**UNIVERSITY OF PÉCS**

Doctoral School of Biology and Sportbiology

# Investigation of cholinergic-glutamatergic interactions in rodent models of cognitive impairment

**PhD Thesis**

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## 1. INTRODUCTION

The various kinds of learning and memory functions, which can be distinguished on the basis of their temporal properties and the aspects of the stored inner representations are considered as the most important skills of animals and humans for serving environmental adaptation. Psychological and neurophysiological evidences support the differentiation of short-term (STM) or working memory and long-term memory (LTM), while some models also imply the existence of an intermediate-term memory [1]. Furthermore, different learning processes are involved in the acquisition of explicit informations about objective facts or events (declarative memory) [2,3], and in acquiring skills through exercise (non-declarative or implicit memory). Short-term memory is supposed to be dependent on maintained activity of neuronal loops in neocortical areas (especially, in the prefrontal cortex, PFC), while the consolidation of declarative LTM is related to structures of the medial temporal lobe (MTL), e.g., the entorhinal cortex and hippocampus [4–6]. On the other hand, MTL also plays an essential role in spatial navigation (cognitive map concept) [7–9], therefore, spatial tasks of rodents are frequently used in the experimental study of learning and memory [10–12].

Currently, neurocognitive disorders and other illnesses accompanied with cognitive deficits are among the most significant public health issues in the developed societies. In neurocognitive disorders (e.g., Alzheimer's disease, AD), a main symptom is the deterioration of learning and memory abilities [13], while cognitive decline may also follow psychiatric disorders, such as schizophrenia [14].

The glutamatergic and the cholinergic neurotransmission are significantly involved in physiological processes of learning and memory, as well as in the pathophysiology of cognitive disorders. Some special groups of glutamatergic neurons are involved in memory, such as place- and grid-cells of the MTL [7,8], and delay-cells located in the PFC [15]. It is well known, that glutamate receptors (especially the NMDA-subtype) play important role in long-term potentiation (LTP), which is supposed to be the cellular mechanism of memory formation [16–18]. On the other hand, acetylcholine (ACh) has a substantial modulatory role in the functioning of memory-related brain structures. Neurons in the nucleus basalis of Meynert provide cholinergic projections to neocortical areas, and supposedly control attention [19], while cholinergic cells of the medial septum and the diagonal band project to MTL structures, and modulate hippocampus-dependent declarative learning processes [20–22].

In AD, marked decrease has been found in the number of memory-related glutamatergic neurons and basal forebrain cholinergic cells. Furthermore, brain tissue of AD patients also shows lower density of ACh and glutamate receptors as well as lower activity of ACh-related metabolic enzymes

(for a review, see [23]). It is noteworthy, that deficits in the expression and function of NMDA-type glutamate receptors (NMDAR) and α7 nicotinic ACh receptors (nAChR) are common features of both AD and schizophrenia [24].

Animal models of diseases play important role both in drug-development and in the study of the physiology and pathology of learning and memory. Good models mimic several aspects of a given disease especially of the etiology, pathophysiology, symptoms and responses to treatments. Cognitive impairment can be induced in several ways in rodents, e.g., with genetic modification, lesions or neurotoxins, while spontaneous and comorbidity dementia models also exist [25]. Although pharmacologically induced transient amnesia models do not mimic specific pathomechanism of AD, they are popular models of cognitive impairments especially in behavioral pharmacology and in drug-development [26,27]. Transient amnesia models are cheap and reversible, thus suitable for extensive screening of potential cognitive enhancer compounds. Moreover, pharmacological models provide useful tool for the selective investigation of deficits in certain neurotransmitter systems. Most frequently used pharmacological compounds for inducing cognitive deficits are muscarinic AChR (mAChR) antagonists (e.g., scopolamine), nAChR antagonists (e.g., MLA), and NMDAR antagonists (e.g., MK-801).

