THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Morphological study on the neuronal networks related to the vestibular system in the frog

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1. INTRODUCTION

The living organisms have continuous relations with their environment. The external stimuli are detected and transformed by receptors and specific sense organs. One of these organs is the vestibular system or organ of sense of balance. It provides information about the displacement of head and body and consequently it maintains the body position against the gravity and coordinates the eye movements in line with the head movements. The very fine and continuous tuning of gaze fixation and the rapid control of movements requires a very fast and precise modification of the motor activity in order to obtain the proper muscle contraction.

The vestibular receptors appear first in the aquatic animals (fish, amphibians before metamorphosis) as the lateral line organ. It is located in a longitudinally running streak on the body surface and its hair cells are mechanoreceptors in function. The hair cells are able to detect the displacement of body in the water. The vestibular sense organs develop later in the phylogenesis and they are located inside the body. Both in non-mammalian and mammalian species, the receptors sensitive for the linear acceleration are found in the saccule and utricule and in lower vertebrates in the lagena, too. The angular acceleration is detected by the hair cells of the crista ampullares of the semicircular canals. The vestibular hair cells are innervated by the peripheral branches of the bipolar neurons of the vestibular ganglion and their central branches form the vestibular part (pars vestibulare) of the vestibulocochlear nerve. The vestibular afferent fibers terminate in the four vestibular nuclear complex of brainstem containing the nucleus vestibularis superior, medialis, lateralis and descendens. These nuclei play a central role in the function of vestibular system and they have widespread connections with various structures of the brainstem.

In spite of the large number of experiments, the structure and function of neuronal network of the brainstem related to the vestibular system is not yet fully understood. In the present study, two parts of this neuronal network was examined with neuromorphological methods. In the first part, the morphological background of the integration and synchronization of the antagonistic and synergistic external eye moving muscles was examined. During body displacement the gaze has to be fixed related to the body position and it needs the synchronized function of motoneurons innervating the eye muscles.

In the second part, the effect of the vestibular system on the vegetative centers of the brainstem was examined. It is known in the clinical practice that the excitation of the

vestibular system results in changes in the function of the cardiovascular, respiratory and gastrointestinal system. It is not known, whether the information of the vestibular system reach the vegetative centers directly or indirectly. The expected results may provide new insights into fundamental mechanisms of the vestibular system related to the vegetative functions. The data can contribute to understand how the symptoms of the vestibular lesions develop and what mechanisms play a rule in the vestibular compensation.

2. AIMS OF THE STUDY

The direct and indirect connections of the vestibular system play an important role in the maintenance of posture and balance to keep the position of the body. During the head movements, the vestibular system coordinates the eye movements to fix the gaze according to the body position. The vestibular system sends information to the autonomic centers during the body displacement and consequently it can modify the function of the vital organs. The following questions were examined:

1. Do the motoneurons of the oculomotor nerve establish direct connections with the motoneurons of the trochlear nerve?

2. What are the morphological characteristics of these connections?

3. Do the vestibular afferent fibers establish direct connections with the motoneurons of glossopharyngeal and vagus nerve?

4. What is the morphological characteristic of these connections?

3. MATERIALS AND METHODS

3.1. **Animals used in experiments**

The experiments were carried out on 43 common water frogs, Rana esculenta in accordance with European Community guidelines and state regulations and with the approval of the University Animal Care Committee (18/2006/DE MÁB). The animals were anaesthetized with 0,01% MS-222 solution (tricaine methane-sulfonate, Sigma, St. Louis, MO).

