Scopolamine-based pharmacological MRI model for testing procognitive agents

Ph.D. thesis

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Introduction

Cognition is a complex mental process, which requires attention, problem-solving, decision-making, and working memory. Several brain areas are involved in cognitive processes; among others prefrontal cortex (PFC), hippocampus, temporal and parietal cortex have prominent roles. Neurological (Alzheimer's disease – AD, Parkinson's disease – PD) and psychiatric disorders (schizophrenia, depression) are frequently accompanied by cognitive deficits.

Neurodegenerative dementias are usually irreversible and progressive due to the decay of neurons. There is a huge unmet medical need for an efficient disease-modifying treatment. The drug research has not been able to produce proven disease-modifying drugs since the understanding of Alzheimer's disease pathomechanism and suitable disease models are still lacking. Therefore, the research and development of procognitive compounds are one of the most challenging areas in drug research. Until today, several disappointing clinical trials have disqualified targets that were believed to be in the main pathway of the disease, such as amyloid β (Aβ), tau, β-side amyloid precursor protein cleaving enzyme (BACE) etc. These negative clinical trials draw attention to the inefficient translational power of current animal models and call for the improvement of predictive value.

Fortunately, the highly expanding field of small animal magnetic resonance imaging (MRI) seems to be a robust method, which may provide a new approach with higher translational power. The functional MRI blood-oxygenation-level dependent (BOLD) technique is suitable for the complex testing of brain functions and also the circulation. It is an important non-invasive tool in preclinical drug discovery with excellent temporal and spatial resolution.

Rat BOLD pharmacological MRI (phMRI) is gaining popularity in studies aiming at the central effect of various pharmacological agents. There are several human phMRI studies as well that verify its translational role in neuroscience. The key advantage of MRI over other imaging modalities in the neuroscience is its ability to follow changes in brain neurobiology after pharmacological, electrical or environmental insult. The reconstructed 'fingerprint' of the brain activity can characterize the activity and function of new psychotherapeutics in preclinical development and study the neurobiology of cognition.

The cholinergic neurotransmitter system undoubtedly plays a central role in cognitive processes. The muscarinergic antagonist scopolamine is a widely used memory disturbing amnestic agent in models of cognition by human and preclinical studies as well. Its reversible memory impairment property makes it a widely used model for the characterization of cognitive enhancers. Beside its negative cognitive effect, it also has a strong cerebrovascular action, which is at least partially responsible for the negative cognitive effect as was proved by the non-brain penetrating analog butylscopolamine.

Objectives

During my Ph.D. work, I aimed to develop a new, small animal pharmacological MRI provocation model, which is applicable for the testing of procognitive agents. The major steps of the experimental protocol are summarized in the following points:

- In the first part of the study we tried to set up a scopolamine-based provocation model in our MRI system. First of all, we should find the optimal administration route of amnestic agents and procognitive enhancers, furthermore we should calculate the optimal length of the pre and post injection periods. After that we registered dose-response curves of amnestic agent scopolamine (in the range between 1-5 mg/kg doses) to identify its maximal pharmacological effect.

- In the second part of the study, we were to test the most often prescribed procognitive agents (donepezil, piracetam and vinpocetine) against AD in our scopolamine-based provocation model.

- Furthermore, we aimed to test two cognitive enhancer candidates (EVP-6124, PNU-120596) which are still in developing phase. In this case, we expected improving cognitive functions and consequently increasing BOLD signals in cognition-related brain areas.

- We had only limited information about the pharmacodynamic and pharmacokinetic activities of the newly developed Richter compound, RG2260. We had to register its dose-response curve and calculate its minimal effective dose in our scopolamine-based provocation model.

- Based on the similar chemical structures of the two Richter compounds (vinpocetine and RG2260), we aimed to compare their efficiencies together in the

same testing system. We were to determine the doses in both cases which leads to similar cognitive improvement.

- In the following part of the study, we tried to examine the vascular components' contribution to cognitive impairment. The amnestic agent was buscopan, which is an antimuscarinic molecule with quaternary structure, therefore it does not cross BBB. Assuming the presence of vascular effects in cognitive decline, we expected decreasing BOLD signals in cognition-related brain areas.

- In the next part of the study, we tried to examine the vascular components' contribution to cognitive improvement. The presumed cognitive enhancer was neostigmine, which is an acetylcholinesterase inhibitor with quaternary structure, therefore it does not cross BBB. In this case, we expected BOLD signal intensity increase in cognition-related brain areas.

- Finally, we wanted to compare the results of phMRI experiments to the results of behavioral pharmacological tests (water labyrinth test). This labyrinth test was one of the most important parts of the whole experiment series to prove the relevance of our phMRI results. In the water labyrinth tests we followed a very similar experimental protocol as in fMRI experiments. Therefore, we expected similar results as in the case of fMRI studies.

