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# Scopolamine-based pharmacological MRI model for testing procognitive agents

**Ph.D. thesis**


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# LIST OF ABBREVIATIONS

| | |
|---|---|
| Aβ | amyloid beta |
| ABCA7 | ATP-binding cassette transporter A |
| Ach | acetylcholine |
| AchEi | acetylcholinesterase inhibitor |
| AchR | acetylcholine receptor |
| AD | Alzheimer’s disease |
| ADRDA | Alzheimer's Disease and Related Disorders Association |
| ANOVA | Analysis of variance |
| APP | amyloid precursor protein |
| ATP | adenosine triphosphate |
| BACE | beta-side amyloid precursor protein cleaving enzyme |
| BBB | blood-brain barrier |
| BIN1 | bridging integrator 1 |
| BOLD | blood-oxygenation-level dependent |
| CAA | cerebral amyloid angiopathy |
| cAMP | cyclic adenosine monophosphate |
| cAVA | cis-apovincaminic acid |
| CBF | cerebral blood flow |
| CBV | cerebral blood volume |
| CD2AP | CD2-associated protein |
| CD33 | CD33 molecule |
| ChAT | choline acetyltransferase |
| CMRO | cerebral metabolic rate of oxygen |
| CNS | central nervous system |
| CLU | clusterin |
| CR1 | complement component (3b/4b) receptor 1 |
| CSF | cerebrospinal fluid |
| CT | computedtomography |
| CTF | C-terminal fragment |
| DMN | default mode network |

| | |
|---|---|
| DTPA | diethylene triamine pentaacetic acid |
| EPHA1 | EPH receptor A1 |
| EPI | echo planar imaging |
| fMRI | functional magnetic resonance imaging |
| FOV | field of view |
| GABA | gamma-aminobutyric acid |
| GAG | glycosaminoglycan |
| GEMS | gradient echo multi slice sequence |
| GM | gray matter |
| GSK3β | glycogen synthase kinase 3β |
| GWAS | genom-wide association study |
| HDR | hemodynamic response |
| i.v. | intravenous |
| i.p. | intraperitoneal |
| LRP1 | lipoprotein receptor related protein 1 |
| M | net magnetization |
| mAchR | muscarinic acetylcholine receptor |
| MCI | mild cognitive impairment |
| MED | minimal effective dose |
| mGluR2 | metabotropic glutamate receptor 2 |
| mPFC | medial prefrontal cortex |
| MRI | magnetic resonance imaging |
| MRS | magnetic resonance spectroscopy |
| MS4A6A | membrane-spanning 4-domains, subfamily A |
| MTC | methylthioninium chloride |
| nAchR | nicotinic acetylcholine receptor |
| nbM | nucleus basalis of Meynert |
| NIFTI | neuroimaging informatics technology initiative |
| NINCDS | National Institute of Neurological and Communicative Disorders and Stroke |
| NMDA | N-methyl-D-aspartate |
| NFT | neurofibrillary tangle |

| | |
|---|---|
| NSAID | nonsteroidal anti-inflammatory drug |
| PAM | positive allosteric modulator |
| PCP | phencyclidine |
| PD | Parkinson’s disease |
| PEG | polyethylene glycol |
| PET | positron emitting tomography |
| PFC | prefrontal cortex |
| phMRI | pharmacological magnetic resonance imaging |
| PICALM | phosphatidylinositol-binding clathrin assembly protein |
| p.o. | per os |
| PSEN1 | presenilin-1 |
| PSEN2 | presenilin-2 |
| RF | radiofrequency pulse |
| ROI | region-of-interest |
| SALA | selective Aβ42 lowering agent |
| sAPP | soluble amyloid precursor protein |
| SIPP | superparamagnetic iron-platinum particle |
| SORL1 | sortilin-related receptor-1 |
| SP | senile plaque |
| SPIO | superparamagnetic iron oxide |
| TE | echo time |
| TR | repetition time |
| USPIO | ultrasmall superparamagnetic iron oxide |
| VSMC | vascular smooth muscle cell |
| WM | white matter |

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# I INTRODUCTION

The brain is the most energy-demanding organ in the body. The high energy requirement is met exclusively by the oxidative breakdown of glucose. The biological oxidation of carbon and hydrogen in glucose demands a great amount of molecular oxygen. Some 20-25 % of the body-wide consumed oxygen is used by the brain. Glucose is not stored in the brain tissue (apart from approximately 10 min supply in astrocytes) and no storage capacity exists for oxygen at all. The function of the brain depends on the availability of glucose and oxygen and therefore, it is greatly susceptible to minor changes in blood supply, which is the carrier of nutrients. An intact cerebral circulation is a precondition for satisfactory glucose and oxygen supply (Wang et al., 2013).

Whenever the presence of these resources is below optimal, the most delicate and most complex functions will be harmed first. Therefore, the deficiency of energy supply is inevitably followed by the deficiency of cognition. Nowadays, it is commonly recognized that ischemic deprivation (oxygen and glucose restriction) exerts detrimental effects on the cognitive function. A small fluctuation in cerebral oxygen delivery also has an impact on cognitive performance. With age, the cortical blood supply is reduced by up to 30 %. Further reduction of regional blood flow was demonstrated in patients with memory impairment. Wang et al. (2013) concluded from a multicenter study that decreases in cerebral blood flow is the first step towards Alzheimer's disease.

As a consequence of insufficient ATP production, neurons depolarize which render membrane ion-transports inefficient. The increased calcium influx leads to the release of various neurotransmitters among others glutamate. The high calcium concentration activates destructive enzymes causing cellular degradation. In this way the cognitive deficit is not the only sign of inefficient energy production: destructive pathways will be soon activated as a consequence of energy restriction.

This vicious circle may be prevented by the maintenance of good cerebral blood circulation. Most importantly, improving blood circulation, especially in regions associated with cognitive processes, may reverse the cognitive impairment caused by insufficient cellular energy production. This may happen with or without the direct modulation of neurotransmitters or their receptors. The restoration of blood supply will restore cognitive functions and also prevents secondary damage (Palmer and Love, 2011).

## I.1 Cognition and Alzheimer's disease (AD)

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 (AD, Parkinson's disease – PD) and psychiatric disorders (schizophrenia, depression) are frequently accompanied by cognitive deficits (Heidbreder and Groenewegen, 2003).

### I.1.1 Cognitive disorders

Cognitive disorders are classified by DSM-V as delirium, dementia, amnestic disorder, and other cognitive disorders. Delirium is a disturbance of consciousness and a change in cognitive function that develops over a short period of time. Dementia is characterized by long lasting multiple cognitive deficits with memory impairment. The amnestic disorder is characterized by memory impairment in the absence of other significant accompanying cognitive impairments (DSM-V, 2013). While all of the above-mentioned disorders could be due to various etiologies, it is generally assumed that more or less common reasons, namely degenerative processes and neuronal dysfunctions are in the background of these diseases. Despite the intense research and development of memory enhancer drugs, no robustly effective agents have reached the market. Unfortunately, the prevalence of dementia is continuously rising in parallel with the increasing life span.

### I.1.2 Alzheimer's disease

The most common cause of dementia is Alzheimer's disease or AD. AD is an irreversible, progressive neurodegenerative disease that adversely affects cortical functions: memory, thinking, and orientation. An estimated 44 million people worldwide have Alzheimer's disease and other dementias. So economically, AD is a major public health problem (Prince et al., 2014). Postmortem studies reported that

60-90 % of patients with Alzheimer's disease show varying degrees of cerebrovascular pathology at autopsy. Furthermore, it is speculated that vascular lesions contribute to the development of clinical symptoms in patients with AD and may lead to more rapid clinical decline (Kalaria, 2000).

AD includes the interaction of amyloid β (Aβ) aggregation with plaque development and hyperphosphorylation and aggregation of tau protein with tangles. The production of amyloid β is a critical step in AD pathogenesis. Aβ is a ~ 4kDa peptide, which is built up of 36 to 43 amino acids (Armstrong, 2009). It is the final product of cleavage of APP (amyloid precursor protein). The most common variety of Aβ ($A\beta_{1\text{-}42}$) is predominantly found in plaques, whereas a more soluble form ($A\beta_{1\text{-}40}$) is found in association with blood vessels (Armstrong, 1998).

Amyloid β forms insoluble and protease resistant fibrils, called senile plaques (SP). These plaques are lesions in the cerebral cortex. SPs have various types: diffuse (pre-amyloid), neuritic (primitive), classic (dense-cored), and compact-type (burned-out) plaques (Armstrong, 2009). These amyloid plaques differ from each other in the level of amyloid aggregation but there is no convincing evidence to support their transformation procedure to each other (Armstrong, 1998).

Neurofibrillary tangles (NFTs) consist of tau protein. In normal healthy subjects, tau is an essential component of the microtubule system, which plays a crucial role in intracellular transport. In patients with AD, tau protein is abnormally hyperphosphorylated and forms insoluble fibrils within the cell. These changes with additional inflammation and oxidative stress, such as a pathological cascade initiate the loss of synaptic integrity and progressive neurodegeneration (Galimberti and Scarpini, 2011).

Until now, the only way to make a precise diagnosis of AD was an autopsy or brain biopsy. In clinical practice, the diagnosis was usually based on the family history and results of on Mental Status Examination (e.g. NINCDS-ADRDA). The time from diagnosis to death varies from as little as 3 years to as long as 10 or more years. Patients with early-onset AD tend to have a more aggressive, rapid course than those with late-onset AD. Nowadays, there are other non-invasive, modern imaging techniques to detect AD. It is possible to diagnose cerebrovascular diseases with computed tomography (CT), positron emitting tomography (PET), or magnetic resonance imaging (MRI) (DSM-V, 2013).

## I.1.3 The pathophysiology and disease-modifying drugs of AD

The exact pathomechanism of Alzheimer's disease is still not understood. Several theories have been proposed about the triggers or origin of the disease.

### I.1.3.1 Amyloid hypothesis

In the last 20-25 years, „amyloid hypothesis“ was the leading scientific explanation of Alzheimer's disease. This hypothesis proposed that the abnormally high level of accumulated amyloid β was the main cause of AD. The APP, as a precursor molecule of Aβ, plays a crucial role in AD pathogenesis, which was the heart of the amyloid hypothesis. Amyloid precursor protein is a type I membrane protein. Two enzymatic cleavages (β-secretase cleavage in the extracellular domain; γ-secretase cleavage in the transmembrane region) are needed to release the neurotoxic Aβ peptide. Indeed, the transformation of APP to Aβ is a sequential proteolytic process: at first, the α-secretase (non-amyloidogenic) or β-secretase (amyloidogenic) cleaves the APP and generates the α- or β- C-terminal fragments (CTFs) of Aβ. The β-secretase, named BACE1 (β-site APP cleaving enzyme) cleaves the APP within the ectodomain and generates the N-terminus of Aβ. After that, the γ-secretase cleaves the α- or β-CTFs and releases the neurotoxic Aβ peptide into the extracellular space (Vassar, 2004).

Lots of studies and clinical trials with newly developed anti-AD agents tried to influence the progression of the disease assuming the above-mentioned pathomechanism of AD.

#### *I.1.3.1.1 Anti-amyloid β agents*

The glycosaminoglycan (GAG) mimetic agent, tramiprosate (Alzhemed$^{TM}$, Neurochem Inc.) was able to decrease the Aβ level in human CSF by up to 70 % but did not show any significant effect on cognitive functions (Gervais et al., 2001; Aisen et al., 2006). The other tested agent, colostrinin (O-CLN; ReGen Therapeutics) could prevent amyloid β aggregation and neurotoxicity in animal models but these positive effects were only temporary during human trials (Leszek et al., 1999; Bilikiewicz and Gaus, 2004).

#### *I.1.3.1.2 Vaccination*

Active immunization with Aβ showed some protective effects against AD in young transgenic mice, e.g. reduced Aβ aggregation and plaque formation, neuritic dystrophy and astrogliosis. In older animals, the antigen therapy was able to reduce the progression of the disease. Unfortunately, this active immunization therapy was not successful in human clinical trials. Some of the treated patients (6 %) had developed meningoencephalitis. In addition, the cognitive functions of the placebo group and antibody responders did not differ significantly (Gilman et al., 2005). To avoid complications due to the excess T-cell activation, passive immunization seemed to be a better therapy. However, the humanized monoclonal anti-Aβ antibodies – bapineuzumab, solanezumab – have not demonstrated the expected results (Grundman and Black, 2008).

#### *I.1.3.1.3 Selective Aβ42-lowering agent (SALA)*

Tarenflurbil (MPC-7869; Myriad Pharmaceuticals; Flurizan™) is a pure R-enantiomer of the NSAID flurbiprofen. It can change the conformation of γ-secretase and make the final cleaved Aβ peptide shorter, more water soluble, and less toxic (Beher et al., 2004). Despite the promising results of preclinical studies (Kukar et al., 2007), tarenflurbil had failed in human phase III trial, so its development was stopped (Green et al., 2009).

#### *I.1.3.1.4 β- and γ-secretase inhibitors*

β- and γ-secretase play important roles in the formation of toxic Aβ plaques, thus their inhibition seemed to be an ideal therapeutic target in AD. These enzymes are responsible for the sequential cleavages of APP. Sadly, in human clinical trials, neither the BACE inhibitor nor the γ-secretase inhibitor was able to show any statistically significant difference in Aβ level or in cognitive functions (active treatment group vs. placebo group) (Siemers et al., 2005; Siemers et al., 2006; Fleisher et al., 2008).

#### *I.1.3.1.5 α-secretase potentiator*

The α-secretase enzyme is the most important proteinase of the nonamyloidogenic pathway, which transforms the amyloid precursor protein to its soluble form (sAPPα). Etazolate (EHT 0202; ExonHit Therapeutics) can stimulate α-secretase, protect cells from toxic Aβ plaques in the CNS, and prevent the neuronal death in rats (Marcade et al., 2008).

Altogether, the amyloid hypothesis has failed in several unsuccessful clinical trials. Nowadays, more and more authors have questioned this causality and suppose that Aβ accumulation in the brain is not the cause of AD but a consequence of it (Drachman, 2014).

### I.1.3.2 Tau hypothesis

In regard to the above-mentioned pathophysiological changes in AD, the inhibition of tau hyperphosphorylation or oxidative processes seemed another good therapeutic choice.

Methylthioninium chloride (MTC; TauRx Therapeutics; Rember ™) or methylene blue, is a well-known indicator molecule in analytical chemistry. In human phase II trials, MTC was able to block the phosphorylation process of tau protein but further studies are required to gain convincing results (Wischik et al., 1996). Lithium, sodium valproate, pyrazolopyrazines, and pyrazolopyridines can inhibit some protein kinases (presumably glycogen synthase kinase 3 or GSK3β) which are responsible for tau hyperphosphorylation. These agents are still in development (Balaraman et al., 2006; Martinez and Perez, 2008; Schneider and Mandelkow, 2008).

### I.1.3.3 Neurotransmitter-based model

This model presumes some kind of injury in the neurotransmitter system (mostly in cholinergic and glutamatergic system). Based on this model, currently, the only available standard medical treatment in AD is the symptomatic therapy. It includes cholinesterase inhibitors (AchEi) – e.g. donepezil, galantamine, rivastigmine, tacrine –, a partial N-methyl-D-aspartate (NMDA) antagonist – memantine – or the combination of these drugs. Acetylcholinesterase inhibitors were the first anti-Alzheimer drugs, which could increase the acetylcholine levels. From these, tacrine – the first drug approved for AD – is rarely used nowadays due to its hepatotoxicity; donepezil, galantamine, and rivastigmine are approved for mild-to-moderate AD. Donepezil is the most often prescribed anti-AD agent by patients with severe AD. In severe cases (for moderate-to-severe AD), memantine seems to be a good additional therapeutic option. This is believed to protect neurons from the excitotoxic activity. But unfortunately, all of these therapies are only marginally effective and preserve cognitive function temporarily. Psychotropic medications are often used as adjuvants to treat secondary symptoms of AD, such as depression, agitation, and sleep disorders (Seltzer, 2005).

#### I.1.3.4 Genetic-based model

The genetic-based model assumes the presence of some alleles or genes in the human genome that can predict the onset of AD. Most people suffering from AD start showing symptoms after age 65 or later. But in rare cases, the disease develops much earlier, between ages of 30 and 60. This early-onset form of AD is caused by one of three genes mutations inherited from parents which cause aberrant protein formation. Mutation of chromosome 21 causes abnormal APP formation, alteration of chromosome 14 induces abnormal PSEN1 (presenilin 1), while changes in chomosome 1 leads to abnormal PSEN2 (presenilin 2) formation (Van Cauwenberghe et al., 2015).

The more common late-onset form of AD usually develops after age 65. Its hereditary component is mounted because no single gene mutation is known to cause this late-onset form of the disease. The expression of the APOEε4 allele in an individual’s genome unambiguously classifies the risk of AD development into three groups. Individuals who are APOEε4 homozygotes have a very high risk to develop the disease while APOEε4 heterozygotes have a moderate chance to it. People who do not carry this gene have very low risk to suffer from this neurodegenerative disease (Hampel et al., 2010).

Researchers are hardly working to determine further genes that may influence the risk of late-onset AD. Until this time, numerous genes (PICALM, CLU, CR1, BIN1, MS4A6A locus, CD2AP, SORL1, CD33, EPHA1 and ABC7) have been identified by

GWAS (genom-wide association study) that may increase the people's risks for AD. These genes are clustered within three main groups depending on their roles in cholesterol and lipid metabolism, immune system and inflammatory response, and endosomal vesicle cycling (Van Cauwenberghe et al., 2015).

#### I.1.3.5 Other environmental and human-related risks

Environmental and other lifestyle factors may also contribute to the developing late-onset AD. Several researches promote that physical activity can slow down or prevent functional decline associated with aging, improves health in older individuals and reduces the risk for developing AD (Hakim et al., 1998; Middleton et al., 2008; Scarmeas et al., 2009). Animal studies have been shown that even a small amount of physical activity decreases cortical amyloid burden in mice, possibly mediated by changes in the processing of amyloid precursor protein (Adlard et al., 2005).

People who regularly do some physical activities are often pay attention to their eating habits. Following mediterranean diet can further decrease the risk for developing AD or mild cognitive impairment (MCI) (Scarmeas et al., 2009).

## I.2 A new approach with high translational power is required in drug development

In spite of the aforementioned scientific exploration, the successful treatment of Alzheimer's disease is not yet developed, so 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 AD 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. As a result, researchers have to face a relative shortage of relevant targets (Armstrong, 2011; Braak and Braak, 1991; Drachman, 2006; Fjell and Walhovd, 2012; Herrup, 2010; Pimplikar, 2009; Pimplikar et al., 2010; Reitz, 2012). These negative clinical trials draw attention to the inefficient translational power of current animal models and call for the improvement of predictive value (Sarter et al., 2009).

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 (Schwarz et al., 2006). The functional MRI (fMRI) blood-oxygenation-level dependent (BOLD) technique is suitable for the complex testing of brain functions and also the circulation (Marota et al., 2000). It is an important non-invasive tool for preclinical drug discovery with excellent temporal and spatial resolution (Leslie and James, 2000).

Rat BOLD pharmacological MRI (phMRI) is gaining popularity in studies aiming at the central effect of various pharmacological agents (Canese et al., 2009; Chen et al., 1997; Sekar et al., 2011; Stark et al., 2006). There are several human phMRI studies as well that verify its translational role in neuroscience (Anderson et al., 2002; Deakin et al., 2008; Stein et al., 1998). The key advantage of MRI over other imaging modalities in neuroscience is its ability to follow changes in brain neurobiology after pharmacological, electrical, or environmental insult (Chang and Shyu, 2001; Reese et al., 2000; Stark et al., 2008). 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 (Ferris et al., 2011).

