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FINAL REPORT

Having concluded the current project period, the main results can be summarized as follows.

I. Pathways and neural mechanisms of the nucleus accumbens (NAc)

1. The majority of source neurons of the descending pathway arising from the NAc core of the domestic chicken and terminating in the parabrachial nuclei contain neurotensin (NT). Involvement of the NAc in this pathway is a novel finding. In mammals, a similar descending pathway originates from the bed nucleus of stria terminalis, lateral part (BSTl). This difference in the higher regulatory circuit of gustatory sensation might reveal a fundamental element of the neural organization of feeding, and is also relevant to different feeding stategies and food-related memory formation in the mammalian and avian brains. The results have been published (Bálint E, Balázsa T, Zachar G, Mezey S, Csillag A, Brain Structure and Function 2014 *epub*, *Print version:* Vol. 221, 605-616 (2016).

2. Selective mapping of amydalo-accumbens connectivity of chick brain revealed by retrograde and anterograde pathway tracing.
Based on our previous study (Hanics et al, 2012), where the termination pattern of the amygdalo-accumbens pathways in the domestic chick and in the rat had been compared, current research elaborated on the source region of the amygdalo-accumbens pathway in the (amygdala equivalent) arcopallium of the domestic chicken.

Retrograde tracing with Cholera toxin subunit B (Ctb), injected into the NAc, yielded labeled perikarya in a ring-shaped area of arcopallium, including Aid, Aiv (v), Ap (PoA) with a wedge-shaped node of dense accumulation in a laterodorsal subunit. Injections into the BSTl and the extended amygdala (EA) led to similar distribution of labeled cells in the arcopallium.

NAc-bound arcopallial neurons were devoid of the dopaminoceptive signal molecule, DARPP-32. However, DARPP-32+ immunoreactivity was present in all striatal regions (including the NAc but excluding the BSTl) and also in the lateral nidopallium bordering the dorsal arcopallium. The position of source neurons for the arcopallial-accumbens pathway was verified also by anterograde pathway tracing.

The study enabled precise topographic description of the two main output fiber streams: one presumably corresponding to the stria terminalis of mammals, and another putative equivalent of the ansa peduncularis of mammals. These fibers merge in the ventral amygdalofugal tract (vaf) and, having bypassed the amygdaloid taenial nucleus, they traverse the subpallial (extended) amygdala before reaching the shell, then the core of NAc and, finally, the BSTl. An attempt was made to reconstruct the course of amygdalofugal pathway in 3D. This study was published by Hanics J, Teleki G, Alpár A, Székely AD, Csillag A (2016) in Brain Structure and Function (in press, *epub* version). It also constituted an important part of the Ph.D. Thesis of János Hanics, successfully defended in 2017.
Part of this work, summarized in a lecture by our medical student and research associate Gyöngyi Teleki was awarded 1st prize at the TDK student researchers' conference of Semmelweis University, 2014, and it was presented also in the nationwide student researchers' conference OTDK in 2015.

3. Convergent amygdalar input to the extended amygdala (EA) and BSTl, as well as to NAc subregions likely transmits fear- and aggression-related signals to both viscerolimbic (EA) and learned reward- and motivation-related (NAc) ventrobasal forebrain regions. Based on evidence from previous and present observations, this pathway is excitatory, with potential

cotransmission of Glu and Asp. Analysis of the precise localization of this forebrain pathway also enabled placement of targeted lesions in the vaf, in association with microdialysis studies, to see if the amount of Asp and Glu (and their relative percentage) change after transection of the main amygdalofugal pathway.

While, due to a high initial variability of excitatory amino acid (EAA) release, these experiments did not produce conclusive results, lesions to the NAc, combined with socially driven behaviors in young domestic chicks, yielded unexpectedly promising data. We found that social group preference was not affected by bilateral NAc lesions, however, under conditions of social isolation stress, the NAc-lesioned chicks responded with more intense and lasting distress vocalization, as compared with sham-operated control chicks. Yet distress calls were strongly attenuated both in NAc-lesioned and sham birds on the presentation of an overhead silhouette of predatory bird. The results indicated that one possible role of the NAc could be learned suppression of fear-related behaviors under conditions of expected (but not clear and present) danger. More importantly, the findings are consistent with a novel interpretation of the role of NAc: decision-making in the absence of detectable rewarding or repulsive cues. This study has been published (Zachar G, Tóth AS, Balogh M, Csillag A, 2016, Eur. J. Neurosci.). Part of this study, presented by our medical student research associate, András Tóth, was awarded 1st prize at the TDK student researchers' conference of Semmelweis University, 2016, and it was awarded a sponsor's prize of the Hungarian Anatomical Society, at the nationwide student researchers' conference OTDK in 2017.

