Investigation of the efficacy of combined cognitive enhancer treatments in different rat models of neurocognitive disorders

Doctoral (PhD) thesis

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1. INTRODUCTION

Aging-related neurocognitive disorders (NCDs) poses a serious public health problem worldwide. These age-related disorders are characterized by progressive neuronal dysfunction and marked deterioration of cognitive abilities and daily living. Although aging is the main risk factor for NCDs the exact mechanisms through which aging is associated with neurodegeneration have not yet been identified. Therefore, it would be important to understand the basic mechanisms of aging and their role in the development and progression of NCDs. Furthermore, in the absence of suitable therapies, it would be necessary to develop new and more effective therapeutic strategies.

The most common type of severe NCD is Alzheimer's disease. The primary pathological markers of AD are brain atrophy resulting by synaptic and neuronal loss (typically in the medial temporal lobe and hippocampus), extracellular aggregated Aβ plaques and p- tau neurofibrillary tangles. Large body of evidence supports the idea that neuritic plaques have a primary role in the development of AD (Haass and Selkoe 2022). However, some research contradicts the amyloid hypothesis of AD, claiming that the neuronal damage of AD can occur independently of Aβ plaques deposition (Morris et al. 2014), and therapies targeting Aβ plaques have not proven effective so far. However many studies assume the key role of increased neuroinflammation underlying these pathological (Castellani et al. 2010; Kinney et al. 2018). Thus, promising future treatment strategies should address neuroinflammatory mechanisms underlying the neurophysiological dysfunctions of the aging brain.

Currently, the non-competitive NMDA receptor antagonist memantine and different cholinesterase inhibitors (e.g., donepezil) can be offered as therapeutic options for patients with NCDs (Yiannopoulou and Papageorgiou 2020), however, several clinical observations indicate that these medications may provide only moderate and transient symptomatic benefits with very limited or no disease-modifying potential (Raina et al. 2008; Tsoi et al. 2016). Therefore, the development of new treatment strategies is still an unmet medical need and the discovery of new avenues for the treatment of NCDs would be crucial in the field. The alpha7 nicotinic acetylcholine receptor (α7 nAChR) is considered a promising target to treat NCDs because it is primarily involved in learning and memory processes (Wallace and Porter 2011) and also plays a role in immune regulation. In recent years, stimulation of α7 nAChRs expressed by glial cells has been shown to reduce neuroinflammation by activating the cholinergic anti-inflammatory pathway (Egea et al. 2015; Maurer and Williams 2017) and, thus, also contributing to the reported therapeutic efficacy of α7 nAChRs in preclinical models of NCD.

Combination therapies may be highly promising novel approaches in the treatment of age-related NCDs, since the co-application of pharmacological agents with different mechanisms of action may result in a superadditive (synergistic) increase in efficacy and/or enables the use of lower doses, while achieving the same effectiveness with less possible side-effects. Furthermore, combination treatments may offer a complex influence on the dysfunction of signaling pathways and pathological processes involved in NCDs (Parsons et al. 2013), which may add up in beneficial disease-modifying pharmacological effects. Although the combination of acetylcholine esterase inhibitor (AChEI) donepezil with glutamatergic antagonist memantine is already in use in the treatment of Alzheimer's disease, clinical evidence is ambiguous about its superior cognitive outcomes over the corresponding monotherapies (Deardorff and Grossberg 2016; Knorz and Quante 2021). In contrast, available results suggest a more promising outcome in cases when memantine is combined with compounds acting directly on the α7 nAChRs (Koola et al. 2018). For example, co-administration of memantine with galantamine (which acts not only as an AChEI but also as a positive allosteric modulator (PAM) of α7 nAChRs) alleviates scopolamine-induced memory deficits in mice (Busquet et al. 2012) and delays natural forgetting in rats (Nikiforuk et al. 2016) with higher efficacy compared to the corresponding monotreatments.

Based on the above findings, the aim of the current study was to further investigate the behavioral level interaction between memantine and selective α7 nAChR agents in both pharmacological induced and naturally aged pre-clinical rat models. Our further aim was to assess key molecular-level indicators of pathological and healthy aging animals by investigating the mRNA and protein expression of inflammatory biomarkers (IL-1β, MIP-1α), ciliary neurotrophic factor (CNTF), and α7 nAChR in both memory-impaired and unimpaired animals.

2. AIMS

I. Investigation of ligand interactions targeting α7-nAChRs in scopolamine-induced pharmacological rat model of NCDs using the Morris water maze (MWM) and T-maze behavioral test paradigms:

1. Determination of the dose-effect relationship of memantine and the selective α7-nAChR agonist PHA-543613.

2. Investigation of the combined effect of memantine and PHA-543613 in subeffective doses on different components of episodic memory (spatial navigation, short-term memory, long-term memory) compared to the corresponding mono-treatments.

II. Investigation of ligand interactions targeting α7-nAChRs in a naturally aged rat model of NCDs using the NOR paradigm:

1. Determination of the dose-effect relationship of memantine, the selective α7-nAChR agonist PHA-543613 and a PAM compound.

