# Morphological analysis of dorsal and ventral striatal neuronal systems of the domestic chick (*Gallus domesticus*)

Ph.D. thesis

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# Introduction

Similar to mammals, the avian striato-tegmental projection arises from a wide region of basal ganglia, in particular the medial striatum (MSt) and lateral striatum (LSt), and reaches two main dopaminergic centers of the midbrain, the substantia nigra (SN) and ventral tegmental area (VTA). The striatonigral projection contains GABA colocalizing either with substance P (SP) or enkephalin (ENK). Synaptic contacts between dopaminergic nigral and VTA neurons and striatal fibers have been verified in mammals and also in birds. The dopamine- (DA) containing projection arising from the brainstem profusely innervates the basal ganglia including the MSt and the LSt. These axon terminals establish synaptic connections with the medium spiny neurons of the striatum, which receive mainly glutamatergic input from cortical equivalent areas such as hyperpallium apicale, amygdaloid arcopallium (PoA) and pallium externum, and contain, at least in part, DARPP-32, a dopamine- and cAMP-regulated phosphoprotein. In the avian striatum DARPP-32 has been found to be present in many SP-containing and fewer ENK-containing neurons, whilst interneurons were devoid of this marker. DARPP-32 may participate in the interaction between the dopaminergic and glutamatergic afferents of striatum by the cAMP-dependent regulation of striatal NMDA-currents. Such interaction has been found to be essential in the induction of long term potentiation (LTP) or long term depression (LTD) of excitatory synaptic transmission in the mammalian striatum and, as a result, it may play a crucial role in learning processes and the DA-dependent reinforcement of behaviour. Previous studies have shown that D1 dopaminereceptor activation increase the amount of cAMP, which leads to phosphorylation of DARPP-32 on threonine-34 through activation of proteinkinase A (PKA). Phosphorylated DARPP-32 (at T34) inhibits protein phosphatase-1 (PP1), thereby modulating NMDA glutamate receptor function. Depending on the site of phosphorylation, DARPP-32 can either: (1) increase the phosphorylation of various substrates (e.g. membrane receptors) following D1 dependent activation of PKA, phosphorylation of T34 and inhibition of PP1; or (2) decrease phosphorylation through inhibition of PKA, and disinhibition of PP1, after phosphorylation of threonine-75 by cycline-dependent kinase 5. Conversely, stimulation of D2 receptor reduces the amount of both cAMP and phospho-T34 DARPP-32 by decreased phosphorylation of DARPP-32 via the cAMP/PKA pathway and by an increased dephosphorylation of DARPP-32 through the Ca/calcineurin pathway. Based on

these regulatory pathways elucidated in mammals, a similar mechanism may be operational in avian systems. Several avian model systems, which involve MSt, are relevant to memory acquisition and storage. Although DA is important for passive avoidance learning (PAL), its quantity does not change in the MSt during the learning process. Conversely, D1 receptor is upregulated during memory acquisition in MSt. A preferential role of the D1 receptor has also been suggested by the inhibiting effect of D1 (but not D2) antagonist on memory retention in passive avoidance training of chicks. D1 receptors facilitate the NMDA-related burst firing of striatal neurons, whereas NMDA activation selectively recruits D1 receptors to be incorporated from internalized pools to the plasma membrane. MSt is needed for the acquisition but not the storage of memory, and the anticipation of reward proximity. The strong reciprocal connection between the dopaminoceptive neurons of the striatum and the dopaminergic cells of the SN/VTA may represent a feed-forward mechanism, which underlies the anticipation of forthcoming reinforcement during operant conditioning. One potential site for the fine-tuning of this neural circuit could be the putative synaptic contact between striato-nigral fibers and Dopaminergic neurons in the brainstem.

The intermediate medial mesopallium (IMM) – PoA – MSt loop is known to serve as an essential brain circuit for the PAL. The MSt receives pallial signals (e.g. visual from hyperpallium, fear-related from PoA) as excitatory terminals, operating mainly by L-glutamate (GLU). Critically timed and balanced coincidence between the glutamatergic and dopaminergic synaptic activities in the ventral/medial striatum, including the nucl. accumbens (Ac), is required for memory to be formed for a given pairing of stimulus and a hedonic quality or behavioural salience. The underlying mechanism involves the activation of NMDA and D1 receptors, as well as the phosphorylation of DARPP-32. Thus, DARPP-32 is responsible for the signal transduction between the converging glutamatergic and dopaminergic striatal afferent pathways.

Former results of our group demonstrated that a considerable part of the arcopallial afferents of MSt, forming asymmetric synapses, were devoid of GLU. Since about 15 % of all projection neurons of arcopallium were found to be aspartatergic, the GLU-negative afferents likely express L-aspartate (ASP) as transmitter.

