MEDICAL UNIVERSITY OF SOFIA

DEPARTMENT OF ANATOMY, HISTOLOGY AND EMBRYOLOGY

ANDREY VALKOV IVANOV, MD

MORPHO-FUNCTIONAL AND NEUROCHEMICAL CHARACTERISTICS OF THE SPINAL NUCLEUS OF THE TRIGEMINAL NERVE IN RATS

A B S T R A C T

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A B S T R A C T

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Prof. Dr. Nikolai Elenkov Lazarov, MD, PhD, DSc

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The dissertation is typewritten on 211 A4 pages and contains 36 figures (26 microphotographs and 13 graphs) and 3 tables. The references include 398 sources, 3 of them in Bulgarian and 395 in English.

The dissertation has been discussed and scheduled for defense by the Department Council of the Department of Anatomy, Histology and Embryology at the Medical University of Sofia.

The experiments from the dissertation have been conducted in the "Functional neuroanatomy" lab at the Institute of Neurobiology at the Bulgarian Academy of Sciences and the Department of Anatomy, Histology and Embryology at the Medical University of Sofia.

The scientific jury made of 5 habilitated persons consists of:
1. Prof. Lina Malinova, MD, PhD – internal member for MU-Sofia, Department of Anatomy, Histology and Embryology at the Faculty of Medicine at MU-Sofia;
2. Assoc. Prof. Dr. Angel Dandov, MD, PhD – internal member for MU-Sofia, Department of Anatomy, Histology and Embryology at the Faculty of Medicine at MU-Sofia;
3. Prof. Dr. Stefan Sivkov, MD, PhD – external member for MU-Sofia, Medical University-Plovdiv;
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5. Assoc. Prof. Dr. Stoyan Pavlov, MD, PhD – external member for MU-Sofia, Medical University-Varna.

Reserve members:
1. Assoc. Prof. Dr. Dimo Krastev, MD, PhD – internal reserve member for MU-Sofia, MK "Yordanka Filaretova" at MU-Sofia;
2. Assoc. Prof. Dr. Nikolay Dimitrov, MD, PhD – external reserve member for MU-Sofia, Trakia University-Stara Zagora.

The defense of the dissertation will take place on the 4th of June, 2024 at 1 pm in the Anatomy auditorium at the Department of Anatomy, Histology, and Embryology at the Medical University of Sofia with address: Sofia, 52A Pencho Slaveykov Blvd.

The dissertation is available in the secretary´s office of the Department of Anatomy, Histology, and Embryology at the Medical University of Sofia, 2 Zdrave Str., floor 3, room 1.

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To each of you, I am deeply grateful for your contributions, support, and belief in me. This thesis is dedicated to you, with love and appreciation, for being an integral part of my academic journey.

TABLE OF CONTENTS

ABBREVIATIONS

PNS	peripheral nervous system
HE	hematoxylin and eosin
CNS	central nervous system
5-HT	5-hydroxytryptamine
ABC	avidin-biotin complex
AChE	acetylcholinesterase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ATP	adenosine triphosphate
BChE	butyrylcholinesterase
BDNF	brain-derived neurotrophic factor
CGRP	calcitonin gene-related peptide
CLR	calcitonin-like receptor
CN V	trigeminal nerve, *nervus trigeminus*
CN V1	ophthalmic nerve, *nervus ophthalmicus*
CN V2	maxillary nerve, *nervus maxillaris*
CN V3	mandicular nerve, *nervus mandibularis*
CN VII	facial nerve, *nervus facialis*
CN IX	glossopharyngeal nerve, *nervus glossopharyngeus*
CN X	vagus nerve, *nervus vagus*
GABA	gamma amino butyric acid
GAD	glutamic acid decarboxylase
GDNF	glial-derived neurotrophic factor
GFRα1	glial family receptor α1
KCC2	K+/Cl cotransporter
NADPH	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factor
NKCC1	$Na^+/K^+/2Cl^-$ cotransporter
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NPY	neuropeptide Y
NT-3	neurotrophin-3
NT-4	neurotrophin-4
p75NTR	neurotrophic receptor p75
PBS	phosphate-buffered saline
PPT-A	pro-tachykinin-A
PRiMA	proline-rich membrane anchor
PYY	peptide YY
RAMP	receptor activity modyfing proteins
SP	substance P
SpV	spinal trigeminal nucleus
SpVc	caudal subnucleus of the spinal trigeminal nucleus
SpVi	interpolar subnucleus of the spinal trigeminal nucleus
SpVo	oral subnucleus of the spinal trigeminal nucleus
SSADH	succinic semialdehyde dehydrogenase deficiency
TCA cycle	tricarboxylic acid cycle
TNF	tumor necrosis factor
TPH	tryptophan hydroxylase
TrkA	tropomysosin kinase A
TrkB	tropomyosin kinase B
TrkC	tropomyosin kinase C
VGLUT2	vesicular glutamate transporter 2
VPM	ventral posteromedial thalamic nucleus, *nucleus ventralis posteromedial thalami*

I. INTRODUCTION

The spinal trigeminal nucleus (SpV) is one of the sensory nuclei of the trigeminal nerve, the fifth cranial nerve (CN V). This nerve is the largest of all the cranial nerves. Its name (trigeminal = tri-, three and Latin geminus, twin: three twins) derives from the fact that this nerve has three main branches: the ophthalmic nerve, *nervus ophthalmicus* (CNV1), the maxillary nerve, *nervus maxillaris* (CNV2) and the mandibular nerve, *nervus mandibularis* (CNV3). The ophthalmic and maxillary nerves are purely sensory, while the mandibular nerve has both motor and sensory functions (Bradley, 2014).

The spinal trigeminal nucleus is subdivided in the rostrocaudal direction into three subnuclei, designated as the oral, interpolar, and caudal portions, respectively. The first of these is associated with the transmission of discriminative (fine) tactile sensations from the orofacial region and is an extension of the main sensory nucleus of the *nervus trigeminus*. The interpolar subnucleus is also functionally associated with the transmission of tactile information and dental pain, while the caudal subnucleus transmits pain stimuli and temperature sensations from the head.

Research on the spinal trigeminal nucleus in recent years has focused mainly on elucidating its structure, functional organization, and somatotopy as an important integral part of the trigeminal sensory nuclear complex. On the other hand, neurochemical studies in experimental animals have established the presence of a wide range of endogenous neuroactive ligands such as classical neurotransmitters, gaseous transporters, neuropeptides, and modulators in neurons in the spinal trigeminal nucleus, which represents a remarkable feature for the cells. These studies have specifically demonstrated the presence of chemical mediators such as serotonin, gamma-aminobutyric acid, dopamine, noradrenaline, and glutamate in spinal trigeminal neurons (Grzanna et al., 1987; Bereiter and Gann, 1988; Costa, 1994; Viggiano et al., 2004; Liu et al, 2019).In addition to these classical mediators, some neuroactive peptides, such as substance P, enkephalin, and neuropeptide Y, have been found in spinal trigeminal nucleus neurons and their outgrowths (Hökfelt et al., 1977; Priestley et al., 1982; Chronwall et al., 1985).

The present study provides detailed data on the structural features of the rat spinal trigeminal nucleus, provides extensive insight into its cytoarchitectonics, and extends the available knowledge of the neurotransmitter profile of neurons in its three subnuclei with an emphasis on elucidating their functional modalities.

II. AIM AND OBJECTIVES

The present study aimed to establish the structural organization and the cytoarchitectonics of the rat spinal trigeminal nucleus, and to reveal the neurotransmitter/neuromodulatory and neurotrophic nature of its neurons.

To accomplish this goal, we set the following main objectives:

1. Use of classical histological techniques to represent the normal morphology of the rat spinal nucleus, including the cytoarchitectonics of neurons in its subnuclei.
2. Using immunohistochemical techniques at the light microscopic level to reveal the neurotransmitter, neuropeptide, and neurotrophic profile of neurons in the rat spinal trigeminal nucleus.
3. Based on the immunohistochemical experiments to quantitatively analyze the neurochemical profile of the individual subnuclei and to make a comparative statistical analysis of the distribution of the studied bioactive substances in the three subnuclei.
4. To provide a functional explanation of the role of the identified neuroactive substances in the individual subnuclei of the rat spinal trigeminal nucleus.

III. MATERIALS AND METHODS

3.1 Experimental Animals

The experiments in the present study were performed on material from sexually mature Wistar rats. A total of twenty-six adult male rats with a body weight of 180–300 g were studied. The experiments were conducted mainly at the Institute of Neurobiology of the Bulgarian Academy of Sciences (BAS), and a small part of them was performed in the laboratories of the Department of Anatomy, Histology and Embryology of the Medical University (MU)-Sofia. All animals were regularly monitored to be in good health, were provided with unlimited access to food and water, and were dewormed by the staff of the Vivarium at the Institute of Neurobiology at BAS.

All studies were conducted according to the regulations for work with experimental animals in Bulgaria in compliance with the rules of the Ethics Committee of the Institute of Neurobiology, BAS (registration FWA 00003059 US Department of Health and Human Services) and those of the Research Ethics Committee of MU-Sofia (KENIMUS).

3.2 Histological Techniques

3.2.1 Collection and Preparation of Material

Experimental animals first received superficial anesthesia with ether and then were injected with thiopental at a dose of 40 mg/kg intraperitoneally. They received anesthesia and the ascending aorta was used for perfusion, with the cannula inserted through the left ventricle of the heart. After perfusion and removal of the brain from the cranial cavity, we dissected the area of interest under a magnifying glass. The material was embedded in paraffin.
Using a Leica RM 2125 RTF microtome, the paraffin blocks were cut into 7 µm-thick sections and the serial sections were placed on chrome-gelatinized slides.

3.2.2 Hematoxylin and Eosin Staining

3.2.3 Toluidine Blue Staining

3.2.4 Staining with Neutral Red

3.3. Immunohistochemical Methods

3.3.1 Immunohistochemical Procedure

The immunohistochemical reactions in this work used the avidin-biotin-peroxidase complex (ABC) method according to Hsu et al (Hsu et al., 1981). Each immunohistochemical reaction was performed on paraffin sections 7 µm thick in a humidified medium.

Table 1. List of the primary antibodies used in the immunohistochemical reactions.

Primary antibody	Catalog number	Supplier	Host/Type	Dilution
GABA	A-2052	Sigma BioSciences	rabbit/polyclonal	1:1000
AChE	PA5-86086	Thermo Fisher Scientific	rabbit/polyclonal	1:500
5-HT	S 5545	Sigma-Aldrich	rabbit/polyclonalo	1:5000
SP	8834033	INCSTAR	rabbit/polyclonal	1:1000
NPY	PRN 1702	Amersham	rabbit/polyclonal	1:500
CGRP	PEPA27	Serotec	rabbit/polyclonal	1:1000
nNOS	610308	BD Biosciences	mouse/monoclonal	1:100
NGF	sc-548	Santa Cruz	rabbit/polyclonal	1:500
BDNF	sc-546	Santa Cruz	rabbit/polyclonal	1:500
NT-3	sc-13380	Santa Cruz	goat/polyclonal	1:500
TrkA	sc-118	Santa Cruz	rabbit/polyclonal	1:500
TrkB	sc-8316	Santa Cruz	rabbit/polyclonal	1:500
TrkC	sc-117	Santa Cruz	rabbit/polyclonal	1:500
GDNF	sc-13147	Santa Cruz	mouse/monoclonal	1:500
GFRα1	sc-10716	Santa Cruz	rabbit/polyclonal	1:500

Table 2. List of secondary antibodies, used in the immunohistochemical reactions.

Secondary antibody	Supplier	Dilution
Horse anti-mouse IgG, biotinylated	Vector	1:250
Goat anti-rabbit IgG, biotinylated	Sigma	1:250
Rabbit anti-goat IgG, biotinylated	Santa Cruz	1:250

IV. RESULTS

4.1 Morphology of the Spinal Trigeminal Nucleus

4.1.1 Subnuclei of the Spinal Trigeminal Nucleus

By systematically examining the neural bodies, nerve fibers, and capillaries within the oral, interpolar, and caudal subnuclei, information is presented that provides a basic understanding of the normal morphologic features within the spinal trigeminal nucleus. A comparative analysis of these subnuclei, whose precise localization was determined according to coordinates from Paxinos and Watson's stereotaxic atlas of the rat brain, enhances our understanding of their complex microarchitecture at specific levels (Paxinos and Watson, 2014).

The spinal trigeminal nucleus represents the largest trigeminal nucleus and extends into the lateral tegmentum of the medulla oblongata and caudal part of the bridge. The nucleus is composed of neurons that have a distinct cell body with sporadic Nile bodies surrounded by a network of myelinated axons. Myelinated fibres can also be observed around the nucleus. These fibers connect to the pain signal from peripheral nociceptors, which are carried by cranial nerves V, VII, IX and X. Upon entering the brainstem, the sensory fibers group and penetrate the spinal trigeminal nucleus. Along its course from rostral to caudal, the spinal trigeminal nucleus is subdivided into three anatomically distinct subnuclei: oral, interpolar, and caudal (Figs. 4.1-4.3).

4.1.1.1 Caudal Trigeminal Nucleus

The most prominent sensory trigeminal subnucleus spans from the lowermost part of the medulla oblongata, reaching from the caudal pole of the inferior olive and obex to the second cervical spinal cord segment. It seamlessly connects with the interpolar trigeminal subnucleus towards the rostral end, while blending with the posterior horn of the spinal cord caudally. Traditionally, this caudal trigeminal subnucleus is subdivided into three distinct regions, mirroring the architecture of the apex of the spinal dorsal horn: the lateralmost area known as the *subnucleus zonalis*, followed by the *subnucleus gelatinosus*, and finally, the medial

subnucleus magnocellularis. The *subnucleus zonalis* presents as a thin layer housing a sparse population of neurons, notably characterized by the presence of large irregularly elongated multipolar cells, some reaching diameters of up to 21 µm. Amidst these, medium-sized neurons with discernible *Nissl* bodies are interspersed, alongside smaller neurons. Transitioning medially into the *subnucleus gelatinosus*, neuron density notably increases. Here, the *subnucleus gelatinosus* comprises predominantly small oval or elongated neurons, with typical diameters of up to 15 µm, displaying relatively large nuclei and scant cytoplasm forming a thin pale perinuclear ring. The magnocellular subnucleus, albeit relatively larger compared to the *gelatinosus*, primarily consists of irregularly oval medium-sized cells,

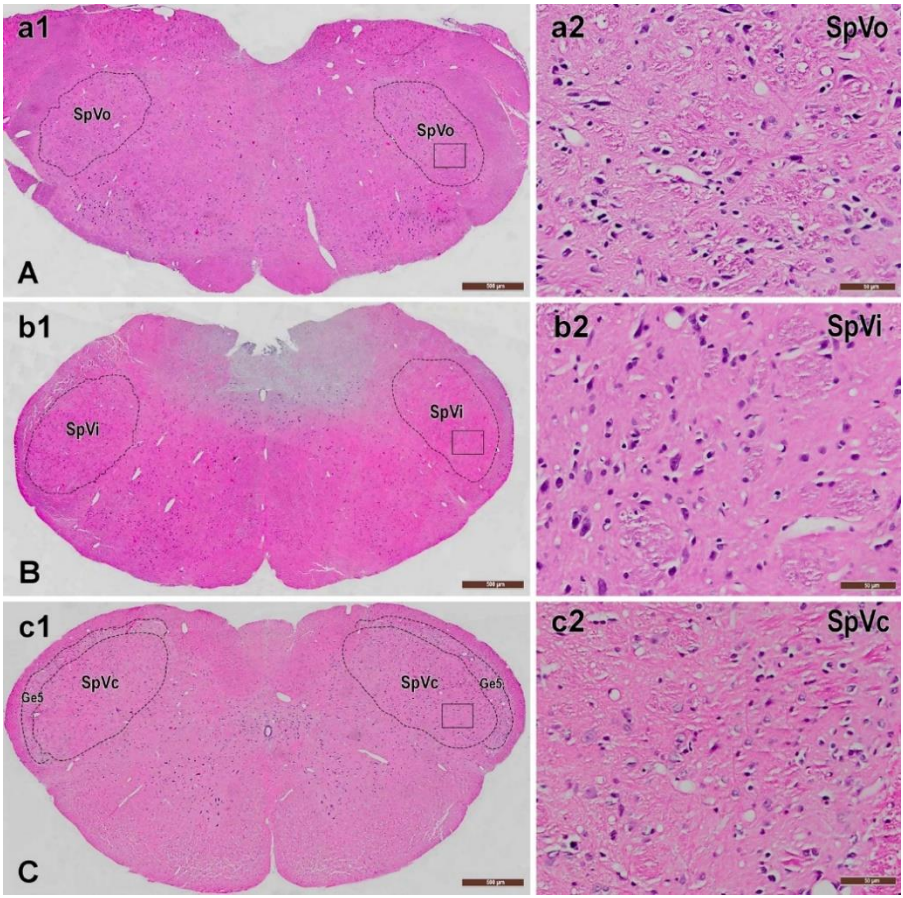

*Figure 4.1. Histological view of the spinal trigeminal nucleus stained with HE. The three distinct subnuclei, labelled as oral SpVo (**A**), interpolar SpVi (**B**) and caudal, SpVc (**C**), are delineated, together with a subnucleus of the latter labelled subnucleus genalitnosus (Ge5). Scale: 200 µm (a1, b1, c1), 50 µm (a2, b2, c2).*

approximately 12 µm in diameter, with distinct basophilia. Despite its name, only a fraction of neurons are notably large, with diameters seldom exceeding 21 µm. Along its course, the caudal spinal trigeminal subnucleus is bordered laterally by the caudal part of the spinal tract of the trigeminal nerve. Dorsally, it is flanked by the cuneate fasciculus, while medially and ventromedially lies the dorsal subnucleus of the central reticular nucleus of the medulla oblongata, together with the ascending spinal tracts, marking its ventral boundary. At its transition into the spinal cord, the spinal trigeminal subnucleus forms a distinct lump-like apex, extending medially from *lamina V* of the spinal cord

4.1.1.2 Interpolar Trigeminal Subnucleus

This nucleus is located in the medulla oblongata. Rostrally it is continuous with the oral spinal trigeminal nucleus and caudally with the caudal spinal trigeminal nucleus. The caudal pole of the interpolar trigeminal nucleus is located somewhat rostral to the caudal pole of the *nucleus olivarius inferior*. The population of neurons is heterogeneous. The majority of cells
are small to medium in size (between 6 and 15 µm), irregularly oval or elongated, with moderate, diffuse basophilia. They are similar to cells in the oral trigeminal subnucleus. Characteristics of this nucleus are large neurons (20 - 35 µm) elliptical perikaryons, with strong, diffuse basophilia. Such large neurons are usually singly scattered among small cells. On transverse sections, the interpolar trigeminal subnucleus has an irregular oval shape with a long axis directed dorsomedially. Rostrally it is a thin lamina that gradually increases in size in a caudal direction. Along its entire length rostrocaudally, the interpolar trigeminal subnucleus borders the t*ractus spinalis nervi trigemini* laterally. In the caudal direction, the *nucleus solitarius* moves medially and is replaced by the *nucleus cuneatus*. Medially, the interpolar trigeminal subnucleus is bounded by the *nucleus reticualris parvocelullaris*, a nucleus of the medulla oblongata, and near the transition to the caudal trigeminal subnucleus by the central reticular nucleus of the medulla oblongata. There are relatively few neuronal perikaryons along the entire medial border, as this area is traversed by prominent fiber bundles.

4.1.1.3 Oral Trigeminal Subnucleus

Located in the caudal pons and rostral medulla oblongata, this subnucleus extends rostrally into the main sensory trigeminal nucleus and caudally into the interpolar trigeminal subnucleus. Neuronal perikarya exhibit a spectrum of sizes, ranging from small to medium. The rostral segment of the nucleus predominantly features medium-sized perikarya, contributing to its larger overall size, while small irregularly oval perikarya are also present. These small cells possess barely discernible dendritic poles, with relatively large nuclei enveloped by thinly stained cytoplasm. Conversely, medium-sized neurons may exhibit either oval or fusiform morphology, with the latter potentially reaching diameters of up to 20 µm. Characterized by cytoplasm with diffuse basophilia, these neurons contribute to the diverse cellular composition of the subnucleus. In the pontine region, the oral trigeminal subnucleus abuts laterally against the *tractus spinalis nervi trigemini*, often intersected by its fascicles, leading to uneven division into distinct groups across individual sections. Dorsally adjacent to the oral trigeminal subnucleus lie the vestibular nuclei, while ventromedially, a narrow oligocellular and parvocellular strip, representing the ventrolateral extension of the *nucleus reticularis parvocellularis*, separates it from the facial nucleus.

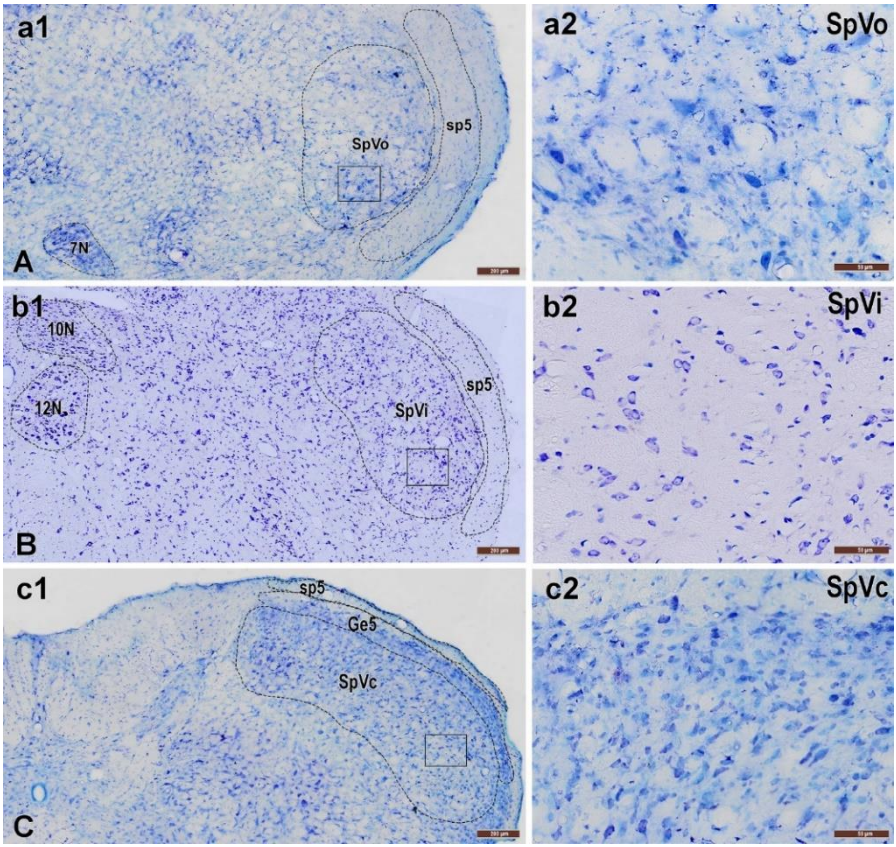

*Figure 4.2. Histological view of spinal trigeminal nucleus stained with toluidine blue. The image highlights the three distinct subnuclei: SpVc (**C**), SpVi (**B**), and SpVo (**A**). Ge5, subnucleus gelatinosus; sp5, tractus spinalis nervi trigemini; 10N, nucleus dorsalis nervi vagi; 12N, nucleus nervi hypoglossi. Scale: 200 µm (a1, b1, c1), 50 µm (a2, b2, c2).*

4.1.2 Morphological Types of Neurons in the Spinal Trigeminal Nucleus

Sections of the brainstem at the level of SpVc, SpVi, and SpVo were stained with toluidine blue to differentiate the various neurons in the individual subnuclei of the spinal trigeminal nucleus. It was found that the three subdivisions include cells with similar external morphological characteristics but with distinct variations among them. Neurons were characterized based on the shape and size of their perikarya and categorized into distinct morphological types, including seven types of neurons (Fig. 4.4, 4.5).

