Glutamatergic component in the raphe-hippocampal connection

PhD thesis outline

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Introduction

Serotonin (5-HT) containing neurons are intermingled with significant number of non-serotonergic projection cells in the raphe nuclei of the brainstem. Both serotonergic and non-serotonergic raphe cells may contain the third isoform of the vesicular glutamate transporter (VGluT3), supposing glutamatergic transmission in the raphe projections. Traditionally, raphe nuclei are classified by anatomical localization: the dorsal raphe nucleus (DR), the median raphe nucleus (MR), the caudal linear nucleus and the group B9 of serotonergic cells are located in the midbrain, the caudal raphe nuclei (nucleus raphe magnus, obscurus and pallidus) belong to the medulla. Our understanding how serotonergic neurons are organized was largely influenced by fate-mapping of neurons deriving from different rhombomeres. DR neurons have origin in the first rostral rhombomere, whereas the MR consists of neurons from the three most rostral rhombomeres. Caudal group of raphe nuclei originates from the fifth or more caudal rhombomeres and give rise to descending serotonergic pathways that innervate the spinal cord. The origin from different rhombomeres may fundamentally determine the expression level of various genes resulting in neurones with disparate functional characteristics. For example, the MR cells deriving from the second rhombomere sit at more depolarized resting potential and are more excitable than other cells of this nucleus. Divergent expression pattern of crucial genes influencing the transmissive capabilities or membrane potential can be detected even among neurons originating from the same rhombomere: the cells deriving from the second rhombomere can be divided on the basis of VGluT3-expression into two distinct subgroups, between which the levels of VGluT3- and tryptophan hydroxylase (catalyzing the serotonin synthesis) expression correlate negatively.

The projection preference of different raphe nuclei is also divergent. The DR, the MR and the goup B9 give rise to ascending pathways innervating the midbrain and forebrain. The cortical targeting fibers are morphologically also heterogeneous: MR fibers are typically thick with big spherical varicosities, whereas DR fibers are thin with numerous small granular or fusiform varicosities. DR neurons preferentially innervate for example the superficial layers of the neocortex, the striatum, the hypothalamus or the basolateral amygdala. The caudal portion of DR (identical with serotonergic group B6) characteristically innervates the limbic structures. The projections of the MR are more restricted than that of the DR, terminating for example in the medial prefrontal cortex, perirhinal cortex and in limbic structures like the hippocampus, the medial septum, the diagonal band of Broca or the tegmental nuclei of Gudden. VGluT3 is present in the majority of terminals forming synapses in the hippocampus, the medial septum and the

medial prefrontal cortex and was found also in the raphe projections reaching the ventral tegmental area, substantia nigra and several hypothalamic nuclei. The complexity of VGluT3 containing raphe fibers is elaborated by the non-uniform presence of VGluT3 in the local or distant terminals even of the same axon. The MR and DR contain also a large fraction of GABAergic cells and some dopaminergic neurons non-overlapping with serotonergic or glutamatergic cells. GABAergic cells are not exclusively locally arborising neurons, some of them project to the prefrontal cortex or the nucleus accumbens, for example.

The typical afferents of DR and MR originate also from different brain regions. The MR – in contrast to the DR – is innervated by fibers from the medial septum, diagonal band of Broca. In turn, DR is strongly innervated by the amygdala, the bed nucleus of stria terminalis, the striatum or basal forebrain. An additional difference between the two raphe nuclei is a result of strong input to MR from lateral and medial habenula and from interpeduncular nucleus. The divergent connections of the two raphe nuclei have functional consequences, the DR is involved in suppression of impulsive behavior and tolerating even longer reward waiting periods. That means, DR may help to overcome the contradictions between the actual and expected situation, therefore, serotonergic depletion may disrupt the recognition of future outcome of actual conduct, and may induce hopelessness or helplessness, and so depressive behavior. In contrast, the MR efferents (and projection from the caudal part of DR) targeting the limbic system or functionally related brain areas may fundamentally alter the operation of the hippocampal network during navigation and episodic memory formation. The medial habenula – as a relay output from the limbic system – and the lateral habenula – involved in generating anxiety and aversive reactions – may regulate the raphe-hippocampal neuromodulation directly or via the interpeduncular nucleus, therefore these nuclei may affect the consolidation of aversive memory and contribute to formation of anxious behavior. In order to implement an efficient and reliable modulation of hippocampal information processing, the raphe-hippocampal connection should use a temporally precise transmission. As VGluT3, present also in raphe fibers, can provide such a reliable and fast glutamatergic transmission, it can play a crucial role in the raphe-dependent regulation of hippocampal processes.