2. Investigation of the combined effect of memantine and α7-nAChR ligands (PHA-543613 and PAM) in subeffective doses compared to the corresponding mono-treatments.

III. Examination of mRNA and protein expression levels of two inflammatory mediators (IL-1β, MIP-1α), a neurotrophic factor (CNTF) and the α7-nAChRs in brain areas responsible for memory (hippocampus, neocortex, striatum).

3. MATERIALS AND METHODS

3.1. Investigation of interactions between memantine and PHA-543613 in a pharmacological NCD model

In the first study we tested the combined effect of the selective α7-nAChR agonist PHA-543613 and memantine in a scopolamine-induced rat model of NCDs. The efficacy of the co-administration treatment on different domains of spatial episodic memory (navigation, short-term memory and long-term memory) was investigated in the Morris water maze (MWM) task. Furthermore, the pharmacological effects of the memantine-PHA-543613 combination treatment on short-term memory were also examined in the T-maze spontaneous alternation task.

The protocol of the MWM navigation task consisted of four training days and an additional probe trial day. During the training days a platform was placed in the center of the SW quadrant (submerged 1 cm below the surface), and the rats had to learn the location of the hidden platform with the help of visual cues placed around the maze. All animals had to perform

four trials per training day. During the trials, the escape latency (the time until the platform was found), the swimming path length to the platform and the swimming speed of the animals were measured and analyzed using Ethovision XT10 software (Noldus, Wageningen, Netherlands). The performance of the animals on the first training day was used to evaluate their short-term memory performance. On the fifth day, a single probe trial was performed for testing the recall of long-term memory. The platform was removed from the pool and the animals were allowed to search the pool for 2 min. During the probe trial, the time spent in the target quadrant was measured as an index of long-term memory recall.

The first series of experiments were established for the determination of the dose–response relationships for memantine and PHA-543613. Memantine was administered in the doses of 0.1 mg/kg, 0.3 mg/kg and 1.0 mg/kg. PHA-543613 was administered in the doses of 0.3 mg/kg, 1.0 mg/kg and 3.0 mg/kg. Efficacy of the applied drugs in different doses was tested on cognitive performance of the rats using the scopolamine-induced (0.1 mg/kg, Scop) transient amnesia model. The monotreatments with different doses of memantine and PHA-543613 were compared to the same vehicle-treated and scopolamine-treated groups. In the second series of experiments, pharmacological interactions between subeffective doses of memantine (0.1 mg/kg) and PHA-543613 (0.3 mg/kg) and the cognitive enhancer effects of their co-administration were tested and were compared with the effects of monotreatments. Animals received the following treatments: vehicle alone, scopolamine alone, memantine monotreatment in 0.1 mg/kg dose followed by scopolamine, PHA-543613 monotreatment in 0.3 mg/kg dose followed by scopolamine, and co-administration treatment followed by scopolamine.

The short-term memory of the animals was also tested in the T-maze task with a spontaneous alternation paradigm. The rat was placed in the start arm and after the opening of the guillotine door, he had to make a choice between the right and left goal arms. After exploring the goal arm and returning to the start arm, the rat was confined in the start arm for 10 s between two trials. Then, the trial was repeated. In each trial, we primarily recorded which goal arm was chosen by the rat. If the rat entered the opposite arm compared to the previous trial, the choice was considered a correct choice (alternation). Otherwise, it was considered an erroneous choice. Alternation rate was determined as the proportion of correct arm choices (alternations) and the total number of trials offered for alternation (a maximum of 14 if all 15 trials were run).

The study consisted of two phases: (1) an experiment for the determination of subeffective and effective doses of memantine; and (2) a set of experiments testing the interactive effects of memantine and PHA-543613. The doses applied in the memantine efficacy test were chosen on the basis of preceding pilot experiments and were the following: 0.001, 0.003, 0.01, 0.03, and 0.1 mg/kg. Subeffective doses of PHA-543613 were determined according to our previous study (Bali et al. 2015). In the experiments testing co-administration of memantine and PHA-543613, the following pharmacological treatments were applied: scopolamine alone (further referred to as Scop), memantine monotreatment in 0.1 mg/kg dose followed by scopolamine, PHA monotreatment in subeffective doses followed by scopolamine, and co-administration treatment with memantine and PHA followed by scopolamine.

3.2. Investigation of interactions between memantine and α7-nAChR ligands in a spontaneous aging model

In the second study we investigate the interaction between memantine and two different α7 nAChR agents (the orthosteric agonist PHA-543613 and a novel compound with PAM characteristics: CompoundX, a proprietary compound of Gedeon Richter Plc., Hungary) in a preclinical animal model of age-related cognitive deficit. The declarative memory of the animals was tested in the novel object recognition paradigm. Our further aim was to investigate the mRNA and protein expression of inflammatory biomarkers (IL-1β, MIP-1α), ciliary neurotrophic factor (CNTF), and α7 nAChR in both memory-impaired and unimpaired animals.

