

**SHORT THESIS FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY (PhD)**

**Neuronal background of the prey-catching behavior of
the frog: sensory-motor integration in the brainstem**

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NEURONAL BACKGROUND OF THE PREY-CATCHING BEHAVIOR OF THE FROG: SENSORY-MOTOR INTEGRATION IN THE BRAINSTEM

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The Examination takes place at Department of Neurology, Faculty of Medicine, University of Debrecen, at 11:00 a.m. 1st June, 2015.

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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, at 1:30 p.m. 1st June, 2015.

1. INTRODUCTION

Prey-catching behavior of the frog consists of a sequence of coordinated activity of muscles corresponding to different stages of feeding. The frog exhibits a remarkable diversity of feeding behavior which provides a unique opportunity to study the sensory-motor integration.

Frogs are excellent animal models, because their nervous system is simple for neuromorphological studies, but the brainstem is rather conservative throughout vertebrate phylogeny.

Based on their phylogeny the frogs are classified into three families (*Archeobatrachia*, *Mesobatrachia*, *Neobatrachia*). The feeding behavior and the morphology of the feeding apparatus show differences among the families.

The motor program of the frog prey-catching behavior is based on a complex neuronal network. The visualization of the prey releases information via the optic tectum toward the reticular formation in the brainstem, where the neurons of the motor pattern generator receive this information, followed by the activation of the motoneurons via the last order premotor interneurons. This relatively long polysynaptic pathway is not efficient for fast reflex-like movement responses. We presume that monosynaptic connections between sensory and motor systems can provide the morphological basis of the immediate fast responses. Although the organization of the neuronal network underlying the prey-catching behavior has been studied with physiological and morphological methods, the morphological substrates of sensory-motor connections are involved in the fast response during feeding have not yet been elucidated.

The efferent components of the prey-catching behavior

In anurans, the sticky tongue plays an important role in ingesting, transporting and swallowing the prey, thus one of the important efferent components

of feeding motor program is the **hypoglossal nucleus**, organizing the contraction of tongue muscles. Three groups of muscles are responsible for movements of the tongue. The geniohyoid and genioglossus muscles are involved in protraction by moving the tongue and hyoid bone forward. Hyoglossus, omohyoid and sternohyoid muscles pull the tongue backward therefore regarded as retractor muscles. The inner or intrinsic tongue muscles change the shape of the tongue during protraction and retraction.

Our previous results demonstrated that the morphology of the protractor, retractor and inner motoneurons can be separated by using multivariate statistics based on their somato-dendritic morphology and orientation of the dendritic arborization. The differences were detected in dendritic geometry among the groups may indicate that functionally different motoneurons use various inputs to control proper timing of movements during feeding. Our group also described that motoneurons involved in different movements of the tongue occupy three distinct areas in the hypoglossal nucleus: the dorsomedial subnucleus (DM) extends rostral to the obex and contains dominantly the motoneurons of protractor tongue muscles. The ventrolateral (VL) subnucleus is mainly localized caudal to the obex, most of the motoneurons innervate the retractor muscles of the tongue, and the intermediate (IM) subnucleus forms a small column 300 μm rostrally from the obex, contains functionally mixed motoneurons.

The majority of muscles involved in swallowing are innervated by the glossopharyngeal-vagal nerves. In the last phase of prey-catching behavior, the captured prey object stimulates the trigeminal afferents in the oropharyngeal cavity which initiates swallowing of the prey. In the frog four groups of motoneurons are distinguished in the **nucleus ambiguus**. The pharyngomotor cells which occupy the rostral 800 μm of the cell column give origin the motor nerves innervating the petrohyoideus muscles. In the middle part of the ambiguus nucleus the visceromotor subdivision consists of cells which were labeled by way of the branches of the vagus

originating from the lung, heart, and part of the gastrointestinal tract. The laryngomotor neurons located in the caudal part of the ambiguus nucleus supply the sphincter muscles of the larynx. The accessory motoneurons which occupy the most caudal part of the nucleus innervate the cucullaris muscle of the frog.

The afferent components of the prey-catching behavior

The appropriate pattern of motoneuron activity during prey-catching behavior is produced by motor pattern generators in the medial reticular formation of the brainstem. The motor command is directed towards the motoneurons and can be modified by sensory inputs exciting the trigeminal, facial, glossopharyngeal-vagal, hypoglossal and second cervical spinal afferent terminals.

