Romanian Academy

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Ph.D. Thesis

Morphofunctional researches on the trigeminal nervous system at the level of the skull base

ABSTRACT

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Key words

trigeminal ganglion; immunohistochemistry; transmission electron microscopy; stem niches; stem cells; progenitor cells; telocytes; Schwann cells; mitochondria; ribosomes; periaxolemmal ribosomal plaques.

Potenţialul stem/progenitor la nivelul ganglionului trigeminal uman adult

Ipotezele de lucru

(1) There are studies identifying the expression of c-kit in some peripheral sensory neurons. Even though the role of c-kit receptors is tightly related to nociception there are no sufficient studies to extrapolate in humans the results of experiments performed in animals. It was thus proposed this hypothesis and the aim of study was to evaluate the expression of c-kit in the human trigeminal ganglion, in neurons which could be related with nociceptive functional circuits and in non-neuronal intraganglionic cell types. (2) Expression of nestin corresponds to an undifferentiated cellular status. A subpopulation of progenitor cells in dorsal root ganglia expresses nestin and was indicated playing role in neuronogenesis. It is also known that resident stem cells in sensory ganglia can differentiate either in neurons, or in Schwann cells. The aim of this study was testing the expression of nestin in the human adult trigeminal ganglion. (3) Experimental studies have demonstrated the presence in sensory ganglia of nestin-positive progenitor cells deriving from the neural crest. This evidence was not documented in transmission electron microscopy (TEM). So, the aim of this study was to evaluate in TEM whether, or not, a subset of satellite glial cells (SGC) could qualify ultrastructurally for a progenitor phenotype, different of the ultrastructural standards of SGC. (4) In adult, telocytes (TC), which are fibroblastoid cells, could be considered multipotent mesenchymal stem (stromal) cells (MSC). It was aimed performing a study to test the expression of CD34 and c-kit in the human adult trigeminal ganglion; a TEM study of the fibroblastoid cells of the trigeminal ganglion was added. (5) Telocytes, defined in 2010 as a new cell type, are cells which are characterized exclusively morphologically, as „*cells with telopodes*", the telopodes being long, thin and moniliform prolongations. Up to present were not identified specific markers of TC, although numerous studies associate them with markers expressed also in endothelial cells, such as CD34, vimentin, endoglin, VEGF; there were also found positively expressed in TCs α-SMA and, inconstantly, c-kit. It was also discussed the active role of TC in processes of neovessels formation. The inconsistency of evaluation of a specific molecular phenotype of TC is also demonstrated by the fact that experiments which attempted to evaluate TC sort them exclusively on a morphological basis and not based on a peculiar immunohistochemical phenotype. It was raised the hypothesis that TC, more than being in fact cells with stem or progenitor potential, could be endothelial progenitors. It was thus aimed to test the hypothesis at molecular and ultrastructural levels.

