THE STRUCTURE OF THE ACCESSORY OPTIC NUCLEI: THE NUCLEUS OF THE BASAL OPTIC ROOT (nBOR) AND THE NUCLEUS LENTIFORMIS MESENCEPHALI MAGNOCELLULARIS (nLMmc) IN THE CHICK

Thesis

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INTRODUCTION

In contempt of constantly altering environment or motion, or rather of the movements of head and neck, actions guiding and harmonizing eye movements maintain a stable retinal picture, which results in a “fixative look” of birds and probably of most vertebrates. Behavioural responses that stabilize the movements of the eye are optokinetic and vestibulo-ocular reflexes whereas optomotor and vestibulo-collicular reflexes are responsible for the relative fixation of the head. Although these four eye-stabilizing reflexes experimentally are experimentally separable, they act simultaneously in vivo. Whenever a body or visual environment makes a motion a head’s and an eye’s compensatory – equalizing, additional – actions step in, which stabilize a retinal picture. The compensatory motions of head and neck are particularly well observable in the typical head-neck’s “mobile keeping” during preying of waders.

Accessory optic system (AOS) offers an auxiliary control in the optic system in eye-keeping, in optomotor and optokinetic reflexes that maintain a stable retinal picture maintaining as well as in vestibulo-ocular reflexes. However, accessory optic centres are less known compared to primary and secondary optic pathways or structures.

The relay-stations of the retinal fibre, i.e. the accessory optic nuclei contribute to optokinetic, optomotor, vestibulo-ocular reflexes. They are situated at the border of diencephalon and mesencephalon and connected to the optic tract. The centres of optic system, that were named accessory optic system, became known later than the primary (retino-geniculo-cortical) or secondary (retino-colliculo-tegmental) visual paths, although they are found in vertebrates, i.e. in mammals, birds, reptilians, amphibians and fish, as well. The first studies were anatomical and histological investigations. Gudden was the first who showed the bunch of optic fibres, which athwartly run along the ventral surface of optic tract in humans and mammals. The insular retinal fibres branch off the optic tract before the meso-diencephalic connection. Gillian, Giolli, Hayhow et al., Hoffmann et al., Shopmann, and Winfield et al. have done morphological examinations on accessory nuclei and their connections. In rabbits and in cats accessory optic fibres end in the medial, lateral and dorsal terminal nuclei, whereas in primates they end in nucleus transpeduncularis that is situated similarly to rabbit’s medial terminal nucleus. Further advances reported series of physiological findings in mammals.

The morphological examination of accessory optic nuclei in mammals resulted in the separation of terminal lateral, medial and dorsal nuclei. These nuclei are small groups of cells connected to the lateral and
medial ends of pedunculus cerebri, in which basal accessory fibres of optic tract end. After first guesses and physiological results it has been established that in mammals (rabbits, monkeys) contralateral terminal nuclei contribute to optokinetic reflexes of eye movements. Maekawa and Simpson have investigated vestibulo-cerebellar connections and proved that accessory nuclei are directly connected to cerebellum. In rats the cooperation of accessory optic nuclei has been shown by the shift of head and neck or rather by eye movements.

In birds, especially in chicks and pigeons, the examination of accessory optic nuclei became the popular area of optic system’s studies. The contribution of accessory nuclei to optomotor reactions and optokinetic nystagmus has been proved in frogs by Lázár, in pigeons by Fite and others. Wallmann and Burns, Morgan and Frost have established the impact of certain cells in AOS on the direction and velocity of motion, and observed no adaptation while continuous stimulation of those.

The investigation of accessory optic nuclei in birds started with the comparative examination of terminal nuclei. The fibres AOS that arise from the retina extraordinary thick bunches of fibres while entering nucleus of the basal optic root (nBOR), are composed of extra-thick optic fibres. The exact retinal origin of optic fibres that ended in AOS was shown by Karten et al.. Thick optic fibres derive from so-called outplaced ganglion cells which are situated in the external part of inner plexiform layer, on the border of internal granular layer. Dogiel and Raymon Cajal described the outplaced ganglion cells.

The efferent projections of accessory optic nuclei form variant connections. The connection of AOS cells and oculomotor complex was established first by Huber and Crosby, Shanklin and later by Herrick. New studies confirmed early observations of oculomotor endings. AOS neurons have established connections with cerebellum, certain nuclei of brainstem, oliva inferior, and vestibular nuclei. Yet Ramon identified certain connections between bilateral accessory nuclei, the possibility of those and similar connections has been proven for most of the accessory nuclei.