The NOR protocol consisted of two trials: an acquisition trial followed by a test trial after 24 hours retention interval. In the acquisition trial, two identical objects were placed near the left and right corners of the box, and the rats were allowed to explore the environment and the objects for 3 min. After 24 hours, one of the identical objects was changed with a novel one and rats were allowed to explore the objects for 3 min.

Because of the innate novelty-seeking behavior of rats, successful recognition of the familiar object (normal memory function) is supposed to be manifested in the preference to explore the novel object. Thus, the time spent with familiar (Ef) and novel (En) objects was recorded, and a discrimination index (DI) was calculated based on the following formula:

$$DI = (En - Ef) / (En + Ef)$$

In the first part of the study, PHA-543613, or CompoundX, as well as co-administrations of memantine and one of the cholinergic agents, were tested in aged animals. Monotreatments with memantine were applied in the following doses: 0.1 mg/kg, 0,3 mg/kg, and 1.0 mg/kg.

PHA-543613 was administered in doses of 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg and CompoundX was administered in the doses of 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg. Then, co-administration of subeffective memantine and PHA-543613 doses were tested against the effects of the corresponding monotreatments by applying the following treatments in a counterbalanced Latin-square design: vehicle alone, memantine monotreatment in 0.01 mg/kg dose, PHA-543613 monotreatment in 0.1 mg/kg dose, and co-administration treatment. Similarly, the efficacy of memantine-CompoundX co-administration in comparison to the monotreatments was tested in a separate experiment using the following treatments: vehicle alone, memantine monotreatment in 0.01 mg/kg dose, CompoundX monotreatment in 0.1 mg/kg dose, and co-administration treatment.

3.3. Post-mortem investigations

After completing the behavioral experiments, animals were anesthetized with an overdose of pentobarbital and were transcardially perfused. A subgroup of young and aged rats was perfused with 0.9% saline, their brains were rapidly removed and dissected into left and right neocortex (CTX), striatum (STR), and hippocampus (HC). Target gene expressions were measured using real-time PCR using Maxima SYBRGreen MasterMix (Applied Biosystems, Waltham, USA) with an ABI Prism 7500 instrument (Applied Biosystems). Cyclophilin A (CycA) was used as a housekeeping gene for the quantification of RNA. Quantification of IL-1β, MIP-1α, CNTF, and α7 nAChR was carried out using Abbexa (Cambridge, UK) ELISA kits with the following catalog numbers, respectively: abx155713, abx155822, abx155360, abx556026. ELISAs were performed according to the manufacturer's protocols.

4. RESULTS

4.1. Combined effect of memantine and PHA-543613 on learning and memory performance of rats

First, the dose–effect relationships of memantine and PHA-543613 monotreatments were analyzed. During the training phase (days 1–4), the mean escape latency of the animals significantly decreased (DAY: $F(3, 213) = 177.313$, $p < 0.001$). There was no significant difference in the overall escape latency between vehicle, scopolamine, memantine (0.1–1.0 mg/kg) and PHA-543613-treated groups (TREATMENT: $F(7, 71) = 0.845$, $p = 0.554$). Learning performance of the animals was analyzed also in terms of swimming path length which showed a profound decrease over the training days (DAY: $F(3, 213) = 153.072$,

$p < 0.001$), and significant effect of treatments on the learning progress was not revealed (TREATMENT: $F(7, 71) = 1.281$, $p = 0.272$) similarly to results based on escape latency.

In the probe trial, the time spent in the target quadrant represented long-term memory. In contrast with the results on the training days, in the probe trial, a significant main effect of pharmacological treatments was detected (TREATMENT: $F(7, 71) = 3.917$, $p = 0.001$). Results showed that scopolamine-treated animals spent significantly less time in the target quadrant than the vehicle-treated group (Control vs. Scop: 43.2 ± 2.8 s vs. 28.0 ± 2.8 s, $p = 0.002$). Although memantine in lower doses (0.1 mg/kg and 0.3 mg/kg) did not improve long-term memory against scopolamine, the highest memantine dose (1.0 mg/kg) successfully reversed the memory deteriorating effect of scopolamine (Scop vs Mem1.0: 28.0 ± 2.8 s vs 41.7 ± 3.3 s, $p = 0.009$). However, PHA-543613 did not alleviate scopolamine-induced memory deficits in the applied doses.