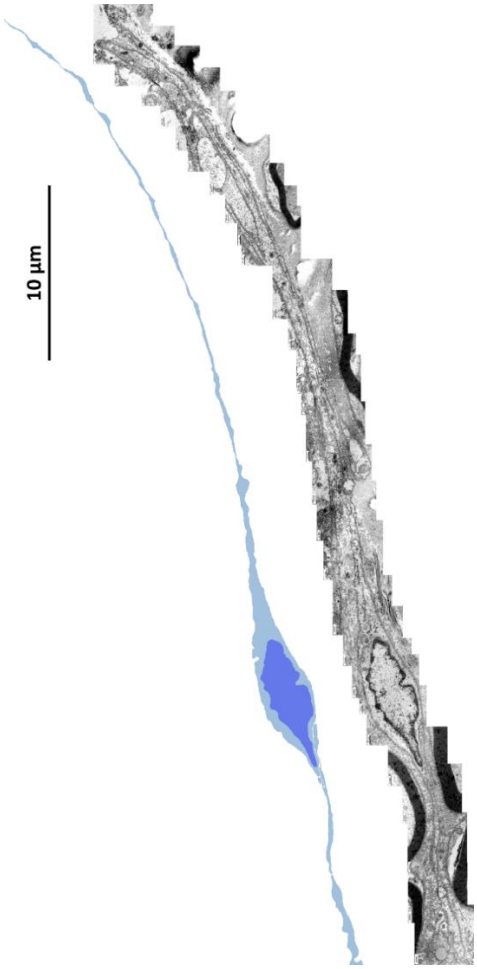
Material and methods

For this study were obtained postautopsic samples of human trigeminal ganglia. Methods: immunohistochemistry on paraffin-embedded samples (IHC-P) and transmission electron microscopy (TEM). There were used the primary antibodies: CD10; CD117/c-kit; CD146; CD31; CD34; CD45; CD68; c-erbB2; cytokeratin 7 (CK7); von Willebrand factor (vWF); the smooth muscle myosin heavy chain (SMM); nestin; neurofilaments; Stro-1; VEGFR-2; vimentin; α-smooth muscle actin (α-SMA). TEM: standard preparation, semithin and ultrathin cuts. Grids were explored and documented in TEM.

Neuronoglial progenitors

Primary trigeminal neurons were nestin-negative. A positive nestin phenotype was identified in cells of satellite glial sheaths, as well as in endothelial cells of the microvessels in the trigeminal ganglion. In TEM were identified cells contacting the neurons and belonging to the neuronal envelopes, which were

ultrastructurally different of the SGC and were characterized by an inactive status: (a) small cell size; (b) small heterochromatic nucleus; (c) a large amount of free cytoplasmic ribosomes; (d) few mitochondria in perinuclear location; (e) few cisterns, occasionally dilated, of rough endoplasmic reticulum; (f) absent Golgi complexes; (g) bundles of intermediate filaments were occasionally observed, usually in perinuclear location. I also observed the presence in the perinuclear region of the neuronoglial progenitors of multivesicular bodies. Some neuronoglial progenitor cells presented intracytoplasmic chromatin fragments, suggestive for the proliferative character of these cells.



Human adult trigeminal ganglion. The image resulted after digital concatenation of 28 micrographs of ultrathin cut; the diagram was made by supercoloration and resizing. It is presented a spindle-shaped telocyte, with long, thin and moniliform telopodes.

c-kit expression in interstitial cells and peripheral trigeminal neurons

By immune labeling with CD117/c-kit I identified within the trigeminal ganglion two neuronal types: (i) c-kit positive trigeminal neurons and (ii) c-kit negative trigeminal neurons. Neuronal processes also expressed c-kit. I have also identified c-kit positive neural fibers in the interneuronoglial interstitia. I found also c-kit positive mast cells, usually neighboring blood microvessels. I identified expression of c-kit and neurofilaments (clone RT97) on successive slides, in the same neuronal somata. I found that neurons expressing c-kit were negative for RT97 and c-kit negative neurons were clearly labeled for RT97. I clearly made the difference between the c-kit positive neurons and those c-kit negative which contained lipofuscin. I also identified thin cellular processes, moniliform, with a circumferential course over the

periphery of the neuronoglial units containing either c-kit positive or c-kit negative neurons. Most of such processes were intermingled on the immunohistochemical slides with the neuronal envelopes consisting of SGC. In some instances the cell body of these cells was found, it was spindle-shaped, distinctively of SGC. These spindle-shaped cells did not express neurofilaments.

The trigeminal telocytes

The immunohistochemical evaluation of CD34 expression leaded to identification of interstitial cells with long and moniliform prolongations which formed a veritable intraganglionic network; these cells were distinguished of the CD34 positive endothelial cells of the intraganglionic microvascular bed, these last cells building a double row containing red blood cells. The stromal networks of spindle-shaped cells with long prolongations were neighboring nerve bundles and intraganglionic microvessels. There were identified TC long of 15-53 μm. These had either a neutral stromal position, or were neighboring microvessels, intraganglionic nerve fibers and neuronoglial units. It was frequently observed a discontinuous basal lamina covering the cellular somata and telopodes, separating the plasmalema of the pericellular collagen fibers. Telopodes were clearly identified. There were identified spindle-shaped cell bodies with two telopodes leaving their extremities and also triangular cell bodies of the TC with three telopodes. The nucleus was ovoidal, with condensed marginal chromatin. There were occasionally observed 1-2 nucleoli. The trigeminal TC displayed plasmalemmal caveolae.

