# CHALLENGING THE CHOLINERGIC SYSTEM:

## AGEING, COGNITION & INFLAMMATION

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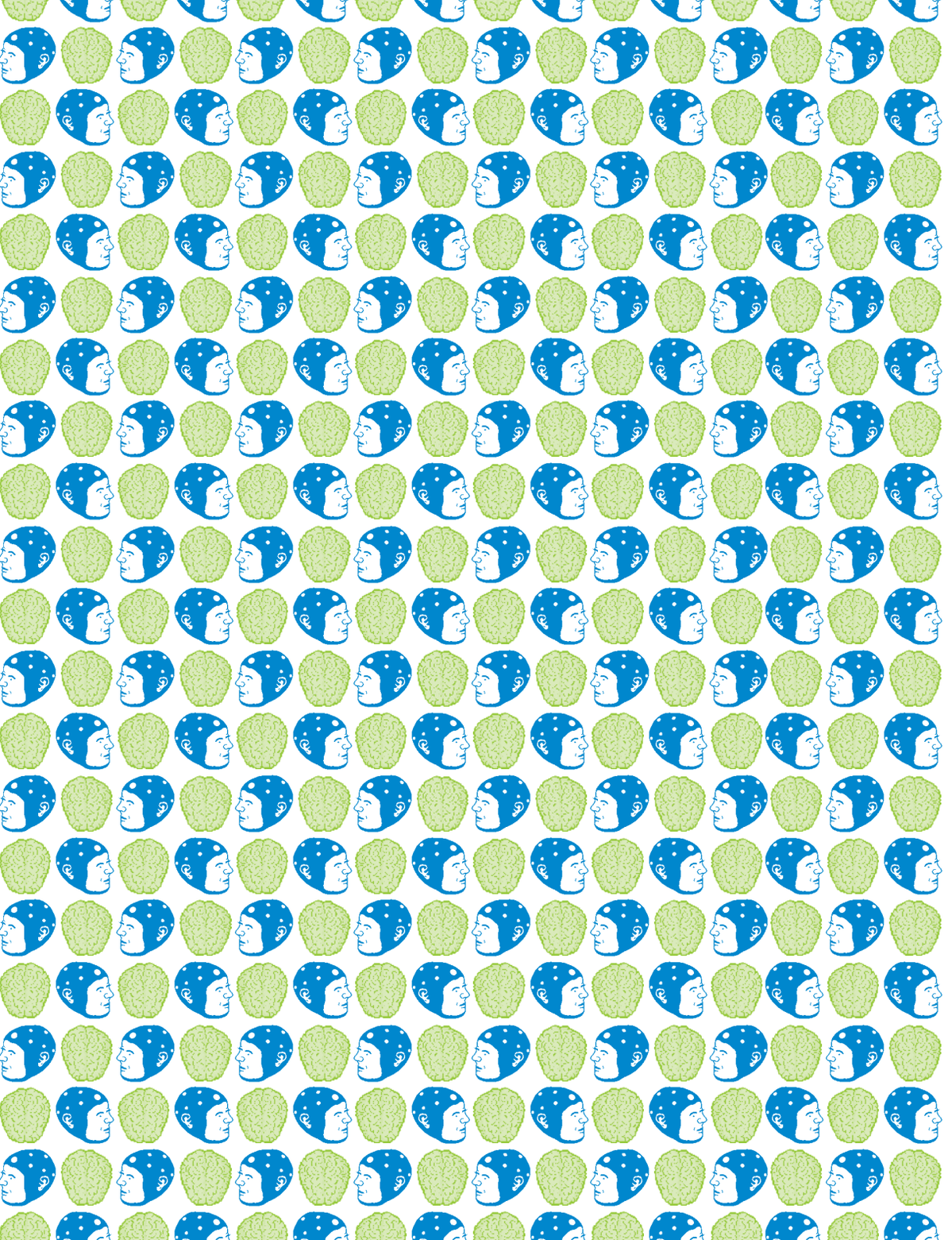

CHALLENGING THE CHOLINERGIC SYSTEM: AGEING, COGNITION & INFLAMMATION

*A MIS PADRES Y ABUELOS*

# CHALLENGING THE CHOLINERGIC SYSTEM: *AGEING, COGNITION & INFLAMMATION*

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# GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

## CHOLINERGIC SYSTEM DISCOVERY AND PHYSIOLOGY

In 1921 Otto Loewi and his co-workers discovered the chemical transmission of nerve impulses, demonstrating that the parasympathetic substance (Vagusstoff, translated from German as 'Vagus Substance'), today known as acetylcholine, played an important role at the sympathetic nerve endings (Loewi, 1921). Based on these observations he received the Nobel Prize of Medicine in 1936, jointly with Sir Henry Dale, who actually identified acetylcholine in the first place with his colleague Arthur Ewins (Ewins, 1914). It was Loewi, however, who showed its important role in the nervous system.

The cholinergic system comprises organized nerve cells that use the neurotransmitter acetylcholine to activate other neurons, mostly by containing and releasing acetylcholine, propagating a nerve impulse. Initial experiments to investigate acetylcholine's central origin were performed. Acetylcholine or diisopropylfluorophosphonate (an acetylcholinesterase inhibitor) induced effects when the compounds were administered in an experimental *cerveau isolé* animal model via carotid injection, but not via de isolated hemisphere preparation, suggesting that acetylcholine production occurred in the brainstem (Jasper, 1965; Karczmar, 1967). Later on, the concepts were supported by the observations that brain electric stimulation increased cortical acetylcholine concentrations (MacIntosh and Oborin, 1953) and, thereafter with immunohistochemistry techniques (Koelle and Friedenwald, 1949). These investigations gave rise to the discovery of the cholinergic alerting mesodiencephalic system or, as we know it today, the (ascending) reticular activating system (ARAS), a network containing the cholinergic and adrenergic system that regulates wakefulness and sleep-awake transitions (Figure 1.1).

Acetylcholine is produced in several brain structures including the basal forebrain (nucleus basalis, diagonal band, medial septum and substantia innominata), ventral tegmental area, raphe and locus ceruleus. All these conglomerates of cholinergic cells project their axons to different areas of the brain exerting mainly an excitatory effect, binding to different acetylcholine receptors (AChRs) in the thalamus and cortex (Mesulam, 2013). The cholinergic system has been associated with a number of cognitive functions, including memory, selective attention, language, reaction time to stimuli and emotional processing (Furey, 2011). In the periphery, acetylcholine also has important functions binding to AChRs in autonomic ganglia and in the neuro-muscular junction (Brunton *et al*, 2011). The functions of acetylcholine are sub served by two different types of receptor: muscarinic and nicotinic.

