Sensory control of jaw movements in the prey-catching behaviour of frogs

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen;
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1   INTRODUCTION

The sensory information gained from the muscles, the joints, the skin, vestibular sense organs and photoreceptors are of vital importance to regulating processes of movements. The afferent stimuli are simultaneously processed in various places of the central nervous system. The central integrating role of the brain stem in organizing the sensory-motor system is also of primary importance. Through its rich afferent and efferent connections, it takes part in the regulation of vitally essential functions as well as in the direction of motor functions that contain stereotype elements of motion. It is here that the nuclei of cranial nerves are also to be found.

The study of the frogs' (*Anuran*) prey-catching behavior has considerably contributed to the understanding of the functioning and structure of the neuronal networks of the brain stem. Since the organization and functioning of their central nervous system has reached a rather high level and, furthermore, the structure of their brain stem can be considered to be phylogenetically conservative, the study of their neuroanatomy may contribute to the cognition of how the central nervous system of mammals and even humans are structured.

The prey-catching behavior of frogs consists of a series of extremely rapid and coordinated movements. Depending on the prey size, ranid frogs can catch their prey by using two different mechanisms. Small prey are captured with the tongue and transported to the esophagus without contacting the jaws (tongue prehension behavior -TPB) whereas large prey are first contacted with the tongue but the head rotates downward, the prey are captured in the closing jaws, and are transported into the oral cavity with the forelimbs (jaw prehension behavior, -JPB). The choice between the two techniques occurs in milliseconds, then the whole series of reflex-like movements occur.

Performing stereotypical movements and oromotor activities requires the maintaining of body balance, the synchronization of head movements with those of the jaw bones, of the tongue and with swallowing movements. To carry out these vitally important movements, the proper timing of motor activities participating in the coordination of the series of these movements is to be ensured. It is the central neuronal network in the brain stem (Motor Pattern Generator, MPG) that is responsible for performing the stereotypical series of movement of prey catching. The visualization of the prey releases the information via the optic tectum toward the reticular formation in the brainstem, where the neurons of the motor pattern generator (MPG) receive that information. The motoneurons responsible for the contraction of the muscles, participating in the given movement, are reached by the pattern generated by the MPG, either directly or via the last order premotor interneurons (LOPI) that are located in the reticular formation. This predetermined program, however, can be modified by the sensory of motor inputs conveyed either directly or indirectly from the central and peripheral nervous systems, which ensures the proper coordination of the extremely rapid goal-oriented movements during the prey-catching behavior.

The motoneurons of the trigeminal and facial nerve responsible for the jaw movements play an important role in the processes of food intake. During jaw movements, its position and the maintenance of the tension of the muscles moving the jaw are controlled by the proprioceptive receptors, mechanoreceptors and vestibular receptors. Depending on the features of the prey and the phases of opening and closing the mouth, a very quick correction of the muscle tension (of the muscles helping open and close the mouth) may be necessary to produce the optimal muscle contraction in order for the prey to be successfully caught. The functioning of facial motoneurons, playing a decisive role in the opening of the mouth, can be

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influenced by the sensory information from the proprioceptive receptors in the muscles spindles and the Golgi tendon organs of the jaw muscles and from the mechanoreceptors of the oral cavity and the tongue, conveyed by the trigeminal nerve.

Our experiments were aimed at studying the connections between the trigeminal nerve participating in opening and closing the mouth and the vestibular system on the one hand. On the other hand, we wanted to investigate whether the excitation of the receptors of the trigeminal nerve could directly affect the activity of motoneurons of the facial nerve that contribute to the opening of the mouth.

Efferent components of jaw movements

The primary mandibular joint is typical of amphibians, reptiles and birds. The joint acts as the hinge of a two-armed lever, and it permits only the opening and closing of the mouth. Of the motor nuclei of the cranial nerve providing for the muscles participating in jaw movements, **the motor nucleus of trigeminal nerve** contains the muscles instrumental to the opening (submaxillaris muscle) and closing (masseter, pterygoideus, temporalis muscle) of the mouth as well as the motoneurons providing for the levator bulbi muscle. In the brain stem it extends from the level of the cerebellar nuclei to the entrance zone of the vestibulocochlear nerve. The nucleus has three morphologically distinct motoneuron types. The motoneurons of the first type occupy the rostral two thirds of the nucleus and provide innervation for the muscles that close the mouth. The perikarya are of large size (40-50 µm), slightly elongated or star-shaped. The second type of motoneurons is to be found in the caudal part of the nucleus and has a small (25-32µm), rounded or oval-shaped perikarya. They supply the submaxillary muscles. The cell bodies of the motoneurons belonging to the third type are polygonal and are about the same size as

those in the second type. They form a dorsomedially uniform group in the rostral part of the motor nucleus of the trigeminal nerve and innervate the levator bulbi muscule which separates the orbit from the oral cavity. This motoneuron type and the muscle are present only in amphibians. The three neuron types are somatotopically located within the motor nucleus, but there is some overlapping in the localization of the motoneurons providing for the closing muscles.

