Final report: ***OTKA K 119521 – PI:*** **Nyiri Gábor**

Control of the limbic network via ascending brain stem-septo-hippocampal pathways

As described in the original research plan, the septum and the hippocampus (HIPP) are both part of the limbic system, are crucial for different types of learning and memory processes, attention, anxiety, fear, fear extinction, aggression and emotional processing. Anatomically and functionally, the septum and the HIPP are heavily interconnected and several connected subcortical nuclei affect these septo-hippocampal (SH) functions, including the median raphe region (MRR). However, it still remains unclear how ascending pathways influence memory formation and convey emotional or motivational contexts. Even the neurotransmitter content of some of the corticopetal afferents are unclear. Our aim was to better understand the function of these unique brain stem-septo-hippocampal connections, primarily focusing on fast and effective GABAergic and glutamatergic pathways. Using state-of-the-art anatomical, electrophysiological and behavioral methods in combination with optogenetics, our 3 proposed aims were completed successfully.

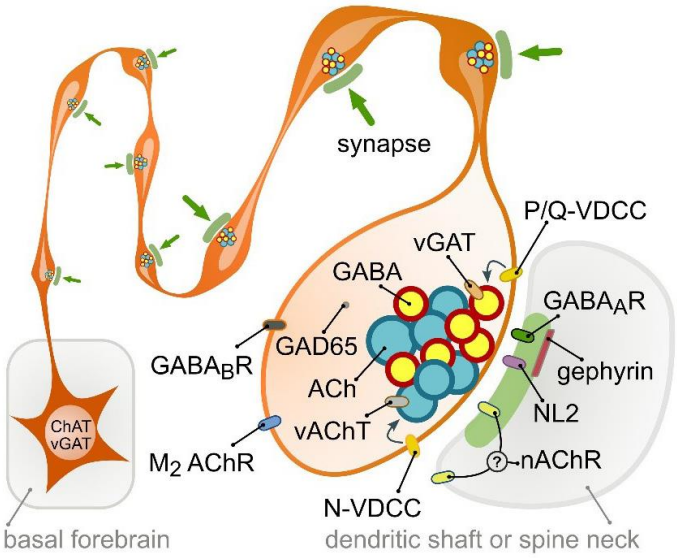
Co-transmission of acetylcholine and GABA regulates hippocampal states

The cholinergic system plays an important role in most of the above mentioned SH functions. Contemporary models of the basal forebrain cholinergic system and efforts to develop pro-cholinergic treatments have however been based largely on the assumption that cholinergic cells release only a single transmitter and it is released non-synaptically. The seemingly rare synapses on cholinergic fibres supported the concept of non-synaptic transmission. However, highly precise cholinergic transmission during reward and punishment, recordings of phasic release, and the dependence of hippocampal synaptic plasticity on the millisecond-scale timing of the cholinergic input challenged this textbook model of non-synaptic transmission by cholinergic fibres.

Therefore, we hypothesized that all cholinergic terminals establish synapses. After immunolabeling, we analysed the real incidence of synapses, localized vesicle pools using STORM super-resolution imaging and we also localized membrane-docked neurotransmitter vesicles using electron tomography. Because previous data suggested the co-localization of acetylcholine and GABA in retina and other brain areas, we also hypothesized that hippocampal cholinergic fibers may be GABAergic as well. Using immunolabelling and optogenetics combined with in vitro electrophysiology, we investigated the possible presence and subcellular regulation of hippocampal co-transmission of acetylcholine and GABA, and the role of its GABAergic component in controlling hippocampal network activity.

Challenging a decades-old model, we showed that all hippocampal cholinergic terminals established GABAergic and cholinergic synapses, and these synapses evoked composite (hyperpolarizing and depolarizing) postsynaptic potentials. Our data suggested synaptic release and action of GABA and synaptic release and a focal, synaptic and/or peri-synaptic action of acetylcholine. GABA and acetylcholine transmissions are modulated by distinct calcium channels and were mutually regulated by presynaptic auto-receptors. We demonstrated that synaptic release of GABA from cholinergic terminals alone can suppress hippocampal sharp wave-ripples effectively and it can attenuate hippocampal epileptiform activity as well.

Illustration of cholinergic terminals and their synaptic architecture



Summary illustration of some of the findings. All cholinergic terminals establish synapses, they are fully equipped with GABAergic-cholinergic co-transmission signalling machinery. GABAergic and cholinergic vesicles are regulated by different voltage-dependent Calcium channels (VDCCs). Although postsynaptic cholinergic receptor (nAChR) distribution cannot be investigated, their response latencies (that are at least and order of magnitude faster that typical non-synaptic transmission) suggest a focal, intra- and/or peri-synaptic localisation, while GABA$_A$ receptors are detected intra-synaptically. Synapses are established on both dendritic shafts and spines in hippocampus. NL2 – neuroligin 2, vAChT – vesicular acetylcholine trasporter, GAD 65 – glutamic acid decarboxylase, vGAT – vesicular GABA transporter.