Currently available pharmacological treatments in Alzheimer's disease are based on either the inhibition of acetylcholine-estherase (AChE) enzyme activity [28], or the enhancement of signal-to-noise ratio of glutamatergic signaling by weak competitive NMDAR antagonist memantine [29]. Identification of further pharmacological targets for AD is still an important topic of drug-development. Potential novel drug-candidates are the agonists and allosteric modulators acting on α7 nAChRs, which showed potent cognitive enhancing effect in preclinical models of AD and schizophrenia [30,31]. Thus, development of α7 nAChR agonists may provide common treatment for the cognitive symptoms of both AD and schizophrenia. However, mechanisms underlying the procognitive effects of α7 nAChR agonists have not been clarified in details yet. Therefore, here we aimed to study the role of α7 nAChRs and NMDARs in learning and memory both with behavioral pharmacological and *in vivo* electrophysiological methods in laboratory rats.

## 2. Aims and Objectives

The aims of the thesis were

I. To test the cognitive enhancer potential of the α7 nAChR agonist PHA-543613 in a hippocampus-dependent spatial working memory test in rats

II. To compare the influence of glutamatergic and cholinergic deficit on the efficacy of the α7 nAChR agonist by testing it in two distinct behavioral pharmacological amnesia models (i.e., scopolamine- and MK-801-induced models)

III. To investigate the neuronal activity of CA1 hippocampal pyramidal cells in connection with glutamatergic and cholinergic neurotransmission using *in vivo* electrophysiological methods. The following objectives were defined in the electrophysiological experiments:

  1. Optimization of single-channel extracellular recordings and microiontophoresis for the selective investigation of hippocampal pyramidal cells and interneurons
  2. Investigation of the local effects of NMDA and ACh on the firing activity of pyramidal cells
  3. Identification of interaction between glutamatergic and cholinergic neurotransmission on the level of neuronal activity by the combined administration of NMDA and ACh
  4. Assessment of the role of different AChR subtypes in the effects of NMDA and ACh as well as in the interaction of the two neurotransmitter systems

IV. To constitute a model based on the electrophysiological results which explains the cognitive effects of the tested pharmacological compounds in the behavioral paradigm

# 3. Materials and Methods

## 3.1. Testing the effects of α7 nAChR agonist PHA-543613 on spatial working memory in two distinct pharmacological amnesia models

For the assessment of spatial working memory performance of rats, spontaneous alternation task was used, which is based on the natural exploratory tendency of rodents, therefore, no reinforcement was used during the experiments. The experiments were performed in a T-maze constructed according to Deacon and Rawlins (2006) [11], and the applied protocol was based on Spowart-Manning and van der Staay (2004) [32]. At the start of a session, the rat was positioned in the start arm, and was allowed to freely choose one of the two goal arms after the opening of the guillotine door. When the rat returned to the start arm after exploring one of the goal arms, the guillotine door was lowered, and the rat was confined in the start arm for 10 s before he began the next trial. Rats with normal spatial working memory tend to visit the opposite goal arm compared to the previous trial, which is considered as a correct choice (alternation). Each session consisted from 15 trials or was terminated after 25 minutes.

To estimate the memory performance of a rat in a given session, alternation rate was calculated using the following formula:

$$Alternation\ rate = \frac{Number\ of\ alternations}{Number\ of\ trials - 1}$$

Effects of PHA-543613 on the performance in the spontaneous alternation task was investigated in two distinct pharmacological amnesia models: 1) in the first experiment cognitive impairment was evoked with mAChR antagonist scopolamine (dose: 0.5 mg/kg, i.p., 10 min before testing, Scop), while 2) in the second experiment NMDAR antagonist MK-801 (0.1 mg/kg, s.c., 35 min before testing, MK) was used as amnestic agent. In both experiments, PHA-543613 was administered s.c. in 1 and 3 mg/kg doses (PHA1.0 and PHA3.0, respectively) 40 min prior to the start of the session. In the first experiment, the applied treatments were the following: Scop alone, PHA1.0+Scop, PHA3.0+Scop. In the second experiment, the following treatments were applied: MK alone, PHA1.0+MK, PHA3.0+MK. When Scop or MK were used alone, physiological saline was injected in replacement of PHA in the same volume. Both experiments were carried out in a counterbalanced (latin-square) design, thus, every animal was subjected to all of the treatments. Effects of different treatments were compared using repeated measures ANOVA, while binomial test was used to compare alternation performance to the chance level in a certain treatment condition.