The **motornucleus of facial nerve** is a well defined motoneuron group of the ventrolateral motor column about 170 µm caudal to the motor nucleus of the trigeminal nerve. Its caudal pole extends almost to the abducent nucleus. Its motoneurons innervate the depressor mandibulae muscl and the subhyoideus, therefore, open the jaw. The nucleus consists of motoneurons which are the exact counterparts of the second type neurons in the trigeminal motor nucleus, which also participate in the opening of the mouth.

Organization of the sensory system of the brain stem forming the background to jaw movements

The sensory root of the **trigeminal nerve** carries general sensory information from the mechano-, thermo-, and chemoreceptors of the head skin and the mucous membrane of the oral cavity, as well as proprioceptive informations from the jaw closing muscles, and sensory informations from Golgi tendon organs of the jaw muscles. In the brain stem the general somatic afferent fibres continue in the spinal tract of the trigeminal nerve. The proprioceptive fibres of mouth closing muscles contribute to axons of the mesencephalicus nucleus of the trigeminal nerve (nVmes). In anurans the sensory fibres of the trigeminal nerve terminate in three different areas of the brain stem. (1)) The termination area of the thin fibres of the tspV is the spinal nucleus of trigeminals nerve i (nspV). The nspV is also the

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somatosensory nucleus of cranial nerves VII, IX and X, so it also accommodates the terminals of these cranial nerves. (2) Thick fibres of the tspV terminate in the principal sensory nucleus of the trigeminal nerve (npV), which is the main sensory nucleus of the trigeminal nerve, but the axon terminals establish a dense meshwork even in the areas of the motor nucleus of trigeminal, facial, hypoglossal nervese and nucleus ambiguus. (3) The large pseudounipolar cells of the mesencephalic nucleus of trigeminal nerve (nVmes) are primary sensory neurons and can be found in the second and fourth layers of the optic tectum. The peripheral axons of these cells ascend in the tegmentum of the midbrain, whereas the descending fibres form the Probst tracts, which runs caudally in a separate bundle at the ventral aspect of the tspV. The axons of Probst tract emit collaterals at the level of the trigeminal and facial motor nuclei as well as at the level of nucleus ambiguous. The termination areas of the collaterals coincide with location of the tspV terminals in the brain stem. The terminals establish a number of direct contacts on the perykaria of the trigeminal motoneurons. The sensory ganglion of the trigeminal nerve is the ganglion trigeminale, which corresponds to the Gasserian ganglion of mammals. The central extensions of its pseudounipolar cells terminate in the npV.

The sensory branches of the **facial nerve** convey general sensory information from the mucous membrane of the throat, the oral cavity, the palate and the nasal cavity. Its hyomandibular sensory branch transmits mainly stimuli generated by acidic and saline chemicals to the central nervous system from the palate. After entering the brain stem, part of the facial afferents run in the solitary tract and their ascending bundle reaches the level of the motor nucleus of trigeminal nerve, while the descending bundle terminates caudally to the level of the obex. The larger part of the fibres terminates in the nucleus tractus solitarii, whereas their smaller part

ends in the comissura infima. Another part of the afferents that consist of thick fibres only progress caudally and join the spinal tract of trigeminal nerve (tspV) and decussate at the medullo-spinal border. The termination area of their collaterals coincides with the terminals of the afferent fibres of the trigeminal nerve and those of the glossopharyngeus-vagus nerve.

The sensory fibres of the glossopharyngeal and the vagus nerve also enter the solitary tract. The terminals of the fibres of all the three nerves can be found along the entire rostrocaudal extension of the solitary nucleus.

The overlapping of afferents within the two sensory systems is typical, and several facial collaterals terminate in the termination areas of the nspV and the thick trigeminal fibres. A significant amount of trigeminal fibres reaches the solitary nucleus in the medulla. The sensory fibres of the trigeminal nerve are also richly distributed in the territory of the brain stem occupied by the facial motoneurons, which points to the possibility of monosynaptic contacts.

The **vestibular system** sends information to the central nervous system about the position of the body and the head, thus maintaining the balance of the body. Through its central connections it takes part in the regulation of muscular tension and is indispensable for fixing the gaze during movements.