In the next experiment, a subeffective dose of memantine (0.1 mg/kg) was co-administered with a subeffective dose of PHA-543613 (0.3 mg/kg). Doses were chosen on the basis of the probe trial in the dose–response experiments. The memory enhancer effect of the co-treatment was compared with the monotreatments using the same doses of PHA-543613 and memantine. In the learning phase (days 1–4), a significant main effect of training was found, as both the escape latency and the swimming path length significantly decreased during the four days (DAY: escape latency: $F(3, 180) = 133.533$, $p < 0.001$; swimming path length: $F(3, 180) = 110.325$, $p < 0.001$). Neither monotreatments, nor co-administration of PHA-543613 and memantine exerted a significant main effect on escape latency (TREATMENT: $F(4, 60) = 1.144$, $p = 0.345$) or swimming path length (TREATMENT: $F(4, 60) = 0.426$, $p = 0.789$)

Short-term memory of the animals was also analyzed which was based on the performance of the animals on the first training day. Results showed a significant interaction between treatments and trials on the first training day, thus treatments significantly affected the performance during training (TREATMENT × TRIAL: escape latency: $F(12, 180) = 1.952$, $p = 0.031$; swimming path length: $F(12, 177) = 2.305$, $p = 0.009$). While almost all experimental groups showed gradual improvement during the four training trials on the first day, animals treated with scopolamine-only did not learn the location of the platform.

Contrasts between pairs of treatment groups and the first and last trials (trial1 vs. trial4) on the first day were also analyzed, and results showed that changes in escape latency and swimming path length were significantly different in the group treated with scopolamine only

compared to the control group ([Control vs. Scop] × [trial1 vs. trial4]: escape latency: 45.7 ± 18.4 (contrast estimate ± SEM), $p = 0.016$; swimming path length: 1475.0 ± 515.7, $p = 0.006$). These results indicate that scopolamine predominantly impaired the short-term component of spatial episodic learning. Furthermore, the scopolamine-induced learning deficit was significantly improved by memantine and PHA-543613 both in monotreatments and in co-administration of the same doses ([Scop vs. Mem0.1] × [trial1 vs. trial4]: escape latency: 65.4 ± 16.3, $p < 0.001$; swimming path length: 1939.4 ± 456.2, $p < 0.001$; [Scop vs. PHA0.3] × [trial1 vs. trial4]: escape latency: 65.3 ± 16.3, $p < 0.001$; swimming path length: 1884.0 ± 464.9, $p < 0.001$; [Scop vs. Mem0.1&PHA0.3] × [trial1 vs. trial4]: escape latency: 79.0 ± 16.3; $p < 0.001$; swimming path length: 2524.0 ± 456.2, $p < 0.001$).

Consequently, rats treated only with scopolamine still showed poor performance on the second training day. Here, a significant main effect of pharmacological treatments was found on escape latency on the second training day (TREATMENT: $F(4, 60) = 2.932$, $p = 0.028$) but not on path length (TREATMENT: $F(4, 60) = 1.661$, $p = 0.171$). Pairwise comparisons of escape latency data showed that control animals and animals who received the low memantine dose and low PHA-543613 dose in monotreatments or in co-administration performed better than rats treated with scopolamine only (control vs. Scop: 37.4 ± 6.7 s vs. 58.6 ± 9.4 s, $p = 0.054$; Scop vs. Mem0.1: 58.6 ± 9.4 s vs 39.3 ± 6.0 s, $p = 0.048$; Scop vs. PHA0.3: 58.6 ± 9.4 s vs. 28.3 ± 6.2 s, $p = 0.002$; Scop vs. Mem0.1&PHA0.3: 58.6 ± 9.4 s vs. 33.0 ± 5.5 s, $p = 0.009$). Here, no significant interaction was found between groups and trials (TREATMENT × TRIAL: escape latency: $F(12, 180) = 1.020$, $p = 0.432$; swimming path length: $F(12, 180) = 0.852$, $p = 0.597$), indicating that scopolamine-treated animals started to show a tendency of learning the platform location on the second training day.

In the probe trial, pharmacological treatments revealed a significant main effect on the time spent in the target quadrant (TREATMENT: $F(4, 60) = 2.805$, $p = 0.033$). According to post-hoc analysis, the scopolamine-treated group spent less time in the target quadrant compared to the vehicle-treated control group (Control vs. Scop: 47.9 ± 3.5 s vs. 38.3 ± 2.1 s, $p = 0.035$) and subeffective doses of memantine and PHA-543613 in monotreatments did not attenuate scopolamine-induced amnesia (Scop vs Mem0.1: 38.3 ± 2.1 s vs 37.1 ± 2.8 s, $p = 0.766$; Scop vs PHA0.3: 38.3 ± 2.1 s vs. 43.3 ± 3.2 s, $p = 0.210$). However, the co-administration treatment with memantine and PHA-543613 effectively reversed the scopolamine-induced long-term memory deficit (Scop vs. Mem0.1&PHA0.3: 38.3 ± 2.1 s vs.

47.1 ± 2.9 s, $p = 0.029$), suggesting a beneficial interaction between memantine and the alpha7 nicotinic acetylcholine receptor agonist PHA-543613.