AOS can be found in variant groups of vertebrates from fish to mammals. In birds two nuclei are responsible for eye movement related, retinal picture fixing and keeping reflexes: nucleus of the basal optic root (nBOR) and nucleus lentiformis mesencephali magnocellularis (nLMmc).
AIMS

Morphological, physiological and behavioural evidences have highlighted the importance of accessory optic nuclei. Despite numerous advances, however, many details of the morphology of accessory optic nuclei which seem to be important in the understanding of certain functions and in completing our knowledge of morphology are still unknown.

The aim of this work was to gain information about the basic intrinsic neuronal organisation and connections of accessory optic nuclei of the chick using immunohistochemistry, electron microscopy and tracing methods.

The light- and electron microscopic analyses of chicks two accessory optic nuclei - nucleus of the basal optic root (nBOR) and nucleus lentiformis mesencephali magnocellularis (nLMmc) - has been performed. Firstly, the investigation of Golgi preparations was carried out followed by the analysis of optic terminals in the nuclei using anterograde tracing. Immunohistochemical investigation at the electron microscopic level was used to shed light upon the local, intrinsic structural connections between the labelled optic fibres, the axons of local neurons, and/or the endings of afferent fibres in nBOR and in the nLMmc.

Investigations addressing the intrinsic organisation of nBOR and nLMmc nuclei have been carried out by completing the following steps:

1. The neuronal structure of nBOR has been investigated using Golgi impregnation. The neurons have been classified as projection or local circuit neurons. The fibres and terminals have been analysed and compared with the biotinylated dextran-amine (BDA) labelled optic fibres and terminals.
2. Applying GABA immuno-staining GABA immuno-positive cells, fibres and terminals have been labelled and compared with the findings of Golgi study.
3. BDA anterograd labelling has been used to reveal the retinal fibres and their termination. The synaptic connections of labelled optic fibre terminals and GABA immuno-positive terminals have been analysed and the arrangement of these connections has been investigated.
4. The structure of the cells and fibres in nLMmc has been examined using Golgi impregnation. Different types of neurons have been distinguished and analysed.
5. Applying GABA immuno-staining GABA-positive neurons have been labelled and compared to those of Golgi preparations.
6. The course of BDA labelled retinal fibres, and the arrangement of their terminals have been observed using light microscopy. The connections of terminals have been examined by electron microscopy extended with GABA immunogold-staining.

7. The intrinsic structural arrangements of the neurons and their synaptic connections have been compared in the two investigated nuclei of the AOS. The intrinsic structure has been functionally analysed in the context of the execution of different physiological tasks.

**MATERIALS AND METHODS**

**Golgi impregnation**

One-month-old domestic chicks (Gallus domesticus) were given a lethal dose of anaesthetic (Calypsol:Rompun 2:1) and perfused through the left cardiac ventricle with a rapid flush of 50 ml of physiological saline, followed by 500 ml of 2% paraformaldehyde in 0,1 M phosphate buffer (pH 7,4) in the birds which were destined to be used for rapid Golgi impregnation. Each brain was then removed from its skull and immersed in the fixation fluid for 2-3 hours prior to further processing. Serial, coronal slices (5-6 mm thick) were cut freehand through the optic tecta. The slices for the rapid Golgi method were then impregnated using a modification of Valverde (1962), and finally embedded in celloidin. Serial 100 µm sections were cut mounted on glass slides and examined by light microscopy. These Golgi-impregnated sections were compared with reference sets of coronal sections of chick optic tectum stained with luxol fast blue and cresyl violet, to aid histological orientation.

