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Small-sized neurons of trigeminal ganglia express multiple voltage-sensitive calcium channels: A qualitative immunohistochemical study

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The cell bodies of pseudounipolar neurons of the trigeminal ganglia have been presumed to play a supportive role to neurites, which transmit various sensations like pain from the periphery to the brain stem. However, several studies have recently shown that these neuronal cell bodies could modulate the afferent stimuli by up-regulating various ion channels and also by increasing the synthesis of neuropeptides like calcitonin gene-related peptide (CGRP). Since voltage-sensitive calcium ion channels (VSCCs) determine neuropeptides/ neurotransmitters released by neurons, the aim of the present study was to localize the various VSCCs (N-, P/Q-, L-, T- and R-types) in the trigeminal ganglia neurons by immunohistochemistry. The results showed that all the VSCCs are expressed by the cell bodies of neurons though the small-sized neurons showed higher expression of these channels. The small-sized neurons were identified by immunohistochemical localization of CGRP, the most common neuropeptide for pain transmission in the trigeminal ganglia neurons. Some of these channels (N, P/Q and T types) were also expressed on the cell surface though previous electrophysiological studies have shown the expression of all the channels on the cell surface. It is suggested that the cell bodies could play a more active role than hereto ascribed to these, in the modulation of sensory stimuli.

Keywords: Calcium, Ganglia, Immunohistochemistry, Ion channel, Rat, Trigeminal

The trigeminal ganglion is chiefly formed by cell bodies (somata) of pseudounipolar neurons and nerve fibers[1]. The cell bodies, which predominantly occupy the peripheral part of the ganglion, are surrounded by satellite cells while the nerve fibers are surrounded by Schwann cells. The single neurite arising from each cell body divides into central and peripheral processes, which transmits sensations like touch and pain from the head and face to the trigeminal nuclei in the brain stem. Structurally and electrophysiologically, both these processes show characteristic features of axons[2]. Specifically, pain and temperature is carried by thinly myelinated (Aδ) and unmyelinated (C) nerve fibers arising from small-sized trigeminal neurons[3].

It has been generally presumed that within the ganglia, nerve impulses are transmitted directly from peripheral to the central processes without involvement of the cell bodies[4]. The cell bodies play a supportive role and are mainly concerned with the maintenance of the neurites. This passive role is highlighted by the fact that they do not interconnect with each other by synaptic junctions. However, recent evidence shows that the neuronal somata could modulate nerve impulses transmitting sensations like pain. The pain following teeth extraction has been shown to be related to the up-regulation of specific sodium ion channels (Nav1.8 and 1.9) and P/Q-type calcium ion channels in the somata of trigeminal neurons[5,6]. Also, the beneficial effect of botulinum toxin type A 150 kDa (BoNT/A) in trigeminal neuropathy may be due to decreased neurotransmitter release from the neuronal cell bodies of the trigeminal ganglia[7]. Similarly, in a model of orofacial inflammation in the rat, increased release of substance P was noted within the ganglia[4]. Substance P is a key neuropeptide of the pain transmitting pathway in the nervous system. The exact mechanism governing the release of neurotransmitters/neuropeptides within the ganglia is still unknown. However, at synapses, release of neurotransmitters/neuropeptides is dependent on calcium ion influx through specific voltage-sensitive calcium channels (VSCCs), which open in response to depolarization[8]. To date, there are no morphological studies on the expression of these channels by the neurons of the trigeminal ganglia.

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VSCCs, which are expressed on the surface of neurons, open in response to depolarization[9]. Calcium ion influx through these channels regulates neurotransmitter release, synaptic plasticity and gene transcription. VSCCs have been classified into high-voltage activated (N-, L-, P/Q-, R types) and low-voltage activated (T-type) channels[10]. Among these, N-, P/Q- and R-types are specifically expressed in presynaptic nerve terminals and regulate the release of neurotransmitters. L-VSCCs are located in postsynaptic terminals and control synaptic plasticity and gene transcription. T-VSCCs are present on dendrites, where they amplify local low-threshold signals and thus, could play a role in driving epileptogenic bursting activity[11].