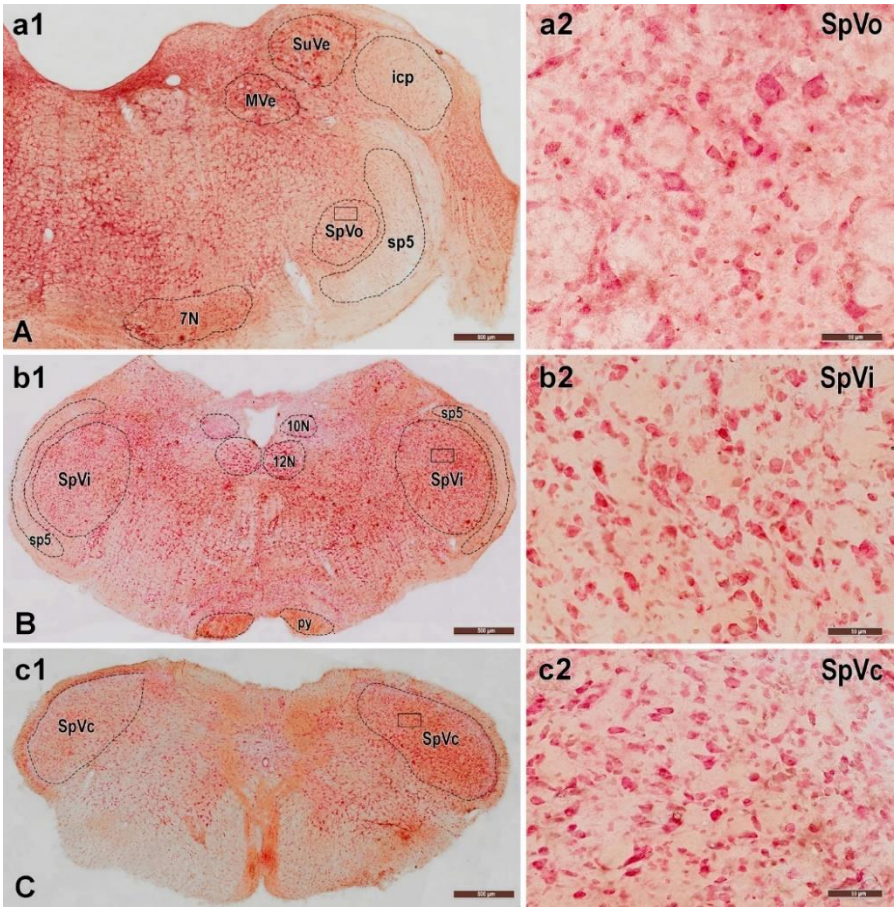

*Figure 4.3. Histological view of the spinal trigeminal nucleus stained with neutral red. The image highlights the three distinct subnuclei: SpVc (**C**), SpVi (**B**), and SpVo (**A**). SuVe, nucleus vestibularis superior; MVe, nucleus vestibularis medialis; icp, pedunculus cerebellaris inferior; 7N, nucleus nervi facialis; 10N, nucleus dorsalis nervi vagi; 12N, nucleus nervi hypoglossi; sp5, tractus spinalis nervi trigemini; py, tractus pyramidalis. Scale: 500 µm (a1, b1, c1), 50 µm (a2, b2, c2).*

4.1.2.1 Boat-shaped Neurons

Our study on the morphological characteristics of neurons in the spinal trigeminal nucleus identified a unique phenotype of neurons characterized by unusually shaped cell bodies resembling boats (Fig. 4.4A). When observed under a light microscope, the perikarya of these neurons resemble boats; they have a wide, elongated shape and a smooth surface structure.

Morphometric measurements of cell dimensions revealed that their perikarya range from 20 to 30 μm, with an average diameter of 25 μm ± 3.8 (n = 11), indicating a significant level of somatic variability among this neuron group.

4.1.2.2 Elongated Neurons

Another morphological type of neuron with unique anatomical features is characteristic of medium-sized neurons in the spinal trigeminal nucleus. This population of spinal trigeminal neurons is characterized by fusiform or elongated cell bodies (Fig. 4.4B), with perikarya ranging in size from 14 to 21 μm and an average diameter of 15 μm ± 2.1 (n = 24).

4.1.2.3 Lobulated Neurons

Our study also revealed the presence of another population of spinal trigeminal neurons with lobulated cell bodies and uneven surface protrusions or indentations (Fig. 4.4C). These lobulated neurons have an average diameter of 16 μm ± 1.3 (n = 9), with perikaryal diameters ranging from 15 to 21 μm.

4*.1.2.4 Neurons with Dilated Axonal Hillocks*

We also identified neurons whose bodies exhibited expanded regions, particularly noticeable near the axonal hillocks and sites of branching of their dendritic processes. Likely, these profiles involve significant dilatation of the dendritic architecture, as the expanded regions often exhibit a width similar to or almost equal to the diameter of the neuronal cell bodies (Fig. 4.4D). The perikarya of neurons with dilations significantly differ from each other in terms of their sizes and shapes. This structural heterogeneity represents a significant distinguishing feature in the morphology of this type of neuron within the spinal trigeminal nucleus.

4.1.2.7 Polymorphic (Multimorphic) Neurons

In the spinal trigeminal nucleus, we sporadically observed the presence of a separate population of very large neurons (Fig. 4.5). Generally, these neurons have diameters exceeding 21 μm in the perikaryal region. Within this population of neurons, the average diameter of their cell bodies is 26.7 μm ± 5.5 (n = 33), suggesting considerable variability in soma size. The largest neuronal cell body measured had a diameter of nearly 44 μm (43.92 μm). The diversity in somatic morphology among these neurons relates to differences in the shapes of their cell bodies, including round, oval, and pear-shaped forms (Fig. 4.5A, B).

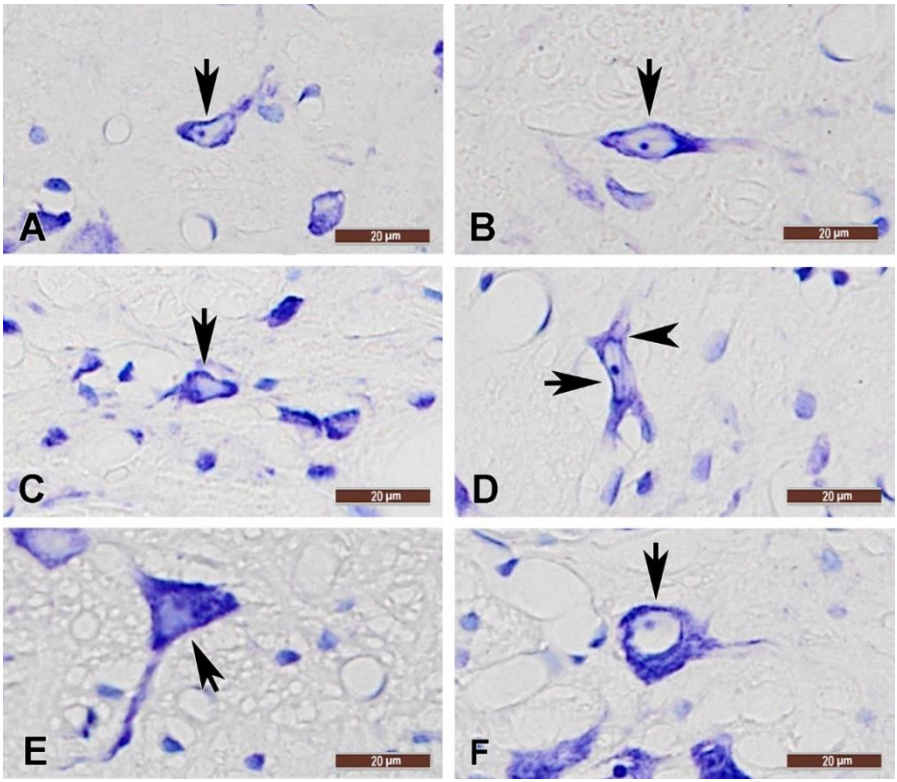

*Figure 4.4. Morphological types of neurons in the spinal trigeminal nucleus, stained with toluidine blue for visualization of their cell bodies. (**A**) Neuron with fusiform body (arrow); (**B**) Neuron with bipolar profile and elongated cell body (arrow); (**C**) Neuron with lobulated cell body (arrow); (**D**) Neuron with dilation (arrowhead) at the beginning of one of its processes; (**E**) Neuron with characteristic pyramidal shape of the perikaryon; (**F**) Typical representative of neurons with oval-shaped cell body (arrow). Scale bar 20 µm.*

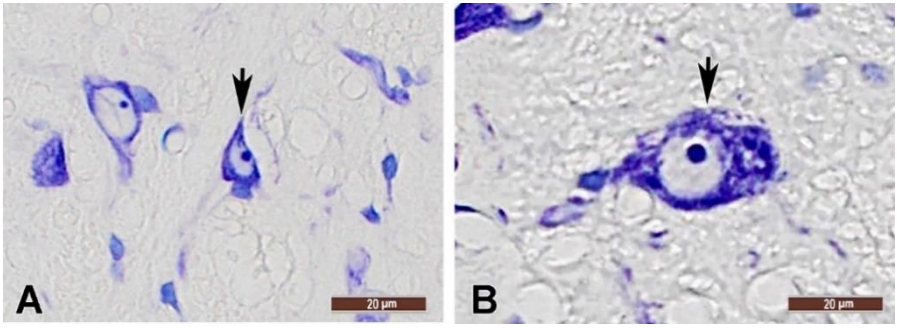

*Figure 4.5. Neurons with large perikaryal sizes. Visible diversity in somatic morphology with different profiles of cell bodies, described with pear-shaped (**A**) and oval forms (**B**). Scale bar 20 µm.*

4.1.3 Morphometric Analysis of Neurons in the Spinal Trigeminal Nucleus

Our morphometric analysis conducted using the Fiji program identified three distinct subgroups of neurons within the spinal trigeminal nucleus, distributed based on the sizes of their cell bodies, as shown in Figure 4.6. Neurons classified as small have soma diameters ranging from 5 to 15 μm, with an average diameter of 9.89 μm ± 2.16 (n = 798). These neurons represent approximately 79.7% of the total neuron population within the spinal trigeminal nucleus (Fig. 4.6). Medium-sized neurons have perikarya with an average diameter of 16.66 μm ± 1.51 (n = 151), ranging from 15 to 20 μm. These neurons constitute approximately 15% of the total neuron population in the nucleus. Neurons categorized as large have soma diameters exceeding 25 μm, with an average diameter of 26.68 μm ± 4.58 (n = 50). Additionally, one very large (giant) neuron was observed with soma dimensions exceeding 40 μm in diameter (n = 1). Neurons with large sizes collectively represent approximately 5% of the total neuron population within the spinal trigeminal nucleus in rats.

Further analysis revealed different size distributions of neurons in the individual subnuclei of the spinal trigeminal nucleus. Specifically, in the caudal spinal trigeminal subnucleus (SpVc), small cells constitute the majority of neurons (almost 85%), medium-sized neurons account for 14.93%, followed by large neurons, which are less than 1% of all neurons in the nucleus. In the interpolar subnucleus (SpVi), small neurons predominate (86%), followed by medium-sized neurons (7.46%), large neurons (6.47%), and sporadic giant neurons (less than 1%). The largest diameter of a neuronal cell body was measured in SpVi – 43.93μm. Particularly striking are the enormous sizes of neurons scattered among smaller ones in this subnucleus. In the oral subnucleus (SpVo), small neurons are almost three times more numerous than medium-sized ones, constituting approximately 70% and around 23%, respectively, while large neurons make up almost 7% of all cells in it. The average diameter of small neurons in the rat spinal trigeminal nucleus ranges from 8.84 to 10.52 μm, that of medium neurons from 16.39 to 16.80 μm, and that of large neurons varies from 25.45 to 27.82 μm across all subnuclei.

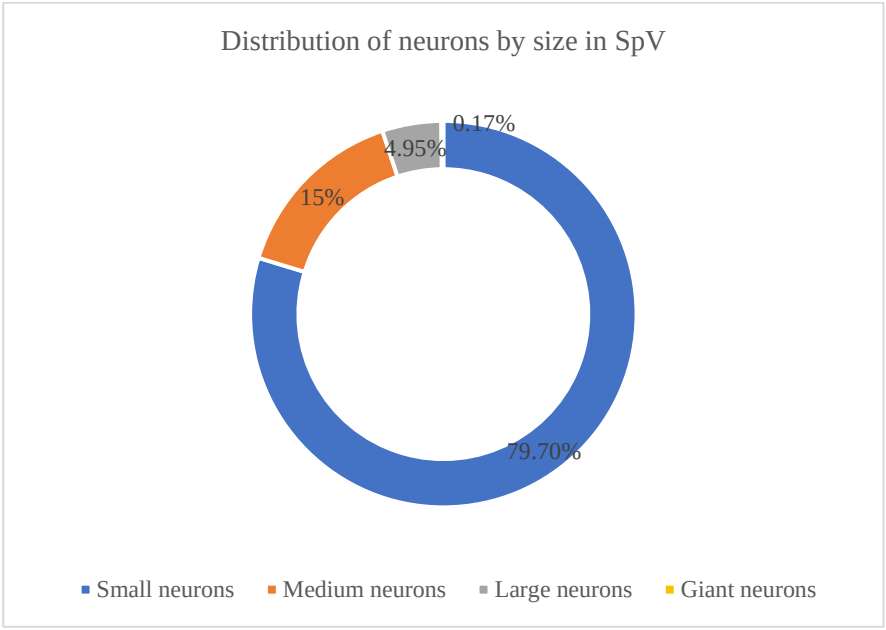

Figure 4.6. Percentage distribution of different-sized neurons in spinal trigeminal nucleus.

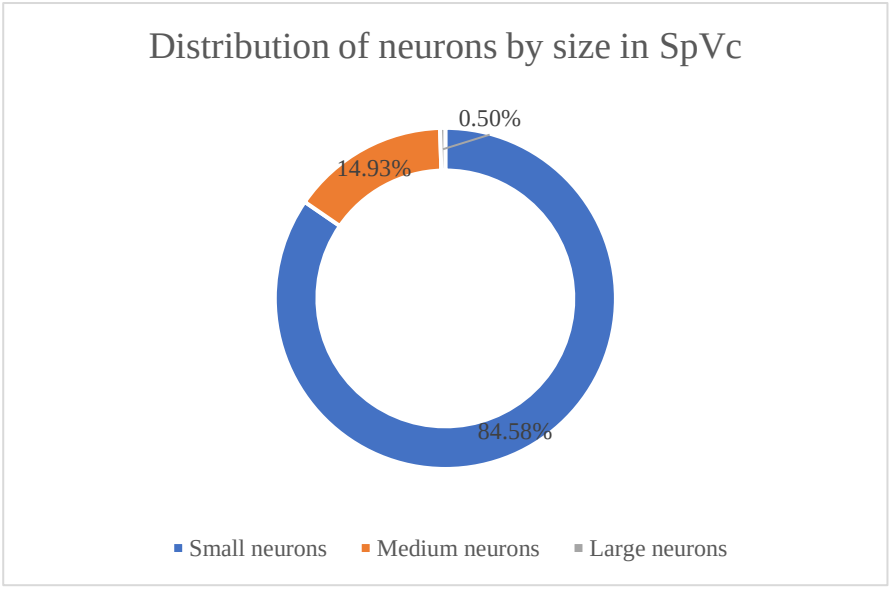

Figure 4.7. Percentage distribution of different-sized neurons in SpVc.

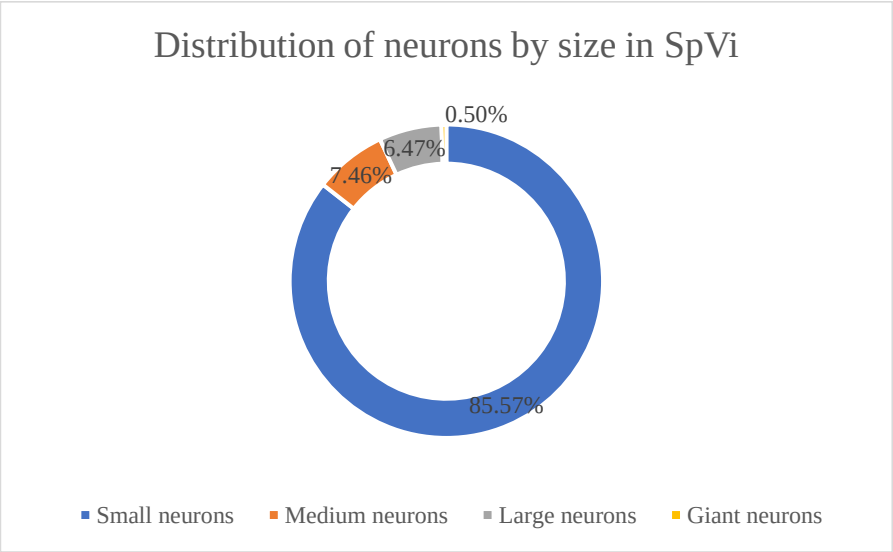

Figure 4.8. Percentage distribution of different-sized neurons in SpVi.

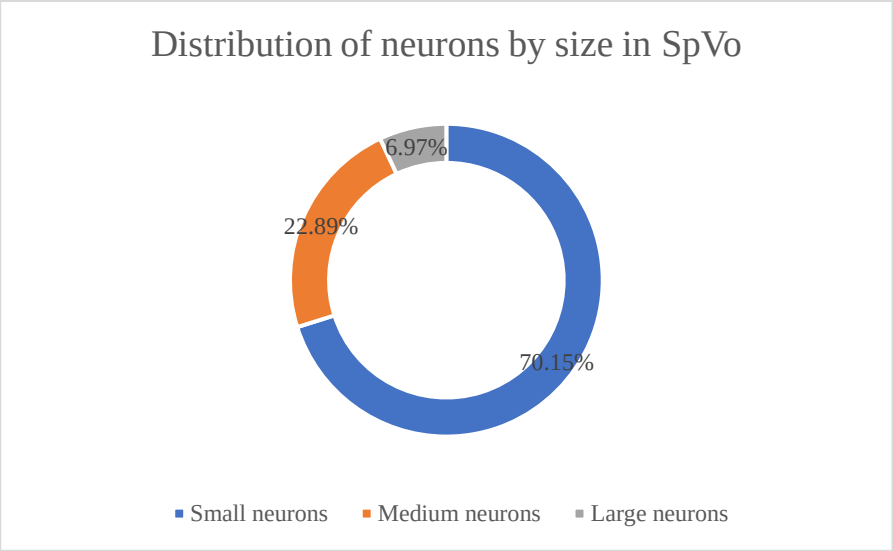

Figure 4.9. Percentage distribution of different-sized neurons in SpVo.

4.2 Neurochemical Characteristics of the Spinal Trigeminal Nucleus

4.2.1 Neurotransmitters and Neuropeptides in the Spinal Trigeminal Nucleus

4.2.1.1 Gamma-Aminobutyric Acid (GABA)

Using immunohistochemical methods at the light microscopic level, we were able to establish the expression of gamma-aminobutyric acid (GABA) in the three subdivisions of the spinal trigeminal nucleus. GABA-immunoreactive spinal trigeminal neurons were observed in the oral, interpolar, and caudal subnuclei, with no apparent differences in the intensity of their immunostaining and their topographical distribution (Fig. 4.10). The majority of immunopositive neurons are small in size, suggesting that a precisely defined cellular subpopulation in the spinal trigeminal nucleus is involved in inhibitory GABAergic neurotransmission. Clear GABA immunoreactivity was observed only in the perikarya of neurons in the spinal trigeminal nucleus, while their processes remained unlabeled. A similar pattern of immunoreactivity was found in all three subnuclei. In SpVc, numerous small-sized neurons were observed, located in the marginal part of the subnucleus, specifically in the subnucleus zonalis. Additionally, isolated thin, varicose GABA-immunopositive nerve fibers passing through the spinal trigeminal nucleus were observed.

After detecting the presence of scattered GABA-immunoreactive neurons in the caudal, interpolar, and oral anatomical divisions of the spinal trigeminal nucleus, we applied the Kruskal-Wallis non-parametric test to objectively compare the staining intensity for GABA in the three parts of the nucleus. The test data showed significant differences in GABA expression $H(2) = 32.10$, $^{****}p < 0.0001$ and median values (Mdn) in the three subnuclei studied, namely: oral subnucleus (GABA-SpVo) (Mdn = 148), interpolar subnucleus (GABA-SpVi) (Mdn = 128.5), and caudal subnucleus (GABA-SpVc) (Mdn = 114). Additionally, we conducted ed post-hoc analysis of these data using Dunn's test. The Dunn test revealed statistically significant differences in GABA expression between SpVo and SpVi subnuclei ($^{**}p < 0.0096$) and between GABA-SpVo and GABA-SpVc subnuclei of the spinal trigeminal nucleus ($^{****}p < 0.0001$). The strongest GABA expression was observed in the caudal subnucleus, followed by the interpolar subnucleus, and the weakest in the oral subnucleus. Statistically significant differences were also observed between the interpolar and caudal subnuclei ($^{*}p < 0.024$).

4.2.1.2 Acetylcholine (ACh)

Significant information about the expression and distribution of this neurotransmitter was obtained by demonstrating its hydrolytic enzyme acetylcholinesterase (AChE) in the structural divisions of the nucleus in rats. Light microscopic examination of immunostained sections showed that numerous nerve fibers in the spinal trigeminal nucleus of rats contained AChE. AChE-immunoreactive fibers were clustered in dense bundles passing through the entire length of the nucleus (Fig. 4.12). An interesting finding was the identification of some of these immunolabeled nerve bundles near blood vessels in the nucleus, suggesting a possible functional relationship between AChE-containing nerve fibers and vascular components in the spinal trigeminal nucleus. Another notable observation related to the extensive expression of AChE in almost all neuronal cell bodies in the spinal trigeminal nucleus. This widespread expression pattern suggests that the acetylcholine- degrading enzyme is widely involved in

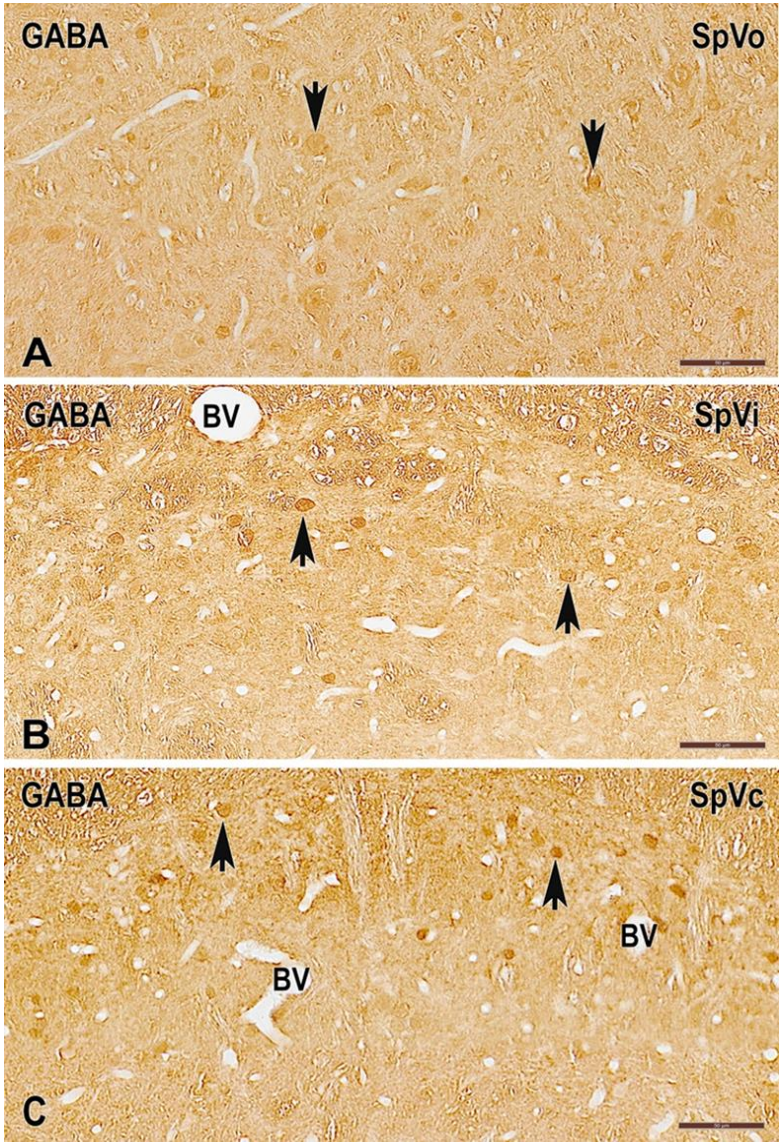

*Figure 4.10. Immunohistochemical reaction for gamma-aminobutyric acid (GABA) in (**A**) the oral (SpVo), (**B**) the interpolar (SpVi) and (**C**) caudal (SpVc) subnucleus of the spinal trigeminal nucleus. Small sized immunoreactive neurons are shown with arrows. BV, blood vessels. Scale = 50 μm.*

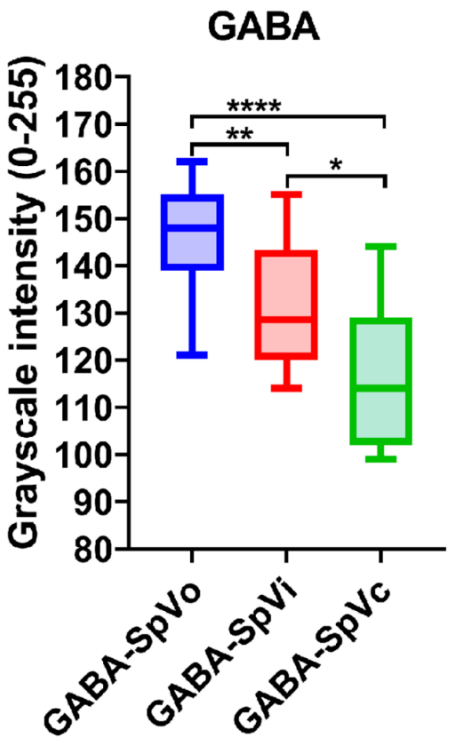

Figure 4.11. Box plot diagrams representing immunostaining intensities for GABA in the oral (SpVo), interpolar (SpVi) and caudal (SpVc) subnucleus of SpV.

the control of nerve activity in various populations of spinal trigeminal neurons. The topographical distribution of AChE-immunopositive neurons in the caudal, interpolar, and oral subnuclei of the spinal trigeminal nucleus was also a focus of our study (Fig. 4.12). The consistent identification of AChE-immunoreactive neuronal cell bodies in each subnucleus underscores the widespread presence of acetylcholine in the spinal trigeminal nucleus, which would imply its real involvement in the regulation and processing of sensory information at the level of the spinal trigeminal nucleus in different functional areas of the brain.