**GABA immunostaining**

28-day-old and 14-day-old chiks were used for GABA immunostaining. The chicks were perfused under deep anaesthesia (Calypsol:Rompun 2:1) with fixative (2 % paraformaldehyde+2 % glutaraldehyde in 0,1 M phosphate buffer, pH 7,4). Brains were removed from the skull and postfixed for 2-3 hours in glutaraldehyde-free fixative. After fixation, the brains were washed and kept in 10 % and 20 % sucrose until they sank. They were then sectioned coronally on a Vibratome at 60-70 µm. Background staining was suppressed by incubating the sections with 20 % normal goat serum (NGS) (Human, Gödöllő) in 0,1 M
phosphate buffer for 1 h, and endogenous peroxidase activity was reduced by treating the sections with 1 % borohydrate sodium (Sigma). The sections were then incubated with the primary antibody (anti GABA antiserum, Sigma), which was raised and characterised by Somogyi and Hodgson (1985), diluted 1:10000 in 1 % NGS for 48 hours at 4 °C. Subsequently, the sections were exposed to biotinylated goat anti-rabbit IgG (Vector Laboratories Inc.) diluted 1:250 in 1 % NGS for 24 h and then in avidin-biotin complex diluted 1:200 in 0,1 M phosphate buffer for 18 hours. The end product was visualised by placing the sections in a 0,025 % solution of 3’3’diamino-benzidine for 20 min and in 0,01 % H$_2$O$_2$ for 6-8 min. After thorough washing in 0,1 M phosphate buffer the sections were dehydrated, mounted on glass and covered by Durcupan (Fluka).

**GABA immunogold labelling**

Blocks had been prepared and ultrathin sections were cut which were treated for postembedding GABA immunogold labelling, following the method of Somogyi and Hudgson (1985). The ultrathin sections were mounted on nickel grids, and pretreated with 1 % periodic acid and 1 % sodium periodate to etch the resin and remove the osmium. They were then sequentially placed into 1 % goat serum for 2x10 minuts. Thereafter they were treated with rabbit anti GABA antiserum (Sigma) diluted 1:1000 in normal goat serum, and then in colloidal gold particles (15 nm) coated with goat anti-rabbit IgG (British Biocell) diluted 1:10 in TRIS bufered saline (pH 7,4), for 2 h. Between the steps, the grids were washed three times in Millipore-filtered distilled water and finally stained with uranyl acetate and lead citrate. The ultrathin sections were examined in a JEOL 1200 EMX electron microscope.

**Anterograde biotinylated dextran-amine (BDA) tracing**

Two and three weeks old chicks were deeply anaesthetised (Calypsol:Rompun 2:1) and mounted in a stereotaxic apparatus. The anterograde tracer biotinylated dextran amine (BDA; Molecular Probes; 20 % diluted in physiological saline) was delivered by iontophoresis into the optic nerve. The roof of the orbit and the optic canal was opened. BDA was delivered under visual control into the optic nerve, using a current of 6-7 µA, cycling on and off every 7 s, for periods of 30-40 minutes. The cannula was left in situ for 5-10 min after iontophoresis of the tracer to minimize the reflux of the tracer along the cannula tract as it was withdrawn. The
dura mater and the skull flap were replaced, the scalp was closed and the chicks were allowed to recover.

Seven to eight days later the birds were intracardially perfused under deep anaesthesia first with saline (0.9 % NaCl; 50 ml) and then with a fixative containing 2 % paraformaldehyde, 1 % glutaraldehyde, and 0.2 % picric acid in 0.1 M phosphate buffer (pH 7.4). The brains were immersed in 10 % and 20 % sucrose, freeze thawed in liquid nitrogen, sectioned on a vibratome (50 μm) and extensively washed in 0.1 M phosphate buffer (pH 7.4). The free-floating sections were rinsed in TRIS-buffered saline (TBS, pH 7.4), and reacted with the avidin-biotin complex (1:100 ABC, Vector) for 24 hours. The precipitate was visualised by using 3’3’diaminobenzidine (DAB, Sigma) in the presence of 1 % hydrogen peroxide. The sections were mounted on glass slides. Those sections destined for electron microscopy were first postfixed with osmium tetroxide (1 % in distilled water, Sigma) for 10 min, then dehydrated and mounted on glass slides and covered with Durcupan (ACM, FLUCA). Ultrathin sections were cut and investigated in a JEOL 12,000 EMX electron microscope.