4.2. Combined effect of memantine and PHA-543613 on alternation performance of rats

In the T-maze test first the relationship between the dose of memantine and its cognitive enhancer effect against scopolamine was determined. Control performance of rats was above the chance level (one-sample $t = 4.745$, $p < 0.001$) and was significantly higher than after scopolamine treatment [Control vs. Scop: 0.63 ± 0.03 (mean ± SEM) vs. 0.43 ± 0.05, $p = 0.047$), indicating that rats showed good control memory performance and alternating behavior. Memantine dose-dependently attenuated scopolamine-induced memory impairment and increased the average alternation rate of rats. Although the memory enhancing effect of memantine in the dose of 0.1 mg/kg was only marginally significant compared with the scopolamine alone treatment according to the corrected *p*-value (Mem0.1 vs. Scop: 0.62 ± 0.04 vs. 0.43 ± 0.05, $p = 0.073$), Mem0.1 treatment restored normal alternating behavior of animals (one-sample $t = 3.011$, $p = 0.008$). Therefore, 0.1 mg/kg dose of memantine was considered as an effective dose for cognitive enhancement.

Then the subeffective 0.003 mg/kg dose of memantine was tested in co-administration with the subeffective 0.1 mg/kg dose of PHA-543613 against scopolamine-induced amnesia of rats. Monotreatments with memantine or PHA-543613 were not effective enough to attenuate the scopolamine-induced memory deficit (0.53 ± 0.07 and 0.45 ± 0.04, respectively; Mem0.003 vs. Scop: $p > 0.1$, $d_{rm} = 0.162$; PHA0.1 vs. Scop: $p > 0.1$), and alternation performance did not significantly exceed the chance level neither after Mem0.003 nor after PHA0.1 treatment.

The dose-response relationship of procognitive effects of memantine and the α7 nAChR ligands PHA-543613 and CompoundX was studied as the modulation of natural memory decline caused by aging. Unlike vehicle treatment, the administration of memantine (0.1 and 1.0 mg/kg) significantly increased the time spent with the exploration of the novel object as compared to the familiar object (time spent observing the novel vs. familiar: VEH: 9.3±1.6 vs. 7.3±1.1, t=1.489, df=10, p=0.167; Mem0.1: 9.0±1.3 vs. 5.5±0.5, t =2.492, df = 11, p = 0.030; Mem1.0: 8.7±1.2 vs. 5.7±1.0, t =2.737, df =10, p = 0.021). Thus, 0.1 and 1.0 mg doses of memantine reversed the age-related recognition memory deficit in rats. However, the effect on DI was not significant after any of the memantine monotreatments (0.1-1.0 mg/kg; F(3, 32) = 0.384; p = 0.765). However, the co-administration of these subeffective doses of

memantine and PHA-543613 resulted in a significant increase in the alternation rate (Mem0.003+PHA0.1: 0.64 ± 0.04; Mem0.003+PHA0.1 vs. Scop: $p = 0.043$), and it restored the normal memory performance of the animals (one-sample $t = 3.506$, $p = 0.004$). Thus, an interaction between subeffective doses of memantine and PHA-543613 was found to be beneficial for the enhancement of short-term memory of rats.

4.3. Investigation of interactions between memantine and α7-nAChR ligands in aged rats

Aged animals receiving PHA-543613 in 0.3 mg/kg and 1.0 mg/kg doses showed a preference towards the novel object (novel vs. familiar: PHA0.3: 10.4±0.8 vs. 5.0±0.8, t =6.354, df = 12, p < 0.001; PHA1.0: 9.3±1.2 vs. 6.7±0.8, t =2.684, df = 12, p = 0.020), while vehicle treated aged rats did not discriminate between the objects (novel vs. familiar: VEH: 9.4±1.7 vs. 7.8±1.1, t=0.799, df=10, p=0.443). Furthermore, PHA-543613 at the lowest dose (0.3 mg/kg) improved the DI of the aged animals compared to the vehicle-treatment (F(3, 34.7) =4.189; p =0.012; PHA0.3 vs. VEH: 0.38±0.07 vs. 0.05±0.12, p =0.012). CompoundX also enhanced memory performance of rats by restoring their ability to discriminate between the novel and familiar objects at 0.3 mg/kg dose (novel vs. familiar: CPDX0.3: 10.9±1.4 vs. 5.3±0.6, t =5.245, df = 9, p = 0.001). Significant main effect of CompoundX monotreatments was not observed on the DI (F(3, 26.6) =1.093; p = 0.369). However, the lowest dose of CompoundX (0.3 mg/kg) increased the mean DI similar to the lowest dose of PHA-543613 (0.3 mg/kg).

In the next series of experiments, a subeffective dose of memantine (0.01 mg/kg) was co-administered with subeffective doses of α7 nAChR compounds PHA-543613 (0.1 mg/kg) or CompoundX (0.1 mg/kg). First, the cognitive enhancing effect of memantine-PHA-543613 combination was compared to the effects of the corresponding monotreatments. Results showed that rats after the treatment with memantine alone and in combination with PHA-543613 spent more time exploring the novel object than the familiar object (novel vs. familiar: Mem0.01: 8.1±0.8 vs. 5.4±0.5, t =2.549, df = 10, p = 0.029; Mem0.01&PHA01: 10.1±1.2 vs. 5.0±0.6, t =3.544, df = 11, p = 0.005) in contrast with vehicle treatment (novel vs. familiar: VEH: 6.5±1.0 vs. 7.2±1.1, t =-0.465, df = 10, p =0.652). However, the monotreatment with PHA-543613 was not found effective (novel vs. familiar: PHA0.1: 8.2±1.3 vs. 6.7±1.1, t =1.604, df = 10, p = 0.140). The applied treatments resulted in a marginal effect on DI (F(3, 30) =2.727; p = 0.062). As expected, pairwise comparisons did not show any significant increase of DI by the low-dose monotreatments with memantine and PHA-543613 compared to the vehicle-treated aged control group (Mem0.01 vs. VEH: 0.19±0.07 vs. -0.06±0.12, p=0.081; PHA01 vs.