The aim of the present study is to investigate the expression of the various VSCCs in the trigeminal ganglia by immunohistochemistry, particularly with reference to the small-sized neurons. As mentioned earlier, the small-sized neurons are mainly concerned with the transmission of pain and temperature from the periphery. These neurons were identified by localization of calcitonin-gene related peptide (CGRP), an important neuropeptide associated with transmission of pain[12].

Materials and Methods

Adult male Wistar rats (6), weighing between 200-225 g were used. Prior permission for animal experimentation was obtained from the Institutional Animal Ethics Committee of All India Institute of Medical Sciences. These rats were anaesthetized by pentobarbitone injection (100 mg/kg, ip) and perfused with 4% paraformaldehyde through the left ventricle. Later, the calvaria were removed and the brains lifted up. The trigeminal ganglia were observed in the floor of the cranial cavity. Its connections were severed and the ganglion kept in paraformaldehyde solution for 3 days. At the end of this period, these were cryoprotected in 30% sucrose solution. Finally, cryostat sections (20 µm thick) of the ganglia were cut at -20°C. Every fifth section from a total of 90-120 sections/ganglion was collected as a free-floating section in 0.1*M* phosphate buffer saline (PBS) and processed for immunohistochemical localization of CGRP and N-, P/Q-, L-, T- and R-types of VSCCs. Few sections were stained with 1% Cresyl violet for localizing the histological components of the ganglia.

Endogenous peroxidase activity in the sections was quenched by 0.3% hydrogen peroxide. Then, non-specific binding was blocked by incubating sections in 10% normal goat serum. Labeling with primary antibody (polyclonal) was done as follows – (1) Anti-CGRP (1:1000) (2) Anti-N-type (1:100) (3) Anti-L-type (1:200) (4) Anti-P/Q-type (1:100) (5) Anti-T-type (1:200) and (6) Anti-R-type (1:150) (All the antibodies were from Alomone labs, Israel except Anti-CGRP, which was from Sigma, U.S.A). Next, the antigens in the sections were visualized by the ABC method (Vectastain ABC kit, USA) using 3,3-diaminobenzidine tetrahydrochloride as a chromogen. Control experiments were also carried out by either omitting the primary antibody (negative control) or adding the antibody after neutralizing it with the cognate peptide (positive control) as per the manufacturer's instructions. These were done to determine the specificity of the immunostaining. The immunostained sections were photographed using low (4X and 10X) and high power (100X) objectives under an E-600 Nikon compound light microscope.

Results

Cresyl violet stained sections of the trigeminal ganglia showed that the majority of the neuronal cell bodies were aggregated peripherally (Fig. 1A). Those situated more centrally were separated by nerve fibers. The cell bodies varied in size. The neurons, particularly the smaller ones, showed darkly staining granular Nissl substance (Fig. 1B). All the cell bodies were surrounded by satellite cells, which could be identified by their smaller polyhedral nuclei.

Neurons showing positive immunostaining for CGRP were relatively small in size (Fig. 1C). Staining was noted to be granular in nature (Fig. 1D).

Almost all the neurons showed high intensity of staining for L-type VSCCs, particularly around the perinuclear region. The small-sized neurons demonstrated higher staining intensity. Cross-sections of nerve fibers were also intensely stained. At higher magnification, a granular pattern was noted, particularly in small sized neurons. Smaller neurons showed more intense staining than larger ones for N-type VSCCs. The immunostained areas within the cytoplasm showed a coarse granular pattern dispersed focally while those on the surface formed ring-like pattern. P/Q-type VSCCs were expressed on the surface as well as in the cytoplasm of the neurons. The cytoplasmic staining was uniform in nature. Higher intensity of staining was noted in the cytoplasm of smaller neurons. Low intensity staining was noted in the nerve fibers. Immunohistochemical localization of

