CONNECTIONS OF THE VESTIBULAR NUCLEAR COMPLEX IN THE FROG AND RAT

TIMEA BACSKAI

Ph.D. THESIS

TUTOR: DR. CLARA MATESZ

2004, DEBRECEN

Introduction

The nervous system and its specialized parts, the sense organs receive information from the external and internal environment. The signals are conveyed from the receptors to the specific regions of the nervous system where the information processing takes place. The nervous system coordinates the function of different organs and maintains the homeostasis of the whole organism. The knowledge of normal structure and function of the nervous system is essential to understand the different pathological processes of neural disorders. Different methods, including the neuronal tracing techniques, have been recently developed to study the neuromorphological and neurophysiological characteristics of the nervous system; however, our knowledge is far from being perfect.

The aim of this work was to study some morphological aspects of the vestibular system that plays an important role not only in the coordination of the body movements but also in the regulation of different vegetative functions. Despite many investigations on the central connections of the vestibular system, a lot of contradictions can be found in the description of morphological background of the vestibular function in the physiological circumstances and during the compensatory processes of the vestibular lesion.

Primary afferent fibers originating in the vestibular sense organs terminate on second-order vestibular neurons of the superior (SVN), medial (MVN), lateral or Deiters (LVN) and descending (DVN) vestibular nuclei of the lower and higher vertebrates. Primary afferent vestibular fibers are connected to the secondary neurons with chemical and electrical couplings. The electrical synapses may enhance impulse transmission, and recruit inactive afferent fibers impinging on the same neuron. In anamniotes the electrical transmission was found in those parts of the central nervous system in which very fast reactions are essentials for the survival of animals. The morpological substrate of electrical coupling is the gap junction. It is not known wheater the primary afferent vestibular fibers form electrical couplings in their cerebellar termination areas. Secondary vestibular projections originating from the vestibular nuclei can be classified as ascending, descending and cerebellar pathways. In addition, we can find projections to the ipsi-, and contralateral vestibular

nuclear complexes. Among the vestibular nuclei, the superior vestibular nucleus has an important role in the vestibuloocular reflex (VOR). This nucleus sends ascending fibers towards the mesencephalic eye-movnig motor nuclei. There are several physiological studies on the inhibitory nature of this connection, but the underlying morphological and neurochemical network is unknown. The descending fibers originating in the vestibular nuclei play an important role in the regulation of motor functions. The origin, projection and the termination areas of these vestibulospinal pathways are not yet studied with modern neuronal tracing methods. The descending and ascending projections have widespread connections with those parts of the reticular formation which can regulate not only the motor but also the vegetative functions. In spite of the large number of physiological experiments, the connections between the individual vestibular nuclei and the different parts of the reticular formation are not unequivocally described in different species.

Aims of the studies

We decided to investigate the following aspects of the vestibular system.

1. Revealing the connections between the primary afferent vestibular fibers and their postsynaptic neurons in the cerebellum of the frog.
2. Mapping the antero-, and retrograde connections between the lateral vestibular nucleus and different parts of the central nervous system of the frog.
3. Mapping the ascending and descending connections between the individual vestibular nuclei and the central nevous system of the rat.
4. Revealing the neurochemical features of synapses between the superior vestibular nucleus and the oculomotor nucleus of the mesencephalon.

Materials and methods

Our experiments were carried out on common water frogs, Rana esculenta and Wistar rats.

1. Dye-coupled connections:

'Dye-coupling' refers to the passage of low molecular weight neuronal tracers through gap junctions, and these tracers are used as indicators of gap junctional couplings. Our experiments were carried out on 35 water frogs (Rana esculenta) under MS 222 anaesthesia. The trigeminal and vestibulocochlear nerves were approached from ventral position, the dorsal surface of the spinal cord was exposed by laminectomy over C2 and L9 dorsal roots. The appropriate nerve was cut and its proximal end was introduced into a small glass tube containing Neurobiotn (NB; 5%), or Lucifer yellow (2-4%). The tubes were closed by silicone oil and stuck to the surrounding tissues. After the survival period (3-6 days) the animals were reanaesthetized and perfused transcardially with isotonic saline followed by fixative containing 2% paraformaldehyde, and 0.25% glutaraldehyde. The 60 μm sections of the spinal cord and brainstem were incubated with ABC reagent then with nickel-DAB solution. For electronmicroscopical experiments we embedded the sections of the cerebellum (see it below).

