Amygdaloid input to the ventromedial forebrain in the young domestic chicken (Gallus gallus domesticus)

PhD thesis

dr. János Hanics

Semmelweis University
János Szentágothai Doctoral School of Neurosciences

Supervisor:	**Dr. András Csillag, D.Sc.**
Official reviewer:	**Dr. Lajos Rudolf Kozák, Ph.D.**
	Dr. Klára Matesz, D.Sc.
Chairman of examination board:	**Dr. Imre Szirmai, D.Sc.**
Members of examination board:	**Dr. Katalin Halasy, D.Sc.**
	Dr. László Tretter, D.Sc.

Budapest

2016

INTRODUCTION

It has been established that, of all telencephalic regions, the basal ganglia show the highest degree of homology between birds and mammals. This may serve as justification for a comparative approach in the investigation of neural mechanisms, such as motivation of elementary actions, which have been conserved throughout vertebral evolution both in mammals and sauropsida (diverging over 200 million years ago). As a model system, young domestic chicks offer an unique opportunity for studying learning and motivation because of their early maturation (precocial development of a nidifugous species).

The ventrobasal forebrain nuclei, including the nucleus accumbens (Ac), bed nucleus of stria terminalis, lateral part (BSTL) and other components of extended amygdala (EA), have been implicated in the initiation and reinforcement of movements, motivation and emotion, reward and aversion These regions are extensively connected with the amygdala, whose involvement in emotional responses is also well established. While the majority of relevant studies focused on mammalian species, our laboratory has been active in revealing a similar role of relevant systems in birds.

Several afferents of the avian ventrobasal forebrain nuclei (including EA, BSTL, Ac, and MSt - medial striatum) originate from the arcopallium. Most of the arcopallium is now considered to represent the amygdalar complex of birds. The arcopallio-striatal (Ac, MSt) projection of birds corresponds, in fact, to the amygdalo-striatal (amygdalo-accumbens) pathway of mammals. Early studies have shown, that the afferents from arcopallium terminate in the MSt, forming varicosities with asymmetrical axospinous synapses, which typically did not contain Glu despite the suggestive morphology. Therefore, it was surmised that some of the afferent terminals might contain another transmitter of the excitatory type. Results, based on electronmicroscopic immunocytochemistry, have indicated the presence of L-Asp and L-Glu in excitatory axodendritic boutons in the striatal region of chickens, however, the source of these terminals remained unrevealed. To further investigate the specificity of the ventrobasal forebrain input pathways in the domestic chicken, it was necessary to carry out a detailed pathway tracing study, combined with light and electron microscopic immunocytochemistry relevant to the chemical nature of arcopallial source regions and of potential target areas. To map the arcopallial output onto the avian ventrobasal forebrain regions including the Ac, EA, BSTL, we applied high-precision region-specific in vivo fluorescent anterograde and retrograde tracing experiments combined with

immunohistochemistry, including dual tracing by simultaneous use of different dyes.

AIMS

- Description of amygdaloid (arcopallial) excitatory input fibers and their temination pattern in ventrobasal forebrain regions of the domestic chicken, combined with anterograde tract tracing and postembedding immunolabelling methods.

- Mapping of those arcopallial 'source regions' (amygdaloid arcopallium) which project to the avian ventromedial forebrain areas by using fluorescent retrograde tracers.

- Mapping of the arcopalliofugal pathways, by comparative retrograde and anterograde tracing.

- Mapping of the terminal subregions of the arcopalliofugal axons in the vetromedial forebrain, including those of the nucleus accumbens and extended amygdala.

- Characterization of the arcopallial 'source neurons', by investigation of DARPP-32 content of putative dopaminoceptive neurons, and expression of calcium binding proteins calbindin, calretinin and parvalbumin, using immunohistochemistry combined with retrograde tracing.

METHODS

Animals

For ultrastructural examination of the ventromedial forebrain thirty two adult male albino rats (Wistar Crl-S.NET Wi Br) and twenty five domestic chicks (*Gallus gallus domesticus Hunnia broilers*) of either sex were used, and further thirty chicks were applied for mapping experiments of the arcopalliofugal pathways.