## THE MUSCARINIC ACETYLCHOLINE RECEPTOR

### RECEPTOR CONFORMATION AND PHYSIOLOGY

Five subtypes ($M_1$-$M_5$) have been characterized. All 5 types are expressed in the CNS, where they play a role in learning and memory, arousal, rapid eye movement sleep, control of movement, thermoregulation and reward behaviour (Pennartz *et al*, 1994; Picciotto *et al*, 2012; Vazquez and Baghdoyan, 2001). In the periphery, muscarinic activation is associated with parasympathic autonomic functions such as a reduction in heart rate ($M_2$) and vasodilatation ($M_1$, $M_2$, $M_3$), increases in exocrine secretions from sweat, salivary and lacrimal glands ($M_1$, $M_3$), and contraction of smooth muscle in the gastrointestinal tract ($M_2$, $M_3$) or airways ($M_3$, $M_4$) (Caulfield, 1993). Muscarinic acetylcholine receptors (mAChR) are members of the 7 transmembrane guanine nucleotide-binding protein (G protein)-coupled receptor (GPCR) superfamily. In general, $M_1$, $M_3$, $M_5$ receptors act via activation of G proteins while $M_2$, $M_4$ receptors perform inhibitory functions. Activation of the receptor coupled G proteins result in activation of an enzyme to generate a second messenger or producing a response by interaction directly with an effector, usually an ion channel (Birnbaumer *et al*, 1990). Knock-out mice of the muscarinic receptor have provided information on the executive functions of the receptor in the CNS. $M_1$ knock-out mice have demonstrated subtle detrimental effects in learning and memory, however none significant effects on behaviour or motor and coordination functions (Miyakawa *et al*, 2001). $M_4$ mice showed a significant increase in basal motor activity, interestingly these mice were also hyper-responsive to stimulation with a $D_1$ dopamine receptor agonist, suggesting that $M_4$ AChRs play a predominant role in dampening $D_1$ -mediated effects on locomotor activity (Gomeza *et al*, 1999). $M_3$ AChR has been related to centrally mediated regulation of feeding behavior and body weight (Yamada *et al*, 2001) and $M_5$ might have an important role in addiction and motivational behaviour (Basile *et al*, 2002).

### RECEPTOR LOCALIZATION, RELATED COGNITIVE FUNCTIONS, LINK TO DISEASES AND THERAPEUTIC POSSIBILITIES

Table 1.1 shows the localization and muscarinic receptor distribution in the rat brain. The highest density of $M_1$ receptors is located mainly in cortex, hippocampus and striatum, which is consistent with effects on memory and cognition (Flynn *et al*, 1997). Interest in cholinergic agonists to treat Alzheimer's disease (AD) has predominantly been driven by the 'cholinergic hypothesis', relying on the fact that a degeneration of cholinergic neurons is associated with the memory and cognitive loss observed with the disease (Coyle *et al*, 1983; Whitehouse *et al*, 1982). Interest in specific $M_1$AChRs agonists grew with the discovery that $M_1$AChRs has a major role in hippocampal-based memory and learning regulation of cognition and short-term memory, all functions affected in AD. Also, post-mortem studies have demonstrated decreased levels of $M_1$ and $M_4$ AChRs in patients diagnosed with schizophrenia (Dean *et al*, 1996, 2002). The first $M_1$ and $M_4$ agonists to reach late clinical development, xanomeline, provided evidence of the cognitive improvement after muscarinic stimulation in patients with AD (Bodick *et al*, 1997) and schizophrenia (Shekhar *et al*, 2008), however due to adverse events secondary to peripheral muscarinic stimulation the compound development was abandoned. Aiming for a more selective central mechanism of action led to the development of allosteric agonists and positive allosteric modulators such as TBPB, 77-LH-28-1, AC260584 and thereafter VU0186470 and VU0357017, which are currently in clinical development with promising early development results (Jones *et al*, 2012). More recently, interest increased even more after chronic use of $M_1$AChR agonists, namely AF102B and talsaclidine, in AD patients compared to controls resulted in a significant decrease of CSF A$\beta$ in AD patients, whereas the acetylcholinesterase inhibitor (AChEIs) physostigmine, galantamine and donepezil did not (Hock *et al*, 2003; Nitsch *et al*, 2000; Parnetti *et al*, 2002). Additionally, $M_1$ localization in the striatum is consistent with its role in control of movement and the noted therapeutic benefit of $M_1$ antagonists for movement disorders like Parkinson's disease (PD) (Xiang *et al*, 2012). As shown in Table 1.1, the $M_4$AChR is highly expressed in the striatum, hippocampus, and neocortex and can modulate dopaminergic signaling, being able to reduce striatal dopamine release (Threlfell *et al*, 2010). $M_4$ allosteric modulators (LY2033298, VU0152099 and VU0152100) are in early phase development for cognitive symptoms of neurodegenerative diseases. $M_5$ positive allosteric modulators are still in preclinical development as potential cognitive enhancers (Bridges *et al*, 2009).

## THE NICOTINIC ACETYLCHOLINE RECEPTOR

### RECEPTOR CONFORMATION

Nicotinic acetylcholine receptors (nAChR) are ligand-gated neurotransmitter coupled ion channel receptors composed of eleven different subunits. The subunits can be sub-classified into two groups, containing eight $\alpha$ and three $\beta$ subunits. The nAChR situated in the muscle is $\alpha_1$, which is the key calcium channel receptor in the neuromuscular junction. The neuronal subunits are $\alpha_2$-$\alpha_9$, all of them determine the agonist binding (functional) subunits. The significance of $\beta$ subunits which are categorized in $\beta_2$-$\beta_4$ is primarily structural, rather than functional. However it has been demonstrated that both $\alpha$ and $\beta$ subunits contribute to the pharmaceutical specificity of the receptors (Gotti *et al*, 2006). Each subtype differs in properties such as localization, up-regulation, channel kinetics and desensitization. Each receptor is formed by 5 homologous subunits, either combining $\alpha$ and $\beta$ or only $\alpha$ subunits ($\alpha_7$, $\alpha_8$, and $\alpha_9$ are the only subunits that can form homomeric receptors). $\alpha_7$ is the predominant homomeric nAChR distributed in the mammalian brain (Dani and Bertrand, 2007). Table 1.2 shows the predominant localization of the main nAChRs in the brain.