Methods

fMRI measurement

Male Wistar rats weighing 240-260 g were used in the in vivo experiments. All the procedures carried out on animals had been approved by the local ethical committee and are in accordance with the rules and principles of the 86/609/EEC Directive. Rats were anaesthetized with isoflurane (Sigma-Aldrich; 5 % induction and then reduced to 1-1.5 % for maintenance of anesthesia during scanning) administered in compressed air. To facilitate drug administration during scanning a cannula line was inserted into the rat tail vein. We use a 9.4T 21ASZ Varian MRI system. Radiofrequency (RF) pulses were transmitted using an actively RF-decoupled two-channel volume coil. A fix-tuned receive-only phased array rat brain coil was placed on the dorsal surface of the rat's head to maximize the signal-to-noise ratio. We made T1-weighted anatomical images and T2*-weighted functional images.

After 1000 sec (16 min 40 sec) baseline period the amnestic agent (scopolamine hydrobromide; scopolamine N-butyl bromide or butylscopolamine – Sigma-Aldrich) was administered intravenously (i.v.) at 1 mg/kg dose. The administration was performed with an infusion pump controlled by optical signals when the animal was in the scanner. Procognitive agents or their vehiculum (Sigma-Aldrich; except vinpocetine and RG2260) were applied intraperitoneally (i.p.) as pretreatment 1 hour before scopolamine/butylscopolamine. The administration of procognitive agents was performed in 1 ml/kg volume in saline (donepezil hydrochloride, piracetam, neostigmine bromide) or in 5% Tween 80 containing vehiculum (vinpocetine, EVP-6124, PNU-120596, RG2260), depending on their solubility. Only one measurement was performed with each animal. The exact administration protocol can be seen in Table 1.

Table 1   The administration protocol of cognitive enhancers during fMRI measurement

	Solvent	Admin.	Doses (mg/kg)
Donepezil	Saline	i.p.	1; 4; 10
Piracetam	Saline	i.p.	100; 500; 1000
Vinpocetine	5% Tween 80	i.p.	5
PNU-120596	5% Tween 80	i.p.	3
EVP-6124	5% Tween 80	i.p.	1
Neostigmine	Saline	i.p.	0.01; 0.03, 0.1; 0.3
RG2260	5% Tween 80	i.p.	0.01; 0.1; 0.625; 1.25; 2.5; 5; 10

The analysis and the visualization of the data were performed using a custom-made Matlab (The Mathworks, Natick, MA, USA) script. Movement was checked, and measurements with a higher than one voxel movement were rejected. For statistical evaluation t-maps and region of interest (ROI) analysis were also performed according to the Rat Brain Atlas of Paxinos and Watson. The t-test was corrected by the Benjamini and Hochberg false discovery rate (FDR) method. Statistical significance of drug effects was calculated using parametric one-way ANOVA and post-hoc Fisher test (Statistica 8.0, StatSoft, USA, Tulsa). Random effect analysis was not considered necessary because the animals formed a homogeneous group.

Water labyrinth test

In the water labyrinth task, male Wistar rats (n = 10 per group) had to maneuver through three choice points of a labyrinth system in order to reach a platform that allowed them to escape from the water. The rats were habituated to the test environment, the platform and the labyrinth. The number of directional turning errors committed at all the three choice points was measured as a variable reflecting learning performance.

Thirty minutes before the start of the first daily trial, 3 mg/kg (2 ml/kg) i.p. scopolamine hydrobromide or buscopan (Sigma-Aldrich) was injected as an amnestic agent. Procognitive agents (Sigma-Aldrich; except vinpocetine and RG2260) were administered p.o. in 5 ml/kg volume in distilled water (piracetam), in Tween 80 containing vehiculum (donepezil hydrochloride, EVP-6124, neostigmine bromide, and RG2260) or polyethylene glycol (PEG) 400 (PNU-120596) according to their solubility. For p.o. vinpocetine administration ascorbic acid solution was used, as the ideal vehiculum based on several previous animal studies. The test drugs or their vehiculum were administered orally (5 ml/kg) 1 hour before the first swimming except for donepezil and EVP-6124, which were given just before the amnestic agent treatment. The reversible cholinesterase inhibitor neostigmine and the newly developed vinpocetine analog RG2260 were administered intraperitoneally, 30 min prior to the first daily swim, likely donepezil and EVP-6124. Each study included a solvent control group, a memory impaired group (induced by 3 mg/kg dose of scopolamine or buscopan), and one or two impaired groups treated with the test drug. The exact administration protocol can be read in Table 2.