The mammalian Ac is a ventral forebrain structure associated with the striatal complex and it plays a crucial role in limbic neural circuits. It is responsible for

motivated goal-directed behavior, reward mechanisms, and emotionality and has been envisaged as an interface between the limbic and motor systems based on its input from the limbic forebrain, viz., basolateral amygdala, hippocampal formation, anterior cingulate cortex, medial prefrontal cortex, and an output to the ventral pallidum (VP). In mammals, Ac has three distinct subdivisions. Whereas the rostral aspect of the nucleus is of largely uniform appearance (termed rostral pole), the caudal two-thirds of the Ac is divided into two subregions: the Ac core (AcC) and Ac shell (AcS). AcS has connections with limbic structures such as the VP, VTA, lateral hypothalamus, bed nucleus of the stria terminalis (BST), and periaqueductal gray. AcC is similar to the surrounding striatal areas and has strong connections with the structures of the basal ganglia including the nucleus subthalamicus, globus pallidus, and SN pars compacta. The AcS and AcC are histochemically separable by the differential distribution of neurochemical markers, such as immunoreactivity to calbindin (CB), calretinin, neuropeptide Y (NPY), and tyrosine hydroxylase (TH). Overall, the core can be regarded as similar to the caudatoputamen, and the shell as being more closely related to the extended amygdala and other limbic structures. Earlier studies have demonstrated the presence of noradrenaline (NA)-containing fibers in ventral striatal structures, including the shell, but not in the core of the Ac. The primary source of these NA-immunoreactive afferents to the AcS is the A2 region of nucleus of the solitary tract (NTS).

Since the evolution of basal ganglia in amniotes seems highly conservative, the question arises as to whether the Ac of the domestic chick also contains separable subregions, similarly to the caudal Ac of mammals. The original concept of the location of the chicken Ac has been revised according to more recent data suggesting that, at rostral levels, the Ac extends to the entire medioventral part of the MSt. At more caudal levels, the ventral tip of the lateral ventricle is surrounded by the BST, pars lateralis (BSTl), rather than the Ac, whereas the Ac proper is shifted laterally from the BSTl. A hodological difference has been found between the Ac and MSt in the chick, i.e., the accumbal neurons project to the VTA, whereas the rest of the MSt projects mainly to the SN. The latter study has suggested that the avian Ac has no distinct boundary with the MSt, the accumbal neurons extending into the anatomically defined medial and ventral MSt and colocalizing with MSt neurons This region receives input from a variety of evidently limbic forebrain structures: the hippocampus of zebra finch and pigeon, the septum of pigeon, the piriform cortex and caudal VP of pigeon, and parts of the arcopallium of pigeon and mallard. Earlier

investigation has shown that the Ac of parrots can be divided into two subregions, representing the AcC and AcS, on the basis of histological markers, viz. staining for TH, CB, SP and acethylcholine esterase. In the budgerigar, CB+ neurons are present in the AcS but not within the AcC. These patterns are similar to those found in marmosets but differ from the pattern described in rats and humans in which staining for CB is more intense in the core than in the shell.

# Objectives

1. To describe the potentially reciprocal anatomical connection between neuronal pairs of the medial striatum and the dopaminergic nuclei of brainstem.

2. To analyse the relationship between the projections from the MSt to the SN and VTA, and the dopaminergic afferents terminating in MSt by combination of retrograde tracing and double fluorescence immunohistochemistry against DARPP-32 and TH. To determine the amount of DARPP-32-containing cells as a percentage of all striato-ventrotegmental and striatonigral projection neurons

3. To describe the synaptic architecture underlying the interaction between dopaminergic and glutamatergic signaling mechanisms in the MSt, using pre-embedding ultrastructural immunocytochemistry against DARPP-32 and postembedding electronmicroscopic immunocytochemistry against GLU.

4. To demonstrate the presence of ASP in medial striatal afferents of arcopallial origin, using a combination of anterograde pathway tracing and ultrastructural immunocytochemistry.

5. To define the precise location of subregions of the Ac in domestic chick, and to harmonize these observations with the current views on the location of and presumable homology with mammalian Ac subterritories, using anterograde pathway tracing and immunocytochemistry.

# Materials and methods

### Animals

Hunnia broiler hybrid domestic chicks (*Gallus domesticus*) 1–14 days of age were used in our experiments.

### Tissue preparation for fluorescence immunohistochemistry and retrograde pathway tracing

The brain tissue was fixed by transcardial perfusion using 4% paraformaldehyde and 3% picric acid solution in 0.1M phosphate buffer (PB). The brains were postfixed and cut on a vibratome into 60 μm sections in a coronal plane.