3.2. Double labeling with fluorescence tracers

3.2.1. Labeling of the oculomotor and trochlear nerves

In the first group of animals (n=15) the oculomotor (III) and trochlear (IV) nerves were prepared from an oropharyngeal approach by an incision of the mucosa on the roof of oral cavity. Under an operating microscope the cranial cavity was opened by removal of parts of the bilateral parasphenoidal bone. After incision of meninges the right oculomotor and the left trochlear nerves were prepared and sharply transected. Crystals of fluorescein binding dextran amine (FDA, 3000 W, Molecular Probes) were applied to cut end of the oculomotor nerve and tetramethylrhodamine dextran amine (RDA, 3000 MW, Molecular Probes) was put on the cut end of the contralateral trochlear nerve. The animals were kept in a refrigerator for 5 days, reanaesthetized and transcardially perfused with isotonic saline for 2-3 min, then fixed by 4% paraformaldehyde in 0,1 M phosphate buffer (pH 7.4). Cross-section from the brainstem were made with a Vibratome at a thickness of 50 μm. Images were recorded using an Olympus FV 1000 confocal laser scanning microscope (40x oil immersion lens, NA 1,3). For the latter analysis we used series of 1 μm thick optical slices. Close appositions were considered if there was no discernable gap between the two profiles, and, if the contact surfaces were at the same focal plane. The close appositions between the oculomotor and trochlear motoneurons were counted manually along Z image series.

3.2.2. Labeling of vestibulocochlear and glossopharyngeal, nervus vagus and accessory nerves

In the second group of animals (n=16) the nerves were prepared from an oropharyngeal approach by an incision of the mucosa on the roof of oral cavity. The cranial cavity and otic capsule were opened at right side of the animals followed by sharp trans-section of the vestibulocochlear (VIII) and common root of the glossopharyngeal, vagus and accessory (IX, X, XI) nerves. Crystals of fluorescein binding dextran amine (FDA, 3000 W, Molecular Probes) were applied to cut the end of the VIII nerve while the crystals of tetramethylrhodamine dextran amine (RDA, 3000 MW, Molecular Probes) was put on the cut end of the IX, X, XI nerves. The animals were kept in a refrigerator at a temperature of 12 °C. 5 days later and the same procedure was applied as it was described above.

3.3. Labeling with neurobiotin

It is known that low molecular weight tracers are transported through the gap junctions and it can be detected in the pre- and postsynaptic neurons. This phenomenon is referred to as dye-coupled connection and the method indicative of the gap junctional coupling at light microscope level. In order to determine the possible electrotonic coupling between the III and IV motoneurons, as well as between VIII fibers and X-XI motoneurons, we have performed the following experiments.

3.3.1. Labeling of oculomotor and trochlear nerve with neurobiotin

In these experiments only one of the cranial nerves was labeled in each animal (n=3 for the oculomotor and for the trochlear nerve, respectively). The animals were anaesthetized and the appropriate cranial nerve was prepared and cut as described above. Neurobiotin crystals were applied to the cut end of the nerve and covered with the mixture of silicone oil and grease in order to prevent the leakage of the tracer. The animals were kept in a refrigerator for 5 days at a temperature of 12 °C, and then reanaesthetized as described above, perfused transcardially with physiological saline and subsequently with a solution of 2% paraformaldehyde and 1.25% glutaraldehyde on 0.1% phosphate buffer, pH 7.4. The brain

was removed and immersed in the same fixative for overnight. The specimens were washed in 0.1 M phosphate buffer (PB), followed by 10% and 20% sucrose dissolved in the same buffer solution. 60 μm serial cross-sections were made with a freezing microtome. The sections were washed for 10 min in 0.1 M PB and phosphate buffer saline (PBS) and incubated for 1 hour in PBS containing 0.001% Extravidin (Sigma, St. Louis, MO). The sections were then washed in for 10 min in PBS, 0.1 M PB, and 0.05 M Tris buffer (pH 8) and incubated in 0.05 M Tris buffer containing 0.075% diaminobenzidine (Sigma, St. Louis, MO), 0.6% nickel-ammonium sulfate and 0.015% hydrogen peroxide until the reaction product was visualized as a black precipitate. Finally, sections were washed in Tris buffer, mounted on gelatin-coated slides, dried overnight, and cover slipped with DPX (Sigma, St Louis, MO). Photographs were taken with a Nicon Eclipse 800 microscope.

3.3.2. Labeling of the vestibulocochlear nerve with neurobiotin

In order to examine the possible electronic coupling between vestibular afferent fibers and motoneurons of the ambiguous nucleus, the vestibulocochlear nerve was prepared as described above and transected proximal to the vestibulocochlear ganglion (n=3). The method of the application of neurobiotin was similar to that of the oculomotor and trochlear nerve.