Tracer injections

The animals were anesthetized intramuscularly with a mixture of ketamine and xylazine and placed in a Kopf stereotaxic instrument. Tracers were injected stereotaxically using a 1,0 µl Hamilton syringe mounted on a Kopf microinjector unit.

Anterograde tracer injection (BDA and HRP). Chicks received unilaterally 10 kDa MW biotinylated dextran amine (BDA) injection into the dorsal arcopallium. We used the following surgical coordinates: 6,2 mm from midline, 1,7 mm rostral from bregma, 4,2 mm ventral from dura; the beakbar was set 5 mm below horizontal. Rats received two types of anterograde tracers: BDA or horseradish peroxidase (HRP). Stereotaxic coordinates were: 4,6-5,0 mm from midline, 2-3 mm rostral from bregma, 7,8 mm ventral from dura, corresponding to the basolateral amygdala (BLA).

Fluorescent retrograde tracer injections in chicks. Animals received unilateral dual or single injections of two different retrograde tracers, Alexa Fluor® 488 (or 594) conjugated choleratoxin B subunit (CTb) and Fast Blue (FB), into the ventrobasal forebrain. In dual retrograde tracing experiments CTb was injected first into the BSTL-Ac at the stereotaxic coordinates 4,30-4,40 mm rostral from bregma, 0,79-0,82 mm from midline, 5,57-6,07 mm ventral from dura, which was followed by the administration of FB further laterally (by 0,5 mm) to the previous injection coordinates (medial striatum - MSt). Single CTb injections were applied at the BSTL-Ac coordinates.

Fluorescent anterograde tracer injection was carried out by using Alexa Fluor® 594 conjugated dextran 10kDa (D594). Chicks received injection of D594 into the dorsolateral (APir, coordinates 1,50 mm rostral from bregma, 6,74 mm from midline, 3,55 mm ventral from dura), dorsal (ADo, coordinates 1,50 mm rostral from bregma, 5,50 mm from midline, 5,00 mm ventral from dura) and hilar (AHil, coordinates 1,50 mm rostral from bregma, 4,60 mm from midline, 6,00 mm ventral from dura) parts of the arcopallium.

Perfusion and sectioning

Ten days after BDA injections chicks were deeply anesthetized and transcardially perfused. Following survival of 48 h (HRP experiments) or three weeks (BDA experiments) rats were transcardially perfused under terminal anesthesia. The brains were removed from the skull and postfixed overnight. Seventy-µm-thick coronal sections were cut on a vibrating microtome.

Four and seven days after fluorescent retrograde and anterograde tracing, respectively, animals were deeply anesthetized and transcardially perfused. The brains were removed from the skull, postfixed overnight and subsequently transferred to 30 % sucrose (4 °C, for 2 days) for cryoprotection. Brains were sectioned at 70 µm on a freezing microtome in the coronal plane.

Tissue processing, immunohistochemistry

For visualization of (BDA and HRP) tracers, the free-floating sections were treated with the following reagents: in the case of BDA, avidine-biotin-HRP conjugate (ABC) followed by 3,3′-diaminobenzidine tetrahydrochloride (DAB); in the case of HRP, only the DAB step was applied. For light microscopic observation, the sections were mounted on gelatin-subbed slides and coverslipped with DePeX.

For electron microscopy, representative regions containing the ventral striatum/nucleus accumbens were dissected and the tissue blocks were incubated in OsO_4 and flat-embedded in Durcupan. Ultrathin sections were cut with an ultramicrotome and placed on Formvar-coated single slot nickel specimen holders.

The electron microscopic procedure was followed by immunostaining against L-Glutamate (Glu) or L-Aspartate (Asp). The staining protocol followed the colloidal gold method. The sections were counterstained with uranyl acetate and lead citrate, viewed and photographed on a JEOL 100S electron microscope equipped with a digital camera.

For multiple-labeling experiments, free-floating sections were exposed to select combinations of primary antibodies: DARPP-32, calbindin, calretinin, parvalbumin. Immunoreactivities were revealed by species-specific carbocyanine (Cy) 2 or 5-tagged secondary antibodies raised in donkey. Glass-mounted sections were coverslipped with Surgipath Micromount mounting medium.