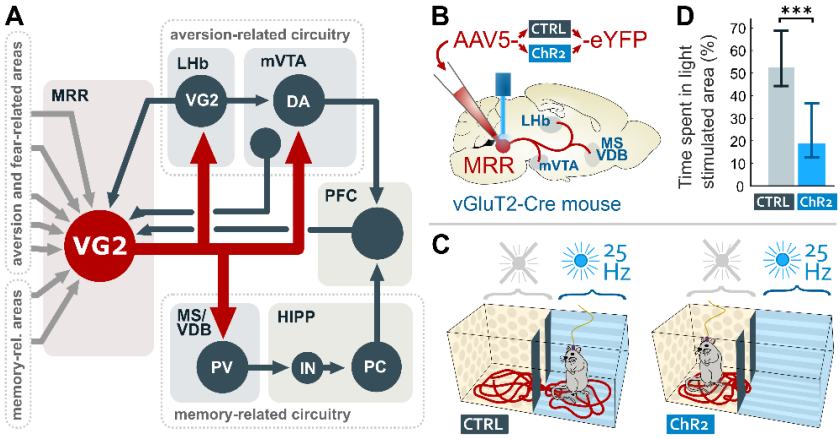
Our data that, published in Nature Communications (Takács, V. T. *et al.* Co-transmission of acetylcholine and GABA regulates hippocampal states. *Nat. Commun.* **9**, 2848, 2018), urged the reinterpretation of previous studies about the basal forebrain cholinergic system and offer a new explanation for the emergence of hippocampal epileptiform activity associated with Alzheimer's disease related loss of cholinergic innervation.

Median raphe controls acquisition of negative experience in the mouse

Coping with negative experience is essential for survival. Animals must quickly recognize a harmful situation, produce an adequate response, and learn its context, so that they can predict the reoccurrences of similar experiences. This process requires the lateral habenula (LHb) and the medial ventral tegmental area (mVTA) for evaluating and predicting aversive stimuli. LHb neurons promote encoding of aversive behavior, learn to respond to cues that predict aversive stimuli and activate negative experience-processing mVTA dopaminergic neurons (DA). Over-excitation of LHb neurons lead to depression-like symptoms, whereas their inactivation has an anti-depressant effect. Coping with negative experience also requires the septo-hippocampal system to record and recall contextual memories of events. This process

necessitates increased firing of pacemaker parvalbumin (PV)-positive neurons in the medial septum and the vertical limbs of the diagonal bands of Broca (MS/VDB) and subsequent theta-oscillations in the hippocampus. However, how all these brain centers coordinate their activity during adverse events is poorly understood.

MRR vGluT2-neurons serve as a key hub for aversive behavior.



MRR vGluT2 (VG2)-neurons process aversive events by activating neurons of LHb and mVTA, and hippocampus (HIPP)-projecting memory acquisition-promoting parvalbumin (PV)-positive cells in MS/VDB (A). After viruses made MRR vGluT2-neurons light-sensitive (B), mice were light-stimulated in a specific area (C) that caused significant avoidance of that area, compared to control mice (D). (PFC: prefrontal cortex)

Because LHb does not project directly to the septo-hippocampal system, the brainstem median raphe region (MRR) has been proposed to coordinate their activity. Although MRR plays an important role in regulating mood, fear and anxiety and neuronal projections from it have been extensively studied for decades, yet it was still unclear how MRR neurons processed these negative experiences. Using cell type-specific neuronal tract-tracing, monosynaptic rabies-tracing, block-face scanning immuno-electron microscopy, in vivo and in vitro electrophysiological methods, we investigated the neurons of mouse MRR that are responsible for these functions. We used in vivo optogenetics combined with behavioral experiments or electrophysiological recordings to explore the role of MRR neurons responsible for the acquisition of negative experience.

We discovered that the MRR harbors a vesicular glutamate transporter 2 (vGluT2)-positive cell population that gave rise to the largest ascending output of the MRR. These neurons received extensive inputs from negative sensory experience-related brain centers, whereas their excitatory fibers projected to LHb, mVTA and MS/VDB (Fig. A). MRR vGluT2-neurons mainly innervated MRR- or mVTA-projecting cells in medial ("limbic") LHb, creating a direct feedback in the MRR-LHb-mVTA axis. MRR vGluT2-neurons were selectively activated by aversive but not rewarding stimuli in vivo. Stimulation of MRR vGluT2-neurons induced strong aversion (Fig. B-D), agitation and aggression and suppressed reward-seeking behavior, whereas their chronic activation induced depression-related anhedonia. The latter can at least partly be explained by our 3D electron microscopy data showing highly effective synaptic targeting of LHb neurons and by our in vitro data showing that MRR vGluT2-terminals can trigger depressive behavior-related bursting activity of LHb neurons. MRR vGluT2-neurons seem to be involved in active responses to negative experience, therefore they induced aggression or

avoidance, classical fight or flight responses. Suppression of MRR vGluT2-neurons precisely at the moment of the aversive stimulus presentation strongly disrupted the expression of both contextual and cued fear memories and prevented fear generalization. MRR vGluT2-neurons could facilitate the learning of negative experience, because their LHb-projecting axons bifurcated and selectively innervated pacemaker MS/VDB PV-positive neurons that projected to the hippocampus. Consequently, in vivo stimulation of MRR vGluT2-neurons instantly evoked memory acquisition-promoting hippocampal theta-oscillations in mice, in vivo.