II. Amino acid transmitters and the role of D-aspartate

1. As a continuation of our previous work on the proportional release of L-glutamate (L-glu) and L-aspartate (L-asp) in striatal regions, we began to investigate the role of D-aspartate (D-asp). This amino acid is implicated in the promotion of neurogenesis. Based on collaboration with the Department of Pharmacodynamics, Semmelweis University, the conditions for chiral separation and detection of D-asp in biological samples have been optimized using the CE-LIF method (Jakó et al, Electrophoresis 2014).
As a next step, D-amino acids (together with L-enantiomers) were determined in tissue extracts from various brain regions of the domestic chick. First, it was assumed that the D-asp content would follow known modifications of neurogenesis upon avoidance learning. This part of the work had been demonstrated in a conference abstract (Zachar G, Wagner Z, Tábi T, Jakó T, Szabó A, Szökő É, Csillag A: Is the elevation of D-aspartate in brain regions of high proliferative activity specific for learning?, Society for Neuroscience Annual Meeting, San Diego, USA, 2013).
Although an elevation of D-asp was not consistently correlated with avoidance training, it clearly depended on the age of the animal (D-asp decreasing with age according to a decline of early neurogenesis) and also on the distance of the measured region from the ventricle. Thus, the results prove a correlation between 'natural' neurogenesis and D-asp, rather than between learning-induced neurogenesis and D-asp. Preliminary findings have been shown at international conferences (Zachar et al., 2014, Cortical Development Conference, Chania, Crete, Greece; Zachar et al, IBRO Workshop Debrecen 2014; Varga et al. 15th Biennial Conference of Hungarian Neuroscience Society, Budapest, 2015), measuring D-asp in tissue extracts from juxtaventricular and apoventricular regions of one telencephalic hemisphere, together with immunocytochemistry of BrdU and doublecortin (DCX) in the contralateral hemisphere, to show the link between newly generated and migrating neurons, and the tissue content of D-asp.
2. For any functionally relevant changes of D-asp to be detected, it was essential to systematically measure its resting level in tissue extracts, depending on the age of the animal,

and also on the brain region. Preliminary findings had been reported at the 14th International Congress on Amino Acids, Peptides and Proteins, Vienna (August 3-7, G. Zachar et al. 2015), and now this work was carried further, also comparing the results between domestic chicks and rats. We now have a consistent method in hand for selective measurement of D and L enantiomers of aspartate not only in tissue extracts but, under certain conditions, also in microdialysis samples. The recent data revealed that most of the D-asp was present in the extracellular fluid (also detectable by in vivo microdialysis, with an evoked release by high potassium), whereas the predominant part of L-asp (as, indeed, of L-glu) is found in non-extracellular form (as measured in tissue extracts). We found a clear age dependence in D- (but not L-) asp, some regional differences, and overall a remarkably similar level of D-asp in rats and chicks. In addition to an elevation of L-EAAs on aversive stimulus (handling stress, reported previously by Zachar et al., 2012), we now showed similar elevation in response to reward stimulus. It appears that D-asp contributes substantially to the elevation of extracellular aspartate on stimulation.
These findings have been written up in an *in extenso* report ('Age-related and region-specific alterations of free D- and L-aspartate in postembryonic chick brain', by Gergely Zachar, Tamás Jakó, István Vince, Zsolt Wagner, Tamás Tábi, Eszter Bálint, Szilvia Mezey, Éva Szökő, András Csillag), submitted to Biochim. Biophys. Acta, Proteins and Proteomics, Special Issue (2017).
3. D-Asp and spatial learning of mice.
Chronic administration of D-Asp has been reported to improve performance of rats in the Morris water maze (MWM) paradigm (Topo et al. 2010). The effect was expected to be due to enhanced neurogenesis. In our experiments, learning improvement by D-asp was confirmed also in mice, involving faster acquisition of the task mainly in the intermediate-term memory range but also with perseverance at reversal learning. D-serine (D-ser) did not improve learning as compared to controls. Contrary to expectation, the behavioral effects were not accompanied by altered neurogenesis (detecting newly born neurons by BrdU incorporation and migratory cells by DCX). There was a tendency for increased gliogenesis (based on combined BrdU – GFAP staining) after D-ser treatment. Some of the recent results will be demonstrated at the Conference of Spanish Neuroscience Society (SENC), September 2017 (Zachar G et al.), and prepared for publication shortly thereafter.