### RECEPTOR PHARMACOLOGY

Brain nAChRs are expressed in postsynaptic terminals at neurons where binding exerts a fast excitatory synaptic transmission. On the other hand, most of the receptors are located presynaptically, where binding mainly modulates neurotransmitter release into the synaptic cleft (Clarke, 1993). For example, both $\alpha_4\beta_2$ and $\alpha_7$ AChRs modulate the release of GABA in hippocampal CA1 interneurons (Alkondon and Albuquerque, 2001). Instead

of terminating in synaptic targets, it has been postulated that the majority of cortical and hippocampal cholinergic projections release sites are non-synaptic and contribute to diffuse volume transmission. In other words, activation of the system causes an effect in large areas of the brain (Descarries *et al*, 1997). Functionally, central nAChRs have high calcium permeability and the receptor is able to rectify the inward currents when the membrane is depolarized (Mathie *et al*, 1990). All nAChRs in the presence of agonist first open the ion channel in several millisecond bursts followed by a desensitized conformation characterized by a closed channel and higher binding affinity conformational change (Quick and Lester, 2002). The receptors are also able to induce allosteric modulation, which are conformational changes, which lead to a response caused by binding of ligands to sites different to the agonist-binding sites (Changeux and Edelstein, 1998). Steroids, specifically 17$\beta$-estradiol, are examples of ligands that modulate the human $\alpha_4\beta_2$ nAChR (Paradiso *et al*, 2001). Adding complexity to understanding the system, nAChR up- or down-regulation occurs independently of the receptor in the presence of a ligand. For example, nicotine-induced up-regulation of $\alpha_3$-associated (predominantly $\alpha_3\beta_2$) and $\alpha_7$ nAChRs in the same hippocampal neurons differ under the same nicotine concentration (Ridley *et al*, 2001). Furthermore, during the development of new compounds with $\alpha_7$ nAChR agonistic activity, namely EVP-6124 and PHA543613, evidence of a bell-shaped or inversed-U concentration-effect curve has been described in *in vitro* experiments (Prickaerts *et al*, 2012; Yang *et al*, 2013) and also in the $\alpha_4\beta_2$ nAChR for nicotine and partial nicotinic receptor agonist varenicline (Rollema *et al*, 2007). This unique nAChR property might result from the afore-mentioned mechanisms and represents a challenge to determine the effective dose of subtype selective nicotinic receptor agonists.

## REGULATION OF COGNITIVE FUNCTIONS

For the above-mentioned reasons, determining the nicotinic function in cognition only by localization and by isolated receptor physiology experiments is not possible. Several experiments with agonists and antagonists *in vivo* have been needed to investigate the role the nicotinic system has in cognition. Memory is improved by nicotinic agonists, and impaired by antagonists and lesions in cholinergic nuclei or tracts impair memory (Levin *et al*, 2006). Local infusion of methyllycaconitine (an $\alpha_7$ antagonist) or dihydro-$\beta$-erythroidine (an $\alpha_4\beta_2$ antagonist) into the basolateral amygdala, the ventral and dorsal hippocampus impaired the working memory of rats in a 16-arm radial maze (Nott and Levin, 2006). In monkeys, while nicotine improved performance in visuo-spatial memory and spatial working memory tests, mecamylamine (a known non-selective nAChR antagonist) impaired visuo-spatial memory and fine motor performance (Katner *et al*, 2004).

## DYSFUNCTION AND THERAPEUTIC OPTIONS OF THE NICOTINIC SYSTEM

Plaques originating from Amyloid-$\beta$ peptides and neurofibrillary tangles are hallmarks of Alzheimer's disease (AD). Even though A$\beta$ plaque deposition is pathognomonic for AD, treatments directed to eliminate the plaques have not yet proven effective as disease modifying or delaying strategies regardless of the fact that the plaques are effectively cleared in the parenchymal brain tissue of treated patients (Paquet *et al*, 2015). AD is characterized by progressive and irreversible cognitive dysfunction, particularly in learning and memory. Anatomically, AD affects limbic structures, subcortical nuclei, and cortical regions. As mentioned in the previous section, the most important neuronal loss in AD is in the cholinergic system, particularly cholinergic neurons in the basal forebrain, the medial septal nucleus, the horizontal and vertical diagonal bands of Broca, and the nucleus basalis of Meynert (Auld *et al*, 2002). Several nAChR subtypes, including the $\alpha_4\beta_2$, $\alpha_7$ and $\alpha_3$-related nicotinic receptors, are reduced in AD (Perry *et al*, 1998). Even though the underlying etiologic mechanisms (link between the presence of Amyloid $\beta$ and cholinergic dysfunction) are unknown, it has been shown that Amyloid $\beta$ binds to the $\alpha_7$ nAChR producing its inactivation and down-regulation (Wang *et al*, 2000). Compounds with nAChR agonist activity have been considered not only as cognitive enhancers, but also as mediators of the innate inflammatory response. They may therefore have a therapeutic possibility as disease modifying treatment for AD (Hurst *et al*,

2013; Vallés *et al*, 2014). Vagus nerve electrical stimulation decreased tumour necrosis factor (TNF-$\alpha$) secretion in macrophages induced by bacterial lipopolysaccharide by acting on $\alpha_7$ nAChRs (Wang *et al*, 2003). Alpha$_7$ nAChRs are also expressed in microglia and therefore might also play an important role in the inflammation response in AD (Conejero-Goldberg *et al*, 2008). Functional changes in the nAChR have been reported in several diseases including schizophrenia (Court *et al*, 2000). Nicotinic agonists have also been tested for the treatment of negative symptoms derived from schizophrenia (Beinat *et al*, 2015). Cholinergic-dopaminergic interactions might be related to improvement in the symptoms related to patients with Attention Deficit Hyperactivity Disorder and Parkinson's Disease (Potter *et al*, 2006; Quik and Kulak, 2002). Administration of nicotine to adolescents diagnosed with Attention Deficit Hyperactivity Disorder improved cognition (Potter and Newhouse, 2004). The nAChRs on dopaminergic neurons of the substantia nigra may be especially sensitive to loss in Parkinson's Disease (PD), suggesting that nAChRs may be especially important in the etiology of PD and might be a potential drug target (Perez-Lloret and Barrantes, 2016; Pérez and Quik, 2011). Recently, a phase III clinical trial with AQW051, an $\alpha_7$ nicotinic agonist, reported no improvement in dyskinesia, however administration of AQW051 50 mg significantly improved cognitive memory tests when compared to placebo (Trenkwalder *et al*, 2016). On the other hand, pre-clinical results are promising for other two $\alpha_7$ nicotinic agonists, ABT-107 and ABT-126, which reduced Levodopa-induced dyskinesia (LID) in an experimental model of PD (Zhang *et al*, 2014, 2015). To date, several novel nicotinic agonists compounds have shown promising early clinical results but are still in development as treatments for neurodegenerative diseases such as AD, PD and for schizophrenia ( Jones *et al*, 2012; Toyohara and Hashimoto, 2010; Vallés *et al*, 2014), and the scientific community waits impatiently the results of the phase III studies.

### CHOLINERGIC PHARMACOLOGIC CHALLENGE MODELS

A pharmacologic challenge influences, preferably selectively, a system by means of a pharmacological agent, with a resulting transitory physiological change that may resemble a pathologic state. During this period the researcher can measure the resulting effect, repetitively if needed, under controlled conditions and obtain important information about the underlying mediating process. Pharmacological challenge test are also used to study the roles of pharmacological systems in health and disease. Pharmacological challenge tests are often used for research purposes but they are also used, mostly for diagnostic purposes, in clinical practice.