The sensory root of the **trigeminal nerve** carries general sensory information from the mechano-, chemo-, and thermoreceptors of the mucous membrane of the oropharyngeal cavity, as well as proprioceptive informations from the jaw closing muscles. The **facial nerve** conveys the information from the mucous membrane of the throat, oral cavity, palate, and nasal cavity. Several branches are responded for chemical stimuli, salty or acidic solutions in the palate region. Proprioceptors of the facial nerve originate in the mouth opening muscles. The activation of **vestibular** afferents is necessary to stabilize the gaze and to fix the position of the head and body during movement of the tongue. The **glossopharyngeal-vagal** autonomic afferents convey information from the alimentary canal and internal organs to the brainstem. The afferents originating from receptors of the heart, lungs, larynx, stomach and baroreceptors of the arteries. The fungiform papillae of the tongue which are stimulated by chemical stimuli are innervated by the peripheral branches of the IX nerve. The axons from proprioceptors carry stretch information of the muscles of pharynx and larynx. The **hypoglossal nerve** in frog contains mechanosensory fibers from the epithelium of dorsal fungiform papillae of the anterior tip of the tongue. Coordinated forelimb

movements during prey-catching demands proprioceptive input sending information about the position of the limbs during movements; in adult frogs the afferents are mainly conveyed in the dorsal root of the **second cervical spinal nerve**.

2. OBJECTIVES

The hypoglossal and ambiguus somatomotor nuclei are essential for prey-catching behavior, because they contain motoneurons innervating muscles for catching and swallowing the prey. The motor command produced by the pattern generators and directed towards the hypoglossal or ambiguus motoneurons can be modified by wide variety of sensory inputs. In order to understand the neural basis of the prey-catching behavior, we need to know how the sensory receptors interact with motoneurons to produce motor output. Recent studies in anurans focused on identifying the afferent pathways involved in prey-catching behavioral pattern in the frog. Our goal was to investigate by using retrograde and anterograde tracing techniques combined with confocal microscopy whether the afferent fibers establish direct connections with hypoglossal and ambiguus motoneurons and to provide a detailed quantitative description of these contacts as well. These monosynaptic connections may give the morpho-functional unit responsible for coding snapping-gulping behavioral pattern during prey capturing.

The first question we addressed was whether the trigeminal afferents of the oral cavity establish direct connections with the functionally different pharyngeal, laryngeal or visceral motoneurons of glossopharyngeal-vagal nerves, and to reveal the spatial distribution of these connections within the ambiguus nucleus.

The aim of our second experiment was to provide a complex connection map of the hypoglossal nucleus, focused on the direct connections of the afferent fibers from the trigeminal, facial, vestibular, glossopharyngeal-vagal, hypoglossal and second cervical spinal nerves. Our quantitative description may provide how sensory information may influence the movements of the tongue.

3. MATERIALS AND METHODS

Preparation and labeling of the nerves

Our experiments were conducted on 95 medium-sized adult common water frogs, *Rana esculenta*. The animals were anesthetized with MS-222 solution applied to the wet skin. The cranial nerves were approached through the oropharyngeal cavity. Under Olympus SZX7 operating microscope, the mucous membrane on the roof of the mouth was sagittally incised, the underlying muscles were cut out, and a portion of the parasphenoidal bone was carefully clipped away to open the cranial cavity. To label the dorsal root of C2 nerve, dorsal approach was performed by cutting the back muscles under the scapula. To study the connections between the ambiguous motoneurons and trigeminal axons, crystals of fluorescein dextran amine (FDA) were applied to the proximal cut end of the trigeminal nerve and tetramethyl-rhodamine dextran amine (RDA) was put on the proximal stump of the IX-X-XI nerves. For mapping the connections of the hypoglossal motoneurons the XII proximal stumps were labeled with FDA, and the rootlets of V, VII, VIII, IX-X, and C2 nerves were labeled with RDA. In one animal one motor nerve and one sensory nerve were labeled simultaneously on the same side by using crystals of the dye. Each sensory-motor combinations were established in three animals.

The operated animals were kept for 3-5 days at the temperature of 12 °C in a wet chamber. During this survival period the dyes were carried by anterograde or retrograde transport into the axons and dendritic trees of neurons.

Histotechniques and microscopic examinations

After the 3-5 survival days the animals were reanesthetized and transcardially perfused with physiological saline and 4% paraformaldehyde fixative in 0.1 M phosphate buffer. The brains and spinal cords were removed and postfixed in 4%

paraformaldehyde then transverse serial sections were cut using freezing microtome at the thickness of 50 μm , the slides are coverslipped with Vectashield Mounting Medium for Fluorescence.

In order to show the location of labeled afferents and neurons in the brainstem, low magnification images were photographed using Olympus camera DP 72 at different levels of the brainstem. On the images we could detect the subnuclei of the motor nuclei and the positions of the afferent tracts.

