Final report: ***OTKA NN 125643 – PI:*** **Tamás F. Freund**

Synaptic connectivity changes in Alzheimer's disease

As described in the original research plan, the basal forebrain (BF), the hippocampus (HIPP) and in general the cholinergic pathways to cortical areas are notoriously affected in human cases and animal models of Alzheimer's and some other neurodegenerative diseases. The HIPP is crucial for different types of learning and memory processes and its dysfunction may lead to dementia. The septal nuclei i.e. the medial septum and the diagonal band of Broca nuclei of BF that are important relay stations of the ascending sensory pathways that connect brainstem nuclei to fore-brain circuits. The limbic hippocampo-septal pathway contains GABAergic projections, while the BF-hippocampal (BF-H) pathway is composed of GABAergic, glutamatergic and cholinergic projections. The cholinergic system plays an important role in most BF and HIPP functions and more generally, in the normal control of neuronal excitability and in higher cognitive processes in cortical areas. Anatomically and functionally, the BF and the HIPP are heavily interconnected. Several connected subcortical nuclei affect these BF-hippocampal (BF-H) functions, including the median raphe region (MRR) and the nucleus incertus (NI). However, it still remains unclear how ascending pathways influence BF and hippocampal functions and even the neurotransmitter content of some of the corticopetal afferents were unclear.

Our aim was to better understand the function of these unique brain stem –> BF –> HIPP connections, primarily focusing on fast and effective cholinergic, GABAergic and glutamatergic pathways. Using state-of-the-art anatomical, electrophysiological and behavioral methods in combination with optogenetics, our research group published several papers during the funding period and we are planning to finish other papers in the near future. Three papers are published about the involvement of the cholinergic system in inflammation and Alzheimer's disease (AD)-related changes in the brain. We also made excellent progress in other two cholinergic system-related projects that will be published in the coming year and we also published our discoveries about important subcortical pathways that can directly influence the septo-hippocampal memory related circuitry and may lead to a better understanding of these dementia-related diseases.

Project 1: Amyloid β induces interneuron-specific changes in the hippocampus of APP^{NL-F} mice

For decades, it was hypothesized that the primary trigger for the pathogenesis of AD was the accumulation of Aβ that was also responsible for Tau-pathology. Numerous studies have proposed that removal of Aβ could prevent the disease. Indeed, removal of Aβ from the brains of transgenic mice with increased levels of Aβ was associated with behavioral improvements. For a long time, several clinical trials aimed to eliminate accumulated Aβ from patient's brains, however, most of these clinical efforts have failed. In addition, some postmortem brains of people who have died in old age without apparent cognitive dysfunction show at least as heavy a plaque load as brains from patients with advanced symptoms of AD.

These apparent discrepancies make it especially important to better understand both the natural course of Aβ accumulation and the mouse models used for AD research.

More than one hundred mouse models were developed for investigating the effects of Aβ accumulation. Expression of their mutated amyloid precursor protein (APP) was controlled by an artificially strong non-specific promoter that forced the overexpression of APP even in populations of cells that would not necessarily express APP, especially not in a large quantity. Overexpression could generate effects in neurons that otherwise would have never occurred in AD. Furthermore, these mice also show an increased level of the proteolytical fragments of APP that may induce Aβ-independent artefacts. To mitigate these issues, here we used APP^{NL-F} mice, which express APP driven by its endogenous mouse promoter. To closely mimic human phenotype, the APP^{NL-F} mouse APP gene was first humanized and then it was modified with a Swedish and an Iberian familial AD mutation. APP^{NL-F} mice also closely mimic the critical ratio of Aβ isoforms found in human patients. Previous behavioral experiments showed modest abnormalities in APP^{NL-F} mice most often only beyond the age of one year.