In control experiments we used 1% of glycyrrhetinic acid (GRA) solution which blocks the gap junctions. The application of Neurobiotin (see above) was preceeded by pressure.

In other parts of the control experiments we used bitotinylated-dextrane-amine (BDA), or cobalt-lysine on the proximal end of the appropriate nerve. The BDA labeling was visualized by ABC reagent and nickel-DAB after the survival period of 5 days. The Co^{3+}Lys labeling was precipitated by hydrogen sulphide and then visualized by physical developer.

2. Neuronal tracing techniques:

In these experiments we used a lectin Phaseolus-vulgaris leukoagglutinin (PHA-L) (Gerfen and Sawchenko, 1984) as a neuronal tracer. This lectin can be transported both in antero-, and retrograde directions in the frog and in anterograde direction in mammalian species. The tracer was injected by glass micropipette filled with PHA-L. A positve direct current of 5 μA was used for the injection, with a pulse duration of 7s, followed by 3s intervals for a period of 10-20 minutes. The injection sites were determined with the aid of the stereotactic atlas of Paxinos and Watson (1986) in the rat, and the coordinates of Kemali and Braitenberg (1969) were used in the frogs. After a survival period of 5-14 days (depending on the transport of the tracers) the animals were transcardially perfused by isotonic saline, followed by fixative solution. In some experiments the BDA and NB were used for the injections. The 60 μm sections of the brainstem and spinal cord were incubated in appropriate solutions. The reactions were completed by Ni-DAB chromogen in all cases.

3. Electronmicroscopical studies:

We used the 60 μm sections of the rat mesencephalon described above. During preembedding immunhistochemical procedure we incubated the sections in biotinylated anti-PHA-L dissolved in normal goat serum (1:2000), for two days. The reaction was completed by DAB chromgen. After the osmium-tetroxide treatment we embedded the sections into Araldite. After the polimerization of the Araldite, we made semithin (0.5 μm) sections and, than ultrathin (60 nm) serial sections. During postembedding immunohistochemical process – after removing of the osmium and the Araldite – we used anti-GABA (1:1000). After incubation in 1% bovine serum albumin the reaction was visualized by secondary antibody (immunogold conjugated goat anti-rabbit, 1:20) which was connected with golden granules (20 nm).

The fibers, terminals and cells were reconstructed by camera lucida or Neurolucida. The microscopic photographs were taken with Nikon Eclipse microscope, and JEOL Electronmicroscope.

Results and conclusions

Frog

Dye-coupled connections of the primary afferent vestibular fibers:

In the literature those neurons which can be labeled from one another by low molecular weight tracers through the gap junctions are called dye-coupled neurons. Application of the Neurobiotin (NB) to the vestibulocochlear nerve resulted in labelling the mossy fibers and granule cells in the auricular lobe of the cerebellum. The axons of the granule cells could be followed to the molecular layer, where they formed synaptic connections with the Purkinje cells. Revealing the presence of „dye-coupled" connections in the vestibular system we applied the low molecular weight tracers to those parts of the nervous system that are also involved in the coordination of movements, e. i. to the primary afferent fibers of the trigeminal nerve and to the dorsal roots of the cervical and lumbar spinal nerves. We could detect dye-coupled neurons in the dorsal horn of spinal cord, in the cerebellum and in the brainstem. Application of Lucifer yellow to the appropriate nerve neurons were detected in the same positions as they were labeled in the case of NB labeling. In control experiments – when we applied high molecular weight tracers to the primary afferent fibers – we could not detect any postsynaptic neurons, only the primary afferent fibers and terminals were labelled. Our results suggest that in addititon to chemical synaptic connections established between the primary afferent fibers and their target cells, information is also transmitted via electrical couplings.

