Original Research Article

Localisation of the gap junctions in the trigeminal ganglia of the rat using various techniques

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Abstract

Background: Gap junctions form the important connecting channels among the neurons and the surrounding cells. Various neurotransmitters pass through them. Gap junctions were made up of Connexons. These connexon subunits of two cells joined with each other to form the gap junctions. In the present study, localization of gap junctions was done in trigeminal ganglia using various techniques. This ganglion consists of neurons of varied sizes, which were surrounded by satellite glial cells. Satellite glial cells from the envelope like structure around the glial cells and in turn responsible for controlling the internal homeostasis of the neuron. Increase activity of these cells were seen in many of the pathological conditions such as trigeminal neuralgia **Material Methods:** The present study was done on the adult male wistar rats (n=18). These rats were randomly divided into three groups, depending upon the procedure performed. Group I (n=6) was utilized for the cresyl violet staining to identify various sizes of neurons, group II (n=6) had undergone immunohistochemistry for the connexin-43 antibody and the group III, electron microscopic study was done to identify and confirm the location of gap junctions **Results:** In the present study, trigeminal ganglia from both the left and right sides were examined for morphological, immunohistochemical and electron microscopic localization of gap junctions. Morphological studies showed that ganglia were composed of neurons surrounded by satellite cells, nerve fibers along with Schwann cells and connective tissue containing blood vessel. Immunohistochemistry showed prominent gap junctions between the neurons and satellite glial cells of the ganglia. Electron microscopy revealed the presence of gap junctions in the trigeminal ganglion. No difference was noted between the trigeminal ganglia of the left and right sides. **Conclusion:** Gap junctions forms the important channels for the transmission of various neurotransmitters. Cresyl violet staining did identification of cells in the trigeminal ganglion. After identification, further confirmation was done by the immunohistochemistry and electronmicrosopic study. Localization of the gap junctions is important to identify the sites for exchange and in future these sites can be utilized for various gap junction blocker studies.

Key Words: Ganglia, Satellite Cells, Neurons, Gap junctions.

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INTRODUCTION

Gap junctions initially were thought to be a part of the common "Junctional Complex" which is comprised of zonulaoccludens, zonulaadherens, and macula adherens but later studies confirmed that gap junctions were separate structures from the junctional complex. They are 'plaque-like' entity composed of an ordered array of subunits called connexon, which extend beyond the cell surface into the gap to keep the opposing plasma membranes approximately 3 nm apart[1]. Connexon consist of 6 cylindrical subunits (composed of proteins called connexins), which are arranged radially around a central channel with a diameter of 1.5 nm. Precise alignment of connexon on adjacent cells produces a junction where cell-to-cell channels permit passage of ions and small molecules with a molecular weight of fewer than 1000 Daltons (1 kDa)[2]. Connexins may exhibit some conformational changes to shut off communication between cells. Glia represents the main cell type

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connected via gap junctions in the CNS. The amount of glial cell gap junctions, if compared to that of neurons, is greater and also persists in the adult. For astrocytes and oligodendrocytes, tens of connexins have been mapped. Glial cell gap junctions may be homologous (astrocyte-astrocyte), heterologous (astrocyte- oligodendrocyte) or even autologous (homocellular) between structures of the same cell (myelin lamellae). Connexin 43 (Cx-43) is the most conspicuous protein in mammalian gap junctions and it is found in most tissues. It is the main constituent of astrocyte gap junctions and is found in neuroectodermal cells at the beginning of embryonic development[3].Within the mature CNS, the more prevalent connexin is connexin 43, expressed in astrocytes and endothelial cells, ependymal cells, leptomeningeal cells and microglia[4]. Trigeminal ganglia, also known as the Semilunar or Gasserian ganglia are the largest sensory ganglia in the human body. It is located in the middle cranial fossa in the base of the skull in the rats. A ganglion is mainly composed of cell bodies of pseudounipolar neurons surrounded by satellite cells and the nerve fibers. The trigeminal ganglion contains the first-order neurons of the somatosensory pathways arising from receptors and free nerve endings in the facial and oral tissues, scalp and part of the Dura mater[5]. It transmits general somatic sensation like pressure, touch, temperature, pain, and proprioception from head and neck region to the trigeminal nuclei in the brain stem. Pain and temperature are carried by thinly myelinated (type Aδ) and unmyelinated (type C) nerve fibers while touch, joint sense and pressure are carried by thicker (types Aα- Aβ) nerve fibers[6]. The two main morphological types of sensory neurons in the trigeminal ganglia are large light (LL) and small dark (SD) neurons. SD neurons give rise to C-fibers (non-myelinated slow conducting) whereas the fibers of LL neurons are of A- type (myelinated, fast conducting). Many of the SD cells contain substance P or calcitonin gene-related peptide and they are concerned with thermo- and mechanoreceptor and many of them were high- threshold nociceptive[7]. Glial cells, which surround the neurons directly, modulate neuronal function and activity by changing the ionic concentrations in and around the neurons[8]. Interestingly, neuron- glia interactions have been shown to be involved in all stages of inflammation and pain associated with several CNS diseases[9]. Glial cells express characteristic substances in common with immune cells by which they respond to viruses and bacteria, releasing proinflammatory cytokines, which create pathological pain[10]. Hence, the present study focus on the localization and the identification of the gap junctions between the neurons and the satellite cells surrounding them.