4.2.1.3 Serotonin (5-Hydroxytryptamine)

Using light microscopic immunohistochemical methods, we were able to demonstrate the expression of serotonin (5-HT) in all subnuclei of the spinal trigeminal nucleus. Detailed examination under the microscope of immunostained sections at the level of the spinal trigeminal nucleus revealed pronounced and diffuse expression of serotonin in many neuron perikarya within the nucleus. Virtually every immunolabeled neuron in the spinal trigeminal nucleus, regardless of the size of its perikaryon, exhibited a similar pattern and intensity of immunostaining.

We also characterized the localization and spatial organization of serotonin-containing cells in the caudal, interpolar, and oral subdivisions of the spinal trigeminal nucleus. Specifically, serotonin-immunoreactive cells were observed in each subnucleus along the entire rostrocaudal axis of the spinal trigeminal nucleus, suggesting a possible role of this monoaminergic transmitter in serving various functional modalities in the orofacial region, as highlighted by their widespread presence in this nucleus. Furthermore, immunopositive cell bodies were observed in all three subnuclei, with the sizes of 5-HT-immunolabeled neurons varying. Both small and large neurons, whose cytoplasm was positive for the presence of 5-HT, were observed. The reaction for serotonin remained poorly expressed in the nerve fibers in the three subnuclei.

The Kruskal-Wallis test was applied to compare the intensity of immunostaining for 5-HT in the three different subnuclei of the spinal trigeminal nucleus (Fig. 4.15). Specifically,

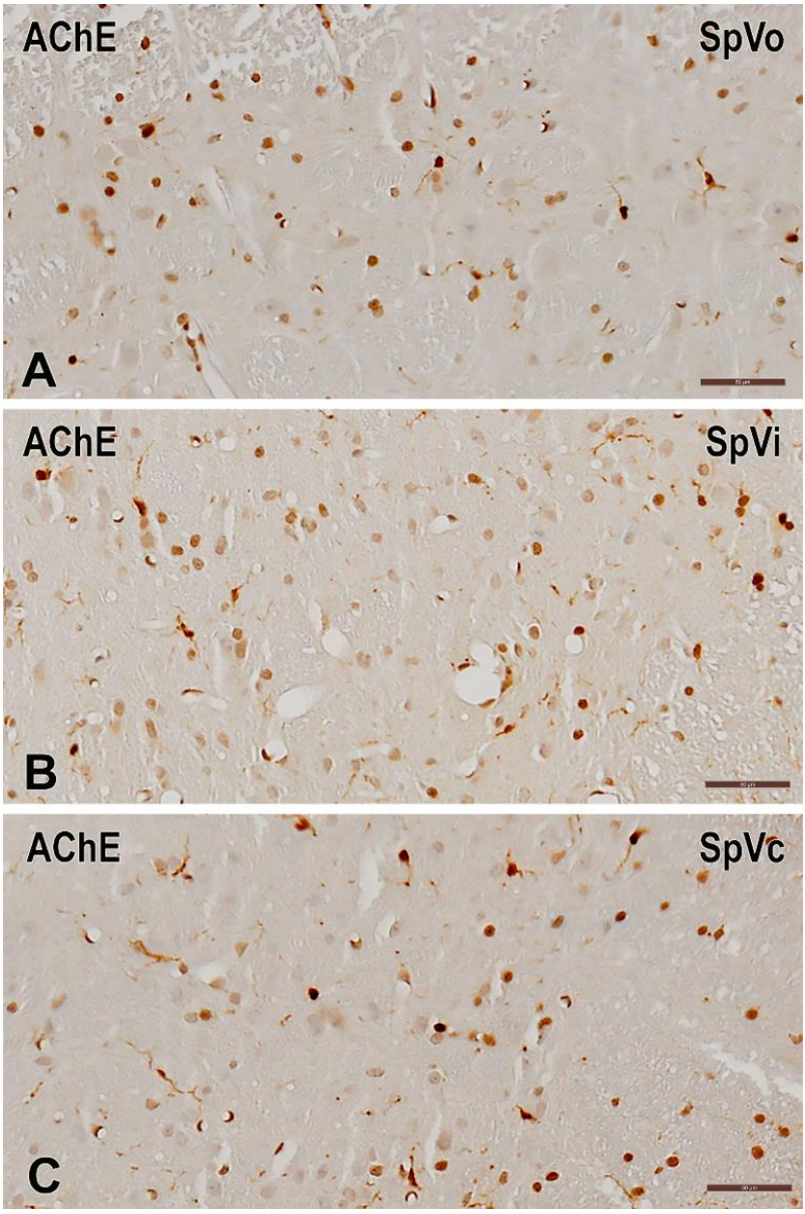

*Figure 4.12. Expression of acetylcholinesterase (AChE) in neuronal cell bodies in (**A**) the oral (SpVo), (**B**) the interpolar (SpVi) and (**C**) caudal (SpVc) subnucleus of SpV. Immunopositive cell bodies of neurons and their growths in the nucleus were observed. Scale = 50 μm.*

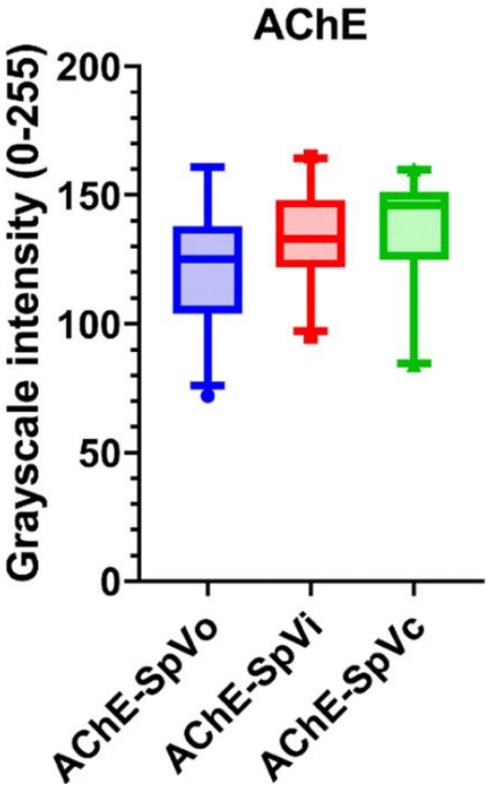

Figure 4.13. Box-plot charts graphically representing the staining intensity for AChE in the oral (SpVo), interpolar (SpVi) and caudal (SpVc) subnucleus of SpV.

we evaluated the levels of expression of 5-HT in the oral subnucleus (5-HT-SpVo), the interpolar subnucleus (5-HT-SpVi), and the caudal subnucleus (5-HT-SpVc). The results of this test did not reveal statistically significant differences in the distribution and expression of 5-HT among these subnuclei ($H(2) = 0.5416$, $p = 0.7628$).

This subsequently necessitated further investigation into potential variations in the expression of 5-HT between individual subnuclei, for which a post hoc analysis was conducted using the Dunn test. This test confirmed the lack of statistically significant differences in the expression of 5-HT between the oral and interpolar subnuclei ($p > 0.9999$), as well as between the oral (5-HT-SpVo) and caudal subnuclei (5-HT-SpVc) ($p > 0.9999$). Moreover, no significant differences were observed between the interpolar and caudal subnuclei ($p > 0.9999$). Taken together, these results demonstrate a consistent pattern of 5-HT expression in the oral, interpolar, and caudal subnuclei of the spinal cord, suggesting potential functional similarities or regulatory mechanisms governing serotonergic signaling in these anatomical subdivisions.

4.2.1.4 Substance P

To investigate the presence and distribution of substance P-immunoreactivity in the individual anatomical divisions of the spinal trigeminal nucleus, indirect immunohistochemical techniques with an antibody against this undecapeptide were used in the present study. The results showed that substance P was highly expressed throughout the extent of the spinal trigeminal nucleus, including its three different subnuclei (caudal, interpolar, and oral) (Fig. 4.16).

Light microscopic immunohistochemistry undoubtedly demonstrated the presence of substance P-immunopositive neurons in the spinal trigeminal nucleus, with varying sizes of their perikarya. Furthermore, immunoreactive cell bodies of spinal trigeminal neurons were

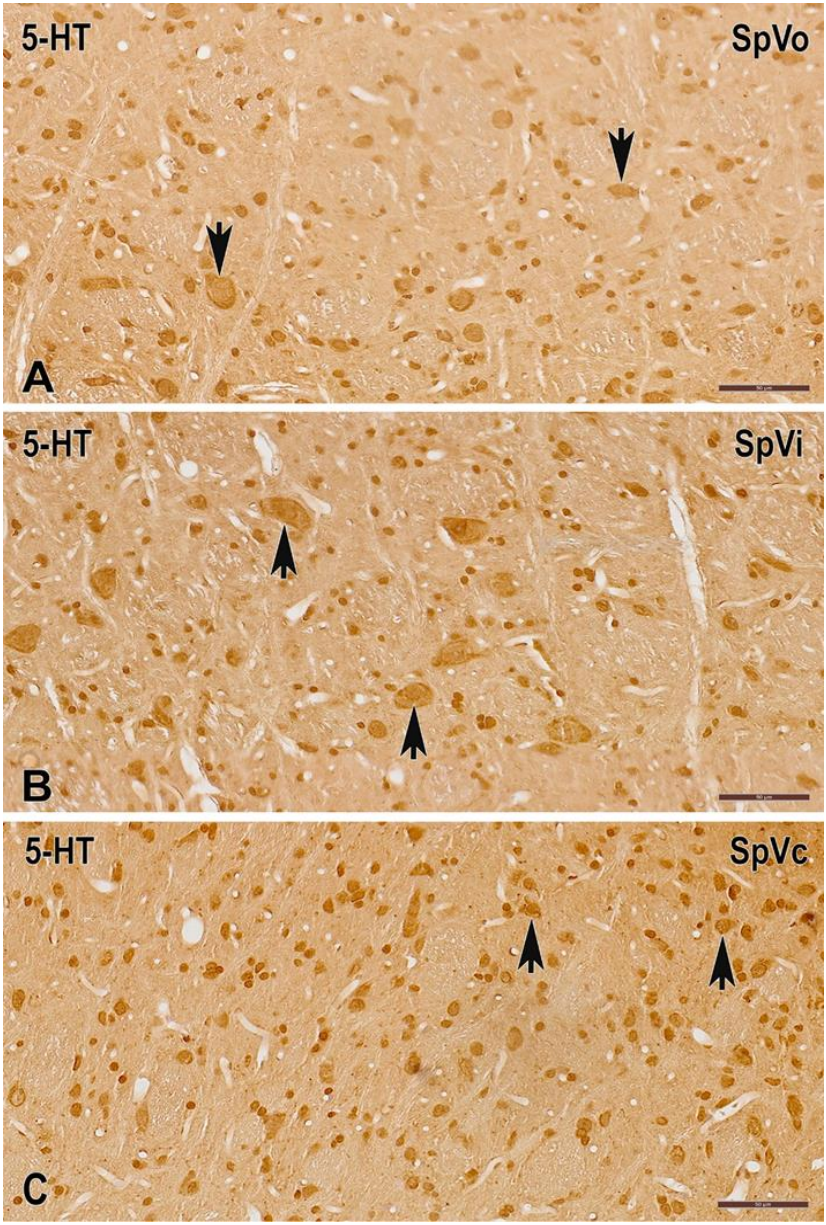

*Figure 4.14. Expression of serotonin (5-HT) in the (**A**) oral (SpVo), (**B**) interpolar (SpVi), and (**C**) caudal (SpVc) subnucleus of the spinal trigeminal nucleus. Immunoreactive neurons of different sizes and varying shape are indicated by arrows. Scale = 50 µm.*

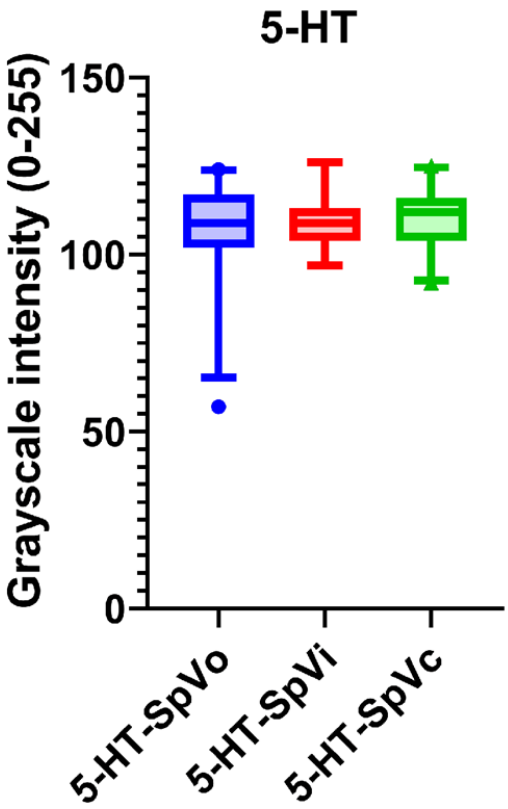

Figure 4.15: Box-top diagrams graphically representing the staining intensity for 5-HT in the oral (SpVo), interpolar (SpVi) and caudal (SpVc) subnucleus of SpV.

observed throughout the extent and in all three subnuclei.

It is noteworthy that the reaction was more intense in the caudal subnucleus compared to the others (Fig. 4.16C). Additionally, it appears that smaller-sized spinal trigeminal neurons exhibit a stronger immunoreaction compared to those with larger sizes.

To compare the expression of substance P (SP) in the three parts of the spinal trigeminal nucleus in experimental animals, since the obtained data were normally distributed, a one-way analysis of variance (ANOVA) was performed. The ANOVA test calculates the F-statistic, which is a ratio between the variation among the individual parts of the nucleus and the variation within the part of the nucleus itself. The ANOVA test showed a statistically significant difference in the mean value between the groups [$F(2, 65) = 121.9$, $p < 0.0001$]. Since the F-statistic is sufficiently large and its associated p-value is below a pre-defined level of significance ($p < 0.05$), this means that there is strong evidence that at least one of the mean values in the different parts of the nucleus differs significantly from the others. In this case, additional post hoc tests were used to determine which specific parts of the nucleus differed from each other. The Tukey's honestly significant difference (HSD) post hoc test showed that the expression of SP in the oral part of the nucleus (SP-SpVo) was significantly lower compared to the interpolar part (SP-SpVi) ($p < 0.0001$, 95% C.I. = [12.75 to 23.43]) and the caudal SP-SpVc ($p < 0.0001$, 95% C.I. = [29.07 to 39.63]). The intensity of the grayscale for SP in the interpolar and caudal parts of the nucleus differed significantly, with the interpolar area exhibiting weaker expression of the neuropeptide SP compared to the caudal area ($p < 0.0001$, 95% C.I. = [10.62 to 21.60]). Tukey's test determines whether our sample consists of areas that differ from each other. Each mean value is compared to the mean value of all other groups, using the "honestly significant difference" to show how far apart the areas are from each other. The 95% C.I. is

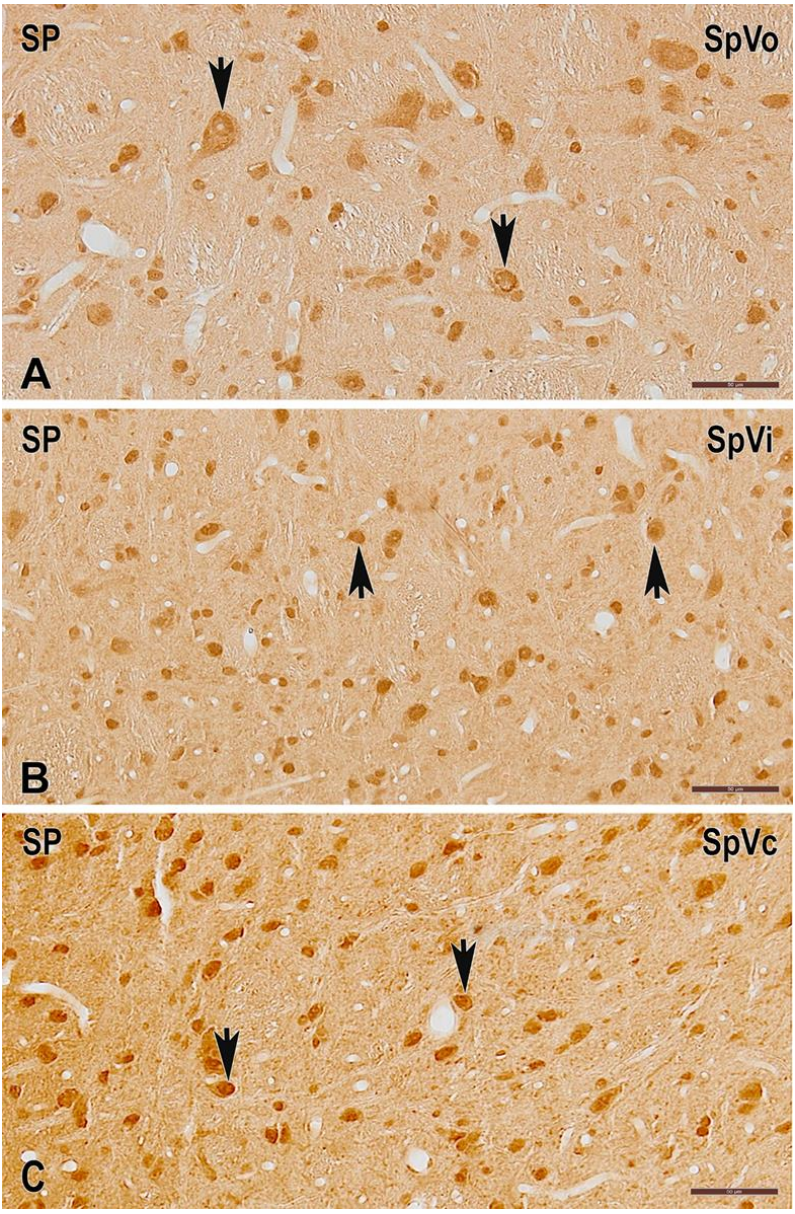

*Figure 4.16. Expression of substance P (SP) in the (**A**) oral (SpVo), (**B**) interpolar (SpVi) and (**C**) caudal (SpVc) subnucleus of the spinal trigeminal nucleus. Immunoreactive neurons of different sizes and shape-varying pericarions are shown with arrows. Scale = 50 µm.*

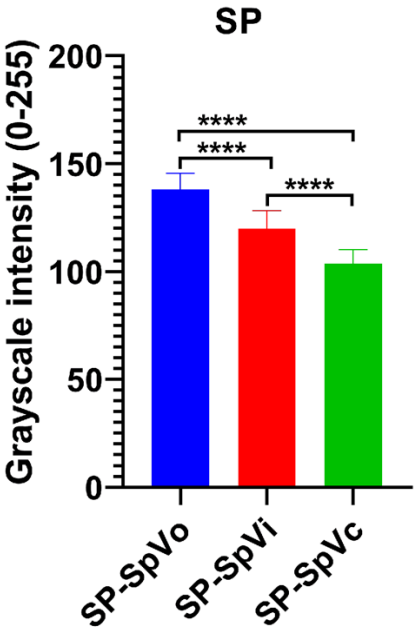

Figure 4.17. Bar chart representing the staining intensity for substance P (SP) in the three subnuclei of the spinal trigeminal nucleus.

the confidence interval. The mean values and standard deviation (SD) in the individual parts of the nucleus were as follows: SP-SpVo (mean value = 138, SD = 7.498), SP-SpVi (mean value = 120, SD = 8.369), and SpVc (mean value = 103.7, SD = 6.442) (Fig. 4.17).

4.2.1.5 Calcitonin Gene-Related Peptide (CGRP)

Investigating the patterns of CGRP expression provided important new information about the neurochemical composition of the spinal trigeminal nucleus. Our analysis at the cellular level shows that calcitonin gene-related peptide is expressed sequentially in the bodies of specific neurons located throughout the extent of the spinal trigeminal nucleus (Fig. 4.18).

In addition to recognizable expression in neuronal bodies, our study revealed the presence of immunopositive nerve fibers. Many nerve fibers in the subnuclei were found to be CGRP-immunopositive. We also observed some neurons to be immunopositive for this peptide. These neurons are oval and belong to cells with larger sizes. Small neurons ranging in size from 5 to 15 µm remain immunonegative. Our knowledge of the established presence of CGRP-immunoreactive fibers in the nucleus contributes further to understanding the role of this mediator in the processing and transmission of pain information in the spinal trigeminal nucleus. The distribution of immunoreactive neurons and their nerve fibers is similar, with no visible deviations in the three subnuclei of the spinal trigeminal nucleus.

We applied the Kruskal-Wallis test as a reliable non-parametric test when comparing the staining intensity for CGRP in the three parts of the spinal trigeminal nucleus. The test showed significant differences in CGRP expression and median values (Mdn) in the three subnuclei studied, namely: oral subnucleus (CGRP-SpVo) (Mdn = 175), interpolar subnucleus (CGRP-SpVi) (Mdn = 158), and caudal subnucleus (CGRP-SpVc) (Mdn = 153), $H(2) = 26.41$, ****$p < 0.0001$. Following the Kruskal-Wallis test, we performed a post hoc analysis using Dunn's test. The Dunn's test showed statistically significant differences in CGRP expression between the oral and interpolar subnuclei of the spinal trigeminal nucleus (***$p < 0.0004$) and between the oral (CGRP-SpVo) and caudal subnuclei (CGRP-SpVc) of the spinal trigeminal nucleus (****$p < 0.0001$). The expression of CGRP is strongest in the caudal subnucleus, weaker in the interpolar subnucleus, and weakest in the oral subnucleus (Fig. 4.19). The staining intensity in the interpolar and caudal subnuclei showed no statistically significant differences ($p = 0.9373$).