For confocal analysis three specimens were selected where labeling of the dendritic arbor and afferent terminals seemed to be complete. Confocal images were recorded along the length of the ambiguous nucleus, whereas in the hypoglossal nucleus five sections from the dorsomedial and ventrolateral subnuclei were chosen in each animal. Series of 1 μm thick optical sections were captured from each slide covering the areas where the labeled afferents overlapped the dendritic trees of the motoneurons, through 40x oil-immersion objective. Close appositions between primary afferents and cell bodies or dendrites of hypoglossal or ambiguous motoneurons were identified on individual optical slices, which were considered as direct contacts if there was no discernible gap between the two labeled profiles, and if the contact surfaces were at the same focal plane.

Reconstruction of the ambiguous and hypoglossal nuclei

In order to illustrate the location of motoneurons in the ambiguous and hypoglossal nuclei, the nerves were retrogradely labeled with neurobiotin crystals, after the frogs were transcardially perfused with 2% paraformaldehyde and 1.25% glutaraldehyde. Transverse serial sections were cut with a freezing microtome at a thickness of 50 μm . Sections were visualized with nickel-enhanced diaminobenzidine chromogen (DAB) reaction. The contour of the medulla and the position of the somata of motoneurons in the subdivisions were three-dimensionally reconstructed from serial sections with Neurolucida 8.0 software.

4. RESULTS

Termination of trigeminal primary afferents on glossopharyngeal-vagal motoneurons

Application of RDA to the cut end of the glossopharyngeal, vagus, and accessory nerves resulted in excellent retrograde labeling of the cell bodies and dendritic trees of visceromotor- laryngo- and pharyngo-motoneurons in the ambiguus nucleus. Application of FDA to the trigeminal nerve showed the spinal tract of the trigeminal nerve (tspV), and ventromedially the mesencephalic tract (mesV). Axon collaterals from the spinal and mesencephalic tracts of the trigeminal nerve could be followed into the ambiguus nucleus where they established an overlapping area with the dorsolateral dendritic array of the labeled motoneurons.

Functionally different motoneurons of the ambiguus nucleus

Distribution of labeled trigeminal axon collaterals within the ambiguus nucleus was revealed by spatial reconstruction of the frog's medulla where the V and IX-X-XI nerves were simultaneously labeled with neurobiotin. The overlap between trigeminal afferents and dendrites of IX-X motoneurons is well recognizable along all subdivisions of the ambiguus nucleus, pharyngo-, visceromotor- and laryngomotor subnuclei. In view of the fact that trigeminal axons could not be detected in the caudal part of the nucleus, we excluded the accessory motoneurons from the further examination.

Although different subdivisions of the ambiguus nucleus were established, it was also stated that motoneurons do not form homogenous groups within these areas: the visceromotor neurons populated mainly the middle part of the ambiguus, but they also extended rostrally into the pharyngomotor area where they form 30% of

the total population of the motoneurons and caudally into the laryngomotor subdivisions establishing 17% of the motoneurons.

Trigeminal afferent terminals on the ambiguous motoneurons

In order to confirm close appositions between anterogradely labeled trigeminal axon terminals and retrogradely stained glossopharyngeal-vagal motoneurons, we traced the cell bodies and dendritic trees of motoneurons by using confocal laser scanning microscope. The contacts were identified on 1 μ m thick optical slices of the confocal z-series of the brainstem. Careful analysis of 8079 optical slices obtained from three animals revealed altogether 2285 contacts between trigeminal axon terminals and motoneurons in the ambiguous nucleus. The vast majority of these identified appositions were located on the dendrites (95%) whereas only 5% were encountered on the somata of motoneurons. Regarding the rostrocaudal distribution of the identified contacts, most of the contacted motoneurons were encountered in the pharyngomotor (44%) and visceromotor (45%) parts of the nucleus, whereas the caudally located laryngomotor neurons received only a few axon terminals (11%) from the trigeminal nerve.

Assuming that trigeminal axon terminals established close appositions on the dendritic trees of motoneurons with equal probability, we shared out the number of the trigeminal contacts between the somatomotor and visceromotor neurons according to their proportion in the subnuclei of the ambiguous nucleus. Our data indicated that motoneurons are involved in movements of the pharynx received only one fourth of the trigeminal axons, whereas the remaining axons established connections with the dendritic trees of the motoneurons supplying the stomach, heart and lung. The visceromotor neurons constituted about half of the total population of cells in the ambiguous nucleus, and they obtained about two thirds of the trigeminal contacts. On the other hand, the individual motoneurons are responsible for

innervations of the viscera, received less trigeminal terminals than the neurons supplying the muscles of the pharynx.