**Double-label fluorescence immunocytochemistry for DARPP-32 and TH**

Sixty μm coronal sections were incubated at first with mouse monoclonal anti-DARPP-32 antibody (1:10 000), then with Cy3-conjugated anti-mouse IgG (1:200). Then sections were incubated in rabbit anti-TH (1:1000) followed by incubation in FITC-conjugated anti-rabbit IgG (1:250). Sections were mounted in glycerol-PBS (mixture of 1:1) mounting medium. In some cases, the DARPP-32 labeled sections were incubated with 4,6-diamidino-2-phenylindol dihydrochloride (DAPI, 0.01%) for fluorescent counterstaining of nuclei. On several sections we carried out triple fluorescent labeling of DARPP-32, TH and DAPI simultaneously. These specimens were used for high resolution observation under the confocal laser scanning microscope. To achieve a better separation of fluorescent labels, Alexa 488-conjugated donkey anti-rabbit IgG diluted 1:500 was used as green fluorophore for the labeling of TH in these experiments.

**Microscopic observation and quantitative analysis of TH- and DARPP-32-immunolabeled and DAPI+ structures in the MSt**

Sections were viewed and photographed under a BIO-RAD MRC1000 confocal laser scanning microscope, using LaserSharp image capturing and processing software. We observed specimens containing the MSt by using an Olympus BX50 fluorescent microscope. From the regions of interest (MSt, VTA, SN) quasi-random images were taken and, on each image, 10 cells were randomly selected for counting the TH+ axon terminals on DARPP-32+ neurons (in MSt) or the DARPP-32+ fibers juxtaposed to TH+ cell bodies (in the VTA and SN).

All DAPI labeled cell nuclei of neuronal character were counted in the MSt. Then, the double labeled (DAPI + Cy3), i.e. DARPP-32+ cells were also counted in the same region. The mean ratio of the DARPP-32+ somata as percent of total was calculated from 15 sections of five brains.

**Confocal microscopy for DARPP-32-TH-DAPI triple labeling**

Confocal laser scanning microscopy was used to visualise DAPI-staining combined with double immunofluorescent labeling. We used Green HeNe laser for Cy3-

(DARPP-32), Ar laser for Alexa 488- (TH) and UV laser for detection of DAPI-labeling.

**Retrograde tracing and correlative analysis of Fast Blue labeled and DARPP-32 immunoreactive neurons in the MSt**

0.1 µl of the retrograde tracer Fast Blue (FB, 3%) was injected stereotaxically into the VTA or SN, five animals each, using a 1.0 µl Hamilton syringe, mounted on a Kopf microinjector unit. The sections were viewed and charted on an Olympus BX40 fluorescent microscope connected to a Neurolucida image analysis system (Version 3.0, Microbrightfield). From each brain four sections were selected for counting FB+ and DARPP-32+ cells using a dual absorption filter for simultaneous detection of Cy3 and FB. On every section a set of sampling frames of 100 µm × 100µm was projected over the MSt. The distance between the frames was 250µm. The DARPP-32+ and FB+ cells falling in the squares were counted only if their perikarya did not touch the 'forbidden lines'. For statistical analysis of data, repeated measures ANOVA followed by post-hoc Student's *t*-tests were used.

**Tissue preparation for electronmicroscopy**

Chicks were transcardially perfused using 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1M PB. The brains were postfixed and 50 µm thick coronal sections were cut on a vibrating microtome.

**Preembedding EM immunocytochemisty against DARPP-32**

Coronal sections were incubated with mouse monoclonal antibody to bovine DARPP-32 (1:10000) followed by biotinylated anti-mouse IgG (1:100), and avidin-biotin-horseradish peroxidase (ABC)-reaction (1:100). The immunocomplex was visualised by 0.05M diaminobenzidine (DAB) and 0.001% hydrogen peroxide.

**Postembedding EM immunocytochemistry against glutamate**

Sections were osmicated (1% buffered $OsO_4$), dehydrated in a graded series of ethanol and propylene oxide, and flat-embedded in Durcupan. Representative samples from the MSt were re-embedded in gelatin capsules. Ultrathin sections were cut with a Reichert ultramicrotome, and placed on Formvar-coated single slot nickel specimen supports. These were then incubated on droplets of the following reagents: 1% periodic acid, 1% sodium metaperiodate, bovine serum albumine (BSA) in 0.01M phosphate-buffered saline. Then the sections were incubated with rabbit

polyclonal anti-GLU antibody (1:5000–7000), followed by anti-rabbit IgG conjugated with colloidal gold (15nm particle size, 1:40). The sections were post-stained with aqueous uranyl acetate and lead citrate.

