the corresponding reaction area was extracted. Each grayscale image underwent automatic binarization, wherein foreground pixels were

converted to black, and background pixels to white, representing the analyzed reaction area for positive measurements. The positive immunoreaction area was quantified based on the number of black pixels within the range of gray levels in neuronal bodies. A minimum of 200 neurons were randomly measured. Neuronal outlines and cross-sectional areas were integrated with the respective immunoreactivity images. Quantitative assessments of immunostaining intensity were conducted on colour images, visually represented using Box-and-Whisker plots to illustrate data distribution. Statistical analyses were performed using Excel 2016 (Microsoft Inc.), employing the Kruskal–Wallis test followed by a post hoc analysis using the Dunn multiple comparison test. Statistical significance was determined at $p < 0.05$.

Results. To reveal the presence of NO in the SpV of rats, we demonstrated the immunohistochemical expression of the neuronal isoform of its synthesizing enzyme, nNOS, in the nucleus. Our immunohistochemical analysis showed that nNOS was strongly expressed in the SpV, with immunostaining mainly detected in the perikarya and nerve processes of some neurons therein. Additionally, we found that glial cells surrounding the blood vessels in the nucleus exhibited strong immunohistochemical staining for nNOS (Fig. 1). The nNOS-immunoreactive nerve fibres were observed to intersect the SpV. Immunopositive neurons are typically small-sized with varied shapes of their perikarya. Their processes displayed intense immunostaining. A large number of blood vessels were surrounded by immunopositive for nNOS cells and their processes. Furthermore, we mapped the distribution of nNOS-positive cells in the caudal, interpolar, and oral subdivisions of the SpV. This broad expression pattern underscored the widespread presence of nNOS along the entire longitudinal axis of the SpV. This distributional pattern was highly similar in the three subnuclei: nerve processes remained immunopositive near the cell bodies of neurons, perikarya of small cells, and cells surrounding the blood vessels.

To assess the intensity of nNOS staining in the three different subnuclei of the SpV, we applied the Kruskal–Wallis test. This test revealed significant differences in the expression of nNOS among the three spinal trigeminal subnuclei ($H(2) = 39.60$, **** $p < 0.0001$). The median values (Mdn) varied across the individual parts, with the highest value observed in the interpolar subnucleus (Mdn = 143), followed by the oral subnucleus (Mdn = 138), and the lowest median was observed in the caudal subnucleus (Mdn = 103). A higher median value indicates a weaker expression; conversely, a lower value indicates a higher expression.

Following the Kruskal–Wallis test, we conducted a post hoc analysis using the Dunn multiple comparison test to further elucidate specific differences in nNOS expression between the subnuclei. The Dunn test showed that there were no statistically significant differences in nNOS expression between the oral and interpolar subnuclei ($p > 0.9999$). However, a statistically significant difference was observed between the oral (nNOS-SpVo) and caudal (nNOS-SpVc) subnuclei of