Termination of primary afferents on hypoglossal motoneurons

Distribution of functionally different motoneurons in the hypoglossal motor nucleus

We counted 216 ± 20 motoneurons in the DM subnucleus ($n=3$) which was dominated by motoneurons responsible for protraction of the tongue. These motoneurons provide approximately three-fourth of the whole population of this subnucleus, whereas the remaining one-fourth is almost exclusively composed of the retractors, which innervate the hyoglossus muscle. The VL subnucleus contained about the same number of motoneurons as the DM one (180 ± 39). This part of the hypoglossal nucleus comprises mainly the motoneurons of muscles that retract the tongue, and only 12% of motoneurons were responsible for protraction of the tongue. The motoneurons of the inner muscles of the tongue comprise the smallest number of cells in each subnucleus. The intermediate subnucleus is included only 5% of the whole population of hypoglossal motoneurons (21 ± 2).

For quantitative analysis of the orientation of the dendritic trees we calculated the length of the dendritic segments in four quadrants around the soma with the help of Neuroexplorer software. In the DM subnucleus about 75% of the whole dendritic arborization occupied the area located lateral to the somata of the motoneurons, half of these dendritic segments run dorsolaterally, whereas the other half was situated ventrolaterally from the cell body. Quantitative comparison of the length of dendritic trees showed that half of the dendrites originated from the cells supplying the inner muscles of the tongue, one-third of them were owned by protractor motoneurons and the remaining of the dendrites came from motoneurons innervating the retractor muscle. In the VL subnucleus the orientation of the

dendritic arborization seemed to be different according to the function of the motoneurons: in the protractor and inner cell groups about half of the dendrites were found in the dorsolateral quadrant and one-fourth in the ventrolateral one. In contrast, the retractor motoneurons mostly projected dorsolaterally and dorsomedially from their cell bodies. Quantitative analysis showed that 50% of the dendrites were owned by the retractors, and the other 25-25% was shared equally between the protractor and inner cells.

In order to estimate the contribution of functionally different (protractor, retractor, inner) motoneurons to the total length of dendrites we calculated the total length of the dendritic segments possessed by motoneurons in the subnuclei, then we distributed these values according to the number of the functionally different motoneurons in the subnuclei. Our data showed that more than 80% of the dendrites belong to the protractor motoneurons in the DM, and to the retractor motoneurons in the VL, while the inner cells bear less the 5% of the dendrites in both subnuclei.

Distribution of afferent terminals on hypoglossal motoneurons

The labeled axons of **trigeminal nerve** emitted large number of collaterals which branched profusely and covered a large area in the dorsolateral part of the brainstem. These labeled collaterals were observed in both subnuclei where they established closed appositions on dendritic segments of the hypoglossal motoneurons. By using confocal microscopic analysis, 118 direct connections were identified. About two-third of them were encountered in the VL subnucleus which were located on distal segments of the dorsal dendritic arborization. In the DM subnucleus, the connections were situated laterally and significantly closer to the somata.

Labeling of the **facial** nerve is resulted a thin afferent bundle. Since we could not detect any overlap between the labeled facial afferents and the dendritic

trees of hypoglossal motoneurons, the facial afferents were excluded from the further analysis.

The labeled **vestibular** terminals established on average 58 connections with the hypoglossal motoneurons, more than 60% were found on the lateral dendrites of the DM subnucleus and very few terminals were detected on the dorsal dendrites of the VL motoneurons.

In case of labeled **glossopharyngeal-vagal** afferents on average 368 close appositions were identified, more than half were found in the DM, whereas 42% in the VL subnucleus. In both subnuclei most of these connections were detected in close vicinity or on the surface of the cell bodies. Concerning the orientation of these connections, the terminals preferred the lateral dendritic segments in the DM subnucleus, and the dorsal dendritic trees of the motoneurons in the VL subnucleus. The statistical analysis did not show significant differences between the two hypoglossal subnuclei regarding the number and distribution of the glossopharyngeal-vagal terminals.

In the DM subnucleus the **hypoglossal** collaterals established few contacts (41 ± 10) with the dorsolateral dendritic trees. In the VL subnucleus they formed over 300 close appositions on the proximal dendritic segments of the dorsally oriented dendritic trees, which significantly differs between the two subnuclei.

In the DM subnucleus the labeled **second cervical spinal** terminals formed large number of contacts with the labeled dendrites in the VL subnucleus (118), which was distributed almost evenly along the dorsal dendritic segments. In the DM subnucleus there were significantly less terminals.

Comparison of different nerve terminals on hypoglossal motoneurons

For quantitative description we investigated 14250 optical slices from 15 brainstems and detected altogether 3034 connections on the hypoglossal

motoneurons. In one animal we found on average 1026 close appositions. About one third of them were located in the DM and two-third in the VL subnucleus.