The pial stem niche, stromal stem cells and endothelial progenitors in the trigeminal ganglion

In light microscopy the trigeminal ganglion appeared covered by a HER-2 positive pial mesothelium. Beneath the mesothelial covering was identified a HER-2 positive microstroma embedding endothelial tubes. The mesothelial layer equally covered trigeminal nervous bundles and pial arterioles. The neuronoglial units were covered by a HER-2 positive microstroma embedding TC. Within the intraganglionic interstitia expression of HER-2 was identified in fibroblastoid isolated cells which, in some instances, were lining to configure a capillary-like tubular morphology. CD31 was expressed in endothelial cells of the intraganglionic microvessels; when cut longitudinally, the CD31 positive cells had a morphology resembling TC. Beneath the pial mesothelium were identified clusters of CD31 positive cells projecting filopodes and forming vasculogenic networks. Microstroma around the neuronoglial units also expressed CD31. The cells of these intraganglionic vasculogenic units (IVU) had variable morphologies, including the TC ones. Such cells, as wells as endothelial cells, also expressed VEGFR-2. At the level of the IVU were also identified nestin positive cells of the endothelial lineage; expression of nestin was also found in cells of the neuronal glial sheaths. Nestin positive cells with TC morphology were applied on the neuronal sheaths. Expression of CD10 was encountered in IVU and in cells with TC morphologies applied on the neuronal sheaths. Immune labeling with c-kit demonstrated the consistent positive expression of it in the subpial layer, the intraganglionic mast cells and in progenitor cells embedded within the IVU. CD34 labeled the microvascular endothelia and IVU. The vasculogenic networks were linked by CD34 positive cells with TC morphologies, cells which were also identified in the perineural sheaths. In the ganglion interstitia were also found isolated small-sized cells, with supraunitary nucleocytoplasmic ratio, which expressed CD34 and were projecting eventually short and thin filopodia; such cells were considered

stem/progenitor cells. Histologically similar cells also expressed CD68, positive expression of this antigen being also found in TC applied on the neuronal sheaths. Isolated expression of Stro-1 was found in stem/progenitor stromal cells located between the neuronoglial units and within the intraganglionic nervous bundles. The cell membranes of the trigeminal neurons expressed CD146; the SGC were CD146 negative. Microvascular endothelial cells were also CD146 positive, the respective antigen being also expressed in IVU. There were also found CD146 positive intraganglionic and intraneural stem/progenitor cells. Pericytes and vascular smooth muscle cells also expressed CD146. Alpha-smooth muscle actin (α-SMA) was exclusively present in vascular smooth muscle cells and pericytes, the IVU being α-SMA negative. Expression of CK7 was found in mesothelial pial cells, in adventitial cells of large vessels and in isolated intraganglionic stromal cells. Evaluation in transmission electron microscopy of ganglionic interstitia brought evidence supporting the immunohistochemical results. There were so objectivised cells with morphology and ultrastructure characteristic for TC. The anatomical diagnosis of capillary lumina allowed the differential diagnosis between TC and endothelial cells which, from an ultrastructural point of view, are quite similar. Ultrastructural differences between TC and fibroblasts were evident, the fibroblasts presenting a well-configured machinery of synthesis. At the level of the intraganglionic neurovascular bundles with vessels of large calibre were identified in perivascular sites cells with progenitor ultrastructural phenotype. Within the microvascular lumina were found different cells embedded within the red blood cells bed; some of these intravascular cells, thus circulating, presented an ultrastructural phenotype suggesting their quality of stem/progenitor cells. Endothelial cells of the resident microvessels, as well as the pericytes which were applied on the endothelial cells, specifically displayed plasmalemmal dense plaques. There were adherens and tight interendothelial junctions. There were also found endothelial-like cells displaying large vacuoles, which were considered nascent capillaries; their allocation to the endothelial lineage was proofed by the intracytoplasmic evidence of the Weibel-Palade bodies which are absolutely specific for the endothelial lineage. Such Weibel-Palade bodies were also found in mature endothelial cells. The evidence of a Weibel-Palade body-like structure within a neuronoglial progenitor cell allowed the hypothesis of the angiogenic potential of these cells embedded among the SGC in the neuronal sheaths. Within the intraganglionic stroma were relatively frequent identified cells with a peculiar ultrastructural phenotype. These had euchromatic nuclei, with condensed marginal chromatin, cytoplasm and well-represented prolongations, their plasmalemma specifically displaying plasmalemmal dense plaques. Other peculiar trait of these cells was the cytoskeleton consisting of abundant intermediate filaments appearing as an almost exclusive cytoplasmic content. There were scarcely found mitochondria and dilated cisterns of rough endoplasmic reticulum. The signaling machinery associated with plasmalemmas included vesicles and subplasmalemmal caveolae. Within the pericellular matrix were identified multivesicular bodies. There was also observed a basal lamina applied on the cell membrane of these cells. Identification of Weibel-Palade bodies in these stromal cells with undifferentiated phenotype indicated them as being endothelial progenitor cells. The progenitor characteristics of these cells were also supported by their nucleolar morphology, as well as by the evidence within their cytoplasm microtubular organizing centers in which were only squelched the centrioles characteristic conformations. Evidence of the intraganglionic vasculogenic processes was also demonstrated by the formation of characteristic networks by endothelial progenitors.