MATERIAL AND METHODS

Male albino wistar rats (n=18) of weight ranging from 200g to 250 g were used in the present study. The rats were obtained from Experimental Animal Facility of All India Institute of Medical Sciences after prior approval of the experimental procedure by Institutional Animal Ethics Committee (IEAC). The animals were kept in cages with no more than three animals in one cage. They were maintained at 12h: 12h light/dark cycles with water and food available ad libitum. Rats were randomly divided into three groups for the present study:

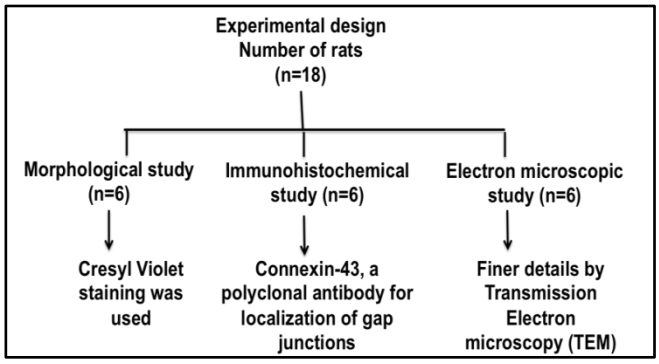

Figure 1: Flow chart representing the study plan for the present study

Perfusion fixation: On the day of the experiment, the rats were deeply anesthetized by intraperitoneal injection of pentobarbital (100 mg/kg). The thoracic cavity was opened and a metal cannula inserted through the apex of the left ventricle into the ascending aorta. After connecting the cannula to a perfusion pump (Masterflex Company), 100 ml of 0.1M Phosphate buffer saline (PBS) was slowly perfused into the aorta. This was followed by perfusion of 4% Paraformaldehyde (Sigma Chemicals, USA) in 0.1M PBS over the time period of about 1 hour a) Dissection Protocol: (Figure:2) After completing perfusion, the skin over the head was incised and reflected laterally. The skull was opened without damaging the brain. Meningeal coverings were then incised and reflected laterally. Using blunt forceps, the brain was lifted up to expose the trigeminal nerve and its ganglion emerging from the brain stem. Then the cranial end of the trigeminal nerve was cut near the brain stem and cleared of adhesions. Distal end of the trigeminal ganglion was cut at the region where it divided into branches and the ganglion was removed carefully.

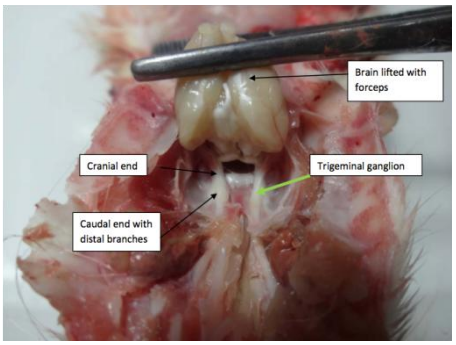

Figure 2: Photograph representing the location of trigeminal ganglia in the rats

A) Cresyl Violet staining: Blocks were made in paraffin wax using plastic molds. Serial sections of 7µm thickness were then cut from these blocks on the rotatory microtome (MICROM HM 315R). The sections were made to float in a water bath (40^{o}C) for spreading out and then mounted on clean glass slides pre-smeared with egg albumin. The sections on the slides were allowed to dry and then subjected to Cresyl Violet (C.V.) staining. Standard protocol for the staining was followed.