Vestibular receptors are mechanoreceptors. In frogs the ganglion cells of the vestibular part of the vestibulocochlear nerve can be found in the vestibulocochlear ganglion. The peripheral processes of the bipolar neurons of the ganglion terminate in the receptors of the sense of balance, whereas their central afferent fibres terminate in the four vestibular nuclei (nucleus vestibularis superior -SVN, medialis -MVN, lateralis -LVN, descendens -DVN) of the brain stem. The primary afferent vestibular fibres reach the cerebellum as well. Besides these, terminals of the fibres can

also be found in the reticular formation of the brain stem, in the sensory and motor nuclei of the cranial nerves and in the nuclei of the dorsal column and can be followed caudally as far as the superficial part of the grey matter of the cervical spinal cord. In the vestibular nuclei, after a complex processing of information, whose details are still unknown, the secondary vestibular neurons are projected to several areas of the central nervous system. The ascending fibres reach the nuclei of the thalamus and the motoneurons of the oculomotor nerve and those of the trochlear nerve. The descending fibres terminate in the ipsilateral and contralateral sides in the ventral horn of the spinal cord, mainly at the L3 level. The cerebellar projection occupies the greater part of the vestibulocerebellum. The individual vestibular nuclei project to the members of the vestibular nuclear complex on the same as well as on the opposite side.

With neuronal labeling examinations terminals of afferent vestibular fibres were detected in the area of the spinal nucleus of trigeminal nerve. Primary afferent trigeminal fibres were found in vestibular nuclei, too.

2 OBJECTIVES

The motor nuclei of the facial nerve and those of the trigeminal nerve play a crucial role in the functioning of the jaw movements in the prey-catching behavior of frogs, since they contain the motoneurons of the muscles that carry out the opening and the closing of the mouth. The process of opening and closing the mouth is a very delicate and genetically determined series of movements that are built on one another and contain stereotypical elements. The process can be influenced by afferent information conveyed by different nerves.

The previous neuronal labeling experiments are suggestive of the influence of vestibular input to the trigeminal system. The extension of the dendrites of the trigeminal motoneurons into the vestibular nuclear complex may indicate the monosynaptic vestibulotrigeminal connection in the frog. This can also be claimed about position of the motoneurons of the facial nerve and that of the primary afferent fibres of the trigeminal nerve in the brain stem as stated by previous cobalt labeling experiments. As in the course of these experiments the cranial nerves were labeled for each animal separately, it was not possible to determine whether there actually existed direct connections between the neuronal elements of the two nerves in the brain stem.

By using in vivo retrograde and anterograde tracing techniques our goal was to investigate the possible anatomical substrates underlying the vestibulotrigeminal neuronal circuitry in the frog, and whether the afferent fibers of trigeminal nerve can establish direct contacts with the facial motoneurons in the brain stem.

In the first part of our experiment we localized the overlapping areas between the neuronal elements of the trigeminal nerve and the vestibular nerve. We also addressed the question of whether the vestibular stimulus did exert a direct influence on the functioning of the muscles innervated by

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the trigeminal nerve and taking part in the movements of the jaw, therefore we also examined the morphological properties of the contacts.

Our next series of experiments was aimed at defining the distance of the contacts from the soma and their distribution on the dendritic segments branching off in different directions so that we could detect their exact position in the brain stem. Since there are motoneurons of different functions in the motor nucleus of the trigeminal nerve, we paid special attention to how the contacts of the primary vestibular afferents were distributed between the motoneurons participating in opening and closing the mouth.

Similarly to our two previous experiments, with studying the connections between the afferent fibres of trigeminal nerve and the facial motoneurons our aim was to provide quantitative description concerning the location and spatial distribution of these connections on the somato-dendritic compartments of facial motoneurons in the frog.

3   MATERIALS AND METHODS

Preparation and labeling of the cranial nerves

Our experiments were conducted on 22 adult common water frogs, *Rana esculenta* (*Pelophylax esculentus*). The animals were anaesthetized with MS 222 solution applied to the wet skin. The examined cranial nerves were approached from an oropharyngeal view. The operation was performed under an Olympus SZX7 operating microscope. The mucous membrane on the roof of the mouth was sagittally incised, and a portion of the parasphenoidal bone was carefully clipped away to open the cranial cavity. The exposure made the trigeminal nerve, the facial nerve and the vestibulocochlear nerve visible. The cranial nerves were prepared and sharply transected with microsurgical scissors and folded onto the bone rim.