3.4. Electron microscopic experiments

In order to determine the dendrodendritic and dendrosomatic connections between the motoneurons of the oculomotor and trochlear nerves, electron microscopic experiments were performed. Three animals were anaesthetized as described above and perfused transcardially with isotonic saline followed by fixative containing 1.25% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The cross-sections containing the oculomotor nucleus were postfixes in 0.5% osmium-peroxide (TAAB, in 0.1 M PB, pH 7.4) for 40 min, and then dehydrated in ethanol line and embedded in epoxy-resin (Durcupan ACM, Fluka). Ultrathin sections were cut from the oculomotor and trochlear nuclei with a Reichert ultramicrotome and mounted on Formwar-coated nickel grids, and then in order to increase the contrast uranyl-acetate for 20 min and Reynolds solution for 10 min were applied. Electron micrographs were taken with Jeol 100B electron microscope.

4. RESULTS AND DISCUSSION

4.1. Connections of the oculomotor motoneurons with the trochlear motoneurons

4.1.1. Simultaneous labeling of the oculomotor and trochlear nerves with fluorochroms

Labeling the oculomotor and the trochlear nerve with fluorescent dyes revealed the motoneurons in the same localization and with the same morphological characteristics that we have obtained previously from the cobalt labeling technique. Oculomotor dendrites were observed among the perikarya of the trochlear nerve, and vice versa, the trochlear dendrites were distributed between oculomotor cell bodies. In addition, many long dendrites of the oculomotor and trochlear neurons were intermingled outside the confines of the nuclei. Using confocal laser scanning microscope we detected a large number of close appositions between the oculomotor and trochlear motoneurons, the contacts appeared more often in the oculomotor nucleus. Of 2210 contacts counted in the oculomotor nucleus, 86% were dendrodendritic connections and the rest of dendrites (14%) were engaged in dendrosomatic contacts. We have found similar ratio of different contacts in the trochlear nucleus: 82% of 485 close appositions appeared as dendrodendritic connection and 18% of them were present as dendrosomatic contact. The number of contacts and the ratios were similar in sections taken from individual animals. The distance between the neighboring profiles suggested close membrane appositions without intercalating glial or neuronal elements.

4.1.2. Application of neurobiotin to the oculomotor and trochlear nerves

Application of Neurobiotin to the oculomotor nerve resulted in labeling of neurons exclusively within the oculomotor nucleus indicating the absence of dye-coupled connections. Similarly, when the trochlear nerve was labeled, the tracer was detected only within the trochlear motoneurons. It indicates the lack of gap junctions between the III and IV motoneurons.

4.1.3. Electron microscopical experiments

Both in dendrodendritic and dendrosomatic appositions, 2–5 dendrites of 0.5–4.5 µm in diameter were in direct contact with adjacent profiles. The apposed membranes did not show any morphological specialization similarly that was demonstrated at the dendrodendritic contact of the cat spinal motoneurons. In the nucleus and in the neuropil, numerous dendrites were present close to each other without intervening glial processes. It was frequently visible that 4–8 neighboring dendritic profiles were grouped in clusters. Such formations of aggregated profiles, called dendrodendritic "thickets", established long membrane appositions (2–3µm). Dendrites within "thickets" run parallel or perpendicular to each other. We did not observe morphological specialization indicative of gap junctions either in dendrodendritic or dendrosomatic appositions, only in a few cases we have detected the narrowing of intercellular space between the adjacent membranes of dendritic profiles.