It is also an important and non-negligable fact about the efficiency of the raphe-hippocampal connection that the raphe fibers form synapses selectively with calbindin containing interneurons, cholecystokinin containing basket cells and some interneuron-selective interneurons, which enables to powerfully interact with principal cells' activity. This synaptic connection is formed by thick raphe fibers, whereas the thin ones allow

serotonergic volume transmission acting on synaptically not innervated hippocampal cells as well. Serotonin shunts out small excitatory inputs onto pyramidal cell by strongly hyperpolarizing them, however, it also promotes the spiking related to strong inputs by inhibiting the currents of slow afterhyperpolarization. In addition, serotonin modulates the information flow and content from the entorhinal cortex to hippocampus, because it can suppress inputs from the lateral perforant path in the distal portion of pyramidal dendrites while augmenting the inputs carried by the medial perforant path reaching mid-portion of dendrites. By its various metabotropic receptors, serotonin can facilitate the processing of newly acquired contextual information instead of recalling previous experiences. Basket cells and interneuron-selective interneurons activated by ionotropic 5-HT_3 receptors may provide an appropriate temporal resolution of this modulation, however, the possible glutamatergic (co-)transmission as a consequence of the VGluT3 present in these contacts may significantly enhance the temporal precision of hippocampal regulation by the raphe nuclei.

The MR – as its most widely studied effect – desynchronises the hippocampal theta oscillation connected with neural coding during navigation. Experiments in awake animals, however, resulted in ambiguous findings, maybe because the electrically stimulated brainstem region was slightly differenet among studies and, also importantly, because the type I and type II theta activity were not distinguished in most of the cases. Type I theta occurs when animal is exploring its environment, not present in urethane-anesthesia and resistant to cholinergic blockers. Type II theta oscillates at lower frequencies than type I theta, it is related to sensory activation and highly dependent on cholinergic transmission. In urethane-anesthesia, theta oscillations similar to type II periodically and spontaneously occur, but the difference between this activity and type II theta is not defined unequivocally. The raphe manipulation has different effects on the two types of theta oscillations. Projections from the DR and the dorsal-medial part of MR suppress the theta in urethane-anesthesia, but facilitates type I theta when animal is moving and exploring. This effect requires intact serotonergic transmission. In contrast, the stimulation of the ventral part of MR containing mostly non-serotonergic projection cells evokes or strengthens theta oscillation in urethane-anesthesia. Based on the electrical and pharmacological raphe manipulation, a model was formed considering the serotonergic pathways as facilitator of type I theta during exploration and suppressing cholinergic theta. This idea is further supported by cellular level effects that may augment the flow of spatial information from the medial entorhinal cortex to hippocampal pyramidal cells. In turn, the non-serotonergic projections from raphe nuclei may promote type II theta activity connected to salient sensory

stimuli. This model is oversimplified, because the electrical stimulation is not selective for any population of raphe projection cells, and even pharmacological manipalution of serotonergic cells can't avoid their glutamatergic component depending on coexpressed VGluT3.

One possible role of the glutamatergic component of raphe fibers is to support the genesis of type II theta oscillations, and to counteract the concurrent type II theta-inhibiting serotonergic effects. Type II theta activity – although there is no clear evidence demonstrating the details how hippocampal neurons participate in generation of this oscillation – is potentially involved in hippocampal processing of salient sensory stimuli dependent on the cholinergic modulation. A second possible way in which the glutamatergic component of raphe fibers can regulate the processing of sensory information is the fine tuning of the hippocampal network at high temporal resolution on top of a background serotonergic tone. This would allow the linking of position-related and salient sensory representations within the hippocampal network. To fullfill this proposed role, the VGluT3-dependent raphe-hippocampal signaling should mediate a truly fast and temporally focused transmission. First, in order to deepen our understanding of the raphe-hippocampal modulation, the latter possibility should be elucidated, that may result in the identification of a novel, powerful form of subcortical modulation. This idea is supported by a recent finding that low-frequency (0.5 Hz) or theta-frequency electric stimulation of MR resets the phase of hippocampal theta oscillation, uncovering that this connection can manipulate the target network within a time frame shorter than one theta cycle (cca. 200 ms). While the contribution of VGluT3 to signaling mechanisms in the raphe cells and projections is still mostly unknown, there are recent data about its role in some GABAergic, glycinergic or cholinergic neurons. VGluT3 can facilitate the storage of a transmitter different from glutamate by vesicular synergy or provide glutamatergic transmission. Hence, we can suppose that VGluT3 in raphe fibers also takes part in glutamatergic signaling that may be the bases of a fast and efficient subcortical fine-tuning.