## 3.2. Investigation of cholinergic and glutamatergic effects and their interaction on the neuronal activity of hippocampal pyramidal cells using *in vivo* electrophysiology

Extracellular spike signals were recorded from the hippocampal CA1 pyramidal layer (AP: −3.5-5.0, ML: 1.5-2.5, DV: 1.8-3.4 mm from Bregma according to Paxinos and Watson, 2014 [33]) of Wistar rats anesthetized with chloral-hydrate (400 mg/kg, i.p.). Recordings were performed through the central carbon fiber of multi-barrel microelectrodes, while neuroactive compounds (NMDA, ACh) were administered through the sorrounding micropipettes by the means of microiontophoresis.

### *3.2.1. Optimization and validation of an electrophysiological separation of CA1 hippocampal pyramidal cells and interneurons*

Interneurons are also located in the pyramidal layer of the CA1 region, which may respond significantly differently to neuroactive compounds compared to pyramidal cells. Therefore, in the first part of the electrophysiological study, we needed to optimize and validate our sorting processes for the reliable separation of different neuronal populations. According to earlier results, two distinct neuronal populations (i.e., single-spiking and complex-spiking neurons) can be distinguished on the basis of electrophysiological properties, such as their firing patterns and the shape of spike waveforms. Thus, single-spiking neurons correspond to interneurons, while complex-spiking neurons represent

pyramidal cells of the CA1 region [34]. For the purpose of the electrophysiological separation of putative interneurons and pyramidal cells, spike activity was analyzed using Klusters software (Lynn Hazan, Rutgers University, NJ, USA) [35]. Data conversions between Spike2 recording software and Klusters were performed using a self-written script[1].

Next, the reliability of the separation was validated on electrophysiological and pharmacological bases, comparing the properties of neuronal populations defined in our experiments with earlier literature data. Therefore, the following spike shape parameters of single- and complex-spiking neurons were determined: 1) peak and trough (P and T, respectively), 2) peak to trough amplitude (P-T amplitude), 3) ratio of peak and trough amplitude (P/T), 4) peak to trough time (P-T time), 5) half-peak duration (HPD). Furthermore, we compared the spontaneous firing rate (Hz) of single-spiking and complex-spiking neurons as well as their firing responses to iontophoretically applied NMDA. Spike shape parameters and firing properties of the two distinct neuronal populations were compared using paired statistical tests (Student's T-test or Wilcoxon signed-rank test).

### *3.2.2. Investigating local effects of NMDA and ACh on neuronal activity, and comparing the influence of muscarinic and α7 nAChR antagonists on cholinergic-glutamatergic interplay*

In further electrophysiological experiments, only complex-spiking neurons (putative pyramidal cells) were tested for pharmacological responses to cholinergic and glutamatergic drug stimulations. Firing frequency (Hz) of the recorded neurons was measured in four distinct recording conditions: 1) spontaneous firing activity (Sp), 2) firing activity evoked by iontophoretic delivery of NMDA (NMDA), 3) firing activity evoked by iontophoretic delivery of ACh (ACh), 4) firing activity as a result of simultaneous delivery of NMDA and ACh (ACh_NMDA). Furthermore, we analyzed, whether the combined effect of NMDA and ACh during their simultaneous iontophoretic delivery resulted in an additive or in a superadditive increase of firing frequency. The presence of a superadditive interaction was tested using the following null hypothesis:

$$\mathrm{H_0}: (NMDA - Sp) + (ACh - Sp) = ACh_NMDA - Sp$$

After acquiring pretreatment control data, animals were systemically injected with mAChR antagonist scopolamine (1 mg/kg, i.p.) or α7 nAChR antagonist methyllycaconitine (MLA, 1 mg/kg, i.p.). Changes in the above described firing conditions and derived variables were examined as an effect of the amnestic agents.

[1] http://www.neurobio.pte.hu/kutatas/sites/elfiz.html
https://www.researchgate.net/publication/268980233_Export_data_from_Spike2_to_Klusters_Spike2_script
https://www.researchgate.net/publication/268980057_Import_data_from_Klusters_to_Spike2_Spike2_script

For the statistical analysis of the results, linear mixed-effects models were used. In pairwise comparisons, p values were adjusted using the method of Holm. All analyses and graphs were made in R environment (packages: *lme4*, *lmerTest*, *lsmeans*, and *ggplot2*).