In one set of experiments, to study the connection between the the **trigeminal and the vestibular system**, green fluoresceindextran-amin (FDA) was put on the transected proximal stump of the trigeminal nerve, while crystals of red tetramethylrhodamine dextran-amin (RDA) were applied to the proximal cut end of vestibulocochlear nerve. *In another set of experiments*, the mapping of the contacts between the **vestibular system and the trigeminal motoneurons** participating in opening and closing the mouth, the VIII nerve proximal stump was labeled with neurobiotin (NB), whereas dextranamin-Alexa Fluor 555 was put on the proximal stump of trigeminalnerve. The labeling was carried out in 6 animals in both of the experiments (12 altogether). To examine the hypothesized contacts between **the sensory fibres of the trigeminal nerve and the motoneurons of the facial nerve,** neurobiotin (NB) was put on the proximal stump of V nerve and the rootlet of studied VII nerve were labeled with dextranamin-Alexa Fluor 555. The labeling was carried out in 10 adult common water

frogs. Both the sensory and the motor nerves of each animal were labeled on the same side.

To avoid nonspecific labeling and prevent the leakage of the tracer, the stumps of the nerves to be studied were covered with a mixture of silicone oil and grease after their being prepared and labeled. At the end of the intervention the edges of the incision made on the mucosa were joined with tissue adhesive. Then the animals were kept in a wet chamber at a temperature of 10 ºC. During the survival period of 5-7 days the labeling materials reached the brain stem through anterograde and retrograde axonal transport, so the motor and sensory neuronal elements of the brain stem, the objects of our investigations, became visible.

Histotechniques and microscopy studies

After the survival period the frogs were reanaesthesized and the animals were transcardially perfused with isotonic saline solution of 0.65% followed by 4% paraformaldehyde fixative in 0.1 M phosphate buffer (pH 7.4). Then the skull was opened, the brain removed and postfixed overnight in 4% paraformaldehyde solution at a temperature of 4 °C. The brain stem areas were excised and immersed in 20% sucrose until they precipitated. Transverse serial sections of the brain stem were cut at a thickness of 50 µm. The sections were cut with Vibratome after cranial nerves V-VIII were labeled with FDA-RDA, for the samples of cranial nerves V-VIII and V-VII labeled with NB-dextranamin-Alexa Fluor 555 freezing microtome was used. The sections were collected in a 0.1 phosphate buffer solution (PB) and their order of sequence carefully retained. The visualization of the FDA, RDA and dextrananim-Alexa labelling materials was not necessary, because of their fluorescent features, with other histochemical or immune histochemical processes. For the visualization of neurobiotin, the sections were treated with

streptavidin-Alexa 488 for two hours at room temperature. Finally, the sections were mounted on slides and coverslipped with Vectashield Mounting Medium for Fluorescence.

To localize the labeled neuronal elements, images were recorded with an Olympus DP 72 camera (10 x and 20 x lenses) at the different rostro-caudal levels of the brain stem. The images recorded in such a way were projected on one another in order to determine the overlapping areas of the sensory and motor parts of the cranial nerves studied. The identification of the hypothesized contacts of the **vestibulo-trigeminal pathway** was performed through the study of the samples labeled with RDA-FDA. Images of the sections were recorded using an Olympus FV1000 confocal laser scanning microscope equipped with a 40x oil immersion objective. Series of 1 µm thick optical slices were used to determine the contacts of the neuronal elements labeled with RDA and FDA. The optical slices were examined one by one. The contacts were identified on individual optical slices, which were considered as direct contacts if there was no discernable gap between the two labeled profile, and if the contact surfaces were at the same focal plane.

In our second series of experiments, the serial sections made from the brain stems of animals labeled with dextranamin-Alexa Fluor 555 and neurobiotin were used to study the distribution of the connections between the **vestibular nerve and the motor nucleus of the trigeminal nerve** on the trigeminal motoneurons participating in opening and closing the mouth.

During the microscopic examination, brain stem specimens of those animals were used in which the afferent collaterals and the dendritic trees of the motoneurons were clearly visible in their full rostro-caudal extension. The search for and the counting of direct connections as well as

the measuring of the distance from the cell body were carried out with a DSD2-Andor Zyla camera.

The contacts between **the sensory fibres of the trigeminal nerve and the motoneurons of the facial nerve** were also examined with an Olympus FV1000 confocal laser scanning microscope equipped with a 40x oil immersion objective. Close appositions were identified and counted. The radial distance of the connections from the motoneuron cell body was also measured with the help of the Olympus Fluo View Software.

The spatial reconstruction of the overlapping areas of the facial, trigeminal and vestibular neuronal elements with the help of neurolucida

The reconstructions were made with Neurolucida 8. Software. After the conture of the brain stem was outlined, the position of the cell bodies of the trigeminal motoneurons in the brain stem was determined, then the extension of the dendritic arborization of the trigeminal afferents and facial motoneurons were reconstructed in three dimensions. During the reconstruction the serial sections of our previous investigations were used, in which the two cranial nerves were separately labelled with cobalt chloride. Similarly, the reconstruction of the cell bodies and dendritic trees as well as that of the spatial position and extension of the vestibular afferents was also carried out with the help of the previous serial sections labelled with cobalt.