VEH: 0.09±0.07 vs. -0.06±0.12, p= 0.298). In contrast, DI was significantly increased by the combination treatment (Mem0.01&PHA01 vs. VEH: 0.31±0.10 vs. -0.06±0.12, p=0.01) indicating that the combination of the two distinct mechanisms passed a threshold to successfully reverse age-related recognition memory decline.

Similarly, the effects of the co-administration of memantine and CompoundX were also tested. As expected, animals who received low-dose memantine or CompoundX in monotreatments did not discriminate between the novel and the familiar objects (novel vs. familiar: Mem0.01: 6.5±0.8 vs. 5.2±0.6, $t = 1.716$, $df = 14$, $p = 0.108$; CPDX0.1: 6.9±0.7 vs. 6.1±0.8, $t = 0.965$, $df = 13$, $p = 0.352$). However, co-administration of memantine and CompoundX reversed age-related memory deficit as rats spent significantly more time with the exploration of the novel object after the combination treatment (novel vs. familiar: 9.8±1.2 vs. 4.5±0.6, $t = 4.560$, $df = 14$, $p < 0.001$). In addition, the combination of memantine and CompoundX also reached a significant effect on DI ($F(3, 41.7) = 3.281$; $p = 0.030$). Regarding monotherapies, age-related decrease of DI was not significantly attenuated by either memantine or CompoundX monotreatments (Mem0.01 vs. VEH: 0.11±0.08 vs. 0.17±0.06, p=0.565; CPDX0.1 vs. VEH: 0.07±0.08 vs. 0.17±0.06, p=0.378). However, the mean DI was marginally significantly increased by memantine-CompoundX co-administration (Mem0.01&PHA01 vs. VEH: 0.37±0.07 vs. 0.17±0.06, p=0.055).

4.4. Effects of aging on mRNA and protein expression levels of inflammatory factors and α7-nAChR in the brain

For further biochemical investigations, 5 young and 10 aged animals were sacrificed. Aged animals were further divided into memory-impaired (AI) and unimpaired (AU) aged groups, with 5 animals in each group, depending on their baseline cognitive performance. Rats who performed above the median DI were considered AU (DI range: 0.17 - 0.68), while rats with a DI lower than the median were considered AI rats (DI range: –0.66 - –0.01).

To evaluate the inflammatory profile and cholinergic aging, mRNA expression levels of various cytokines and α7 nAChR were assessed in neocortical, striatal, and hippocampal brain tissue samples of young, AI and AU rats.

Results of qRT-PCR analysis revealed that both AI and AU animals showed significantly higher IL-1β mRNA levels in the neocortex and the striatum compared to young control rats

(CTX: $F(2, 11) = 5.631$, $p = 0.021$; AI vs. young: 26.9±4.7 vs. 9.7±2.1, p=0.008; AU vs. young: 22.2±4.5 vs. 9.7±2.1, p=0.047; STR: $F(2, 11) = 8.753$, $p = 0.005$, AI vs. young: 55.3±8.3 vs. 18.9±2.4, p=0.002; AU vs. young: 40.8±7.2 vs. 18.9±2.4, p=0.038). On the contrary, only AI animals exhibited a significant increase of IL-1β mRNA in the hippocampus compared to young animals ($F(2, 12) = 6.708$, $p = 0.013$; AI vs. young: 35.6±3.1 vs. 19.1±3.6, p=0.005), and a difference between AU and young groups was not found (AU vs. Young: 23.7±3.4 vs 19.1±3.6, p=0.349).

MIP-1α mRNA expression was upregulated in both AI and AU rats in all selected brain areas compared to young animals (CTX: $F(2, 12) = 5.860$, $p=0.017$; AI vs. young: 75.6±20.7 vs. 14.5±3.8, p=0.006; AU vs. young: 58.3±8.0 vs. 14.5±3.8, p=0.035; STR: $F(2, 12) = 45.5$, $p < 0.001$; AI vs. young: 184.8±22.7 vs. 42.6±5.8, $p<0.001$; AU vs. young: 232.1±9.7 vs. 42.6±5.8, $p<0.001$; HC: $F(2, 12) = 16.121$, $p<0.001$; AI vs. young: 122.1±21.1 vs. 18.8±1.6, $p > 0.001$; AU vs. young: 96.2±9.5 vs. 18.8±1.6, p=0.001). In the neocortex and the hippocampus the AI group showed the highest mean expression of MIP-1α mRNA.