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. MRR vGluT2-neurons could activate the aversion- and negative prediction-related LHb-mVTA axis and could swiftly transform the state of the septo-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.

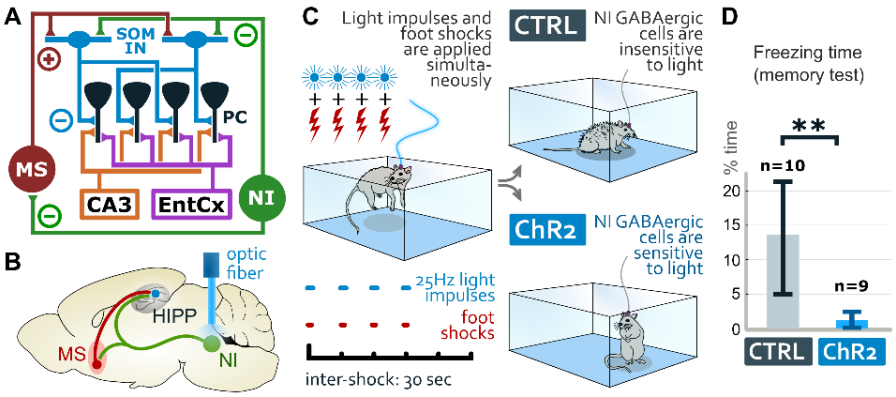
Brainstem nucleus incertus controls contextual memory formation

Associative learning is essential for survival and the mammalian hippocampal neurocircuitry has been shown to play a central role in the formation of specific contextual memories. Contrary to the slow, neuromodulatory role commonly associated with brainstem systems, we discovered a highly specific, spatiotemporally precise, inhibitory ascending brainstem pathway that effectively controls hippocampal fear memory formation. Pyramidal neurons of the dorsal hippocampus CA1 region pair multisensory contextual information (see on the next page: Fig. A, CA3) with direct sensory-related inputs (Fig. A, EntCx). Each memory trace is encoded by a specific subset of pyramidal neurons. Remaining pyramidal cells must be actively excluded from the given memory encoding process by direct dendritic inhibition, which is executed by somatostatin-positive (SOM) dendrite-targeting interneurons. SOM interneurons are activated by excitatory inputs from the medial septum (MS) upon salient environmental stimuli. Previous models suggested that the subset of memory-forming pyramidal cells escape this dendritic inhibition only by a stochastic, self-regulatory process, in which some SOM interneurons become inactive. However, we hypothesized that this process must be regulated more actively, and SOM interneurons should be inhibited precisely in time, based on subcortical information, otherwise, under-recruitment of pyramidal neurons would lead to unstable memory formation.

GABAergic inhibitory neurons of the brainstem nucleus incertus (NI) seemed well suited to counter-balance the activation of SOM interneurons as they specifically project to the stratum oriens of the hippocampus where most SOM cells arborize. Using cell type-specific neuronal tract-tracing, immuno-electron microscopy and electrophysiological methods, we

investigated the targets of NI in the mouse hippocampus, and in MS where excitation of SOM cells originates. We also used monosynaptic rabies-tracing to identify the inputs of GABAergic NI neurons. Two-photon calcium imaging was used to analyze the response of GABAergic NI fibers to sensory stimuli in vivo. Finally, we used in vivo optogenetics combined with behavioral experiments or electrophysiological recordings to explore the role of NI in contextual memory formation and hippocampal network activity.

Nucleus incertus (NI) activation prevents memory formation.



NI GABAergic neurons regulate contextual memory formation by inhibiting somatostatin interneurons (SOM IN) directly in hippocampus (A) and indirectly via inhibition of their excitatory inputs in the medial septum (MS). Pairing optical stimulation (B) with aversive stimuli (C), eliminates fear memory-formation, while control mice display normal fear (freezing) after exposure to the same environment a day later (D).

We discovered that NI GABAergic neurons selectively inhibit hippocampal SOM interneurons in stratum oriens both directly and also indirectly via inhibition of excitatory neurons in MS (Fig. A, B). We observed that NI GABAergic neurons receive direct inputs from several brain areas that process salient environmental stimuli, including the prefrontal cortex and lateral habenula and these salient sensory stimuli (e.g. air-puffs, water rewards) rapidly activated hippocampal fibers of NI GABAergic neurons in vivo. Behavioral experiments revealed that optogenetic stimulation of NI GABAergic neurons or their fibers in hippocampus, precisely at the moment of aversive stimuli (Fig. C), prevented the formation of fear memories, while this effect was absent if light stimulation was not aligned with the stimuli. However, optogenetic inhibition of NI GABAergic neurons during fear conditioning resulted in the formation of excessively enhanced contextual memories. Optogenetic stimulation of NI GABAergic neurons also changed memory encoding-related hippocampal theta rhythms.

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.

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.