Although the membrane specializations were extremely rare or not present, electrotonic couplings were recorded between the spinal cord motoneurons of the frog in electrophysiological experiments. The discrepancy between the morphological and physiological results may be solved by postulating ephaptic interactions between two neurons with apposing membranes of sufficiently large surface and low resistance. The long membrane appositions presented here satisfy the condition of electrotonic coupling between the oculomotor and trochlear motoneurons. Dendritic bundles of motoneurons extending into the adjacent spinal segment were demonstrated in different mammalian species and the electron microscopical studies revealed membrane appositions with or without gap junctions in these cases. Electrophysiological experiments performed on adult cat provided evidence for short latency couplings of motoneurons suggesting the significance of dendritic bundles in the synchronization of the activity of motoneurons. Direct membrane appositions between the dendritic bundles of the oculomotor and trochlear motoneurons presented here may be the morphological background of the synchronized contraction of the bilateral eye muscles. Activation of ipsilateral motoneurons of the inferior rectus muscle located in the oculomotor nucleus may spread very rapidly by way of electrically coupled dendritic bundles to the trochlear motoneurons. The trochlear nerve innervates the contralateral superior oblique muscle, which is contracted in synchrony with the inferior rectus muscle during vertical eye movements.

4.2. Connections between the vestibular afferents and the motoneurons of the glossopharyngeal and vagus nerves

4.2.1. Simultaneous labeling of the vestibulocochlear, glossopharyngeal and vagus nerves with different fluorochroms

Using of FDA to the vestibulocochlear nerve resulted in labeling of afferent fibers with a pattern similarly to that found in our earlier neuronal labeling experiments. Correspondingly, the RDA labeled afferent and efferent components of IX, X, and XI nerves were detected in the same extent and morphological pattern that it was found by using of cobalt chloride or cobalt-lysine tracer. The rostrocaudal level of each section and terminology used for different parts of glossopharyngeal and vagus motoneurons was applied from our previous work when we performed detailed cytoarchitectonical analysis on the ambiguous nucleus after separate labeling of individual branches of IX, X and XI nerves. These branches related to the IX and X nerves were the posterior inferior pharyngeal branch, the long and short laryngeal nerves, the gastric nerve, the pulmonary nerve and the cardiac branches. In the present work we have used this map for identification of different types of neurons at a given cross section of brainstem. For the sake of simplicity we applied the visceromotor terminology for those motoneurons that supply smooth or cardiac muscles (VMN), and motoneurons with skeletal muscle target were named as somatomotor neurons (SMN). The long diameter of somatomotor neurons varied between 25.4 -30.6 μm, whereas the cell bodies of visceromotor neurons measured 14.8-24.5 μm in their long diameter. The number of smaller neurons varied 230-250; the larger neurons excluding those of XI nerve, was 450-470 in each animal.

Combination of RDA and FDA images displayed that the accessory motoneurons, including their dendrites, were out of the termination areas of vestibular fibers. Therefore, the accessory motoneurons were excluded from the further examination. The overlapping areas of vestibular and IX-X nerves started about 2400 μm rostral to the obex and continued caudally in 1400 μm length. Throughout this rostrocaudal extent the fascicles of FDA labeled vestibular fibers, having left behind the vestibular nuclear complex, turned medially around the dorsal or dorsomedial aspect of nucleus of solitary tract and continued into the gray matter. The majority of vestibular fibers were thicker and upon reaching the motoneuron pool and nucleus of solitary tract they emitted a large number of thin collaterals. At the level of the entrance of glossopharyngeal root of ambiguous nucleus, the vestibular fibers and terminals

intermingled with large perikarya and dendrites representing the pharyngeal motoneurons. Caudally to the glossopharyngeal root the bundles of vestibular fibers reached the lateral reticular zone (LRZ) of reticular formation, a small-celled area near to the nucleus of solitary tract. Within this area RDA labeled neurons were also detected. Neurons in similar position and with similar size were labeled from the gastric, cardiac and pulmonary branches of the IX and X nerves. The vestibular fibers were also found among the dorsomedial dendrites and perikarya of the large cells of ambiguous nucleus supplying the muscles of pharynx and larynx. We could follow the FDA labeled vestibular fibers into the nucleus of solitary tract.