Unfortunately, the direct examination of drug effects is not always possible by fMRI. Frequently occurring problems are the weak or slowly evolving BOLD effects and the BOLD effect of pharmaceutical formulations that do not contain assumed active ingredients. Some of the studied procognitive agents intended for intravenous (i.v.) application have poor aqueous solubility; they are not soluble in an indifferent vehiculum. Since the BOLD readings coincide with the i.v. treatment of animals, the experimenter has to make sure that changes due to osmotic pressure, high dose volume, differing pH, or auxiliary pharmaceutical materials do not interfere with the BOLD effect of the test compound. As it is not always possible to formulate test materials in an indifferent formulation, pretreatment of animals with the test compound and testing its effect by a neutrally formulated challenging drug one hour later can solve the problem of unwanted formulation effect. This type of experimental approach was used in the paper of Chin et al. (2011), where the effect of metabotropic glutamate 2/3 receptor agonist LY379268 was tested against ketamine. In another study, the effect of an allosteric potentiator or agonist of mGluR2 against phencyclidine (PCP) was measured (Gozzi et al., 2008; Hackler et al., 2010) and this sort of approach was used in our studies as well.

## I.3 The basis of suitable disease-modifying treatments in the future

Nowadays, more and more publications emphasize the role of intact brain circulation and microcirculation in cognitive functions (Drachman, 2014; Dotti and De Strooper, 2009; Hall et al., 2014; Hunter et al., 2012). Even a small fluctuation in cerebral oxygen or glucose delivery may have an impact on cognitive performance. Animal studies also prove that cerebral hypoperfusion by itself can cause increased oxidative stress, damage to cholinergic neurotransmitter system and cognitive impairment (Bink et al., 2013; Iadecola, 2013; Xi et al., 2014; Zlokovic, 2011).

Regional cerebral hypoperfusion is a pronounced sign of the cognitive failure and the clinical manifestation of AD (de la Torre, 2000; de la Torre and Stefano, 2000). Furthermore, in advanced age, the cortical blood supply may be reduced by up to 30 %. Altogether, it can cause critically low cerebral perfusion, when the energy demand exceeds the capacity of metabolic processes (de la Torre, 2000; Hunter et al., 2012; Lu et al., 2011). The insufficient energy supply (e.g. due to a circulatory problem) may lead to amyloid β-peptide (Aβ) deposition in cerebral vessel walls and in brain parenchyma as well, termed cerebral amyloid angiopathy (CAA). Under intact energy supply, the low-density lipoprotein receptor-related protein 1 (LRP1) eliminates the soluble form of amyloid protein from the brain parenchyma to the blood. LRP1 is a potent amyloid β efflux transporter across the blood-brain barrier (BBB). It is easy to see that some kind of defects in vascular smooth muscle cells (VSMCs) lead to disrupted Aβ clearance. Hypoperfusion downregulates the clearance transporter which leads to disrupted Aβ clearance and amyloid deposition. The excess amyloid protein inhibits the synaptic transmission and basic neural functions; it worsens further the circulation and aggravates the energy deficit (Bell et al., 2009; Dotti and De Strooper, 2009). The Aβ deposition impairs the cholinergic system directly, which initiates a vicious circle (Olivero et al., 2014).

Improving blood circulation, especially in regions associated with cognitive processes, may reverse the cognitive impairment caused by insufficient cellular energy production. This may happen without direct modulation of the level of neurotransmitters or their receptors (Palmer and Love, 2011).

Following neuronal stimuli, increased blood flow and prompt relaxation of capillaries can be seen (Hall et al., 2014) due to the bidirectional neurovascular coupling (Peppiatt et al., 2006). The cerebral capillary flow is regulated mostly by pericytes. This type of cells covers mainly the brain microvessels; some 80 % of microvessels are enveloped in the brain while other organs contain only a few percent (Armulik et al., 2011; ElAli et al., 2014; Winkler et al., 2012). The abundance of pericytes seems to be proportional to the barrier function of the organ (Winkler et al., 2010; Winkler et al., 2012). Pericytes have lots of neurochemical receptors (Dore-Duffy, 2008; Hamilton et al., 2010) and receive paracrine stimuli either from other neurons through astrocytes and from endothelial cells that convey luminal signals (Peppiatt et al., 2006). Long-term hypoxia causes pericyte migration or its death around the capillaries (de la Torre and Stefano, 2000; Gonul et al., 2002). Therefore, the capillary system becomes unresponsive to physiological, neural, and luminal stimuli, which impairs irreversibly the neuronal system (Cleary et al., 2005).

These changes can also be observed in AD patients who did not suffer a pericyte degeneration (Baloyannis and Baloyannis, 2012; Farkas and Luiten, 2001; Sengillo et al., 2013). Targeting the pericytes – which could provide selective brain-specific action – in the prevention and therapy of dementias, such as AD is suggested in our and other studies as well (ElAli et al., 2014; Hamilton et al., 2010).

## I.4 Cognition and the cholinergic system

### I.4.1 Acetylcholine

Acetylcholine (Ach) is an important neurotransmitter, which undoubtedly plays a role in cognitive processes. It is located in synapses of peripheral nervous system, in vegetative ganglions, in parasympathetic postganglionic fibers, and in neuromuscular junctions as well. Acetylcholine is considered to be a physiological agonist of nicotinic and muscarinic acetylcholine receptors (Brunton et al., 2005).

#### I.4.1.1 Nicotinic acetylcholine receptors (nAchRs)

Nicotinic acetylcholine receptors (nAchR) are ionotropic receptors with high sodium, potassium, and calcium permeability. nAchR has lots of subunits; the most important one related to cognitive functions is the α7 subunit (Brunton et al., 2005).

The α7 subunit containing nAchRs are highly expressed in brain regions implicated in cognitive processes, among others in the hippocampus, cortex, and several subcortical limbic regions (Gotti et al., 2006; Marks and Collins, 1982; Rubboli et al., 1994). Low expression level can be seen in thalamic regions and in basal ganglia (Clarke et al., 1985; Poisik et al., 2008; Tribollet et al., 2004). Therefore, targeting α7 nAchRs with pharmacological agents seems to be an effective treatment in cognitive decline. Unfortunately, the uncommon properties of α7 nAchRs (low probability of channel opening and rapid desensitization) make the conventional agonist useless (Williams et al., 2011a,b). Positive allosteric modulators (PAMs) can solve these problems; they do not possess any direct agonist effects on the target but they increase the effect of classical orthosteric agonists (Gill et al., 2011). Type I PAMs increase the peak amplitude of Ach current while Type II PAMs increase the current response due to the inhibition of desensitization (Hu et al., 2009; Schrattenholz et al., 1996; Timmermann et al., 2007).

#### I.4.1.2 Muscarinic acetylcholine receptors (mAchRs)

Muscarinic acetylcholine receptors (mAchR) are metabotropic G-protein coupled receptors, which have five subtypes (M1-M5). The neocortex, hippocampal, and striatal neurons are rich in M1 and M4 AchRs; M3 subtypes are located in thalamus and brainstem nuclei; M5 mAchRs are expressed in low levels in CNS, where they mediate the dilatation of arteries and arterioles; M2 receptor subtype can be found presynaptically, where it controls the transmitter release (Brunton et al., 2005).

### I.4.2 Scopolamine

Pharmacological manipulation is a valuable technique to demonstrate the importance of the cholinergic system to memory functions in intact and diseased conditions. Scopolamine as a non-selective muscarinic receptor antagonist is a widely used memory disturbing amnestic agent in models of cognition in human and preclinical studies as well (Beatty et al., 1986; Caine et al., 1981; Drachman and Leavitt, 1974; Flicker et al., 1990; Ghoneim and Mewaldt, 1975; Klinkenberg and Blokland, 2010; Lenz et al., 2012). The effect of scopolamine might be mediated by the M1 and M5 receptor subtypes based on their special distribution within the brain (predominantly located in cortex, hippocampus and striatum) (Caulfield, 1993). First, scopolamine was used clinically as an adjunct to surgical or obstetric procedures to induce sedation and anterograde amnesia (Drachman and Leavitt, 1974). Nowadays, its application is less frequent due to the cholinergic cognitive deficit it evokes and which cholinergic deficit has a high resemblance to those associated with Alzheimer's disease. This cholinergic blockade causes impairment in tasks of working memory, declarative memory, visual sustained attention, and psychomotor function (Wesnes and Revell, 1984; Wesnes and Warburton, 1983, 1984). Older people are more sensitive to scopolamine treatment than younger people, which is in accordance with animal studies (Molchan et al., 1992). The reversible memory impairment property of scopolamine makes it a widely used model for the characterization of cognitive enhancers (Ebert and Kirch, 1998). Besides its negative cognitive effect, it also has a strong cerebrovascular action. Based on the fact that the cerebral vasculature is rich in cholinergic fibers and muscarinic receptors, scopolamine as an antimuscarinerg agent could act as a vasoconstrictor (Honer et al., 1988). Its peripherial administration mostly affects muscarinic receptors in the septo-hippocampal area, in the amygdala and in the parietal cortex (Klinkenberg et al., 2011). Scopolamine decreases the neuronal activity and metabolism in the indicated brain regions and consequently reduces the CBF (Honer et al., 1988). These statements are in accordance with the results of lesion studies related to the nucleus basalis of Meynert (nbM), which is the origin of cholinergic projections to the cerebral cortex. Lesioning of nbM leads to decreased cerebral metabolism via the changed expression level of presynaptic choline acetyltransferase (ChAT) (Kiyosawa et al., 1987; Orzi et al., 1987).

All of the above-mentioned cerebrovascular effects are partially responsible for the negative cognitive effect of scopolamine as were proved by the non-brain penetrating analog butylscopolamine in our previous study.

### I.4.3 Butylscopolamine

Butylscopolamine (buscopan) is a quaternary nitrogen-containing muscarinergic antagonist. Its use as premedication for gastrointestinal endoscopy reportedly causes anterograde amnesia in patients (Lee et al., 2007). This temporary memory impairment shows similarities to human dementias where vascular problems often precede cognitive deficit (Wang et al., 2013) or have prominent importance (Ortner et al., 2014). Recently, several studies have pointed out the importance of intact brain circulation in humans (Dotti and De Strooper, 2009) or even suggest its improvement as the only way to prevent Alzheimer’s disease (Drachman, 2014).

## I.5 Cognitive enhancers in therapy and in active development phases

Unfortunately, there are no proven medication treatments to inhibit the developing of AD until this time. Various cholinergic and non-cholinergic agents have been tested clinically until today but the existing medication protocols have only modest potential. The clinical relevance of the first-line medications AchE inhibitors are not clear. They positively influence the cognitive functions, behaviour and activities of daily living. AchE inhibitors are often prescribed with additional cognitive enhancers, e.g. gingko biloba extractum, memantine, piracetam and vinpocetine to enhance their therapeutic effects (Atri et al., 2008; Stein et al., 2015)

There are further procognitive agents which are still in development phase. PNU-120596 and EVP-6124, the two alpha 7 nicotinerg agents, can positively influence the cholinergic neurotransmission system through nicotinergic AchRs (McLean et al., 2012; Prickaerts et al., 2012; Wallace and Bertrand, 2013). The newly discovered

Richter compound, RG2260 can enhance the brain perfusion presumably via pericytes (results not published).

In the last years, our research group started to investigate the central effects of neostigmine. As a quaternary AchE inhibitor, its peripheral actions have been identified but its role in CNS-related processes was totally unknown (Brunton et al., 2005).

Based on the progressive nature of dementias and neurodegenerative disorders, there are no available pharmacological agents or complex medications today those can reverse the progession of AD. The applied medications are able to delay the progression of diseases but these positive effects are only temporary (Winslow et al., 2011).

### I.5.1 Donepezil

Donepezil, a reversible, selective acetylcholinesterase inhibitor (AchEi) is the most prescribed medicine in Alzheimer's disease (Burns et al., 1999; Rogers and Friedhoff, 1996; Rogers and Friedhoff, 1998; Seltzer et al., 2004); often applied in vascular dementia (Black et al., 2003; Wilkinson et al., 2003) and in dementia associated with Parkinson's disease (Aarsland et al., 2002; Leroi et al., 2004). Its brain specificity was proved by preclinical studies (Seltzer, 2005).

Generally, it is administered orally, once a day at 5-10 mg dose in human therapy. Donepezil has long absorption time; it reaches its plasma peak concentration within 3-5 hours (Rogers and Friedhoff, 1998). Its bioavailability is 100 %; no food effect or circadian effect are of known status (Tiseo et al., 1998a, b). It is a well-tolerated drug with few side effects: nausea, vomiting, diarrhea, increased hydrochloric acid secretion, insomnia, and vivid dreams (Burns et al., 1999; Galligan and Burks, 1986; Lewin, 1999; Pratt et al., 2002; Rogers and Friedhoff, 1996). Its elimination is slow (half-life time ~70 hours, 96 % plasma albumin binding) via urine and feces (Tiseo et al., 1998a,b).

There are three other commercially available cholinesterase inhibitors, namely tacrine, rivastigmine, and galantamine. Unfortunately, none of them are able to stop or reverse the progression of AD (Galimberti and Scarpini, 2011).

### I.5.2 Piracetam

Piracetam is a cyclic product of the neurotransmitter γ-aminobutyric acid (GABA) but its effect is totally independent of GABA neurotransmitter system. The exact mechanism of action has not been fully understood yet; presumably, piracetam influences positively the membrane fluidity. It has neural and vascular effects as well. At neural level, it restores the normal neurotransmission – influences the cholinergic (Pilch and Müller, 1988; Stoll et al., 1992; Wurtman et al., 1981), serotonergic (Valzelli et al., 1980), noradrenergic (Olpe and Steinmann, 1982), and glutamatergic systems (Cohen and Müller, 1993) –, enhances the neuroplasticity (Brandão et al., 1995; Brandão et al., 1996), has neuroprotective (Mingeot-Leclercq et al., 2003) and anticonvulsant effects (Hawkins and Mellanby, 1986; Kulkarni and Jog, 1983; Mondadori and Schmutz, 1986; Mondadori et al., 1984). At vascular level, it decreases the erythrocyte adhesion to the endothelium (Nalbandian et al., 1983), stimulates prostacyclin synthesis, so in this way it causes vasodilatation (Moncada et al., 1976; Moncada et al., 1977a,b; Moriau et al., 1993; Schrör et al., 1980), reduces the plasma fibrinogen and von Willebrand factor level in dose-dependent manner (Moriau et al., 1993), increases the cerebral blood flow (CBF) and the peripheral microcirculation (Herrschaft, 1978; Sato and Heiss, 1985). Piracetam was the first nootropic on the market without any sedative or stimulating side effect. Nowadays, its indication area involves cognitive disorders, dementia, vertigo, cortical myoclonus, dyslexia, and sickle cell anemia (Giurgea, 1972).

Generally, piracetam is administered orally; it achieves the peak plasma concentration within 30 min due to the rapid and full (its bioavailability is close to 100 %) absorption (Gobert and Baltès, 1977). Elimination of piracetam from the circulation is rapid (no metabolites and no bounding to plasma albumin) via urine (Gobert, 1972). Altogether, piracetam is a well-tolerated nootropic, which has no irreversible toxicity after single oral dose up to 10 g/kg in small animals. In human studies, the applied dose depends on the indication area and varies between 2.5-24 g (Winblad, 2005).

### I.5.3 Vinpocetine

Vinpocetine (Cavinton®) is a synthetic derivative of Vinca Minor alkaloid, vincamine. This nootropic agent has been used for the treatment of various cerebrovascular diseases, such as cognitive decline and dementias for over 30 years (Szatmári and Whitehouse, 2003). Vinpocetine possesses a rather complex mechanism of action which has not been fully elucidated yet. Several studies emphasize that vinpocetine decreases the effect of hypoxia in the brain by enhancing the energy metabolism (Erdö et al., 1990). Furthermore, it improves the energy balance and the cerebral microcirculation due to the reduced cerebral vascular resistance (Kuzuya, 1985). Other scientists proved that vinpocetine points to molecular targets associated with voltage-dependent sodium and calcium channels, glutamate receptors, calcium- and calmodulin-dependent cGMP phosphodiesterases (Bönöczk et al., 2000; Erdő et al., 1996; Kiss et al., 1991; Vas and Gulyás, 2005).

In human studies, vinpocetine is administered orally at 5-10 mg dose range; after rapid absorption process vinpocetine is hydrolyzed into its major metabolite, cis-apovincaminic acid (cAVA). This metabolite has been formed during the intense first pass metabolism (Chen et al., 2006; Miskolczi et al., 1987; Szakács et al., 2001). Vinpocetine is undoubtedly responsible for several pharmacological effects, but its major metabolite, cAVA, also has pharmacological activity. Animal experiments proved that both compounds, parent and its metabolite as well, play important roles in neuroprotection (Nyakas et al., 2009).

### I.5.4 PNU-120596

PNU-120596 (N-(5-chloro-2,4-dimethoxyphenyl)-N‘-(5-methyl-3-isoxazolyl)-urea) is an α7 selective nicotinic acetylcholine receptor (nAchR) positive allosteric modulator (Type II). It directly increases the cerebral blood flow (Si and Lee, 2002), the peak current, the opening time of the ion channels, and destabilizes the desensitized state of the receptors (Grønlien et al., 2007; Hurst et al., 2005). This agent is one of the most effective PAMs until this time but it is still in development phase (Williams et al., 2011a).

### I.5.5 EVP-6124

EVP-6124 ((R)-7-chloro-N-quinuclidin-3-yl)benzo[b]thiophene-2-carboxamide) is a partial agonist of the neuronal α7 nicotinic acetylcholine receptor. After oral administration, it moderately binds to plasma proteins and easily passes through the blood-brain barrier (Bitner et al., 2007; Wallace et al., 2011; Wishka et al., 2006). Several studies emphasize its memory improving property after short-term memory impairment in sub-to-low nanomolar dose range, which seems to be dose-dependent. It can directly enhance the cerebral blood flow as well (Si and Lee, 2002). EVP-6124 is still in development phase, but the interim results are encouraging. Therefore, EVP-6124 has a chance to become an effective cognitive enhancer in the future (Prickaerts et al., 2012).

### I.5.6 RG2260

RG2260 is a newly invented, synthetic eburnane alkaloid by Gedeon Richter Plc. It is still in preclinical phase. This cognitive enhancer candidate does not have any affinity to usual molecular targets that are associated with cognitive functions. RG2260 is not able to cross the blood-brain barrier, so its mechanism of action seems to be evoked only vascularly. It is presumed, that the endothelial pericytes are the targets of this drug, which are abundant in the proximity of cerebral capillaries. These findings explain its selectivity in the cerebral vascular system (not published data).

### I.5.7 Neostigmine

Neostigmine is a reversible acetylcholinesterase inhibitor. The quaternary nitrogen-containing inhibitor exerts its effect only peripherally and it is unable to enter into the CNS. Thus, its therapeutic application is only peripheral; it improves the muscle tone in people with autoimmune myasthenia gravis (Brunton et al., 2005) and non-autoimmune congenital myasthenic syndromes. Neostigmine is used as a prophylactic agent for organophosphate poisoning (Yu et al., 2010) and it can reverse the effect of the non-depolarizing muscle relaxant agent at the end of anesthesia (Brunton et al., 2005).

Neostigmine has high aqueous solubility and usually administered orally. It has a moderate duration of action; its half-life time is approximately 50-90 min. After hepatic metabolism, it is excreted mostly via urine (Barber and Bourne 1974; Yu et al., 2010). Since neostigmine does not cross the blood-brain barrier, its possible central effect has not been investigated before.

# I.6 Overview of Magnetic Resonance Imaging

## I.6.1 What is Magnetic Resonance Imaging?

Magnetic Resonance Imaging (MRI) is a widely used complex imaging modality in medicine. First of all, the most important features of MRI are its non-invasive and ionising radiation free character. Furthermore, most MRI measurements do not require any contrast agent or preparation.

There are only some unpleasant consequences of the measurements (claustrophobic feeling, dizziness, nausea, and headache) but altogether no harmful effects of MRI have been proved until now. (Huettel et al., 2008).

Modern human MRI scanners have a stable magnetic field in the range of 1.5 to 11 T while in animal studies up to 24 T scanners also appear. They have very good spatial (human scanner: 1-3 mm; small animal scanner: ~0.1 mm) and temporal (~1sec) resolution compared to the other imaging modalities.

MRI is a collective name of several main imaging modalities that use strong static magnetic field:

- **Spectroscopy** (MRS) measures the presence and concentration of several MR active metabolites or nuclei (1H, 13C, 19F, 23Na, 31P) in tissues.
- **Structural MR imaging** measures the static anatomical structure of the dedicated tissue.
- **Functional MR imaging** (fMRI) measures the activation or deactivation pattern in tissues (Jezzard et al., 2001).