The role of Schwann cells in regeneration and survival of trigeminal peripheral neurons

Maintaining stable the axon structure is critical for its function. The traditional concept is that macromolecules, such as proteins, are synthesized in the neuronal soma and delivered in the axon by axoplasmic transport. There are however research groups which defeat this theory and support the local axonal synthesis of the necessary proteins for augmenting the input from the neuronal soma. The third concept, modern, indicates as a mechanism of supplemental supply of proteins in the axon the transfer from the adaxonal glia to the axon by extracellular vesicles. Schwann cells (SC) regulate several axonal functions. The answer of SC to nervous lesions represents the basis of nerve regeneration and repair in the peripheral nervous system.

There was identified the presence of multivesicular bodies in the adaxonal cytoplasm of SC, indicating the role of SC in glioaxonal signaling by the ability of releasing exosomes. There were frequently identified axonal dilatations and spheroids. The respective morphologies were associated with alternating layers, of microvesicles and thin myelin, covering the axons. The presence of the axon and lack, equally of compaction and of a complete contour of the myelin sheath, indicated ongoing processes of regeneration at axonal level.

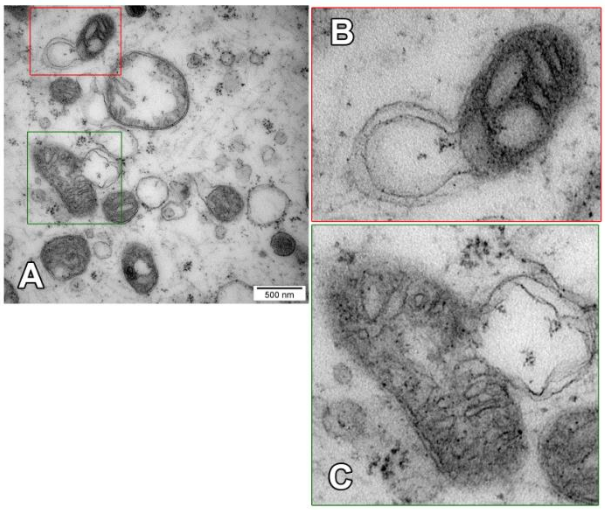
Vesicles similar to the adaxonal ones populated the abaxonal perinuclear cytoplasm of SC. The regenerative pattern was also supported by lack of an axolemma separating the axon and the adaxonal glial microvesicular layer. At the level of spheroids axons were lacking and the myelin sheath which was thin and circumferentially incomplete, contained only microvesicles and large vacuoles. Inside the myelin sheaths were found axons occupying a diminished space as compared to the higher intramyelinic content of microvesicles. An ultrastructural configuration different of the discussed regenerative structures was represented at the level of the myelin fibers with seemingly unaltered myelin and axons unaltered anatomically by the morphology of the adaxonal parts of the SC. At this level were identified either (a) unique cellular narrowed segments between the axolemma and the myelin sheath, or (b) vesicle-like protrusions of the adaxonal cytoplasm of SC, or (c) seemingly nanotubular prolongations. Such vesicles were forming at the level of the axolemma pockets of endocytosis to which were corresponding subaxolemmal vesicles with double membranes. There was found the granular content, of ribosomes-like particles, of these protrusions/vesicles, seemingly having the role of transferring to the axons glial cytoplasmic content. There was also identified the axonal presence of ribosomes, usually grouped as polysomes. The subaxolemmal density of ribosomes was obvious in some instances. There were identified periaxolemmal ribosomal plaques. It was found the polarity of ribosomes in SC, to the neighbour myelin sheaths. This evidence indicates the morphological availability of SC to transfer ribosomal material to axons, possibility having a positive anatomical determinant, that of a null extracellular space through which the glioaxonal signaling evolves. At the level of the axonal spheroids the myelin sheaths appeared immature and incomplete, being split by microvesicular layers and associated with absent or incomplete axolemmas, which was indicative for active processes of axonal regeneration.