Children with growth hormone deficiency may undergo a challenge with the $\alpha_2$-adrenergic agonist clonidine to guide de pediatric endocrinologist to the right diagnosis (Lanes *et al*, 1985). A challenge model can give the additional benefit of quantifying the magnitude of the effect and relate the disturbing stimulus (e.g.: plasma concentrations of the pharmaceutical compound) to the effect (Klein *et al*, 2013).

A pharmacological challenge model may also be used to test new pharmaceutical compounds in humans and animals, as a disease model or to study the impact of a drug on a pharmacological system. Challenge tests can therefore be important translational tools in drug development and to test and quantify pharmacokinetic and pharmacodynamic interactions. Scopolamine is a selective competitive muscarinic antagonist and has been widely used in the field of neuropharmacology as a standard test to induce dementia- and age-related temporary cognitive impairments to healthy subjects, patients and animals (Klinkenberg and Blokland, 2010). Recently, the effects of scopolamine as a challenge model have been successfully quantified in order to better understand the effect of muscarinic blockade in the human brain (Liem-Moolenaar *et al*, 2011) and these advances have helped to test novel compounds acting on the cholinergic system (Buccafusco, 2009; Liem-Moolenaar *et al*, 2010a, 2010b; Lines *et al*, 1993; Preston *et al*, 1988). Several authors have reported reversal of scopolamine effects in cognition using two acetylcholinesterase inhibitors, galantamine and donepezil (Baraka and Harik, 1977; Thomas *et al*, 2008). Mecamylamine is a selective and competitive nicotinic antagonist widely used in animal experimental models but it has not been validated in humans as a model of cognitive impairment and therefore, to date, a specific nicotinic challenge test lacks.

The aim of the thesis was to study the functional effects of muscarinic and nicotinic pharmacological blockage using different cholinergic challenge models. The challenge models were also evaluated in order to test compounds with cholinergic activity. In order to differentiate nicotinic and muscarinic effects in humans a trial comparing a muscarinic pharmacologic challenge (using scopolamine) and a nicotinic challenge (using 10 and 20 mg of mecamylamine, a nicotinic antagonist) was performed (chapter 3). The trial was also an exploratory study to determine a tolerable and safe mecamylamine dose to be used in a later validation study (chapter 4). The mecamylamine validation study presents the results of the validation study using a higher mecamylamine dose, namely 30 mg, and in order to validate this pharmacological challenge model to be used in drug development, nicotine (a nAChR agonist) and galantamine (an acetylcholinesterase inhibitor) were separately co-administered with mecamylamine to attenuate or even reverse the disturbances induced by mecamylamine administration. The effects of mecamylamine administration in the previously two mentioned studies (chapter 3 and 4) were analyzed using a PK-PD model (chapter 5). The objective of the analysis was to quantify the relationships between the plasma mecamylamine concentration (pharmacokinetics) and the measured effects (pharmacodynamics). None of the measured effects reflected muscarinic activity closely enough to serve as a good biomarker. Therefore, a composite muscarinic cholinergic index using scopolamine effects on the EEG with biomarker algorithms in the different frequency bands and used machine-learning techniques was developed (chapter 7). The index integrates information from multiple EEG biomarkers, which on one side increased the sensitivity of drug-induced changes in the EEG and might be a useful biomarker in clinical trials. Finally, chapter 6 contains *in vitro* experiments performed with lipopolysaccharide plus an adjuvant (aluminium hydroxide, adenosine tri-phosphate) to explore the immune-modulating effect of choline (a nicotinic agonist) in human whole blood and monocytic THP-1 cells.

### CHOLINERGIC HYPOTHESIS OF AGEING

Added to the previously mentioned role of the AChR in disease, a cholinergic dysfunction has also been suggested as aetiology for the age-related cognitive impairment in healthy elderly (Bartus *et al*, 1982). Acquisition, processing and recall of information and the speed this is performed seems to be most affected by ageing (Hedden and Gabrieli, 2004). Longitudinal morphological studies have also provided evidence that brain areas rich in acetylcholine (*e.g.*: hippocampus, entorhinal and temporal cortex) shrink significantly with age in healthy subjects (Raz *et al*, 2005). Attempts have been made to quantify the extent of cholinergic disturbances with an anticholinergic challenge test to indirectly measure cholinergic impairment. A significant difference in performance between young adults compared to elderly subjects during scopolamine administration was observed in several cognitive tests evaluating short-term verbal and numeric (working) memory (Molchan *et al*, 1992; Ray *et al*, 1992; Zemishlany and Thorne, 1991), attention (Zemishlany and Thorne, 1991), acquisition (Zemishlany and Thorne, 1991), visuo-spatial praxis (Flicker *et al*, 1992) and episodic memory (Molchan *et al*, 1992) and have finally led to the hypothesis that a cholinergic dysfunction takes place in healthy subjects with increasing age as a process of healthy or normal ageing (Dumas and Newhouse, 2011; Ellis *et al*, 2009; Terry and Buccafusco, 2003). Similarly but less extensively studied, mecamylamine also induced a significantly greater cognitive impairment measured as an increase in reaction time and number of incorrect answers in a recognition memory task (Newhouse *et al*, 1994). Concentration-effects relationships were not performed in any of the previously mentioned trials and therefore it is still unknown if the exposure or the sensitivity across different ages is responsible for this differences.

In order to study these differences in effects between healthy subjects, the effects of a previously validated and broadly used cholinergic pharmacologic model with scopolamine were compared between subjects in a wide age range (between 18 and 78 years). For this analysis, a PK-PD model relating plasma scopolamine concentration and effects was used to detect differences in exposure (pharmacokinetics) and differences in sensitivity (pharmacodynamics) to scopolamine using age as covariate (chapter 2).

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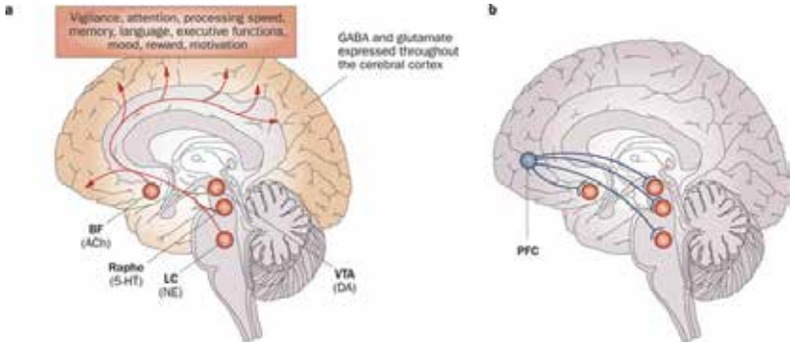
**FIGURE 1.1 Cholinergic pathways in the human brain.**