Here we investigated morphological alterations, synaptic $GABA_A$ receptor content, synaptic areas, changes in hippocampal interneuronal connections and numbers, and behavioral alterations in APP^{NL-F} mice. We found that most investigated parameters were not different in APP^{NL-F} mice. However, we found that the area of synapses of axon initial segment (AIS)-targeting axo-axonic INs were enlarged significantly. Contrary to data from other models, we found that PV INs are particularly resistant to amyloidosis in APP^{NL-F} mice. The expression of perineuronal nets around PV-cells seemed to be unaffected and APP^{NL-F} mice did not show a significant difference in the density of PV and SOM positive INs. In addition, PV-positive axonal fibers were rarely dystrophic.

Our results showed that even at old age APP^{NL-F} mice display only mild anatomical signs and behavioral symptoms. This suggests that even massive amyloid plaque-formations can have relatively moderate effects alone on neural network function, similar to patients with high Aβ plaque density without cognitive impairment, which may be due to the effective protection provided by glial cells. However, our results also suggest that amyloid-induced increase in excitatory activity may change the inhibitory balance in hippocampus. This is realized by increasing a compensatory inhibition of pyramidal cells on their AIS by PV-positive axo-axonic cells that seem to be resistant to Aβ.

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Project 2: Chronic Amyloid beta Oligomer Infusion Evokes Sustained Inflammation and Microglial Changes in the Rat Hippocampus via Nod-like receptor family, pyrin domain-containing 3 (NLRP3)

Microglia are instrumental for recognition and elimination of amyloid β1-42 oligomers (AβOs), but the long-term consequences of AβO-induced inflammatory changes in the brain are unclear. Here, we explored microglial responses and transciptome-level inflammatory signatures in the rat hippocampus after chronic AβO challenge. Middle-aged Long Evans rats received intracerebroventricular infusion of AβO or vehicle for 4 weeks, followed by treatment with artificial cerebrospinal fluid or NLRP3 Inhibitor, MCC950 for the subsequent 4 weeks. AβO

infusion evoked a sustained inflammatory response including activation of NF-κB, triggered microglia activation and increased the expression of pattern recognition and phagocytic receptors. Aβ1-42 plaques were not detectable likely due to microglial elimination of infused oligomers. In addition, we found upregulation of neuronal inhibitory ligands and their cognate microglial receptors, while downregulation of Esr1 and Scn1a, encoding estrogen receptor alpha and voltage-gated sodium-channel Na(v)1.1, respectively, was observed. These changes were associated with impaired hippocampus-dependent spatial memory and resembled early neurological changes seen in Alzheimer's disease. To investigate the role of inflammatory actions in memory deterioration, we performed MCC950 infusion, which specifically blocks the NLRP3 inflammasome. MCC950 attenuated AβO-evoked microglia reactivity, restored expression of neuronal inhibitory ligands, reversed downregulation of ERα, and abolished memory impairments. Furthermore, MCC950 abrogated AβO-invoked reduction of serum IL-10. These findings provide evidence that in response to AβO infusion microglia change their phenotype, but the resulting inflammatory changes are sustained for at least one month after the end of AβO challenge. Lasting NLRP3-driven inflammatory alterations and altered hippocampal gene expression contribute to spatial memory decline.

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Project 3: Interleukin-1 mediates ischaemic brain injury via distinct actions on endothelial cells and cholinergic neurons

The cytokine interleukin-1 (IL-1) is a key contributor to neuroinflammation and brain injury, yet mechanisms by which IL-1 triggers neuronal injury remain unknown. Here we induced conditional deletion of IL-1R1 in brain endothelial cells, neurons and blood cells to assess site-specific IL-1 actions in a model of cerebral ischaemia in mice. Tamoxifen treatment of IL-1R1 floxed (fl/fl) mice crossed with mice expressing tamoxifen-inducible Cre-recombinase under the Slco1c1 promoter resulted in brain endothelium-specific deletion of IL-1R1 and a significant decrease in infarct size (29%), blood-brain barrier (BBB) breakdown (53%) and neurological deficit (40%) compared to vehicle-treated or control (IL-1R1fl/fl) mice. Absence of brain endothelial IL-1 signalling improved cerebral blood flow, followed by reduced neutrophil infiltration and vascular activation 24 h after brain injury. Conditional IL-1R1 deletion in neurons using tamoxifen inducible nestin-Cre mice resulted in reduced neuronal injury (25%) and altered microglia-neuron interactions, without affecting cerebral perfusion or vascular activation.