## 4. RESULTS AND DISCUSSION

### 4.1. Effects of α7 nAChR agonist PHA-543613 on spatial working memory in two distinct pharmacological amnesia models

The mAChR antagonist scopolamine markedly impaired spatial working memory of rats (N=10) in comparison with the control performance: alternation rate significantly decreased from 0.71 ± 0.03 (mean ± SEM) to 0.25 ± 0.03 ($p<0.01$). Thus, after treatment with scopolamine alone, animals did not show alternating behavior: frequency of correct choices did not exceed the chance level (0.5). Alpha7 nAChR agonist PHA-543613 dose-dependently reversed amnestic effect of scopolamine: while lower dose of PHA-543613 (PHA1.0) did not induce a significant increase in the alternation rate compared to scopolamine alone treatment (Scop vs. PHA1.0+Scop: 0.25 ± 0.03 vs. 0.45 ± 0.07, n.s.), in the higher dose (PHA3.0), PHA-543613 potently enhanced memory performance of the animals (Scop vs. PHA3.0+Scop: 0.25 ± 0.03 vs. 0.59 ± 0.06, $p<0.01$). Furthermore, PHA3.0 treatment restored normal memory function indicated by significantly higher number of alternating choices compared to chance level.

Similarly to scopolamine, NMDAR antagonist MK-801 also significantly decreased the alternation rate (Control vs. MK: 0.71 ± 0.02 vs. 0.43 ± 0.03, $p<0.001$) and impaired spatial working memory performance of rats (alternating by chance). Lower dose of PHA-543613 increased the alternation rate compared to MK-801 alone treatment (MK vs. PHA1.0+MK: 0.43 ± 0.03 vs. 0.56 ± 0.02, $p<0.05$), however, it was not potent enough to restore memory performance to the control level (Control vs. PHA1.0+MK: 0.71 ± 0.02 vs. 0.56 ± 0.02, $p<0.001$). Furthermore, alternation performance of animals after the treatment with PHA1.0+MK did not exceed the chance level ($p=0.1$). Moreover, higher doses of PHA-543613 was not effective at all againts MK-801 induced transient amnesia (MK vs. PHA3.0+MK: 0.43 ± 0.03 vs. 0.44 ± 0.03, $p=0.95$). Thus, in the pharmacological amnesia model induced with NMDAR antagonist MK-801, PHA-543613 showed an inverted U-shaped dose-effect curve. These results suggest that activation of α7 nAChR may potently reverse memory impairment originated in cholinergic deficits, while the same memory enhancement strategy is apparently less effective, when NMDA-dependent glutamatergic transmission is directly impaired.

## 4.2. Cholinergic and glutamatergic effects and their interaction on the neuronal activity of hippocampal pyramidal cells: *in vivo* electrophysiological experiments

### *4.2.1. Optimization and validation of an electrophysiological seperation of CA1 hippocampal pyramidal cells and interneurons*

In the first part of our electrophysiological study, we recorded extracellular activity of 44 hippocampal neurons. On 11 recordings, both single-spiking and complex-spiking clusters were found, thus, validation analyses of the separation process were performed on these recordings.

No significant differences were found between single-spiking and complex-spiking neurons in the following spike shape parameters: 1) peak and trough amplitude, 2) P-T amplitude. However, the two neuronal populations showed marked differences 1) in P/T ratio (single-spiking vs. complex-spiking: 2.22 ± 0.13 vs. 1.54 ± 0.10; $p<0.001$), 2) in P-T time (0.283 ± 0.016 ms vs. 0.538 ± 0.043 ms; $p<0.001$), and 3) in HPD (0.150 ± 0.009 ms vs. 0.201 ± 0.015 ms; $p<0.01$). These differences in the spike shape of single- and complex-spiking neurons are in line with previous data in the literature [37,38], which confirm the reliability of the separation process.