**A**. Cortical, cognitive and behavioral functions are regulated by ACh, NE, 5-HT and DA neurotransmitter systems. **B**. The prefrontal cortex in turn exerts top-down regulatory control over ascending modulatory systems, favouring the processing of incoming information and filtering of irrelevant stimuli. Damage, for example to the cholinergic system, may interfere with the processing of incoming verbal stimuli and favour the emergence of perseverations, omissions and semantic errors. Damage to the cholinergic system might be amenable to pharmacological treatment with cholinergic agonists. *Reprinted by permission from Macmillan Publishers Ltd: Nature reviews. Neurology (Berthier and Pulvermüller, 2011), copyright 2011.*




*Abbreviations: 5-HT, 5-hydroxytryptamine; BF, basal forebrain; ACh, acetylcholine; DA, dopamine; GABA, γ-aminobutyric acid; LC, locus coeruleus; NE, norepinephrine; PFC, prefrontal cortex; VTA, ventral tegmental area.*

**TABLE 1.1 Distribution of muscarinic receptors in the brain.**

Determined using **A**. high affinity radioligand [3 H] N-methylscopolamine (Flynn et al, 1997) or **B**. complementary nucleic acid sequences able to hybridize with parts of muscarinic receptor mRNA (Caulfield, 1993) in the rat brain.

| Location | Receptor | | | | |
|---|---|---|---|---|---|
| | M1 | M2 | M3 | M4 | M5 |
| Cerebral cortex | +++++[a,b] | ++[a] mainly occipital region | - | +[a] mainly occipital region | +[a] mainly outermost layer |
| Hippocampus | ++++[a,b] mainly CA1 and CA2, followed by CA3 | NA[b] | +[a,b] | +[a,b] CA1 | +[a,b] CA1 and CA2 |
| Striatum (caudate and putamen) | ++++[a,b] | ++ caudate (dorsal region)[a,b]; NA[b] putamen | + [a,b] cortical laminae, CA1, CA2, and CA3 | ++++[a,b] | +[a,b] |
| Nucleus accumbens | +++[a] | ++[a] | - | +[a] | +[a] |
| Dentate gyrus | +++[a] molecular layer | - | - | - | +[a] polymorphic layer |
| Olfactory bulb, olfactory tubercle | ++a,b | ++[a] | NA[b] | +[a,b] mainly anterior | - |
| Brainstem | +[a] | +++[a] parabrachial nuclei; ++[a,b] trigeminal motor and the facial nuclei | - | ++[a] mainly pons, facial, and trigeminal motor nuclei; great overlap of M4 with M2 labelling | - |
| Amygdala | NA[b] | - | - | - | - |
| Superior and inferior colliculi | - | +++[a] superficial layers | - | - | +[a] only superior colliculus |
| Cerebellum | - | +[a,b] | - | - | - |
| Hypothalamus | - | NA[b] | - | - | - |
| Thalamus | - | - | NA[b] | - | - |
| Basal forebrain | - | - | - | - | ++[a] |
| Substantia nigra | - | - | - | - | NA[b] pars compacta |

*NA: present but quantitative information not available.*

**TABLE 1.2 Distribution of nicotinic receptors in the brain.**

Distribution of complementary nucleic acid sequences able to hybridize with parts of nicotinic receptor mRNA in human brain.

| Location | Receptor | | | | | | |
|---|---|---|---|---|---|---|---|
| | β2 | β3 | β4 | α3 | α4 | α5 | α7 |
| Cerebral cortex | + prefrontal, motor, entorhinal, cingular and temporal | + | + | ++ prefrontal, motor and entorhina;+ cingular and temporal | +, ++ temporal | + | + e ntorhinal, ++ prefrontal, +++ motor |
| Thalamus | + dorsomedial and ventro-posterolateral | + | +, ++ reticular, +++ lateropos-terior | +++ dorsomedial and ventro-posterolateral | - | +, +(+) reticular, ++ geniculate bodies | ++ dorsome-dial |
| Hippocampus | +(+) | - | - | + | - | ++ | ++ |
| Dentate gyrus | +(+) | - | - | + | - | - | ++ |
| Striatum (caudate and putamen) | +(+) | + | + | - | - | + | ++ |
| Cerebellum | + | + | + | + | +(+) | + | - |

*Adapted from Paterson and Nordberg, 2000.*



# MODEL-BASED EXPOSURE-RESPONSE ANALYSIS TO QUANTIFY AGE RELATED DIFFERENCES IN THE RESPONSE TO SCOPOLAMINE IN HEALTHY SUBJECTS

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## ABSTRACT

Subjects with increasing age are more sensitive to the effects of the anti-muscarinic agent scopolamine, which is used (among other indications) to induce temporary cognitive dysfunction in early phase drug studies with cognition enhancing compounds. The enhanced sensitivity has always been attributed to incipient cholinergic neuronal dysfunction, as a part of the normal aging process. The aim of the study was to correlate age-dependent pharmacodynamic neuro-physiologic effects of scopolamine after correcting for differences in individual exposure. We applied a pharmacokinetic and pharmacodynamic modelling approach to describe individual exposure and neuro-cognitive effects of intravenous scopolamine administration in healthy subjects. A two-compartment linear kinetics model best described the plasma concentrations of scopolamine. The estimated scopolamine population mean apparent central and peripheral volume of distribution was 2.66 ± 1.050 L and 62.10 ± 10.100 L, respectively and the clearance was 1.09 ± 0.096 $L \cdot min^{-1}$. Age was not related to a decrease of performance in the tests following scopolamine administration in older subjects. Only the saccadic peak velocity showed a positive correlation between age and sensitivity to scopolamine. Age was however correlated at baseline with an estimated slower reaction time while performing the cognitive tests and to higher global δ and frontal ϑ frequency bands measured with the surface EEG. Most of the differences in response to scopolamine administration between young and older subjects could be explained by pharmacokinetic differences (lower clearance) and not to an enhanced sensitivity when corrected for exposure levels.


## INTRODUCTION

The involvement of the cholinergic system in the etiology of geriatric cognitive dysfunction was already suggested in the early eighties (Bartus *et al*, 1982). At that time, evidence from different sources using mostly semi-parametric statistical methods, suggested that cholinergic neuronal dysfunction played a major role in the brains of healthy older subjects, subjects with Mild Cognitive Impairment (MCI) and to a greater extent in those of patients with dementia (Coyle *et al*, 1983). Research in older subjects show that cognitive abilities such as encoding new memories of episodes or facts, working memory (the simultaneous short-term maintenance and manipulation of information involving executive processes) and processing speed seem to be the most affected by ageing (Hedden and Gabrieli, 2004). Recently, the age-related decrease in specific areas in the brain of healthy subjects was quantified prospectively with MRI imaging. Acetylcholine-rich regions in the brain including the hippocampus, the entorhinal cortex, the inferior temporal cortex and the prefrontal white matter, decreased with age. The hippocampus was the only structure that showed a non-linear increased rate of shrinkage with increasing age, providing evidence of an age-related decline in hippocampal volume in subjects with increasing age and indirectly suggesting a decline in hippocampal neurons, an area rich in cholinergic neurons (Raz *et al*, 2005).