Connections of the lateral vestibular nucleus:

We have chosen to study the connections of the lateral vestibular nucleus for the following reasons. The LVN is the main recipient of the primary vestibular afferents from the utricular macule that plays a main role in equilibrium. The strongest vestibular influence on the frog spinal cord is mediated by way of the LVN. Most of the physiological experiments in the frog have been performed on this nucleus. The LVN shows the closest similarity in its structure and fiber connections between the mammalian and amphibian LVN. After the PHA-L injection into the LVN, the anterograde fibers could be followed to the level of the

diencephalon. This kind of diencephalic connection was unknown earlier. To confirm these results and to exclude the possibility of false labelling we injected BDA, a retrograde tracer into the thalamus. After BDA injection we could detect labeled cells only in the LVN and other vestibular nuclei and we have never found retrogradely labeled neurons in the cochlear nucleus, suggesting that the vestibular neurons directly reach the thalamus. Most of the fibers of the LVN origin projected into the dorsal thalamus, and a somewhat weaker projection was detected onto the ventral thalamus. The same thalamic nuclei are recipients of acoustic and somatosensory impulses in the frog. There is, therefore, an extensive overlap in the thalamic projections of the acoustic, somatosensory and vestibular information in anuran species suggesting the integration of these sensory modalities. The other functional significance of the vestibulothalamic projection is its involvement in the motor function. The central and anterior thalamic nuclei of the dorsal thalamus, that have also a robust connection to the striatum, may have a significant contribution to the afferent inflow of the motor system providing multimodal information. The results of our study have demonstrated reciprocal connection between the thalamus and the LVN of the frog.

At the level of the **mesencephalon** the majority of the ascending fibers from the LVN terminate within the ipsilateral oculomotor and trochlear nucleus. It suggests that the LVN has an important role in the coordinated eye movements in the frog. We have showed that the mesencecphalic tegmental nuclei are reciprocally connected with the LVN. The functions of these tegmental nuclei are almost entirely unknown in the anuran species, they probably take part in the transmission of proprioceptive information. We have described the strongest anterograde and retrograde labeling in the **rhombencephalon**. At this level the LVN has widespread connections with the different parts of the reticular formation especially with the lateral reticular zone. This area may represent the mammalian lateral tegmental field (LTF). The LTF is regarded as an integrative center of vegetative functions insofar as it is involved in cardiovascular control, and in respiration. We could detect antero-, and retrograde labeling in the ventrolateral part of the caudal medulla this area was designated as the inferior olive in mammalian species. We could find connections of the LVN with the other ipsilateral vestibular nuclei. These so-called intrinsic connections have not been

demonstrated previously in lower vertebrates. We have also demonstrated the terminals of LVN origin in the contralateral vestibular nuclei establishing commissural pathways. According to our results the LVN has only a minor contribution to the vestibular commissural connections. The descending fibers originating in LVN were found in all funiculi of the **spinal cord**. The majority of the PHA-L labeled terminals could be detected in the ventral horn of cervical segments, and they gradually decreased in the caudal direction. Another part of the descending vestibular boutons were detected in that part of the spinal grey matter which has been designated as the triangular area. This area receives large calibre dorsal root fibers which probably represent muscle afferent fibers. The retrogradely labeled neurons may represent the origin of the spinovestibular tract. From our observations we conclude that the triangular area is the major site of origin of the spinovestibular tract. We have found bilateral reciprocal connections of the LVN with the **cerebellum**. In the frog, primary afferent fibers of the vestibular nerve terminate in the auricular lobe this was the major termination area of the fibers of LVN origin. We could find retrograde connections between the LVN and deep cerebellar nucleus.

Rat

Afferent and efferent connections of the vestibular nuclei:

The efferent connections of the SVN and MVN were studied by applying of PHA-L, whereas the afferent and efferent projections of DVN and LVN were examined following the NB into the corresponding nuclei. The rostralmost projection from all vestibular nuclei was detected in the **diencephalon** bilaterally. Most of the fibers originated in SVN and MVN and terminated in the ventral posteromedial nucleus (VPM) of the thalamus which receive somatosensory pathways (medial lemniscus, spinothalamic tract). It suggests that the VPM has an important role in the integration of the proprioceptive and vestibular inputs that are important to maintain the position of the body. In the **mesencephalon** the strongest projection was demonstrated onto the ipsilateral eye moving motor nuclei. Earlier physiological experiments described, that the SVN has main role in the vestibuloocular reflex (VOR), and GABA antagonists block the inhibitory