Table 2 **The administration protocol of cognitive enhancers during behavioral pharmacological measurement**

	Solvent	Admin.	Dose (mg/kg)
Donepezil	5 % Tween 80	p.o.	0.25; 0.5; 1; 4;
Piracetam	Distilled water	p.o.	500; 750; 1000
Vinpocetine	Ascorbic acid	p.o.	5; 7.5; 10
PNU-120596	PEG 400	p.o.	3; 10
EVP-6124	5 % Tween 80	p.o.	0.3; 1; 3
Neostigmine	5 % Tween 80	i.p.	0.3
RG2260	5 % Tween 80	i.p.	0.625; 1.25; 2.5; 5; 10; 20

Statistical comparisons between parameters of each group were carried out by three-way repeated measures ANOVA (Statistica 8.0, StatSoft, USA, Tulsa) using 'groups' as the independent between groups factor and 'days' and 'trials' as the repeated measures factors. Post-hoc comparisons (Duncan-test) were performed in the case of a significant between-groups effect or group interaction. The percent reversal by the compounds of the amnesia was calculated from the group-means of pooled errors for all the trials in the training days using the formula:

$$\% = \frac{N_{err} \text{ of amnestic-} N_{err} \text{ of compound}}{N_{err} \text{ of amnestic-} N_{err} \text{ of control}} x100$$

where N_{err} means the number of errors.

Results

fMRI measurement

In the first part of the study, the effect of **scopolamine** was tested on the BOLD response. It significantly (n=85, $p<0.001$) decreased the BOLD signal in the prefrontal cortex at 1 mg/kg dose but it had no visible effect in other brain regions (Figure 1). The effect of scopolamine was not dose-dependent in the dose range 1-5 mg/kg.
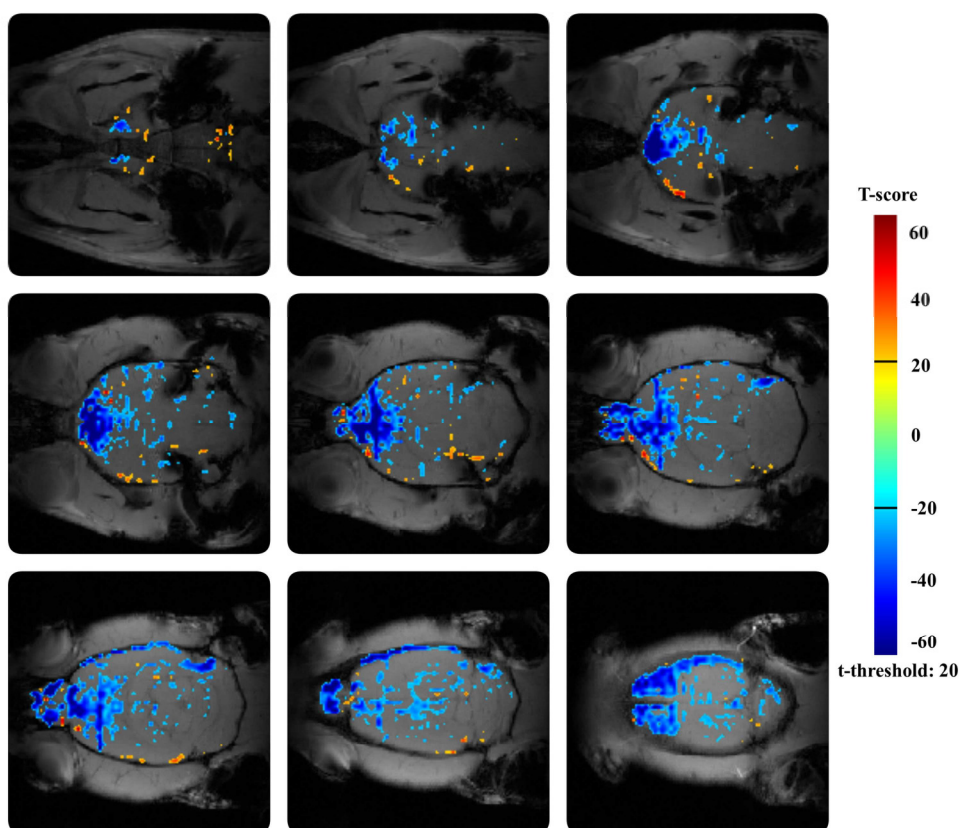
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Figure 1   Effect of scopolamine on BOLD response in the rat brain after vehiculum pretreatment.

We tested the butyl analog of scopolamine, **buscopan** as well. It contains quaternary nitrogen, so it cannot enter into the central nervous system (CNS). Surprisingly, buscopan at 1 mg/kg intravenous dose caused a marked (n=7, $p<0.05$) negative BOLD response in the prefrontal cortex (PFC) while other brain areas remained essentially unchanged. The effect of butylscopolamine was highly similar to that of scopolamine; it showed an identical inhibitory pattern and BOLD signal reduction at 1 mg/kg dose.

In the second part of the study the effects of procognitive compounds – administered alone – were tested.

Donepezil had no visible effect at 1 mg/kg i.p. dose. At 4 mg/kg dose, a non-significant weak changes were noticed 10-15 minutes after drug administration in the cortex and hippocampus. At 10 mg/kg, it caused some short and weak increase of BOLD response in most brain areas followed by a long descent. In the PFC and septum, the initial increase of BOLD response was absent.

The direct administration of **piracetam** caused a dose-dependent, fast and short BOLD response increase in the most studied brain areas. **Neostigmine** (0.1 mg/kg dose), similar to the low dose of donepezil, did not evoke any significant changes in the BOLD response when administered alone.