Although no significant difference was found in the spontaneous (baseline) firing rate of the two neuronal populations, NMDA-evoked firing frequency of complex-spiking neurons was significantly higher compared to single-spiking neurons. (40.04 ± 6.27 Hz vs. 9.86 ± 2.06 Hz, respectively; $p<0.001$). Thus, iontophoretically delivered NMDA induced a 58.0 ± 23.0-fold increase in the firing rate of complex-spiking neurons, while single-spiking neurons responded only with a 11.4 ± 3.6-fold increase of firing rate compared to the spontaneous activity (response to NMDA of single-spiking vs. complex-spiking neurons: $p<0.01$). According to earlier results, higher responsiveness of complex-spiking neurons to NMDA suggests that this electrophysiologically separated neuronal population correspond to hippocampal pyramidal cells. These results are in line with earlier *in vitro* studies on hippocampal pyramidal cells using electrophysiological and calcium-imaging methods [39,40]. Furthermore, higher expression of NMDA and AMPA receptors and greater sensitivity to neurotoxic events [41,42] also confirms the presently reported stronger excitability of pyramidal cells with NMDA.

### *4.2.2. Local effects of NMDA and ACh on neuronal activity. Comparing the influence of muscarinic and α7 nAChR antagonists on cholinergic-glutamatergic interplay*

In the second part of the electrophysiological experiments, recordings were performed from the hippocampal CA1 region of altogether 15 rats. After the examination of firing responses to local

iontophoretic delivery of NMDA and ACh, effects of i.p. administered scopolamine (N=7) and MLA (N=8) were investigated.

In the pretreatment control state, iontophoretically applied NMDA and ACh evoked a similar degree of firing rate increase compared to the spontaneous firing frequency (Sp vs. NMDA: 5.6 ± 2.0 Hz vs. 58.5 ± 8.9 Hz, $p<0.001$; Sp vs. ACh: 5.6 ± 2.0 Hz vs. 59.3 ± 9.2 Hz, $p<0.001$), while the simultaneous delivery of NMDA and ACh resulted in a more pronounced evoked firing response (ACh_NMDA: 125.4 ± 13.2 Hz; Sp vs. ACh_NMDA: $p<0.001$, NMDA vs. ACh_NMDA: $p<0.001$, ACh vs. ACh_NMDA: $p<0.001$). Moreover, simultaneous iontophoresis of NMDA and ACh exerted significantly higher increase of firing rate compared to the sum of the two mono-treatment effects (ACh_NMDA−Sp: 121.3±13.6 Hz vs. (NMDA−Sp)+(ACh−Sp): 99.6±12.5 Hz; $F(1, 11)= 5.95$, $p<0.05$), thus, statistical analysis confirmed the superadditive nature of the combined effect of NMDA and ACh. These results suggest an additional mechanism which requires the activation of both NMDARs and AChRs, and may be explained by the cholinergic facilitation of glutamatergic neurotransmission [43,44].

After the systemic administration of scopolamine, no changes were observed in the spontaneous firing frequency of pyramidal cells, while the mAChR antagonist markedly decreased the firing responses to NMDA, to ACh and to the simultaneous delivery of NMDA and ACh. Namely, in 30 min after scopolamine administration, NMDA-evoked firing frequency decreased from 64.6±13.2 Hz to 39.9±19.3 Hz ($p<0.05$), ACh-evoked firing frequency decreased from 51.8±12.9 Hz to 3.0±1.6 Hz ($p<0.001$), while the firing frequency evoked by the combined treatment (ACh_NMDA) decreased from 117.0±18.1 Hz to 56.5±20.1 Hz ($p<0.001$). However, scopolamine did not affect the superadditive effect evoked by the simultaneous delivery of NMDA and ACh, as the combined effect of NMDA and ACh (ACh_NMDA−Sp) paralelly decreased with the simple sum of mono-treatment effects [(NMDA-Sp)+(ACh-Sp)]. Thus, simultaneous delivery of NMDA and ACh exerted a super-additive increase of firing frequency compared to the summarized effect of mono-treatments even at 30 min after scopolamine administration [ACh_NMDA-Sp vs. (NMDA-Sp)+(ACh-Sp): 55.0±19.9 Hz vs. 40.0±19.3 Hz; $F(1, 6)= 5.78$; $p=0.05$]. To sum up, scopolamine markedly blocked tonic firing rate increasing effect of locally delivered ACh, while it had no effect on the cholinergic enhancement of firing responses to NMDA. These results imply that in our experimental paradigm, mAChRs did not contribute to the cholinergic potentiation of glutamatergic neurotransmission, or presynaptic mAChRs were not blocked by this pharmacologically relevant dose of scopolamine (i.e., 1 mg/kg).