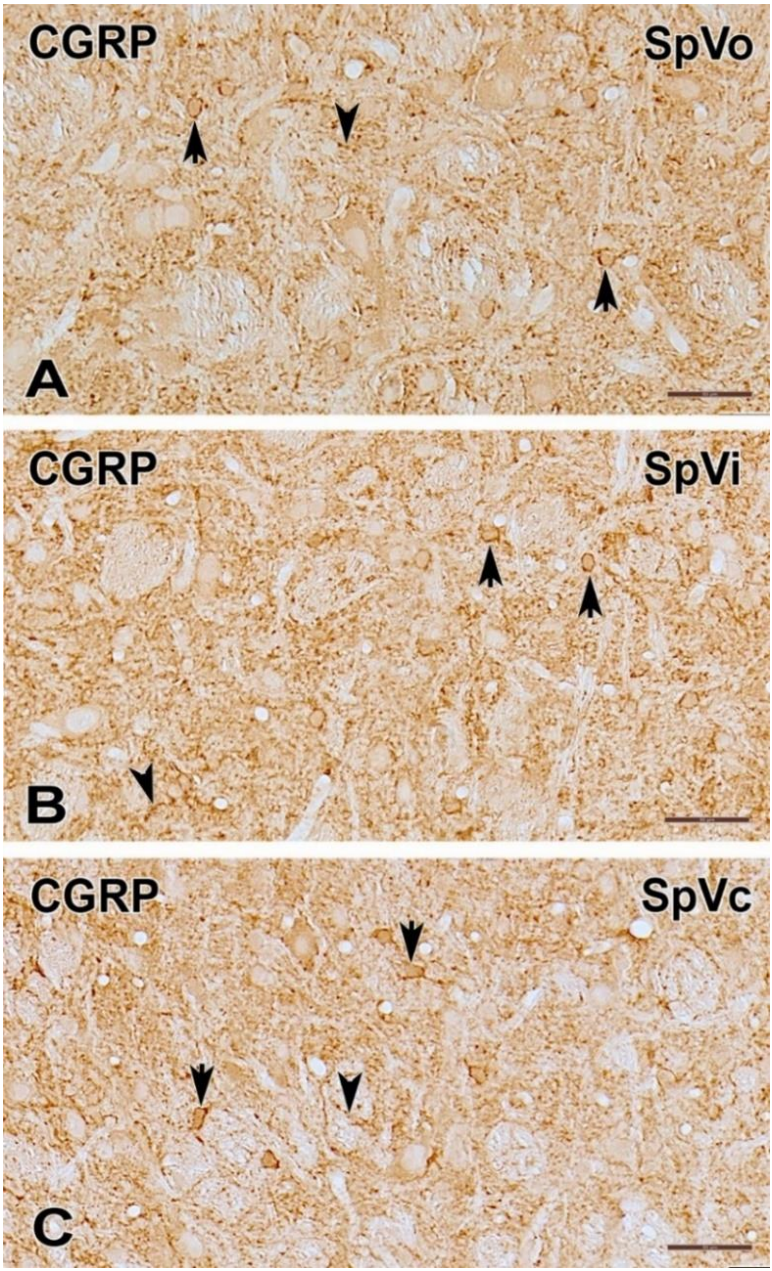

*Figure 4.18. Immunohistochemical staining of pericarions (arrows) and nerve fibres (arrow heads) for calcitonin gene-related peptide (CGRP) in the oral (**A**), interpolar (**B**) and caudal (**C**) subnucleus of the spinal trigeminal nucleus. Scale = 50 μm.*

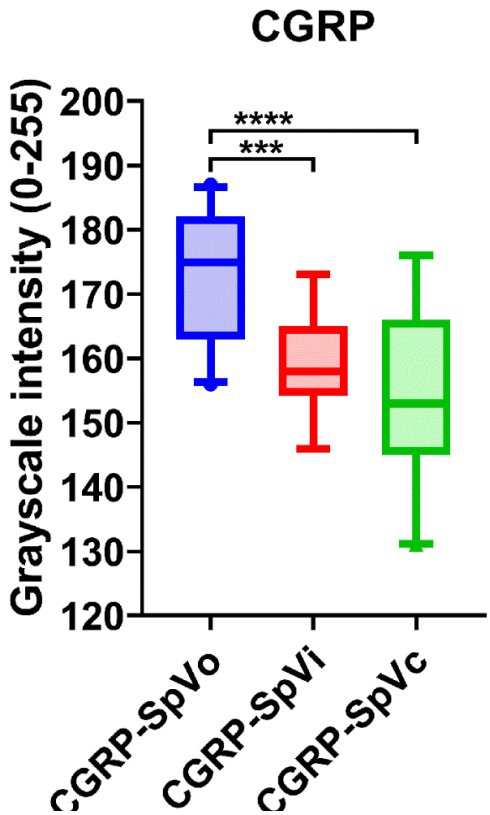

Figure 4.19. Box-plot charts graphically representing the staining intensity for CGRP in the oral (SpVo), interpolar (SpVi) and caudal (SpVc) subnucleus of the spinal trigeminal nucleus.

4.2.1.6 Neuropeptide Y (NPY)

Light microscopic examination of immunostained sections provided valuable information about the presence and distribution of neuropeptide Y (NPY) in the structural components of the rat spinal trigeminal nucleus. Our results confirm that neurons in all three subnuclei contain NPY (Fig. 4.20).

Immunopositivity for this peptide is present in a relatively small percentage of spinal trigeminal neurons, with the reaction product detected in some neuronal perikarya in all subnuclei of the spinal trigeminal nucleus, albeit with a different pattern of distribution of immunoreactive structures. The reaction product was deposited in the peripheral zones of neuronal perikarya in the three spinal trigeminal subnuclei. Additionally, a large number of varicose nerve fibers and their terminals in the nucleus were observed. This complex pattern of immunoreactivity adds another level of complexity to the neurochemical profile of this nucleus, showing selective involvement of NPY in specific populations of spinal trigeminal neurons.

The topographic distribution of NPY expression along the rostrocaudal axis of SpV revealed a similar pattern of immunoreactivity in the three subnuclei – SpVo, SpVi, and SpVc, where sequentially occurring patterns of distribution of immunopositive cell bodies and nerve fibers were observed. The widespread distribution of NPY suggests a more important role for this peptide in regulating sensory processing in multiple functional zones within the spinal trigeminal nucleus.

To compare the intensity of staining for NPY in the three parts of the spinal trigeminal nucleus, we used the non-parametric Kruskal-Wallis test. In the three tested subnuclei of the spinal trigeminal nucleus – the oral subnucleus (NPY-SpVo) (Mdn = 171), the interpolar subnucleus (NPY-SpVi) (Mdn = 168), and the caudal subnucleus (NPY-SpVc) (Mdn = 157), this test revealed significant differences in NPY expression and median values (Mdn). H(2)

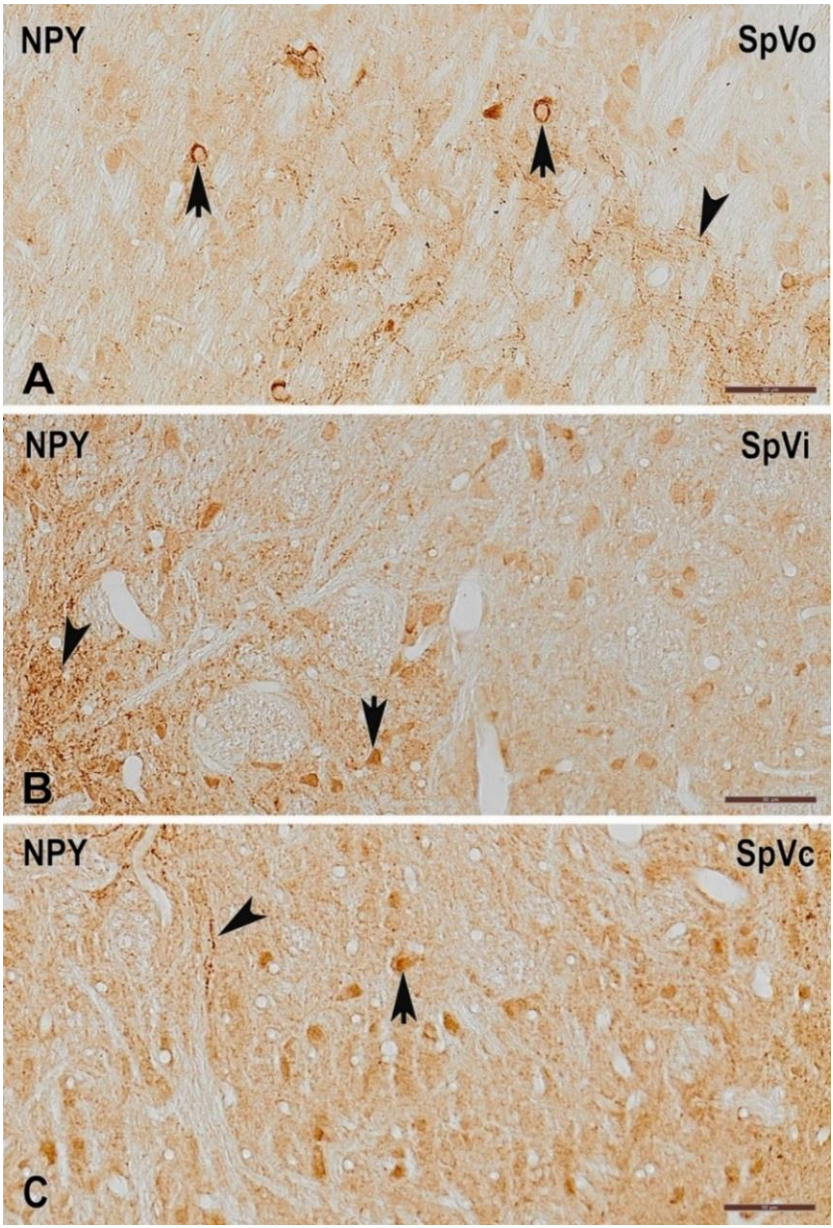

*Figure 4.20. Immunohistochemical staining for neuropeptide Y (NPY) in (**A**) the oral (SpVo), (**B**) the interpolar (SpVi) and (**C**) caudal (SpVc) subnucleus of the spinal trigeminal nucleus. Multiple immunopositive for NPY pericariya (arrow) and nerve fibres (arrowhead) were observed. Scale = 50 µm.*

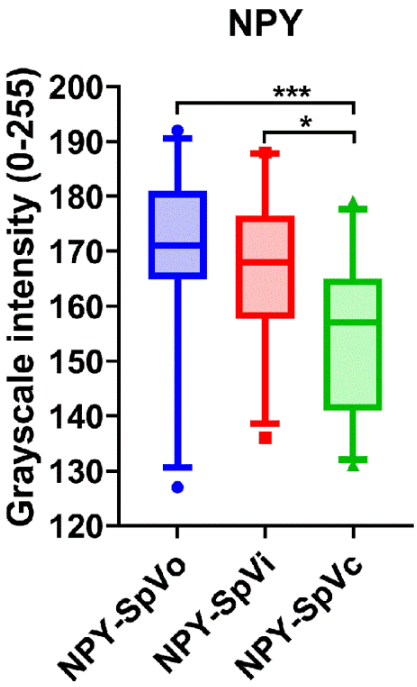

Figure 4.21. Box-plot charts graphically representing the staining intensity for NPY in the oral (SpVo), interpolar (SpVi) and caudal (SpVc) subnuclei of SpV.

= 15.78, ***p = 0.0004. We used Dunn's test to conduct post hoc analysis after the Kruskal-Wallis test. Between the oral and interpolar subnuclei of SpV, Dunn's test did not show statistically significant differences in NPY expression (p > 0.9999). However, the results of this test showed a statistically significant difference in NPY immunopositivity between the caudal (NPY-SpVc) and oral (NPY-SpVo) subnuclei of SpV (***p = 0.0004). Additionally, it was found that the differences in staining intensity between the caudal and interpolar subnuclei were statistically significant (*p = 0.0115) (Fig. 4.21).

4.2.2. Gas Neuromodulators

4.2.2.1 Nitric Oxide

To reveal the presence of this gaseous molecule in the spinal trigeminal nucleus of rats, we demonstrated, through immunohistochemical methods, the expression of the neuronal isoform of the synthesizing enzyme, nitric oxide synthase (nNOS), in the nucleus.

Our immunohistochemical analysis showed that nNOS is strongly expressed in the spinal trigeminal nucleus, 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 exhibit strong immunohistochemical staining for nNOS (Fig. 4.22).

The nNOS-immunoreactive nerve fibers identified by us were observed to intersect the spinal trigeminal nucleus. This finding emphasizes the presence of the enzyme in neuronal chains responsible for transmitting sensory data through the nucleus, thus linking nNOS to the regulation of nociceptive signals as they pass through this important brain center. Immunopositive neurons are typically small-sized with varied shapes of their perikarya. Their processes exhibit intense staining. A large portion of blood vessels are surrounded by immunopositive cells and their processes for nNOS.

Furthermore, we mapped the distribution of nNOS-positive cells in the caudal, interpolar, and oral subdivisions of the spinal trigeminal nucleus. This broad expression pattern underscores the widespread presence of nNOS along the entire longitudinal axis of the spinal trigeminal nucleus. The distribution of the reaction for this neuroactive substance is highly similar in the three subnuclei – nerve processes remain immunopositive near the cell bodies of neurons, perikarya of small cells, and glial cells surrounding the blood vessels.

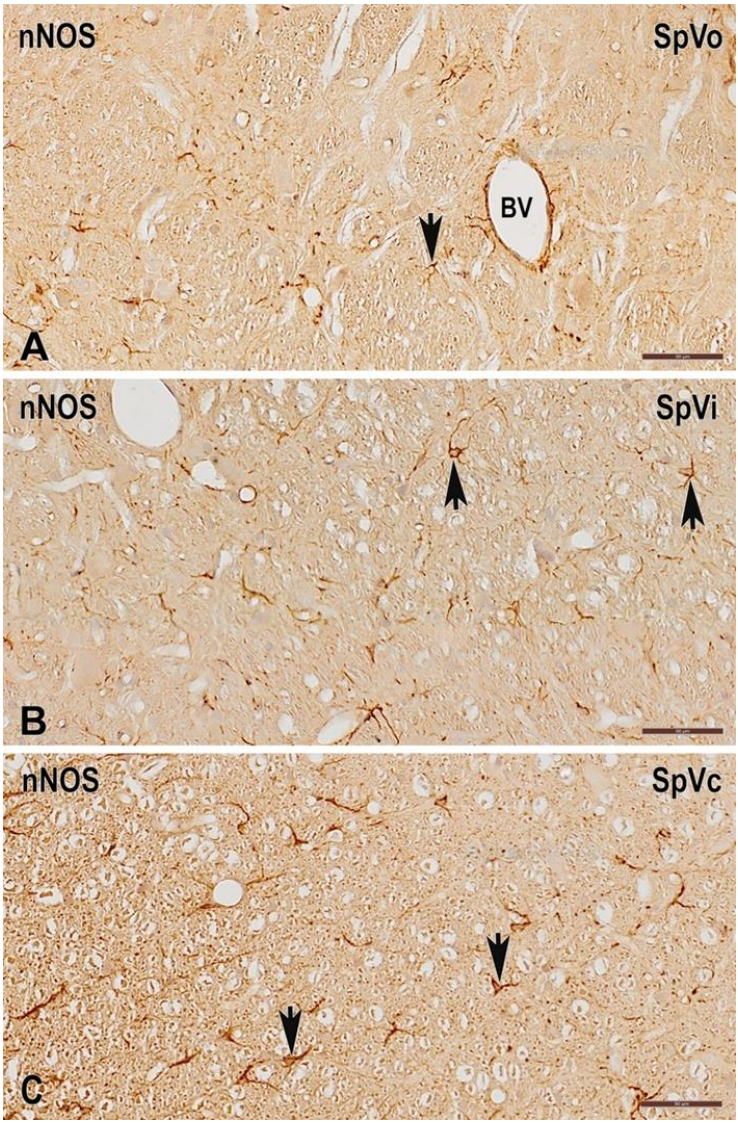

*Figure 4.22. Expression of the neuronal form of nitric oxide synthase (nNOS) in nerve cells around blood vessels (BV) and bodies of neurons (arrows) in the oral (SpVo) (**A**), the interpolar (SpVi) (**B**), and the caudal (SpVc) (**C**) subnucleus of the spinal trigeminal nucleus. Scale = 50 μm.*

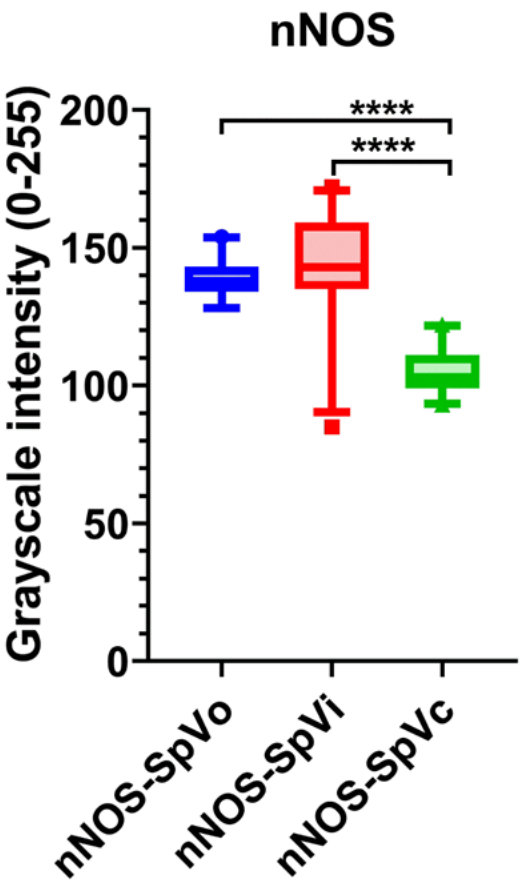

Figure 4.23. Box-plot diagrams graphically representing the staining intensity for the nNOS in the oral (SpVo), interpolar (SpVi), and caudal (SpVc) subnucleus of the SpV.

In the next stage of the study, we applied the Kruskal-Wallis test to assess the intensity of nNOS staining in the three different subnuclei of the SpV. These subnuclei include the oral (nNOS-SpVo), interpolar (nNOS-SpVi), and caudal (nNOS-SpVc) subnuclei. The Kruskal-Wallis 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, and 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 the spinal trigeminal nucleus (****$p < 0.0001$), indicating a difference in the levels of nNOS expression between these regions (Fig. 4.23). 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.

4.2.3. Neurotrophic Factors and Their Receptors

The nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) are members of the neurotrophic factor family. By initiating signaling through the low-affinity (pan) neurotrophic receptor p75NTR, as well as through the high-affinity transmembrane receptors belonging to the receptor tyrosine kinase (Trk) family, which are proto-oncogenes, these substances promote the development, survival, and plasticity of neurons. This receptor family includes TrkA, TrkB, and TrkC, which exhibit binding specificity. NGF interacts with the TrkA receptor, BDNF and NT-4 with the TrkB receptor, and NT-3 with the TrkC receptor.

4.2.3.1 Nerve Growth Factor

The nerve growth factor (NGF), a key neurotrophic factor involved in the survival, differentiation, and synaptic plasticity of neurons, exhibits different patterns of immunoreactivity in the three subnuclei of the spinal trigeminal nucleus. Our immunohistochemical experiments and subsequent statistical analysis of the results provide a detailed and reliable representation of the spatial distribution of NGF expression in these anatomical subdivisions (Fig. 4.24). In the caudal subnucleus, NGF immunoreactivity is manifested as specific staining, mainly localized in neuronal cell bodies. High-intensity immunopositivity is observed in the perikarya of spinal trigeminal neurons, indicating significant NGF expression in this region.The expression of NGF in the interpolar subnucleus exhibits a distribution pattern similar to that observed in SpVc, in terms of staining intensity and localization of the reaction product. Immunoreactivity for NGF is observed in neuronal perikarya, indicating strong synthesis and secretion of NGF in SpVi.Within the oral spinal trigeminal subnucleus, NGF immunoreactivity outlines a similar profile of protein expression as in the other two subnuclei, characterized by moderate staining intensity, primarily localized in neuronal perikarya. The presence of NGF-positive nerve fibers further emphasizes the involvement of NGF signaling in modulating synaptic connectivity and plasticity in this structural part of the spinal trigeminal nucleus.

4.2.3.2 Brain-Derived Neurotrophic Factor (BDNF)

Within the three subnuclei of the spinal trigeminal nucleus, BDNF exhibits characteristic patterns of immunoreactivity. The spatial distribution and levels of BDNF expression in the anatomical subdivisions of the nucleus are demonstrated in our immunohistochemical studies (Fig. 4.25).Specifically, we found that in the caudal spinal trigeminal subnucleus, strong immunostaining is observed, mainly visible in the proximal dendritic fibers and neuronal perikarya, indicating immunoreactivity towards BDNF. The strong immunopositivity in these areas indicates significant production and release of BDNF, emphasizing the critical role of the protein in controlling synaptic plasticity and neuron survival in SpVc.