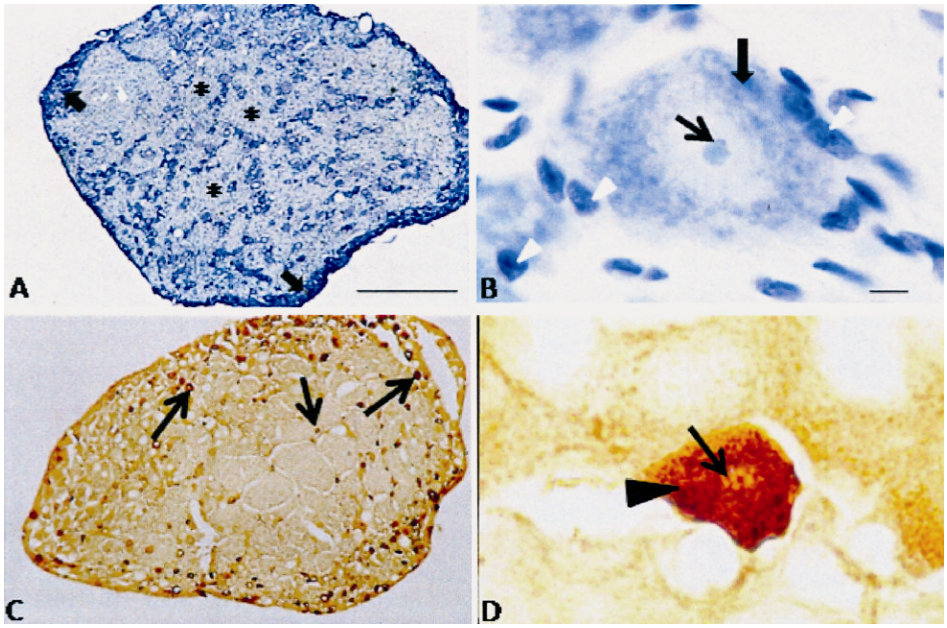

Fig. 1—Cresyl violet stained section of the trigeminal ganglion showing the majority of the cell bodies of the neurons aggregated towards the periphery (arrows). The central portion consists of nerve fibers (*) separating fewer cell bodies (A). A neuronal cell body with prominent Nissl granules in the cytoplasm (block arrow) and the nucleus with a prominent nucleolus (line arrow). The nuclei of the satellite cells surrounding the neuronal cell bodies are also seen (arrow head) (B). Localization of CGRP in the trigeminal ganglion. Only the small-sized cell bodies show positive immunostaining for CGRP (arrows) (C). A neuronal cell body showing CGRP immunostaining within the cytoplasm in a granular pattern (arrow head). The outline of the nucleus (arrow) is indistinct due to overlapping CGRP positive granules (D). [Bar = 250 µm for A and C; Bar = 2.41 mm for B and D]

T-VSCCs showed ring-like expression on the cell membrane of all the neurons though the smaller neurons showed a more intense stain. The cytoplasm, which was stained less intensely, showed a granular pattern. Cross-sections of nerve fibers also showed expression of these channels. R-VSCCs too showed a ring like expression at the surface of the cell bodies of the neurons. However, it was due to immunostaining of the satellite cells – both their nuclei and cytoplasm. The nuclei of neurons were also stained. Besides, nerve fibers were also intensely stained. The smaller neurons showed higher intensity of staining.

Thus, every neuronal cell body showed expression of all the different VSCCs. The smaller-sized cell bodies showed higher expression of these channels. Some VSCCs (N-, P/Q- and T-types) were also prominently expressed on the cell membrane. The satellite cells showed expression of R-VSCCs.

Control experiments, where the antibody was initially neutralized with the cognate antigen (positive control) (Fig. 2) and those where the antibody was omitted (negative control) showed little non-specific binding in the tissue sections.