B) Immunohistochemical analysis: The trigeminal ganglion was placed in the chuck and embedded in optimum cutting temperature (OCT) medium and blocks were made. Then the tissue was sectioned using cryostat (LEICA CM 1950) of 10µm thickness at -22^{o}C. For each tissue, the sections are collected separately in the multivial culture plates and labeled. For free-floating immunohistochemical localization, the antibodies for Connexin 43 were obtained from Sigma Laboratories (USA). The procedure was standardized for this antibody at different dilution ratios. For Connexin 43, the optimal ratio of antibody (1:400) was determined.

C) Electron microscopic study: Initial fixation was already done by perfusion-fixation by 4% paraformaldehyde. After removing the tissue samples, further fixation was done in 2.5% glutaraldehyde and 2% paraformaldehyde (Karnovsky's Fixative) in 0.1M sodium phosphate buffer (PH 7.3) for 8- 10 hours at 4^{o}C. The tissues were embedded in pure embedding medium (resin) using molds. Embedded blocks were kept in an oven at 50°C for 12-24 hours. The temperature was raised to 60°C to continue polymerization for 48-72 hours. After polymerization, blocks were removed by bending the mold sideways or by cutting the plastic BEEM capsules with a razor blade. Before proceeding with ultrathin sectioning, thick sections (0.5 to 1.0µm) were cut for scanning the tissue under an optical microscope and selecting appropriate sections. The semithin sections were stained with Toluidine Blue (1%) for 0.5 to 2.0 min (depending on the thickness). Sections were washed thoroughly in running water. Sections were dried and mounted with DPX. After scanning the sections under the light microscope, the area to be examined under TEM was selected and the blocks were further trimmed. Fresh glass knives were used for cutting ultrathin sections. Ultrathin sections show interference colors while floating on the liquid of the prepared knife-boat. The sections were lifted from below on specially made metal grids. A double staining method using uranyl acetate and lead citrate was followed. The sections were finally observed under transmission electron microscope (TEM) (Morgagni 268D Fei Company, the Netherlands) and selected images were digitally captured on a desktop computer and viewed using SI Viewer (OLYMPUS).

RESULTS:

A): Morphological study: (Figure.3): Pseudounipolar neurons of varying sizes were observed ranging from large round cell bodies too much smaller ones. The cell bodies were present in lacunae, which became prominent after staining, due to retraction of cell bodies towards the center. Most of the neurons were present in the periphery rather than in the center. The cell bodies were surrounded by a uniform row of satellite glial cells. A centrally placed nucleus with a prominent nucleolus was also noted. The Nissl bodies are granular and distributed uniformly within the cytoplasm. Several blood vessels mainly capillaries were also seen. **B):** Immunohistochemical study: (Figure.4) Immunohistochemical labeling for connexin 43 showed higher aggregation of neurons towards the surface of ganglia. Most of the neurons had a ring like staining around their periphery indicating the existence of gap junctions. Under higher power, the immunohistochemical staining was seen well. In some cases, the satellite glial cells could be also seen. Under still higher magnification dot like gap junctions could be seen at the periphery of neurons and the adjacent periphery of glial cells. These small punctuate immunostained areas actually represent the areas where connexin 43 is present and hence the localization of gap junctions. **C):** Electron microscopic study: (Figure.5) On electron microscopic examination, the neuronal and the satellite glial cells were noted to be placed adjacent to one another with no evidence of connective tissue in the intervening space. The ganglion cell contained various intracellular organelles like Nissl substance, mitochondria and lipofuscin granules. Gap junctions could be seen where the cell membrane of the neuron and satellite cell became adherent. Away from the gap junction, the plasma membranes of both the cells were wider apart. Schwann cells (SC) are also seen frequently enveloping several unmyelinated axons or enveloping a single axon and forming the myelin sheath. Processes of the Schwann cell are also noted extending

outwards enclosing a nerve fiber. The satellite glial cell was seen to contain rows of the rough surface endoplasmic reticulum, vesicles, and mitochondria, lipofuscin granules that give it a darker color than the lighter ganglion neurons in the vicinity.