The cholinergic hypothesis was further strengthened by evidence that the severity of cognitive deficits appeared to be related to the extent of cholinergic impairment (Mufson *et al*, 2000), and to the observation that anti-cholinergic compounds induce cognitive dysfunction (Broks *et al*, 1988; Liem-Moolenaar *et al*, 2011; Newhouse *et al*, 1994). The most commonly used cholinergic challenge utilizes scopolamine as a selective competitive muscarinic antagonist. Scopolamine has a high affinity for all five muscarinic receptor subtypes ($M_1$– $M_5$) and a negligible affinity for histaminergic and dopaminergic receptors (Ali-Melkkilä *et al*, 1993). Intravenous administration of scopolamine leads to a reduction in attention, arousal, verbal and non-verbal working memory and episodic memory. The reduction in cognitive functioning was observed in both healthy young and healthy older subjects,

but appeared to be more severe in healthy older subjects. A significant difference between young adults compared to older subjects was observed in several cognitive tests evaluating short-term verbal and numeric working memory (Molchan *et al*, 1992; Ray *et al*, 1992; Zemishlany and Thorne, 1991), attention (Zemishlany and Thorne, 1991), acquisition (Zemishlany and Thorne, 1991), visuo-spatial praxis (Flicker *et al*, 1992) and episodic memory (Molchan *et al*, 1992). Additionally, some authors have suggested that scopolamine use in clinical practice in older patients might induce a higher risk of cognitive impairment when compared to younger subjects (Flicker *et al*, 1992; Seo *et al*, 2009). It is hypothesized that this difference between young adults and older subjects receiving scopolamine is due to incipient cholinergic neuronal dysfunction in the healthy older subjects and hence an enhanced sensitivity to anti-cholinergic drug effects (Dumas and Newhouse, 2011; Terry and Buccafusco, 2003). Consequently, a lower scopolamine dose in the older subjects will result in similar effects compared to younger subjects. However, none of these studies, measured age-related difference in plasma scopolamine concentrations.

In the current paper, a non-linear mixed effects (NLME) approach was used to quantitatively correlate the pharmacodynamic neuro-physiologic effects of scopolamine as an indirect measure of muscarinic brain activity after correcting for differences in exposure (pharmacokinetics). This allows the identification of pharmacokinetic (PK) and pharmacodynamic (PD) differences at population and individual level, and their relationships with covariates like age. NLME methods are a useful tool when differences in exposure explain a different measurable effect, while biological differences such as inter-subject and intra-subject random variability, are also taken into consideration and may be explained by covariates (e.g.: body weight, height, BMI, age, etc.).

## METHODS

### STUDY POPULATION

A total of 135 healthy subjects participated in four separate clinical studies performed at the Centre of Human Drug Research (Leiden, the Netherlands). Subjects' demographics can be found in Table 2.1. A medical ethics committee approved the four different study protocols. After giving written informed consent, all subjects were medically screened prior to study participation. Exclusion criteria included the use of agents or drugs known to influence CNS performance (including smoking and drug or alcohol abuse), consuming more than five cups of caffeine-containing drinks per day and evidence of relevant medical abnormalities. Older subjects were required to score 28 points (or higher) on the Mini-Mental State Examination (Folstein *et al*, 1975) (MMSE) at screening.

### STUDY DESIGN

Data for this analysis were obtained from all four studies. In the first two trials the effects of two different glycinergic compounds were studied using a scopolamine challenge model (Liem-Moolenaar *et al*, 2010a, 2010b), the third one was a study in which the effects of scopolamine on cognitive functions were compared with those of mecamylamine (a nicotinic acetylcholine receptor antagonist). In the fourth study, the effects of an $a_7$ nicotinic acetylcholine receptor agonist were examined in older subjects using a scopolamine challenge without a placebo-control. Only the data from the occasions where scopolamine, scopolamine in combination with placebo or only placebo (comparison placebo group) was administered were included for analysis.

An intravenous dose of 0.5 mg of scopolamine hydrobromide was administered to subjects below 65 years and an intravenous dose of 0.3 mg to the older subjects group (≥ 65 years), according to previous recommendations (Flicker *et al*, 1992; Snyder *et al*, 2005; Thomas *et al*, 2008). In all four studies, scopolamine was administered as an intravenous infusion over 15 minutes. Plasma scopolamine concentrations were determined using a validated, selective and sensitive liquid LC-MS/MS method with a Lower Limit of Quantification of 10 pg · $mL^{-1}$. Across the study sample analytical runs, the accuracy (expressed as the percentage of bias) of scopolamine QC samples ranged from 2.0 to 3.3% and assay precision (expressed as the percentage of CV) ranged from 2.5 to 4.0%.

A battery of CNS tests (neurophysiological, psychomotor and cognitive tests, subjective drug effects) was performed to quantify the pharmacodynamics effects of scopolamine, which provided information on the effects of scopolamine on different functional CNS domains. All pharmacodynamic tests were performed in a quiet room with ambient illumination with only one subject in the same room with a research assistant per session. All subjects were thoroughly trained and familiarized with the psychometric tests within 14 days preceding study start to minimize learning effects during the study. Each baseline or pre-dose assessment was performed twice at the beginning of each occasion. The lack of placebo arm in the older subjects group was handled by using the PD baseline measurements to estimate the scopolamine effects. A combination of tests evaluating both neurophysiological and cognitive variables on which an effect was previously reported (Liem-Moolenaar *et al*, 2011) were analysed:

**N-BACK TEST** ✳ Subjects were asked to remember and correlate a sequence of letters presented in a random order, thereby allowing evaluation of (short-term) working memory. This test evaluates working memory (Lim *et al*, 2008). Subjects were asked to remember and correlate a sequence of letters presented in a random order. Performance was expressed as the percentage of correct answers on the 0-, 1- and 2-back paradigms, and as reaction time on the 0-back paradigm. N-back ratio data was analysed as count data and therefore the data was logit-transformed.

**ADAPTIVE TRACKER TEST** ✳ The test evaluates attention and executive skills as visuo-motor coordination (Borland and Nicholson, 1984; van Steveninck *et al*, 1991). Subjects were asked to use a joystick to keep a randomly moving target on the screen inside a circle. The percentage accuracy was recorded.

**SACCADIC EYE MOVEMENTS TEST** ✳ This is one of the most sensitive tests for sedation (van Steveninck *et al*, 1991). Subjects were requested to look at a randomly moving target in the computer screen. With help of two electrodes, the horizontal peak velocity eye movements and inaccuracy were measured.



**ELECTRO-ENCEPHALOGRAM** ✳ Four cranial superficial electrodes (Fz, Cz, Pz, Oz) were placed following the 10-20 system and subjects were asked to close their eyes. Fast Fourier transformed absolute power (μV) was calculated from the raw measurements in the $\alpha$ [7.5 – 11.5 Hz], $\beta$ [11.5 – 30 Hz], $\delta$ [0.5–3.5 Hz] and $\vartheta$ [3.5 – 7.5 Hz] frequency ranges in two bipolar leads: Fz-Cz and Pz-Oz).