RESULTS

nBOR

The nBOR has a significant role in eye movement connected to optokinetic nystagmus. This nucleus is one of the so-called accessory optic nuclei, and it is able to assist with complementary eye movements, i.e. the eye’s exact positioning on the score of incoming optic stimuli procession. nBOR is directly connected to vestibulo-cerebellum and through this to vestibulo-ocular and vestibulo-spinal reflexes. Apart from distant connections it is necessary to come to know the intrinsic system of afferents and efferents in order to understand the function of the nucleus. Supposedly, the intrinsic structure of nBOR is substantial in the processing of optical information. Through exploring the internal construction of the nucleus, it was found that:

1. Neurons within the Golgi-impregnated nBOR could be classified into two groups on the basis of morphology of their perikarya and the pattern of their dendrites and axon arborisation: projection and local circuit neurons.
2. According to the size and dendritic ramification pattern projection neurons could be further classified in two subtypes, i.e. large and medium-sized neurons. These presumably project to different centres
(oculomotor nuclei, vestibulo-cerebellum, nLMmc, contralateral nBOR) which have been identified as targets of nBOR.

3. The dendritic trees of two types of projection neurons and the ramification of optic fibres in the nBOR spread over wide, but corresponding areas. The terminal dendritic branches of both neuron types form common dendritic nests which guarantees identical visual input of different projective neurons. This morphological feature may provide the basis for the transmission of the same visual information into different centres. (Figure 18)

4. Using electron microscopy and BDA-labelling, numerous endings of optic fibres have been noted on the terminal dendritic trees of projective neurons.

5. On the basis of the morphology and the arrangement pattern of dendrites local circuit neurons could be classified into two groups: small ovoid and elongated or small fusiform neurons. Both of these types were proved to be GABA-positive. Their axon terminals end on the dendrites of projective neurons and form symmetric synapses.

6. GABA-positive myelinated afferents were observed which branched into nBOR and probably ended on dendrites of projection neurons.

7. Two types of GABA-positive terminals were distinguished which originated from local circuit neurons and from the myelinated GABA-positive afferents: one with flat synaptic vesicles and the other with pleomorphic vesicles. These terminals typically contacted dendritic branches of projective neurons. At the origin of the principal dendrites of projective neurons only terminals with flat synaptic vesicles were present.

**nLMmc**

The accessory optic nucleus nLMmc is according to physiological examinations, responsible for horizontal nystagmus. Interacting with nBOR, nLMmc carries out the precise adjustment of the eyes. The majority of its afferents stem from retinal ganglion cells. The various connections of nLMmc are provided by numerous efferens paths according to their functions. This nucleus has a connection to vestibulo-cerebellum, to folia VI, VII and VIII, to lateral pontine nucleus, to inferior olive, to optic tectum and to the contralateral nBOR. These diverse contacts are possible because of the intrinsic structure of nLMmc, since efferents transmit the same information to the different effector centres. Through performing analysis using Golgi impregnation the possibility of such a hierarchy has been proved. Golgi staining has shown the correlated topographical
position of nLMmc’s two – huge and large - projective neurons. It is concluded that the close relationship of dendritic arbours of the two neurons makes the reception of the same terminals possible. The same optic fibres project onto both types of projective neurons as shown by BDA-labelling of optic fibres. Electron microscopical observations confirmed the existence the synaptic connections. This topographical relationship has major functional relevance, i.e. the exact same optic information can be delivered to the different eye moving centres of the nervous system.

The light and electron microscopic examination of nLMmc has shown the followings:

1. Neurons identified in the Golgi preparations could be classified into two groups: projection and local circuit neurons. The projection neurons established distant connections and local circuit neurons provided intrinsic connections.

2. Projection neurons could be classified into further subtypes: huge, large and medium-large neurons.

3. The large neurons were located in the close vicinity of the huge neurons, and their dendrites were directed similarly to that of the huge neurons, i.e. the dendrites lie parallel with each other. This topographical situation suggested that the two types of projection neurons had contacts with the same optic fibres and might also receive the same optic impulses.

4. Two types of medium sized neurons were observed in nLMmc: multiangular and elongated neurons. The elongated medium-sized neurons were GABA-ergic and might inhibit the nLMmc and nBOR of the contralateral side.

5. Small round or ovoid local circuit neurons had also been impregnated in the nLMmc which turned out to be GABA-ergic. Supposedly, these short-axon neurons modulate the transaction of signals locally, i.e. within the nucleus.

6. The optic fibres have been anterogradely labelled with BDA. Small bunches of labelled optic fibres formed knitting bulbs of terminals, which had a tendency to group small grape-like formations of 3-5 bulbs.