In the DM subnucleus more than half of the identified contacts (356) were established by axon terminals of the glossopharyngeal-vagal nerves, whereas the other nerves provided approximately 10%-10% of the terminals. Apart from the large numbers, the terminals of the glossopharyngeal-vagal nerves differed from the other ones concerning their distributions. The majority of the glossopharyngeal-vagal terminals (83%) concentrated within less than 300 μm distance from the cell body resulting significantly smaller average distance than any other terminals. The VIII, XII and C2 terminals were located mainly on the middle part of the dendritic trees, whereas the trigeminal contacts were located farther from the cell bodies than any other terminals. When we compared the position of the contacted dendrites, we found that most of the terminals were located on the lateral dendritic segments. The glossopharyngeal-vagal nerves established contacts with the dorsolateral and ventrolateral dendrites in almost equal number, whereas the other nerves preferred the dorsolateral area where more than 70% of their terminals were located. The majority of axon terminals targeted at the protractor motoneurons. Almost half of the identified contacts were established between the axon terminals of the glossopharyngeal-vagal nerves and the dendritic segments of protractor motoneurons. As a result of the high number of contacts on the proximal dendritic segments, we can suppose that stimulation of the glossopharyngeal-vagal nerves can strongly influence the function of the hypoglossal motoneurons. Comparing to the glossopharyngeal-vagal nerves, all other afferents established significantly smaller number of connections which were situated farther from the cell body. The trigeminal, vestibular and hypoglossal nerves contributed their terminals equally on the protractor, retractor and inner hypoglossal motoneurons. The motoneurons innervating the inner muscles of tongue received very few (usually 1-2) direct contacts from terminals of any nerves studied.

The dendritic trees of motoneurons in the VL subnucleus received on average 670 axon terminals. About half of them belonged to the afferent fibers of the hypoglossal other nerve, one-fourth of the terminals were owned by the glossopharyngeal-vagal nerves, whereas the remaining boutons were distributed among V, C2 and VIII nerves. The axon collaterals from the glossopharyngeal-vagal nerves terminated on the proximal dendrites of the hypoglossal motoneurons, significantly closer than terminals of V and VIII nerves. The XII and C2 axons contacted the middle part of the dendritic arborization, whereas the V and VIII boutons were located significantly farther from the cell bodies than the hypoglossal ones. The majority of connections were found on the dorsolateral and dorsomedial dendritic arrays. When we calculated how the identified connections could be shared among functionally different VL motoneurons, we found that more than 80% of terminals were located on the somata and dendritic segments of motoneurons retracting the tongue and the majority of them were established by axon terminals of the hypoglossal, glossopharyngeal-vagal and C2 afferents. The protractor motoneurons received only 14% of the terminals.

In essence, the motoneurons in the VL subnucleus received two-third of the afferent terminals, and most of them originated from three nerves: the hypoglossal (45%), the glossopharyngeal-vagal (23%) and the C2 (18%). These axons terminated mostly on the dorsal dendritic segments of the retractor motoneurons. The dendritic segments receiving the IX-X and XII terminals were located rather close to the cell body, therefore they can modify the electrical properties of the motoneurons relatively easily. Based on the position of the C2 terminals on the middle part of the dendritic trees, we suppose their moderate effect on the activity of hypoglossal motoneurons. Due to the small number of connections which were very far from the cell body, the V and VIII nerve can induce only small changes on the somata of the motoneurons.

5. DISCUSSION

In our experiments we studied the sensory-motor pathways modifying the prey-catching behavior of the frog. Our investigations focused on the affect of the sensory afferents of the oral cavity or the proprioceptors of the mouth closing muscles on the ambiguous motoneurons. We also studied the distribution of the different terminals on the functionally different motoneurons of the hypoglossal motor nucleus.

Connections between trigeminal terminals and ambiguous motoneurons

In *Ranid* frogs, the key stimulus of prey-catching behavior is provided by a visual input. The frogs recognize, localize and analyze the prey. Once these sensory processes have been carried out, the immediate motor response occurs within a very short period of time. Although the spatio-temporal pattern of activity of motoneurons is determined, various sensory modalities interact with the motor commands to achieve the optimal motor responses. One of the sources of sensory modulation is the trigeminal nerve, which is known to be important in the proper coordination of jaw and tongue muscles. In anurans, the sensory fibers of the trigeminal nerve related to the prey-catching behavior originate in the mechanosensory receptors of the oral mucosa including the tongue, and in the proprioceptors of jaw muscles. The oral mechanoreceptors are stimulated by the captured prey during the initial phase of tongue retraction whereas the proprioceptors of the jaw closing muscles are stimulated continuously during tongue protraction and retraction.