By using confocal laser scanning microscope we could detect a large number of close appositions between the vestibular afferent fibers and dendrites or perikarya of the ambiguous nucleus. There was no visible gap in the XY images between the neighboring FDA/RDA labeled profiles suggesting close membrane appositions without intercalating glial or neuronal elements. We have counted the close appositions in the rostral part of the ambiguous nucleus containing exclusively the SMNs of the pharyngeal muscles where the number of close contacts was 48.3±3.6 in a 0.1 mm^2 area. In the middle part of the ambiguous nucleus, app. 1300 µm rostral to the obex, the number of close appositions was 59.4±3.4 in a 0.1 mm^2 area containing the VMN neurons of stomach, heart and the lung. At this level the SMNs innervate the laryngeal muscles and the number of close appositions of these motoneurons with the vestibular fibers was 46.5±3.8 in a 0.1 mm^2 area.

4.2.2. Application of neurobiotin to the vestibulocochlear nerve

Application of neurobiotin to the vestibulocochlear nerve resulted in labeling of central vestibulocochlear fibers and terminals similarly to that of previous results. In addition, the perikarya and stem dendrites of second order vestibular neurons were also labeled in the vestibular nuclear complex indicating their dye-coupled connections with the vestibular afferent fibers. However, we have never detected neurobiotin labeled cells in the ambiguous nucleus suggesting the absence of dye-coupled connections between the vestibular fibers and the motoneurons of glossopharyngeal and vagus nerves.

4.2.3. Functional correlations

Based on present findings and data from literature we outline the possible neuronal circuit underlying the interaction of vestibular afferent fibers and efferent neurons of glossopharyngeal and vagus nerves of the frog. Stimulation of vestibular receptors activates the afferent vestibular fibers that terminate principally on the second order neurons of vestibular nuclei through gap junctional and chemical synapses. Neurons of the vestibular nuclei activate the VM and SM neurons of IX and X nerves, neurons of the nucleus of solitary tract and reticular formation. In addition, the labyrinthine stimulus can reach first the reticular formation and the nucleus of solitary tract and then it is transmitted to the VM and SM neurons. All these connections provide polysynaptic pathways from the labyrinthine sense organs to the efferent neurons of the IX and X nerves. Due to the combination of gap junctional and chemical synapses of the vestibular afferent fibers to the second order vestibular neurons and because of the various routes in the polysynaptic pathways, the motoneurons of IX and X nerves can be activated sequentially. However, in case of rapid displacement of body the propagation of impulse transmission via the polysynaptic pathway may be too slow to modify the activity of motoneurons in time and properly. The monosynaptic connection between the afferent vestibular fibers and efferent neurons of IX and X nerves presented here may provide a quick and immediate response of motoneurons in order to obtain the required muscle contraction. Since this contact is established largely by the thick vestibular fibers, their fast conduction velocity is also in favor of rapid impulse propagation and subsequent synaptic transmission.

Due to the lack of experimental data on the vestibulo-autonomic interaction in non-mammalian species we can interpret our results in the light of studies on mammalian species. In the mammalian brainstem the nucleus of solitary tract and different areas of reticular formation such as the lateral tegmental field (LTF) respond to electrical stimulation of vestibular nerve via the vestibular nuclei. Spare projections of primary vestibular fibers were also detected in the LTF of the monkey and gerbil. Topographically the LTF of mammalian species corresponds to the lateral reticular zone of the frog reticular formation where we have found not only the primary vestibular afferent fibers but also the VM efferent neurons of the IX and X nerves. Scattered VM neurons of IX and X nerves were identified in the LTF of various mammalian species. Since these small VM neurons cannot be distinguished from the neurons of reticular formation without specific neuronal tracing we can assume that the possible direct interaction between the primary vestibular afferents and VM neurons of IX and

X nerves remained undiscovered. The LTF of mammalian species is regarded as an important integrative center of vegetative functions as it is involved in cardiovascular control, respiration and in the act of vomiting. There are certainly differences in vegetative functions between mammals and amphibian, the LRZ of the frog and the LTF of mammalian species may be homologous structures regarding the vestibulo-autonomic interaction.

5. SUMMARY

Applying different neuronal labeling techniques we have studied the morphological background of the neuronal network underlying the eye movements and the brainstem control of vegetative functions of the frog.