**SOFTWARE** ✳ Pharmacokinetics and pharmacodynamics modelling analyses were performed using non-linear mixed-effect (NLME) modelling in NONMEM v7.2 and v7.3 (Beal *et al*, 2009) (ICON Development Solution, Hanover, MD). The database and all graphs were created using R v2.13.1 (R Core Team, 2013) (R Foundation for Statistical Computing, Vienna, Austria).

## MODEL DEVELOPMENT AND EVALUATION

Plasma scopolamine concentration-time dependent data was analysed using a consecutive NLME modelling approach; once the best pharmacokinetic model was obtained, the individual PK parameter estimates were fixed to develop the pharmacodynamic models. The first order conditional estimation method with interaction (FOCE-I) was used for all data except for the N-back ratio data where a Laplacian method for count data was also compared to FOCE-I. Several compartment models were explored for the pharmacokinetic model, before inter-individual variability (IIV) was tested for PK parameters, as were additive, proportional or combined error models. Body weight, height and age were tested as potential covariates for parameters on which IIV could be identified.

For the PD endpoints, indirect $E_{max}$ model structures were preferred to assess the age-related sensitivity to scopolamine, in terms of $EC_{50}$ and $E_{max}$. Delay compartments were taken into consideration for the pharmacodynamics models. Once the structural model was defined, IIV was tested in each parameter estimate, as were additive, proportional or combined error models. Correlations between post-hoc Bayesian estimates and between post-hoc Bayesian estimates and potential covariates were explored using Pearson's correlation coefficients ($r^2$). A coefficient of

determination ≥ 0.4 was considered relevant and taken forward in testing of the omega block structures and covariate analysis (body weight, age and height). Age was tested as a covariate in all models regardless of correlation. Competing models were compared based on their Goodness of Fit (GOF) plots, improvement in OFV, plausibility of parameter estimates, residual error, parameter precision (in terms of residual standard error, RSE), shrinkage and parameter distribution. A decrease in the OFV of 3.84 units ($p<0.05$) was considered statistically significant. GOF plots included population and individual predictions vs. observations, time- and observations-dependent conditional weighted residuals (with interaction) and IIV distribution graphs. Visual Predictive Checks (VPCs) were obtained by simulating 1000 subjects, using the population parameter estimates and the full variance-covariance matrix. Covariates were sampled from the observed population distribution (assuming a normal distribution, with resampling).

## RESULTS

### MODEL DEVELOPMENT – PLASMA SCOPOLAMINE CONCENTRATIONS

Pharmacokinetic model graphical representation and parameter estimates can be found in Figure 2.1 and Table 2.2, respectively. A two-compartment linear model structure proved superior compared to a one-compartment linear plasma scopolamine pharmacokinetic model (ΔOFV = -1474 points). Inter-individual variability could be identified on the central ($V_c$) and peripheral ($V_p$) volumes of distribution and clearance. As displayed in Equation 2.1, age (in years) and body weight (in kilograms) were identified as covariates on clearance (ΔOFV = -29 and -38 points, respectively). Body weight was also identified as a covariate on peripheral volume of distribution (ΔOFV = -13 points; Equation 2.2).

$$CL = \alpha \cdot e^{\{[CAC \cdot \log(\frac{AGE}{28})] + [CWC \cdot \log(\frac{WGT}{78.5})]\}} \quad (2.1)$$

$$Vp = \beta \cdot e^{\{CWV \cdot \log(\frac{WGT}{78.5})\}} \quad (2.2)$$





From the estimated clearance and volumes of distribution we computed the second order rate elimination and inter-compartmental distribution, which are shown in Equations 2.3.

$$k_{10} = \frac{CL}{V_c}, k_{12} = \frac{Q}{V_c} \text{and } k_{21} = \frac{Q}{V_p} \quad (2.3)$$

Finally, the best model's differential equations (2.4 and 2.5) are shown below for the central $A_c$ and peripheral $A_p$ compartments.

$$\frac{dA_c}{dt} = -k_{10} \cdot A_1 - k_{12} \cdot A_1 + k_{21} \cdot A_2 \quad (2.4)$$

$$\frac{dAp}{dt} = k_{12} \cdot A_1 - k_{21} \cdot A_2 \quad (2.5)$$

### MODEL DEVELOPMENT – SCOPOLAMINE EFFECTS

The scopolamine effect on the $\beta$ frequency in the EEG (in both Fz-Cz and Pz-Oz leads) was negligible and therefore the parameter was excluded from the analysis. An indirect $E_{max}$ model accurately described scopolamine's effect on the other pharmacodynamics tests in the CNS test battery.

#### REACTION TIME IN THE 0-BACK PARADIGM OF THE N-BACK TEST

✳ Older subjects had a slower (prolonged) reaction time at baseline when compared to young subjects while performing the 0-back paradigm of the N-back test (average per group 402 ms for young vs. 476 ms for older subjects). Following scopolamine administration, the Reaction Time of the 0-Back paradigm increased significantly from baseline. Adding IIV to $E_{max}$ provided a non-significant decrease in the OFV (3 points) and therefore was abandoned. Age was identified as covariate for the baseline (presented in Equation 2.6; $K_{in}$) estimated value (Figure 2.4), which provided a significant decrease in the OFV (24 points) and a better fit.

$$k_{IN} = \lambda \cdot e^{\{\frac{AGE}{CAB}\}} \quad (2.6)$$

**SACCADIC EYE MOVEMENTS (SEM) TEST – INACCURACY** ✳ Scopolamine increased the saccadic inaccuracy in the SEM test. IIV was identified for $K_{in}$ (baseline), $EC_{50}$ and $E_{max}$. An omega block structure between $EC_{50}$ and $E_{max}$ was tested. This did not reduce the OFV but provided a reasonable shrinkage reduction from 90.3 to 36.9 % and improved the GOF of the model, and was therefore kept in the model.

**SACCADIC EYE MOVEMENTS (SEM) TEST** ✳ Saccadic Peak Velocity decreased the saccadic peak velocity in the SEM test. IIV was identified for baseline ($K_{in}$) and $EC_{50}$. IIV on $E_{max}$ did not significantly improve the model fit. Age was identified as covariate for $EC_{50}$, which decreased the OFV (25 points), improved the GOF, and the IIV on the parameter decreased (CV = 3.5 vs. 2.8). Figure 2.3 provides a visual representation of the correlation between age and estimated $EC_{50}$ values. This was the only model from the battery of tests were age was correlated with the estimated $EC_{50}$ (Equation 2.7), indicating a higher sensitivity to scopolamine with increasing age where $\varepsilon$ and CAE are estimated coefficients.

$$EC_{50} = \varepsilon \cdot e^{\left\{-\frac{AGE}{CAB}\right\}} \quad (2.7)$$

**ADAPTIVE TRACKER TEST (PERCENTAGE OF ACCURACY)** ✳ Shortly after scopolamine was administered, an abrupt decrease on the performance in the Adaptive Tracker test was observed. In previous models where $E_{max}$ was estimated, the result was consistently 1 (100% decrease in performance). Fixing $E_{max}$ at 1 resulted in a reduction of the parameter uncertainty, improved the model's stability and resulted in no change in the OFV or GOF. Therefore $E_{max}$ was fixed at 1. The addition of an exponent in the $E_{max}$ function ($\gamma$=1.1 ± 0.063), even though this value is near to 1 and has a low variability, improved the model fit and decreased the OFV by approximately 7 points and therefore was accepted. IIV was identified for $k_{in}$ and $EC_{50}$.