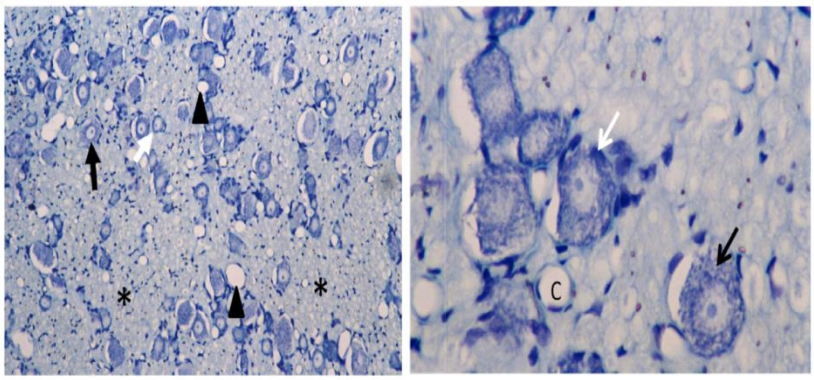

Figure 3: Cresyl violet stained section of the trigeminal ganglion. A) Both, large and small-sized neurons were observed (large neuron-black arrow; small neuron- white arrow).The cell bodies contain dense Nissl substance and are surrounded by satellite glial cells. The tissue between the cell bodies is composed of nerve fibers (*) and occasional capillaries and even larger blood vessels (arrow heads).Magnification-260. B) Higher magnification showed cytoplasm is filled with the Nissl substance (black arrow). Satellite cells are observed around the periphery of the cell bodies (white arrow). Magnification – X1300.C – Capillary lumen.

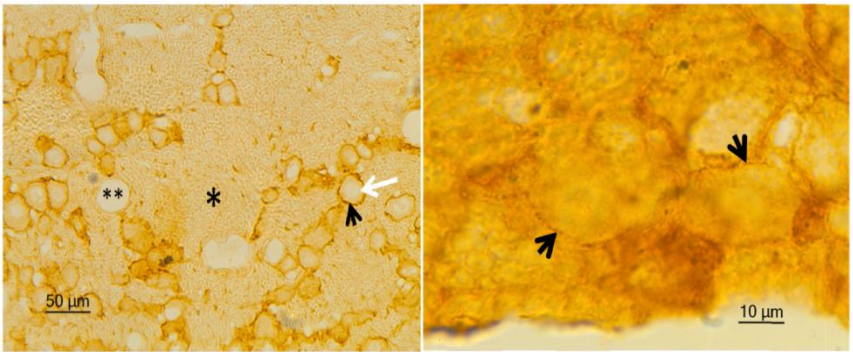

Figure 4: Immunostained sections of trigeminal ganglia with connexin-43. A) Several neurons are seen (white arrow) surrounded by satellite glial cells. The junctional area (black arrow) between the neuron and glia has been stained indicating the existence of gap junctions. Myelinated nerve fibers were also seen (*). Blood vessels were marked (**) B) Higher magnification of immunostained section showed that the stained area between the neuron and glia is composed of discontinuous small dot like structures (arrow). Each of these dots like structure represents gap junctions.

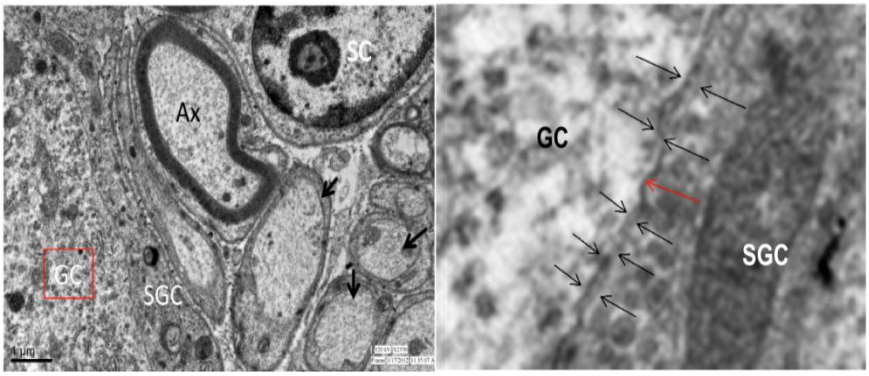

Figure 5: Electron microscopic localization of gap junctions in the trigeminal ganglion. A) It shows lighter color ganglion cell (GC) covered by darker satellite glial cells (SGC). A Schwann cell (SC) is seen in the upper right corner along with a myelinated axon(Ax).Several other unmyelinated axons were seen in the lower right corner enveloped by sheaths of Schwann cells (arrows). B) A gap junction between the cell body of trigeminal ganglion neuron (GC) and a satellite glial cell (SGC) is shown (red arrow) and in the adjoining region, the cell membranes of the neuron and the glial cell are separated by a well-defined interval (black arrows).Magnification- X3825.