Vinpocetine, **EVP-6124**, **PNU-120596**, and **RG2260** could not be tested directly in aqueous solution because of their poor solubility.

In scopolamine-based fMRI provocation model, the reversibility of the memory disturbing effect of scopolamine was tested by procognitive compounds. The effect of donepezil, piracetam, and neostigmine were compared to that of saline solution used as solvent. Vinpocetine, EVP-6124, PNU-120596, and RG2260 were dissolved in 5% Tween 80 containing solution; therefore, their effects were compared to that of 5% Tween 80 containing saline. The Tween 80 agent was not inert in this model; it increased significantly the effect of the amnestic agent (ANOVA, post-hoc Fisher test, $p<0.05$), which points out the importance of adequate control in this very sensitive approach.

Piracetam was not effective at doses of 100 (n=5) and 500 mg/kg (n=6) against scopolamine-induced BOLD response decrease, but at 1000 mg/kg dose (n=5) a clear trend was seen. It enhanced the detected BOLD response but this reversal was too small to distinguish from the noise (Figure 2).

The effect of **donepezil** at 1 mg/kg dose (n=6) was not different from that of the control but at higher dose (4 mg/kg, n=11, $p<0.01$) significantly reduced the inhibitory effect of scopolamine. Donepezil at 10 mg/kg dose (n=3) provoked several side effects, such as tremor and seizures, therefore, the experiment was discontinued after testing three animals (Figure 2).
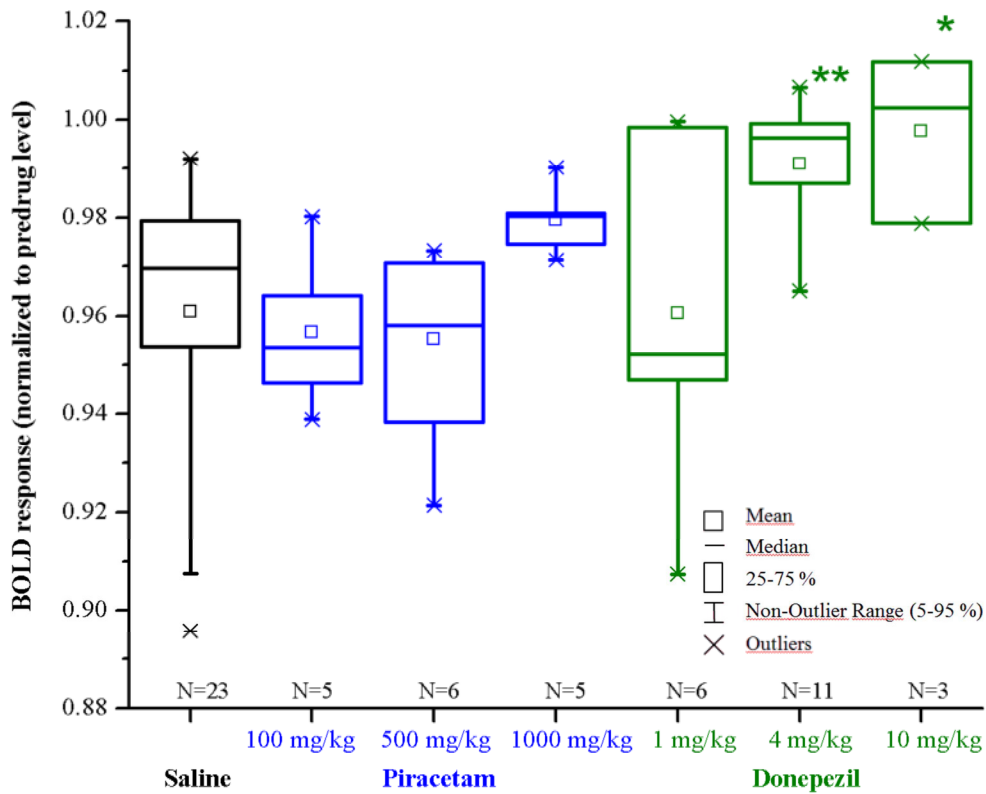
Figure 2 **Effects of piracetam and donepezil pretreatment on scopolamine evoked BOLD response changes in the prefrontal cortex.** Piracetam was administered at 100, 500 and 1000 mg/kg intraperitoneally. Donepezil was injected at 1, 4, and 10 mg/kg intraperitoneally. Scopolamine was tested at 1 mg/kg intravenously. (ANOVA, Fisher post-hoc test; *$p<0.05$; **$p<0.01$)