## I.6.2 Basic physical background

MRI records the absorption of radio frequency electromagnetic radiation by the atomic nuclei of the sample placed in a magnetic field. The behavior of a nucleus in magnetic field is determined by its spin. Spin is the intrinsic angular momentum of elementary particles. It is one of the basic physical parameters of the particle, among other parameters such as mass or charge. Charged particles with non zero spin have an intrinsic magnetic momentum. In living systems, the most abundant nucleus is that of the hydrogen atom: the proton. The magnetic momentum ($M_N$) of the proton spin is:

$$M_N = g_N \cdot \mu_N \sqrt{S(S+1)}$$

where $g_N$ is the nuclear factor, $\mu_N$ is the Bohr magneton, $S$=1/2 is the spin of the proton. The value of the Bohr magneton can be calculated as:

$$\mu_N = \frac{e \cdot h}{4 \cdot \pi \cdot m_p}$$

where $e$ is the elementary charge, $h$ is Planck's constant, $m_P$ is the mass of the proton.

### I.6.2.1 The introduction of an external magnetic field ($B_0$)

After the introduction of an external magnetic field ($B_0$), spins initiate a gyroscopic motion, known as precession (ν). They rotate along their axes with a well-determined frequency, called the Larmor frequency. This frequency depends on the type of the nucleus and the strength of the external magnetic field. The Larmor frequency can be calculated from the following equation for each type of nucleus:

$$\nu = \frac{\gamma B_0}{2\pi}$$

where $\gamma$ is the gyromagnetic constant.

In the presence of an external magnetic field, the interaction between the magnetic moment of the particle and the external magnetic field causes the energy level of the proton to split into two levels, one of which will correspond to the ground state of the particle, and the other to the excited state. This splitting is called Zeeman effect. Some protons will be parallel, while others opposite (antiparallel) to the magnetic field. The energy difference between the two states depends linearly on the strength of the applied external magnetic field.

Under these conditions the net magnetization vector (M; it represents the sum of the magnetic momentums within the spin system) and the main magnetic field are in the same direction, so the imaging process is impossible. The net magnetization vector (M) needs to be turned out from longitudinal (z-direction) to transversal direction (x-y plane) to be measurable (Huettel et al., 2008).

#### I.6.2.2 Excitation

Excitation in MRI refers to the process in which atomic nuclei absorb electromagnetic energy to jump from a lower- to a higher-energy state and the spin populations of the two energy levels redistribute. Electromagnetic excitation happens at the same frequency as the frequency of the Larmor precession of the spins.

Consequently, the net magnetization vector rotates away from the z-direction induced by $B_0$ static magentic field. The longitudinal component of the net magnetization vector tends to be zero and the transversal component will be measurable.

In general, **90-degree excitation pulse** means a pulse of electromagnetic radiation which transfers the net magnetization vector from the z-direction into the x-y plane.

A **180-degree excitation pulse** means an electromagnetic pulse that exactly inverses the direction of the net magnetization vector (Huettel et al., 2008).

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### I.6.2.3 Relaxation

The MR signal is not stable over time. After excitation, the spin system relaxes back to thermal equilibrium. The transversal magnetization (the projection of the magnetization vector in the x-y plane) slowly tips back into the longitudinal direction (z-direction) due to the rapid loss of phase coherence of spins. These changes are called relaxation. There are two major mechanisms which contribute to the loss of MR signal: the longitudinal relaxation (T1 relaxation) and the transversal relaxation (T2 relaxation) (Huettel et al., 2008).

#### *I.6.2.3.1 T1 relaxation*

T1 relaxation time (tipically a few hundred milliseconds - a few seconds) is the time constant of the longitudinal relaxation (longitudinal recovery, or spin-lattice relaxation). It means that the net magnetization vector recovers to the longitudinal direction (Figure 1) (Huettel et al., 2008).

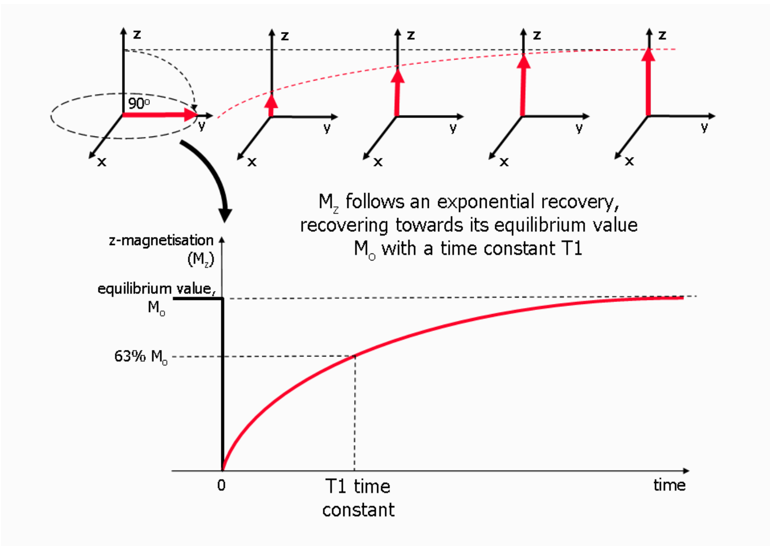


**Figure 1 The overview of T1 relaxation process (T1 recovery).** The net magnetization vector (M) has been tipped into the transversal plane with a 90-degree excitation pulse. The spins begin to relax after the excitation and release the excess energy into the surrounding environment. At the end, the net magnetization vector recovers to the longitudinal direction (adapted from Ridgway, 2010).

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#### *I.6.2.3.2 T2 relaxation*

T2 relaxation time (a few hundred milliseconds) is the time constant of the transverse relaxation (transverse decay, or spin-spin relaxation). The precessing frequency of spins will be different from each other during T2 relaxation process, so they fall out of the same phase. This causes exponential reduction of the net magnetization in the transverse plane (Figure 2) (Huettel et al., 2008).

#### *I.6.2.3.3 T2* relaxation*

T2* relaxation is a type of T2 relaxation process which is sensitive to the inhomogeneity of the magnetic field as well. This additive inhomogeneity decreases the net magnetization vector faster than T2 relaxation. Thus, the T2* relaxation time is always shorter (a few tens of milliseconds) than the T2 relaxation time (Figure 2). This type of relaxation is the most important and usually used in functional measurements (Huettel et al., 2008).

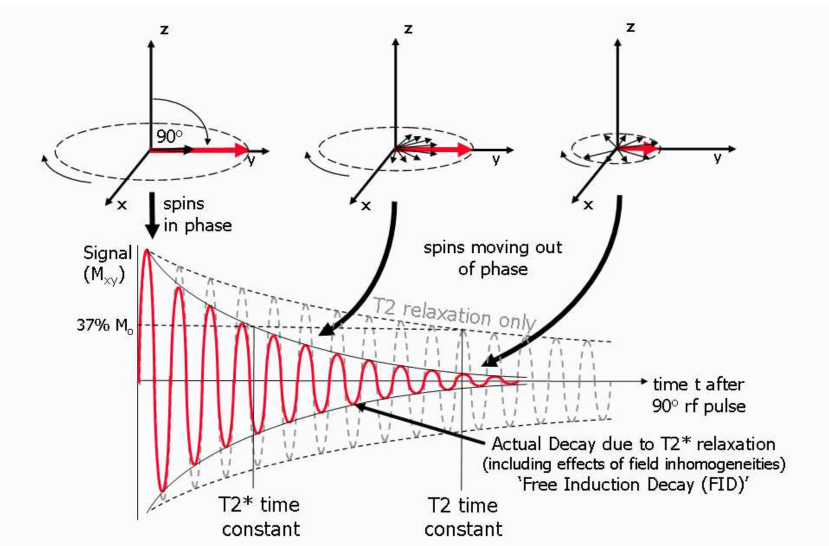


**Figure 2 The overview of T2 decay and T2* relaxation process.** The net magnetization vector (M) has been tipped into the transversal plane with a 90-degree excitation pulse. After the excitation, this vector decays (T2 relaxation) or rapidly decays (T2* relaxation, including effects of field inhomogeneity) as a result of the loss of phase coherence (adapted from Ridgway, 2010).

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### I.6.2.4 Image formation

A basic one dimensional magnetic resonance spectroscopy (MRS) makes possible the chemical analysis of the sample without any spatial coding. The resulting spectrum carries qualitative and quantitative information. Increasing the number of dimensions with the application of gradient coils, the chemical analysis of every single unit (pixel, voxel) will be measureable.

The final goal of medical magnetic resonance imaging is image formation. Three dimesional MR images are the combinations of many 2D representations, which are basically spatial distribution maps of spins within planar cross-sections of the samples. To allow 3D image formation, in addition to the main static magnetic field ($B_0$), 3 gradient magnetic fields (x, y, z-gradients) are used. The strong static magnetic field ($B_0$) arranges the atomic nuclei along the two determined directions while the gradient magnetic fields are used to gain spatially resolved information about the sample. The application of gradient coils ensures that atomic nuclei precess at different rates at various spatial location.

The **slice selecting gradient** ($G_z$ - parallel with the scanner bore and the $B_0$ magnetic field) is essential to index a slice within the 3D object in the z-direction. The **frequency encoding gradient** ($G_x$) changes the precession frequency of spins along the x-axis while the **phase encoding gradient** ($G_y$) has effect on spins along the y-axis. The acquired MR signal depends on the consecutive combination of the appropriate frequency and phase encoding gradients. The schematic overview of the applied radiofrequency (RF) pulse and magnetic gradients can be seen on the next diagram (Figure 3) (Huettel et al., 2008).

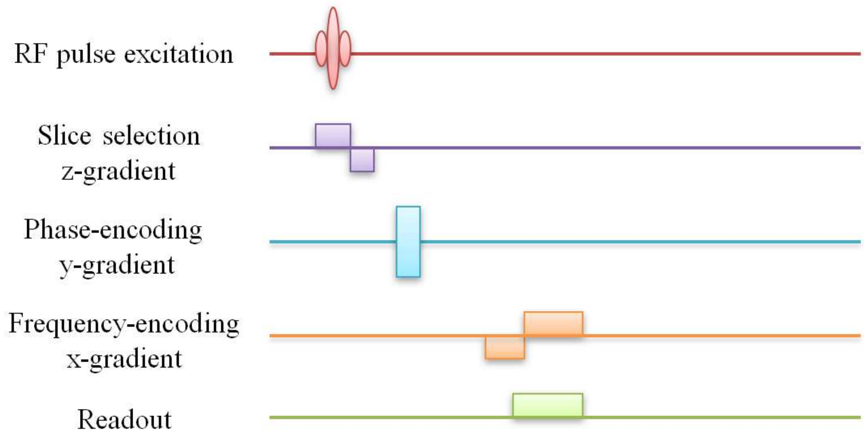


**Figure 3 The illustration of a general pulse sequence which is necessary to obtain a two-dimensional image of a given slice.** The application of slice selecting gradient determines the two-dimensional slices in the z-direction. An appropriate RF excitation pulse flips the net magnetization vector into the x-y plane within the determined slice. After the slice selection and the excitation procedure, the $G_y$ and $G_x$ gradients change the precessing frequency of the spins. Altogether, these gradients ensure that the atomic nuclei precess at different rates at various spatial location (adapted from Huettel et al., 2008).

#### I.6.2.5 MRI contrast mechanism

MRI is a widespread and flexible neuroimaging technique, which uses various contrast mechanisms to create images. First of all, it is important to define two major factors: **repetition time** (TR) and **echo time** (TE). TR is the time interval between two consecutive excitations, expressed in seconds. TE is that time which goes by between the excitation and the data acquisition, usually expressed in milliseconds. These values depend on the strength and the homogeneity of the magnetic field (higher field strength causes slower longitudinal relaxation and longer T1 value, faster transverse relaxation and shorter T2 and T2* values), the type of atomic nuclei and several other factors.

There are four large contrast mechanism groups:

- **Endogenous contrast** represents the intrinsic properties of biological tissues. The most frequently applied endogen contrast agent is the hemoglobin molecule. During BOLD fMRI measurements the changes of oxy-deoxy hemoglobin ratio cause the determinable MR signal (Huettel et al., 2008).
- **Exogenous contrast** mechanism requires external contrast agent. These compounds increase the statical and motion contrast as well, so these are

frequently used in clinical MRI experiments. There are lots of agents applied in daily routine e.g. Gd-DTPA, superparamagnetic iron oxide (SPIO), ultrasmall superparamagnetic iron oxide (USPIO) (Nakamura et al., 2000), superparamagnetic iron-platinum particles (SIPPs) (Taylor et al., 2011), and manganese-based nanoparticles (Koretsky and Silva, 2004).

- **Motion contrast** depends on the diffusional (diffusion contrast) and perfusional motion (perfusion contrast) of the atomic nuclei within the sample (Huettel et al., 2008).
- **Static contrast** is a commonly used contrast mechanism. MRI measurements are based on this technique to determine the brain anatomy. It is sensitive to the type, number, relaxation time, and resonance properties of the spins (Huettel et al., 2008).

##### *I.6.2.5.1 Proton-density contrast*

It is the simplest static MR contrast based on the different numbers of spins within voxels. The suppression of T1 and T2 contrasts are required to maximize the proton-density contrast (very long TR and very short TE values). The most powerful signals come from the cerebrospinal fluid (CSF) and ventricles. Gray matter (GM) gives a less intensive signal while white matter is only barely distinguishable from the air (Huettel et al., 2008).

##### *I.6.2.5.2 T1 contrast*

This contrast mechanism is based on the different relaxation properties of atomic nuclei in tissues. Parallel with the above-mentioned proton-density contrast, varying the repetition time and echo time can create the ideal T1 contrast (intermediate TR and short TE). The most forceful signals come from the white matter (WM) and bone marrow, due to their short T1 values. The area of the gray matter (GM) seems to be less intense while the water content of the CSF provides no signal (dark area on the images) from the air (Huettel et al., 2008).

#### *I.6.2.5.3 T2 contrast*

The basis of this contrast type is similar to the T1 contrast. In this case, only the T2 or transverse relaxation is examined, so the T1 contrast must be suppressed (long TR and intermediate TE values). The maximal signals come from the water containing regions (CSF and ventricles), these are bright on the images. The gray matter (GM) seems to be moderately bright while the white matter (WM) is totally black.

T2* contrast is a special type of T2 contrast, that is susceptible to the magnetic field inhomogeneity. T2* contrast forms the basis of BOLD fMRI measurements because it is sensitive to the actual level of deoxyhemoglobin concentration in the blood (Huettel et al., 2008).

## I.6.3 Functional Magnetic Resonance Imaging

In the 19th century, Angelo Mosso performed his famous 'human circulation balance' experiment. He measured the redistribution of blood in the brain noninvasively during emotional and intellectual activities (James, 1890; Sandrone et al., 2012).

In the further development of functional MR imaging, Charles Roy and Charles Sherrington played unquestionable roles (Roy and Sherrington, 1890). They were the first ones who could prove the connection between cerebral blood flow (CBF) and the brain functions (Raichle et al, 2001). In 1936, Linus Pauling and Charles Coryell realized that deoxygenated hemoglobin molecules can affect the homogeneity of the magnetic field.

After this revelation, Seiji Ogawa extended the usage of MRI from structural images to functional ones. He recognized the role of deoxyhemoglobin molecules as endogen contrast agent. Thereby, changes in neuronal activity in the brain could be seen as alteration of MR signal (Ogawa et al., 1990).

Presently, fMRI techniques rely on the fact that cerebral blood flow and neuronal activation are coupled (Huettel et al., 2008). Functional MRI is kept a modern, flexible, and indirect functional neuroimaging technique, which uses a high static magnetic field. To create images about the physiological activity, the scanner uses continuously changing magnetic gradient fields and radiofrequency (RF) pulses, known as pulse sequence. The exact frequency of the RF excitation determines which MR active nucleus will be excited. Most scanners are tuned to the frequency of hydrogen nucleus (42.574 MHz/T) due to the high prevalence of water is biological system (Pykett et al., 1982).

### I.6.3.1 Physiological background of fMRI measurements

#### *I.6.3.1.1 Neurovascular coupling*

The human brain consists of approximately 100 billion neurons, which have nearly 100 trillion synaptic connections with each other. Neurons are the basic information-processing units in the brain, which have complex roles in brain integration and signaling pathways. However, neurons and other brain structures do not have any energy storing system. So, their energy demands (the maintenance and restoration of the ion concentration gradient consume lots of energy after neuronal activations) are directly covered by glucose and oxygen via the vascular system.

Currently, it is well known that the neural and the vascular system are coupled. Neural activations generate strong determined alteration in the brain vasculature and increase the energy consumption in the given part of the brain tissue. The glucose and oxygenated hemoglobin concentration in the blood decreases (due to the increased cerebral metabolic rate of oxygen or CMRO) and the quantity of deoxygenated hemoglobin molecules increases. Fortunately, the brain is able to cover these temporary deficits via vasodilatation. The locally enhanced cerebral blood flow (CBF) and cerebral blood volume (CBV) are sufficient to increase the oxygenated-deoxygenated hemoglobin ratio. During this process, the blood volume remains unchanged within the brain, only the local blood distribution changes (Huettel et al., 2008). These relative oxygenated- and deoxygenated hemoglobin concentration changes are overviewed in Figure 4.

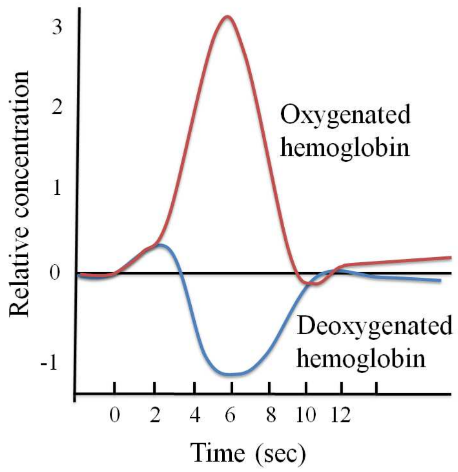


**Figure 4 The changes of oxygenated- and deoxygenated hemoglobin concentration during neuronal activation.** Neuronal activation causes a quick increase in the concentration of deoxygenated hemoglobin. 2 sec after the stimulus onset the time-curve achieves its maximum value and then rapidly declines (6 sec after the onset). This mechanism forms the basis of the BOLD hemodynamic response. Meanwhile, the oxygenated hemoglobin concentration time-curve shows slight delay compared to the stimulus onset. It rises its peak much slower (5-6 sec) and recovers to the basic level in ~10 sec (adapted from Huettel et al., 2008).

#### *I.6.3.1.2 The blood-oxygenation-level dependent (BOLD) contrast*

The BOLD contrast or BOLD imaging is a functional MRI technique for non-invasive, indirect detection of neuronal activities. It relies on the surrogate signal, resulting from regional differences in CBF, CBV, and changes in CMRO.

The main reason fMRI is able to detect these changes is due to the oxygen-transporting hemoglobin molecule in the blood, which has significant magnetic character. Its oxygenated form, the diamagnetic hemoglobin has no unpaired electrons, so its magnetic moment is zero, while the deoxygenated, paramagnetic hemoglobin molecules have unpaired electrons and remarkable magnetic moment. In general, paramagnetic materials are able to change the strength of the surrounding magnetic field and cause notable effects on the precessing frequency of spins. Blood-oxygenation-level dependent (BOLD) fMRI measurements are based on these distinct magnetic properties of hemoglobin molecules. As the hemoglobin becomes deoxygenated, the T2* relaxation time of the blood (after neuronal activation) becomes shorter than the ambient tissues and the measurable MR signal is decreased (Huettel et al., 2008).

Ogawa assumed that increased neuronal activity leads to increased oxygen consumption, raise the relative concentration of deoxygenated hemoglobin and consequently the remarkable decrease in the MR signal (Ogawa et al., 1990). In practice, however, greater MR signal was detected following neuronal stimulation. Fox and Raichle argued that more oxygen is transferred into the active brain regions than it is necessary, so the oxygen-rich blood flushes out the deoxyhemoglobin rich blood and the measurable MRI signal will be greater (Huettel et al., 2008).