Objectives

During my studies, I investigated the glutamatergic component of the serotonergic system using *in vivo* electrophysiological approach. The goal of my first series of experiments was to clarify the distinguishing electrophysiological properties of VGluT3-expressing MR (later on referred to as MRR meaning median raphe region that involves the paramedian raphe, as well) neurons from their serotonergic counterparts – since the capability of glutamatergic (co-)transmission may be associated with a different mode of operation. *Our specific*

questions were: **1)** Do VGluT3-containing and serotonergic MRR neurons fire at different frequencies or with different variability? **2)** Is the firing frequency of VGluT3-expressing and serotonergic MRR cells dependent on the hippocampal theta and non-theta states, and on the sensory stimulation? **3)** Are the action potentials of VGluT3-expressing and serotonergic MRR neurons coupled to the phase of hippocampal or prefrontal cortical oscillations?

In our second series of experiments, we investigated the cellular effects of the glutamatergic component of the serotonergic projection by electrically stimulating the MRR. First of all, we aimed to answered whether hippocampal interneurons that receive synaptic input from MRR fibers are activated by a fast glutamatergic transmission. *Our specific questions were:* **1)** What are the basic electrophysiological characteristics of fast activation of hippocampal interneurons evoked by MRR stimulation? **2)** Is the reaction of hippocampal interneurons dependent on glutamatergic receptors? How is the reaction of hippocampal interneurons changed by the local application of an AMPA-type glutamate receptor antagonist? What is the role of ionotropic 5-HT_3 receptors in the rapid response of target neurons? **3)** Is there any correlation between the type of reaction to MRR stimulation and the neurochemical identity or electrophysiological properties of the responding interneurons?

Methods

Our experiments were carried out in the Laboratory of Cerebral Cortex Research, in the Institute of Experimental Medicine of Hungarian Academy of Sciences. All our experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee of the Institute of Experimental Medicine and of the Animal Health and Food Control Station in Budapest. During design and implementation of our research, all efforts were made to minimise pain and potential suffering of involved animals, and we used the lowest possible number of animals. In case of both electrophysiological characterization of MRR cells and investigation of effects of raphe-hippocampal fibers on interneurons, we used adult male Wistar rats. In both series of experiments, the rats were anesthetized by intraperitoneally injected 20% urethane (dose: 0.007 ml/g body weight). Using a stereotaxic frame, animals were head-fixed and the skull was cleaned for surgical procedures. During the experiments, the animals' temperature was held constant at 37 degrees of Celsius by a homeothermic heating pad.

In parallel with juxtacellular recording of MRR cells' activity, the local field potential (LFP) in the hippocampus and the medial prefrontal cortex was also monitored. After a 2–10 minutes long control recording and a subsequent 30 seconds long tail pinch (as a sensory

stimulation) period, the recorded neuron was attempted to be labeled with Neurobiotin and the brain was perfused by fixative for further immunohistochemical identification. In case of 78 animals out of 166, we recovered the labeled neuron. In a few experiments, either the recording position and the location of the labeled neuron did not match perfectly or multiple somata were recovered close to each other. For all these cases, the recordings were excluded from further analysis resulting in a pool of 60 MRR neurons. Only the 23 cells with unequivocal immunofluorescent identification were involved in the study. The electrophysiological characteristics of these neurons divided into four groups based on their 5-HT- and VGluT3-content were compared. Spikes were detected using Spike2 software, controlled by visual inspection. Data were exported into MATLAB environment where the custom-written scripts and routins for further analysis run. The average spike width, the firing frequency and variability during hippocampal theta and non-theta states, as well as during the sensory stimulation were determined. Parameters representing the transient and permanent firing changes evoked by sensory stimulation were introduced. Furthermore, signs of rhythmic firing and coupling to hippocampal and prefrontal cortical oscillations were searched for. The group differences between the four neurochemical groups were detrmined using one-way non-parametric Kruskal-Wallis ANOVA, pairwise significant divergence was uncovered by Wilcoxon signed rank test. For comaprison of firing of a given population during two states, sign test was used.

