Ultrastructural transneuronal degeneration study of axonal elements within the paratrigeminal nucleus in sinoaortic deafferented rats

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ABSTRACT

Objective: Morphological study that searched to authenticate the presence of sinoaortic baroreceptor inputs within the dorsolateral medullary nucleus under electron microscopy analysis. Methods: After a 5-day survival period, 9 baroreceptor-denervated rats deeply anaesthetized with equithesin were transcardially perfused and their brains were histologically processed. Results: The neuronal cytoarchitecture of the paratrigeminal nucleus comprehends afferent projections from other nuclei that have a distributive character regarding visceral and nociceptive functions in the cardiovascular reflex integration response. Conclusion: The medial portion of the nucleus receives afferent projections of the rostral ventrolateral medulla, as shown by retrograde neurotracing studies. The present results show that the medial extent of the paratrigeminal nucleus contains degenerated axoplasmic cellular components in sinoaortic deafferented rats. The number of degenerated axonal fibers was also larger in this area of the nucleus.

Keywords: Nerve degeneration; Trigeminal nucleus, spinal; Baroreflex; Denervation; Microscopy, electron; Medulla oblongata

INTRODUCTION

The paratrigeminal nucleus (Pa5) cytoarchitecture is typified by a fusiform neuronal collection with distributive characteristics regarding visceral and nociceptive functions in the cardiovascular reflex integration response. The Pa5, a dorsal lateral medullary structure within the spinal trigeminal complex, has been studied with emphasis to morphological and functional aspects. The latest participates in cardiovascular, respiratory and thermoregulatory reflex mechanisms.

The Pa5 was primarily physiologically identified in rats by means of bradykinin microinjected into the dorsolateral medulla, which significantly increased the arterial blood pressure response. The B₂ bradykinin receptors were consequently characterized and classified through autoradiography and gene expression. The principal density and distribution were...
carefully identified in the spinal trigeminal tract, Pa5 and nucleus tractus solitarius (NTS)\(^{13,14}\). As known, arterial blood pressure modulation is physiologically regulated by negative feedback of the cardiovascular reflex through baroreceptors located in the carotid sinus and aortic arc. Neural mechanisms underlie the arterial blood pressure (AP) to the sympathetic nervous system, and those referred to peripheral including the sympathetic nervous system to AP\(^{15}\).

These biological sensors are activated by each cardiac systole, producing action potentials transmitted by the vagal (aortic baroreceptors) and glossopharyngeal (carotid baroreceptors) nerves to the NTS\(^{16,17}\). Vagal and glossopharyngeal nerve fibers have excitatory synapses in the NTS, considered the primary medullary or central terminal site for baroreceptor afferents\(^{18-20}\).

The excitatory fibers of the NTS project to the caudal ventrolateral nucleus (CVL) and activate inhibitory preganglionic cardiovascular neurons within the rostroventrolateral reticular nucleus (RVL)\(^{21}\). This regulatory mechanism responds to a hypertensive situation and is always activated in order to provide biological homeostasis, by causing reduction of mean arterial pressure\(^{22,23}\). On the other hand, if a hypotensive condition occurs, the sympathetic cardiovascular tone activity increases the arterial pressure since the baroreceptors are not triggered. Consequently, NTS and CVL neuronal activities are diminished and RVL cardiac cycle-locked neurons are not inhibited\(^{24}\), up regulating the cardiovascular physiological system.

Retrograde tract-tracing studies have evidenced distinct projection fields in the Pa5\(^{2,5}\). Microinjections of latex microspheres containing fluorescent substances have revealed sets of labeled Pa5 neurons organized along the extent of the rostral-caudal tract. Ultrastructure investigation based on a degenerated nerve fiber technique has great value in tracing interneuronal connections. The aim of the present investigation was to verify the existence of sinoaortic baroreceptor inputs within the paratrigeminal nucleus in deafferented rats. Electron microscopy was performed to confirm the identification of degenerated axon terminals characterized by increased electron opacity of the axoplasm. The morphology and distribution of degenerating synapses in the target nucleus were analyzed from distinct locations, although a quantification of terminals seen in these samples taken from different parts of the nucleus was not performed in the present study. Our results validate the presence of degenerated synaptic elements to describe the distribution of the terminals with respect to the somata, and proximal and distal dendrites of the target cells.

**OBJECTIVE**

The aim of this morphological study was to authenticate the presence of sinoaortic baroreceptor inputs within this dorsolateral medullary nucleus under electron microscopy analysis.

**METHODS**

**Experimental animal handling**

The experimental design was approved by the Research Ethics Committee of the *Universidade Federal de São Paulo* (UNIFESP) and stands in accordance with the guide for the care and use of laboratory animals, as adopted and promulgated by the US National Institutes of Health. Care was taken to ensure that animals did not suffer at any stage in the experimental procedure. The animals were housed individually, with a 12-hour dark-light cycle. Food and water were available *ad libitum*.