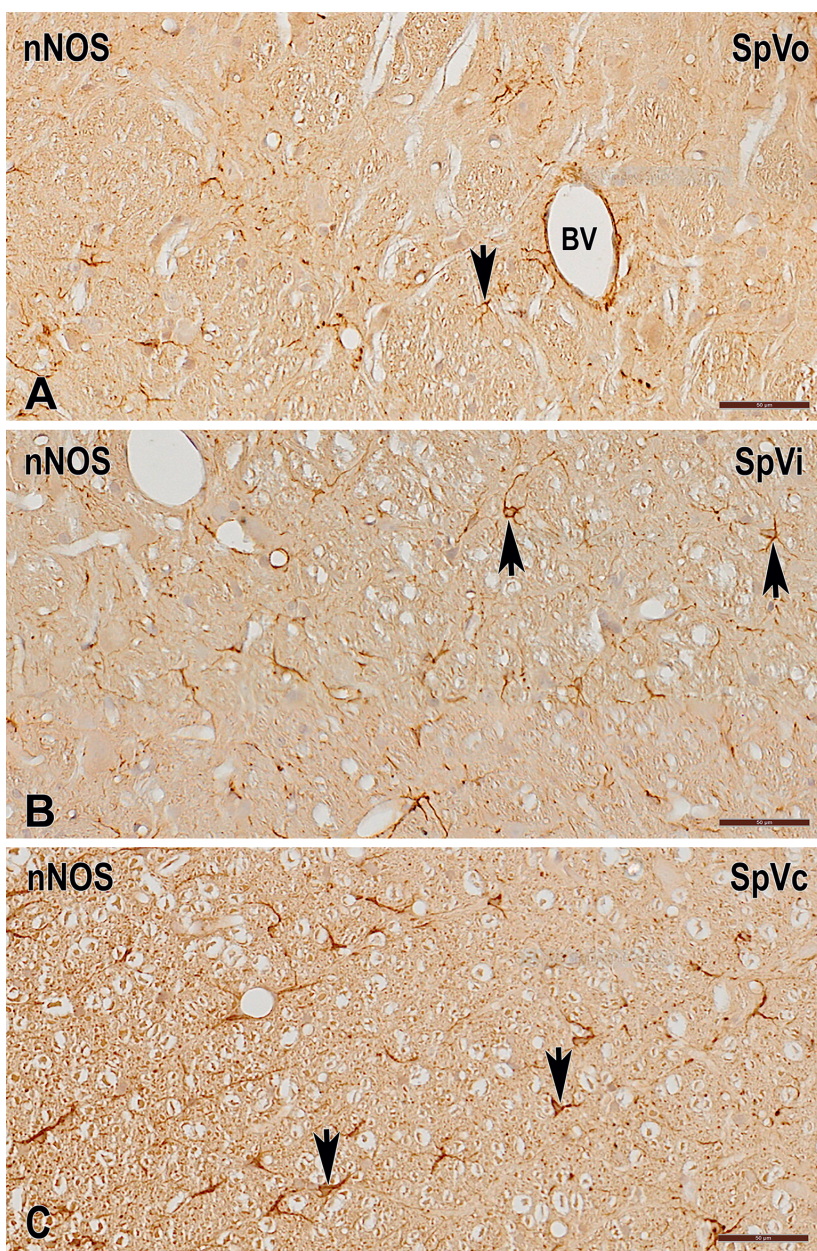


Fig. 1. Expression of the neuronal form of nitric oxide synthase (nNOS) in nerve cells around blood vessels (BV) and neuronal perikarya (arrows) in the oral (SpVo) (A), the interpolar (SpVi) (B), and the caudal (SpVc) (C) subnucleus of the spinal trigeminal nucleus (SpV). Perivascular nNOS immunostaining is present in the proximal parts of the nerve fibres in all three subnuclei. Immunopositive neurons were typically small in size with varied perikaryal shapes, showing intense process staining. The distribution pattern is consistent across subnuclei, with immunopositive nerve processes near neuronal cell bodies, perikarya of small cells, and cells around blood vessels, underscoring its widespread presence along its entire axis. Scale bar = 50 μm

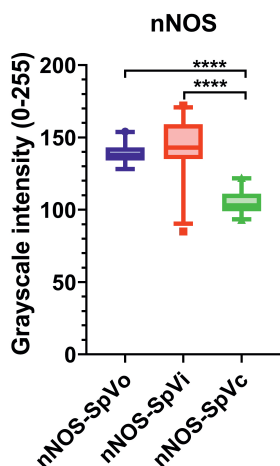


Fig. 2. Box-plot diagrams graphically representing the staining intensity for the nNOS in the oral (SpVo), interpolar (SpVi), and caudal (SpVc) subnucleus of the spinal trigeminal nucleus (SpV). Median values (Mdn) varied, with the highest observed in the interpolar subnucleus (Mdn = 143), followed by the oral subnucleus (Mdn = 138), and the lowest in the caudal subnucleus (Mdn = 103). A post hoc analysis revealed no statistically significant differences in nNOS expression between the oral and interpolar subnuclei ($p > 0.9999$). However, a significant difference was found between the oral (nNOS-SpVo) and caudal (nNOS-SpVc) subnuclei of the spinal trigeminal nucleus (**** $p < 0.0001$)

the spinal trigeminal nucleus (**** $p < 0.0001$), indicating a difference in the levels of nNOS expression between these regions (Fig. 2). Notably, nNOS expression was strongest in the caudal subnucleus. Furthermore, statistically significant differences were found between the interpolar and caudal subnuclei (**** $p < 0.0001$), underscoring the distinct expression profiles of nNOS in these anatomical subdivisions of the spinal trigeminal nucleus.