#### *I.6.3.1.3 The shape of hemodynamic response (HDR) curve*

The MR signal changes that follow neuronal activation are called hemodynamic response or HDR. The shape of the HDR curve depends on the evoking stimulus: the firing rate of neurons determines the amplitude of the HDR curve, whereas its width, namely the plateau, is based on the duration of the stimulus.

The cortical neuronal responses appear within tens of milliseconds following the stimulus onset. One or two seconds later (lag the neuronal events) develops the determinable HDR curve. At the beginning, there is an initial, short negative-going dip (not always seen). This reflects the transiently rised level of deoxygenated hemoglobin after neuronal activation. Then the metabolic demands will be compensated by the circulation system. The increased CBF, CBV, and the huge quantity of oxygenated hemoglobin molecules flush out the deoxygenated blood from the active brain regions. This process forms the basis of the increasing HDR signal. In the absence of stimulation, the system returns to the normal equilibrium state. The CBF and CBV decrease towards the basic levels with different characteristics. Nevertheless, the decrease of CBF is much stronger than the decline of CBV. So, CBV is still above the baseline level, when the blood flow has already been returned to normal level. As a result, the HDR curve decreases below the baseline (undershoot) and remains there until the blood volume returns to the initial level again (Huettel et al., 2008). Two canonical HDR curves can be seen in Figure 5.

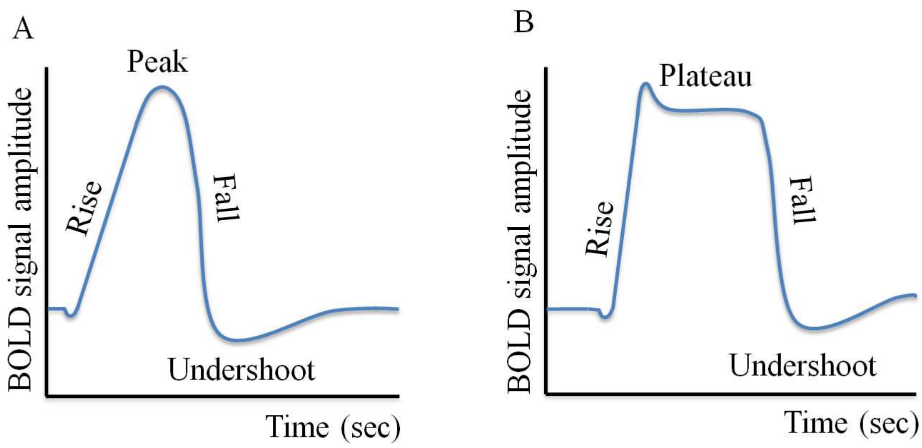


**Figure 5** **The illustration of two canonical HDR curves.** The first curve (A) is a simple, short duration stimulus-evoked HDR while the second one (B) is an extended block of time stimulus response (adapted from Huettel et. al, 2008).

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# II 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). Due to the small BOLD signal changes (~1 %) during animal fMRI experiments, the relevance of this techniques is often questioned by neuroscientists and other researchers. Therefore, the 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.

# III MATERIALS AND METHODS

## III.1 fMRI measurement

Male Wistar rats weighing 240-260 g were used in the in vivo experiments. The animals were purchased from Harlan and kept in polycarbonate cages (Lignifer Kft. Isaszeg, Hungary) in a thermostatically controlled room at 21 ± 1 °C. The room was artificially illuminated from 6 a.m. to 6 p.m. The rats were fed with conventional laboratory rat food (ssniff R/M+H Spezieldiäten GmbH D-59494 Soest). (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.)

In small animal imaging, the anesthesia is a crucial point of the measurement. Stable and deep anesthesia level decreases or eliminates the full motion artifacts from the data but it also inhibits the BOLD activation. In contrast, lower concentration of narcotics does not inhibit the BOLD signal but also does not decrease the motion artifacts (Kocsis et al., 2013). In our study, the rats were anesthetized 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 rats tail vein.

The anesthetized rat was transferred into the magnet. Body temperature was monitored using a rectal probe and maintained at 37 ± 1 ºC via a thermostatically controlled airflow around the rat. The ventilation of the animals was also controlled. We used a 9.4T 21ASZ Varian MRI system with a free bore diameter of 210 mm, fitted with a 120 mm inner size gradient coil (180 µs rise time). 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.

### III.1.1 Parameters of anatomical and functional images

**Anatomical images** (used parameters: gradient echo multi slice sequence (gems), repetition time (TR) = 200 ms, echo time (TE) = 3.83 ms, flip angle = 45º, averages = 3, dummy scans = 4, data matrix = 192x192, total scan time = 2 minutes, field of view (FOV): same as functional FOV) were acquired both before and after the functional echo planar imaging (EPI) sequences. The poor quality EPI pictures are fitted to these anatomical scans to localize the activity changes.

**BOLD contrast images** were received with EPI (gradient echo, TR = 3000 ms, TE = 10.0 ms, flip angle = 90º, shots = 3, triple reference scan, averages = 1, dummy scans = 4, data matrix = 64x64, 1000 repetitions, FOV: oriented Axial 90, 35x35 mm, 9 interleaved slices, thickness 1 mm, gap 0.2 mm).

### III.1.2 The experimental protocol

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 the dose of 1 mg/kg. 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 (Figure 6). 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 (Table 1). Only one measurement was performed with each animal. The exact experimental design and administration protocol can be seen in the following figure and table:

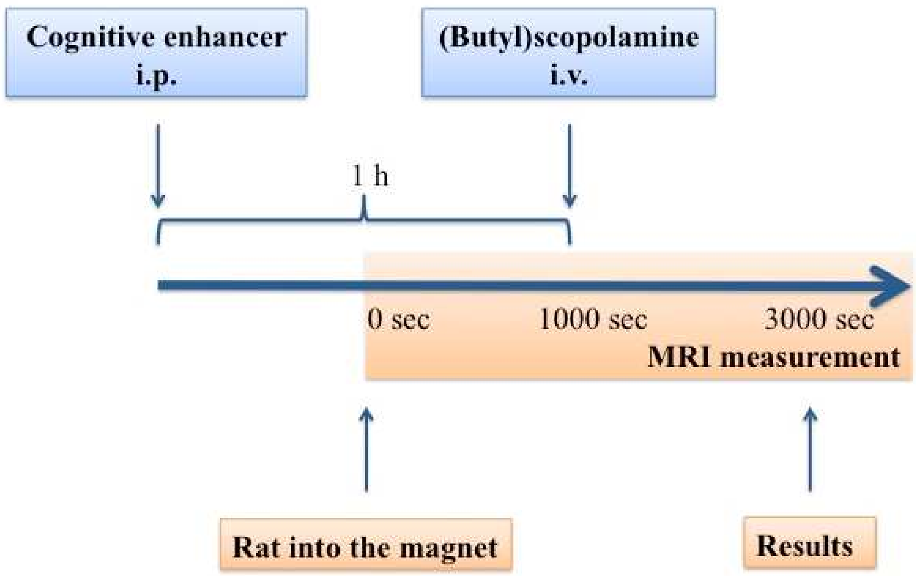


**Figure 6** **The experimental design of fMRI experiments**

**Table 1** **The administration protocol of cognitive enhancers during fMRI measurements.**

| | 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 |
| **EVP-6124** | 5% Tween 80 | i.p. | 1 |
| **PNU-120596** | 5% Tween 80 | i.p. | 3 |
| **RG2260** | 5% Tween 80 | i.p. | 0.01; 0.1; 0.625; 1.25; 2.5; 5; 10 |
| **Neostigmine** | Saline | i.p. | 0.01; 0.03, 0.1; 0.3 |

### III.1.3 Data analysis

The results of each measurement were stored in the scanner's own file format (fdf-files). These files were converted into the widely used nifty-format (Neuroimaging Informatics Technology Initiative) using a proprietary custom-made Matlab (The Mathworks, Natick, MA, USA) script. The analysis and the visualization of the data were also performed using a proprietary Matlab script.

To create individual t-maps in the pharmacological magnetic resonance imaging experiments, one-way within-subjects one-factor analysis of variance (ANOVA) was performed on each voxel's two levels (with and without drug administration) for each scan. In this case, ANOVA is a voxel-wise paired t-test with samples from the pre-injection baseline and post-injection period, respectively (Friston et al., 1994). The t-test was corrected using the Benjamini and Hochberg false discovery rate method (Friston et al., 1994). A t-value was assigned to each voxel, and voxels over the t-value limit were highlighted. A random effect analysis was not considered necessary because the animals formed a homogeneous group (males only, same species, same weight and other circumstances were also standard) and the onset of blood-oxygenation-level dependent (BOLD) change coincided with the application of test drug and the change was compared with the pre-drug level. Movement was checked, and measurements with a higher than one voxel movement were rejected.

Region of interest (ROI) analysis was also performed using a proprietary custom-made Matlab script. The ROIs were determined on the basis of the Rat Brain Atlas of Paxinos & Watson. The drug effects were calculated from BOLD signal changes of the dedicated ROIs. The efficiency of the actual drug was estimated from an averaged 300 msec long period after the drug administration. This time period was determined separately for each agent due to the different pharmacokinetic properties of the applied drugs. This post-injection period (where the maximal biological effect was registered) was normalized to the pre-injection baseline (an averaged 300 msec long time period immediately before the drug administration). The statistical significance of the drug effects was calculated using parametric one-way ANOVA and a post-hoc Fisher test (Statistica 8.0, StatSoft, USA, Tulsa).

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## III.2 Water labyrinth test

In the water labyrinth task, male Wistar rats (n = 10 per group; 180-200 g) 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 test was carried out as previously described in detail in the paper of Laszy et al. (2005). The water tank (1 m long, 60 cm wide and 60 cm deep) was filled with water of $24 \pm 1^{o}C$ to a depth of 30 cm. The labyrinth system was constructed with vertical metal plates. The escape platform (10 x 7 cm), with its top surface raised 0.5 cm above the water level, was placed in the corner of the tank farthest from the start point (Figure 7). The procedure for testing the water labyrinth acquisition process was performed on four consecutive days as described below.

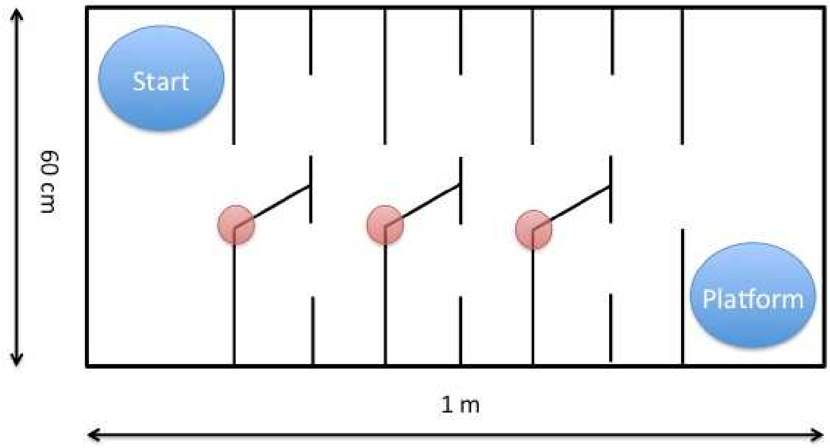


**Figure 7 The schematic illustration of the applied water labyrinth system.**

### III.2.1 The experimental protocol

On Day 1 (pretraining day) rats were habituated to the test environment without plates. They were conditioned three times to swim in the tank from the start point to the platform. The animals were left on the platform for 20 sec and they were allowed to rest in their cage for 20 minutes between each swim.

During the next days (Days 2-4) the labyrinth system was in place and the animals were trained in three daily trials separated by intertrial rest periods of approximately 25 min. The rats were placed into the water at the start point of the labyrinth system and had to reach the escape platform. The animals spent 20 sec on the platform, and then they were placed into a shared cage for rest periods between trials. The number of directional turning errors was measured as a variable reflecting learning performance. An error was defined as swimming through a choice point in the direction that did not allow the animal to reach the platform (blind alley). When the rat made an error, it was allowed to swim back to the choice point and try again. However, once a rat swam over a choice point in the correct direction (leading out of the labyrinth), the way back was manualy closed by a metal plate. The swimming time (the time interval from the entry into the labyrinth until the exit from the water) could not exceed 5 min for any trial. If the rat did not find the platform during this period, it was assisted to the end of the labyrinth by the experimenter, and the number of errors was recorded as 12.

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 (Figure 8, Table 2). 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.

I.p. administration may have effects on cognition and behavior of animals in behavioral pharmacological experience, probably due to slight pain and peritoneal irritation. Therefore, per os (p.o.) administration is preferred for cognitive tests in our study. During fMRI measurements the animals are put in light anesthesia one hour before the enhancer administration, so per os application is not predicable. The intrinsic sensitivity of the two above-mentioned methods is different, which explains the different doses and various pharmaceutical formulations. The exact experimental design and behavioral pharmacological administration protocol can be seen in the following figure and table:

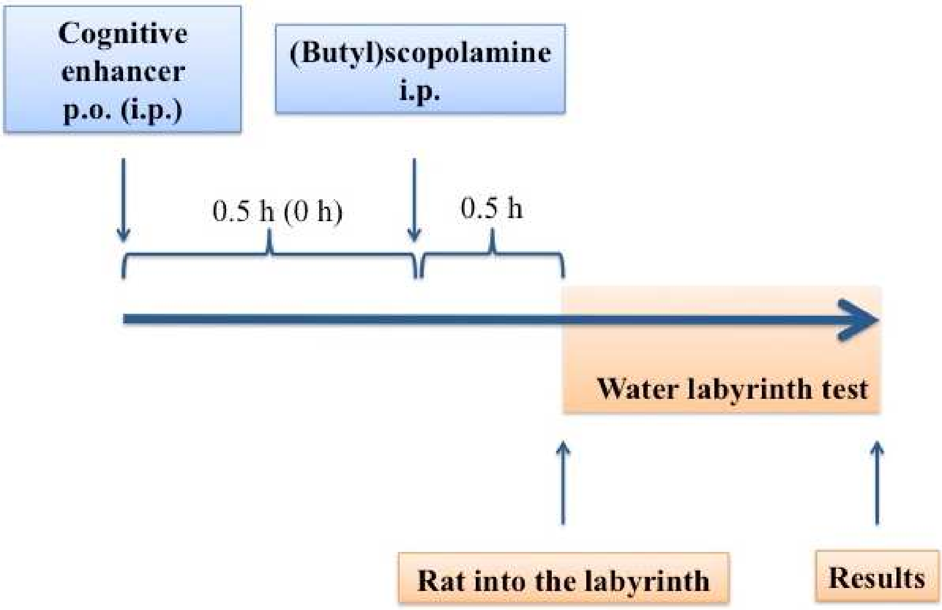


**Figure 8 The experimental design of water labyrinth tests**

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

| | 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 |
| **EVP-6124** | 5 % Tween 80 | p.o. | 0.3; 1; 3 |
| **PNU-120596** | Polyeth. glycol (PEG) 400 | p.o. | 3; 10 |
| **RG2260** | 5 % Tween 80 | i.p. | 0.625; 1.25; 2.5; 5; 10; 20 |
| **Neostigmine** | 5 % Tween 80 | i.p. | 0.3 |

### III.2.2 Data analysis

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:

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

where $N_{err}$ means the number of errors.

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# IV RESULTS

## IV.1 fMRI measurement

### IV.1.1 The effect of amnestic agent scopolamine

In the first part of the study, the effect of scopolamine was tested on the BOLD response (see details in Figure 9). 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 10). The effect of scopolamine was not dose-dependent in the dose range 1-5 mg/kg (not illustrated).

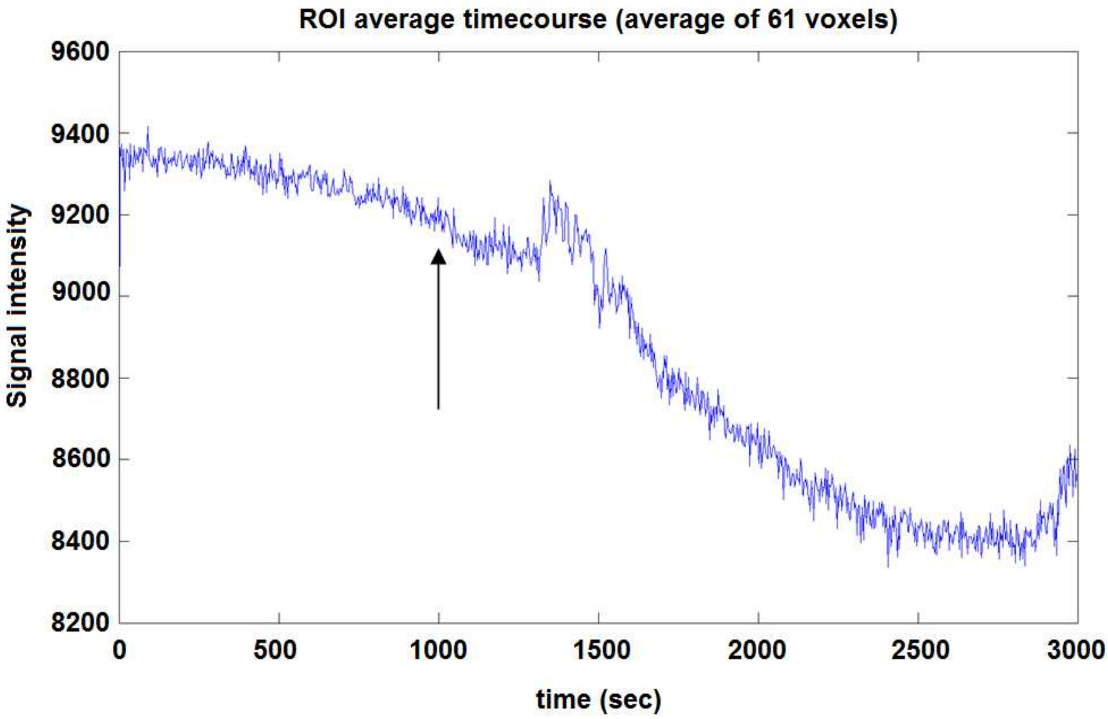


**Figure 9 The BOLD signal decrease after scopolamine treatment.** This curve demonstrates the signal intensity changes in the area of PFC after scopolamine treatment (average of 61 voxels) during 3000 seconds. Scopolamine (1 mg/kg dose) is administered at 1000 seconds, as the arrow indicates. The analysis of this curve is difficult due to the scanner drift (low-frequency change in the MR signal) but the effect of scopolamine can be clearly seen (slow BOLD signal decrease).