**Quantitative morphometric analysis**

Digital electronmicrographs were taken from selected brain tissue of the MSt (30 images). The images were visually scanned in full and a total of 41 asymmetric synapses were identified in the MSt. The total area of the analysed images was 104.2 $\mu m^2$. The areas of the dendritic and axonal profiles with a visible synapse were identified and measured, and the gold particles falling over the areas manually counted. In order to verify the selective accumulation of gold particles over axon terminals as compared to dendrites we carried out Kruskal–Wallis test followed by *post hoc* Mann–Whitney *U*-tests. Presynaptic axon terminals were categorised according to whether the postsynaptic dendrite contained an electron dense precipitate, highlighting immunoreactivity to DARPP-32, and whether these elements represented dendritic spines or shafts. The particle density values were compared by one-way ANOVA. For comparison of frequency data to a hypothetical uniform distribution $\chi^2$ tests or Fischer exact tests were used. The statistical analyses were carried out using SPSS software package.

**Anterograde pathway tracing from the arcopallium**

Biotinylated dextran amine (0.15µl 20% BDA in distilled water) was unilaterally injected via a Hamilton micropipette into the dorsal intermediate arcopallium of anesthetised chicks using a stereotaxic frame. Sections were incubated in ABC. The signal was amplified by biotinylated tyramin, followed by a subsequent ABC reaction. The stain was developed with diaminobenzidine (DAB, 0,03%) reaction.

**Postembedding EM immunocytochemistry against glutamate and aspartate**

The method of the post-embedding immunocytochemistry was the same we used in the case of GLU-immunocytochemistry. The sections were incubated with mouse monoclonal anti-GLU antibody (1:1500), followed by anti-mouse IgG conjugated with colloidal gold (10nm particle size, 1:20). For ASP-immunocytochemistry the sections were incubated with rabbit polyclonal anti-ASP antibody (1:7000), followed by anti-rabbit IgG conjugated with colloidal gold (15nm particle size, 1:25).

**Tissue preparation for anterograde pathway tracing from the NTS, and for NPY and CB-immunohistochemistry**

Animals were transcardially perfused with 4% paraformaldehyde in 0.1M PB. The brains were postfixed and sectioned at 60μm on a Leica vibratome in the coronal plane.

### Anterograde pathway tracing from the NTS

BDA (0.1μl, 20 %) was unilaterally injected via a Hamilton micropipette into the NTS using stereotaxic frame.

Sections were incubated in ABC, then the stain was developed with nickel-enhanced diaminobenzidine (DAB) reaction (0.015% DAB and 0.25% nickel-ammoniumsulphate). Sections were mounted on gelatin/chromalum-subbed slides, air-dried, dehydrated through graded alcohols, cleared with xylene, coverslipped with Depex, and viewed under an Olympus BX 51 microscope by employing the image-capturing and processing programs Viewfinder Lite and Studio Lite.

### Immunohistochemistry against CB and NPY

Tissue sections were treated with rabbit polyclonal antibody against NPY (1:16 000) or with the mouse monoclonal antiserum against calbindin $D_{28K}$ (1:500). Then the sections were incubated with biotinylated anti-rabbit IgG (1:100) or with biotinylated anti-mouse IgG, respectively, followed by ABC-reaction.The stain was developed by using the nickel-enhanced DAB reaction, as above.

## Results

### Connectivity of MSt and tegmental dopaminergic nuclei

*Distribution of DARPP-32 and TH immunoreactive structures in the brainstem*

A considerable proportion of the fibers descending from the telencephalon and radiating from ansa lenticularis contained DARPP-32. These fibers innervated the territory of brainstem dopaminergic nuclei VTA and SN, overlapping with the TH+ positive cell bodies. TH was present in medium to large sized multipolar neurons in the VTA and SN. No cellular co-localisation of DARPP-32 and TH labeling was detected. The DARPP-32+ axons entering the regions of dopaminergic cells, such as VTA and SN, formed varicose terminals. These varicosities were often juxtaposed to the somata or proximal dendrites of TH+ cells. On average, we found 2.2±0.54 (mean±s.e.m., n=4) juxtapositions per TH+ cell body in one optical section.

*Distribution of DARPP-32 and TH immunoreactive structures in the MSt*

We observed DARPP-32-immunoreactive medium sized multipolar neurons and a dense mass of neuropil in the entire avian striatum including MSt. TH+ fibers were also abundant in the MSt. The TH-containing axons formed baskets of varicosities around the somata of the MSt neurons.Virtually all cells of the MSt were surrounded by TH+ fibers but only 80 ± 0.72% (mean ± S.E.M., n = 4) of all DAPI-labeled MSt neurons were immunoreactive to DARPP-32. Juxtapositions were detected between the varicosities of TH+ axons and DARPP-32+ cells: 4.43±0.52. (mean±s.e.m., n=4) juxtaposition per cell per optical slice. Counterstaining with DAPI enabled the DARPP-32 immunonegative neurons to be distinguished from other 'empty baskets' such as capillaries.