Although differences in staining intensity and localization of the reaction product were observed, the pattern of BDNF expression distribution within the interpolar subnucleus is comparable to that observed in SpVc. Strong immunoreactivity is observed in the initial segments of the nerve fibers and the neuronal bodies, suggesting significant production and release of BDNF from neurons in SpVi. Additionally, the presumed involvement of BDNF

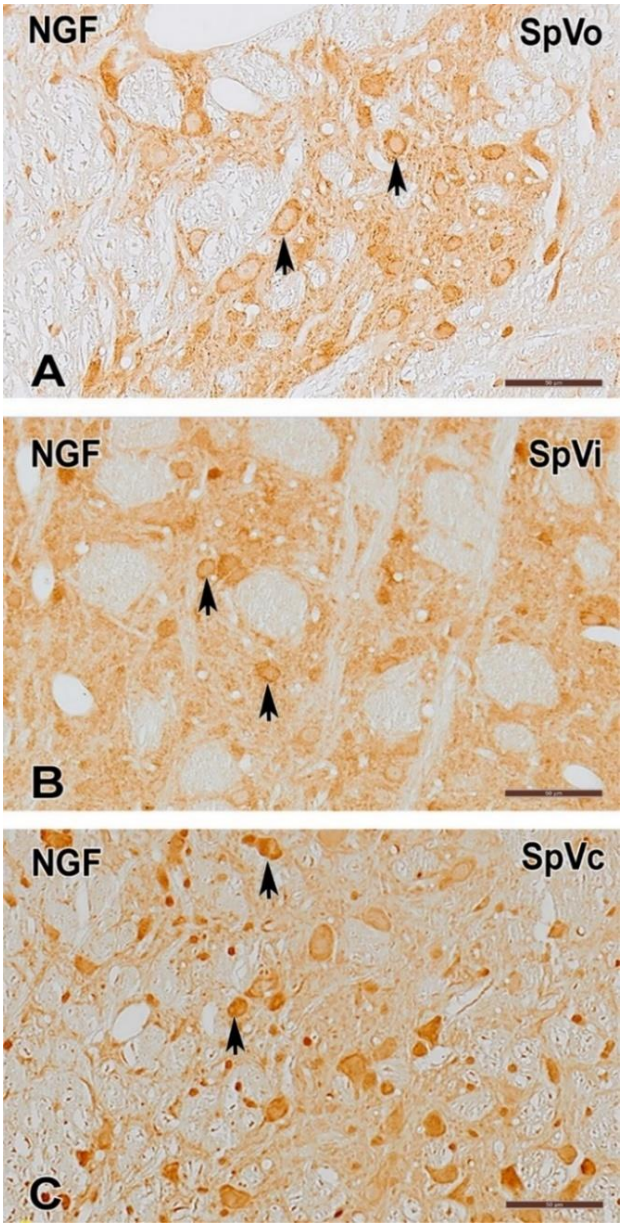

*Figure 4.24. Expression of NGF in cell neuronal bodies (arrow) in the oral (SpVo) (**A**), interpolar (SpVi) (**B**) and caudal (SpVc) (**C**) subnucleus of the spinal trigeminal nucleus. Scale = 50 µm.*

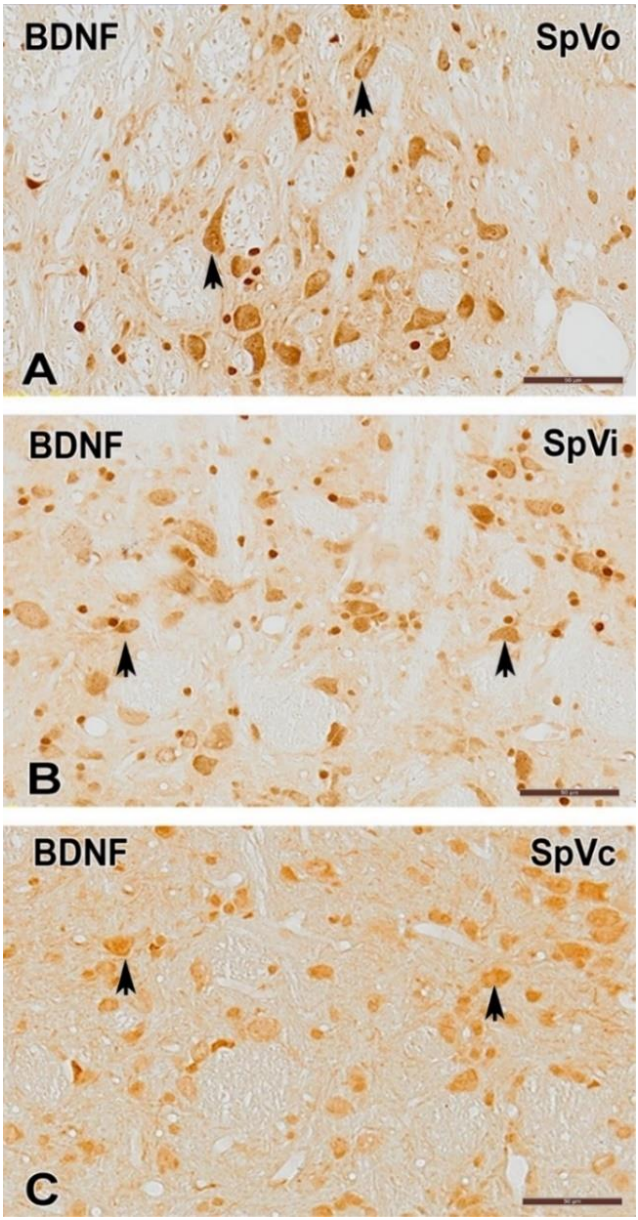

*Figure 4.25. Immunohistochemical expression of BDNF in proximal dendritic processes and perikaryons of neurons (arrows) in the oral (SpVo) (**A**), interpolar (SpVi) (**B**), and caudal (SpVc) (**C**) subnucleus of the spinal trigeminal nucleus. The immunoreactive neurons in the three subnuclei were indicated by arrows. Scale = 50 μm.*

in controlling synaptic transmission and plasticity in the interpolar subnucleus is underscored by the presence of BDNF-positive nerve fibers in this anatomical area.
The expression profile of BDNF in the oral subnucleus is characterized by high staining intensity, predominantly recognizable in the proximal nerve fibers and neuronal perikarya. SpVo shows noticeably higher levels of BDNF expression compared to SpVc and SpVi, emphasizing the role of the protein in regulating synaptic connectivity and plasticity in this subnuclear area.

4.2.3.3 Neurotrophin-3

Another member of the neurotrophin family, NT-3, is crucial for synaptic plasticity, neuron survival, and differentiation. The three subnuclei of the spinal trigeminal nucleus exhibit diverse patterns of NT-3 expression, which, based on immunohistochemical findings, may be attributed to its different regulatory functions in these anatomical subdivisions (Fig. 4.26).
Immunoreactivity to NT-3 is observed in neuronal perikarya and is particularly strong in nerve fibers, suggesting higher levels of NT-3 in this area. The cells with positive reactions are predominantly of medium and large sizes. A notably higher intensity of immunoreactivity is observed in nerve fibers compared to perikarya. Moreover, the identification of NT-3-immunopositive nerve fibers crossing SpVc suggests its role in regulating neuroplasticity and synaptic connectivity in this subnuclear region.

Similar to SpVc, NT-3 expression in the interpolar subnucleus exhibits a distribution pattern with no significant differences in staining intensity and localization. Strong immunoreactivity is observed in the bodies of spinal trigeminal neurons, indicating significant synthesis and secretion of NT-3 in SpVi. The presence of NT-3-immunoreactive nerve fibers further emphasizes the role of NT-3 in regulating synaptic connectivity and plasticity in this area of the spinal trigeminal nucleus.The expression profile of NT-3 in the oral subnucleus is characterized by moderate staining intensity, primarily visualized in the nerve fibers crossing the subnucleus. The presence of NT-3-immunoreactive nerve fibers underscores the role that NT-3 plays in regulating synaptic connectivity and plasticity in this area of the spinal trigeminal nucleus.

4.2.3.4 Glial-Derived Neurotrophic Factor

Among the members of the transforming growth factor-β superfamily, glial-derived neurotrophic factor (GDNF) is essential for neuron survival and maintenance in the central and peripheral nervous systems. Immunohistochemical analysis revealed different expression patterns of GDNF in each of the three subnuclei of the spinal trigeminal nucleus, highlighting the potential role of this neurotrophic factor in controlling sensory processing and neuronal plasticity in the facial region.

Immunopositivity for GDNF is visible in neuronal perikarya and proximal processes in the caudal subnucleus. The strong staining intensity indicates significant production and secretion of GDNF in SpVc, suggesting its role in controlling synaptic plasticity and neuron survival in this anatomical region (Fig. 4.27). During the experiments, we did not establish significant quantitative differences in staining intensity and localization; therefore, the expression of GDNF in the interpolar subnucleus shows a distribution pattern similar to that observed in SpVc. Moderate immunoreactivity is observed in the cell bodies of various-sized

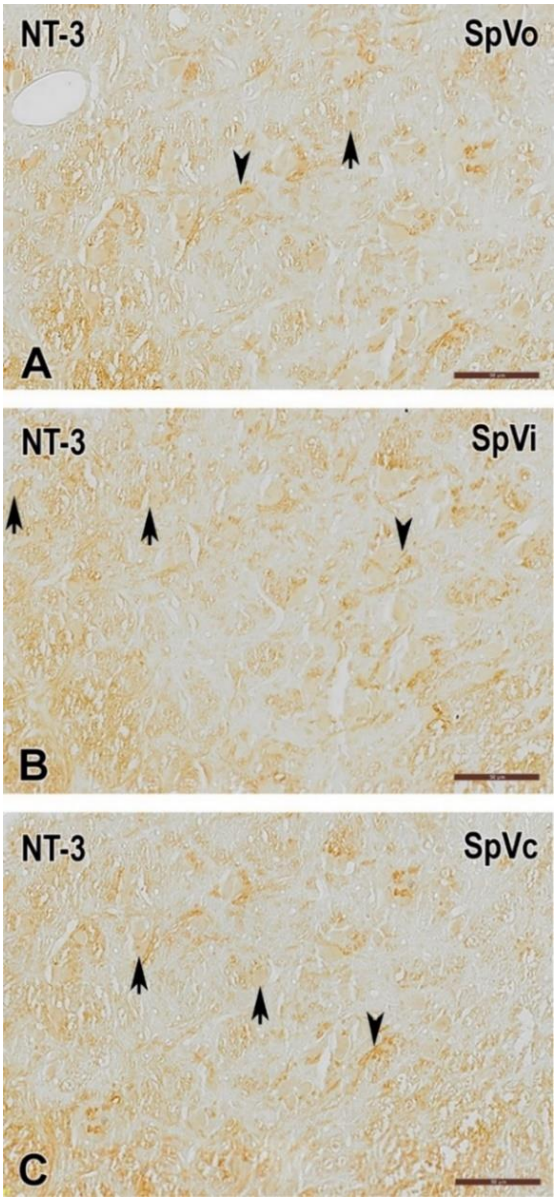

*Figure 4.26. Immunohistochemical localization of NT-3 in SpVo (**A**), SpVi (**B**) and SpVc (**C**) of spinal trigeminal nucleus. NT-3-immunopositive fibers were indicated with an arrowhead and pericarions with a weak immunoresponse were indicated with an arrow. Scale = 50 μm.*

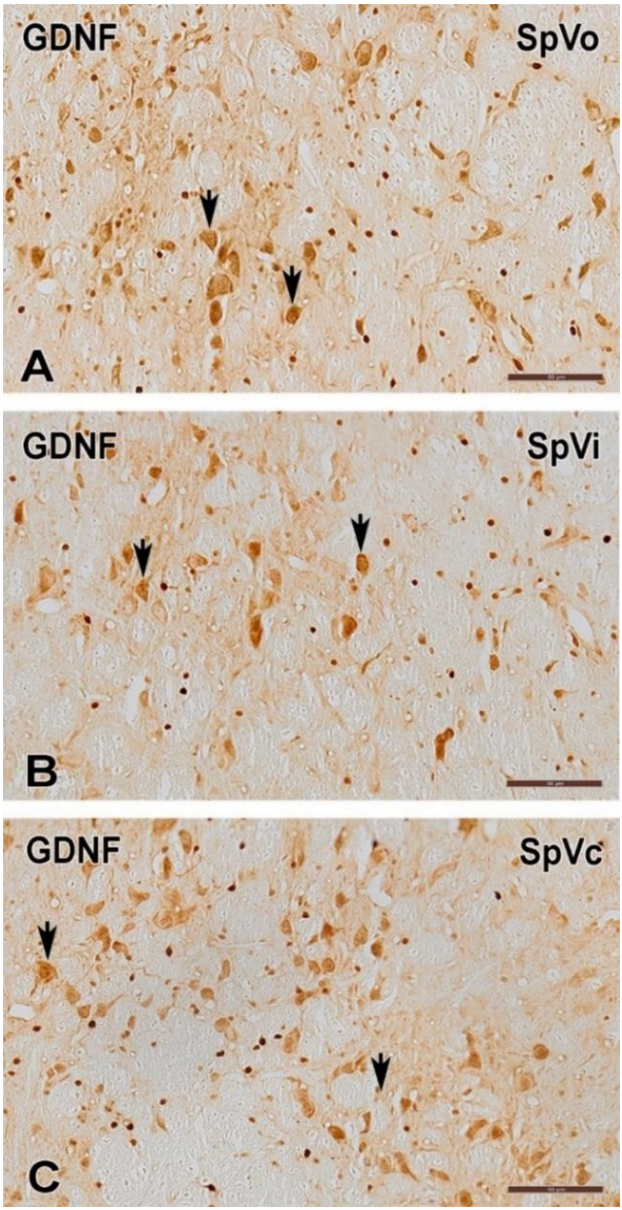

*Figure 4.27. Immunohistochemical localization of GDNF in SpVo (**A**), SpVi (**B**) and SpVc (**C**) of spinal trigeminal nucleus. With arrows, immunolabeled nerve profiles of different size and shape are shown. A more intense response is observed in perikaryons of small-sized neurons. Scale = 50 μm.*

spinal trigeminal neurons, suggesting significant synthesis and secretion of GDNF in SpVi. The expression profile of GDNF in the oral subnucleus is characterized by high staining intensity, primarily concentrated within neuronal perikarya. SpVo shows higher levels of GDNF expression compared to SpVc and SpVi. The presence of GDNF-immunopositive nerve fibers in the subnuclear domain demonstrates the potential role of this neurotrophin in regulating synaptic connectivity and plasticity.

4.2.3.5 Tropomyosin Receptor Kinase A (TrkA)

The high-affinity receptor for NGF, tropomyosin receptor kinase A (TrkA), is crucial for modulating neuronal survival, differentiation, and synaptic plasticity in the CNS and PNS. The spinal trigeminal nucleus exhibits similar patterns of TrkA immunoreactivity in its three subnuclei, highlighting the potential function of this selective receptor in regulating sensory processing and neuroplasticity in the craniofacial region. Immunoreactivity for TrkA is widely represented in the caudal subnucleus, with pronounced staining in the neuronal perikarya (Fig. 4.28).
The expression of TrkA in the interpolar subnucleus follows a distribution pattern similar to that observed in SpVc but with stronger staining intensity. Strong immunoreactivity is observed in the bodies of spinal trigeminal neurons, suggesting a major signaling mechanism mediated by TrkA in SpVi.
TrkA positivity in the oral subnucleus defines a profile of high staining intensity, primarily concentrated within neuronal perikarya. Immunopositive neurons are of medium and large sizes. Levels of TrkA expression in SpVo are higher than in SpVc and SpVi. In all three subnuclei, a significant portion of nerve fibers crossing the spinal trigeminal nucleus remains immunonegative for TrkA.

4.2.3.6 Tropomyosin Receptor Kinase B (TrkB)

Using light microscopy immunohistochemistry, TrkB expression in the structural subdivisions of the spinal trigeminal nucleus has been identified. The results show that the three subnuclei of the spinal trigeminal nucleus exhibit different patterns of TrkB immunoreactivity distribution (Fig. 4.29).
Immunoreactivity to TrkB is widely distributed in the caudal subnucleus, where it is highly intense in the cell bodies of spinal trigeminal neurons. Interestingly, immunopositive cell bodies are sporadic in this subnucleus and vary in size. The strong expression of TrkB suggests its role in mediating BDNF signaling pathways, which, in turn, affect plasticity, synaptic transmission, and neuron survival in SpVc.
The expression of TrkB in the interpolar subnucleus follows a distribution pattern similar to that observed in SpVc but with differences in staining intensity and localization. Weaker immunoreactivity is observed in the perikarya of smaller-sized spinal trigeminal neurons.
Immunoreactivity to TrkB in the oral subnucleus reveals a characteristic expression profile with moderate staining intensity, primarily concentrated within the neuronal perikarya of smaller-sized cells. Impressively, the levels of TrkB expression in SpVo are not as high as in SpVc and SpVi.

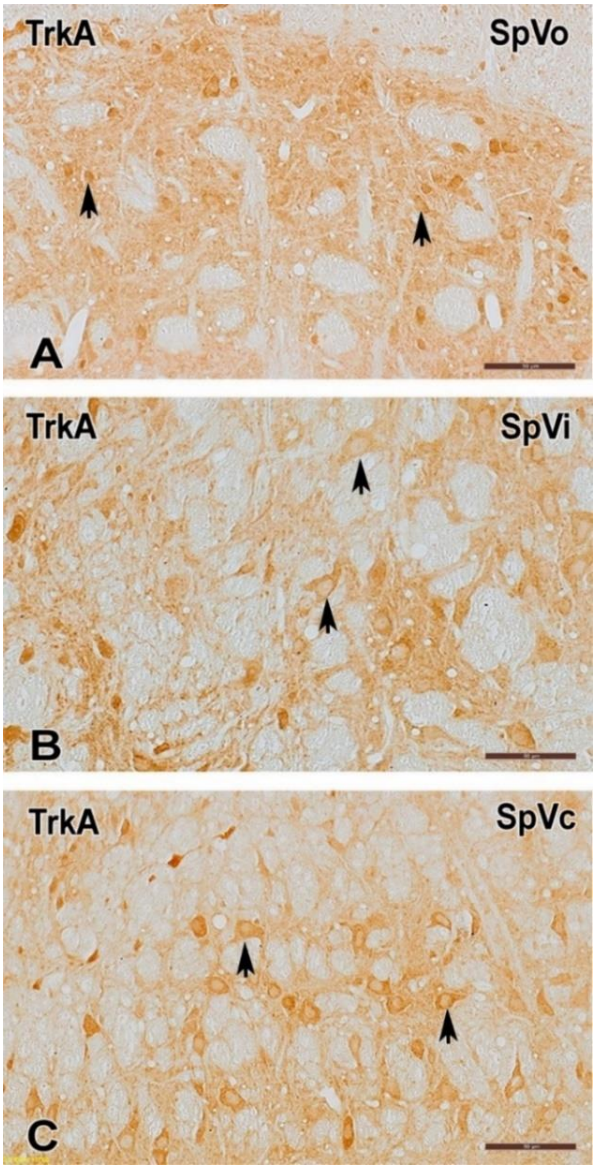

*Figure 4.28. Immunohistochemical localization of TrkA in SpVo (**A**), SpVi (**B**) and SpVc (**C**) of spinal trigeminal nucleus. Spinal trigeminal neuronal bodies with positive immunohistochemical reaction were marked with arrows. Perikaryons fall into the group of medium and large-sized neurons. Scale = 50 μm.*

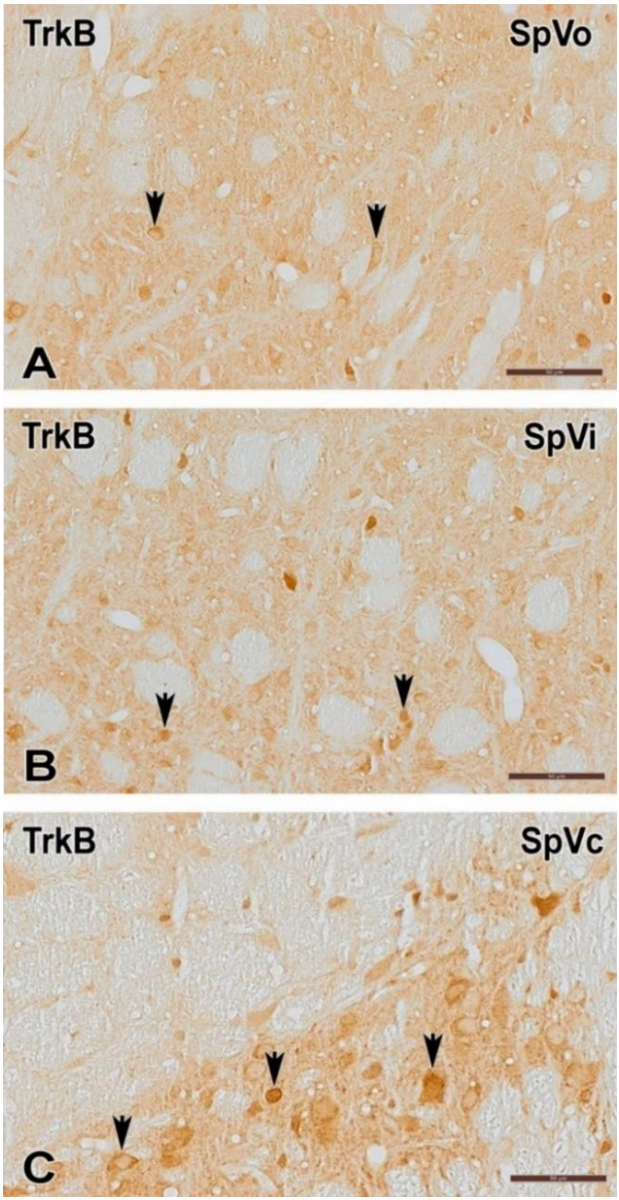

*Figure 4.29. Immunohistochemical localization of TrkB in SpVo (**A**), SpVi (**B**), and SpVc (**C**) of spinal trigeminal nucleus. Immunopositive cells for TrkB are indicated by arrows. The different intensity of the reaction and the varied external morphology of immunoreactive neurons are noteworthy. Scale = 50 µm.*

4.2.3.7 Tropomyosin Receptor Kinase C (TrkC)

The immunoreactivity of TrkC is not widely expressed in the caudal subnucleus, and this expression is relatively weak in intensity. Nerve fibers in the nucleus are most brightly stained (Fig. 4.30). TrkC expression in the interpolar subnucleus shows a distribution pattern similar to that in SpVc, with no visible differences in staining intensity and localization. Immunoreactivity is primarily observed in the processes of spinal trigeminal neurons. TrkC-immunopositive nerve fibers passing through the interpolar subnucleus demonstrate how this anatomical area regulates synaptic transmission and plasticity. Immunoreactivity of TrkC in the oral subnucleus reveals an expression profile with moderate to weak staining intensity, primarily concentrated in nerve processes. Levels of TrkC expression in SpVo qualitatively do not show differences as observed in SpVc and SpVi. Similarities in TrkC expression among the three subnuclei are represented by the presence of only nerve fibers positive for the reaction, while the bodies of spinal trigeminal neurons remain negative.

4.2.3.8 Glial Receptor α1

The expression of glial receptor α1 (GFRα1) in the spinal trigeminal nucleus can be revealed using light microscopy immunohistochemistry, allowing the demonstration of its distribution and potential functional effects in each of the three subnuclei. Impressively, the immunoreactive spinal trigeminal neurons vary in size and shape (Fig. 4.31).

Immunoreactivity of GFRα1 shows a recognizable pattern of expression in the caudal sensory trigeminal subnucleus, characterized by strong staining in the perikarya and proximal segments of the outgoing nerve processes. The strong expression of GFRα1 suggests its critical role in modulating the signaling pathways of GDNF in SpVc. Additionally, the existence of nerve fibers positive for GFRα1 further emphasizes its potential involvement in signaling in this subnuclear area.

The expression pattern of GFRα1 in the interpolar subnucleus resembles that observed in SpVc, with no significant differences in staining intensity and localization. Remarkably strong immunoreactivity is observed in the bodies of spinal trigeminal neurons, suggesting significant GFRα1-mediated signaling in SpVi. Immunoreactivity of GFRα1 in the oral subnucleus reveals a characteristic expression profile with moderate staining intensity, primarily concentrated in neuronal perikarya. SpVo does not show qualitatively different levels of GFRα1 expression compared to the intensity of immunostaining in SpVc and SpVi.

As a result of the conducted experimental immunostainings, we found that the majority of neurons and supporting glia in the spinal trigeminal nucleus of sexually mature rats are NGF-immunopositive. The immunoreactive cells are scattered throughout the nucleus. Specific staining in the cell nucleus is not observed in any of the immunopositive cells. Similarly, BDNF immunostaining is observed in some neurons in the spinal trigeminal nucleus, while NT-3 immunoreactivity is visible in both neurons and glia.