Discussion. In this study, we performed immunohistochemical staining to reveal the distribution of nNOS expression in the individual subnuclei of the rat SpV. Our immunohistochemical data unequivocally demonstrate the presence of nNOS both in the nerve fibres and in the neurons within the SpV. Statistically significant differences in nNOS expression are observed between its subnuclei, with the highest expression being found in the caudal subnucleus, followed by the interpolar subnucleus, and the lowest in the oral subnucleus. Interestingly, no significant difference in nNOS expression was found between the interpolar and oral parts of the SpV.

The observed regional differences in nNOS expression among the subnuclei of the SpV suggest a nuanced regulatory mechanism. The significantly higher expression in the SpVc may indicate a specialized role of nNOS in this region, potentially contributing to the modulation of nociceptive signals or sensory processing specific to the caudal aspect. The lack of significant difference in nNOS expression between the interpolar and oral subnuclei suggests potential functional similarities in these regions. Intriguingly, despite their different locations within the nucleus, these subnuclei exhibit comparable levels of nNOS expression.

The link between NO and the pathophysiology of migraine is emphasized by the well-known effect of inhibiting NO to reduce spontaneous activity in the SpV. The observation that nNOS expression is highest in the SpVc, known for its involvement in the sensory processing of nociceptive information, further supports the idea that NO may enhance migraines. This finding is consistent with existing

literature data indicating the involvement of NO in the generation or modulation of migraine headaches. Infusion of NO donors leads to a spontaneous increase in neuronal activity in the neurons of the SpV in an experimental rat model [11]. For example, the introduction of NO donors may facilitate nociceptive transmission. Using NADPH-diaphorase histochemistry and nNOS immunohistochemistry, SCHLECHTWEIG et al. [12] identified nitric oxide synthase (NOS)-containing neurons in the rat SpV after infusion of the NO donor sodium nitroprusside [13]. Following the infusion, there has been a time-dependent rise in the number of NADPH-diaphorase-positive neurons by a factor of more than two as well as an increase in the quantity of nNOS-immunopositive neurons. Possibly, through the activation of nNOS expression, NO donors increase the number of neurons in the SpV that generate NO. This fact may suggest that NO, as a nitrovasodilator, leads to enhanced transmission of pain information from the head.

When it comes to sensory information reaching and passing through the SpV, inhibiting endogenous NO synthesis reduces spontaneous activity in this nucleus, which should mean that NO has a tonic effect on migraines [11]. This hypothesis has already been confirmed by clinical studies showing increased NO metabolites in the blood in the internal jugular vein in people suffering from migraines during spontaneous migraine attacks [13]. Thus, NO plays an important role in the pathogenesis of migraine attacks, which in turn means that activation of nNOS would lead to increased signal transduction in neurons in the SpV. Considering that according to our results, the levels of nNOS are highest there, it can be hypothesized that the enzyme actively participates in increasing pain transduction. Adding to the theory that specific parts of the head have a columnar distribution in all three parts of the SpV, with sensory information from the lateral parts of the head, specifically the temporal areas reaching the SpVc, it turns out that the higher basal expression of nNOS there compared to other parts of the nucleus may be an important support in tracking the effect of nNOS on the pathogenesis of migraine attacks. The established expression of nNOS in the nucleus can be used for further targeting of the enzyme and its inhibition to mitigate pain signals coming from the head.

Conclusion. In conclusion, our results provide immunohistochemical evidence on the differentiated expression of nNOS in the subnuclei of the rat SpV. The observed regional variations raise intriguing questions about the specific roles of nNOS in pain modulation and sensory processing in this nucleus. Furthermore, the correlation between nNOS expression and the pathophysiology of migraine suggests potential opportunities for further research into the therapeutic targeting of NO in migraine treatment. Overall, these findings contribute to the growing body of knowledge on the neurochemical basis of pain and offer potential avenues for the development of targeted treatments for migraine disorders. Further investigation into the specific roles of nNOS in these regions may reveal common neurochemical pathways or regulatory mechanisms.

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