Discussion

The present study showed that the cell bodies of the trigeminal ganglion neurons (TGN) express all the different types of VSCCs. Considering that calcium ions are an important link in the intracellular signal transduction system (as second messengers) and in the release of neurotransmitters from the presynaptic nerve endings, the findings of the present study suggest that the neuronal cell bodies could play a critical role in the pathophysiological changes occurring during disease processes[13]. The small-sized neurons, which mediate pain and temperature sensation, demonstrated positive immunostaining for CGRP. Possibly, this neuropeptide is synthesized here and then transported to the nerve terminals, as reported earlier[14]. In this earlier study, immunoreactive CGRP (iCGRP) in the dental pulp decreased significantly after transection of the inferior alveolar nerve in rats. Both, CGRP and substance P (sP) immunoreactivity in the trigeminal ganglion showed a significant increase after adjuvant-induced inflammation of the temporomandibular joint[15]. Again, in a different study, sP was noted to be released within the trigeminal ganglion following

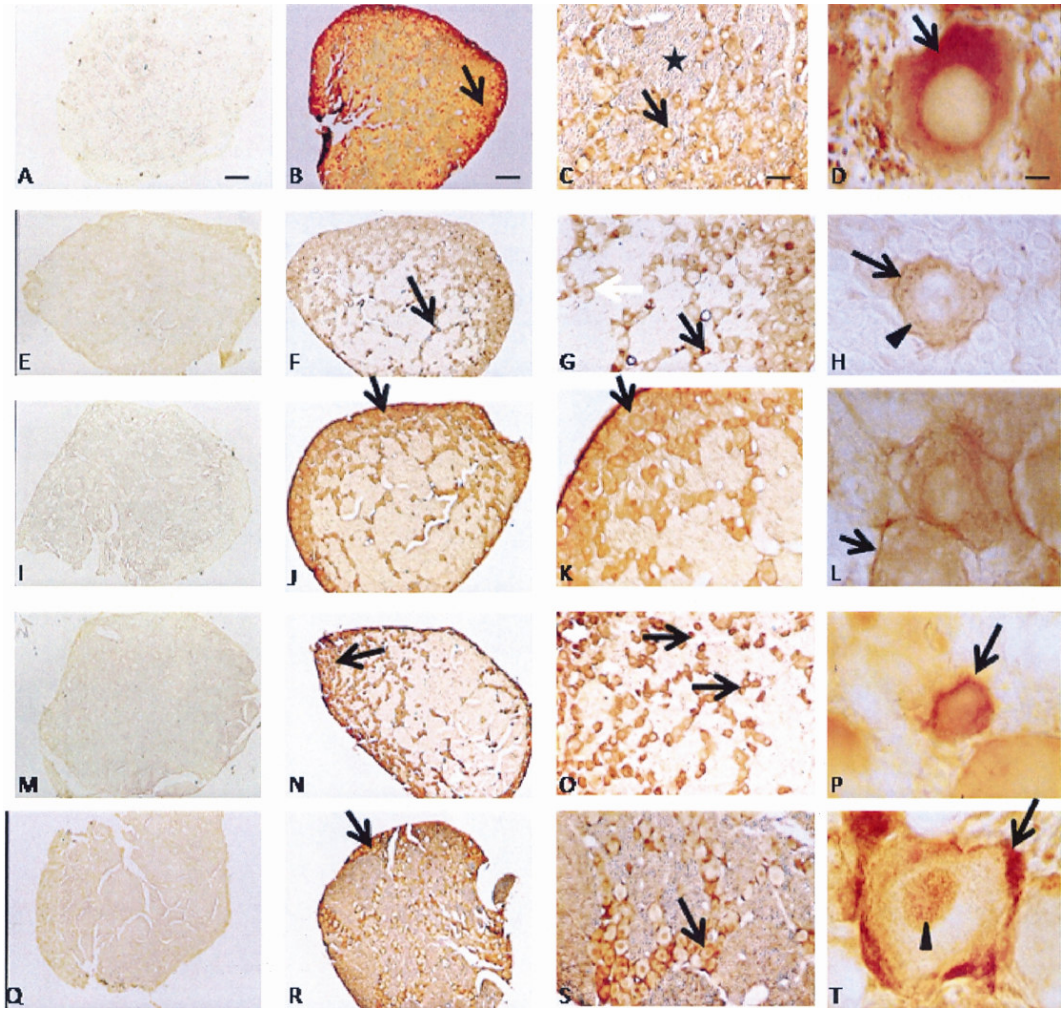

Fig. 2— Immunostaining of the trigeminal ganglia with antibodies directed against L-type (A – D), N-type (E to H), P/Q-type (I to L), T-type (M to P) and the R-type (Q to T). Positive control with antibody neutralized with corresponding antigen for L (A), N (E), P/Q (I), T (M) and R (Q) channels. Immunopositive neurons for L-type channel (arrow) in (B) & (C). Cross-sections of nerve fibers also show intense staining (*). A neuronal cell body with the cytoplasm showing L-type channel (D). (F) and (G) showing small-sized neuronal cell bodies stained for N channel (black arrows) while the larger neurons showed expression in the cell membrane (white arrow). (H) A small-sized neuron showing expression of N channel both on the surface (arrow) and in the cytoplasm (arrow head). (J) and (K) Imuunostaining for P/Q-type channel in neurons (arrow). (L) P/Q channel expressed on the neuronal cell surface. (N) and (O) Immunostained neurons for T-type channel (arrow). (P) Small-sized neuron showing immunostaining in the cytoplasm as well as on the surface (R) & (S) showing immunostained neurons (arrow). (T) The nucleus of a neuron (arrow head) as well as the nuclei and cytoplasm of satellite cells (arrow) show higher intensity of staining. [Bar = 100 µm for A, B, E, F, I, J, M, N, Q and R. Bar = 240 µm for C, G, K, O and S. Bar = 2.4 mm for D, H, L, P and T]