These results allow the objectivation for the trigeminal ganglion of the modern concept regarding the axonal regeneration. After a focal lesion of an axon, distal to the site of lesion occurs the anterograde Wallerian degeneration which affects the axon, its terminations and the myelin sheath; the SC dedifferentiate and with the macrophages phagocytise the rests of the myelin sheath. These changes occur distal to the lesion and propagate long the distal segment of the axon; however, Cajal described a retrograde Wallerian degeneration which extends back along the neural fibre, up to the first Ranvier node

proximal to the site of the lesion. Distally to the lesion the axon initially dilates (axonal varicosities) then it breaks in a series of spheres with membranes (axon spheroids), this process evolving distally to the site of the lesion. If the regeneration of a new axon is possible, distally to the site of the lesion, in the peripheral nervous system, it is needed an intact endoneurial sheath (basal lamina) to guide the new axon towards the peripheral target. Reduplication of the SC begins after lesions and axonal sprouting shortly thereafter. If the tubular continuity of the basal lamina was not altered that will act to guide the axonal sprouts. Initially these are very small and correspond to a single SC; as they grow and dissociate, each one will associate an individual SC. As the axonal sprouts elongate the SC move with them and will get forming also myelin sheaths but if the newly formed axons do not contact the target tissues they will degenerate. There were identified here structures surrounded by thin and circumferentially incomplete myelin sheaths at the level of which were abundant mitochondria and vesicles. These structures correspond to axon growth cones at the level of which were described as specific the abundant mitochondria, different vesicles and vacuoles, neurotubules and a small amount of smooth endoplasmic reticulum; non axonal cells are located between the growth cone and the basal lamina, these later containing a small amount of cell residuum. It should be taken into account that neighbour structures were found, seemingly presenting the ultrastructural characteristics of an axonal growth cone located proximally to a site of axonal interruption/degeneration. However, one of that structures had an incomplete axolemma, while the neighbour one, anatomically comparable from the viewpoint of the mitochondrial layer covering a microvesicular core, did not exhibit an axolemma. This could be explained by the fact that the morphology of the axonal spheroids and growth cones is comparable. A difference favouring the identification of a regenerative process is the immature morphology of the myelin sheath, without the evidence of myelin lesions. There are various tissular insults which can trigger the degeneration of axons which, in turn, respond with different morphologies, topologies and speeds. There are two models of the axonal degeneration: (a) the *dying back* model in which the axonal degenerations begins at the distal end of the axon and has a retrograde evolution and (b) the model of the focal lesion in which such lesions determine the Wallerian degeneration of the axonal segment which is distal to the site of the lesion, the proximal segment of the axon remaining unaltered.

Altered mitochondrial anatomy in peripheral trigeminal neurons, in diabetes

Neurons from sensory ganglia are exposed to oxidative attack in diabetes. Altered mitochondrial morphologies are due to impaired dynamics (fusion, fission) and to cristae remodeling. This study aimed to evaluate using transmission electron microscopy mitochondrial changes in diabetic trigeminal ganglia suggestive for ignition of apoptosis, in absence of “classical” morphological signs of apoptosis. We used samples of trigeminal ganglia (from six type 2 diabetes human donors and five streptozotocin (STZ)-induced diabetic rats). In human diabetic samples we found three main distributions of mitochondria: (a) small “dark” normal mitochondria, seemingly resulted from fission processes; (b) small “dark” damaged mitochondria, with side-vesiculations (single- and double-coated), large matrix vesicles and cytosolic leakage of reactive species, mixed with larger “light” mitochondria, swollen, and with crystolysis; (c) prevailing “light” mitochondria. In STZ-treated rats a type (c) distribution prevailed, except for nociceptive neurons where we found a different distribution: large and giant mitochondria, suggestive for impaired mitochondrial fission, mitochondrial fenestrations, matrix vesicles interconnected by lamellar cristae, and mitochondrial leakage into the cytosol.