CNTF mRNA expression levels were significantly increased in the AI group in the cortex and hippocampus compared to young animals (CTX: $F(2, 11) = 4.167$, $p = 0.045$; AI vs. young: 12.2±2.3 vs. 6.4±0.4, p=0.017; HC: $F(2, 12) = 4.099$, $p = 0.044$; AI vs. young: 46.0±8.2 vs. 28.1±1.7, p=0.029). In contrast, AU rats did not express increased mRNA levels of CNTF in these brain regions (CTX: AU vs. young: 7.9±0.8 vs. 6.4±0.4, p=0.507; HC: AU vs. young: 28.0±2.8 vs. 28.1±1.7, p=0.990). CNTF mRNA expression in the striatum did not differ between the groups ($F(2, 12) = 0.394$, $p = 0.683$).

AI rats also showed upregulated α7 nAChR mRNA levels in the examined brain areas compared to the young group, while the AU group expressed α7 nAChR mRNA levels similar to the young rats (CTX: $F(2, 11) = 3.739$, $p = 0.058$; AI vs. young: 28.6±4.6 vs. 15.8±1.3, p=0.020; AU vs. young: 20.7±3.2 vs. 15.8±1.3, p=0.350; STR: $F(2, 11) = 5.635$, $p = 0.021$; AI vs. young: 16.1±0.9 vs. 13.5±0.7, p=0.027; AU vs. young: 12.7±0.4 vs. 13.5±0.7, p=0.489; HC: $F(2, 12) = 3.085$, $p = 0.083$; AI vs. young: 68.7±3.0 vs. 52.6±5.4, p=0.047; AU vs. young: 53.4±6.5 vs. 52.6±5.4, p=0.907).

Next, we analyzed the protein expression levels of IL-1β, CNTF, MIP-1α, and α7 nAChR in the neocortex, striatum, and hippocampus of young, AI, and AU rats.

Results of ELISA analysis revealed a significant increase of IL-1β protein level in the striatum of AU but not of AI animals compared to the young control group ($F(2, 12) = 3.127$,

p =0.081; AU vs. young: 1.5±0.1 pg/µg vs. 1.1±0.1 pg/µg, p=0.032; AI vs. young: 1.4±0.1 pg/µg vs. 1.1±0.1 pg/µg, p=0.110). In the neocortex and hippocampus no differences in IL-1β expression were found between the groups (CTX: $F(2, 7) = 0.511$, $p = 0.621$; HC: $F(2, 12) = 0.061$, $p = 0.941$).

Surprisingly, neither the AI nor AU groups showed a significant change in the expression of MIP-1α protein in any of the tested brain regions (CTX: $F(2, 7) = 0.652$, $p = 0.550$; STR: $F(2, 12) = 0.051$, $p = 0.950$; HC: $F(2, 12) = 0.173$, $p = 0.843$).

In contrast with mRNA expression levels, protein levels of hippocampal CNTF were significantly downregulated in the AI group compared to the young group ($F(2, 12) = 5.386$, $p = 0.021$; AI vs. young: 0.6±0.1 pg/µg vs. 1.3±0.2 pg/µg, p=0.007). In addition, there was a (non-significant) tendency to decreased CNTF protein expression level in the hippocampus of the AU group compared to young animals (AU vs. young: 0.9±0.1 pg/µg vs. 1.3±0.2 pg/µg, p=0.070). In the cortex and the striatum, no significant main effect of aging could be observed on CNTF protein levels of rats (CTX: $F(2, 7) = 2.298$, $p = 0.171$; STR: $F(2, 12) = 2.167$, $p = 0.151$).

There was no significant main effect on the protein expression level of α7 nAChR (CTX: $F(2, 7) = 1.882$, $p = 0.222$; STR: $F(2, 12) = 2.120$, $p = 0.163$; HC: $F(2, 12) = 0.383$, $p = 0.690$). However, in the striatum, a (non-significant) tendency of upregulation of α7 nAChR was detected in the AU group compared to the young group (AU vs. Young: 2.1±0.1 pg/µg vs. 1.6±0.2 pg/µg, p=0.081).

5. SUMMARY

In the present study, we tested the interactions between memantine, an already approved drug for the treatment of NCDs and selective α7-nAChR ligands at behavioral level in different rat models of NCDs. In our first study, we investigated the interaction between memantine and the α7-nAChR agonist PHA-543613 in a scopolamine-induced NCD rat model. We tested the cognitive enhancer effect of the combined treatment on several components of episodic memory (including spatial navigation, short-term memory, and long-term memory) using MWM and T-mazes spontaneous paradigm. In the second series of experiments, we examined the previously used memantine-PHA-543613 combination treatment, as well as the combination of memantine with the PAM compound on discriminating ability of aged rats using the NOR paradigm. Finally, we investigated the expression of various inflammatory markers at molecular level