**Sinoaortic baroreceptor denervation**

Three-month-old male normotensive Wistar EPM-1 strain\(^{25}\) rats weighing 290 to 320g were anaesthetized with thiopental 20mg.kg\(^{-1}\) and chloral hydrate 300mg.kg\(^{-1}\), i.p.\(^{26}\) Animals that did not attain an adequate anesthetic level within 10 minutes after anesthetic administration were not used. The level of anesthesia was verified recurrently by assessing limb withdrawal reflexes to noxious pinching. Sinoaortic baroreceptor denervation\(^{27}\) was performed in nine rats. The carotid bifurcation area was dissected on both sides of the trachea. The aortic nerve, superior laryngeal nerve and superior cervical chain were isolated and bilaterally transected under a binocular surgical microscope (Carl Zeiss, Germany). The connective tissue and fibers of the carotid artery wall were excised and swabbed with 10% phenol in ethanol solution. Baroreflex response was experienced after sinoaortic denervation by means of a phenylephrine hydrochloride injection (10µg.kg\(^{-1}\), intravenous) through a polyethylene catheter positioned in the external jugular vein. This catheter was connected to a pressure transducer (TP-1, Ampere Electro-Eletrônica, São Paulo, SP, Brazil) and to an analog amplifier (Axon 3080, Axon Instruments, Union City, CA, USA) for arterial blood pressure monitoring. One injection of phenylephrine should be sufficient to increase the arterial pressure by at least 28 to 32mmHg without the occurrence of bradycardia in successfully
deafferented rats. A wide spectrum of veterinary antibiotics was administered by intramuscular route after the surgery.

**Histological procedure**

Within 2 to 4 days survival period, rats were deeply anaesthetized with equithesin (4.5ml/kg, i.p.) and placed on a respirator for intracardiac perfusion (via the apex of the left ventricle) by means of 2% paraformaldehyde and 2% glutaraldehyde in a 0.1M cacodylate buffer at pH 7.35 to 7.45. The perfusion pump was stopped when the upper and lower limbs became rigid. The brain stems were removed from the skull and samples were taken from caudal to rostral areas of the Pa5, secondarily fixed in buffered 1% osmium tetroxide, block-stained with uranyl acetate, dehydrated in a crescent ethanol series followed by propylene oxide transition, and embedded in an Araldite® resin blend. The Pa5 was identified in 0.5µm toluidine-stained sections under light microscopy. Ultrathin sections of silver interference color were cut and samples were collected according to distinct projection fields of the Pa5 and finally mounted on 300-mesh grids. Electron micrographs were taken of Pa5 cross-sections and quantitative observations were based on counts of 20 fields on random grid squares selected from each projection segment. All examinations were performed under a Jeol 1200 EXII transmission electron microscope (Peabody, MA).

**Patterns of degeneration**

Several degeneration patterns at the electron microscopy level have been described by morphological studies. Different chemical or temporal factors can interfere in ultrastructural element analysis, therefore, it is very important to describe the main variables applied herein. The Wallerian axonal degeneration, also known as electron dense degeneration, was the type considered for this study. This kind of degeneration is characterized foremost by augmented electron opacity of the axoplasm. It is worth remembering that electron opacity may vary depending on fixation and animal handling. Other features like cellular element changes were observed accompanying the increase in axoplasmic electron density identification. Early changes were seen particularly in mitochondria, axoplasmic reticulum and synaptic vesicles. The mitochondria became globoid, sometimes crenated, with stacked mitochondrial cristae. The axoplasmic reticulum often swelled and the synaptic vesicles became larger. Also, a floccose appearance of the axoplasm leads to the perceptible darkening observed at later phases. The degenerated fibers commonly lose their continuity and arrangement chains of fragments. Dark synaptic buttons remain allocated to the postsynaptic elements in early stages of degeneration.

**RESULTS**

The fine structure of the entire Pa5, which had ultrathin slices randomly collected, was examined under a transmission electron microscope. Within 2 to 4 days of postoperative period, electron microscopy analysis showed degenerated axon terminal fibers and identified synaptic elements in the medial extent of the paratrigeminal nucleus (Pa5, Figure 1), which projects to other nuclei functionally involved in central neuronal cardiovascular control. The bilateral cross-sections of the Pa5 revealed that the number of degenerated axonal fibers in the vicinity of synaptic elements were strictly distributed within the areas that receive afferent fibers from NTS and RVL as described by Caous et al. Again, the Pa5 region considered the medial extent of the nucleus was properly identified according to its morphological cytoarchitecture under light microscopy analysis of the nissil-stained tissue sections as established by previous studies.