*Distribution of FB-labeled cells and co-localisation with DARPP-32 in the MSt*

The distribution of retrogradely labeled cells was charted in series of coronal sections of the MSt and compared with the distribution of DARPP-32+ cells. The overall density of DARPP-32+ cells was much higher (VTA-injected brains: 781.2 ± 53.6; SN-injected brains: 835.9 ± 52.9, expressed as cell No./mm2; mean ± S.E.M.) than the density of FB+ cells (VTA injected brains: 25.0 ± 4.5; SN injected brains: 13.6 ± 7.1) regardless of whether the injection was placed in the VTA or SN. Neither the injection site nor the rostrocaudal coordinate had a significant statistical effect on the densities of FB+ or double labeled neurons. Significantly more cells projected to the VTA than to the SN ($t = 3.01$, $P < 0.05$) from the rostral part of MSt. However, this difference disappeared in the caudal part of MSt and, therefore, when assessed in the entire MSt, the overall density of retrogradely labeled neurons did not differ according to the placement of FB injection. A similar trend was observed in the case of double labeled cells (interaction: $F = 7.4$; d.f. = 1; $P < 0.05$, $t = 2.56$, $P < 0.05$). Overall, there was a significantly greater percent of FB+ cells co-expressing DARPP-32 in the SN-injected group (60.2 ± 4.1%) than in the VTA-injected group (40.6 ± 2.5%; $t = 4.01$, $P < 0.01$).

**Pallial afferents of striatal projection neurons**

*Glutamatergic afferents of striatal projection neurons*

Using ultrastructural electron microscopy of chick specimens double-labeled against GLU and DARPP-32 we observed direct synaptic connections between GLU-immunoreactive axon terminals and DARPP-32-labeled dendrites in the MSt. The

vast majority of axon terminals with asymmetric synapse were Glu+, and these terminals synapsed with both DARPP-32+ and DARPP-32− dendrites. However, DARPP-32+ dendrites also synapse with axon terminals that do not express GLU. Axon terminals synapsing with dendritic spines tend to have more gold particles than those synapsing with shafts (ANOVA $F$=5.22, d.f.=1, $p$<0.05). No other factors such as the DARPP-32 content of the postsynaptic dendrite had any significant effect on the particle density in axons. We did not find statistically significant colocalisation between DARPP-32 and GLU either in somata or in dendrites, in agreement with the notion that glutamatergic neurons are not present in the striatum.

*Electron microscopy of the fibers arising from arcopallium*

When combining anterograde pathway tracing with electron microscope immunocytochemistry, numerous BDA-containing fibers, anterogradely labeled from intermediate dorsal arcopallium, were detected in the MSt. The axon terminals contained round and clear vesicles and asymmetric synapses corresponding morphologically to the excitatory type. The bulk of anterogradely labeled fibers were immunoreactive to ASP but not to GLU.

**Nucleus accumbens in the domestic chick**

*Anterograde pathway tracing from NTS*

Anterogradely labeled fibers were observed rostrally in the ventrobasal part of telencephalon, i.e., the tuberculum olfactorium (TO) and the region lateroventral to the lateral ventricle (presumed AcS). The presumed AcC was largely devoid of afferent fibers. More such fibers were observed caudally in the medial and in the lateral BST bilaterally, with an ipsilateral dominance. Fibers ascending from the NTS were present primarily in the caudal part of the Ac. At more rostral levels, a number of anterogradely labeled fibers were observed in the ventral part of the Ac. The presence of puncta and extensive branches of fibers in the ventral part of the Ac represented terminal fields.

*CB-, NPY- and DARPP-32- immunohistochemistry*

The ventral field of the Ac (putative shell) was rich in CB+ perikarya and fibers, whereas the dorsal and more medial parts of the Ac (putative core) were almost devoid of CB immunoreactivity, only a few CB+ elements being present in the latter region. The TO and VP were abundantly labeled with CB+ elements. The putative AcS was particularly rich in CB+ fibers, whereas other subpallial (MSt and lateral

striatum) and pallial (nidopallium) regions were characterized primarily by the presence of numerous CB+ perikarya.

Neuronal perikarya immunoreactive to NPY were only rarely observed in the ventral striatal-pallidal regions investigated in the present study. Although NPY-immunoreactive (NPY+) fibers were abundant in the entire ventrobasal forebrain, including the lateral septal nucleus, the density of NPY+ fibers was markedly greater in the areas representing the putative AcS than in the adjacent putative AcC. In the putative shell, the fibers were arranged as a dense meshwork.

For better separation of the BSTl, particularly its rostral aspect, from the putative AcC, we used immunostaining against DARPP-32. Characteristic for dopaminoceptive neurons, this peptide was abundant in the striatal region. In accordance with previous observations, only sparse immunolabeling to DARPP-32 was found in the rostrobasal juxtraventricular area, delineating the BSTl distinctly from the surrounding rostral Ac. However, no clear separation between the Ac and MSt was observed by using DARPP-32 as a marker.