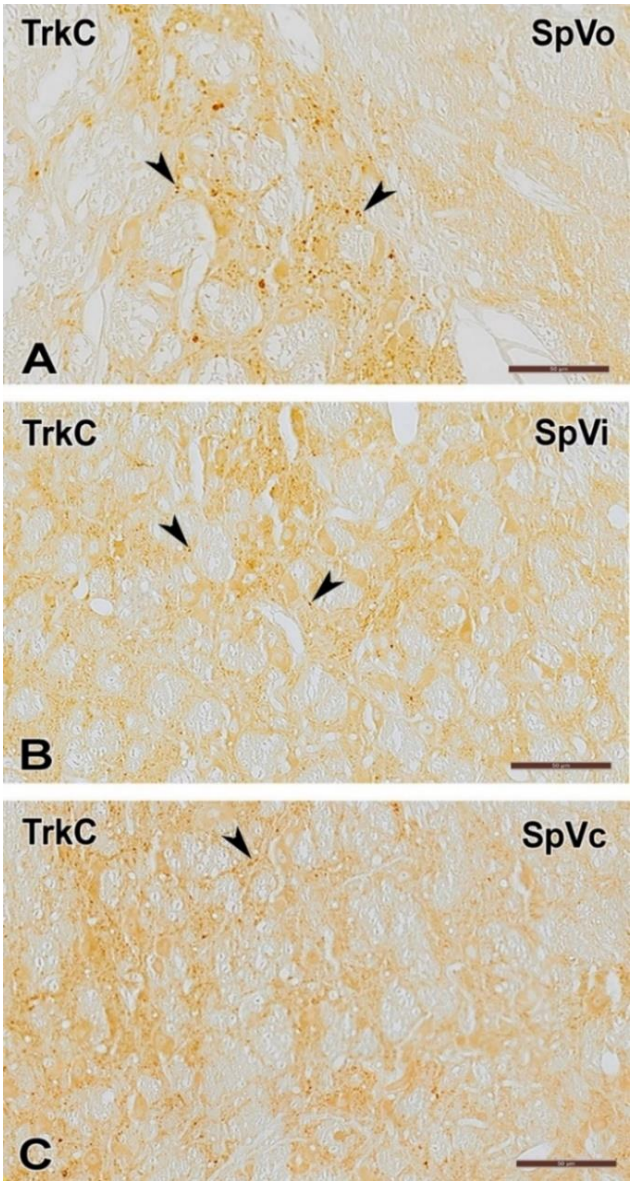

*Figure 4.30. Immunohistochemical localisation of TrkC in SpVo (**A**), SpVi (**B**), and SpVc (**C**) of the spinal trigeminal nucleus. Arrowheads indicate the reaction in immunopositive nerve fibres. The absence of immunolabelled pericarions in the subnuclei is noteworthy. Scale = 50 µm.*

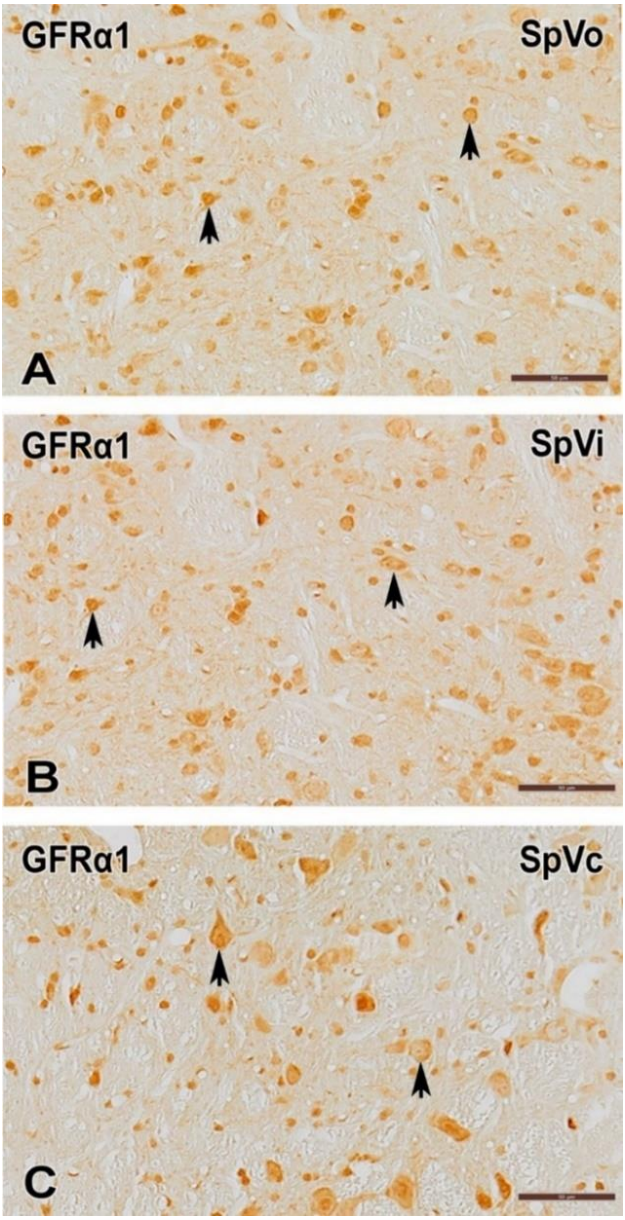

*Figure 4.31. Immunohistochemical localization of GFRα1 in SpVo (**A**), SpVi (**B**), and SpVc (**C**) of the spinal trigeminal nucleus. Several neurons with positive pericarions and the proximal regions of the exiting from the body nerve processes are indicated with arrows. Scale = 50 μm.*

Using descriptive statistical analysis, we were able to demonstrate and compare the levels of expression of neurotrophic factors and their receptors in the three subnuclei of the spinal trigeminal nucleus. The data show that NGF is uniformly expressed by neurons in the nucleus, with no statistically significant difference in the intensity of immunostaining in them. Increased immunoreactivity for TrkA is observed in SpVo compared to SpVi and SpVc. Additionally, TrkA expression in spinal trigeminal neurons in SpVi is slightly stronger compared to SpVc.

For BDNF expression levels, the comparison between the three subnuclei of the spinal trigeminal nucleus revealed different expression levels for this neurotrophic factor. BDNF has the highest expression in the spinal trigeminal neurons of the SpVo subnucleus, followed by those in SpVc, and neuronal expression in SpVi is the lowest. The expression of NT-3 is the lowest among the set of neurotrophic factors investigated in our study, together with the levels of expression of its TrkC receptor. No statistical difference in expression levels was found in neurons in the three subnuclei.

In contrast to NT-3, GDNF expression is intense in neurons in all three subnuclei of the spinal trigeminal nucleus. The highest and statistically significant expression is observed in neurons from the oral subnucleus compared to those in the interpolar and caudal subnuclei. The intensity of immunostaining for the GFRα1 receptor does not differ in the three subnuclei of the spinal trigeminal nucleus.

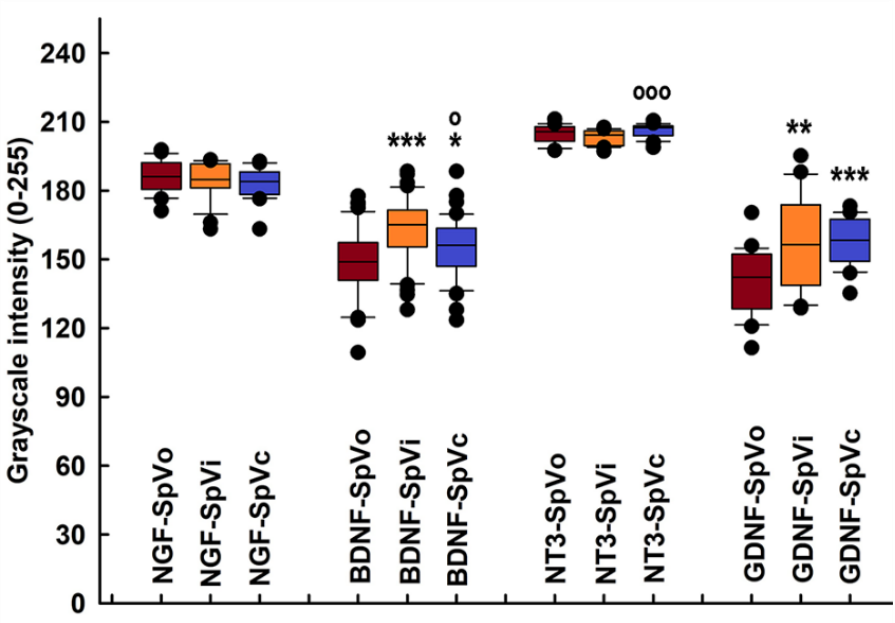

Figure 4.32. Statistical analysis of the expression of neurotrophic factors in the three parts of the spinal trigeminal nucleus.

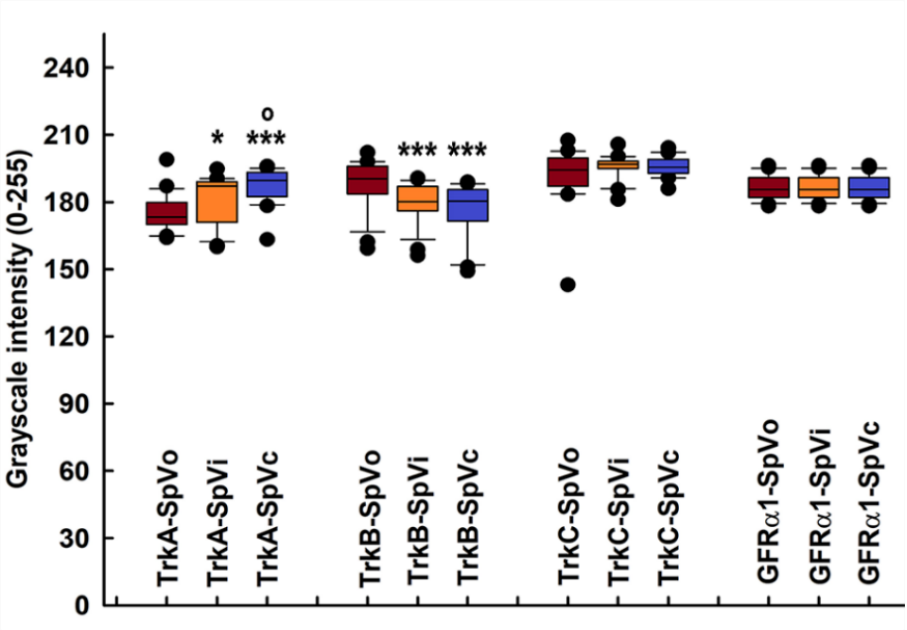

Figure 4.33. Statistical analysis of receptor expression for neurotrophic factors in the three parts of the spinal trigeminal nucleus.

4.2.4 Summary of the Neurochemical Anatomy of the Nucleus

The immunohistochemical results obtained by us for the neurotransmitters and/or neuromodulators studied in this investigation, as well as the data for the tested neurotrophic factors in the rat spinal trigeminal nucleus, have been systematized in Table 3. The table provides an overview of the variants and relative intensity of immunoreactivity of the neuroactive substances identified by us in the three parts of the spinal trigeminal nucleus. The comparative localization of the neuroactive substances in the bodies of the neurons is separately presented. The presented data do not reflect the quantitative differences in the degree of immunostaining between the two cell types. The observed different degrees of qualitative staining are determined by a subjective photometric criterion and are coded from weak to strong staining intensity as follows:

Table 3. Neuroactive substances in the spinal trigeminal nucleus.

Bioactive substances	SpVc	SpVi	SpVo
GABA			
5-HT			
SP			
NPY			
CGRP			
nNOS			
AChE			
NGF			
BDNF			
NT-3			
GDNF			
TrkA			
TrkB			
TrkC			
GFRα1			

 = LOW IMMUNOREACTIVE EXPRESSION

 = MODERATE IMMUNOREACTIVE EXPRESSION

 = HIGH IMMUNOREACTIVE EXPRESSION

V. DISCUSSION

5.1 Structurreal Organization of the Spinal Trigeminal Nucleus

5.1.1 Subnuclei of the Spinal Trigeminal Nucleus

In the first part of the present study, we present morphological data on the neuroanatomy of the spinal trigeminal nucleus which, in turn, would serve to lay the foundation in a more comprehensive understanding of its neurochemistry. This nucleus is the largest trigeminal nucleus and is located in the lateral tegmentum of the brainstem and caudal part of the bridge, adjacent to the spinal trigeminal tract. Caudally, the nucleus grows into the substantia gelatinosa of the spinal cord, while the tract continues with Lissauer's bundle (Paxinos and Watson, 2014). SpinalTrigeminal neurons have a distinct cell body with sporadic Nissl bodies surrounded by a network of myelinated axons.

The topographic-anatomical separation of neurons in the spinal trigeminal nucleus offers researchers two complementary perspectives to consider: a conventional classification based on purely anatomical features and a modern segmentation scheme based on molecular methods. While the classical notion that divides the nucleus into three distinct subnuclei-oral, interpolar, and caudal-is a cornerstone of neuroanatomical research, the molecular approach to segmentation represents a more recent endeavor that addresses the complex molecular characteristics of neurons within this nucleus.

During the early stages of neural tube development in vertebrates, the morphological and functional complexity of the brain emerges as different regions and subregions begin to define and differentiate. One mechanism contributing to this complexity is the process of segmentation, which involves the division of the proneuromeres into transverse regions, known as neuromeres, along the rostrocaudal axis (García-Guillén et al., 2021). These neuromeres display distinct molecular and cellular identities and develop into specific brain regions containing unique neuronal populations through proliferation and neurogenesis (Puelles et al., 1987; Lumsden, 1990; Kiecker and Lumsden, 2005). Although migratory processes during development can disrupt some segmental boundaries, mapping experiments have shown adherence to distinct segmental regions (Birgbauer and Fraser, 1994; Marín and Puelles, 1995; Watson et al., 2017). The current segmental model for the vertebrate brain, known as the prosomere model, specifies seven prosomers in the forebrain and 11 rhombomeres in the hindbrain (Watson et al., 2010; Nieuwenhuys and Puelles, 2015; Ten Donkelaar, 2020). This segmentation process contributes to the formation of distinct brain regions with specific neuronal populations.

The trigeminal sensory column, located in the hindbrain, is an example of such a brain structure, consisting of the main trigeminal sensory nucleus in the rostral part of the hindbrain and the spinal trigeminal nucleus extending caudally. On the other hand, as described, it is further subdivided into oral, interpolar, and caudal subnuclei (SpVo, SpVi, SpVc), each characterized by unique molecular markers and connectivity patterns (Olszewski, 1950). The trigeminal column consists of second-order sensory neurons that receive information from trigeminal nerve fibers. The prosomer model suggests that this column is divided into segmental units derived from the corresponding rhombomeres (Marín and Puelles, 1995; Aroca et al., 2006). Experimental studies have localized the main sensory trigeminal nucleus to midbrain rhombomeres (r2-r3) in mice (Oury et al., 2006). The spinal trigeminal nucleus comprises multiple rhombomeres (r4 to r11 in mice) in the pontine, retropontine, and

medullary regions of the hindbrain (Marín and Puelles,1995; Cambronero and Puelles, 2000). Understanding the organization of these subunits sheds light on the more subtle organization of the trigeminal sensory column, which is potentially influenced by underlying rhombomeric structures.

Despite the emergence of molecular segmentation as a promising way to understand neuronal organization, our current study remains rooted in the traditional framework of the classical division of the spinal trigeminal nucleus. By adhering to this well-established anatomical categorization, we aim to provide a comprehensive study and extensive analysis of the morphological and functional characteristics of spinal trigeminal subnuclei, shedding light on their distinctive roles in neuronal circuits.

Considering the results of the present study in elucidating the morphology and distribution of neurons in the rat spinal trigeminal nucleus, a comparison can be made with the earliest descriptions of its cytoarchitectonics in primates. Prior to Olszewski's work, the spinal trigeminal nucleus was thought to be a continuous structure that was a direct extension of the posterior horns of the spinal cord, reaching the main sensory nucleus of the fifth cranial nerve in the bridge. This manner of presenting with uniformity in structure has given rise to the idea that the entire nucleus also possesses uniformity in function. Thanks to Olszewski's work on material from man and Macaca mulatta, it is now clear that the nucleus can be divided into three parts. He called them "spinalis, medullaris, and pontina," which correspond to the caudal, interpolar, and oral subnuclei of the spinal trigeminal nucleus (Olszewski, 1950). The spinal trigeminal nucleus is a critical component of the trigeminal sensory system responsible for processing sensory information from the face and head. Understanding the structural characteristics, particularly the size and morphology, of neurons in thesesubnuclei, is essential for revealing the functional organization of the trigeminal sensory system.

The above description, although based on observations on material different from that used in the present study, shows similarities with the observed organization of the caudal part of the spinal trigeminal nucleus in the rat. Backing up the descriptions already made with statistical analysis, our results show that this part of the nucleus in fact contains unique neurons that can be divided into three groups according to the size of their bodies. Several studies have investigated the neuronal architecture of the spinal trigeminal nucleus, providing valuable information on the morphological heterogeneity within its subnuclei. In particular, studies using various histological techniques and immunohistochemistry (Gobel, 1975, 1978, 1979; Li et al., 1999; Schoenen, 1982) have consistently reported differences in neuronal size among SpVo, SpVi, and SpVc.

In conclusion, the structural heterogeneity of neurons within the three subnuclei of the spinal trigeminal nucleus is well described in the literature, and there are divergent reports on the size of neurons within them. Moreover, the discrepancies in the external morphology of spinal trigeminal neurons observed in different studies highlight the complexity of this brain structure.

5.1.2 Types of Neurons in the Spinal Trigeminal Nucleus

Neuronal elements in the spinal trigeminal nucleus have been studied in various animal species, including rats (Li et al., 1999), cats (Gobel, 1975, 1978, 1979; Matthews et al., 1989), monkeys (Olszewski, 1950), camels (El-Dwairi et al., 2023), and humans (Rusu, 2004; Schoenen, 1982), using different techniques such as Golgi impregnation, horseradish

peroxidase (HRP) labeling, Nissl staining, and immunocytochemistry (Gobel, 1975, 1978, 1979; Schoenen, 1982; Falls, 1983; Falls et al., 1985; Matthews et al., 1989; Li et al., 1999; Rusu, 2004; El-Dwairi et al., 2023). The investigation of neuronal diversity within the three subdivisions of the spinal trigeminal nucleus reveals multiple cell types. In the cat nucleus, for example, six different types of neurons have been identified, including spiny pyramidal neurons, non-spiny pyramidal neurons, multipolar neurons characterized by dense dendritic trees, multipolar neurons with sparse branching dendrites, cells characterized by small oval or round cell bodies and discrete accumulations of spines on distal dendrites, and fusiform cells showing multiple fine filamentous branches and spines on their dendrites (Gobel, 1975, 1978, 1979).

In rats, a detailed study has also been conducted, which showed the presence of well-stained fusiform cells characterized by extensive dendritic and axonal arborizations, distinguished by a significant number of thin lateral branches emanating from their dendrites (Li et al., 1999). Studies on the human oral subnucleus outline two main categories of neurons (Schoenen, 1982; Rusu, 2004). The first category consists of small rounded or fusiform cells (8-10 μm in diameter), clustered in small clusters or evenly dispersed. The second category includes large neurons (22 μm in diameter) with pear-shaped, fusiform, multipolar, or bipolar morphology.

Two of the subnuclei of the spinal trigeminal nucleus have been extensively studied in various species. For example, in the cat interpolar subnucleus, one study revealed the presence of five different types of nerve cells (Matthews et al., 1989). These types exhibit different morphologies, visualized as smooth pyramidal, smooth multipolar, characterized by spherical dendritic arborizations, bipolar fusiform or oval neurons with small nuclei, stellate neurons characterized by 2-4 extensively branched spiny dendrites, and cells with very small oval bodies and densely arranged dendrites. The diameters of these neurons vary widely from 6-12 μm to 15-25 μm.

On the other hand, it has been found that the oral subnucleus of rats contains three main types of nerve cells (Falls, 1983; Falls et al., 1985), including oval, fusiform, or pyramidal cells. The sizes of neuronal bodies range from 5-15 μm to 25-50 μm in diameter. Previous studies of neurons in the spinal trigeminal nucleus mainly categorize them based on the shape and size of their cell bodies, with limited attention paid to dendritic trees. Notable exceptions include detailed descriptions of stellate neurons in small experimental animals such as rats and cats (Gobel, 1975; Matthews et al., 1989; Li et al., 1999), where neurons are characterized as medium-sized neurons with numerous spiny branches. Two previous studies briefly outlined the dendritic branching of neurons in the human oral subnucleus of the spinal trigeminal nucleus (Schoenen, 1982; Rusu, 2004). However, this is only the beginning of understanding the complex arborization of neurons, and it should be noted that the perception of pain as a whole is associated with specialized sensory neurons characterized by complex dendritic processes for detecting injurious stimuli (Moore et al., 2002; Kim et al., 2006).

Previous studies, especially those focused on type I neurons, commonly referred to as stellate neurons, have provided detailed descriptions characterizing them as complex cells with spiny dendrites and numerous dendritic branches (Gobel, 1975, 1978; Matthews et al., 1989). It is worth noting that their description emphasizes the dynamics of the spines, influenced by neuronal activity and developmental age (Runge et al., 2020). In contrast, other types of neurons, including granule cells, fusiform, pyramidal, and multipolar cells, have only been mentioned in earlier studies with limited details. In a study on cats, for example, both

spiny and non-spiny pyramidal neurons have been identified, and granule cells have been described as small, round or oval with single spines on their dendrites (Gobel, 1979). However, detailed morphological data, such as information on dendritic tree density and the types and distribution of different appendages, have not been reported.

Another recent and similar study focused on the morphological characteristics of neurons in the spinal trigeminal nucleus in camels, which were investigated using the Golgi impregnation method (El-Dwairi et al., 2023). To further characterize the shape of neuronal perikarya, the study classified cells based on the size and shape of the cell body, dendritic tree density, as well as the morphology and distribution of their processes. The study identified at least 12 morphological types of neurons, including stellate, granule, octopod-like, segmented, boat-like, pyramidal, multipolar, round, oval, and elongated neurons (El-Dwairi et al., 2023). The described neurons demonstrate various forms of processes originating from both dendrites and their cell bodies, with some of them exhibiting large dilations at the points of dendritic branching. Together with our study, these are the two investigations for which we know that they successfully demonstrate the specific morphology of the cell bodies of spinal trigeminal neurons, respectively in camels and rats, some of which, such as boat-shaped and lobulated neurons, are unique among the neuronal population in the brain.

The present study represents the first comprehensive investigation of the morphology of neurons in the spinal trigeminal nucleus in rats. Our data further confirm the complex structure of the so-called stellate neurons and provide an in-depth description of other types of typical spinal trigeminal neurons, including fusiform, pyramidal, and multipolar cells, which were only briefly described in previous reports. Our study introduces new data, providing valuable information on the presence of neurons exhibiting characteristic morphological features, which classify them as octopod-like, boat-shaped, and lobulated cells. Specifically, our study identifies and categorizes at least seven different types of nerve cells within the spinal trigeminal nucleus in rats, emphasizing their distribution similarities within the three structural components of the spinal trigeminal nucleus.

Previous studies on the morphology of neurons in the spinal trigeminal nucleus, conducted using various staining techniques in rats, cats, and humans, categorize nerve cells in the spinal trigeminal nucleus mainly based on the size and shape of their bodies. However, these studies often lack detailed descriptions of dendritic trees and somatic/dendritic spines and appendages, with stellate cells being a notable exception among spinal trigeminal neurons and widely documented in previous studies (Gobel, 1975; Matthews et al., 1989; Li et al., 1999;). This approach poses clear challenges when comparing the morphology of neurons in the spinal trigeminal nucleus in rats, classified according to multiple somatic and dendritic characteristics, with analogs from other species, primarily classified based on the size and/or shape of the soma.

Furthermore, our study revealed the presence of pyramidal neurons in the spinal trigeminal nucleus of rats, resembling morphological analogs found in other animal species, which is a novel finding. Additionally, small round or oval neurons were identified, likely corresponding to the small neurons described in other species (Gobel, 1975; Matthews et al., 1989; Li et al., 1999). The study also confirmed the similarity in morphology of multipolar neurons in the spinal trigeminal nucleus of rats with those reported in previous studies in other animal species (Gobel, 1979; Matthews et al., 1989). Along with these commonalities, we described some distinctive types of neurons in the spinal terminal nucleus, characterized by unique features such as dendritic and somatic dilations.

Some previous reports explicitly emphasized the presence of small and stellate neurons, neglecting other types of neurons in the spinal trigeminal nucleus (Gobel, 1975; Bennett et al., 1980). However, the present study, while acknowledging these two types of neurons as most commonly observed, identified other neuronal types in significant numbers in the nucleus. Specifically, we report large neurons with soma diameters in some cases exceeding 40 μm, a characteristic not documented in the spinal trigeminal nucleus of rats before. Additionally, neurons with characteristic shapes such as octopod-like, boat-shaped, and lobulated cells are reported for the first time in the spinal trigeminal nucleus of rats in this study, without precedent for the presence of similar neurons in this nucleus in rats in previous morphological studies.

5.2 Chemical Profile of the Spinal Trigeminal Nucleus

5.2.1 Neurotransmitters and Neuropeptides in the Spinal Trigeminal Nucleus

5.2.1.1 Gamma-Aminobutyric Acid (GABA)

Recent experimental findings provide an intriguing insight into the distribution of gamma-aminobutyric acid (GABA) within the spinal trigeminal nucleus. Immunohistochemical reactions performed with an antibody against GABA reveal that a significant number of cells in the spinal trigeminal nucleus are immunopositive for GABA. This suggests that GABA, as an inhibitory neurotransmitter, may play a significant role in the functioning of this nucleus. Interestingly, the presence of GABA is not uniform throughout the nucleus. A statistically significant difference in the intensity of the immunohistochemical reaction for the amino acid is observed in the caudal part of the nucleus compared to that in the oral and interpolar subnuclei. The most intense reaction is found in the caudal part, indicating a potential gradient in the distribution of GABA-positive cells. As one moves rostrally, this difference decreases and ultimately becomes statistically insignificant. This spatial variation in the presence of GABA may have significance for the functional organization of the spinal trigeminal nucleus. It may suggest that different parts of the nucleus have different roles in processing sensory information, potentially associated with the inhibitory effects of GABA. However, further studies are needed to fully understand the functional significance of these findings.