Deletion of IL-1R1 specifically in cholinergic neurons reduced infarct size, brain oedema and improved functional outcome. Ubiquitous deletion of IL-1R1 had no effect on brain injury, suggesting beneficial compensatory mechanisms on other cells against the detrimental effects of IL-1 on endothelial cells and neurons. We also show that IL-1R1 signalling deletion in platelets or myeloid cells does not contribute to brain injury after experimental stroke. Thus, brain endothelial and neuronal (cholinergic) IL-1R1 mediate detrimental actions of IL-1 in the brain in ischaemic stroke. Cell-specific targeting of IL-1R1 in the brain could therefore have therapeutic benefits in stroke and other cerebrovascular diseases.

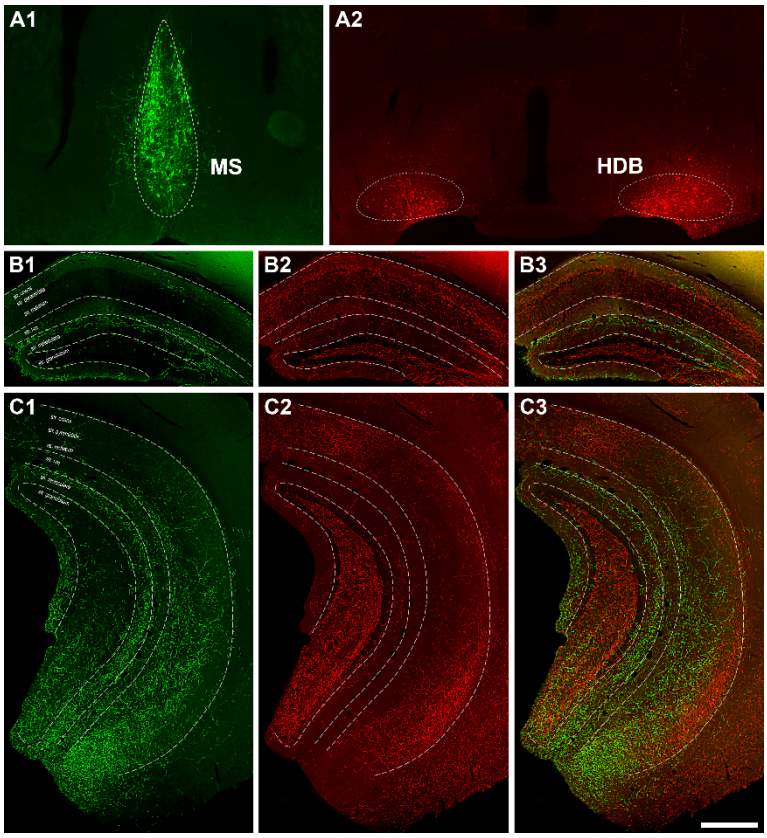
This was published in Brain Behav Immun. 2019 Feb; 76: 126–138.

Changes in the field of AD models during the funding period

The research plan had 4 original aims. Project 1-3 covered Aims 1 and 4. Aim 4 was carried out completely as planned and described in Project 1 above. The plan of Aim 1 was based on the usefulness of the APP^{NL-F} mouse model which model had already been published in prestigious journals by that time. Indeed, the APP^{NL-F} mouse model originally generated a lot of interest worldwide, because it was thought that this is the most precise and useful approach to study the effect of AD-related amyloidosis. However, as our own study and several years of research in other groups clearly demonstrated, amyloidosis in itself is not a good model for AD. Several labs in the world who invested efforts into these types of models had to realize that even by the time these mice grow up they do not show typical AD-related phenotype. And in general even the brain tissue can tolerate amyloid precipitation quite well. Therefore, we also investigated other aspects of the cholinergic system and memory related circuitry that may potentially be related to the patho-mechanism of dementia and Alzheimer's disease. These plans were described in Aims 2 and 3 and will be described here as Project 4 and 5, respectively.