The effect of raphe-hippocampal fibers on interneurons was investigated by juxtacellular recording of hippocampal cells while the MRR was electrically stimulated. The LFP activity of hippocampus was also registered. In a significant portion of our experiments, pharmacological tests were carried out to determine the role of AMPA-type glutamate receptors (locally iontophoretized antagonist) and 5-HT_3 receptors (intraperitoneally injected antagonist) in mediating the effects of MRR stimulation. After recordings, the neurons were attempted to be labeled with Neurobiotin for further neurochemical identification. All the analyses were made in the MATLAB environment running custom-written scripts. The spikes were detected on the basis of their amplitude. In addition to neurochemically identified interneurons, non-labeled hippocampal cells in case of which electrical MRR stimulation was carried out were also included in the analysis. The non-labeled cells were divided into subgroups of putatitve interneurons and putative principal cells, based on their spike width, firing frequency and presence of complex spiking patterns. Similar to the electrophysiological characterization of MRR cells, hippocampal theta and non-theta states were distinguished in this series of experiments, as well. The neurons' firing rate, the index of theta modulation and the theta phase preference were calculated. The effects evoked by

MRR stimulation were characterized by their succes rate, latency and duration. It was also determined, whether these parameters are altered by application of antagonists and whether any such change in success rate of stimulation correlate with change in basic firing rate. Data are presented as the mean and standard error of mean, group differences were uncovered using Mann-Whitney U-test, whereas state-dependent changes were calculated by Wilcoxon signed rank test.

Results

Electrophysiological characterization of MRR cells

The 23 MRR neurons with undoubted neurochemical identity were divided into four subgroups resulting in n = 7 VGluT3(+)/5-HT(-) neurons, n = 5 VGluT3(+)/5-HT(+) neurons, n = 4 VGluT3(-)/5-HT(+) neurons and n = 7 VGluT3(-)/5-HT(-) neurons. The cells lacking serotonin – irrespective of VGluT3-expression – fired significantly narrower spikes and significantly faster during hippocampal theta state than neurons containing serotonin. The firing of VGluT3(+)/5-HT(+) population was statistically not different from the other subgroups, however, their electrophysiological parameters typically fell in between those of VGluT3(+)/5-HT(-) and VGluT3(-)/5-HT(+) subgroups. VGluT3(-)/5-HT(+) neurons fired significantly slower during theta episodes than during non-theta state distinguishing them from the other three MRR populations (which fired more during theta compared to non-theta state). There was no outstanding group difference in firing variability, although the firing of neurons lacking 5-HT was tendentiously more variable. Serotonergic cells characteristically increased their firing activity transiently at the start of sensory stimulation, in addition, the firing of VGluT3(+)/5-HT(+) cells remained elevated during the whole sensory stimulation period. VGluT3(+)/5-HT(-) neurons also exhibited a permanent firing response during sensory stimulation, but the activity of VGluT3(-)/5-HT(-) cells was not affected – taking together, all four subgroups responded to sensory stimulation in a different way. The spikes of MRR neurons lacking 5-HT were coupled to the phase of hippocampal and prefrontal slow waves (frequency range: 0.9–2.5Hz). Among the recorded cells in this study, there were only one VGluT3(+)/5-HT(-) and one VGluT3(-)/5-HT(+) neurons showing rhythmic activity.

Effect of raphe-hippocampal fibers on interneurons

The effect of activating the raphe-hippocampal fibers was examined in 52 hippocampal cells. Eight of them were succesfully labeled by Neurobiotin and identified as interneuron.

The neurochemical identity was proven in case of four cells: two neurons contained cholecystokinin, one expressed somatostatin and an additional one interneuron contained calbindin D28k. The non-labeled neurons (n = 44) were divide into subgroups of putative principal cells (n = 3) and putative interneurons (n = 41), based on their electrophysiological characteristics. The electrical stimulation of the MRR evoked the following response types in hippocampal neurons:

a) fast (latency < 15 ms) activation registered in case of 16 cells,
b) slow (latency between 15–50 ms) activation found in 7 cells,
c) very slow (latency > 50 ms) activation showed by 4 cells,
d) inhibiton without initial activation registered in case of 25 neurons including the putative principal cells, as well.