Simultaneous labeling of the oculomotor and trochlear nerve with different fluorochroms was performed in *in vivo* experiments. By the use of confocal laser scanning microscope we detected a large number of close contacts in both nuclei; the majority of them were dendrodendritic appositions. The distance between the adjacent profiles suggested close membrane contacts without intercalating glial or neuronal elements. At the ultra structural level, the dendrodendritic and dendrosomatic contacts did not show any morphological specialization; the long membrane appositions may provide ephaptic interactions between the neighboring profiles. This electrotonic coupling between the oculomotor and trochlear nerve motoneurons may promote the co-activation of the inferior rectus and superior oblique muscles responsible for vertical eye movements.

We have studied whether the primary vestibular afferent fibers establish direct connections with the motoneurons of glossopharyngeal and vagus nerves of the frog. The vestibulocochlear and the glossopharyngeal-vagus nerves were simultaneously labeled with fluorescein dextran amine and tetramethylrhodamine dextran amine. With a confocal laser scanning microscope we could detect close appositions between the vestibular afferent fibers and somatodendritic components of the somato-and visceromotor neurons of the ambiguous nucleus of IX-X nerves. The direct impulse transmission may provide a quick and immediate response of cardiovascular and gastrointestinal system upon body displacement.

Our results can help to understand the structural organization of complex neural systems related to the sense of balance. The results may also assist in developing new therapeutic strategies for the treatment of symptoms of vestibular lesion.

6. PUBLICATIONS

This thesis is based on these in extenso publication:

1. Tímea Bácskai, Gábor Veress, Gábor Halasi, **Ádám Deák**, Éva Rácz, György Székely, Clara Matesz: Dendrodendritic and dendrosomatic contacts between the oculomotor and trochlear motoneurons of the frog, *Rana esculenta.* Brain Res Bull 75: 419-423. 2008. **IF: 2,281**

2. **Ádám Deák**, Tímea Bácskai, Gábor Veress, Clara Matesz. Vestibular afferents to the motoneurons of glossopharyngeal and vagus nerves in the frog, *Rana esculenta*. Brain Res 1286: 60-65. 2009. **IF: 2,494**

This thesis is based on this book chapter:

1. Matesz, C**.**, Bácskai, T., **Deák, Á.**, Rácz, É., Veress, G., Székely, G.: Using of confocal laser scanning microscope in the examination of neural network underlying the gaze and posture control. In: Fiber Lasers: Research, Technology and Applications. Nova Science Publishers, Inc. New York. pp.1-5.

Other in extenso publications:

1. Clara Matesz, Gabriella Kovalecz, Gábor Veress, **Ádám Deák**, Éva Rácz, Tímea Bácskai: Vestibulotrigeminal pathways in the frog, *Rana esculenta.* Brain Res Bull 75: 371–374. 2008. **IF: 2,281**

2**.** Barna Kelentey, **Adam Deák**, Tivadar Zelles, Klara Matesz, István Foldes, Gabor Veress, Tímea Bácskai. Modification of innervation pattern by fluoroquinolone treatment in the rat salivary glands. Anat. Rec. 293: 271-279. 2010. **IF: 1,569**

3. Zoltan Zaborszky, Klara Matesz, **Ádám Deák**, Marta Jackel, Gellert Karvaly, Jozsef Furesz, Bakity Boldizsár **.**Pathological examination of the abdominal compartment syndrome in animal experiment. Submitted to J. Amer. Surgery 2010.

Cumulative impact factor of published papers: 8,625.

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5. **Deák Á**, Bácskai T, Rácz É, Matesz, K: Vestibular lesion-induced changes in the expression of hyaluronan in the brainstem. Congress of The Hungarian Neuroscience Society, 2007 Szeged, Clin.Neurosci.2007;60(S1):1-72.

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7. **Deák, Á,** Bácskai, T, Rácz, É, Matesz, K Vestibular lesion induced changes in the expression of hyaluronan in the rat brainstem. PENS/Hertie Winter School, "The Design of Neuronal Networks: Contributions from Invertebrates“, Obergurgl, Austria, 2008

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