Imaging

Survey images were captured on an Olympus BX-51 epifluorescent microscope, equipped with a digital camera. Alternatively, overview images were taken on a 780LSM confocal laser-scanning microscope (Zeiss) at 10x magnification and using the auto-tile-and-stitch function. Sections processed for multiple immunofluorescence histochemistry were viewed and the images were acquired on the 780LSM confocal laser-scanning microscope, using minimal optical slice thickness (0,7-0,9 μm) with high power imaging.

For high-fidelity tract tracing, multiple middle-power magnification photos that covered the whole unilateral basal telencephalic section were taken and the images were ’stitched’ together. Altogether 25 multi-tile images were assembled that included the total cranio-caudal extension of the arcopallial-striatal tracts.

RESULTS

The termination pattern of arcopallio-striatal (accumbens) pathway is indicative of excitatory nature.

Following tracer injections, anterogradely labeled fibers were detected in the ventrobasal forebrain regions of both rats and chickens. In rats, the nucleus accumbens core region (AcC) was abundantly invaded after BLA injections, however, in chickens, the tracer had to be deposited at the border of dorsal and ventral arcopallium in order for a sufficient amount of labeled fibers to be revealed within the accumbens core.

In the chick brain, immunostaining against Asp was regularly detected in axospinous asymmetrical axon terminals regardless of their origin. Typical boutons containing the tracer BDA alone, with asymmetrical and axospinous synapse, were also observed. Axon terminals of this type were often conspicuous for the simultaneous occurrence of anterograde tracer and Asp associated gold particles. Similarly to Asp, immunoreactivity for Glu was also common occurrence in the AcC of the chick, regardless of the origin of fibers. These (mostly axospinous) terminals clearly belonged to the asymmetrical type. Simultaneous occurrence of BDA tracer and Glu associated gold particles were also frequent.

Without immunostaining, the appearance of amygdalofugal axon terminals in the AcC of the rat was largely similar to those observed in the chicken brain. Whether detected by HRP or BDA, the boutons were densely packed with uniform clear vesicles and established mainly asymmetrical axospinous synaptic connections. Immunoreactivity for Asp was relatively rarely detected over BDA labeled terminals. Using HRP, a similarly weak correspondence was found between the presence of the anterograde tracer and Asp-immunoreactivity. Conversely, the presence of Glu in the amygdalofugal axons terminating in AcC was more evident in the rat.

The medial striatum is heterogeneous in its afferentation pattern.

To identify the select arcopallial cell group ("amygdaloid arcopallium") establishing an amygdalostriatal (amygdalosubpallial) pathway, we injected two different fluorescent retrograde tracers, CTb and FB, into the medial and lateral divisions of the medial striatum (MSt) of chicks, respectively. When mapping the arcopallium in its complete cranio-caudal extension for retrogradely labeled CTb^+ perikarya, we found a large number of neurons in its peripheral domain with an outstanding density in its dorsolateral part, a region that continued caudally towards a seemingly more ventral position. At the same time, we were unable to identify FB^+ cell

bodies in any region of the arcopallium. In the dorsal thalamus, CTb^+ and FB^+ neurons outlined to minimally overlapping regions, with CTb^+ perikarya populating the anterior dorsomedial nucleus and FB^+ perikarya the medial part of the dorsolateral anterior thalamic nucleus. Thus, the simultaneous retrograde tracing from medial and lateral parts of the medial striatum allowed us to identify a different projection pattern from the arcopallium and thalamus: whilst both adjacent tracer deposits led to markedly separate labeling sites in the dorsomedial thalamus, only one of them (the medial) yielded backfilled cells in the arcopallium. Discrete thalamic labeling could thus serve also as validation of the selective tracing method.

The arcopalliofugal (amygdalofugal) tract terminates in a select domain of the ventrobasal forebrain.