Quantitative analysis on the number of close contacts between the trigeminal axon terminals and functionally different motoneurons of ambiguous nucleus showed that the pharyngomotor neurons have considerably higher number of close contacts than the motoneurons supplying the laryngeal muscles. It is consistent with the pivotal role of pharyngeal musculature in the execution of swallowing. The

laryngeal muscles do not participate directly in the transportation of captured prey, albeit they have important accessory function by narrowing and dilating the laryngeal inlet during swallowing. We have found the largest number of close contacts on the visceromotor neurons. This connection was not yet identified in anurans. In the rat, morphological evidence was given for the monosynaptic connections between the trigeminal proprioceptive afferents and visceromotor neurons of the ambiguus nucleus providing anatomical substrate for the jaw-visceral coordination. Electrophysiological and morphological studies revealed the reflex pathway as polysynaptic projection via the various regions of reticular formation to the visceromotor neurons of ambiguus nucleus.

The identified direct contacts presume monosynaptic pathways, the combination of the direct and indirect trigeminal routes provide the proper coordination of the motoneurons.

Connections between primary afferents and hypoglossal motoneurons

We demonstrated direct connections between trigeminal, vestibular, glossopharyngeal-vagal, hypoglossal and second cervical spinal afferent terminals and hypoglossal motoneurons. The afferent terminals of the facial nerve are missing at this level of the brainstem, presumably the proprioceptive information of the mouth opening muscles is carried on polysynaptic pathways. Presumably these monosynaptic connections may serve as one of the morphological substrates of the fast response during feeding movements of amphibians.

Distribution of primary afferent terminals in the subnuclei of XII nerve

We have counted two thirds of the identified sensory-motor close appositions in the **ventrolateral subnucleus (VL)**; more than 80% of them were found on the dendrites of the retractor motoneurons followed by the protractors and then the inner ones. Our results indicated that the most powerful monosynaptic input

from the afferent fibers to the motoneurons of VL subnucleus is provided by the hypoglossal nerve. Although the IX-X nerves gave rise to only 22% of close contacts, their soma-near position on the proximal dendrites is suggestive of being the second most efficient monosynaptic signal from the afferents studied. The rest of terminals originated in decreasing number from the C2, trigeminal, and vestibular nerves. This finding suggests that the monosynaptic influence of trigeminal and vestibular afferents on the activity of the XII motoneurons in the VL subnucleus is probably less important, especially in case of the VIII nerve with its 3% contribution to the total number of terminals.

The majority of the identified close appositions in the **dorsomedial subnucleus** (DM) (approximately 60%) arrived from the sensory fibers of IX-X nerves. The large number and location of the close appositions, which were distributed almost exclusively on the perikarya and proximal dendrites, are in favor of strong synaptic influence of the IX-X nerves on the activity of tongue motoneurons. The rest of the identified close appositions (approximately 40%) to the motoneurons of the DM subnucleus arrived from the trigeminal, vestibular, hypoglossal, and second cervical spinal afferents. The contacts established by them were located in more distal parts of dendrites and concentrated in the middle zone of the dendritic trees. This finding suggests a less effective monosynaptic activation of XII motoneurons by the trigeminal, vestibular, XII and C2 fibers compared to the IX-X afferents.

Possible role of primary afferent terminals of hypoglossal motoneurons in the prey-catching behavior of the frog

The sensory fibers of **IX-X nerves** are mostly visceral afferents from the oropharyngeal mucosa, respiratory tract, heart and stomach, whereas the lesser amount of fibers might represent the mechanosensitive afferents from the oral and lingual mucosa and proprioceptive afferents from the pharyngeal and laryngeal

muscles. However, no data are available whether the IX afferents have monosynaptic contact with the retractors, and whether the protractor and inner motoneurons can be activated directly by the IX nerve. Our results show that the IX afferent fibers do establish direct contacts with the protractor, retractor and inner motoneurons. According to biomechanical studies, the tongue position has major impact on the ventilation in mammalian species, helps to maintain the pharyngeal airway patency by preventing the tongue from being sucked against the posterior pharyngeal wall during inspiration. The close appositions suggest the possibility of direct activation of hypoglossal motoneurons from the periphery. These monosynaptic contacts make possible a very effective and quick response for the threatening closure of airway apparatus.

The **trigeminal nerve** is important in proper coordination of jaw and tongue muscles during prey-catching behavior in anurans. Activity-dependent dye uptake after stimulation of either the hypoglossal or trigeminal nerve suggested polysynaptic impulse transmission from the trigeminal afferents to the XII motoneurons. Our findings showed that an additional monosynaptic trigemino-hypoglossal pathway can provide sensory feedback on the position of the jaw.

The **XII afferents** control jaw and tongue movements.. Previous neuronal labeling studies revealed polysynaptic routes from XII afferents to the XII motoneurons, our results indicate that the XII afferent fibers can activate both the protractor and retractor motoneurons, however, their influence could be much stronger to the protraction phase of prey-catching behavior. In addition, the XII afferents may also influence the respiration.