# Discussion

### Connectivity of MSt and tegmental dopaminergic nuclei

The present study supports the hypothesis that DARPP-32-containing striato-nigral and striato-ventrotegmental fibers arising from the avian striatum synapse with the dopaminergic neurons of the brainstem. The quantitative analysis of the present study demonstrated a distinct pattern of distribution of striatofugal neurons targeting VTA or SN. Despite a considerable spatial overlap between the two populations shown previously, the neurons projecting to the VTA tend to be accumulated in the rostral MSt, whereas those projecting to the SN are more abundant in the caudal MSt. A similar tendency was evident for the double labeled neurons, co-expressing DARPP-32. The percentage of DARPP-32-expressing neurons was significantly greater in the nigra-projecting group (60%) than in the VTA-projecting group (40%). On the one hand, our findings prove that a considerable percentage of DARPP-32+ fibers contacting TH+ neurons of the brainstem indeed originate from the MSt. On the other hand, surprisingly many striato-tegmental projection neurons, particularly those projecting on VTA, proved to be DARPP-32 negative. Notably, although both SP+ and ENK+ striato-nigral projection neurons are known to possess DARPP-32, a smaller percentage of ENK+ striatal neurons than SP+ striatal neurons has been

found to co-express DARPP-32 in the pigeon. The presence of DARPP-32 in only 7–24% of ENK+ striatal cells may be related to D2 receptors, with a possible modulatory effect on GLU receptors. Since DARPP-32 is more prevalent in SP+ projection neurons, many of the DARPP-32-negative neurons projecting to the SN, and mainly to the VTA, may be ENK+. Conversely, the majority of the DARPP-32+ contingent of striato-tegmental projection neurons likely represent SP+ cells. Previous findings have indicated that DARPP-32 is enriched in those neurons expressing the D1 receptors, which tend to be localized on SP+ striatal projection neurons. Both SP+ and ENK+ striato-nigral/striato-ventrotegmental neurons may contain DARPP-32 and receive direct dopaminergic input. This is consistent with previous ultrastructural studies showing synaptic contacts between dopaminergic terminals and SP+ or ENK+ striatal cells. However, as evidenced by the present study, dopaminergic (TH+) terminals were found to juxtapose also DARPP-32-negative cell bodies, which may represent D2 expressing projection neurons or interneurons of the striatum.

The presence and distribution of DA and DA receptors have been demonstrated also in the brain of different avian species by several techniques: TH-immunocytochemistry, direct DA-immunocytochemistry, DA-receptor autoradiography, DA-receptor mRNA using in situ hybridization. Although the TH+ synaptic baskets in the MSt could also contain other catecholamines such as adrenaline or NA, we categorized them as predominantly dopaminergic, based on earlier neurochemical and anatomical data. The claim that the juxtapositions observed in the present study represent genuine synapses can be substantiated by previous electron microscopic studies demonstrating that TH+ terminals make synaptic contact with the same type of striatal neurons as those containing DARPP-32. Similarly, the juxtapositions observed by us in the tegmental dopaminergic nuclei likely correspond to those SP- or ENK-containing terminals, which have been shown to form synaptic junctions with dopaminergic neurons of the nigra. One earlier study reported the presence of DARPP-32 in the midbrain dopaminergic nuclei, VTA and SN of the quail in the form of ‘fibrous and punctate’ structures. The authors raised the possibility that DARPP-32 immunoreactivity might co-localize with DA in certain structures of the SN and VTA. Alternatively, DARPP-32 and DA could be present in distinct but closely apposed elements (also supported by the present study), similar to the situation found in the pigeon telencephalon.

The presence of D1 receptor in striato-nigral terminals has been described previously in mammals, and activation of D1-receptors was found to modulate GABA release in the SN pars reticulata. Dendritically released DA can inhibit the activity of SN pars reticulata neurons via local stimulation of D1 receptors. However, other studies failed to confirm such an effect of endogenous DA on the striatal input of the nigra. DA is also released from the somata of SN neurons by exocytosis. The presence of close appositions between the DARPP- 32+ and TH+ elements in the chick tegmentum raises the possibility that such contacts are not merely one-way (orthograde) synapses. Endogenous DA may exert a retrograde synaptic effect on the afferent striato-tegmental fibers, apart from the reported extrasynaptic action.

Our findings, in particular the abundance of juxtapositions observed in the avian brainstem and the MSt seem to support the existence of reciprocal circuits connecting pairs of striatal and tegmental neurons.