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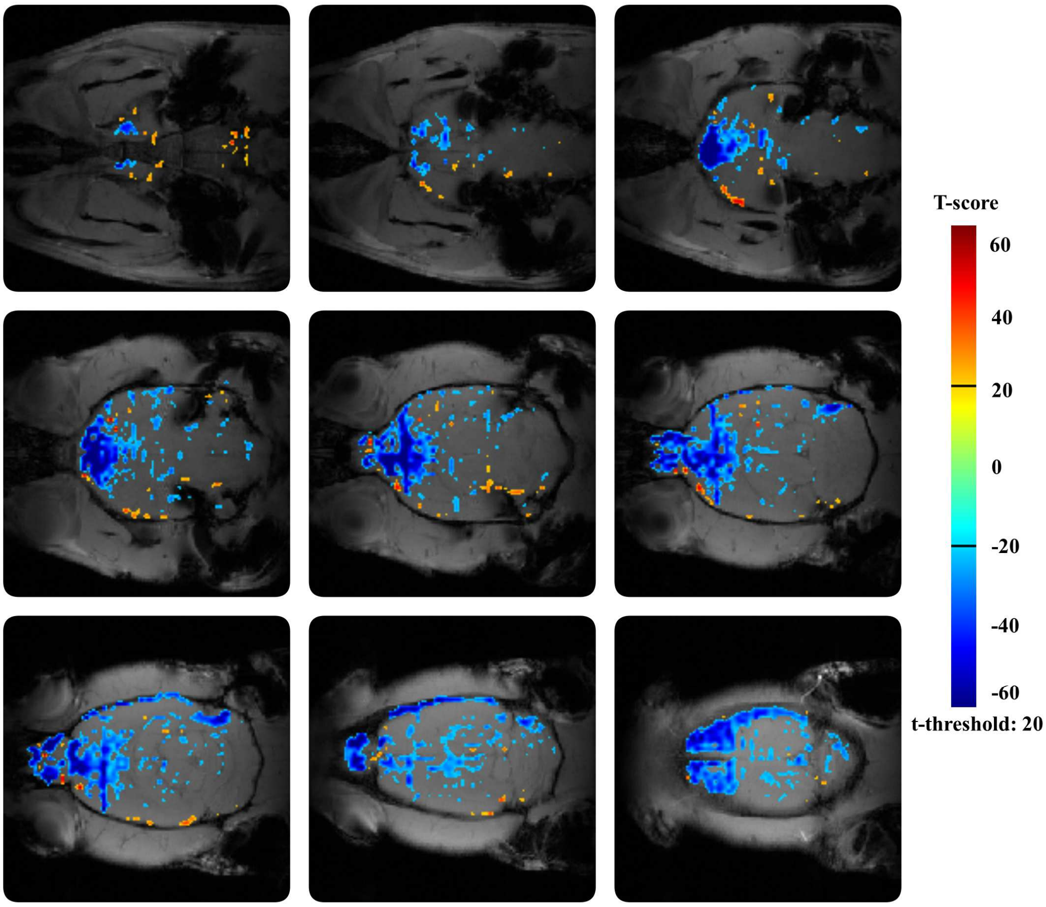


**Figure 10 Effect of scopolamine on BOLD response in the rat brain after vehiculum pretreatment.** Clear negative BOLD effect can be seen in the area of prefrontal cortex. Data are from an individual experiment in which scopolamine (1 mg/kg) was administered intravenously (one-way within-subjects one-factor ANOVA).

### IV.1.2 The effect of amnestic agent buscopan

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 (Figure 11).

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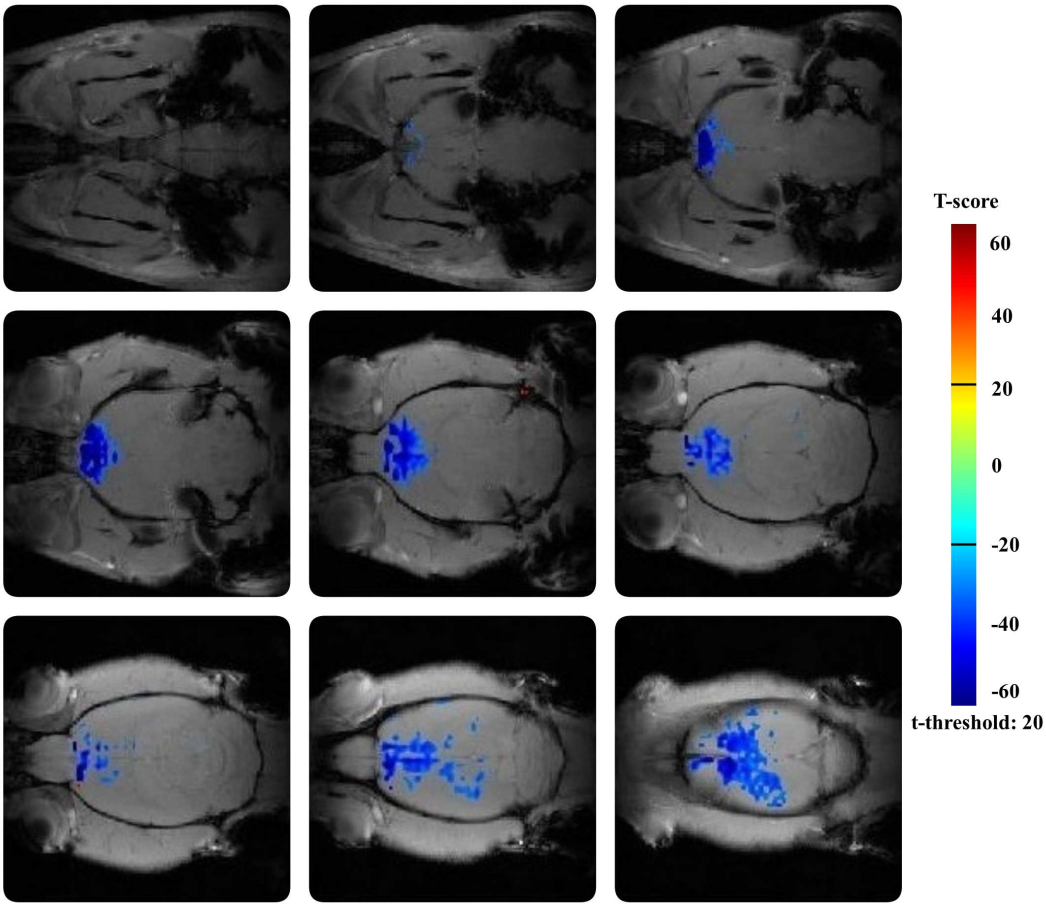


**Figure 11 Effect of buscopan on BOLD response in the rat brain after vehiculum pretreatment.** Surprisingly, clear negative BOLD effect can be seen in the area of prefrontal cortex, similar to that one caused by scopolamine. Data are from an individual experiment in which buscopan (1 mg/kg) was administered intravenously (one-way within-subjects one-factor ANOVA).

### IV.1.3 The effects of cognitive enhancers – direct administration

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 (Figure 12A, in hippocampus). 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 (Figure 12B, in hippocampus).

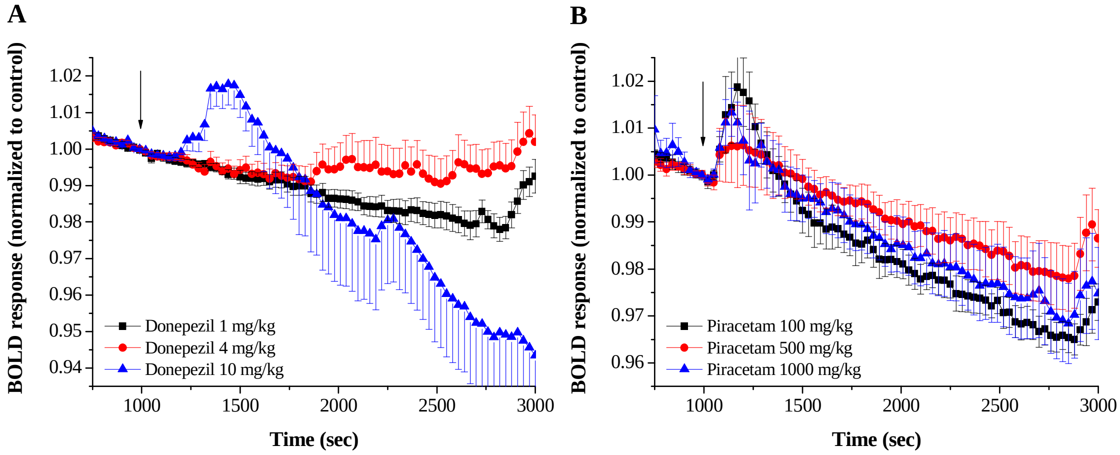


**Figure 12 Effects of donepezil (A) and piracetam (B) on BOLD response in the rat hippocampus.** Donepezil was administered at 1, 4 and 10 mg/kg intraperitoneally. Piracetam was injected at 100, 500 and 1000 mg/kg intraperitoneally. Arrows indicate drug administration.

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

### IV.1.4 The effects of cognitive enhancers in provocation model

Table 3 presents the reversibility of scopolamine-induced BOLD signal changes by procognitive compounds in our provocation fMRI model. 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 ($p<0.05$) the effect of the amnestic agent in the PFC (Figure 19, 21 and 22 compare to Figure 15 and 23; one-way ANOVA, post-hoc Fisher test), which points out the importance of adequate control in this very sensitive approach.

**Table 3 Effect of cognitive enhancers against (butyl)scopolamine induced amnesia in fMRI measurements.**

| Compound | Mean BOLD intensity ± SEM | ANOVA |
|---|---|---|
| Scopolamine (veh.: saline) | 0.961 ± 0.006 | $F(6.52)=3.735$, $p=0.0037$ |
| **Piracetam 100 mg/kg** | 0.957 ± 0.007 | |
| **Piracetam 500 mg/kg** | 0.955 ± 0.009 | |
| **Piracetam 1000 mg/kg** | 0.980 ± 0.003 | |
| **Donepezil 1 mg/kg** | 0.961 ± 0.014 | |
| **Donepezil 4 mg/kg** | $0.991 \pm 0.004^{**}$ | |
| **Donepezil 10 mg/kg** | $0.998 \pm 0.010^{*}$ | |
| Scopolamine (veh.: tween) | 0.940 ± 0.008 | $F(3.41)=3.461$, $p=0.0248$ |
| **Vinpocetine 5 mg/kg** | $0.969 \pm 0.009^{*}$ | |
| **EVP-6124 1 mg/kg** | $0.967 \pm 0.006^{*}$ | |
| **PNU-120596 3 mg/kg** | $0.977 \pm 0.003^{**}$ | |
| Scopolamine (veh.: tween) | 0.928 ± 0.011 | $F(7.48)=1.353$, $p=0.2468$ |
| **RG2260 0.01 mg/kg** | 0.937 ± 0.014 | |
| **RG2260 0.1 mg/kg** | 0.949 ± 0.007 | |
| **RG2260 0.625 mg/kg** | 0.951 ± 0.011 | |
| **RG2260 1.25 mg/kg** | $0.958 \pm 0.007^{*}$ | |
| **RG2260 2.5 mg/kg** | 0.951 ± 0.009 | |
| **RG2260 5 mg/kg** | $0.968 \pm 0.007^{**}$ | |
| **RG2260 10 mg/kg** | 0.955 ± 0.015 | |
| Saline (control) | 0.981 ± 0.003 | |
| Scopolamine (veh.: saline) | $0.955 \pm 0.010^{+++}$ | $F(3.20)=5.225$, $p=0.0079$ |
| **Neostigmine 0.1 mg/kg** | $0.975 \pm 0.010^{*}$ | |
| **Donepezil 4 mg/kg** | $0.994 \pm 0.005^{***}$ | |
| Buscopan (veh.: saline) | $0.962 \pm 0.005^{+}$ | $F(3.20)=15.99$, $p<0.0001$ |
| **Neostigmine 0.1 mg/kg** | $0.992 \pm 0.002^{***}$ | |
| **Donepezil 4 mg/kg** | $0.993 \pm 0.003^{***}$ | |

$+p<0.05$, $+++p<0.001$ vs. control group; $*p<0.05$, $**p<0.01$, $***p<0.001$ vs. (butyl)scopolamine-treated group (one-way ANOVA, post-hoc Fisher test)

**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 13, 15 and Table 3).

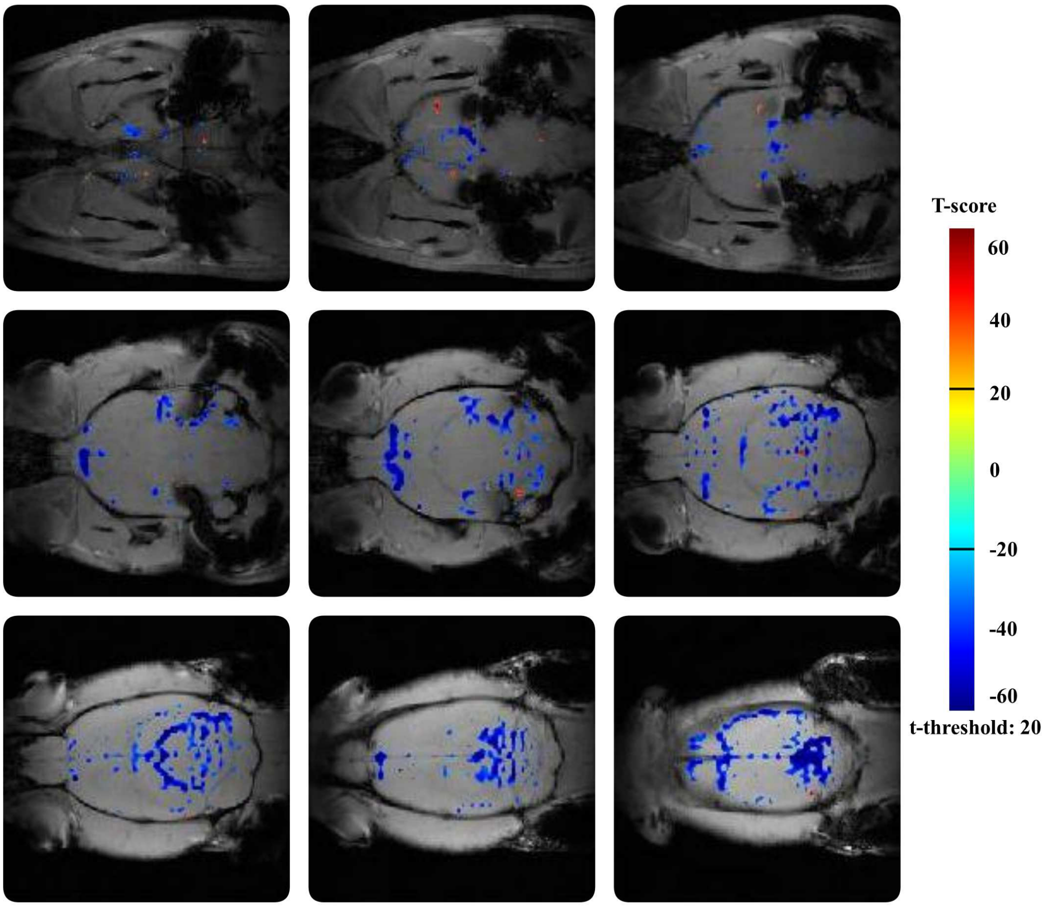


**Figure 13 Effect of piracetam pretreatment on scopolamine evoked BOLD response changes in the rat brain.** Data are from an individual experiment in which scopolamine (1 mg/kg) was injected intravenously. Piracetam was administered (1000 mg/kg) intraperitoneally (one-way within-subjects one-factor ANOVA).

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 (Figure 14 and Table 3). 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 15 and Table 3).

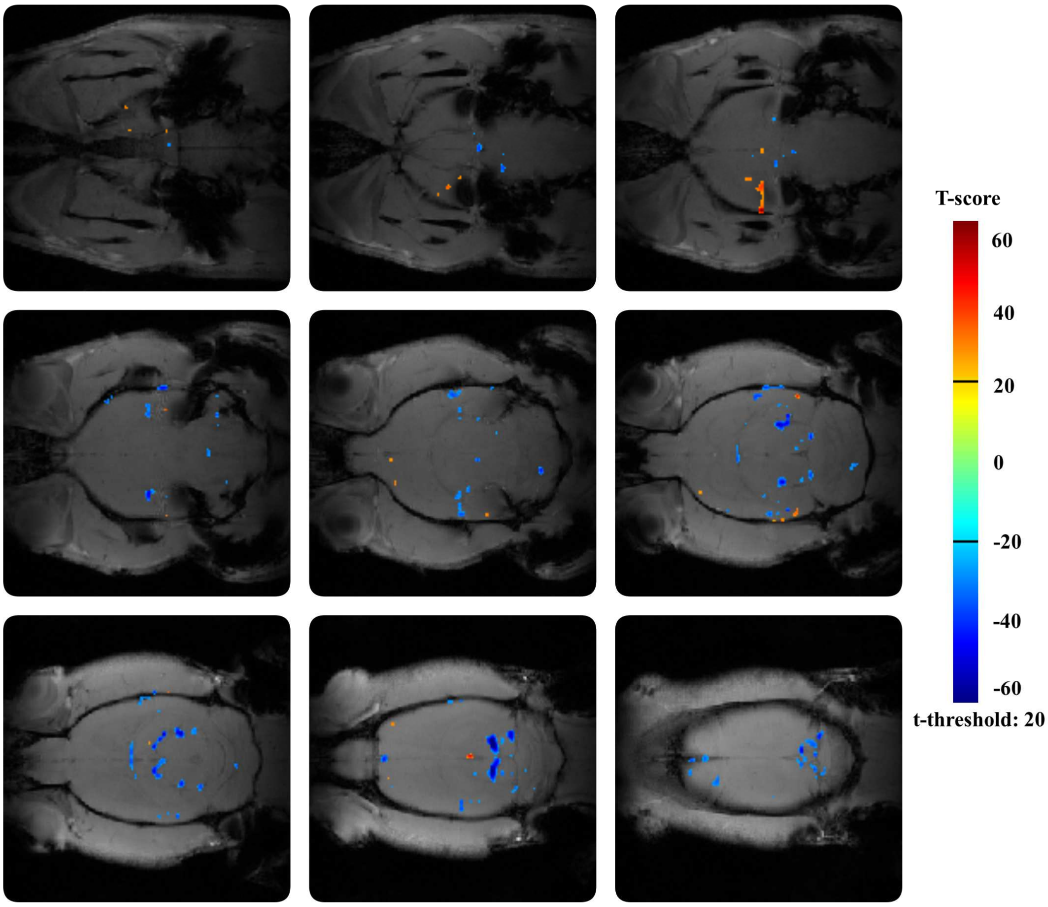


**Figure 14 Effect of donepezil pretreatment on scopolamine evoked BOLD response changes in the rat brain.** Data are from an individual experiment in which scopolamine (1 mg/kg) was injected intravenously. Donepezil was administered (4 mg/kg) intraperitoneally (one-way within-subjects one-factor ANOVA).

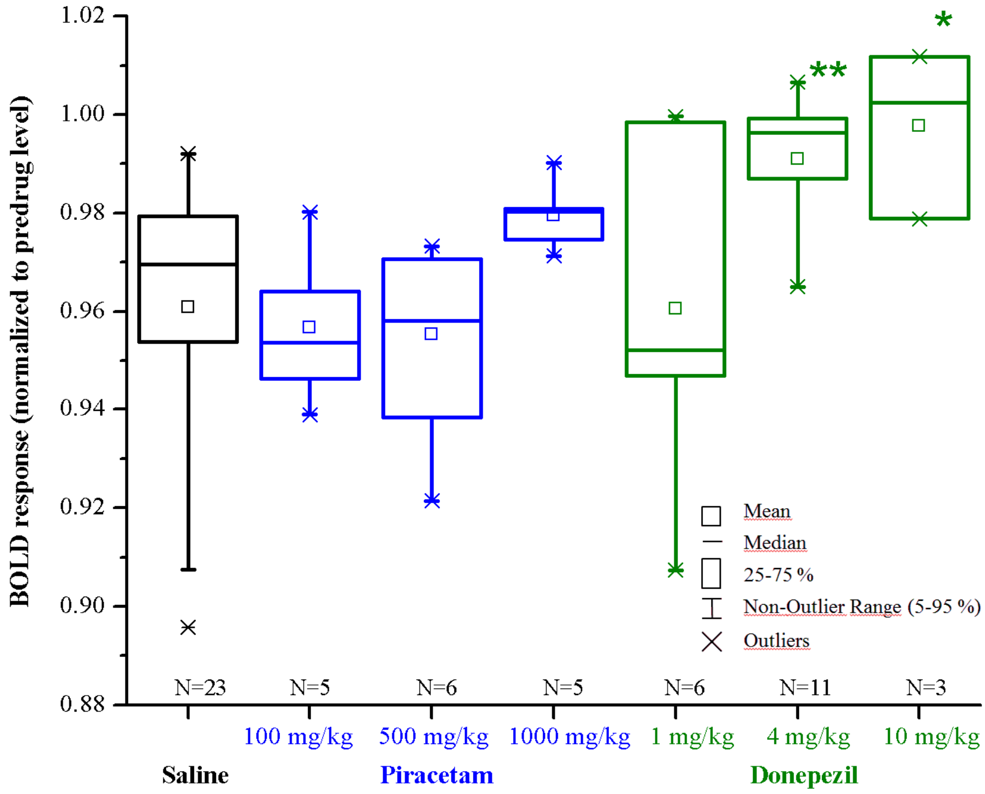


**Figure 15 Effects of piracetam and donepezil pretreatment on scopolamine evoked BOLD response changes in the prefrontal cortex.** Piracetam was administered at 100, 500 and 1000 mg/kg intraperitoneally. Donepezil was injected at 1, 4, and 10 mg/kg intraperitoneally. Scopolamine was tested at 1 mg/kg intravenously. (one-way ANOVA, Fisher post-hoc test; *$p$<0.05; **$p$<0.01)

**Vinpocetine** (5 mg/kg, n=5, $p<0.05$; Figure 16 - result of an individual experiment), **EVP-6124** (1 mg/kg, n=10, $p<0.05$; Figure 17 - result of an individual experiment) and **PNU-120596** (3 mg/kg, n=6, $p<0.01$; Figure 18 - result of an individual experiment) significantly inhibited the effect of scopolamine in the indicated doses (Figure 19 and Table 3).