Despite the main finding of the present investigation that comprehended degenerated axonal elements in the same innervation site that receives projections from RVL or NTS, other detailed observations should be described as follows. Transverse sections of the paratrigeminal nucleus (Figure 2) contain a compact area of a nerve cell that constitutes the nucleus and surrounding cytoplasm depicted by a conspicuous and spherical nucleolus and arising from the top of the cell body is a thick myelinated degenerating axon (A, B and Pa5: paratrigeminal nucleus; ECu: external cuneate; sp5: spinal trigeminal tract. Bar=100µm

**Figure 1.** Photomicrograph of transversal Nissl-stained section of the paratrigeminal nucleus within the spinal trigeminal tract in the rat
The presence of gap junctions could be also observed between dendrites (F) and an oligodendrocyte is shown between neuronal perykaria where two proximal cell bodies (type 2 perykaria) with a prominent nucleolus encompassed by degenerated and non-degenerated fibers were observed (Figure 3). Axon terminals (At) containing pleomorphic clear vesicles form asymmetrical synapses with dendrites, some intact (D) and other presenting a dark degenerated characteristic (E). These buttons contacting larger dendrites were generally small-to-medium in size, made asymmetric-type synaptic contacts, and contained pleomorphic vesicles of medium-to-large size (Figure 4A).

On the basis of synaptic polarity, vesicle size, and the nature of the pre- and postsynaptic elements, fundamentally axo-dendritic and axo-somatic types could be distinguished. Small occurrences of axo-somatic synapses were perceived and axo-dendritic synapses were predominantly identified. All synapses with a non electron-dense core were round and asymmetric with a
thick postsynaptic membrane element. Mitochondria of variable shapes were identified, as well as dispersed rough endoplasmic reticulum throughout the cytoplasm. Synaptic contact on the soma of Pa5 neurons was small and covered, on average, only 4% of the soma surface. The most striking feature of the synaptic structure of the Pa5 was that more than 90% of all synapses were axo-dendritic (Figure 4B). Dense degenerated axon fibers with the same shape and type patterns (Figure 5) were observed in the entire examined target nucleus. Dendritic spines were rare and no synapses between two vesicle-containing structures, axo-axonic or dendro-dendritic synapses, were identified.

**DISCUSSION**

**Cardiovascular and baroreceptor functions of the Pa5**

The Pa5 is already confirmed to be the only extra-solitary structure of the central nervous system which receives primary afferent fibers from vagus, trigeminal, and glosopharyngeal nerves (28). Despite the fact that the nucleus does not present interneuron cells (4), it integrates cardiorespiratory and thermoregulatory or thermosensitive reflexes (29), since the Pa5 lesion with ibotenic acid microinjection impeded the animals’ efficient thermoregulation in a warm environment (unpublished observations). The sympathetic and cardiac baroreflex components of chemical abscission in non-anaesthetized rats within the NTS, display an unyielding reduction of the arterial blood pressure response to phenylephrine-pressor induction with or without bilateral Pa5 ablation. Additional denouement demonstrated that the Pa5 mediates the reflex responses to smaller blood pressure increments, while the NTS would be activated in surges greater than 40mmHg (30).

**Ultrastructure and electrophysiological inferences**

Degenerated cellular components identified herein represent barosensitive neurons recorded in the Pa5 in earlier studies (9). This functional pathway between the Pa5 and RVL was evidenced through cardiocycle-locked neurons recorded in the RVL that respond to bradykinin microinjections in the Pa5 (6, 12). Our findings hypothesize that the medial extent of the Pa5 containing degenerated axoplasmatic cellular components after sinoaortic denervation, may be the same one that receives afferent projections of the rostroventrolateral medulla as shown in previous retrograde neurotracing studies, in which a distributive characteristic of the Pa5 was based on the topographical organization of neuroanatomical primarily afferent connections (2, 5, 31), although this relevant information should be confirmed by neurotracing and electron microscope conjugated experiments.

**General discussion**

After sinoatrial node denervation and ultrastructural inspection of the paratrigeminal nucleus, the described degenerating fibers and terminals allow us to imply that this nucleus has the necessary connections to contribute to cardiovascular reflexes. Also, it can be claimed that the terminals in question are confined to a specific portion of the paratrigeminal nucleus that corresponds mainly to its medial portion. The vagus nerve has sensitive and motor fibers in the cervical region and in the nodose ganglia; both ascend to a medullar level through the dorsolateral medullary region and the islands of neuropil of the Pa5 (32), and the aortic and carotid baroreceptors were first identified in vagal and glossopharyngeal nerves, respectively (33); both nerves have somatic inputs to the Pa5. The afferent projection areas from NTS and RVL inserted in the medial extent of the nucleus contained electron-dense degenerated axonal fibers. This finding may confirm the baroreflex component characteristic of the nucleus. The present neuroanatomical data suggest that the Pa5 can be considered a sensitive information gateway to visceral reflexes.

**CONCLUSION**

This neuroanatomical substrate or pathway that involves baroreceptor elements can be associated with
sympathetic activation and its cardiovascular effects. In conclusion, after the sinoatrial node denervation the paratrigeminal nucleus was inspected at the electron microscopic level, degenerated axonal fibers or terminals were identified in the medial extent of the nucleus. The present result, notwithstanding the morphological work, complements other research on the Pa5 regarding its function as an important relay for pressure-sensitive information to the cardiovascular reflex arc.

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