Our results confirm data from other experiments regarding the presence of GABA in the spinal trigeminal nucleus (Ginestal & Matute, 1993). Not only that, but our results are also in agreement with the distribution of GABA expression, which according to Ginestal and Carlos is also highest in the caudal part of the nucleus (Ginestal & Matute, 1993). Regarding the contribution of the current results, it is related to quantitatively proving the higher expression of GABA through statistical analysis. Additionally, our experiments provide more information about the existence of GABA-positive neurons throughout the length of the spinal trigeminal nucleus and in all three of its subnuclei, while previous studies provided such information focusing on the caudal subnucleus. Moreover, the statistical analysis of GABA immunohistochemical expression sheds light on the extent of the statistically significant difference in the distribution of the neurotransmitter throughout the nucleus in rats.

The presence of GABA in the spinal trigeminal nucleus may be associated with the modulatory role of the neurotransmitter (Almond et al., 1996). Tests by Viggiano show two results; first, GABA increases in the caudal subnucleus of the spinal trigeminal nucleus during

pain tests, and second, increasing GABA leads to behavioral changes in rats that show decreased pain perception (Viggiano et al., 2004). The hypothesis is that nociceptive stimuli reach the caudal part of the nucleus, where they activate GABAergic neurons. The latter, in turn, act on nerve fibers expressing GABAA receptors and reaching the ventroposterior thalamic nuclei (Burton & Craig, 1979). The data cited inevitably show that GABA represents an important neurotransmitter in the spinal trigeminal nucleus for modulating pain sensory information through inhibition.

In conclusion, these results provide valuable insights into the neurochemical organization of the spinal trigeminal nucleus. They emphasize the importance of GABA in this structure and suggest a complex spatial organization that warrants further investigation. Future studies should aim to clarify the functional consequences of these findings and explore the specific roles of GABAergic cells in different parts of the spinal trigeminal nucleus.

5.2.1.2 Acetylcholinesterase

The cholinergic system in the spinal trigeminal nucleus plays a crucial role in modulating pain processing and understanding the distribution of key enzymes, such as acetylcholinesterase (AChE), which significantly contributes to understanding this complex neuronal network. In this study, we investigated the expression of AChE throughout the spinal trigeminal nucleus to elucidate potential variations and their significance for pain modulation. Our studies show that the expression of AChE is uniformly distributed throughout the spinal trigeminal nucleus, with no statistically significant differences observed between its three subnuclei. This uniform basal level of expression suggests a sequential enzymatic regulation of acetylcholine within the nucleus. Remarkably, this uniformity is maintained despite the different locations and functional roles of the caudal, interpolar, and oral subnuclei.

Our results demonstrate the expression of acetylcholinesterase in neurons and nerve fibers in the spinal trigeminal nucleus of rats, consistent with Reid's study of the cholinergic system in the central nervous system of mice (Reid et al., 2013). This expression suggests that the cholinergic system locally within the nucleus may be regulated by acetylcholinesterase. Mesopontine cholinergic neurons in sensory systems are known to modulate thalamic transmission by enhancing sensory information transfer (Timofeeva et al., 2005). Our research contributes to understanding the cholinergic system in the spinal trigeminal nucleus by demonstrating the expression of the enzyme that degrades acetylcholine. It appears that the expression of acetylcholinesterase is uniformly distributed along the course of the spinal trigeminal nucleus, without statistically significant differences.

The observed uniform expression of AChE throughout the spinal trigeminal nucleus provides valuable information about the cholinergic modulation of pain processing in this region. The uniform distribution of AChE may indicate concerted efforts for uniformly regulating acetylcholine levels, regardless of subnuclear location. This finding raises questions about the functional consequences of this uniformity and its role in coordinating pain information processing. The fact that all three subnuclei project to the thalamus underscores the potential functional significance of the observed uniform expression of AChE. The thalamus is a key relay station for sensory information processing in the central nervous system, and the uniform modulation of acetylcholine in the spinal trigeminal nucleus may influence how pain information is transmitted and processed at this higher level. This uniformity in AChE expression may signify coordinated efforts to regulate cholinergic

signaling, ensuring consistent influence on thalamic processing across all subnuclei. While the uniform distribution of AChE may imply basal regulation, further studies are needed to elucidate the specific roles of acetylcholine and AChE in pain modulation in each subnucleus. Investigating interactions between the cholinergic system and other neurotransmitter systems within the spinal trigeminal nucleus may provide a more comprehensive understanding of the complex mechanisms governing pain information processing.

Another reason for focusing on studying the expression levels of this enzyme is related to Timofeeva's studies in rats (Timofeeva et al., 2005). Previous studies by colleagues have shown that acetylcholine depolarizes large neurons in the interpolar part of the spinal trigeminal nucleus. Local application of a cholinergic agonist in the study led to an increase in the receptor field covering the spinal trigeminal nucleus, while administration of scopolamine as an acetylcholine antagonist led to the opposite effect (Timofeeva et al., 2005). The presence of a uniformly distributed acetylcholinesterase would mean that it would have a uniform effect on the activity of a cholinergic agonist or antagonist, whether it is receptor fields associated with the interpolar subnucleus or any other part of the spinal trigeminal nucleus. Further results suggest that cholinergic neurons in the nucleus can be divided into large and small sizes, which, in turn, project to the thalamus or other divisions of the trigeminal complex, respectively (Timofeeva et al., 2005). These lines of thinking show that cholinergic neurons in the nucleus exert effective control over receptor fields at the first station of receiving sensory information in the central nervous system. Even at the level of the spinal trigeminal nucleus, modulation of sensory information occurs under the influence of the cholinergic system. These studies, together with our evidence of the presence of acetylcholinesterase, can enrich our understanding of sensory information processing.

It can be speculated that acetylcholinesterase, as an enzyme degrading acetylcholine, indirectly influences sensory transduction at two levels (the first station of sensory transduction and higher levels of the spinal trigeminal nucleus), decreasing the transmission of sensory information. In conclusion, our study contributes to understanding the cholinergic system in the spinal trigeminal nucleus by demonstrating the equal expression of acetylcholinesterase throughout its course. This finding suggests coordinated regulation of acetylcholine, potentially influencing pain information processing. Future studies should focus on uncovering the specific roles of acetylcholine and AChE in pain modulation in each subnucleus, shedding light on the complex interaction of neurotransmitter systems in this important region of the central nervous system.

5.2.1.3 Serotonin

The central nervous system is the third most common site where serotonin is found, following enterochromaffin cells in the gastrointestinal tract and platelets (Berger et al., 2009). Therefore, its study in the spinal trigeminal nucleus is important for a better understanding of neurotransmitter distribution. Recent experimental findings provide significant insight into serotonin expression in the spinal trigeminal nucleus. The study reveals that neurons in the spinal trigeminal nucleus express serotonin uniformly in all three parts of the nucleus. This uniformity in serotonin expression, showing no statistically significant differences, is an important observation as it suggests a constant level of this bioactive substance in all three subnuclei. For the first time, the distribution of serotonin expression from caudal to rostral

direction in the nucleus has been demonstrated. This distribution pattern suggests that the baseline level of serotonin is equal throughout the three subnuclei.

A primary reason for focusing on the presence of serotonin in the spinal trigeminal nucleus is the hypothesis that it acts locally through its 5HT1B/1D receptor, leading to vasoconstriction. In the context of sensory transduction from the nucleus, serotonin halts acute migraine attacks (Kimball et al., 1960). Goadsby's work shows that serotonin infusion inhibits excitatory impulses from cells in the spinal trigeminal nucleus in the presence of pain in intracranial vascular structures, and this neurotransmitter's effect can be blocked by applying a 5HT1B/1D receptor antagonist (Goadsby & Hoskin, 1998). This indicates that serotonin has an inhibitory effect on the transmission of sensory pain information, while also demonstrating the method of action—through the 5HT1B/1D receptor. When combining our data on the distribution of serotonin expression along the course of the spinal trigeminal nucleus with serotonin's inhibitory role in transmitting nociceptive information, models can be developed to track how changes in substance levels affect the nucleus's physiology.

This finding serves as an important starting point for future studies, especially in investigating changes in serotonin availability in experimental models of various diseases. These results contribute to our understanding of serotonin's role within the spinal trigeminal nucleus and its potential implications in diseased states. Further research is needed to explore how these findings can be applied in the context of disease models and what impact they may have on our understanding of serotonin's role in these conditions. This study initiates such future investigations and opens up new avenues for studying serotonin's role in the nervous system. This knowledge has direct clinical applications with the development of medications such as sumatriptan, which are agonists of 5HT1 receptors (Feniuk et al., 1989). Sumatriptan is effective in relieving acute migraine attacks. Monitoring serotonin expression could be observed as a function of serotonin agonist use and compared with baseline expression levels from our experiments. The conclusion that can be drawn from the presented data is that serotonin may exert an inhibitory effect on trigeminovascular neuronal nociceptive pathways throughout the entire extent of the spinal trigeminal nucleus.

5.2.1.4 Substance P

One of the earliest neuropeptides identified to act as neurotransmitters in both the peripheral and central nervous systems is Substance P. According to structural analysis, it is an undecapeptide, a peptide with 11 amino acid residues (Chang et al., 1971). Experimental results provide significant insight into the distribution and expression of Substance P (SP) in the spinal trigeminal nucleus of rats. Immunohistochemical reactions performed with an antibody against the undecapeptide molecule revealed a network of SP-immunopositive nerve fibers. This observation, made at the light microscopic level, suggests that SP plays a crucial role in the functioning of these neurons. Upon closer examination, it was found that the cell bodies of neurons in the spinal trigeminal nucleus of rats are also immunopositive for SP. This indicates that SP is present not only in nerve fibers but also in the nerve cells themselves. This discovery may have significant implications for our understanding of the role of SP in neuronal communication and function.

Interestingly, the expression of SP varies in the three parts of the nucleus. In the caudal part of the nucleus, peptide expression is highest. As it passes through the interpolar and then the oral part of the nucleus, SP expression decreases. These differences in expression are

statistically significant, suggesting that they are not due to random variations but represent genuine biological phenomena. This expression pattern may reflect the different roles played by the caudal, interpolar, and oral parts of the nucleus in the functioning of the nervous system. The more intense expression of SP in the caudal part of the nucleus may indicate a greater need for the peptide's functions in this region.

Our studies confirm the presence of Substance P in the spinal trigeminal nucleus, as demonstrated by the team of Hokfelt (Hokfelt et al., 1977). The addition to these data from over 40 years, we presented the distribution of Substance P in the three parts of the nucleus. The fact that Substance P has been immunohistochemically demonstrated in the spinal trigeminal nucleus should be taken into account when hypothesizing about its role in sensory transduction at this level in the central nervous system. Substance P is believed to have a stimulating effect (Hokfelt et al., 1977). It is also believed that Substance P increases the release of endogenous opioids, thus participating in pathways leading to analgesia as a result of stimulation (Basbaum et al., 1976). Since Substance P has an excitatory action, it may activate enkephalin-releasing neurons, leading to a decrease in nociception. Although such a hypothesis is not specifically developed for the trigeminal spinal nucleus, based on these data, it would be adequate to make this deduction and to conduct experiments to prove or refute it.

In conclusion, these experimental results provide valuable insight into the distribution and expression of SP in the spinal trigeminal nucleus of rats. They highlight the potential significance of the peptide for neuronal function and pave the way for further research into its role in the nervous system. However, further studies are needed to fully understand the significance of these findings and their potential applications in neurology and medicine.

5.2.1.5 Calcitonin Gene-Related Peptide

The spinal trigeminal nucleus plays a key role as a relay station for pain information coming from the head. Calcitonin gene-related peptide (CGRP) is a bioactive substance known to be involved in nociceptive processing. In this study, we aimed to elucidate the distribution of CGRP in the three different parts of the spinal trigeminal nucleus. Our investigation included statistical analyses to identify significant differences in CGRP expression between the caudal, interpolar, and oral subnuclei.

Statistical analyses showed that the intensity of CGRP expression in the caudal subnucleus did not significantly differ from that in the interpolar region. However, when comparing CGRP expression between the interpolar and oral subnuclei, a notable finding emerged: the intensity of the immunohistochemical reaction to CGRP was significantly higher in the interpolar subnucleus. Similarly, a statistically significant difference was observed between the caudal and oral subnuclei, with CGRP expression decreasing along the nucleus from the caudal to the oral direction. The observed lack of statistical difference in CGRP expression between the caudal and interpolar subnuclei suggests a certain degree of uniformity in the distribution of CGRP in these areas. This raises questions about potential functional similarities in pain modulation processes between these two subnuclei. Intriguingly, while the caudal subnucleus exhibits comparable CGRP expression to the interpolar region, a significant increase is observed in the interpolar subnucleus compared to the oral region.

The gradual decrease of CGRP expression in the direction from the caudal to the oral part of the spinal trigeminal nucleus is a remarkable finding. This spatial pattern may suggest

a regulatory mechanism in which CGRP may play a more significant role in the caudal subnucleus, but with a visibly attenuated effect in the oral part. Understanding this gradient in CGRP expression may provide insight into the nuanced modulation of pain signals within the spinal trigeminal nucleus.

The clinical application of these data extends to potential interventions with therapeutic effect. The observed differences in CGRP expression suggest that inhibitors targeting CGRP may exert different effects in different subnuclei. Our results therefore highlight the importance of accounting for the regional distribution of CGRP in the spinal trigeminal nucleus in the development and implementation of selective inhibitors for pain relief. In this line of thought, CGRP is produced by both neurons in the PNS and those in the CNS, and as a potent peptide vasodilator it can function in the transmission of nociception (Brain et al., 1985). This vasoconstrictive action appears as a milestone in migraine therapy.

In conclusion, while our study represents a significant step toward uncovering the intricacies of CGRP expression in the spinal trigeminal nucleus, it underscores the need for further in-depth research. A deeper investigation into the functional roles, regulatory mechanisms, and clinical implications of CGRP in different subnuclei will undoubtedly contribute to advancing knowledge in this area and may pave the way for innovative therapeutic approaches.

5.2.1.4 Neuropeptide Y

Neuropeptide Y is a member of the large family of pancreatic polypeptides, including several structurally similar peptides. It is a polypeptide composed of 36 amino acid residues and is characterized by the presence of tyrosine at both ends of the molecule, with the tyrosine at the C-terminus being aminated (Tatemoto et al., 1982). Immunohistochemical investigation of neuropeptide Y (NPY) expression at the light microscopic level provides valuable insight into the distribution and intensity of this neuropeptide in the spinal trigeminal nucleus. Neuropeptide Y, a key participant in neuronal signaling, is involved in various physiological and pathological processes. In this study, we aimed to elucidate regional variations in NPY expression in the subnuclei of the rat spinal trigeminal nucleus. Our results reveal a strong expression of neuropeptide Y in nerve fibers, highlighting its important role in neural transmission. Additionally, the discovery of NPY-immunoreactivity in the cytoplasm of selected nerve cells adds a layer of complexity to understanding its cellular localization. Statistically significant differences in NPY immunoreactivity were observed between individual subnuclei of the spinal trigeminal nucleus. Particularly noteworthy is the increased expression of NPY in the caudal subnucleus compared to the oral and interpolar subnuclei. This inconsistency in the intensity of immunohistochemical reaction suggests spatially distinct regulation of neuropeptide Y within the spinal trigeminal nucleus.

Given the expression of NPY in the spinal trigeminal nucleus, hypotheses about its function should be considered. It is known that NPY participates in the modulation of many effects in the CNS and PNS. For example, direct injection of NPY reduces anxiety in behavioral tests (Sajdyk et al., 1999). Similarly, intrathecal administration of NPY leads to a reduction in nociception, and this mechanism is independent of opioid and alpha-2 adrenergic mechanisms in the spinal cord (Xu et al., 1994). Furthermore, it is known that injury or inflammation of peripheral nerves increases peptide expression in the dorsal horn of the spinal cord (Ji et al., 1994). These results indirectly suggest that NPY may also be involved in

modulating pain information processed in the spinal trigeminal nucleus, likely exerting an antinociceptive effect.

The observed regional variability in NPY expression in the subnuclei of the spinal trigeminal nucleus prompts investigation into potential functional consequences. The caudal subnucleus, where the highest expression of NPY is observed, may indicate a specialized role of this neuropeptide in modulating sensory processing or transmitting specific types of signals in this region. The lack of statistical difference in NPY presence between the interpolar and caudal parts suggests a certain level of homogeneity in the distribution of NPY in these subnuclei. This observation raises questions about the functional significance of NPY in these specific regions and whether it plays a role in common neural circuits or signaling pathways. Furthermore, our confirmation of the presence of NPY in the rat brain, specifically within the spinal trigeminal nucleus, aligns with existing literature regarding the diverse roles of NPY in regulating the central nervous system (Y. S. Allen et al., 1983). The complex interplay between NPY expression, subnuclear localization, and potential functional consequences underscores the complexity of neuropeptide Y's involvement in neural processes.

In conclusion, our immunohistochemical studies shed light on the differentiated expression of neuropeptide Y within the various subnuclei of the spinal trigeminal nucleus. These findings contribute to our understanding of the nuanced distribution of NPY in the rat central nervous system and provide a basis for future research investigating the functional consequences of this regional variability in neuropeptide Y expression.

5.2.2 Gas Neuromodulators

5.2.2.1 Nitric Oxide

Nitric oxide is generated endogenously by NOS, and its synthesis depends on the presence of oxygen in the cell, while its biological activity directly correlates with its binding to heme ligands. Endogenous nitric oxide can be synthesized by three isoforms of NOS (constitutive NOS, neuronal NOS, and inducible NOS), with the focus of research being on the nNOS isoform due to its widespread distribution in the structures of the central nervous system.

In this study, we conducted immunohistochemical analyses to investigate the distribution of nNOS expression in the individual subnuclei of the rat spinal trigeminal nucleus. Our immunohistochemical studies unequivocally demonstrate the presence of nNOS both in the nerve fibers and in the neurons within the spinal trigeminal nucleus. Statistically significant differences in nNOS expression are observed between 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 spinal trigeminal nucleus.

The observed regional differences in nNOS expression among the subnuclei of the spinal trigeminal nucleus suggest a nuanced regulatory mechanism. The significantly higher expression in the caudal subnucleus 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 nitric oxide and the pathophysiology of migraine is emphasized by the well-known effect of inhibiting NO to reduce spontaneous activity in the spinal trigeminal nucleus. The observation that nNOS expression is highest in the caudal subnucleus, known for its involvement in sensory processing, further supports the idea that NO may have an enhancing effect on migraines. This finding is consistent with existing literature indicating the involvement of NO in the generation or modulation of migraine headaches. Infusion of nitric oxide donors leads to a spontaneous increase in neuronal activity in the neurons of the spinal trigeminal nucleus in an experimental rat model (Koulchitsky et al., 2004). This fact may suggest that nitric oxide, as a nitrovasodilator, leads to enhanced transmission of pain information from the head.

When it comes to sensory information reaching and passing through the spinal trigeminal nucleus, inhibiting endogenous NO synthesis reduces spontaneous activity in the spinal trigeminal nucleus, which should mean that NO has a tonic effect on migraines (Koulchitsky et al., 2004). This hypothesis has already been confirmed by clinical studies showing that increasing NO metabolites in the blood in the internal jugular vein in people suffering from migraines during spontaneous migraine attacks (Sarchielli et al., 2000). Thus, nitric oxide 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 spinal trigeminal nucleus. 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 spinal trigeminal nucleus, with sensory information from the outer parts of the head to the temporal areas reaching the caudal subnucleus, 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.

In conclusion, our immunohistochemical studies provided valuable information on the differentiated expression of nNOS in the subnuclei of the rat spinal trigeminal nucleus. 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.

5.2.3. Neurotrophic Factors and Their Receptors

The spinal trigeminal nucleus participates in the transmission of head pain. There is compelling evidence that some neurotrophic factors are necessary for neuronal plasticity, and studies on these factors and cells may contribute to a better understanding of the underlying mechanisms leading to nociceptive disorders. Combining this with the subsequent division of the nucleus into three subnuclei and their assignment to various sensory zones of the head, it can be carefully examined how these neurotrophic factors are distributed throughout the spinal

trigeminal nucleus. The present study provides immunohistochemical evidence that different parts of the spinal trigeminal nucleus release trophic factors, helping to explain some of the mechanisms underlying the development of chronic conditions associated with nociception. Our results show that the three subnuclei express neurotrophic factors from the NGF, BDNF, NT-3, and GDNF groups and their respective receptors with high intensity. Here we demonstrate the presence of NGF, BDNF, NT-3, and GDNF proteins in the majority of neurons, which are also equipped with their respective receptors TrkA, TrkB, TrkC, and GFRα1.

Furthermore, our immunohistochemical and semi-quantitative image analysis compares the levels of expression for neurotrophic factors and their receptors in the three subnuclei. Using descriptive statistical analysis, we were able to demonstrate and compare the levels of expression of neurotrophic factors and their receptors in the three subnuclei of the spinal trigeminal nucleus. The data show that NGF is uniformly expressed by neurons in the nucleus without a statistically significant difference at $p<0.05$. The same applies to its TrkA receptor. Conversely, for BDNF expression levels, the comparison reveals that the three subnuclei have their levels of expression for the neurotrophic factor. It turns out that BDNF has the highest expression in the neurons of the oral subnucleus, followed by those in the caudal, with the expression in the interpolar subnucleus being the lowest. The difference in expression between the three locations along the length of the spinal trigeminal nucleus is statistically significant. TrkB as the receptor for BDNF shows differences in all three subnuclei; the expression level is highest in the caudal subnucleus, and the expression in the oral subnucleus is the lowest. The statistically significant difference is between the oral and interpolar subnuclei and between the oral and caudal subnuclei. The expression of NT-3 is the lowest among the set of neurotrophic factors studied in our work, together with the levels of expression of its TrkC receptor, without a statistically significant difference in the three subnuclei.

On the other hand, the immunohistochemical results for GDNF show high levels of expression, and with $p<0.05$, it turns out that the three subnuclei consist of neurons with relatively equal levels of expression without a statistical difference between them. The intensity of immunostaining for the GFRα1 receptor differs only slightly at the level of the interpolar subnucleus compared to the caudal subnucleus, but still enough to detect a statistical difference as is shown in Table 3. The significance of these findings lies in the fact that for the first time, the levels of expression of neurotrophic factors and their receptors have been compared in the three parts of the rat's spinal trigeminal nucleus. Differences in expression between the three subnuclei have never been investigated before. Now we know that BDNF and GDNF, along with their receptors, occupy a large part of the set of neurotrophic factors studied in this research; conversely, NT-3 and its receptor TrkC do not appear to be as well expressed by 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, with the latter conducting sensory impulses to higher zones of the central nervous system (Dreyfus, 1989). Previous studies show that many growth and trophic factors have been found in the cell populations of the mammalian spinal trigeminal nucleus (Ibáñez, 1995). Our results provide immunohistochemical evidence that neurons in the spinal trigeminal nucleus of mature rats express certain neurotrophic factors, namely NGF and NGF GDNF, as well as their respective receptors. These expression patterns correlate with the conclusion that neurons can exert trophic and/or regulatory effects on neighboring cells in the spinal

trigeminal nucleus of mature rats through neurotrophic factors, which in turn play an autocrine and/or paracrine role in promoting the survival, growth, and differentiation of these cells.