Vinpocetine (5 mg/kg, n=5, $p<0.05$), **EVP-6124** (1 mg/kg, n=10, $p<0.05$) and **PNU-120596** (3 mg/kg, n=6, $p<0.01$) significantly inhibited the effect of scopolamine in the indicated doses (Figure 3).
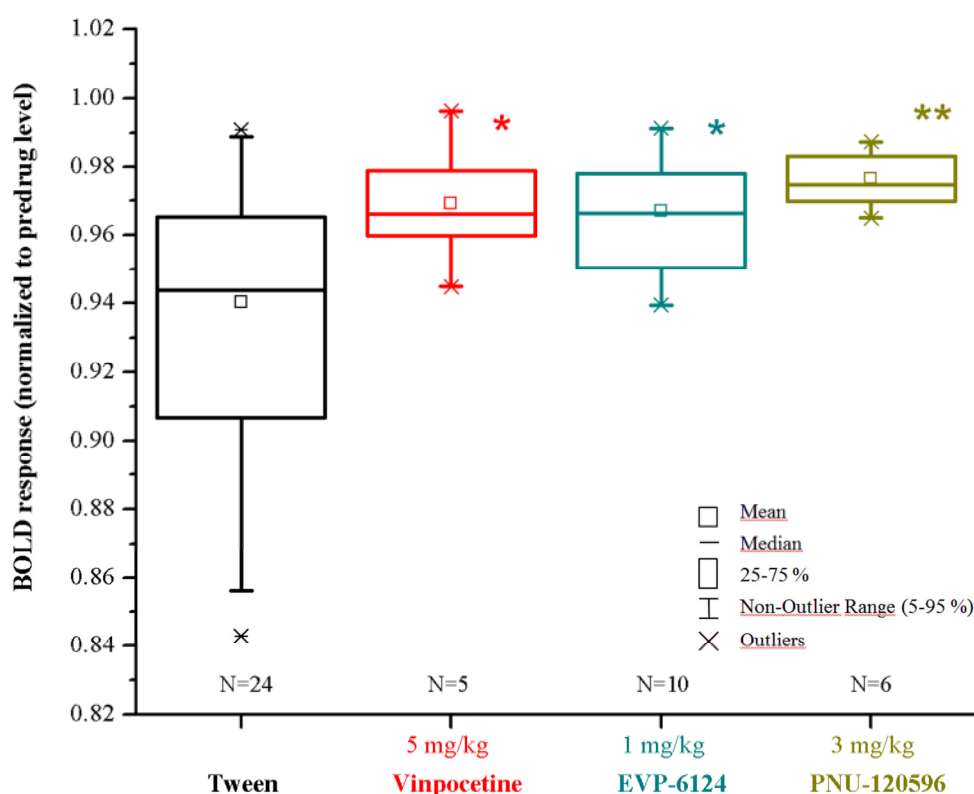
Figure 3 **Effects of vinpocetine, EVP-6124, and PNU-120596 pretreatment on scopolamine evoked BOLD response changes in prefrontal cortex.** Vinpocetine was administered at 5 mg/kg intraperitoneally. EVP-6124 was injected at 1 mg/kg intraperitoneally. PNU-120596 was tested at 3 mg/kg intraperitoneally. Scopolamine was injected at 1 mg/kg intravenously. (ANOVA, Fisher post-hoc test; *p<0.05; **p<0.01)

The newly developed **RG2260** agent was effective in BOLD fMRI study against scopolamine-induced BOLD response decline in the prefrontal cortex in rats. It was formulated and administered in Tween 80 suspension. A dose response curve was generated by RG2260 to determine the minimal effective dose (MED). Stable drug effect was seen at 0.1 mg/kg dose (n=6) but statistically significant difference was only seen at 1.25 (n=6, *p*<0.05) and 5 mg/kg (n=5, *p*<0.01) dose. After one hour pretreatment RG2260 at 10 mg/kg i.p. was able to prevent the inhibitory effect of scopolamine in rat PFC and showed marginally significant result (n=6, p=0.053). The efficacy of the newly reported compound (RG2260) seems to be surprisingly similar to that of the reference compound vinpocetine. The main difference between these two nootropics is their effective doses. Vinpocetine shows significant (n=5, *p*<0.05) inhibitory effect against