Previous physiological studies demonstrated polysynaptic activation of the XII nerve after stimulation of **vestibular nerve** in frog. In various mammalian species, the activity of protractor motoneurons is modified via polysynaptic pathways in relation to the body position in order to help the maintenance of airway patency. Our results indicate the presence of monosynaptic vestibulo-hypoglossal

connection, which provides the morphological background of a quick reaction in response to postural changes during the prey-catching behavior.

The afferents of the **second cervical spinal segment** convey information from the proprioceptors of the forelimb muscles. Sensory information provided by the C2 fibers may be important in the integration between feeding and locomotion. The positioning of the head before capture is achieved by extension of the forelimbs, and during the whole course of prey-catching behavior the fixation of limbs is essential to maintain the body position. The influence of forelimb afferents on XII motoneurons was not yet experimentally established; based on the termination area of C2 fibers in the brainstem both mono- and polysynaptic routes could be postulated in the frog. The small number of direct contacts from the C2 and their position on the dendritic tree suggest that the monosynaptic influence of the C2 fibers is insignificant on those motoneurons of tongue that are located in the DM subnucleus.

Functional implications of the contacts between primary afferent terminals and the motoneurons of XII nerve

The close appositions between terminals of V, VIII, IX-X, XII and C2 fibers and functionally different types of hypoglossal motoneurons suggesting a possible co-activation of protractor, retractor and inner motoneurons. However, our present findings also indicate that the strength of monosynaptic input provided by various afferent fibers to the functionally different XII motoneurons is not identical from many points of view. (1) The total number of close appositions is different from the various sensory fibers. Our results showed that approximately two thirds of total close appositions on the XII motoneurons arrived from the IX-X and XII nerves. During the earlier phase of events the feedforward chemical signal, carried by the IX nerve, is important in choosing between the capture and subsequent swallowing or avoidance and rejection of prey. When the prey contacts with the surface of the tongue and it is palatable, the very quick signal which is produced by the tongue

mechanoreceptors and carried via the XII afferents initiates the retraction of the tongue without delay. (2) The IX-X nerves gave about half of the terminals in the DM subnucleus, where more than 70% of motoneurons innervate the protractor muscles suggesting that the IX-X nerves may exert stronger influence on the activity of protraction, whereas the XII afferents are more important in the retraction. In contrast, the retractor motoneurons comprised about 80% of cells in the VL subnucleus where half of the terminals on the motoneurons arrived from XII terminals. (4) The localization of close appositions along the dendritic tree showed unequal distribution from different nerves and was variable on the functionally different motoneurons of the tongue. Thus, the IX-X terminals were nearest to the perikaryon followed by the XII nerve terminals located mostly in the proximal and middle parts of the dendritic tree. The C2 terminals concentrated on the middle part of dendritic tree in the DM subnucleus, whereas they were evenly distributed on the entire length of dendrites in the VL subnucleus. The close appositions of the trigeminal nerve were located in on the middle and distal parts of the dendritic tree. The terminals of VIII nerve were closer to the cell body in the DM subnucleus where they located in the proximal and middle parts of the dendritic trees, whereas most than half of terminals in the VL subnucleus were located on more remote dendrites.

In case of the IX-X, XII and C2 nerves the distance of direct contacts from the perikaryon did not show significant differences in the two subnuclei, in contrast the distance significantly differs in the V nerve.

Corollary consideration on direct sensory-motor connections in the prey-catching behavior of the frog

During the predator behavior the activity of motor nuclei innervating different muscles can be modified directly via the neurons of the MPG or indirectly via various primary afferents. These polysynaptic pathways with the combination of the monosynaptic ones provide a processed control of the predator movements.

Significant number of direct contacts was found between the glossopharyngeal-vagal or hypoglossal afferents and hypoglossal motoneurons. The influence of trigeminal, vestibular or C2 afferents on the activity of motoneurons is probably less important, while the facial terminals were not established contacts with the motoneurons in our study.

To summarize our data, the trigeminal input contributed to the control of the activity of the ambiguus motoneurons during the swallowing phase of the prey-catching behavior. Direct contacts between the primary afferent terminals and XII motoneurons may provide one of the possible morphological substrates of very quick feedback and feedforward modulation of motor program during various stages of prey-catching behavior. Combination of direct and indirect sensory inputs may contribute to optimize the ongoing motor execution.