postsynaptic potentials in the oculomotor nucleus after stimulation of the vestibular nerve in the cat. This finding suggested strong GABAergic projections from the SVN. In our electron microscopical experiments, we have found that the terminals of SVN origin established symmetrical connections with the oculomotor neurons and the majority of these terminals showed a positive GABA immunoreactivity. In the mesencephalon, the other termination area of all vestibular nuclei was the red nucleus. This nucleus has widespread connections with different parts of the central nervous system, and plays an important role in the coordination of the motor activity. Because this connection of the vestibular nuclei has not been previously described, no data are available about the excitatory or inhibitory nature of the vestibulorubral connection. The red nucleus receives cortical and cerebellar fibers and the rubrospinal, rubrobulbar and rubroolivar pathways are derived from this nucleus. On the basis of our electron microscopical studies we conclude that the vestibular system exerts an inhibitory influence mediated by GABAergic synaptic transmission on the activity of these pathways. We could detect labeled fibers and terminals from all vestibular nuclei in the periaqueductal grey matter (PAG). It suggests that the vestibular system can modify the activity of different sensory and motor parts of the nervous system by way of the widespread connections of the PAG. We could not detect retrogradely labelled cells either in the diencephalon or in the mesencephalon. In the **rhombencephalon**, we have demonstrated a very extensive projection of all vestibular nuclei into the different parts of the reticular formation. The weak connection demonstrated between the vestibular nuclei and the visceromotor and viscerosensory cranial nerve nuclei suggests that the vestibular system can modify the activity of vegetative functions through the reticular formation. We could detect the terminals of vestibular nuclei in those structures of the rhombencephalon that are involved in the integration of proprioceptive (dorsomedial part of the reticular formation, nucleus prepositus hypoglossi, inferior olive, etc.) and somatosensory (dorsal column nuclei) information. We have found a well-developed intrinsic connectivity between the ipsilateral vestibular nuclei. We could also detect strong commissural projections between the bilateral MVN, SVN and DVN, whereas this kind of connection was relatively weak in the case of the LVN. Retrogradely labelled cells originating in LVN and DVN were found in the reticular formation, in vestibular nuclei on both sides and

in the inferior olive. It means that the inferior olive can modify the activity of LVN fibers projecting towards the cerebellum. In the **spinal cord** most of the descending vestibular fibers were found in all funiculi with an ipsilateral dominance. We have found, contrary to the previous data, that the SVN also contribute to the formation of vestibulospinal projection. The terminals were distributed mainly in all Rexed laminae – except the I and II -, and their number decreased in the caudal direction. A small number of retrogradely labelled cells could be followed to the sacral segments of the spinal cord, indicating that the information from the spinal cord into the vestibular nuclear complex is transmitted by way of the reticular formation.

SUMMARY

Applying of low molecular weight neuronal tracers to the primary afferent fibers we could detect labeling of their secondary vestibular neurons and granular cells in the cerebellum of the **frog**. The dye-coupled neurons suggest the presence of electrical (gap junction) couplings.

We have mapped the antero- and retrograde connections of the lateral vestibular nucleus (LVN) in the **frog** by using neuronal tracing techniques. In the diencephalon we could find vestibular efferent terminals in the thalamus, -which was unknown earlier- in a manner similar to that of mammalian species. At the level of the mesencephalon we could detect the termination areas of vestibular fibers in the eye moving motor nuclei, in the tegmental nuclei and the in the nucleus of medial longitudinal fascicle. We could find widespread reciprocal connections between the LVN and the reticular formation. It suggests that the frog reticular formation plays an important role in the transmission of vestibular information. We have described reciprocal connections of the LVN with the areas of central nervous system receiving proprioceptive information. We have found the location of vestibulospinal tract formed by descending fibers of LVN in the whole lenght of the spinal cord on both sides. We have described the cells of origin of the spinovestibular tract.

In the **rat** we could demonstrate the anterograde connections of the vestibular nuclei with different structures of the central nervous system. We have described the previously unknown connections of the superior vestibular nucleus with the spinal cord. At the level of mesencephalon we have described for the first time the termination of secondary vestibular fibers in the red nucleus. The electronmicroscopical studies revealed inhibitory and GABAergic nature of this connection. This result suggests that the NVS can modify the activities of the cortico-rubral and cerebello-rubral pathways by the inhibition of the neurons of the red nucleus. By using of tract tracing techniques we gave a detailed description of the vestibulo-reticular connections. We could detect reciprocal connections between NVL and the rhombencephalon as well as the spinal cord. Our results are supported by earlier physiological observations.

Our experiments revealed widespread connections between the vestibular nuclei and different structures of the central nervous system. These results can help us

to understand the stucture of the vestibular system and the morphological background of the vestibular lesion and the subsequent compensatory mechanism.

PUBLICATIONS

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