In addition to their established role in maintaining the structural and functional organization of the spinal trigeminal nucleus, there is evidence suggesting that neurotrophic factors facilitate the proliferation of neuronal cells and the differentiation of some cells originating from the neuronal crest (Lewin & Barde, 1996). Our studies confirm previous findings and show that NGF and its corresponding TrkA receptor, which binds with high affinity to nerve growth factor, are mainly expressed by neurons in the spinal trigeminal nucleus (Klein et al., 1991). Although there is evidence that neurons do not depend on NGF and BDNF for their survival and thus growth requirements vary for these cells; however, increased survival chances depend on the activation of protein kinase B (Rajagopal et al., 2004). In the present study, we further visualized NGF and TrkA in the cells of the nuclei of the spinal trigeminal nucleus in rats. Our results are consistent with the hypothesis that increased expression of these compounds in the spinal trigeminal nucleus, which regulates its postnatal growth, may influence the way pain is perceived at a central level.

Unlike other neurotrophic factors from the NGF family, NT-3 and NT-4 have been much less studied in the spinal trigeminal nucleus of mature rats. In our study, we demonstrated the presence of NT-3 and its specific TrkC receptor, but not that of NT-4 in the nucleus neurons. However, we were able to show that TrkB, which is a common receptor for BDNF and NT-4, is expressed in almost all neurons. It is also well known that NT-3, despite its lower affinity, binds to TrkA as well as to TrkB (Klein et al., 1991). All these data may explain the somewhat illogical results of TrkB expression in the three subnuclei, which do not correlate with the levels of expression of a single neurotrophic factor. The results rather reveal the complex interaction and thus the difference in receptor expression and neurotrophic factors. Therefore, the survival of neurons seems to depend on TrkB signaling as well.

From the results presented so far, it can be concluded that most of the neurons in the spinal trigeminal nucleus of mature rats contain high levels of NGF, BDNF, and GDNF and their respective receptors. Taken together, our results suggest that cells in the spinal trigeminal nucleus depend on the local production of certain trophic factors. In conclusion, our study suggests that the expression of neurotrophic factors in the three subnuclei of the spinal trigeminal nucleus varies. Specifically, the majority of neurons contain NGF, BDNF, NT-3, GDNF, and their receptors, and their expression varies significantly in different parts of the nucleus. It is also likely that the cell populations in the nucleus may exert trophic and/or regulatory control over neighboring cells in the spinal trigeminal nucleus through the entire cocktail of neurotrophins, implying a possible autocrine and paracrine role. And finally, but not least, the conclusions from the expression of neurotrophic factors can be applied to better understand their role in the genesis and/or temporal evolution of conditions affecting sensory nerve pathways for pain from various areas of the head.

5.3 Study Limitations

5.3.1 Technical Limitations of Staining Methods

The present study demonstrates the presence of small and stubby neurons characteristic of the spinal trigeminal nucleus but also identifies a large number of neuronal types in the nucleus with different external morphologies. Specifically, the study reports the presence of individual

large neurons with a body diameter of up to 45 μm, a characteristic not previously documented in the rat spinal trigeminal nucleus. Furthermore, neurons with characteristic shapes such as octopus-like, fusiform, and lobulated cells are reported for the first time in the rat spinal trigeminal nucleus, without precedent for the presence of such neurons in this nucleus of other species. However, to visualize and characterize neuronal types, it is crucial to consider factors influencing staining methods, including the type and selectivity of the staining method, differences in histological techniques applied in different laboratories, the age of experimental animals, fixation method, among other variables (Zaqout & Kaindl, 2016). Therefore, we logically assume that some of the neuronal types reported for the first time in this study may exist in the spinal trigeminal nucleus of other animal species, but their non-detection so far may be due to some of the aforementioned technical factors affecting the staining process and subsequent visualization of nerve cells.

5.3.2 Limitations of Semi-quantitative Immunohistochemistry

To objectify the immunohistochemical findings in this study, we applied quantitative techniques based on computer-assisted microscopy. However, this technique has several potential limitations that minimize its overall utility. Firstly, differences in tissue processing in batch-specific immunostaining can influence the outcome of the immunohistochemical procedure, especially the intensity of immunostaining, thus compromising the correct interpretation of staining results. Considering that the quality of histological staining depends on the initial tissue processing, differences in the intensity of immunostaining may result from variations in tissue fixation or subsequent chromogenic reaction saturation, which, in turn, may lead to differences in the generated digital images, thus affecting their objective analysis. To overcome this problem, during the reactions, we simultaneously incubated tissue sections taken from the three subnuclei to minimize the risk of any errors caused by variations during the immunohistochemical procedure.

Another methodological problem could be related to the precise calibration of the system before its use for quantitative determination of intensity. For this purpose, the image analysis system must have calibrators suitable for the type of analysis and the parameters being evaluated. Additionally, they must be chosen to be suitable and subsequently adjusted. Due to these issues, digital methods require appropriate validation, and therefore, the system must be precisely calibrated before brightness measurement begins.

Finally, digital photomicroscopy is a major source of potential variability; obtaining a digital image correctly proves difficult unless all factors that may affect image quality are known and strictly controlled. Moreover, the available commercial software is limited to color thresholds and pixel counting; each color is limited to assigning a digital value or grayscale between 0 and 255. Ultimately, image processing and analysis must be performed identically for experimental and control samples.

Despite all potential technical limitations of quantitative immunohistochemistry, our study demonstrates that this approach can be used to accurately determine the amount of bioactive substances and their receptors of interest in specific tissue(s). However, quality control measures are necessary for the final data analysis.

5.3.3 Limitations and Specifications in the Experimental Animal Group

Although the present study contributes valuable information about the level of expression of various neurotransmitters, bioactive substances, enzymes related to the metabolism of some neurotransmitters, as well as neurotrophic factors and their receptors in the three subnuclei of the spinal trigeminal nucleus in the rat, there are some specific limitations of the study that need to be recognized. First of all, the experimental animals used in this study were only male rats. This means that potential sex differences in the structure and neurochemical affiliation of the studied neurons were not accounted for. This aspect is important because a significant focus in such general studies over the past ten years has been the possible presence of sex differences in the pathological mechanism of pain, the biological basis of which has been described in several review scientific papers related to neuropathic pain (Sorge et al., 2015; Mapplebeck et al., 2016). The authors of these studies found, for example, that the involvement of BDNF, released from microglial cells in the spinal cord, in the induction of mechanical allodynia after neuronal damage is specific to men (Sorge et al., 2015). In addition, the influence of 17-β-estradiol on temporomandibular joint disorders was studied, and women with reproductive capacity were reported to be particularly affected by this type of pain (Wu et al., 2015). The data presented indicate that 17-β-estradiol regulates the expression of TRPV1 and NGF in a dose-dependent manner (Wu et al., 2015). In addition, NGF was found to increase the number of neurons that express the NMDA receptor in the trigeminal ganglion of the rat in both sexes, but the expression of some sensory neuropeptides such as CGRP and SP increased in ganglion cells only in female rats (Van Gerven et al., 2017). These studies suggest that there are significant sex differences in the way some of the neuroactive proteins we test are expressed. In view of these existing differences, it is possible that the results of the present study are different if female rats were included.

Secondly, all rats used in this study were of the same breed, which may limit the generalization of the results over the whole species of animals. It is possible that different breeds of rats express the studied neuroactive substances differently in the three subnuclei of the spinal trigeminal nucleus, which means that the results of this study may not be applicable to other breeds of rats or other experimental animals. Hence it must be concluded that the results presented and their interpretation apply to Wistar rats.

Finally, it is important to note that the current study focused only on the levels of immunohistochemical expression of the studied substances in the spinal trigeminal nucleus in the rat, without taking into account any variables. Other potential factors, such as rat diet, environment and previous experience, have not been considered.

Since our study aims to establish the basal levels of many substances whose role proves to be important and complex in the conditions that affect the transmission of pain information from the head, the diet of rats should be taken into account in further studies. It turns out that diet affects conditions that are associated with sensory transduction. So far, the effects of different types of diets with respect to migraine and headache have been investigated (Bellamy et al., 2006; Alpay et al., 2010; Di Lorenzo et al., 2013). It has been suggested that nutritional interventions could influence headache/migraine characteristics through different mechanisms. These mechanisms may include influencing serotonergic dysfunction, neuronal excitability, levels of neurochemical factors such as CGRP and nitric oxide, and also of certain hormones such as adiponectin and leptin with a certain role in migraine pathogenesis, the influence of brain mitochondrial function, neuroinflammation, hypothalamic function and

platelet aggregation (Le May et al., 2000; Bellamy et al., 2006; Peterlin et al., 2016; Deen et al., 2017; Edvinsson et al., 2018; Ramachandran, 2018). These factors could influence the synthesis of bioactive substances in the three subnuclei studied.

Despite these limitations, we believe that the present study provides an important contribution to the field of neuroscience and highlights the potential role of certain neurochemicals in neuronal function and survival of neurons in the spinal trigeminal nucleus. Future research may aim to address these limitations and extend the results of the present study to achieve a more comprehensive understanding of the fundamental aspects in cytoarchitectonics and neurochemistry of this nucleus.

5.3 Future Directions and Perspectives

Given the above, our future scientific endeavors will be directed toward investigating the expression of additional substances that may influence neuronal trophism and sensory transduction in the spinal trigeminal nucleus, as well as continuing studies related to the structure of the three subnuclei. In this context, our team plans to conduct specific experiments to investigate the following characteristics of the spinal trigeminal nucleus:

5.3.1 Investigation of the Degree of Immunohistochemical Expression of Dopamine

At the time of the last update of our knowledge on the subject at the date of completion of this work, the specific role of dopamine in spinal trigeminal nucleus has not been extensively studied or well documented in the literature. Currently, most of the research related to dopamine has traditionally focused on its role in the CNS, especially in areas such as the basal ganglia (Smith & Kieval, 2000) and the mesolimbic pathway (Pierce and Kumaresan, 2006).

Dopamine is a neurotransmitter that plays a crucial role in various physiological functions, such as motor control (Gepshtein et al., 2014), reward mechanisms (Lewis et al., 2021), and mood regulation (Salgado-Pineda et al., 2005). It is primarily associated with dopaminergic pathways in the brain, such as the nigrostriatal pathway and the mesocorticolimbic pathway. Dopamine receptors are found in different parts of the CNS, such as in the spinal cord, where they modulate sensory and motor functions. In the available literature, data are available on expression of dopamine and dopamine receptors at mRNA and protein level in the mesencephalic trigeminal nucleus (Lazarov, 2000; Lazarov, 2002, 2007), but so far, no emphasis has been placed on their expression in the spinal trigeminal nucleus (Liu et al., 2019), as well as on the potential interactions or modulating effects of dopamine in trigeminal sensory processing (Bergerot et al., 2007).

Our future specific intentions are to continue to study the neurochemical profile of the spinal trigeminal nucleus, shedding more light on the delicate interconnections between individual neurotransmitters in the nucleus, which together lead to the reception, processing and transmission of sensory information about pain and temperature.

5.3.2 Stereological Studies on the Cytoarchitecture of the Three Parts of the Spinal Trigeminal Nucleus

Stereology is an extremely important and systematic method for unbiased quantitative analysis of three-dimensional structures, making it a fundamental tool for studying the cellular and structural characteristics of nerve nuclei, including the spinal trigeminal nucleus. Since this nucleus is involved in processing sensory information related to the face and head, it requires precise quantitative assessments to understand in detail its structural features and functional relationships. Stereological methods can be used in the study of the spinal trigeminal nucleus for quantitative assessment of the neurons within it, volume estimation, calculation of the length and density of the capillary network, area of capillary cross-sections, and also to establish correlations between neuronal and vascular parameters. Stereological analysis of the spinal trigeminal nucleus can be particularly valuable in pathological conditions affecting the trigeminal system. Changes in the number of spinal trigeminal neurons, volume, and vascular parameters can serve as quantitative markers for disease progression, contributing to a better understanding of structural changes associated with neurological disorders affecting the face and head.

In conclusion, the application of stereology in our future work on the spinal trigeminal nucleus is crucial for obtaining objective and accurate quantitative data. The combination of neuronal and vascular assessments will provide a comprehensive view of the structural and functional characteristics of different areas within the spinal trigeminal nucleus, contributing to advances in our understanding of sensory processing and potential implications for neurological health.

5.3.3 Investigation of the Ultrastructure of the Spinal Trigeminal Nucleus in Rats

Future studies aimed at elucidating the detailed subcellular structure of the three subdivisions of the spinal trigeminal nucleus would be possible with the application of modern imaging techniques such as scanning electron microscopy and transmission electron microscopy. Successfully implemented, they promise to contribute to a better understanding of the cellular and synaptic organization in this critical brainstem nucleus.

On one hand, scanning electron microscopy offers the opportunity to visualize the surface morphology of neurons, glial cells, and synaptic connections in the finest detail. By investigating the three-dimensional architecture of neuronal cell bodies, dendrites, and axonal terminals, scanning electron microscopy can provide valuable information about the structural characteristics and spatial relationships of cellular elements within the spinal trigeminal nucleus. On the other hand, transmission electron microscopy (TEM) provides visualization of ultrastructural details on a nanometric scale, allowing the study of cellular organelles, synaptic vesicles, and synaptic membranes with unmatched resolution. TEM can provide insight into the subcellular organization of neurons, including the morphology and distribution of synaptic contacts, the localization of neurotransmitter receptors, and the ultrastructural characteristics of synaptic transmission in the nucleus. Additionally, during the studies, additional approaches such as immunoelectron microscopy (CLEM - correlation between light and electron microscopy) can be applied to localize specific proteins, neurotransmitters,

and receptors at the ultrastructural level, providing new information and detailed insight into the molecular composition and synaptic localization of key signaling molecules within the spinal trigeminal nucleus.

Overall, in their entirety, the future studies described above have the potential to elucidate the complex ultrastructural organization of the three subdivisions of the spinal trigeminal nucleus, revealing the synaptic architecture and cellular connectivity of individual components underlying sensory processing, pain modulation, and neurochemical signaling in this critical brainstem nucleus.

VI. CONCLUSION

The spinal trigeminal nucleus is an important structure in the central nervous system, receiving sensory information about pain and temperature from the head and face. The nucleus consists of a bilateral cluster of neurons, located from the most rostral areas of the spinal cord, extending into the medulla oblongata and reaching the pons, with its subdivisions - subnucleus caudalis, interpolaris, and oralis respectively presented from a caudal direction. The nucleus comprises neurons, glial cells, and nerve fibers of various sizes and shapes. This description of the nucleus correlates with its functional specialization, showing that its parts are associated with the reception, processing, and transmission of sensory information from different areas of the face.

The study of neurons in the spinal trigeminal nucleus encompasses various methodologies, each shedding light on different aspects of its neuronal characteristics. This study delves into an expanded examination beyond traditional classifications based on cell size, aiming to provide a nuanced understanding by including assessments of neuronal morphology and revealing the neurochemical profile in this nucleus.

By incorporating morphological descriptors, the present study applies a second approach, carefully examining the diverse forms of neuronal bodies within the spinal trigeminal nucleus. Through delving into the intricacies of neuronal morphology, a more comprehensive description of the different types of neurons present in this nucleus is achieved.

In addition to size and shape, the neurochemical composition of neurons is an important aspect that influences their functional roles. This study elevates the level of investigation by uncovering the neurochemical profile of neurons within the spinal trigeminal nucleus, shedding light on the bioactive substances synthesized by these neuronal units.

In our study, we identified a remarkable neurochemical coding of spinal trigeminal neurons, revealing the presence of a wide spectrum of neuroactive substances, including classical neurotransmitters, neuropeptides, and neurotrophins, which are presumed to play a significant role in pain transduction. It can be assumed that the neurons of the spinal trigeminal nucleus modulate sensory information about pain through their neurotransmitters. Spinal trigeminal neurons also express neurotrophic factors and their corresponding receptors, which likely contribute to their resilience under unusual environmental conditions and support neuronal plasticity in response to incoming pain information.

In light of the aforementioned considerations, the present study presented a more comprehensive characterization of spinal trigeminal neurons, elucidating the interaction between neuronal morphology and neurochemical profiles. Embracing a holistic approach, we aim to contribute new insights into the complex architecture of the spinal trigeminal nucleus and its role in sensory processing.

VII. INFERENCES

1. The spinal trigeminal nucleus possesses a complex internal structure involving a specific arrangement of neurons, glial cells, and their fibers. This structural organization determines the important role of the nucleus in receiving, processing, and transmitting somatosensory information from the face.
2. The presence of spinal trigeminal neurons with different external morphologies of their perikaryons indicates the structural complexity in the organization of the three subnuclei of the spinal trigeminal nucleus.
3. The neurochemical profile of the spinal trigeminal nucleus is characterized by a wide variety of endogenous neuroactive ligands that include classical neurotransmitters such as gamma-aminobutyric acid, serotonin, neuropeptide substance P, neuropeptide Y, and calcitonin gene-related peptide, as well as the enzymes nitric oxide synthase and acetylcholine transferase associated with the metabolism of some of these neurotransmitters/neuromodulators. They presumably play an important role in the transduction, processing, and transmission of pain information.
4. Cell populations in the rat spinal trigeminal nucleus express neurotrophic factors and their corresponding receptors, which are likely to be important for the survival of neurons of this nucleus as an overall structure as well as of its subnuclei.
5. The difference in the morphology of neurons in the three subnuclei of the spinal trigeminal nucleus is related to differences in the expression of the bioactive substances examined. Each subnucleus possesses its unique neurochemical profile that most likely provides it with the ability to process sensory information differently.
6. Neurons in each subnucleus of the spinal trigeminal nucleus express immunoreactivity to neurotrophic factors and their receptors in a differential manner. This most likely means that each subnucleus possesses a specific pattern of response to damaging stimuli, maintaining the trophicity of spinal trigeminal neurons and their neurochemical plasticity. Inevitably, identified neurotrophic factors, neurotransmitters, and neuromodulators interact with each other to maintain the local environment in the subnucleus.

VIII. CONTRIBUTIONS TO DISSERTATION WORK

8.1 Confirmatory Contributions

1. The rat spinal trigeminal nucleus is organized into three rostrocaudal subnuclei, each with characteristic cytoarchitectonics.
2. High expression levels of classical inhibitory mediators such as gamma-aminobutyric acid and the monoamine transporter serotonin have been found in spinal trigeminal nucleus neurons.
3. Increased expression of the neuropeptide substance P and calcitonin gene-related peptide was found in spinal trigeminal neurons, as well as low expression levels of neuropeptide Y in neurons and nerve fibers in the spinal trigeminal nucleus.
4. Activity of metabolic enzymes such as neuronal nitric oxide synthase and acetylcholinesterase was demonstrated in single neurons and nerve fibers in the rat spinal trigeminal nucleus.
5. Marked immunoreactivity for the neurotrophic factors NGF, BDNF, NT-3, and GDNF, and their specific receptors TrkA, TrkB, TrkC, and GFRα1 was observed in rat spinal trigeminal neurons.

8.2 Original Contributions

1. A comprehensive morphological and generalized neurochemical characterization of spinal trigeminal neurons in the rat brain is presented.
2. The detailed structural organization and cytoarchitectonics of the spinal trigeminal nucleus subdivisions in the rat are described.
3. Seven morphological types of neurons in the spinal trigeminal nucleus of the rat brain have been defined.
4. A comparative statistical analysis of the distribution of the studied bioactive substances in the three subnuclei of the rat spinal trigeminal nucleus was performed and the exact neurochemical profile of the neurons in them was established.
5. A direct correlation was found between the defined chemical coding of neuronal subpopulations in structural subdivisions of the spinal trigeminal nucleus and the functional modalities they serve.

IX. ARTICLES BASED ON THE DISSERTATION

1. **Ivanov A.,** Atanasova D., Lazarov N. (2019) Cytoarchitecture of the spinal trigeminal nucleus in rats. *Acta morphologica et anthropologica* **26**: 46-50.
2. **Ivanov A.**, Atanasova D., Lazarov N. (2023) Expression of neurotrophic factors and their receptors in the rat spinal trigeminal nucleus. *Comptes rendus de l'Académie bulgare des Sciences,* in press

   (IF – 0.329)
3. **Ivanov A.**, Atanasova D., Lazarov N. (2024) Neuronal types in the rat spinal trigeminal nucleus. *Acta morphologica et anthropologica*, in press

X. PARTICIPATION IN SCIENTIFIC FORA

1. **Ivanov A.**, Atanasova D., Lazarov N. Neuroanatomy of the trigeminal spinal nucleus in rats. *XXIV National Congress of the Bulgarian Anatomical Society, May 31 – June 2, 2019, Stara Zagora, Abstracts, p. 67.*
2. **Ivanov A.**, Atanasova D., Lazarov N. Cytoarchitecture of the trigeminal spinal nucleus in the rat. *Scientific Conference with International Participation "Neuroscience, Bioinformatics, Microbiome, and Beyond", September 17-19, 2019, Bachinovo, Abstract Book, pp. 57-58.*
3. **Ivanov A.**, Atanasova D., Lazarov N. Expression of some neurotrophic factors in the spinal trigeminal nucleus in rats. *XI International Symposium on Clinical Anatomy, October 2-4, 2020, Varna, Scripta Scientifica Medica* ***52*** *Suppl. 1, 2020:* 40-41.
4. **Ivanov A.**, Atanasova D., Lazarov N. Expression of NGF, BDNF, and NT3 in the spinal trigeminal nucleus in rats. *Third Scientific Conference "Neurosciences – From the Theory to the Experiment and Practice", October 23-25, 2020, Bachinovo, Abstracts, p. 28*
5. **Ivanov A.,** Atanasova D., Lazarov N. Expression of neurotrophic factors and their receptors in the rat spinal trigeminal nucleus. *XXV National Congress of the Bulgarian Anatomical Society with International Participation, May 28-30, 2021, Pleven, Journal of Biomedical & Clinical Research* ***14****(1), Suppl. 1, 2021, 53-54.*

XI. S U M M A R Y

An important part of the brainstem, the spinal trigeminal nucleus, is vital to the intricate processing of sensory data related to the craniofacial region. This nucleus is a complex structure with unique functional and neurochemical properties that are situated cranially to the spinal cord. There are several levels in the brainstem where the spinal trigeminal nucleus is located, starting from the upper levels of the cervical spinal cord and ending in the pons. It has a key location for the integration and transmission of sensory information from the face and head. Typically, the nucleus is separated into the caudal (SpVc), interpolar (SpVi), and oral (SpVo) subnuclei. In the craniofacial area, each subnucleus contributes to complicated sensory processing and is linked to distinct functional characteristics. By methodically analyzing the immunoexpression and differential distribution of important neurotransmitters and neuroactive chemicals in its subnuclei, this study contributes to our knowledge of the spinal trigeminal nucleus. We have found strong evidence for the immunoexpression of serotonin and GABA, with different distribution patterns in each of the three subnuclei. Substance P, neuropeptide Y, and CGRP—three significant neuroactive substances—had their immunoexpression levels well-defined as well. In the craniofacial region, these compounds are crucial for both modulating pain and amplifying sensory inputs. The investigation clarified the immunoexpression levels of two enzymes that are essential for neuronal transmission: acetylcholinesterase (AChE) and neural nitric oxide synthase (nNOS), offering information on their possible functions as well as their distribution. In the spinal trigeminal nucleus, the expression patterns of neurotrophic factors (NGF, BDNF, NT-3, GDNF) and their corresponding receptors (TrkA, TrkB, TrkC, GFRα1) were examined. This clarifies the trophic support processes in this area. The intricate relationship between GABA, serotonin, neuroactive chemicals, enzymes, neurotrophic factors, and receptors demonstrates the spinal trigeminal nucleus's intricate and well-coordinated activity. The spinal trigeminal nucleus is diverse, as evidenced by the different neurochemical profiles found in the three subnuclei. The distinct chemical fingerprints of each subnucleus are probably responsible for their distinct roles and specialized tasks in sensory processing. More sophisticated knowledge of each structure's unique contributions to craniofacial sensory integration and regulation is made possible by the capacity to neurochemically differentiate between them.

In summary, the spinal trigeminal nucleus is revealed to be a neurochemically varied entity in addition to an architecturally complicated area. Understanding the distinct neurochemical profiles seen in each of its subnuclei improves our comprehension of the intricate brain circuits governing craniofacial sensory processes. Future research aiming at determining the specific contribution of each subnucleus to sensory processing in the craniofacial area will build upon this neurochemical variability.