**Pallial afferents of the MSt**

*Connectivity of DARPP-32+ neurons and glutamatergic fibers in the MSt*

The results of our present electron microscopic study indicate that glutamatergic transmission may participate in the postsynaptic regulation following DA receptor binding in the avian brain. In the MSt, DARPP-32 does not colocalise with GLU in the cell bodies or dendrites, in agreement with the observation that the vast majority of cells here represent GABAergic medium spiny neurons (also containing SP or ENK). It is this cell type that seems to contain DARPP-32 together with D1-receptors and to receive simultaneous input from glutamatergic and dopaminergic axon terminals. The former arrive from various parts of the pallium externum, arcopallium and amygdaloid arcopallium, whereas the main sources of dopaminergic terminals are the VTA and SN. The dopaminoceptive medium spiny neurons may act as coincidence detectors and are also capable of forming memory traces. Excitatory amino acid input *via* NMDA, AMPA and metabotropic GLU receptors arises from the PE(carrying, e.g. visual or taste signals) or the amygdaloid pallium (conveying, e.g. fear-related signals), whereas dopaminergic input *via* the D1 receptor, arising from the tegmentum, acts as reinforcer of behaviour. The key role of DARPP-32 as an integrator molecule, whose phosphorylation state is mutually influenced by all of the above mentioned receptors, can be envisaged as a highly sensitive switch capable of steering the medium spiny neuron in a “go” or “no-go” direction.

Our finding that GLU is present in a high percentage of axon terminals forming asymmetric synapses with DARPP-32-containing dendritic elements substantiates that the mechanism outlined above may be operational also in the chick forebrain. However, an apparent lack of GLU-like immunoreactivity in a considerable percentage of excitatory type terminals synapsing with DARPP-32+ dendrites raises the possibility that an excitatory transmitter other than GLU might also be involved.

*Fibers arriving from arcopallium*

ASP could function as an excitatory neurotransmitter in some systems. In a previous study from our laboratory, it has been described that a considerable percentage of arcopallial neurons (15%) was found to contain only ASP. ASP-immunoreactive axon terminals with or without colocalisation of GLU were found to form asymmetrical (putative excitatory) synapses with dendritic profiles of the MSt and nucleus accumbens. We surmised that ASP may play the role of a signalling molecule in the avian MSt/accumbens, potentially associated with the aversive response. The presence of ASP in the intermediate medial mesopallium–arcopallium/amygdala – MSt loop, implicated in passive avoidance learning of domestic chicks, may represent a novel mechanism of neural modulation associated with early adaptive learning and motivation.

**Nucleus accumbens of the domestic chick**

Based on the results of tract tracing, and CB- and NPY-immunohistochemistry, the Ac of the domestic chick can be divided into subregions probably representing the core and shell. The shell subregion is located more ventrally than the core and is larger in its lateral and medial extent than the core subregion. The putative AcS is rich in CB-immunoreactivity, whereas staining in the putative AcC is much lighter. This pattern is similar to that found in the budgerigar, lizard and marmoset but differs from the staining pattern found in rats and humans. NPY-labeling in the chick brain is also stronger in the AcS. This pattern is similar to that reported in mammals. The AcS subregion is continuous with the surrounding TO and the relevant part of the VP, whereas the CB-impoverished AcC subregion is coextensive with part of the MSt, as distinct from other parts of MSt, which contain denser labeling with CB.

The separation of AcS and AcC in the relevant region of chick brain is further specified by our hodological finding, i.e., the presence of anterogradely labeled fibers in the presumed shell subregion, arising from the NTS. Although the

noradrenergic nature of this particular projection has not been specified in birds, the presence of NA-ergic fibers in the region that we define as the AcS has been reported in quail and chicken brains.

One complication in this system is the precise position of the BSTl. This terminology was first used to designate a region previously defined as the Ac, whereas the proposed site of the "genuine" Ac was shifted accordingly. The parcellation of the Ac-relevant region is further supported by physiological observations. No ingestive response has been found when the AMPA-antagonist is injected into the site of the "classic" Ac (now officially renamed the BSTl). A useful marker for the position of BSTl is DARPP-32, which strongly labels all striatal (and presumed accumbal) components, whilst immunolabeling in the BSTl is weak.

Impulsive choice behavior has been found to be a typical consequence of lesions to the AcC of mammals. Accordingly, lesions to the caudal MSt of domestic chicks (AcC, in our study) were also found to elicit impulsive choice behavior.

In summary, an Ac-relevant region can be defined in the ventrobasal forebrain of the domestic chick. Based upon the data of the present report and previous physiological observations, the Ac region extending between coordinates A10.6 and A8.8 can be subdivided into a core and a shell, the core corresponding to the ventromedial and juxtaventricular MSt dorsolateral to BSTl, and the shell representing an arched region situated ventrally and ventrolaterally to the core. Whereas the suggested core lies entirely within the boundary of MSt, the shell seems to partially overlap the VP. In the parrot study, the relative position of suggested core and shell subregions is similar to that described here for the domestic chick. However that study did not show the BSTl, or specify the rostral pole of accumbens. Conversely, in our study, no shell/core parcellation was found in the MSt rostral to A10.6 by immunocytochemical staining. This part of the MSt probably corresponds to the rostral pole of the Ac of mammals.