scopolamine-induced BOLD response at 5 mg/kg dose while RG2260 is effective (n=6, $p<0.05$) in a much lower dose range (Figure 4).
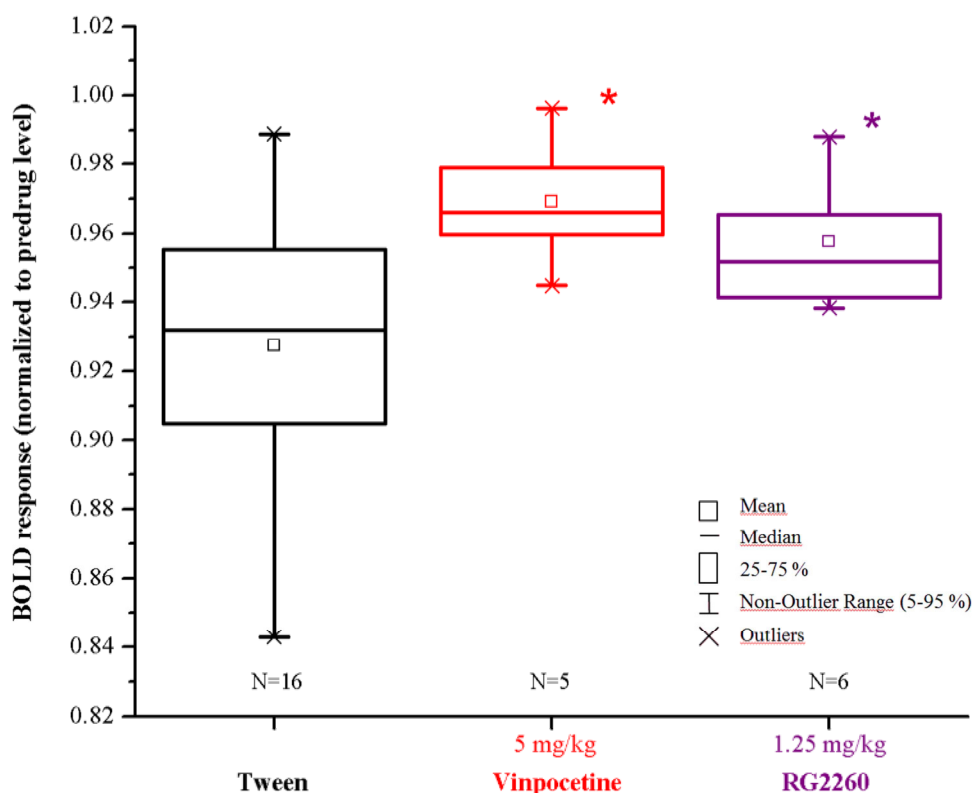

Figure 4   Effects of vinpocetine and RG2260 pretreatment on scopolamine evoked BOLD response changes in the prefrontal cortex. The efficacy of the new nootropic RG2260 compound was similar to that of vinpocetine, but it was effective in a lower dose range. Vinpocetine was administered at 5 mg/kg intraperitoneally. RG2260 was injected at 1.25 mg/kg intraperitoneally. Scopolamine was administered at 1 mg/kg intravenously. (ANOVA, Fisher post-hoc test; *p<0.05)

The peripherally acting cholinesterase inhibitor **neostigmine** was tested in scopolamine and in buscopan model as well. Pretreatment with 0.1 mg/kg intraperitoneal dose had statistically significant (n=5, $p<0.05$) effect on the BOLD response evoked by scopolamine in the PFC. It caused similar, somewhat lower BOLD signal changes in comparison to donepezil at 4 mg/kg dose (n=6). Neostigmine at 0.1 mg/kg dose was able to fully reverse (n=7, $p<0.001$) the negative BOLD effect of buscopan, like donepezil at 4 mg/kg dose (n=4, $p<0.001$). With both provoking agents statistically significant BOLD signal increases were detected when counteracted with either neostigmine or donepezil. Interestingly, with scopolamine provocation significant

(*p*<0.05) difference was found between the effect of neostigmine and donepezil (Figure 5).
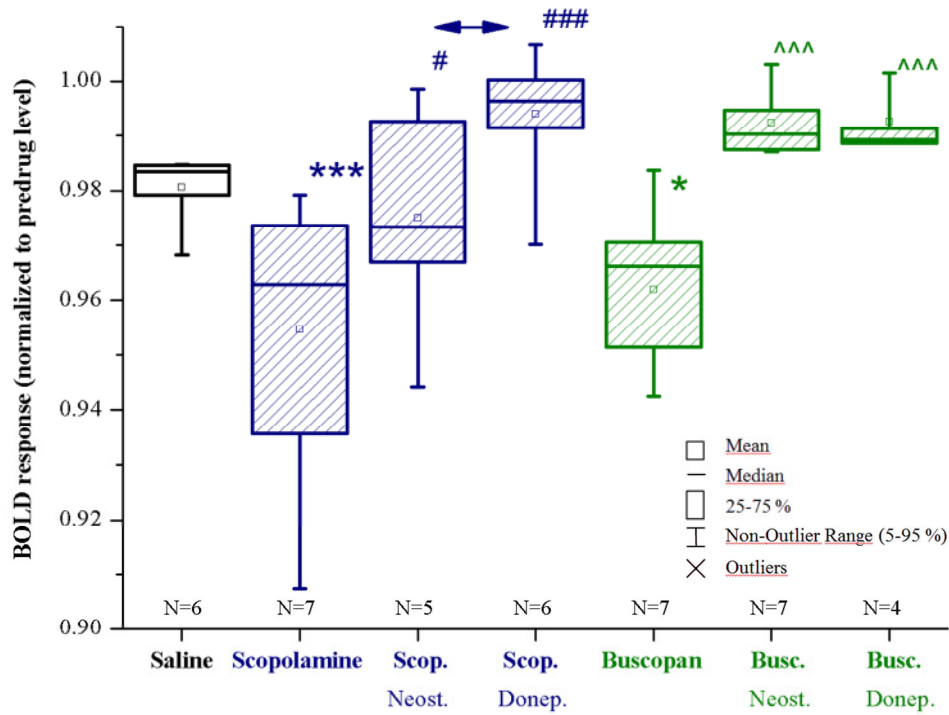