Project 4: Hippocampal target selectivity of BF cholinergic neurons: an alternative cholinergic innervation of the hippocampus

Although it was generally accepted that the hippocampus receives its cholinergic afferents from the medial septal area, our specific viral tracing experiments in the beginning of the funding period demonstrated an abundant cholinergic innervation from the horizontal diagonal band as well. Furthermore, the two basal forebrain nuclei provide cholinergic input to the hippocampus in a complementary, layer specific manner: while cholinergic cells of the medial septum innervate stratum moleculare of the dentate gyrus, the horizontal diagonal band mostly targets the hilus of the dentate gyrus.



After fine-tuning the rabies-virus based tracing technology, we precisely defined the anatomical localization of cells that synaptically target the two different groups of basal forebrain cholinergic neurons mentioned above. Cholinergic cells of the medial septum projecting to the hippocampal formation receive abundant inputs locally, from the medial septum, vertical and horizontal diagonal band, as well as the lateral septum in the forebrain and the median raphe region in the

brainstem. On the other hand, hippocampus projecting cholinergic cells of the horizontal diagonal band rather receive their synaptic inputs from the lateral preoptic area and the lateral hypothalamus, while these cells lack abundant input from the neighboring basal forebrain nuclei or the median raphe region.

Using double retrograde tracing studies, we have also shown that HDB cholinergic cells that innervate the hippocampus are not the same as those that target mPFC, therefore cholinergic modulation of attention and hippocampal memory formation take place through distinct neural pathways. The dorsal hippocampus performs primarily cognitive functions while the ventral contributes to emotional aspects of learning. Using double tracing, we have demonstrated that at least 12% of the hippocampally projecting cholinergic HDB cells targets both parts of the hippocampus. We also found, that 41% of the HDB cells that project to the hippocampus are not cholinergic. We will also investigate the neurochemical identity and functional role of these non-cholinergic cells.

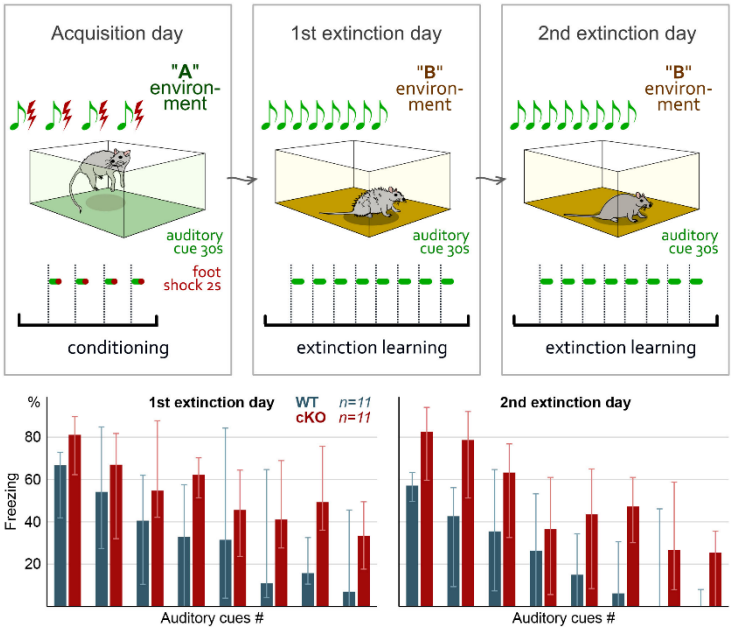
We also investigated the targets of cholinergic fibers ascending from the horizontal diagonal band. Using viral track tracing and immunohistochemistry together with fluorescent and electron microscopy, we found that these fibers make synaptic contacts primarily with calretinin-positive hilar mossy cells of the ventral dentate gyrus. As these neurons play a pivotal role in the coding of environmental novelty and cholinergic cells fire upon unexpected environmental cues, our further aim is to demonstrate the functional relation between these cell types using behavioral studies combined with chemo- and ontogenetics.