III. Hypothalamic peptides in relation to social and reproductive behaviors of songbirds

Based partly on collaboration with Tamás Székely (Univ. of Bath, UK), the distribution of vasotocin and vasoactive intestinal peptide (VIP) has been described in the blue tit *(Cyanistes coeruleus)*. The results are largely consistent with previous reports from other avian species, with minor differences potentially due to specific behavioural features (e.g. biparental care of offspring). This work was published (Montagnese et al., Frontiers in Neuroanatomy, 2015). We also collected a large body of data from another related species, the penduline tit (*Remiz pendulinus*), with a distinctly different mating and nesting strategy, as an attempt to relate the distribution of nonapeptides to the social system of birds. This part of the work has also been published (Montagnese et al, Eur. J. Anat., 2016).

IV. Gestational exposure to 'designer drugs'
Synthetic cathinones known as 'designer drugs' have been under investigation in our lab, in relation to their known interaction with basal ganglia functions, e.g. causing apoptosis in the NAc in mice (Ádám et al., Neurotoxicology, 2014), or enhancing distress vocalization on social isolation of chicks (Zsedényi et al., Neurosci. Lett. 2014). Based partly on the project by Ágota Ádám, and in collaboration with Árpád Dobolyi, pregnant female mice were

chronically treated with methylenedioxypyrovalerone (MDPV), and tested for maternal care (nest building, pup retrieval). The hypothalamic peptides TIP39 and amylin (implicated in maternal behavior and lactation) of mothers were investigated by in situ hybridisation. MDPV treatment caused a distinct impairment of maternal care, whereas TIP39 and amylin expression were unaltered. Thus, the observed behavioral effects are likely due to agitated behavior and attentional disorder, typical of dopaminergic agents. The study will be presented at the conference of Spanish Neuroscience Society (SENC), September 2017 ('Gestational exposure to the designer drug methylenedioxypyrovalerone results in reduced maternal care and behavioral alterations in mouse pups' by Gerecsei L, Csillag A, Zachar G, Gévai L, Simon L, Dobolyi Á, Ádám Á.), to be followed by submission of a full paper.

Experimental work in progress

1. NMDA receptor subunit alterations following treatment with D-asp and cathinone drugs.
We performed experiments on Western blotting (WB) of NMDA receptor subunits in avian and mammalian brain tissues (T. Balázsa). The conditions for a quantitative analysis of NR1, NR2A and NR2B subunits (using actin as loading control) are now set. Subunit alterations are expected to underlie neurodegenerative and proliferative effects and they are also likely to follow behavioral signals (appetitive vs aversive conditioning), likely determining the effectiveness of different L- or D-EAAs. We collected brain samples for measuring NR changes from the dentate gyrus of adult mice, following chronic D-asp treatment and MWM tests (see above), and also from the striatum of 7-day-old mice following acute injection of the designer drug MDPV. WB analysis of the samples is underway.

2. Patch-clamp analysis of single NAc neurons in superfused brain slices.
We continued the previously started electrophysiological study of brain slices from NAc (report by Balázs et al. 2012, resulting from our previous OTKA grant). The experiments involve patch clamp analysis of selected medium spiny neurons of NAc core, with correlative bath application of Glu, together with L-and D-asp, and EAA transporter inhibitors, following aversive and appetitive conditioning (supervised by G. Gerber). These experiments are also being combined with expression pattern changes of NMDA receptor subunits (WB). Preliminary experiments have been carried out to check the consistency of NMDA subunit expression during exposure to in vitro conditions. The first results are promising in that the subunit pattern remains stable for at least 6 hours in brain slices in vitro.

3. Lesions to selective forebrain regions of the 'social brain network'.
Radiofrequency and excitotoxic lesions using stereotaxic approach, targeting the anterior hypothalamic area, BSTm, EA, NAc and amygdala regions in domestic chicks and mice. After histological verification, the behavioral changes (locomotor, anxiety, vigilance) and auditory distress signals will be analysed under conditions of social separation and reinstatement.