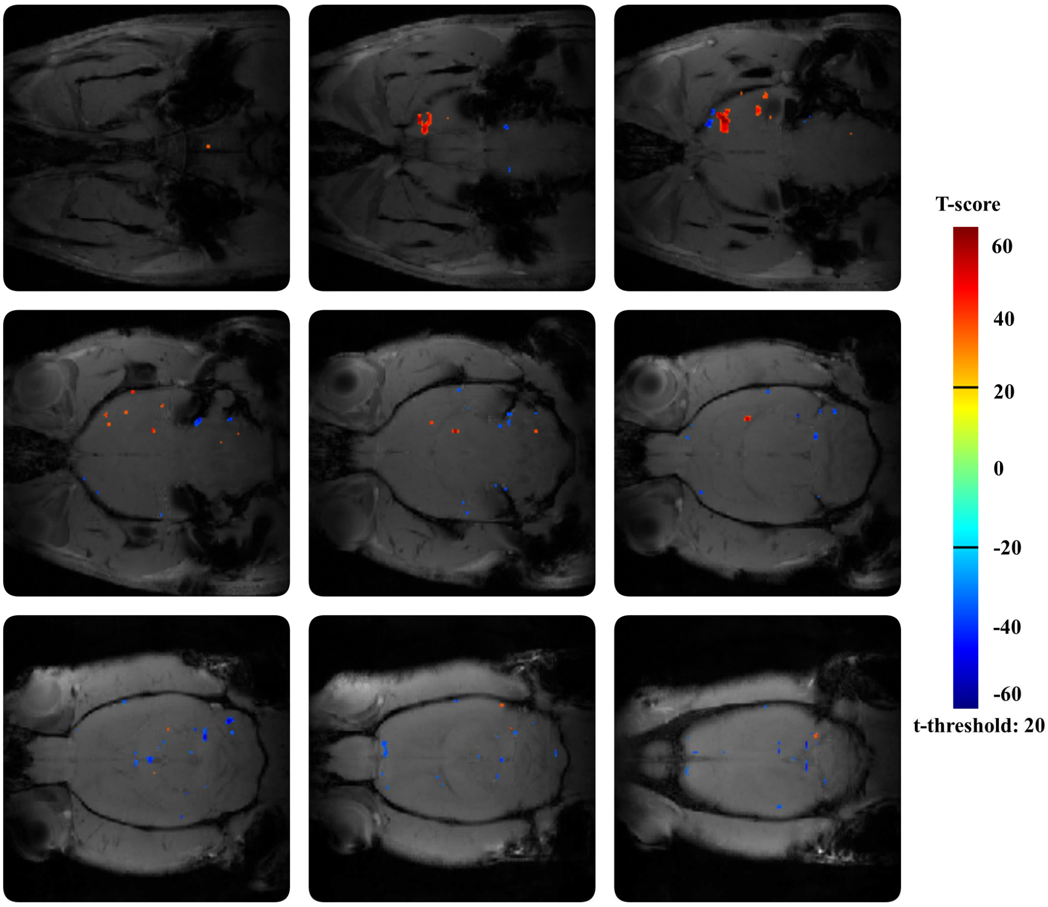


**Figure 16 Effect of vinpocetine pretreatment on scopolamine evoked BOLD response changes in the rat brain.** Data are from an individual experiment in which scopolamine (1 mg/kg) was injected intravenously. Vinpocetine was administered (5 mg/kg) intraperitoneally (one-way within-subjects one-factor ANOVA).

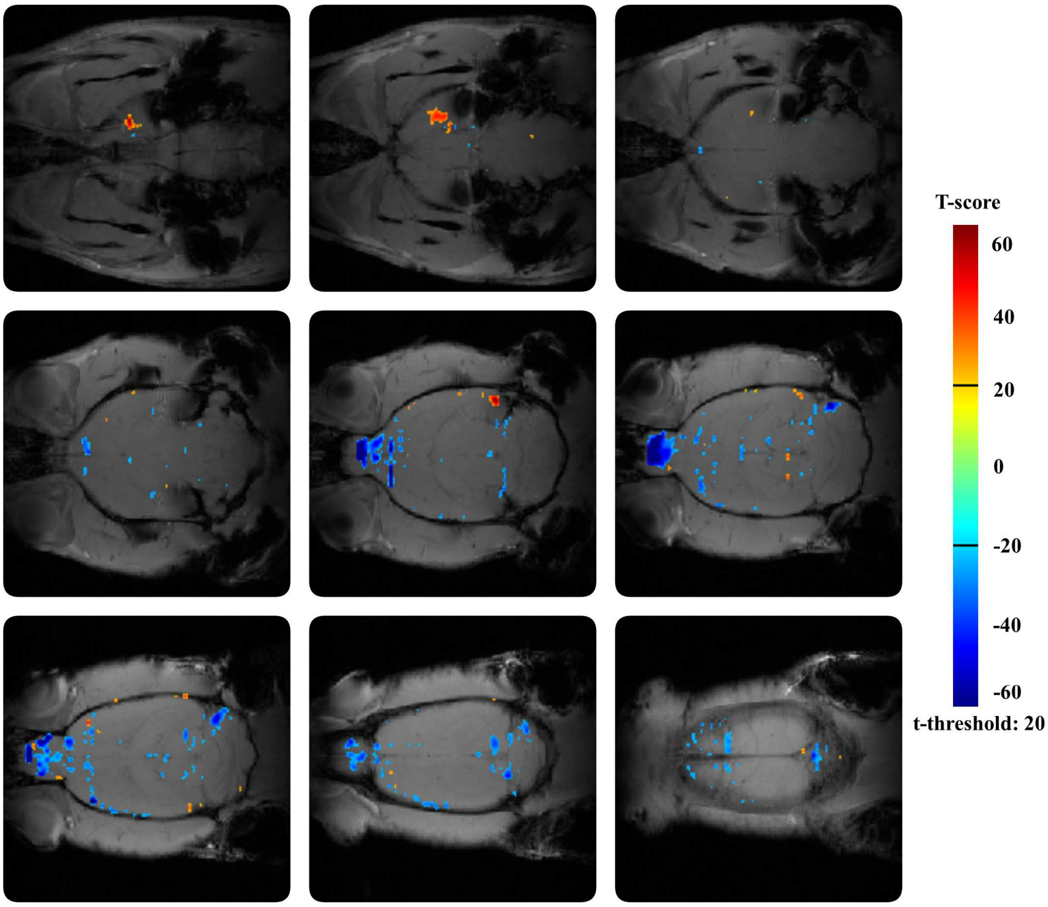


**Figure 17 Effect of EVP-6124 pretreatment on scopolamine evoked BOLD response changes in the rat brain.** Data are from an individual experiment in which scopolamine (1 mg/kg) was injected intravenously. EVP-6124 was administered (1 mg/kg) intraperitoneally (one-way within-subjects one-factor ANOVA).

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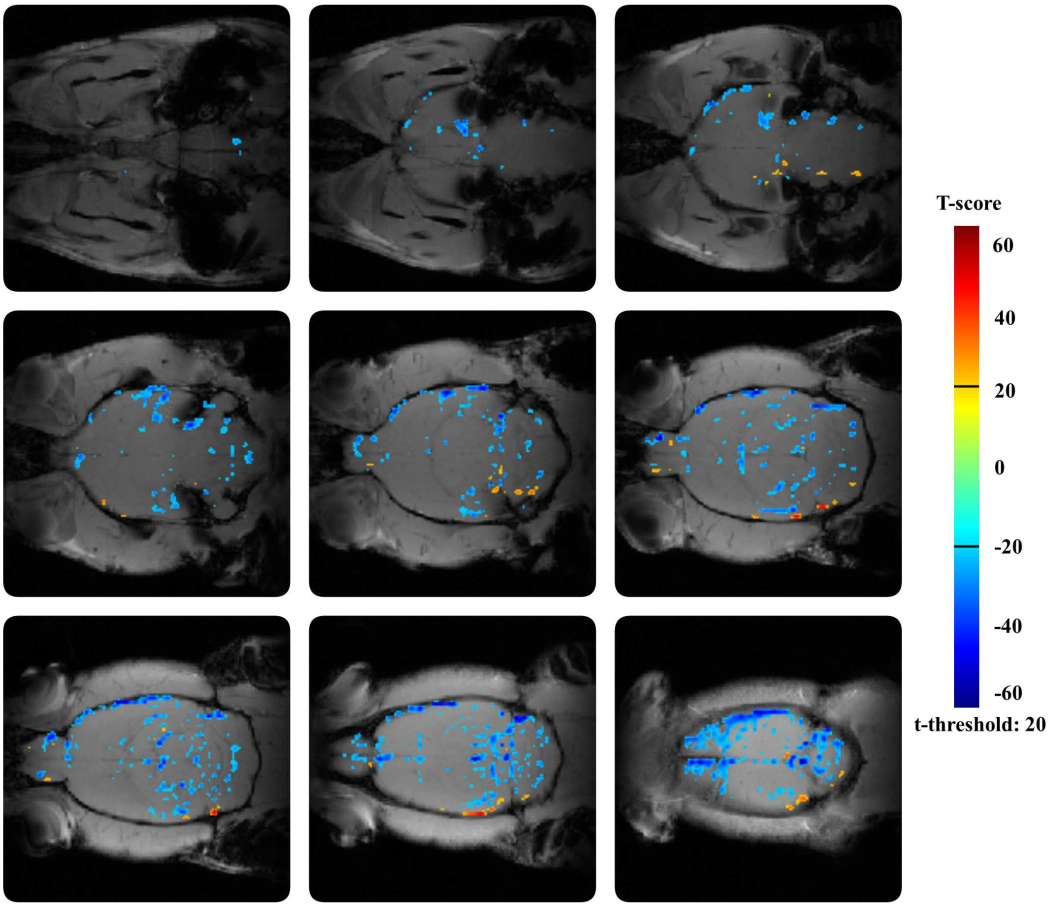


**Figure 18 Effect of PNU-120596 pretreatment on scopolamine evoked BOLD response changes in the rat brain.** Data are from an individual experiment in which scopolamine (1 mg/kg) was injected intravenously. PNU-120596 was administered (3 mg/kg) intraperitoneally (one-way within-subjects one-factor ANOVA).

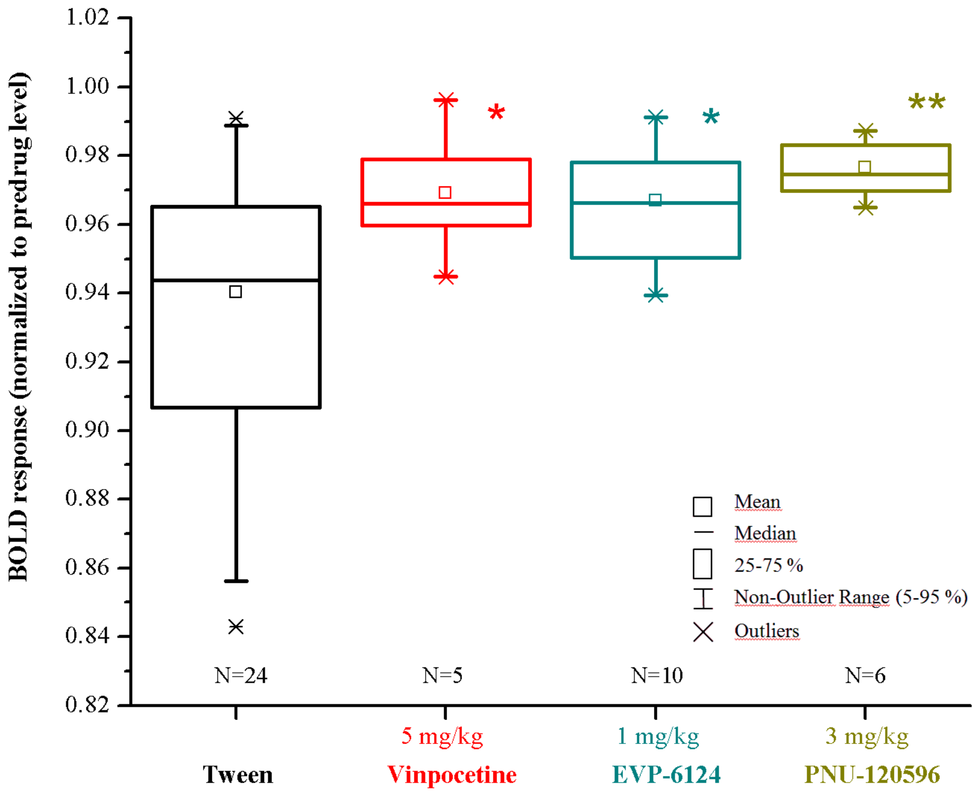


**Figure 19 Effects of vinpocetine, EVP-6124, and PNU-120596 pretreatment on scopolamine evoked BOLD response changes in prefrontal cortex.** Vinpocetine was administered at 5 mg/kg intraperitoneally. EVP-6124 was injected at 1 mg/kg intraperitoneally. PNU-120596 was tested at 3 mg/kg intraperitoneally. Scopolamine was injected at 1 mg/kg intravenously. (one-way 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 (Figure 20).

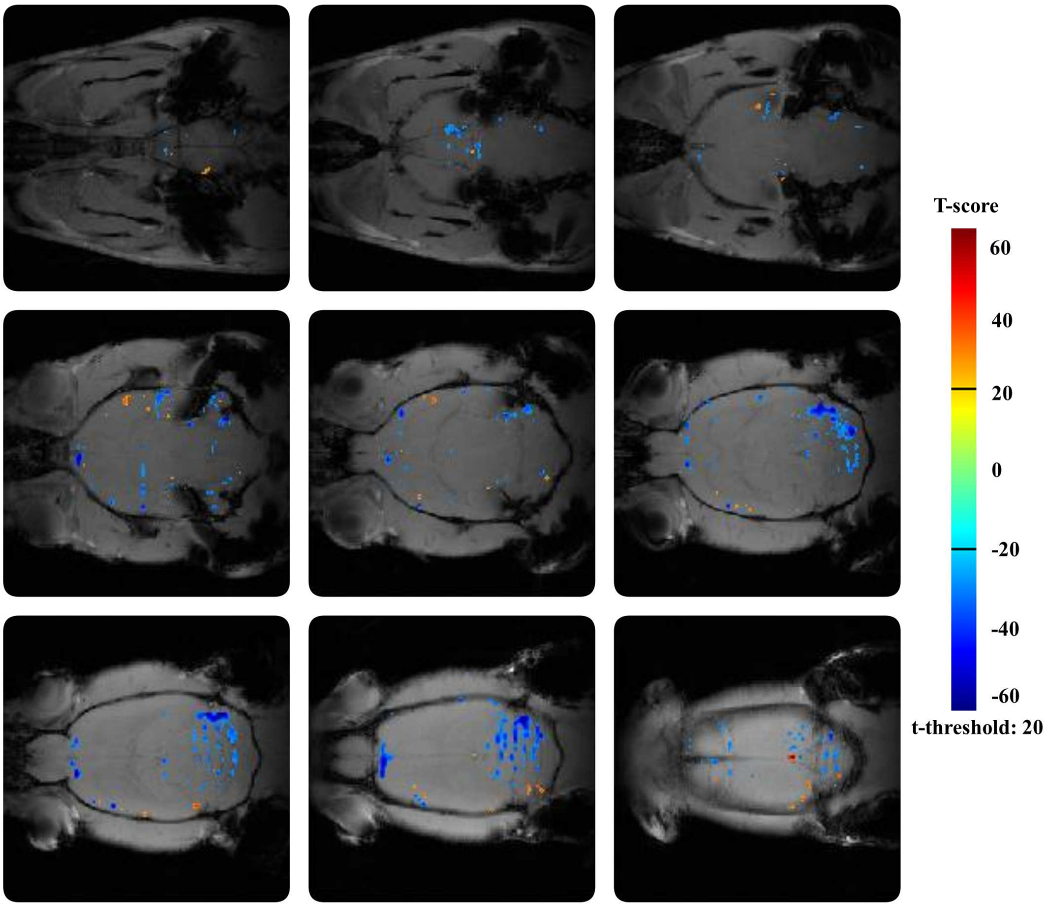


**Figure 20 Effect of RG2260 pretreatment on scopolamine evoked BOLD response changes in the rat brain.** Data are from an individual experiment in which scopolamine (1 mg/kg) was injected intravenously. RG2260 was administered (1.25 mg/kg) intraperitoneally (one-way within-subjects one-factor ANOVA).

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, $p=0.053$; Figure 21 and Table 3).

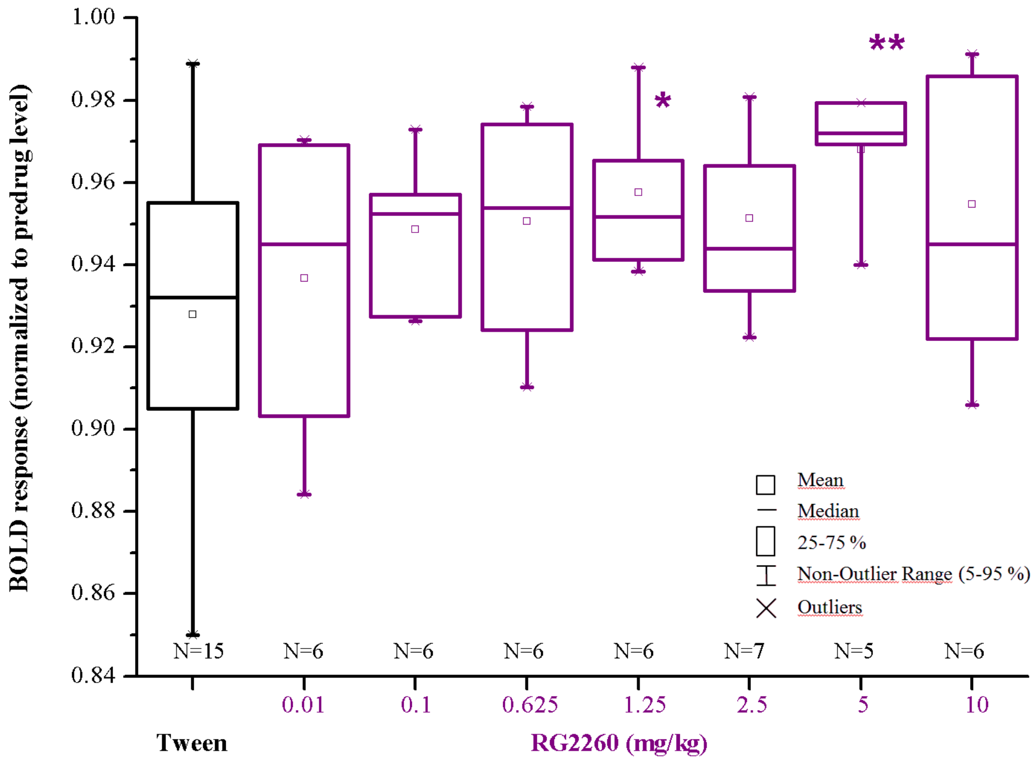


**Figure 21 Effect of RG2260 pretreatment on scopolamine evoked BOLD response changes in the prefrontal cortex.** RG2260 was administered at 0.01, 0.1, 0.625, 1.25, 2.5, 5, and 10 mg/kg intraperitoneally. Scopolamine was injected at 1 mg/kg intravenously. (one-way ANOVA, Fisher post-hoc test; *$p<0.05$; **$p<0.01$)

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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 22 and Table 3).