Figure 5   Effects of saline, scopolamine and butylscopolamine (buscopan) on BOLD response in the rat brain after saline pretreatment, and prevention of the effect of scopolamine and butylscopolamine by neostigmine or donepezil pretreatment. Scopolamine (Scop) was administered at 1 mg/kg intravenously. Butylscopolamine (Busc) was tested at 1 mg/kg intravenously. Neostigmine (Neost) was injected at 0.1 mg/kg intraperitoneally. Donepezil (Donep) was administered at 4 mg/kg intraperitoneally. *p<0.05, ***p<0.005 from saline; #p<0.05, ###p<0.005 from scopolamine; ^^^p<0.005 from the butylscopolamine. The arrow means the significant difference between the reversal effect of neostigmine and donepezil against scopolamine p<0.05 (ANOVA, Fisher post-hoc test).

Water labyrinth test

Scopolamine (3 mg/kg i.p.) had significant inhibitory effect on the learning performance of the animals. It disrupted the normal learning process of rats, resulting in a significant increase in the number of errors (Figure 6).

Piracetam at high doses (750 and 1000 mg/kg, *p*<0.01) significantly inhibited the amnesic effect of scopolamine while the 500 mg/kg dose was inactive (Figure 6).
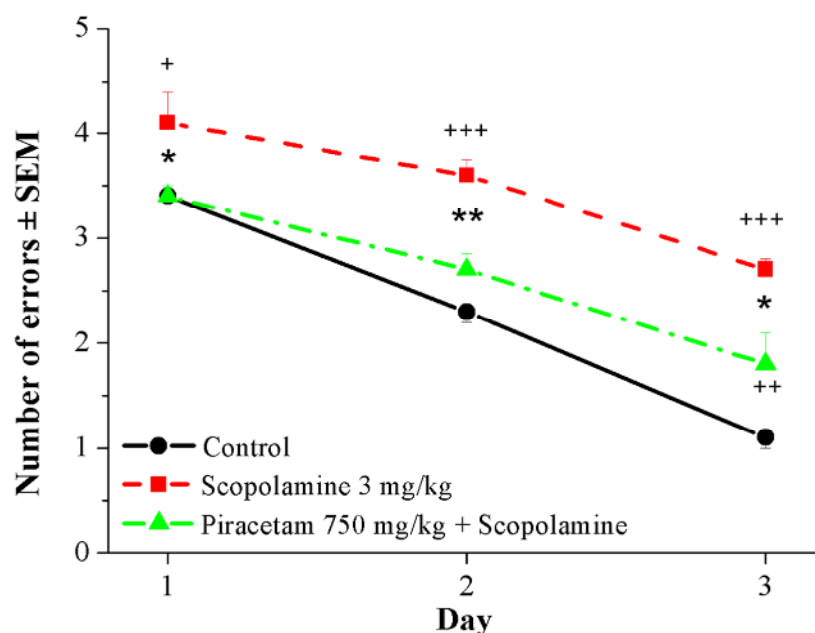
Figure 6   Effects of piracetam on the learning deficit induced by scopolamine in water labyrinth test. Piracetam was adminstered at 750 mg/kg per os. Scopolamine was injected at 3 mg/kg intraperitoneally. $+p<0.05$, $++p<0.01$, $+++p<0.001$ vs. control group; $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. scopolamine-treated group (ANOVA, post-hoc Duncan test)

Vinpocetine proved to be effective at doses of 7.5 ($p<0.05$) and 10 mg/kg ($p<0.001$) in water labyrinth test.

The inhibitors of acetylcholinesterase appeared to be effective in a much lower dose range than other tested agents. **Donepezil** in a dose range of 0.25-4 mg/kg significantly ($p<0.01$) and dose-dependently improved the learning deficit induced by scopolamine. The lowest tested dose (0.25 mg/kg) approximated the minimal effective dose of the compound and caused 50 % reversal. At 0.5, 1, and 4 mg/kg dose produced 63.5 %, 82 %, 71.2 % reversal respectively.

Neostigmine at 0.3 mg/kg dose also caused a highly significant ($p<0.01$) and considerable (76.8 %) reversal.

The nicotinic alpha 7 receptor orthosteric agonist **EVP-6124** compound at 0.3, 1, and 3 mg/kg dose significantly ($p<0.05$) alleviated the scopolamine-induced learning deficit. While **PNU-120596**, an alpha 7 nicotinic positive allosteric modulator (PAM) showed moderate, non-significant improving effect.

The newly developed **RG2260** was effective in the water labyrinth test against scopolamine-induced memory impairment in rats. This compound restored the impaired cognitive performance in the dose range of 0.625-20 mg/kg i.p. with a U-shaped dose-response pattern. The dose of 0.625 mg/kg was inactive (0.00 % reversal); at 1.25 mg/kg dose significant improving effect was registered ($p<0.05$; 44.2 % reversal); the maximal effect ($p<0.001$; 100 % reversal) was obtained at 5 mg/kg dose.