4   RESULTS

The study of the connection system between the trigeminal and vestibular nerve

The fluorescent labeling of the the trigeminal and vestibular nerve

When the trigeminal nerve was labelled with FDA, its sensory fibres as well as the cell bodies of the motoneurons and their dendritic trees became visible. The RDA, placed on the stump of the vestibulocochlear nerve made the central termination fields of the afferent fibres of the nerve and the efferent vestibular neurons identifiable. Projecting the images onto one another, we found overlapping areas between the labelled trigeminal and vestibular neurons in the various structures of the brain stem. One of these areas was detected in the rostral part of the rhombencephalon at the level of the entrance of the root of the trigeminal nerve to the brain stem. The lateral branches of the dorsal dendritic tree of the motoneurons, labelled with FDA, extended into the superior, medial and descending vestibular nuclei. At the same level, the vestibular fibres run medially direction and were distributed on the dorsal dendritic branches of the trigeminal motoneurons.

With a confocal laser scanning microscope, we could detect close appositions between the vestibular terminals and the motoneuron dendrites. The closeness of neighboring profiles is suggestive of direct membrane apposition without intervening glial or neuronal elements.

In the other overlapping region, we could detect the termination fields of the primary sensory neurons of trigeminal nerve throughout the rostrocaudal extent of the vestibular nuclear complex. A relatively abundant number of vestibular fibres and terminals can be identified in the termination areas of the descending limb of the mesencephalic trigeminal nucleus, whereas the vestibular terminals and fibres occurred less

frequently in the territory of the principal and spinali nucleus of trigeminal nerve, i.e. Gasserian ganglion.

The distance in space between the trigeminal and vestibular boutons shown by the confocal microscope was suggestive of the presence of some intervening neuronal or glia element separating them, so they could not enter into direct contact with each other.

The neurolucida reconstruction of the brain stem to illustrate the overlapping areas of the trigeminal motoneurons and the afferent vestibular neurons

One of the objectives of our research was to investigate whether the vestibular afferent areas that overlap with the trigeminal motoneurons cover the full length of the motor nucleus.

The motoneurons of the trigeminal nerve were reconstructed with neurolucida software on the previous serial slices labelled with cobalt, then the extension of the dendritic trees of the motoneurons as well as the location of the vestibular afferents in the brain stem were defined. We found that the vestibular fibres create an area of overlapping along the full rostrocaudal extension of the motor nucleus in parts of the brain stem occupied by the cell bodies and the dendrites of the trigeminal motoneurons that corresponds even to motoneurons participating in the rostrally positioned closing of the mouth and the caudally located opening of the mouth.

The labeling of the trigeminal nerve with dextranamin-Alexa Fluor 555 and the vestibulocochlear nerve with neurobiotin

An additional aim of our research was to determine whether the primary afferent terminals of the vestibular nerve create a direct contact between

the two functionally different motoneuron types and how these contacts are distributed between them.

Microscopic images of our slices were made at different levels of the motor nucleus of the trigeminal nerve with a DSD2-Andor Zyla camera. At the level of rhombencephalon the anterogradely labeled vestibular afferents and the retrogradely labelled trigeminal motoneuron cell bodies as well as the dendrites constituted an extensive overlapping area in the vicinity of the rostral and caudal parts of the motor nucleus.

The vestibular axon collaterales extended into the trigeminal dendritic branches, setting up a great number of direct contacts with the dendritic segments of the trigeminal motoneurons.

The distribution of the vestibular terminals on the functionally different motoneurons of the trigeminal nerve

The contacts were identified on 1 µm thick optical slices of the brainstem made with a DSD2-Andor Zyla camera. Direct connections were identified in the serial sections of those two animals in which the motoneurons cell bodies and the dendritic trees appeared in their full extension, and the caudal motoneurons participating in opening the mouth could be clearly distinguished from the rostral motoneurons instrumental to the closing of the mouth. Examining 1589 optical slices, we could identify 324 direct connections between the trigeminal motoneurons and the primary afferent terminals of the vestibular nerve in the two animals. The overwhelming majority (95%) of the contacts were situated on the dendritic trees, whereas a minor part of them (5%) proved to be axo-somatic. Most of the contacts were located close to the cell bodies of the motoneurons within a 600 µm radial distance from the perikarya. Nearly two thirds of the connnections were localized to the rostral area of the motoneurons participating in the closing of the mouth, but no remarkable

differences were detected in terms of their average distance from the soma between the two parts of the motor nucleus.