7. Electron microscopical analysis showed that both labelled and non-labelled optic fibres terminals and GABA-ergic terminals formed synaptic islands bordered partly with GABA-positive dendrites and partly with thick GABA-negative dendrites and fibres. Some GABA-positive dendrites containing pleomorph synaptic vesicles were observed. These dendrites were identified as dendraxons and they
made contacts with GABA-negative dendritic profiles by forming symmetrical synapses. The optic fibres established asymmetrical synapses with dendrites, dendritic processes and spines. GABA-positive terminals typically joined optic fibres with symmetric synapsis.

**DISCUSSION**

The aim of our work was to investigate the intrinsic structure of birds’ accessory optic nuclei and the endings of optic fibres. Birds have two substantial accessory optic nuclei: nucleus of the basal optic root (nBOR) located in diencephalon and nucleus lentiformis mesencephali magnocellularis (nLMmc) in the latero-rostral connection of diencephalon and mesencephalon i.e. in the pretectum. These nuclei are fundamental in optokinetic nystagmus. The examination of the intrinsic structure of the nuclei was undertaken to understand their neural elements and the terminal endings of their afferens (optic fibres). The neuronal connections that make these two nuclei able to transmit the same optic information to different targets – probably through the collaterals or axons of the different projective neuron types - were identified. Analysis of the intrinsic structure of nuclei provided morphological evidence for creating inhibitory connections between contralateral nBOR and nLMmc. Previous physiologic investigations have shown that in birds there is an inhibitory, contralateral and excitatory, unilateral connection between nuclei. The present findings offer morphological support for the physiological findings suggesting the modulation of the transduction of optic signals.

Observations on Golgi preparations were fundamental to this study. The intranuclear ramification of main afferents, i.e. optic fibres and the specific shape of optic terminals were examined applying anterograde tracing method. In order to distinguish the functional types of neurons GABA immunostaining was used which made the separation of GABA-positive and GABA–negative neurons possible. Further preparations were labelled with GABA immunogold and examined using an electron microscope.

It has been known from previous studies that the ending of optic fibres is in accessory optic nuclei. Numerous essays have proved the efferent connections of nBOR and nLMmc with eye movement controlling motor centres as well as with the vestibulo-cerebellum. However, the intrinsic structure of nuclei – neither light- nor electron microscopically – has remained unexplored apart from preliminary data gained from cell-dyed preparations.
Except of rather few and poor preliminary attempts there have been hardly any studies on the Golgi architecture of accessory optic nuclei of birds. This can be explained by the notoriously ambiguous success of Golgi impregnation. Furthermore, supposedly because of the poor blood supply, this brain areas could be especially poorly visualised by Golgi impregnation. Our preparations had not showed ideal Golgi-proofing results, but the main structure seemed to have been captured. Further successful analyses are required to complete our understanding of the organization of this region.

The results of the Golgi method – in the face of mentioned difficulties – provided extremely important information on the structure of nBOR and nLMmc, that can be used in functional assessments of further morphologic analysis. Numerous types of projection neurons ensure the connection with various regions. The correlating position of different projection neurons makes every type of neurons able to receive the optic information from the same terminals. It is more than probable, that these morphological properties enable the combined movements of the eye, neck and head, i.e. they contribute to the co-ordinated motions of eye and head.

Both accessory optic nuclei (nBOR and nLMmc) are environed by stringy perinuclear capsules that isolate them from their surroundings. In nBOR, large and medium-large projection neurons are connected to each other through their distal, spiny dendrites. Electron-microscopical investigations have proved that terminals of optic fibres net this region. Whilst exploring the ultrastructure of the nuclei, it was noticed that optic terminals almost completely cover dendrite branches which lie side by side. There are no GABA-positive terminals among them, so distal dendritic sections are able to summarize incoming information without any modulation. The function of proximal parts of dendrites is the modulation - that probably follows the first information - in nBOR.

In spite of different topographical structure, the dendrites of large projection neurons and their possible connection to optic fibres were found in nLMmc. The intrinsic connectivity structure of these two nuclei is complicated by the presence of much smaller projection neurons on dendrites of nucleus-constructing neurons and by the fact, that there are GABA-positive neurons among them. The latter are responsible for direct contralateral inhibition.

Results of physiological analysis have proved the cooperation between two nuclei in horizontal and vertical nystagmus; as well as the excitation of unilateral and the inhibition of contralateral nuclei. The basis for this extremely complicated system is provided by the morphological structure, as can be seen from the results of our experiments.
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