Systemic administration of α7 nAChR antagonist MLA did not affect spontaneous firing frequency of the recorded neurons. Moreover, no significant changes were observed in NMDA-evoked and ACh-evoked firing responses of neurons for 30 min after MLA administration. On the other hand,

MLA significantly decreased firing responses to the simultaneous delivery of NMDA and ACh, which decreased from 139.4 ± 17.7 Hz to 91.7 ± 14.7 Hz ($p<0.05$) at 30 min after MLA administration. Furthermore, combined effect of NMDA and ACh decreased to the simple sum of mono-treatment effects, thus, simultaneous iontophoresis of NMDA and ACh did not evoke a superadditive increase in the firing frequency at 30 min after MLA treatment [ACh_NMDA−Sp vs. (NMDA−Sp)+(ACh−Sp): 88.8±15.0 Hz vs. 103.6±18.7 Hz; $F(1, 6)= 1.45$, $p=0.27$, N.S.]. According to the results observed after the systemic administration of MLA, we concluded that α7 nAChRs did not contribute substantially to the tonic firing rate increase evoked by local release of ACh, while α7 nAChRs play essential role in the cholinergic facilitation of glutamatergic neurotransmission.

## 5. SUMMARY

In the present research, we aimed to study the interaction of α7 nAChR and glutamate receptors from the NMDA subtype in the context of cognitive functions. Studies were performed on two levels of organization using behavioral pharmacological and *in vivo* cellular electrophysiological methods.

Results of the behavioral experiments showed that PHA-543613 totally reversed the memory deficit induced by the mAChR antagonist scopolamine in a dose-dependent manner. On the contrary, it was much less effective in the MK-801 model which was based on the blockade of NMDARs, thus the effect of the α7 nAChR agonist was not sufficient to restore the normal working memory function of animals. These findings suggest that the cognitive enhancer potential of α7 nAChR agonists is closely related to the function of NMDARs, as sustained glutamatergic transmission through the NMDARs is required for the efficacy of α7 nAChR agonists.

In the electrophysiological studies, first we optimized the single-channel extracellular recording method and the microiontophoresis technique for measurements in the dense pyramidal cell layer of the hippocampal CA1 region. Next, we performed electrophysiological and pharmacological experiments with this setup to validate proper separation of neuronal signals. Results confirmed reliable distinction of hippocampal pyramidal cells and interneurons on the basis of electrophysiological properties, even when single-channel recordings are used in a combination with microiontophoresis.

In further experiments, we studied the pharmacological responses of pyramidal cells to locally iontophoretized NMDA and ACh, furthermore, we investigated the role of AChR subtypes in the observed local effects of ACh. According to the electrophysiological measurements, we identified two distinct effects of ACh on CA1 hippocampal pyramidal cells: 1) a tonic excitatory effect, which maintain high firing frequency of neurons, and 2) the potentiation of glutamatergic transmission through NMDARs, which was observed as the superadditive increase of firing frequency after

simultaneous delivery of NMDA and ACh. Results from experiments involved systemic administration of mAChR antagonist scopolamine and α7 nAChR antagonist MLA suggested that the tonic excitatory effect of ACh could be attributed mainly to the activation of mAChR, while cholinergic potentiation of glutamatergic neurotransmission depends substantially on the function of α7 nAChRs.

The present results, obtained in behavioral pharmacological and in *in vivo* electrophysiological experiments support the critical role of glutamatergic-cholinergic interactions in cognitive functions, especially in hippocampus-dependent memory. Furthermore, we demonstrated the important role of α7 nAChRs in this interaction. Our results may also provide important data for interpreting results originating from various pharmacologically induced amnesia models (e.g., scopolamine, MK-801, MLA). Additionally, the presently described *in vivo* electrophysiological paradigm may be suitable to investigate the cellular mechanisms of action underlying the cognitive enhancer effects of new drug-candidates.

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