We also studied the distribution of the direct connections on the differently oriented dendritic segments of the motoneurons participating in closing and opening the mouth. The space around the soma was divided into four (dorsomedial, dorsolateral, ventromedial ventrolateral) quadrants with the help of a coordinate axis placed on it. The number of contacts was determined for each quadrant and their distance from the soma established for each quadrant. More than 75 % of direct connections were situated on the dorsomedial and ventrolateral dendrites in both parts of the nucleus. The greatest number of contacts were found on the ventrolateral dendritic trees of the motoneurons carrying out the closing of the mouth and on the dorsomedial dendrite trees of the motoneurons participating in opening the mouth. The distribution of the contacts on the dorsolateral and ventromedial segments was nearly identical. The average distance from the soma of the connections detected on the dendrites branching off in various directions of the two motoneuron groups proved to be nearly identical, but the distance of the contacts on the dendrites branching off in different directions diverged. 74 % of the contacts on the dorsomedial segments were situated within a 400 µm distance from the cell bodies even in the rostral and caudal part of the nucleus. We managed to identify a relatively small number of connections on the ventromedial dendritic trees of the two motoneuron types, but these contacts were situated very close to the soma, which means 200 µm radial distance rostrally and 100 µm caudally. The ventrolateral and dorsolateral dendrite trees the connections were detected mainly in the middle sections of the dendrites.

Study of the sensory-motor circuitry of the trigeminal nerve and the facial nerve

Neurolucida reconstruction of the brain stem to reveal potentional overlapping areas of the trigeminal and facial neuronal elements

The spatial reconstruction of the trigeminal and facial motor nuclei was performed with the Neolucida program. Then the overlapping fields between the territory of trigeminal afferents and area occupied by the somatodendritic compartments of facial motoneurons were represented in three dimensions. We made the reconstructions by making use of previous brain stem serial slices labelled with cobalt.

We have found that the facial motoneurons were more cadually and dorso-medially situated in relation to the trigeminal motor nucleus and parts of the nuclei overlapped. In both cranial nerves the muscles opening the mouth are innervated by the motoneurons of the overlapping area. The trigeminal afferents and their colletarals are distributed between the perykarya of the facial motoneurons and showed a definite overlapping area with the territory of ventrolateral facial dendritic tree.

Fluorescent labeling of the trigeminal nerve with neurobiotin and the facial nerve with dextranamin-Alexa Fluor 555

Application of Alexa Fluor 555 to the cut end of the facial nerve resulted in retrograde labeling of cell bodies and dendritic trees of the facial motoneurons and the anterogradely labeled axons of the solitary tract. The majority of the facial dendrites directed ventrolaterally from the cell bodies and a weaker array of dendritic branches in the dorsomedial direction. Neurobiotin labeling of the trigeminal nerve revealed the cell bodies of motoneurons located rostrally to the column of facial motorneurons, some of the trigeminal somata were intermingled with the

perikarya of the facial motoneurons. The labeled trigeminal nerve constituted well defined bundles of axons running in the spinal tract and the mesencephalic tracts on the medial side of the solitary tract and sent off several collaterals towards the facial motoneurons.

With confocal laser scanning microscopy we could confirm direct connection between anterogradely labeled trigeminal axon terminals and dendritic trees and cell bodies of retrogradely labeled facial motoneurons.

We investigated 5050 optical slices from the brainstem of four animals and we identified 831 direct connections between the trigeminal axon terminals and the facial motoneurons. The majority of the appositions (87%) were detected on the dendritic trees, while 13% proved to be axo-somatic contacts on cell bodies. Most contacts were situated within a 600 µm radial distance from the perikarya of the facial motoneurons.

5   DISCUSSION

In our present study, we examined the morphological background of the sensory-motor pathways modifying the prey-catching behavior of frogs by using different neuron labeling techniques. Within this general framework, we investigated the connections between the vestibular nerve and the sensory and motor nuclei of the trigeminal nerve, the distribution of the primary afferent vestibular terminals that are in direct contact with the functionally different motoneurons of the sensory trigeminal nucleus were also surveyed together with the distribution of the primary afferents of the trigeminal nerve on the motoneurons of the facial nerve.

Connections of the trigeminal and the vestibular nerve

During our experiments, we have demonstrated for the first time unambiguous instances of overlapping areas between the neuronal elements of the trigeminal and vestibular nerve at various levels of the brain stem. These overlaps may form the morphological background to the vestibulo-trigeminal pathway, which can be divided into two groups. One of them can be found in the area of the trigeminal motoneurons, whereas the other is located in the area of the sensory nuclei of the trigeminal nerve. Analysis of confocal images suggested a hitherto unknown monosynaptic connection between motoneurons of the trigeminal nerve and the afferent vestibular fibers of labyrinthine origin.