In this part of our study, we focused on the activation with latency faster than 15 ms supposing fast synaptic excitation. The average latency of this fast response was 8.9 ± 0.58 ms, the success rate was 65.7 ± 5.29%. The activation was temporally focused, the jitter was 1.7 ± 0.19 ms. The slow response was less precise in time. NBQX (a selective antagonist of AMPA-type glutamate receptors) significantly and reversibly decreased the success rate of evoked fast activation, but the jitter was not affected. NBQX had only subtle effect on the slow activation. Ondansetron (a selective blocker of ionotropic 5-HT_3 receptor) decreased the succes rate of fast activation in a lesser extent than the application of NBQX, however, the inhibition following the initial fast activation was strogly affected by ondansetron. There was no correlation between the change of basic firing frequency and the change of succes rate of activation evoked by MRR stimulation during application of both NBQX and ondansetron. The firing of some interneurons exhibiting the fast activation was coupled to the theta rhythm, while others fired irregularly. Some of these interneurons were activated during hippocampal theta state, the activity of other cells was suppressed during theta oscillation.

Conclusions

Our experiments provided the first evidence that VGluT3(+)/5-HT(-) neurons in the MRR are divergent from their VGluT3(-)/5-HT(+) counterparts regarding their basic *in vivo* electrophysiological properties. Glutamatergic cells fired at higher frequency and were activated during hippocampal theta state, in contrast to serotonergic cells firing less during theta. VGluT3(+)/5-HT(+) neurons capable of glutamatergic-serotonergic co-transmission did not differ significantly from previous subgroups, their parameters typically fell in between those of glutamatergic and serotonergic populations. It is an important consequence of our

work, that spike width commonly used for separation of serotonergic MRR cells in blind recordings is not a reliable parameter. Transgenic recombinase-expressing driver lines widely used in brain research are also incapable of selectively differentiate MRR subpopulations beceause of the overlap in 5-HT- and VGluT3-expression. Interestingly, neurons capable of glutamatergic-serotonergic co-transmission represented a transitional firing phenotype (between the glutamatergic and serotonergic populations), the reason of which can be an ongoing transition or switching of transmitter phenotype reported already in various types of neurons. Sensory stimulation evoked different response in case of all four MRR populations, which indicates they may play different role in regulation of sensory processing. Similar to the DR neurons responding to several aspects of sensory stimuli in diverse manner, they can also contribute to the modulation of sensory tuned information. Serotonergic and non-serotonergic modulators of MRR may exert a complementary regulation of hippocampal network, and the physiological differences unraveled by our study may serve the requirements of glutametergic (co-)transmission. In spite of moderate or subtle phase coupling of VGluT3-expressing cells, their firing represents a mode of operation suitable for precise fine-tuning and may be capable of conveying abruptly occurring transient events. Possibly, glutamatergic cotransmission may affect only a subnetwork in the hippocampus, and as such, it may contribute to functionally couple units that code overlapping features of the environment into assemblies. However, this function requires a fast, powerful and reliable signaling, which has been investigated in our second series of experiments.

We discovered a glutamatergic, temporally focused and powerful activation in the raphe-hippocampal projection that selectively targets interneurons. The data presented in my thesis are largely supported and complemented by the work of our collaborators. In the *in vitro* experiments using optogenetics carried out by Attila Losonczy, the same phenomenon was demonstrated as in our *in vivo* work, justifying the specificity of stimulated pathways. VGluT3 was detected in the presynaptic raphe terminals forming synapses with labeled fast activated interneurons by Zsolt Borhegyi. In addition, the postsynaptic presence of glutamate receptors was also proven by work of Gábor Nyíri. These experiments provided the first evidence that MRR fibers containing VGluT3 release glutamate acting on innervated interneurons. Importantly, this connection may influence the pyramidal cells, as showed by our inhibition of pyramidal cells upon MRR stimulation (both in case of *in vivo* and *in vitro* experiments), indicating the significance of glutamatergic transmission from raphe fibers. VGluT3-exressing neurons – regardless of their 5-HT content – are activated strongly and powerfully during sensory stimulation, as we demostrated in our first series of experiments.

Considering their higher firing rate and capability of fast and precise glutamatergic signaling, they may exert a robust activation of target neurons, differing from their purely serotonergic counterparts. This is in concert with our original hypothesis that the glutamatergic component of the MRR projections is capable of modulating the hippocampal processing of salient information. The fast glutamatergic transmission provides a subcortical modulation with a higher than theta timescale precision, and through activating interneurons may exert a powerful effect on the hippocampal network. Our work is the initial step in the quest for identifying the function of a newly discovered, powerful component of subcortical modulation. In future experiments, the contribution of this glutamatergic communication to the hippocampal learning primarily associated with navigation should be tested, using cutting-edge methods for specific and reversible optogenetic silencing of the glutamatergic component.

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