DISCUSSION

Trigeminal ganglia neurons are divided into two types – Type A cells which were large and contain small or medium-sized clumps of Nissl substance, which are abundant in central part of the cytoplasm (LL: Large-Light cells) while Type B were smaller and have coarser clumps of Nissl substance, uniformly distributed in the cytoplasm (SD: Small-Dark cells). In the present study, wide variations were noted in the size of the trigeminal ganglion neurons. The size of cells is supposed to be related to the transcriptional activity[11]. This was first demonstrated in neurons of the frog, where bigger neurons required higher transcriptional activity[12]. The rates of transcription in neurons are positively related to the degree of interactions with their targets[13]. This, in turn, correlates with neuron size and its activity. Ichikawa *et al*. (1993) had measured the size of trigeminal neurons in the rat with respect to area wherein; it was noted to range from about 100 micron to 1400 micron. Maximum neurons were noted to be between 200-300 micron and between 400-500 micron[14]. In humans, the trigeminal ganglion contains about 27,000 neurons and the associated supporting non-neuronal cells are about 100/neuron[15]. However, in the rat, the total numbers of neurons are about 35,300 of which, 66% are of type A and the remaining of type-B[16]. Some of the authors classified the neurons in other sensory ganglia such as dorsal root ganglia in small, medium and large, depending upon the staining with specific antibody such as peripherin. Peripherin antibody specifically stains the small to medium sized neurons[17]. Neurons in sensory ganglia have no dendrites and thus their structure is much simpler than that of most other neurons. They do not receive synapses but are still endowed with receptors for numerous neurotransmitters. Satellite glial cells (SGC) wrap completely around the neurons in the sensory ganglia. They could be recognized clearly in cresyl violet stained paraffin sections, immunohistochemical labeled cryostat sections and the ultrathin sections for electron microscopy. Often, Schwann cells are reported to be the only component of peripheral glia, leaving aside the satellite glial cells. The current opinion is that SGCs were laminar and had no true processes. In general, each sensory neuron has its own SGC sheath, which usually consists of several SGCs and thus, the neuron and its surrounding SGCs form a distinct morphological and probably, the functional unit[18]. The distance between glial and neuronal plasma membranes is about 20 nm and therefore the extracellular space around the neurons is very small. The neurons send numerous fine processes, some of which fit into invaginations of SGCs[19]. The objective of the research work, described in this research paper, was to identify neuron-neuron gap junctions. Immunohistochemistry noted gap junctions between neurons and glia in trigeminal ganglia. Electron microscopy of the trigeminal ganglia showed gap junctions. However, apart from gap junctions between neurons and satellite cells, gap junctions at other sites were not observed. Possibly, immunoelectron microscopy could be helpful in this case. Connexin-43 is the most conspicuous protein in mammalian gap junctions. It is also most prevalent in the astrocytes. During development of the nervous tissue, connexin-26 is the most common. However, with maturation Connexin-43 becomes more prevalent while Connexin-26 is limited to leptomeninges, ependyma, and pineal gland. There is electron microscopic evidence for the presence of gap junction in satellite glial cells in sympathetic ganglia[20]. Gap junctions are clinically relevant. Chemotherapy-induced neuropathic pain, which occurs in about one-third of patients, could be due to gap junctions. Oxaliplatin and taxol were able to increase the gap junction-mediated coupling between satellite glial cells by up to fivefold and two-fold respectively[21]. This, in turn, lowered the pain threshold. The authors investigated the frequency of gap junctions by injecting a fluorescent dye - Lucifer yellow – into satellite cells encircling one neuron cell body. In a recent study, gap junctions were noted to allow bi-directional calcium ion signaling between neurons and satellite glial cells, when one of this cell was stimulated by focal electrical or mechanical stimulatio[22]. Increased expression of connexin-43 was observed in the neurons of the dorsal root ganglia after plantar incision model performed in rats[23]. Finally, the result of the present study, particularly immunohistochemistry showed that gap junctions are widely expressed in the trigeminal ganglia. Qualitative estimation was not done in the present paper. Other researchers have found strong evidence of the linkage of gap junction expression with pain. Pharmacotherapy for reducing the functional status of gap junctions may be helpful in the treatment of pain arising from both somatic and visceral structures.

CONCLUSION

Gap junctions allow the exchange of ions, second messengers and small metabolites between adjacent cells and are formed by two connexin proteins. The present study was undertaken to observe gap junctions between neuron-neuron and neuron-satellite cells in trigeminal ganglia by using the various confirmatory methods. Knowledge regarding the gap junctions was important to identify etiology for various diseases as well as to identify the pharmacokinetic property of new drugs.

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