Diabetic (type 2 diabetes) adult trigeminal neuron. Herniated mitochondrial vesicles (A) detailed at higher magnifications (red and green insets in C, respectively D). It is appreciated the double vesicle membrane formed by the outer mitochondrial membrane and the inner limiting mitochondrial membrane.

Conclusions

1. The peripheral control centre of the trigeminal system, located at the level of the skull base, is represented by the trigeminal ganglion of Gasser. Although numerous studies, especially experimental, investigated this ganglion from the point of view of trigeminal neurotransmission and neuromodulation, it was almost completely overlooked the regenerative potential at this level. This potential certainly exists as it results from the hereby exploratory research.
2. Although in common microscopic anatomy the neuronoglial units of peripheral sensory ganglia are considered consisting of peripheral neurons and satellite glial cells, there was brought evidence by immunohistochemistry and the results were confirmed in transmission electron microscopy that the glial sheaths of trigeminal peripheral neurons also consist of undifferentiated cells which were indicated as neuronoglial progenitors. Although morphologically these progenitors seem oriented towards the glial lineage, it cannot be discarded their potential for peripheral trigeminal neuronogenesis. The presence of Weibel-Palade bodies within neuronoglial progenitors could indicate a process of transdifferentiation of these progenitors, especially if one takes into account the abundant microvascular stroma of the trigeminal ganglion.
3. The peripheral neuronal population is heterogeneous. However, immune labeling for c-kit could represent a useful tool for evaluating the nociceptive trigeminal neurons and to selectively investigate the possible interactions of neurons and the adjacent satellite cells. c-kit immune labeling only could not firmly associate this marker with nociception but taking into account that the nociceptive trigeminal neurons are neurofilaments poor, the evidence brought here link the c-kit positive neurons to the nociceptive trigeminal pathway.
4. The stromal cells of the trigeminal ganglion were studied in immunohistochemistry and transmission electron microscopy. Initially they fulfilled criteria to be indicated as interstitial Cajal-like cells. Further, due to terminology change, they were

indicated as telocytes. Telocytes represent however just a morphological trait of cells and actually was not identified a firm molecular phenotype of them. The hereby researches brought convincing arguments that at least a subset of trigeminal telocytes qualify as endothelial progenitors. Their role for the maintenance of the trigeminal microvascular bed is of utmost importance for the ganglion function.

5. The modern concept regarding the axonal regeneration indicates as a mechanism of supplemental supply of proteins to the axon the transfer from the adaxonal glia to the axon through extracellular vesicles, such as exosomes. The transfer of macromolecules from Schwann cells to axons was reconsidered as being vesicles-mediated. Actually there were brought evidences of the presence of exosomes and microvesicles in the glial cells of the central nervous system but such evidences largely lack in the peripheral nervous system. There was brought here novel evidence of microvesicles and exosomes signaling in the human adult trigeminal ganglion, process of which Schwann cells are responsible. As such axonal regenerative processes involve the dedifferentiation of glial cells, it is mandatory to include the Schwann cells within the adult ganglionic stem niche. Moreover, the ribosomes transfer in the trigeminal axons make from the Schwann cells relevant players in maintaining the functional integrity of the trigeminal nerve. The possibility of glioaxonal communication via nanotubular canals could not be ignored, but further studies should evaluate this feature. Also, newly identified structures, such as argosomes and vesicle-vacuolar organelles should be adequately validated to be associated with Schwann cells.
6. The ultrastructural pattern of mitochondria damage in diabetic samples of sensory neurons may provide clues on the initiation of intrinsic apoptosis, even if the classical morphological signs of apoptosis are not present. Further studies, combining use of biochemical and ultrastructural techniques, may allow a better quantification of the degree in which mitochondrial damage, with membrane alterations and cytosolic leaks, may be used as morphological signs suggesting the point-of-no return for apoptosis.