related to normal and "pathological" aging in the brain areas responsible for memory functions. The results of our behavioral experiments show that the memantine-PHA-543613 combination treatment successfully improved both the short-term and long-term memory functions of rats compared to scopolamine. The mono-treatments also proved to be effective in terms of short-term memory in the MWM test. However, the long-term memory deficit caused by scopolamine was only alleviated by the combined treatment by exceeding the null effects of the corresponding subtherapeutic levels of monotreatments. In our second study we reported a significant cognitive decline in old rats. However, combinations of memantine and α7-nAChR compounds (PHA-543613, or PAM) successfully alleviated aging-related memory impairment in rats, compared to monotreatments. The results of post-mortem examinations showed that pathological changes can also be observed in old animals. Both memory impaired an unimpaired aged animals showed a significant up-regulation of certain inflammatory markers (IL-1β and MIP-1α). The opposite direction of change was observed in terms of neurotrophic support during aging. In addition, we also reported the aging-related expression changes of α 7-nAChRs.

In conclusion, in the present study, we confirmed the beneficial interaction between memantine and α7 nAChR acting agents in both pharmacological induced and naturally aged rat models. Aged rats demonstrating a translationally more relevant cognitive improvement that might be a target for future procognitive therapy. The relevance of naturally aged rats for modelling human NCDs was also demonstrated by the animals showing characteristic neuropathological changes also at the molecular level, such as elevated levels of neuroinflammatory markers and decreased levels of neurotrophic support to the cells. Combination treatment with memantine and PHA-543613 or memantine and CompoundX successfully alleviated the age-related cognitive decline, moreover efficacy of both combinations was superior to the corresponding monotreatments. Based on our results, we further hypothesize a prominent role of α7 nAChR in the cognitive enhancer effects of the combination treatments in clinical conditions with cognitive decline in older ages. Therapeutic potential of combination treatments that are based on pharmacological interactions on the α7 nAChRs is also supported by the finding that expression of the receptors may become highly upregulated in aging, thus, providing well-accessible binding sites for their pharmacological modulation.

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8. PUBLICATIONS

List of journal articles related to thesis:

Bruszt, N., Bali, Zs. K., Nagy, L. V., Bodó, K., Engelmann, P., Némethy, Zs., Lendvai B., Hernádi, I. Combination of memantine and alpha7 nicotinic acetylcholine receptor stimulation exerts superior efficacy over monotreatments to improve cognitive performance of aged rats (under submission)

Bruszt, N., Bali, Z. K., Tadepalli, S. A., Nagy, L. V., Hernádi, I. (2021). Potentiation of cognitive enhancer effects of Alzheimer's disease medication memantine by alpha7 nicotinic acetylcholine receptor agonist PHA-543613 in the Morris water maze task. Psychopharmacology, 238(11), 3273–3281. https://doi.org/10.1007/s00213-021-05942-4 **Q1, IF: 3,4**

Bali, Z. K., **Bruszt, N.**, Tadepalli, S. A., Csurgyók, R., Nagy, L. V., Tompa, M., Hernádi, I. (2019). Cognitive Enhancer Effects of Low Memantine Doses Are Facilitated by an Alpha7 Nicotinic Acetylcholine Receptor Agonist in Scopolamine-Induced Amnesia in Rats. Frontiers in Pharmacology, 10, 73. https://doi.org/10.3389/fphar.2019.00073 **Q1, IF:5,988**

List of conference abstracts related to thesis:

Bruszt, N., Bali, Z. K., Tadepalli, S. A., Nagy, L. V., Kolozsvári, Á., Engelmann, P., Bodó, K., Hernádi, I. (2023). Combinations of memantine and alpha7 nicotinic acetylcholine receptor ligands exert superior efficacy over monotreatments in the improvement of cognitive performance of aged rats. Joint Meeting of the Hungarian Neuroscience Society (MITT) - Austrian Neuroscience Association (ANA), Budapest, Hungary

Bruszt, N., Bali, Z. K., Tadepalli, S. A., Nagy, L. V., Hernádi, I. (2022). Combined application of memantine and alpha7 nicotinic acetylcholine receptor agonist PHA-543613 improves novel object recognition memory in aged rats. International Neuroscience Meeting, Budapest, Hungary

Bruszt, N., Tadepalli, S. A., Bali, Z. K., Nagy, L. V., Hernádi, I. (2019). Combination of memantine and alpha7 nicotinic acetylcholine receptor agonist PHA-543613 improves spatial long term memory in scopolamine induced amnesia in rats. 48th Meeting of the European Brain and Behaviour Society, Prague, Czech Republic

Other journal articles:

Bali, Z. K., Nagy, L. V., **Bruszt, N.**, Bodó, K., Engelmann, P., Hernádi, Zs., Göntér, K., Tadepalli, S. A., Hernádi, I., Increased brain cytokine level associated impairment of vigilance and memory in aged rats can be improved by alpha7 nicotinic acetylcholine receptor agonist treatment (2023) Geroscience, Nov 23. doi: 10.1007/s11357-023-01019-6. **Q1, IF: 5,6**

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