To unequivocally prove and describe the exact projection pattern of the arcopallium onto the striatal region, we carried out correspondingly planned retrograde and anterograde tracing experiments. Retrograde tracer (CTb) deposited into the Ac, including the juxtaventricular zone of the BSTL, labeled a large number of axons in the ventral amygdalofugal tract (vaf) and perikarya in outstanding density in a dorsolateral wedge-shaped region of the arcopallium, termed amygdalopiriform area (APir). Albeit in lower densitites, CTb^+ cell bodies were detected in the hilar (AHil), dorsal (ADo) and postero-lateral (APL) parts of the arcopallium. No (or very few) labeled neurons were identified in the amygdalo-hippocampal (AHi), taenial (ATn) or core domains of the arcopallium at the same time. More cranially, retrogradely labeled neurons appeared in a large number throughout the extended amygdala (EA).

Anterograde tracing using D594 from the APir led to corresponding results, verifying the presence of multiple axons arising from the APir in the vaf and BSTL. To identify the route of the investigated amygdalosubpallial pathway with high precision, we carried out total brain section scanning using high power magnification and subsequent multi-tile-stitching on complete cranio-caudal series of the arcopallium. We found that axons arising from the APir follow two pathways (i) medially along the dorsal border of the arcopallium) to reach the vaf with terminal endings subsequently identified in the BSTL or (ii) a ventral course, passing through the ventrobasal part of EA, and then invading the nucleus basalis and olfactory tubercle.

Arcopallial domains show principal differences in their connectivity with the avian nucleus accumbens.

Retrograde tracing from the Ac labeled perikarya in a dorsolateral wedge-shaped region of the arcopallium in outstanding density, whereas further – far less abundant - cell bodies were detected along the periphery of the arcopallium, leaving the "core" of the arcopallium spared from labeled neurons. To investigate the exact projection pattern of this arcopallial "belt", we injected the anterograde tracer D594 into its dorsolateral (APir), dorsal (ADo) and medial, hilar (AHil) divisions, and mapped the caudal, intermediate and rostral regions of the Ac for terminating axons. D594 injection led to a dense axonal and terminal labeling throughout the Ac when targeting the APir. In contrast, anterogradely labeled fibers within the Ac were identified in a dramatically smaller number when the injection sites were restricted to the ADo or AHil divisions of the arcopallium. In fact, no fibers were detected in the rostralmost part of the Ac when D594 had been administered into the hilar region of the arcopallium.

Arcopallial fibers typically target the medial (DARPP-32$^-$) division, and also invade the lateral (DARPP-32$^+$) division of the ventrobasal forebrain.

Medial and lateral divisions of ventrobasal forebrain are characterized by the absence or presence of dopamine- and cAMP-regulated phosphoprotein containing (DARPP-32$^+$) neurons, respectively, conferring potential functional differences. We hypothesized that the heterogeneity of this brain field might be reflected also by its arcopallial connectivity pattern. To test this, we investigated the ventrobasal juxtaventricular forebrain (including the BSTL and Ac) in serial coronal sections for anterogradely labeled fibers (D594-injection into the APir), and co-stained their target region for DARPP-32-immunoreactivity. In the cranial sectional levels, the medial and lateral divisions of the ventrobasal forebrain could be clearly distinguished according to their DARPP-32-immunoreactivity. The majority of arcopallial axons ended in a medial DARPP-32 negative subregion, corresponding to the BSTL. However, although in lower densities, axons and terminal fields were also identified amongst the DARPP-32$^+$ neurons in the adjacent lateral region, corresponding to the Ac. In more caudal sectional levels, where the Ac has phased out, the axon terminals were found to be distributed in the BSTL and also in the extended amygdala

(EA), ventral pallidum (VP), and the nucleus basalis of Meynert (B), arriving via the vaf.

The arcopallial 'source neurons' of the ventromedial forebrain lie in a DARPP-32 negative region.

Next, we investigated the relationship between DARPP-32-immunoreactivity and the source neurons of the arcopalliosubpallial tract. Using CTb as a retrograde tracer, we identified labeled perikarya within the typical sites of origin in the arcopallium, which were surrounded by, but mostly did not coincide with, DARPP-32^+ neurons. Most of the arcopallium proved to show very weak or negative labeling agaist DARPP-32. In only a few cases did CTb^+ neurons share DARPP-32-immunoreactivity. Conclusively, we identified the amygdalosubpallial pathway which arises from mostly DARPP-32^- neurons to pass and terminate within largely DARPP-32^- regions, but with a select final target area also amongst DARPP-32^+ neurons.