## Summary and conclusions

1. Approximately 80 % of all striatal cells of domestic chicks are immunoreactive to DARPP-32. However, only 40 % of striatonigral projection neurons and 60 % of striato-ventrotegmental neurons contain DARPP-32. Thus, those neurons which are devoid of DARPP-32 may also play an important role in motivation, learning,

reinforcement of behavior and in the neural circuits underlying locomotor regulation.

2. Since the bulk of striatonigral projection neurons express SP as co-transmitter, and the SP-containing neurons likely express D1 receptor, the same neuronal population of striatum may project on the SN and receive afferents from it. This anatomical situation enables the establishment of reciprocal circuits consisting of coupled neuronal pairs.

3. In the MSt of the domestic chick, glutamatergic axon terminals directly synapse with DARPP-32 containing dendrites. This may underly synaptic plasticity, similarly to mammals.

4. A contingent of axon terminals arising from the arcopallium and forming excitatory, asymmetrical synapses are devoid of GLU but are immunoreactive to ASP. Thus ASP may play the role of neurotransmitter in these fibers.

5. Based upon immunohistochemical pattern and the connection with the nucleus of the solitary tract, the caudal part of nucleus accumbens of the domestic chick can be subdivided into core and shell subregions, the former being continuous with the dorsal striatum, and the latter being coextensive with the ventral pallidum, similar to the situation found in mammals. The rostral part of medial striatum, lateral to the telencephalic ventricle and the BSTl accomodates the putative rostral pole of accumbens.

# Publications forming the basis of the dissertation

## *Research reports*

**Bálint E**, Kitka T, Zachar G, Ádám A, Hemmings HC Jr, Csillag A. 2004. Abundance and location of DARPP-32 in striato-tegmental circuits of domestic chicks. J Chem Neuroanat. 28:27-36.

**Bálint E**, Csillag A. 2007. Nucleus accumbens subregions: hodological and immunohistochemical study in the domestic chick (Gallus domesticus). Cell Tissue Res. 327:221-30.

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## *Abstracts*

Zachar G., Ádám Á., **Bálint E**., Kitka T., Csillag A. Correlative analysis of dopaminergic and dopaminoceptive neural elements in the striatum and the

tegmentum of domestic chick (in hungarian). VIII. Conference of Hungarian Neuroscience Association. 2001

**Bálint E**., Kitka T., Csillag A. Quantitative analysis of reciprocal connections between the tegmentum and the medial striatum in the domestic chick (Gallus domesticus). IX. Conference of Hungarian Neuroscience Association. 2003. Clinical Neuroscience 56/2, p. 9. 2003

**Bálint E.**, A. Csillag. Nucleus accumbens subregions: hodological and neurochemical analysis in domestic chick. Avian Brain Conference. 2005. Budapest

**Bálint E.**, A. Csillag. Nucleus accumbens subregions: hodological and immunohistochemical study in the domestic chick (Gallus domesticus). International IBRO Workshop Budapest, Hungary, 26-28 January 2006.

Csillag A., **Bálint E.**, A. Adam, G. Zachar. Basal ganglia organization: relevance to motivation and learning of domestic chicks. IBRO Satellite: Brain Mechanisms, Cognition and Behaviour in Birds Heron Island, Queensland, 19- 23 July, 2007

Csillag A., Adam A., Zachar G., **Bálint E**. Potential signaling role of aspartate in striatal synaptic circuits of the domestic chick. SFN, Neuroscience 2007, San Diego, California, 3-7 November

# Other publications

## *Research reports*

Montagnese CM, Zachar G, **Bálint E**, Csillag A. 2008. Afferent Connections of Septal Nuclei of the Domestic Chick (*Gallus domesticus*). A Retrograde Pathway Tracing Study. J Comp Neurol. 511:109-150.

## *Abstracts*

Csillag A., **Bálint E**. Afferent connectivity of the olfactory tubercle in the domestic chick. IBRO Satellite: Brain Mechanisms, Cognition and Behaviour in Birds Heron Island, Queensland, 19- 23 July, 2007

**Bálint E,** Csillag A. 2007. Efferent connections of the ventrobasal telencephalon in the domestic chick. A Magyar Idegtudományi Társaság (MITT) XI. Konferenciája Szeged, 2007. január 24-27.

**Bálint E.**, Csillag A.. Diencephalic and brainstem projections of the nucleus accumbens in the domestic chick. SFN, Neuroscience 2007, San Diego, California, 3-7 November