intraganglionic application of potassium chloride or after electrical stimulation of peripheral afferent fibers[4]. Interestingly, this release was shown to be dependent on extracellular calcium. Also, this release of sP increased significantly after unilateral orofacial inflammation. The authors had opined that the release of sP by non-synaptic mechanism could fulfil the role of a diffusible chemical messenger that can modulate the excitability of surrounding neurons. Thus, it might be speculated that other neuropeptides like CGRP could also be released within the ganglion in a similar manner. In fact, stimulation of TGN in culture by nitric oxide donors led to > 4-fold increased release of CGRP[16]. The intermediate step was calcium ion influx through possibly R-VSCCs. Similarly, trans-2-pentenal induced an increase in intracellular calcium after interacting with Transient receptor potential A1 (TRPA1) channels[17]. Calcium ion influx was mediated by L- and P/Q-type channels.

In the present study, the small-sized neurons were noted to express P/Q-, N- and T-type channels on the cell surface. However, electrophysiological studies on small-sized neurons (diameter < 30 µm) using whole cell patch clamp technique showed the expression of all the VSCCs[18]. The maximum current was carried by N-type (55%) and L-type (26%) channels. In a different study, N-type (~65%) channels carried a still greater proportion of the current in these neurons (diameter <30 µm)[19]. Zolmitriptan, an antimigraine drug, was found to predominantly inhibit P/Q channels in whole-cell patch experiments on TGN[20]. The plasma membrane calcium-ATPase (PMCA) is involved in removal of the excess calcium ions from the cytosol after depolarization[21].

The small-sized neurons showed higher intensity of staining for the L-, P/Q-, N- and T-channels. The reason for this is not definitely known. However, cresyl violet staining had shown extremely dense Nissl substance in these neurons. The presence of VSCCs in the nerve fibers indicated that the calcium channels were being possibly transported to the nerve endings. Satellite glial cells were noted to express R-VSCCs. Though the functional significance of this is unknown, it has been earlier reported that silencing of a single potassium ion channel (Kir4.1) in these cells by RNA interference produced neuropathic pain in rats[22]. Also, TGN and the satellite cells have been shown to communicate via gap junctions and paracrine signaling[23]. Though, the small-sized TGN conveying pain and temperature use several neuropeptides/neurotransmitters other than CGRP like glutamate and substance P, CGRP is the most abundant mediator of pain in the TGN[24]. Thus, CGRP was used to label the small-sized neurons in the present study.

In conclusion, the results of the present study along with that of earlier studies suggest that TGN could take part in the modulation of various sensory stimuli including that of pain and temperature.

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