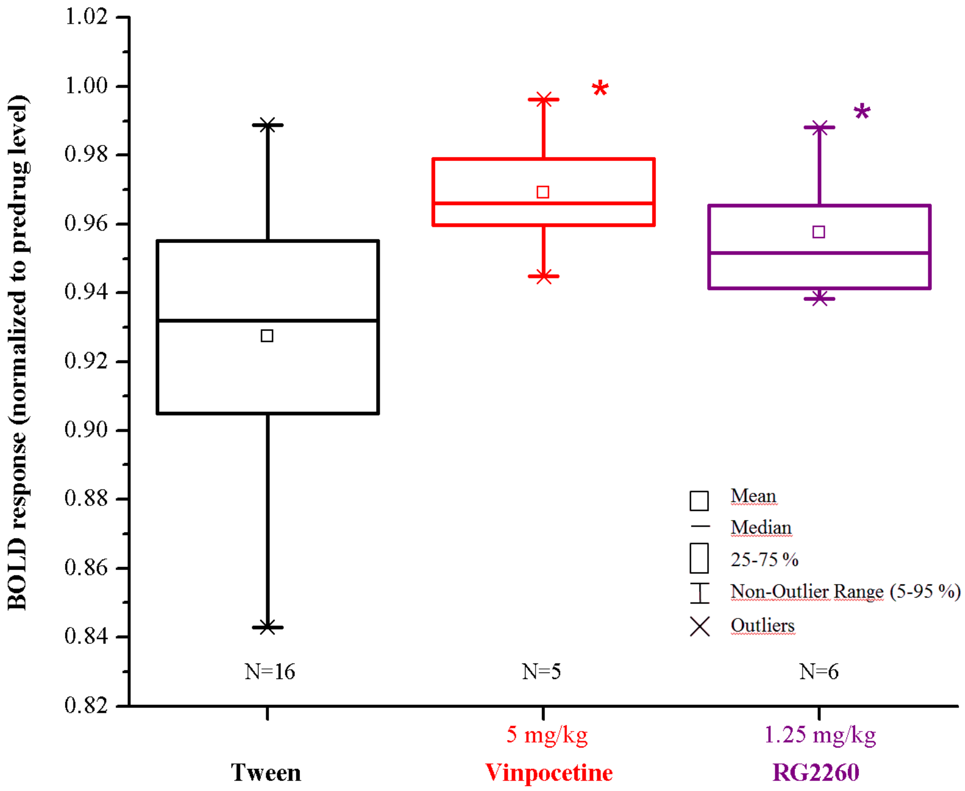


**Figure 22 Effects of vinpocetine and RG2260 pretreatment on scopolamine evoked BOLD response changes in the prefrontal cortex.** The efficacy of the new nootropic RG2260 compound was similar to that of vinpocetine, but it was effective in a lower dose range. Vinpocetine was administered at 5 mg/kg intraperitoneally. RG2260 was injected at 1.25 mg/kg intraperitoneally. Scopolamine was administered at 1 mg/kg intravenously. (one-way 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 23 and Table 3).

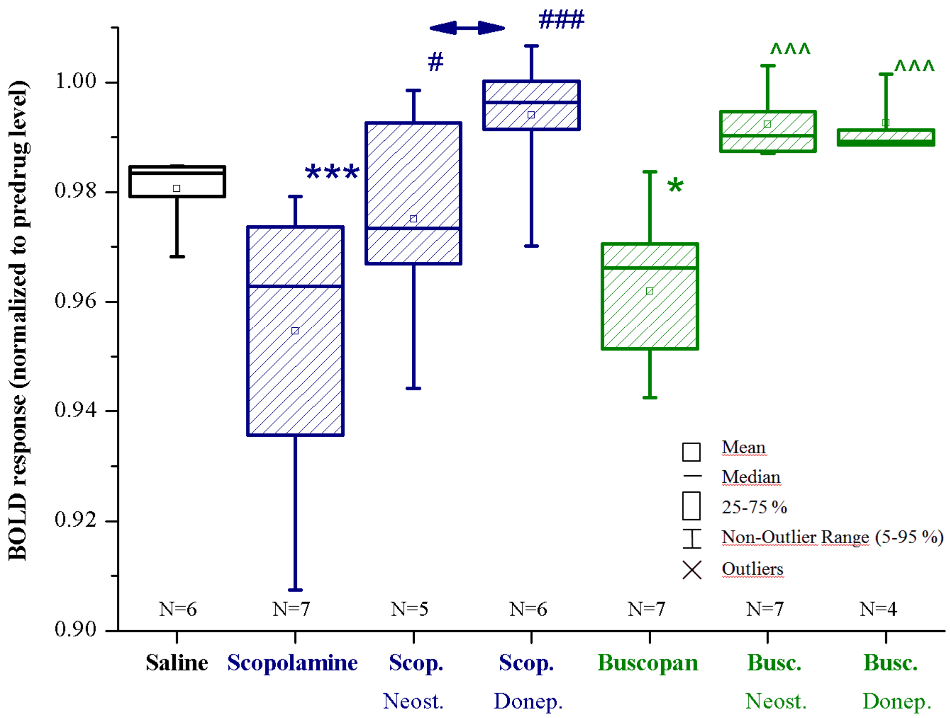


**Figure 23 Effects of saline, scopolamine and butylscopolamine (buscopan) on BOLD response in the rat brain after saline pretreatment, and prevention of the effect of scopolamine and butylscopolamine by neostigmine or donepezil pretreatment.** Scopolamine (Scop) was administered at 1 mg/kg intravenously. 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 $p<0.05$ (one-way ANOVA, Fisher post-hoc test).

## IV.2 Water labyrinth test

Table 4 presents the percent reversal of scopolamine-induced amnesia produced by the tested cognitive enhancers in the water labyrinth test (data are calculated from the group mean ± SEM values of pooled errors for all trials on the training days, as described in Methods). 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 24, 25, 26 and Table 4).

**Table 4 Effect of cognitive enhancers against (butyl)scopolamine induced amnesia in the water labyrinth test.**

| Compound | No. errors ± SEM | % effect | ANOVA |
|---|---|---|---|
| Vehiculum | 2.09 ± 0.31 | | |
| Scopolamine | 4.06 ± 0.45 | | $F(2.27)=1.44$, $p=0.2556$ |
| **Vinpocetine 5 mg/kg** | 3.37 ± 0.38 | **35.0%** | |
| Vehiculum | 1.35 ± 0.17 | | |
| Scopolamine | 5.57 ± 0.39$^{+++}$ | | $F(2.25)=14.93$, $p<0.0001$ |
| **Vinpocetine 7.5 mg/kg** | 3.7 ± 0.3 | **44.3%**$^{*}$ | |
| Vehiculum | 2.22 ± 0.18 | | |
| Scopolamine | 4.71 ± 0.32$^{+++}$ | | $F(2.27)=22.47$, $p<0.0001$ |
| **Vinpocetine 10 mg/kg** | 2.68 ± 0.21 | **81.5%**$^{***}$ | |
| Vehiculum | 2.43 ± 0.18 | | |
| Scopolamine | 3.59 ± 0.2$^{+}$ | | $F(2.27)=4.65$, $p=0.0184$ |
| **Piracetam 500 mg/kg** | 3.51 ± 0.29 | **6.9%** | |
| Vehiculum | 2.29 ± 0.13 | | |
| Scopolamine | 3.41 ± 0.15$^{+++}$ | | $F(2.27)=12.61$, $p<0.0001$ |
| **Piracetam 750 mg/kg** | 2.68 ± 0.13 | **65.2%**$^{**}$ | |
| Vehiculum | 2.02 ± 0.16 | | |
| Scopolamine | 3.39 ± 0.18$^{+++}$ | | $F(2.27)=10.10$, $p=0.0005$ |
| **Piracetam 1000 mg/kg** | 2.49 ± 0.17 | **68.2%**$^{**}$ | |

| **Compound** | **No. errors ± SEM** | **% effect** | **ANOVA** |
|---|---|---|---|
| Vehiculum | 1.59 ± 0.13 | | |
| Scopolamine | 3.07 ± 0.11$^{+++}$ | | $F(3.36)=12.13$, $p<0.0001$ |
| **Donepezil 0.25 mg/kg** | 2.33 ± 0.16$^{++}$ | **50%**$^{**}$ | |
| **Donepezil 0.5 mg/kg** | 2.13 ± 0.16$^{+}$ | **63.5%**$^{***}$ | |
| Vehiculum | 1.88 ± 0.13 | | |
| Scopolamine | 2.99 ± 0.15$^{+++}$ | | $F(3.35)=6.32$, $p=0.0015$ |
| **Donepezil 1 mg/kg** | 2.08 ± 0.13 | **82%**$^{**}$ | |
| **Donepezil 4 mg/kg** | 2.2 ± 0.15 | **71.2%**$^{**}$ | |
| Vehiculum | 1.86 ± 0.24 | | |
| Scopolamine | 4.6 ± 0.45$^{++}$ | | $F(3.35)=4.78$, $p=0.0067$ |
| **PNU-120596 3 mg/kg** | 3.39 ± 0.3$^{+}$ | **44.2%** | |
| **PNU-120596 10 mg/kg** | 3.59 ± 0.15$^{+}$ | **36.9%** | |
| Vehiculum | 1.68 ± 0.17 | | |
| Scopolamine | 4.1 ± 0.28$^{+++}$ | | $F(4.64)=8.23$, $p<0.0001$ |
| **EVP-6124 0.3 mg/kg** | 2.59 ± 0.2 | **62.4%**$^{*}$ | |
| **EVP-6124 1 mg/kg** | 2.66 ± 0.23 | **59.9%**$^{*}$ | |
| **EVP-6124 3 mg/kg** | 2.78 ± 0.33 | **54.5%**$^{*}$ | |
| Vehiculum | 1.54 ± 0.3 | | |
| Scopolamine | 4.38 ± 0.35$^{+++}$ | | $F(2.26)=17.94$, $p<0.0001$ |
| **Neostigmine 0.3 mg/kg** | 2.2 ± 0.21 | **76.8%**$^{**}$ | |
| Vehiculum | 1.42 ± 0.2 | | |
| Buscopan | 2.27 ± 0.28$^{+}$ | | $F(2.52)=4.19$, $p=0.0200$ |
| **Neostigmine 0.3 mg/kg** | 1.2 ± 0.17 | **100.0%**$^{*}$ | |
| Vehiculum | 1.7 ± 0.13 | | |
| Scopolamine | 2.83 ± 0.2$^{+++}$ | | $F(2.27)=11.9$, $p=0.0002$ |
| **RG2260 1.25 mg/kg** | 2.33 ± 0.14 | **44.2%**$^{*}$ | |

| Compound | No. errors ± SEM | % effect | ANOVA |
|---|---|---|---|
| Vehiculum | 1.71 ± 0.2 | | |
| Scopolamine | 2.61 ± 0.21$^{++}$ | | $F$(2.27)=5.79, $p$=0.0080 |
| **RG2260 2.5 mg/kg** | 1.77 ± 0.18 | **93.3%**$^{**}$ | |
| Vehiculum | 1.92 ± 0.17 | | |
| Scopolamine | 3.3 ± 0.2$^{+++}$ | | $F$(4.45)=10.28, $p$<0.0001 |
| **RG2260 5mg/kg** | 1.76± 0.14 | **100.0%**$^{***}$ | |
| **RG2260 10 mg/kg** | 2.27 ± 0.2 | **74.6%**$^{***}$ | |
| **RG2260 20mg/kg** | 2.27± 0.2 | **74.6%**$^{***}$ | |

+$p$<0.05, ++$p$<0.01, +++$p$<0.001 vs. control group; *$p$<0.05, **$p$<0.01, ***$p$<0.001 vs. (butyl)scopolamine-treated group (three-way repeated measures ANOVA, post-hoc Duncan test)

**Piracetam** at high doses (750 and 1000 mg/kg, $p$<0.01) significantly inhibited the amnesic effect of scopolamine (Figure 24A and Table 4) while the 500 mg/kg dose was inactive (Table 4). **Vinpocetine** proved to be effective at doses of 7.5 ($p$<0.05) and 10 mg/kg ($p$<0.001) in water labyrinth test (Figure 24B).

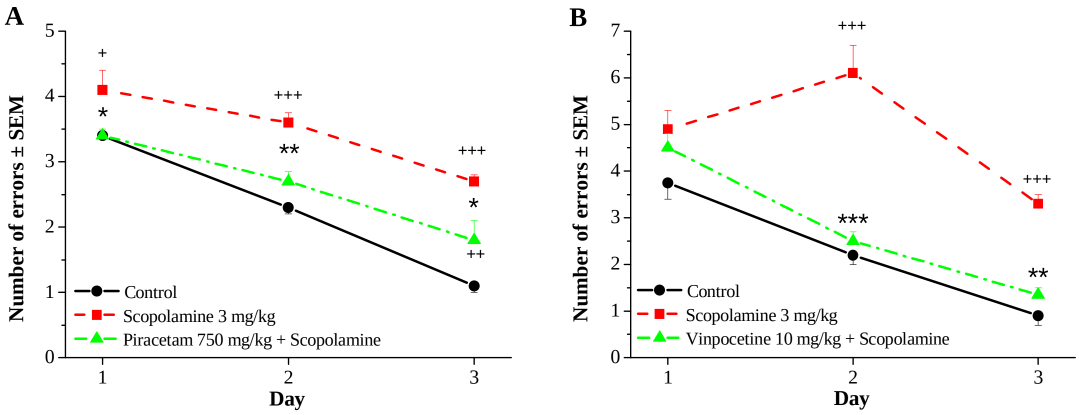


**Figure 24 Effects of piracetam (A) and vinpocetine (B) on the learning deficit induced by scopolamine in water labyrinth test.** Piracetam was adminstered at 750 mg/kg per os. Vinpocetine was tested at 10 mg/kg per os. Scopolamine was injected at 3 mg/kg intraperitoneally. +$p$<0.05, ++$p$<0.01, +++$p$<0.001 vs. control group; *$p$<0.05, **$p$<0.01, ***$p$<0.001 vs. scopolamine-treated group (three-way repeated measures ANOVA, post-hoc Duncan 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 (Figure 25A and Table 4). At 0.5, 1, and 4 mg/kg dose produced 63.5 %, 82 %, 71.2 % reversal respectively (Table 4). **Neostigmine** at 0.3 mg/kg dose also caused a highly significant ($p$<0.01) and considerable (76.8 %) reversal (Figure 25B and Table 4).

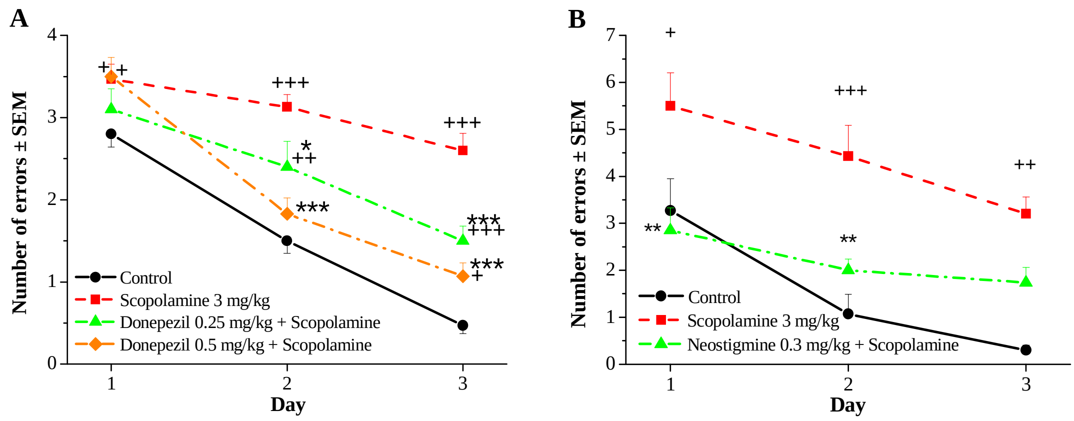


**Figure 25 Effects of donepezil (A) and neostigmine (B) on the learning deficit induced by scopolamine in the water labyrinth test.** Donepezil was administered at 0.25, 0.5 mg/kg per os. Neostigmine was injected at 0.3 mg/kg intraperitoneally. Scopolamine was administered at 3 mg/kg intraperitoneally. +$p$<0.05, ++$p$<0.01, +++$p$<0.001 versus control group; *$p$<0.05, **$p$<0.01, ***$p$<0.001 versus scopolamine-treated group (three-way repeated measures ANOVA, post-hoc Duncan test)

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 (Table 4). While **PNU-120596**, an alpha 7 nicotinic positive allosteric modulator (PAM) showed moderate, non-significant improving effect (Table 4).

The newly developed **RG2260** was effective in the water labyrinth test against scopolamine-induced memory impairment in rats (Figure 26). 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;

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44.2 % reversal); the maximal effect ($p$<0.001; 100 % reversal) was obtained at 5 mg/kg dose (Table 4).

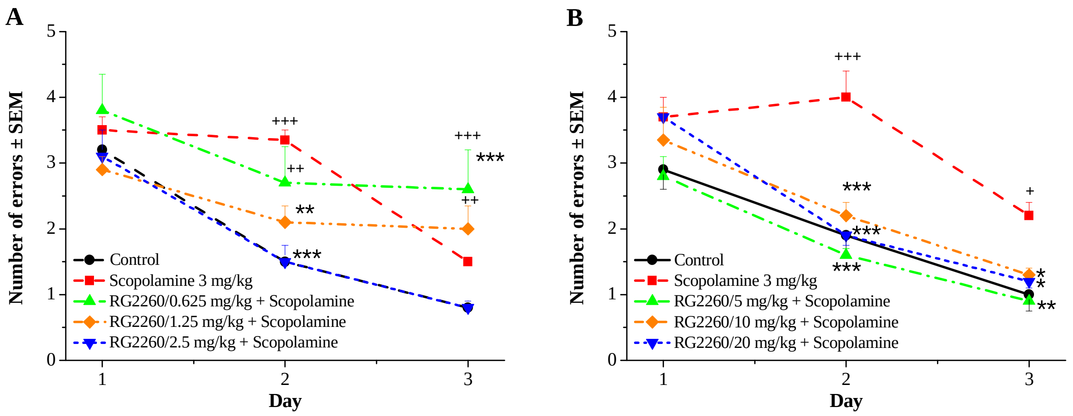


**Figure 26 Effect of RG2260 on the learning deficit induced by scopolamine in the water labyrinth test.** RG2260 was administered at 0.625, 1.25, 2.5, 5, 10, 20 mg/kg intraperitoneally. Scopolamine was injected at 3 mg/kg intraperitonally. +$p$<0.05, ++$p$<0.01, +++$p$<0.001 versus control group; *$p$<0.05, **$p$<0.01, ***$p$<0.001 versus scopolamine-treated group (three-way repeated measures ANOVA, post-hoc Duncan test)

Butylscopolamine showed a dose-dependent effect; it caused significant ($p$<0.05) impairment in the learning performance at 3 mg/kg dose (Figure 27A), which was reversible by 0.3 mg/kg of neostigmine ($p$<0.05; Figure 27B).