The rostral part of the motor trigeminal nucleus contains the motoneurons of the muscles participating in closing the mouth, whereas its caudal area accommodate the motoneurones responsible for the contraction of the mouth-opening muscles. Three quarters of the connections of the vestibule-trigeminal connections were found on the motoneurons of the mouth-closing muscles. It is not surprising at all the majority of contacts

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were detected on the rostrally located neurons of the motor nucleus since the motoneurons participating in the closing of the mouth regulate several muscles, and the number of motoneurons to be found here considerably exceeds that of the mouth-opening motoneurons located in the caudal area of the nucleus. For both categories of motoneurons, 95% of the connections were of the axo-dendritic type. There is a general regularity that seems to be increasingly probable, namely, that, in most cases, the primary afferent fibers controlling the prey-catching behavior of the TPB type establish an axo-dendritic connection with the efferent neurons. A similar distribution of the trigeminal afferents could be observed on the motoneurons of the nucleus ambiguus and on those of the hypoglossus nucleus as well as on the motoneurons of the vestibular, glossopharyngeal vagus and spinal nerve afferents, furthermore, on the motoneurons of the muscles operating the tongue.

On average, the distance of the vestibulotrigeminal contacts from the cell body ranged from 250 to 300 micrometers for the motoneurons of both the mouth-opening and mouth-closing muscles. The contacts located in the ventromedial quadrant, however, were significantly closer to the cell body, their distance being under 100 micrometers in both muscle groups. Although these connections make up only 4-5% of all contacts, it may be justified to say that their role cannot be neglected because of this close proximity. The somato-dendritic distribution of the vestibular afferents has earlier been examined on the motoneurons that move the tongue. It was found that the vestibular afferents, especially those responsible for the retraction of the tongue, were located on the more remote segments of the dendritic arbor. These results suggest that the modulating role of the vestibular system is different in the various efferent elements of the prey-catching behavior.

A large number of vestibular terminals were detected in the somatosensory nuclei of the trigeminal nerve, namely, both in the principal and the spinal nucleus of trigeminal nerve as well as in the termination areas of the descending limb (Probst bundles) of the mesencephalic nucleus of trigeminal nerve. The analysis of the confocal images suggest that they establish a synaptic connection with interneurons, so the vestibular stimulus can reach the motoneurons of the trigeminal nerve along a polysynaptic pathway.

The effect of the vestibular system on jaw movements has not yet been described in animals with a primary mandibular joint which allows the opening and the closing of the mouth only. Elecrophysiological and morphological examinations carried out in mammals (rats, guinea pigs) have shown that the vestibular primary afferent fibers affect the activity of the muscles that move the jaw in a polysynaptic way. Our results suggest that the system of vestibule-trigeminal connections is remarkably complex in amphibians. The direct contacts that we have identified refer to monosynaptic pathways, which means that the combination of the direct and indirect pathways enables the animal to give quick motor responses accompanied by the head movements required by the prey-catching process and to eventually coordinate the activation of motoneurons in time during jaw movements.

Trigeminal contacts on the motor nucleus of the facial nerve

Using confocal laser scanning microscopy we have demonstrated for the first time that a large number of direct contacts exist between the sensory trigeminal fibers and the facial motoneurons innervating the jaw opening muscles in the frog. We assumed that the majority of close appositions should be synaptic contacts, which was confirmed for the various parts of central nervous system at electron microscopic level. The identified direct

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connections may function as the morphological substrate for the very quick feedback and feedforward modulation of motor responses during the prey-catching behavior.

In the frog, the depressor mandibulae muscle lowers the jaw and consequently the mouth opens. High-speed cinematography synchronized with computer-analyzed electromyograms (EMGs) revealed that the first peak of the contraction in the depressor mandibulae muscle appeared in the preparatory phase of the prey-catching behavior prior to mouth opening, and the activity level of muscle contraction changes continuously during the execution of the tongue prehension behavior. Taking into consideration the very quick process of prey-catching, it can be assumed that during the feedback and feedforward modulations that form a background to the rapidly changing activity of the depressor mandibulae muscle sensory input is transported by the trigeminal nerve to the facial motoneurons.

Sensory fibers of the trigeminal nerve related to the prey catching behavior carry information from different receptors associated with different axonal morphology and physiological properties. One of the receptor types includes the proprioceptors in the muscle spindles and the Golgi tendon organs of the jaw muscles that are usually large in diameter. The other group of receptors are the mechanoreceptors of the oral mucosa and those of the tongue with small and medium sized fibers. The wide range of sensory modalities from different receptors which are propagated along the primary afferent axons with different velocity of impulses may be responsible for the fine tuning of the very rapid switch from one phase of tongue prehension to the other. Furthermore, the two muscle spindle types identified in marine toads and in reptiles are also likely to play a role in the adjustment of muscular activity during the various phases of the prey-catching behavior.