Butylscopolamine showed a dose-dependent effect; it caused significant ($p<0.05$) impairment in the learning performance at 3 mg/kg dose, which was reversible by 0.3 mg/kg of neostigmine ($p<0.05$).

Conclusions

During my Ph.D work I successfully developed a small animal scopolamine-based provocation phMRI model in a team work with my collegues, which model is characterized by high translational power and excellent spatial and temporal resolution. The developed model was tested with several cognitive enhancers (donepezil, piracetam, vinpocetine, neostigmine, EVP-6124, PNU-120596, and RG2260).

- First, we set up a small animal scopolamine-based provocation MRI system. The most effective and successfull protocol was when the administration of procognitive agents happened i.p. one hour before the injection of amnestic agent. In this case, we could exclude the noising factor of injection procedure and the significant pharmacodynamic effect of the applied detergent (Tween 80) from the system. The optimal length of the pre-injection baseline was found 1000 sec. After this period, the amnestic agent was administered in a saline-based solution via i.v. injection to achieve immediately forming effect. The optimal length of a complete phMRI experiment was 3000 sec.

- We tested the amnestic agent scopolamine in various doses. It significantly reduced the detected BOLD signal intensity at 1 mg/kg dose in the area of PFC, while other brain areas remained intact. The effect of scopolamine was not dose-dependent in the dose range 1-5 mg/kg. Maximal pharmacological effect could be seen at 1 mg/kg dose already.

- In the second part of the study, we tested commercially available procognitive agents. Piracetam, donepezil and the RG compound vinpocetine produced

increasing BOLD signal in the PFC of scopolamine-treated animals as we previously expected. Significant BOLD signal changes were detected only at 4 and 10 mg/kg doses of donepezil and at 5 mg/kg of vinpocetine.

- The next tested alpha 7 selective nicotinerg agents (EVP-6124 and PNU-120596) were effective in our phMRI model. Both agents significantly increased the registered BOLD signal in cognition-related brain areas as we supposed.

- We tested the newly developed Richter compound, RG2260 in our model system. After the dose-response curve registration in a dose range of 0.01-10 mg/kg, we determined the minimal effective dose of RG2260 at 1.25 mg/kg. Significant BOLD response increase was detected only at 1.25 and 5 mg/kg doses in PFC.

- The comparative measurement of vinpocetine at 5 mg/kg and RG2260 at 1.25 mg/kg dose showed similar results. The detected BOLD signal changes were almost the same in both cases but the effective dose of RG2260 agent could be detected in a much lower dose range compare to the MED of vinpocetine.

- Assuming the presence of vascular effects in cognitive decline, it was important to test the butyl analogue of scopolamine, buscopan in our phMRI model as well. Surprisingly, it caused a similar marked negative BOLD effect at 1 mg/kg dose in the PFC as scopolamine while other brain areas remained intact. Its central effect was totally unexpected based on the fact that buscopan cannot penetrate through the BBB.

- Based on the presence of vascular effects in cognitive decline, we presumed similar mechanisms in the background of cognitive improvement. In this case, the peripherially acting testing agent was neostigmine. It could fully reverse at 0.1 mg/kg dose the scopolamine and the buscopan-evoked negative BOLD signal changes in the PFC. Based on these results, we successfully proved the presence and the central role of vascular effects in cognition-related processes.

- Finally, we compared the results of our phMRI studies to the outcomes of behavioral pharmacological tests to prove the relevance of our phMRI results. In water labyrinth tests the amnestic agents (scopolamine and buscopan) singificantly increased the number of errors by rats, while all of the tested procognitive agents showed reduced number of directional turning errors in this scopolamine-based provocation model.

- Consequently, water labyrinth tests successfully proved the relevance of our phMRI results. The outcomes of the two above-mentioned methods were in good correspondence with each other.

Publications related to the thesis

1. **Hegedűs N**, Laszy J, Gyertyán I, Kocsis P, Gajári D, Dávid S, Deli L, Pozsgay Z, Tihanyi K. (2015) Scopolamine provocation-based pharmacological MRI model for testing procognitive agents. J Psychopharmacol, 29: 447-455.

2. Kocsis P, Gyertyán I, Éles J, Laszy J, **Hegedűs N**, Gajári D, Deli L, Pozsgay Z, Dávid S, Tihanyi K. (2014) Vascular action as the primary mechanism of cognitive effects of cholinergic, CNS-acting drugs, a rat phMRI BOLD study. J Cereb Blood Flow Metab, 34: 995-1000.

Other publication

1. Sárvári M, Kocsis P, Deli L, Gajári D, Dávid S, Pozsgay Z, **Hegedűs N**, Tihanyi K, Liposits Z. (2014) Ghrelin Modulates the fMRI BOLD Response of Homeostatic and Hedonic Brain Centers Regulating Energy Balance in the Rat. PLoS ONE, 9: e97651.

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