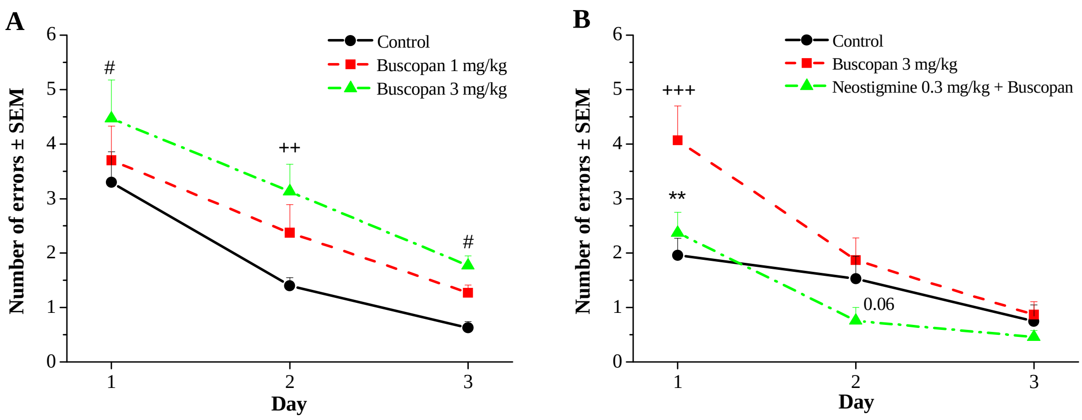


**Figure 27 Effects of butylscopolamine (A) and effect of neostigmine (B) on the learning deficit induced by butylscopolamine in the water labyrinth test.** Butylscopolamine was administered at 1 and 3 mg/kg intraperitoneally. Neostigmine was injected at 0.3 mg/kg intraperitoneally. #$p$<0.1,++$p$<0.01,+++$p$<0.001 versus control group, **$p$<0.01 versus butylscopolamine-treated group (three-way repeated measures ANOVA, post-hoc Duncan test)

To summarize our results, the following table (Table 5) contains the applied doses of procognitive agents in fMRI (left size) and water labyrinth tests (right side). Bold numbers indicate those doses which were able to significantly inhibit the (butyl)scopolamine induced changes.

**Table 5 Cognitive enhancers in fMRI and water labyrinth tests.**

| **Applied doses (mg/kg) in fMRI measurements** | **Cognitive enhancers** | **Applied doses (mg/kg) in water labyrinth tests** |
|---|---|---|
| 1; **4;** 10 | **Donepezil** | **0.25; 0.5; 1; 4** |
| 100; 500; 1000 | **Piracetam** | 500; **750; 1000** |
| **5** | **Vinpocetine** | 5; **7.5; 10** |
| **3** | **PNU-120596** | 3; 10 |
| **1** | **EVP-6124** | **0.3; 1; 3** |
| 0.01; 0.03; **0.1;** 0.3 | **Neostigmine** | **0.3** |
| 0.01; 0.1; 0.625; **1.25;** 2.5; **5;** 10 | **RG2260** | 0.625; **1.25; 2.5; 5; 10;20** |

Bold numbers indicate those doses which were able to significantly inhibit the (butyl)scopolamine induced changes. (fMRI measurements: one-way ANOVA Fisher post-hoc test; $p<0.05$; water labyrinth test: three-way repeated measures ANOVA Duncan post-hoc test; $p<0.05$)

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# V DISCUSSION

Dementias including Alzheimer's disease (AD) are major public health problems worldwide with a very high unmet need for treatment. A variety of theories about the cause or origin of Alzheimer's disease has been proposed and pointed out several molecular targets while a compelling clinical proof is still absent.

Amyloid and tau pathology are undoubtedly associated with AD, yet their causalities have been eroded over time. Probably, Aβ is merely a marker and a later consequence of upstream changes (Drachman, 2014). The focus of research seems to be shifting from the amyloid cascade to vascular and endothelial pathological processes in dementias and more and more such papers can be found nowadays in the literature. Based on these facts, instead of the neuronally acting drugs, the role of neurovascular unit, vascular endothelium and blood supply come into the limelight of the Alzheimer's research (Briyal et al., 2011; Leonard et al., 2011; Palmer and Love, 2011).

## V.1 The neurovascular connection

With age, the brain circulation may be reduced by 30 % which makes the elderly susceptible to AD. Vascular risk factors have long been associated with neurodegeneration and dementia (Marchesi, 2011). There are other associated diseases – atherosclerosis, diabetes, cardiovascular diseases and other conditions –, which may reduce further the brain perfusion. The metabolic energy crisis resulted from circulatory inefficiency initiates abnormal Aβ processing, especially trafficking through brain vascular walls (Bell et al., 2009). This circulatory inefficiency downregulates Aβ transporters at the vascular wall (Bell et al., 2009; Dotti and De Strooper, 2009) and the accumulation of Aβ will aggravate cholinergic losses. In animal study, the anticholinergic scopolamine not only decreases blood flow and cognitive performance (Bihaqi et al., 2012), but also elevates Aβ level (Chen et al., 2014).

The pressure of oxygen saturated arterial blood is largely dissipated when the blood enters the capillaries. Therefore, usual vasodilating pharmacological agents have only little direct effect on brain capillary flow. So as to improve brain function, the capillary circulation needs to be improved or restored. Neuronally acting drugs can increase blood flow via neurovascular coupling. However, in disease state, when the neurovascular coupling is injured, these drugs can exacerbate the metabolic need and accelerate the secondary neurodegenerative processes. So, stimulation of neuronal activity, when energy metabolism is limited by impaired circulation, is probably a false concept.

## V.2 The role of pericytes in microcirculation

In the brain, pericytes cover some 80 % of microvessels, other organs are less abundant in pericytes (ElAli et al., 2014; Winkler et al., 2012). The abundance in pericytes seems to be proportional to the barrier function characterizing an organ (Gonul et al., 2002). On sustained hypoxia, pericytes migrate away from blood vessels or die (Farkas and Luiten, 2001; Hall et al., 2014). In AD subjects, the loss or degeneration of pericytes is described (Farkas and Luiten, 2001). Due to the lacking pericytes, small blood vessels lose responsiveness to physiological, neural or luminal stimuli, which renders functional incompetence of neurons following hypoxia. Pericyte losses also initiate tau pathology (Cleary et al., 2005).

The increase in blood flow after neuronal stimuli is due to the prompt relaxation of capillaries and the subsequent dilatation of small arterioles (Hall et al., 2014). This quick alteration of capillary flow on stimuli seems to be regulated predominantly by pericytes.

Pericytes have a set of neurochemical receptors (Hamilton et al., 2010) and can react by constriction or relaxation on ligand stimuli. Apparently, pericytes can receive paracrine stimuli either from the direction of neurons through astrocytes or from the endothelial cells. These endothelial cells convey luminal signals. The main modulators of CBF (following changed neural activity) are the pericytes. However, the neurovascular coupling is bidirectional (Peppiatt et al., 2006) and luminal signals may also activate neuronal function.

## V.3 Why is BOLD fMRI a good choice to measure neuronal activities?

The great advantage of this fMRI BOLD-based method is its high sensitivity to the vascular action of drugs and its high translational power.

BOLD fMRI detects signals from capillaries and arterioles. The higher the field strength of the magnet the smaller the blood vessel we can detect. Whereas about 70 % of the BOLD signal arises from larger vessels in a 1.5 T scanner, about 70 % arises from smaller vessels in a 4 T scanner (Huettel et al., 2008). Consequently, our 9.4 T MRI equipment predominantly detects changes originating from capillaries.

The translational value of BOLD fMRI is demonstrated by several human studies (Anderson et al., 2002; Deakin et al., 2008; Stein et al., 1998), where scopolamine was used as a memory disturbing agent (Beatty et al., 1986; Caine et el., 1981; Drachman and Leavitt, 1974; Flicker et al., 1990; Ghoneim and Mewaldt, 1975; Klinkenberg and Blokland, 2010; Lenz et al., 2012; Snyder et al., 2005). Generally, small animal fMRI studies could form a bridge between behavioral pharmacological animal models and human clinical studies.

## V.4 How do amnestic agents work?

The blockade of central muscarinic receptors can induce a pattern of cognitive impairment in healthy subjects that is reminiscent of Alzheimer’s disease. Therefore, the scopolamine-based cognitive animal models generally considered suitable for dementia research. Scopolamine also has direct vascular effects (proved by its butyl analog, buscopan), which are responsible for its memory disturbing effect to a remarkable extent.

In our cognitive model, scopolamine and butylscopolamine have significant BOLD effects in the prefrontal cortex (PFC) only. This area of brain implicates working memory, attention, response initiation, and management of autonomic control and emotion (Zhou et al., 2011). In humans, dysfunction of prefrontal cortical areas, with which the medial prefrontal cortex (mPFC) of rats is most likely comparable, is related to psychopathology including schizophrenia, sociopathy, obsessive-compulsive disorder, depression, and drug abuse (Heidbreder and Groenewegen, 2003). The medial prefrontal cortex is one of the main part of the so-called default mode network (DMN), which plays a key role in the mental processes (Broyd et al., 2009). The measured negative effect of (butyl)scopolamine on the BOLD responses in the prefrontal cortex fits well its negative cognitive effect.

## V.5 The advantage of provocation model

Several arguments support the use of provocation model in fMRI studies. The effect of cognitive compounds can be different in a normal and a diseased brain or in pharmacologically or physiologically created conditions. Using scopolamine, the cognitive deficit can be modelled, as is found in several studies (Beatty et al., 1986; Caine et al., 1981; Drachman and Leavitt, 1974; Flicker et al., 1990; Ghoneim and Mewaldt, 1975; Klinkenberg and Blokland, 2010; Lenz et al., 2012). On the other hand, many compounds have poor solubility; adjuvants should be used for their injectable formulation. Since fMRI readings are taken immediately after and during the administration of test material, the effect of treatment itself becomes visible. Such effects are the alteration of the circulating blood volume, osmotic pressure or pH. Adjuvants in the treatment formulation are also often active in phMRI studies; their effects can mimic the effect of test compounds. When the test drug is administered one hour before the provoking agent, those unwanted effects are already not visible by the time of BOLD scanning.

## V.6 How do cognitive enhancers work?

Several cognitive enhancer reference compounds with various mechanisms of action were tested in our provocation model. They could be tested directly if indifferent pharmaceutical formulation was possible, or in a provocation model against scopolamine. Donepezil and piracetam we could test directly.

The acetylcholinesterase inhibitor **donepezil** had a weak effect at 4 mg/kg; it caused some BOLD increase in the hippocampus and temporal cortex. At 10 mg/kg dose, the time dependence of the response was different; donepezil caused a bi-phasic response but in some regions, such as prefrontal cortex, only the negative component could be registered. Similar negative effects of donepezil on healthy brain were reported by Balster et al. (2011) in humans.

**Piracetam** caused some fast but short, dose-independent, non-significant increase of BOLD responses in all studied brain regions.

In contrast, **donepezil** showed a clear dose-dependent effect in the scopolamine model while **piracetam** revealed a clear, but non-significant trend. Piracetam had a much weaker BOLD effect in comparison with that of cognitive animal models and human therapy. The action of donepezil was strong and statistically significant.

**Vinpocetine** (Cavinton®) is a synthetic derivative of Vinca Minor alkaloid, vincamine. The complex mechanism of action has not been fully clarified yet. It presumably decreases the hypoxia in the brain by enhancing the metabolic rate of oxygen and glucose (Erdő et al., 1990), increases the ATP/cAMP levels, and improves the cerebral microcirculation due to the reduced cerebral vascular resistance (Kuzuya, 1985). Cavinton® was tested at only one dose (5 mg/kg) in the scopolamine model. It caused significant reversal of scopolamine-induced BOLD response decrease in the prefrontal cortex.

**EVP-6124** is a partial agonist of neuronal α7 nicotinic acetylcholine receptor. It improves the memory after short-term memory impairment in sub-to low nanomolar dose range, which seems to be dose-dependent. EVP-6124 can increase the cerebral blood flow directly as well (Si and Lee, 2002). This nootropic agent is still in development phase but the results are very convincing; it may become an effective cognitive enhancer in the future (Prickaerts et al., 2012). EVP-6124 was tested at only one dose (1 mg/kg) in our scopolamine model. It caused significant reversal of scopolamine-induced BOLD response decrease in the prefrontal cortex.

**PNU-120596** is a Type II positive allosteric modulator (PAM) molecule of α7 nicotinic acetylcholine receptor. It increases directly the cerebral blood flow (Si and Lee, 2002), the peak current, the opening time of the ion channel, and destabilizes the receptors in their desensitized state (Grønlien et al., 2007; Hurst et al., 2005). This agent is one of the most effective PAMs as of now but, it is still in development phase, too (Williams et al., 2011a). PNU-120596 was tested at only one dose (3 mg/kg) in the scopolamine model. It caused significant reversal of scopolamine-induced BOLD response decrease in the prefrontal cortex.

**Neostigmine**, the quaternary nitrogen-containing inhibitor exerts its effect only peripherally and it is unable to enter the CNS. In our study neostigmine (0.1 mg/kg) was also capable of preventing the effect of both scopolamine and butylscopolamine. These BOLD results suggested that the memory-disturbing effect of scopolamine was mediated to a considerable extent by a vascular mechanism. The impairment of cognition by anticholinergic drugs probably happened via a vascular mechanism. The primary mechanism of procognitive cholinesterase inhibitors was probably also vascular. The cross-checking of brain-penetrating or non-penetrating anticholinergic drugs and cholinesterase inhibitors proved that, at least in the prefrontal and frontal cortex, intact circulation is a determining factor for normal cognition.

**RG2260**, the newly developed Richter compound is a special cognitive enhancer molecule, which may mean a new opportunity for the treatment of dementias or Alzheimer’s disease. The wide receptor profiling of RG2260 did not reveal any substantial affinities to conventional molecular targets that play important roles in cognitive processes. The lack of affinity to any cognition related molecular target and also the brain impenetrability of RG2260 greatly reduces the number of possible mechanisms by which cognitive enhancement could occur. The most likely effector of its action is the contractile cells embracing capillaries, the pericytes. They can be found mostly in cerebral capillaries and missing from peripheral microvessels. The distribution of pericytes explains the RG2260 compound selective cerebrovascular effect (not published data). The capillary dilatation is not a secondary response to increased pressure produced by arteriole dilatation (Hall et al., 2014). Since RG2260 does not modulate cholinergic receptors or cholinesterase activity at all, its molecular mechanism differs that of the widely used cholinesterase inhibitors.

RG2260 prevented the scopolamine-induced BOLD reduction in the PFC of rats in the functional MRI screens. It was tested at a dose range of 0.625-20 mg/kg. From 1.25 mg/kg dose, RG2260 significantly affected the scopolamine evoked BOLD response changes and restored the impaired cognitive performances.

As the above-mentioned two peripherally acting agents showed, neurovascular coupling works in both directions. Increased neuronal activity induces circulation, which is reflected by a positive BOLD answer. Cholinergic inhibition reduces the circulation of prefrontal vasculature which in turn inhibits normal neuronal activity reflected by impaired learning and a negative BOLD while cholinergic stimulation restores normal circulation and neuronal functioning.

## V.7 The water labyrinth tests confirm the result of fMRI studies

The water labyrinth tests confirmed the conclusions drawn from the fMRI BOLD studies. Scopolamine, as well as butylscopolamine, caused deterioration in the learning performance of rats. The effects of tested drugs were somewhat different in the water labyrinth test. **Piracetam** showed statistically significant inhibitory effect above 500 mg/kg dose. **Donepezil** also was more effective in the behavioral model. **Neostigmine**, similar to donepezil, significantly improved the learning deficit induced by scopolamine or buscopan, too. Results of **vinpocetine** and **EVP-6124** studies showed good correspondence with the phMRI results obtained in the provocation model. The optimal vehiculum and drug administration protocol were different in behavioral models (p.o. higher volume) and MRI study (i.p. lower volume). It might have caused the difference between the effect of **PNU-120596** (very low solubility) in phMRI and in the cognitive test. **RG2260** was highly effective in water labyrinth tests as well. From 2.5 mg/kg dose to the examined 20 mg/kg dose restored the impaired performance significantly.

## V.8 Looking to the future

The statement that "Alzheimer's disease is a vascular disorder with neurodegenerative consequences rather than a neurodegenerative disease with vascular consequences" seems to be increasingly justified by accumulating evidence (de la Torre and Stefano, 2000; Dotti and De Strooper. 2009). So the triggering of the well-determined vicious circle may be prevented by the maintenance of good cerebral blood circulation. Most importantly, improving blood circulation, especially in regions associated with cognitive processes, may reverse cognitive impairment initiated by insufficient cellular energy production. This may happen without interfering neurotransmitters or their receptors.

Drugs that have effects on the CNS but do not penetrate BBB are not unprecedented: cholinesterase inhibitors exert their cognitive effects mostly by vascular action, since peripherally acting cholinesterase inhibitors and anticholinergic agents reveal nearly identical efficacy in cognitive and fMRI models. Therefore, it is reasonable to assume that a vascularly acting, BBB non-penetrating compound may have cognitive effects whenever microcirculation limits cognitive performance. Most importantly, in order to exert an effect on pericytes, a drug compound does not need to be present in the brain parenchyma tissue. The targeting of pericytes in the prevention and cure of dementias is suggested by this and other studies (ElAli et al., 2014; Hamilton et al., 2010; Winkler et al., 2014), which would provide a selective brain-specific action.

In summary, our scopolamine-based provocation phMRI model in rats is suitable for testing procognitive agents with various mechanisms of action. Similar studies can be performed on human volunteers, probably in phase 0; translational studies of new compounds in exploratory phase could decrease the chance of false positive results in phase II/III trials, reducing the time and cost of development of new effective cognitive therapeutics.

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# VI 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.

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# VII SUMMARY

There is a huge unmet need to understand and treat pathological cognitive impairment. The development of disease-modifying cognitive enhancers is hindered by the lack of correct pathomechanism and suitable animal models. Fortunately, the highly expanding field of small animal MRI seems to be a robust method, which may provide us with a new approach that has 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 for preclinical drug discovery with excellent temporal and spatial resolution.

Some pharmacological agents such as scopolamine or its butyl analog buscopan evoke similar effects on cognition and cerebral circulation in rodents and humans. The effects of deemed procognitive agents (donepezil, piracetam, vinpocetine, neostigmine, EVP-6124, PNU-120596, RG2260) were compared on BOLD responses and also linked to rodent cognitive models. These drugs revealed significant reversal of negative BOLD signal except for piracetam. In the water labyrinth test, only PNU-120596 did not show any significant effect.

Concordant result of fMRI and behavioral tests prove that blood-brain barrier penetrating molecules and some non-blood-brain barrier penetrating drugs produce robust central nervous system effects as well. Since neurovascular coupling works in both directions, the increased capillary circulation stimulates neuronal activity, which appears in the form of improved cognition.

Altogether our scopolamine-based fMRI provocation model is suitable for testing procognitive compounds. These fMRI experiments can be paralleled with human studies, which may help reduce the number of failed cognitive clinical trials.

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# VIII ÖSSZEFOGLALÁS

A jelenlegi gyógyszerkutatás egyik legfőbb iránya a kognitív funkciók javítása. Több állatmodell ismert, mely alkalmas hatóanyagok kognitív hatásának vizsgálatára, azonban ezek általában kis transzlációs erővel bírnak. Az utóbbi időben elterjedt, kisállatok vizsgálatára alkalmas fMRI (funkcionális mágneses rezonanciavizsgálat) nagyteljesítményű, hatékony transzlációs módszernek mondható, mellyel a humán és állatkísérletek eredményei összehasonlíthatóvá válnak. Az általunk használt állatmodellben szkopolamin és butilszkopolamin BOLD válaszra gyakorolt hatását vizsgáltuk patkányok agyában. A kialakuló negatív BOLD jel a prefrontális kéreg területén jól magyarázza a szer memóriakárosító hatását. A létrejövő reverzibilis memória romlást különböző kognitív javító ágensekkel (donepezil, piracetam, vinpocetin, neosztigmin, EVP-6124, PNU-120596, RG2260) sikeresen visszafordítottuk.

Az általunk végzett fMRI méréseket viselkedésfarmakológiai tesztekkel összevetve hasonló ereményeket kaptunk, melyek alapján kijelenthető, hogy mind vér-agy gáton átjutó, mind pedig a penetrációra képtelen vegyületek rendelkeznek centrális hatással. Mindezen megfigyeléseink a neurovaszkuláris kapcsolatok kétirányúságán alapulnak. Ugyanis a megnövekedett kapilláris áramlás helyreállítja a neurális aktivitást, ami javuló kognitiv képességekben nyilvánul meg.

Eredményeink alapján kijelenthető, hogy az általunk alkalmazott provokációs modell alkalmas különböző prokognitív anyagok tesztelésére, centrális hatású anyagok hatásmechanizmusának vizsgálatára különböző agyterületekre kifejtett hatásaik alapján.

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# X 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.

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# XI 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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