Quantitative analysis on close contacts established by the trigeminal axon terminals revealed that the majority of them were encountered on the dendrites of facial motoneurons and approximately 10% of them located on the perikarya. The axodendritic contacts were within the proximal two thirds of the ventrolateral dendritic trees.

According to the termination areas of the mesencephalic (Vmes) and the spinal tract of the trigeminal nerve (tspV), the results obtained from our earlier cobalt labeling strongly suggest that the Vmes exerts stronger influence on the activity of depressor mandibulae muscle as its fibers terminate in the territory of perikarya and proximal dendritic segments of the motoneurons instrumental to the functioning of the muscle. It is also confirmed by the results of previous physiological findings showing that the proprioceptors of the jaw muscles are continuously active during tongue protraction and retraction, whereas the mechanoreceptors of the oral cavity are stimulated by the captured prey in the initial phase of tongue retraction. As the facial motoneurons are involved in other behaviors like vomiting, respiration and phonation, the direct trigeminal sensory inputs described here may also contribute to the rapid modification of these motor programs.

We can hypothesize that direct contacts of the trigeminal sensory fibers with the trigeminal and facial motoneurons may form the morphological background to the synchronization and timing of the jaw closing and opening during the feeding movements. Combination of the direct and polysynaptic pathways may provide a high degree of plasticity required to optimally carry out the motor activity during the tongue prehension behavior.

6   SUMMARY

During the prey-catching behavior of frogs the coordinated activity of muscles participating in jaw movements is of pivotal importance. This activity can be modified by impulses reaching their motoneurons and generated by the central motor pattern generator as well as influenced by sensory information conveyed by different cranial nerves either directly or via interneurons. Using double neuronal labeling techniques in our research, we studied the possible neuronal connections between the vestibular and trigeminal systems in the brainstem, and our another aims was to find out whether the primary afferent trigeminal fibres enter into direct contact with the motoneurons of the facial nerve, which plays an important role in opening the mouth.

- Our experiments were the first of their kind to demonstrate the indisputable presence of overlapping areas between the neuronal elements of the vestibular and the trigeminal nerve at different levels of the brainstem.

- In the confocal images, direct contacts were identified between the afferent vestibular fibres and the motoneurons of the trigeminal nerve participating in the opening and the closing of the mouth.

- The vestibular terminals detected in the somato-sensory nuclei of of the trigeminal nerve should mean that the vestibular information can reach the motoneurons of trigeminal nerve through polysynaptic pathways.

- In the confocal images, we also demonstrated a large number of direct connections between the sensory fibres of the trigeminal nerve and the motoneurons of the facial nerve.

- It can be concluded from the localization of the axodendritic contacts that the trigeminal input originating from the proprioceptors and conveyed via the mesencephalic tract of trigeminal nerve, play important role in the functioning of facial motoneurons.

The majority of direct contacts identified proved to be axodendritic by both our investigations, which, on the basis of our present and previous research, allows us to conclude that during the tongue prehension behavior the primary afferent fibres establish mainly axodendritic connections with the efferent neurons. The identified monosynaptic connections may serve as one of the neuromorphological substrates to regulating the quick feedback and feedforward mechanisms of motor responses during the prey-catching behavior.

7 LIST OF PUBLICATION

DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR

List of publications related to the dissertation

1. **Kovalecz, G.**, Kecskés, S., Birinyi, A., Matesz, C.: Possible neural network mediating jaw opening during prey-catching behavior of the frog.
   Brain Res. Bull. 119, 19-24, 2015.
   DOI: http://dx.doi.org/10.1016/j.brainresbull.2015.09.012
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2. Matesz, C., **Kovalecz, G.**, Veress, G., Deák, Á., Rácz, É., Bácskai, T.: Vestibulotrigeminal pathways in the frog, Rana esculenta.
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List of other publications

3. Alberth, M., Lampé, K., **Kovalecz, G.**, Nemes, J.: Peso estremamente basso alla nascita e salute orale nella regione nord-orientale dell'Ungheria = Extremely low birth weight and oral health in north-east part of Hungary.
   Il Den. Mod. 33 (9), 76-88, 2015.

4. **Kovalecz, G.**, Alberth, M., Nemes, J.: Trattamento e profilassi del ?den sin dente": Report di quattro casi clinici = Management and profilactic treatment of dens invaginatus: report of four cases.
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5. Alberth, M., Majoros, L., **Kovalecz, G.**, Borbás, E., Szegedi, I., Márton, I., Kiss, C.: Significance of oral Candida infections in children with cancer.
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   Pediatr. Blood Cancer. 43 (1), 88-90, 2004.
   DOI: http://dx.doi.org/10.1002/pbc.20023
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