6. SUMMARY

The prey-catching behaviour of frogs consists of a sequence of coordinated activity of different muscles corresponding to various stages of feeding. The activity of motoneurons responsible for coordinated activity of muscles can be modified indirectly via interneurons or directly via sensorimotor connections between afferent and efferent systems in the brainstem. In the present Thesis the sensorimotor integration was studied in two efferent components of the neuronal networks underlying prey-catching behaviour: the ambiguus nucleus which is necessary for gulping and visceromotor functions, and the hypoglossal nucleus responsible for contraction of tongue muscles. The aim of our experiments was to study whether the afferent fibres establish direct connections with the motoneurons in motor nuclei, and to provide a map on the distribution of the connections in the motor nuclei.

- Confocal micrographs showed direct contacts between sensory terminals of trigeminal nerve and motoneurons of the ambiguus nucleus.
- The contacts were not evenly distributed between the subnuclei: two-third of the afferents terminated on the visceromotor motoneurons of the stomach, heart and lung.
- Large number of terminals were found on the pharyngo-motoneurons, while the number of connections on the laryngo-motoneurons was insignificant.

We also demonstrated direct connections between trigeminal, vestibular, glossopharyngeal-vagal, hypoglossal and second cervical spinal afferent terminals and hypoglossal motoneurons. The collaterals of the facial afferents are absent at the level of hypoglossal motor nucleus, suggesting that the proprioceptive information from the mouth opening muscles is carried through polysynaptic pathways.

- Based on the highest number of the connections and their closest location to the soma, we presume that the glossopharyngeal-vagal afferents can exert the strongest effect on the different motoneurons in the hypoglossal nucleus, and these contacts influence both the protraction and the retraction of the tongue.

- The hypoglossal afferents can activate the retraction, but during protraction they do not play important role.
- The proprioceptive information from the muscles of the upper limb, carried by the C2 afferents, can modify the retraction of the tongue.
- The monosynaptic influence of trigeminal and vestibular afferents on the activity of the hypoglossal motoneurons is probably less important; the stimulus of the oral mucosa by the prey animal and proprioceptive information of mouth closing muscles may contribute to the retraction of tongue via the trigeminal nerve, while the vestibular information may influence the protraction.

We conclude that these monosynaptic connections may serve as one of the neuro-morphological substrates of the fast response during feeding movements of amphibians that gives the reflex-like ability of prey-catching behaviour.

7. LIST OF PUBLICATIONS



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Registry number: DEENK/22/2015.PL
Subject: Ph.D. List of Publications

Candidate: Szilvia Kecskés
Neptun ID: YFDDM0
Doctoral School: Doctoral School of Neurosciences
Mmt ID: 10037409

List of publications related to the dissertation

1. **Kecskés, S.**, Matesz, C., Gaál, B., Birinyi, A.: Neural circuits underlying tongue movements for the prey-catching behavior in frog: Distribution of primary afferent terminals on motoneurons supplying the tongue.
Brain Struct. Funct. Epub ahead of print (2015)
DOI: <http://dx.doi.org/10.1007/s00429-014-0988-1>
IF:4.567 (2013)
2. **Kecskés, S.**, Matesz, C., Birinyi, A.: Termination of trigeminal primary afferents on glossopharyngeal-vagal motoneurons: Possible neural networks underlying the swallowing phase and visceromotor responses of prey-catching behavior.
Brain Res. Bull. 99C, 109-116, 2013.
DOI: <http://dx.doi.org/10.1016/j.brainresbull.2013.09.006>
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List of other publications

3. **Kecskés, S.**, Gaál, B., Rácz, É., Birinyi, A., Hunyadi, A., Matesz, C.: Extracellular matrix molecules exhibit unique expression pattern in the climbing fiber-generating precerebellar nucleus, the inferior olive.
Neuroscience. 284, 412-421, 2015.
DOI: <http://dx.doi.org/10.1016/j.neuroscience.2014.09.080>
IF: 3.327 (2013)
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Brain Behav. Evol. 83 (2), 104-111, 2014.
DOI: <http://dx.doi.org/10.1159/000357751>
IF: 4.288 (2013)
5. Rácz, É., Gaál, B., **Kecskés, S.**, Matesz, C.: Molecular composition of extracellular matrix in the vestibular nuclei of the rat.
Brain Struct. Funct. 219 (4), 1385-1403, 2014.
DOI: <http://dx.doi.org/10.1007/s00429-013-0575-x>
IF: 4.567 (2013)
6. **Kecskés, S.**, Kőszeghy, Á., Szűcs, G., Rusznák, Z., Matesz, C., Birinyi, A.: Three-dimensional reconstruction and quantitative morphometric analysis of pyramidal and giant neurons of the rat dorsal cochlear nucleus.
Brain Struct. Funct. 218 (5), 1279-1292, 2013.
DOI: <http://dx.doi.org/10.1007/s00429-012-0457-7>
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Total IF of journals (all publications): 24,29

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