Projection neurons of the arcopalliofugal (amygdalofugal) tract do not express the major neuronal calcium binding proteins parvalbumin, calbindin or calretinin.

We tested the possibility if arcopallial neurons projecting to the ventrobasal forebrain are distinct by the select expression of a calcium binding protein. In contrast to $calretinin^+$ neurons which occurred, but only sporadically, in the chick arcopallium, $parvalbumin^+$ and $calbindin^+$ neurons were detected throughout the arcopallium. Using retrograde tracing combined with multiple immunolabeling we showed that arcopalliofugal projection neurons did not express any of the major calcium binding proteins: CTb^+ neurons remained invariably immunonegative for calretinin, parvalbumin or calbindin.

CONCLUSIONS

- An amygdalo-accumbens homologue pathway also exists in the domestic chicken forebrain system. These axons terminate with asymmetrical axospinous synaptic connections.
- L-Aspartate and L-Glutamate can be immunocytochemically detected in synapses of homologous amygdalo-striatal (amygdalo-accumbens) pathways of rats and chickens. The anatomical situation appears to be consistent with an Asp – Glu corelease (cotransmission) mechanism.
- Specified neuronal groups (APir, ADo, AHil, APL) of the arcopallium send projections to limbic areas of the ventromedial forebrain in the domestic chicken. These arcopallial regions are parts of the 'amygdaloid arcopallium'. The core of the arcopallium does not project to these ventromedial forebrain areas.
- The fibers from the amygdaloid arcopallium follow two pathways. The first one passing on the dorsal border of the arcopallium, and terminating within the ventromedial forebrain areas (BSTL, Ac, MSt) is a homologue of the mamalian stria terminalis. The second pathway, passing on the ventral forebrain border, and terminating in the basal forebrain areas (including VP, B, TO) is a likely homologue of the mammalian ansa peduncularis.
- The fibers from the amydaloid arcopallium equally reach the neighbouring regions of the ventromedial forebrain (BSTL and Ac).
- Neurons of the amygdaloid arcopallium do not express the calcium binding proteins calbindin, calretinin and parvalbumin.
- The 'source regions' of the amygdaloid arcopallium do not express DARPP-32 signal protein, unlike their surrounding regions.

REFERENCES

Research articles related to the thesis

Hanics J, Balint E, Milanovich D, Zachar G, Adam A, Csillag A. (2012) Amygdalofugal axon terminals immunoreactive for L-aspartate or L-glutamate in the nucleus accumbens of rats and domestic chickens: a comparative electron microscopic immunocytochemical study combined with anterograde pathway tracing. Cell Tissue Res, 350: 409-23.

Hanics J, Teleki G, Alpar A, Szekely AD, Csillag A. (2016) Multiple amygdaloid divisions of arcopallium send convergent projections to the nucleus accumbens and neighboring subpallial amygdala regions in the domestic chicken. A selective pathway tracing and reconstruction study. Brain Struct Funct, DOI 10.1007/s00429-016-1219-8.

Research articles not related to the thesis

Hanics J, Barna J, Xiao J, Millan JL, Fonta C, Negyessy L. (2012) Ablation of TNAP function compromises myelination and synaptogenesis in the mouse brain. Cell Tissue Res, 349: 459-71.

Alpar A, Tortoriello G, Calvigioni D, Niphakis MJ, Milenkovic I, Bakker J, Cameron GA, **Hanics J,** Morris CV, Fuzik J, Kovacs GG, Cravatt BF, Parnavelas JG, Andrews WD, Hurd YL, Keimpema E, Harkany T. (2014) Endocannabinoids modulate cortical development by configuring Slit2/Robo1 signalling. Nat Commun, 5: 4421.

Bullmann T, Seeger G, Stieler J, **Hanics J**, Reimann K, Kretzschmann TP, Hilbrich I, Holzer M, Alpar A, Arendt T. (2016) Tau phosphorylation-associated spine regression does not impair hippocampal-dependent memory in hibernating golden hamsters. Hippocampus, 26: 301-18.