Our chemogenetic experiments suggest that contextual memory formation is inhibited by the inhibition of HDB cholinergic cells. To further identify this as a hippocampal-specific effect, we are carrying out local photostimulation or inhibition of BF cholinergic fibers in the hippocampus. In the past year, we managed to fine-tune the behavioral and optogenetic paradigm, while gaining promising preliminary results. Our main goal is to unveil the functional importance of HDB cholinergic fibers in the hippocampus and publish our data in the next year.

Project 5: The role of GABA release from cholinergic neurons

The basal forebrain cholinergic system comprises several nuclei that provide innervation to cortical areas. It contributes to the regulation of arousal, attention and memory, including fear and extinction learning, and it is implicated in anxiety and post-traumatic stress disorder. We have shown that cholinergic terminals synaptically release not only acetylcholine, but GABA as well, the release of which can be modulated independently. Although previous studies demonstrated that the alteration of GABAergic cotransmission is possible and has functional consequences in other non-cholinergic brain regions, the role of GABA release from forebrain cholinergic cells is unknown. We have created a conditional knockout mouse strain (ChAT-vGAT-cKO) showing decreased GABA release from cholinergic neurons.

Results from behavioral phenotyping of this strain revealed that decreased GABA release from cholinergic neurons led to increased hippocampal theta activity during sleep and increased cognitive performance in an operant learning task, possibly due to a relatively more efficient cholinergic effect. However, ChAT-vGAT-cKO mice showed significant deficits in fear extinction learning after cued fear conditioning (see figure on the right). These results can reveal a formerly unrecognized mechanism for certain pathological conditions.



We have also investigated the possibility of artificially expressing the vesicular GABA transporter (vGAT) specifically in cholinergic cells using AAV. This approach would allow us to enhance the GABAergic cotransmission above normal in a series of gain of function experiments, as well as to rescue the effect of its downregulated state in cKO animals. In fact, the latter could also mean a novel gene therapeutic method of fear related diseases. After a series of anatomical and behavioral experiments, the available vGAT expressing AAVs proved to be ineffective in forebrain cholinergic neurons. Therefore, we need to find a more suitable construct for efficient vGAT expression.

Additionally, as a similar phenomenon could be the basis or contribute to mood disorders, we are testing if decrease in neuronal vGAT levels in cholinergic neurons could occur naturally, in mood disorder model mice. We hope to find physiological or environmental effects that can regulate vGAT expression in BF cholinergic cells and publish our data in the next year.

Project 6 and 7: Brainstem control of memory related BF-hippocampal circuitry

Two other studies were also partially inspired and funded by this grant. These studies describe how the above mentioned BF-hippocampal circuitries are modulated by the brainstem. Our results, published in Science (Szőnyi, A. et al. Median raphe controls acquisition of negative experience in the mouse. Science, 366, 2019), revealed that the MRR harbors a previously unrecognized brainstem center that serves as a key hub for the acquisition of negative experience. The MRR vGluT2-neurons that we discovered could activate the aversion- and negative prediction-related LHb-mVTA axis and could swiftly transform the state of the basal forebrain-hippocampal system for immediate acquisition of episodic memories of the negative experience. Maladaptations in processing negative experience is the basis of several types of mood disorders, which have a huge social and economic impact on individuals and society. Selective targeting of this neural hub may form the basis of new therapies.

Our data, published in Science (Szőnyi, A. et al. Brainstem nucleus incertus controls contextual memory formation. Science 364, 2019), represent an unexpectedly specific role of

an ascending inhibitory pathway from a brainstem nucleus in memory encoding. A role of NI GABAergic neurons may be fine-tuning of the selection of memory-encoding pyramidal cells, based on the relevance and/or modality of environmental inputs. They may also help filter non-relevant everyday experiences (e.g. those to which animals have already accommodated), by regulating the sparsity of memory-encoding dorsal CA1 pyramidal neurons. NI GABAergic neuron dysfunction may also contribute to dementia-like disorders or pathological memory formation in certain types of anxiety or stress disorders.