**ELECTRO-ENCEPHALOGRAM** ✳ Scopolamine induced a decrease of the absolute power in the *α* frequency band in both Fz-Cz and Pz-Oz leads. On the other hand, scopolamine increased the absolute power in both δ and ϑ frequency bands (with a greater effect on the δ frequency band) in the EEG of the analysed bipolar leads (Fz-Cz and Pz-Oz). Addition of $E_{max}$ IIV resulted in a significant decrease of the OFV of 196 points in the *α* Fz-Cz EEG and 22 points in the *α* Pz-Oz models and was therefore accepted. Addition of age as covariate in any of the parameters of the *α* models (both Fz-Cz and Pz-Oz) resulted in an increase in OFV and a worse fit and therefore was abandoned. Addition of $E_{max}$ IIV resulted in a significant decrease of the OFV of 29 points in the δ Fz-Cz EEG and 38 points in the δ Pz-Oz models and therefore was accepted. Age could be identified as covariate for the baseline ($K_{in}$) estimates in the δ frequency band in Fz-Cz (average of the older 1.360 μV and the younger adults group 1.745 μV) and Pz-Oz (older 1.468 μV vs. younger adults 1.776 μV). Once incorporated, the OFV decreased 68 points in the Fz-Cz and 12 points in the Pz-Oz model. In the δ Fz-Cz model, IIV could be identified for $EC_{50}$ and $E_{max}$ and $K_{in}$. An omega block structure between $EC_{50}$ and $E_{max}$ and between $EC_{50}$ and $K_{in}$ was tested. This resulted in a model improvement (ΔOFV = -36 and -29 points respectively). In the ϑ frequency band in the Fz-Cz lead (but not in the Pz-Oz lead) IIV tested for $E_{max}$ did not significantly improve any of the models. Age was used as covariate for the baseline measurements (older group average 1.535 μV vs. younger adults 2.024 μV).

**PERCENTAGE OF CORRECT ANSWERS IN THE 0-, 1- AND 2-BACK PARADIGMS OF THE N-BACK TEST** ✳ Subjects scored at baseline (pre-dose measurements) on average 97.75% for the 0-back, 96.54% for the 1-back and 94.48% for the 2-back test. Following scopolamine administration, the number of correct answers decreased significantly compared to placebo. In all models (0-, 1- and 2-back) IIV was identified on $E_{max}$, however IIV at $EC_{50}$ could not be identified. For the 0- and 2-back models, IIV was identified at baseline ($E_0$) and an omega block (variance –covariance structure) between $E_{max}$ and $E_0$ was required. In the 1-back model IIV was identified at $K_{in}$ and $E_{max}$, but could not be identified in $E_0$. Only for the 0-back test, the addition of transit compartments to create a delay for the effect was required (ΔOFV = -80 points). $E_{max}$ was fixed to 1 as this reduced the parameter uncertainty and improved the model's stability while providing only a marginal increase

of the OFV (a maximum of +10 points). No covariates could be identified in any of the estimated model parameters.

### MODEL EVALUATION

In general, for the pharmacokinetic, Adaptive Tracker, EEG ($\alpha$, $\vartheta$ and $\delta$ frequencies of the Fz-Cz and Pz-Oz leads) and Saccadic Eye Movement (Peak Velocity and Inaccuracy) tests, the GOF plots indicate that the central and individual trend of the data is well described, and that no bias occurs over time or observations. The RSE's are relatively small, indicating acceptable accuracy of the parameter estimates, with acceptable shrinkage (Table 2.2). The VPCs indicate that the variability for these parameters is well described as 95% of the data lie within the 95% prediction interval (Figure 2.1). However, for N-back ratios, even though the VPC graphs indicate that the observations are contained within the 95% prediction interval and the GOF plots show a good description of the central and individual trends of the data, the estimated variability (appreciated by the 95% prediction interval in the VPC graphs shown in Figure 2.1) is considerable, probably related to the great inter- and intra-individual variability observed in the individual observations during the test where a substantial IIV is estimated to describe outliers; this was also reflected in the high estimated shrinkage in the $E_{max}$ (42.1%) of the 0-back and the $K_{in}$ (47.3%) of the 1-back ratio models. Similarly, the shrinkage values for the $\alpha$ frequency $EC_{50}$ (48.4%) in the Fz-Cz lead and $\delta$ frequency $E_{max}$ (48.0%) in the Pz-Oz lead were considerable; probably related to the high inter-individual variability of the test and the modest effect of scopolamine on the EEG. Finally, the central volume of distribution $V_c$ in the PK model showed a high shrinkage (49.3%).

### DISCUSSION

Our results show that age-related increase in scopolamine exposure (pharmacokinetics), rather than previously hypothesized 'higher sensitivity' of older subjects, explains enhanced age-related anti-cholinergic drug effects. The developed structural model is in agreement with the two-exponential equation describing bi-phasic pharmacokinetics (central and peripheral volume of distribution) as reported in previous literature using non-linear regression (non-compartmental analysis) and NLME methods (Ebert *et al*, 2001; Liem-Moolenaar *et al*, 2011; Putcha *et al*, 1996; Renner *et al*, 2005). Also, the relatively large apparent volume of distribution, mainly of the peripheral compartment, is in agreement with the weak basic non-polar alkaloid high lipophilicity physicochemical properties of scopolamine. Body weight and age were not earlier reported as covariates associated with plasma scopolamine pharmacokinetics. Body weight was directly related and age was inversely related to the total clearance. Scopolamine is hardly detected unchanged in urine (~2.6%), and based on *in vitro* and interaction studies in humans, scopolamine seems to be mainly eliminated hepatically, with CYP3A4 as the responsible elimination pathway (Renner *et al*, 2005). Increasing age has been correlated with a diminished blood flow to the liver and a reduced liver volume and functional capacity, explaining reduced clearance with increase in age (Woodhouse and James, 1990; Wynne *et al*, 1989). Medication was controlled in the current studies, however use of concomitant CYP3A inhibitors, thereby further decreasing clearance, could explain the higher incidence of side effects in other studies in older subjects after scopolamine administration, especially if scopolamine exposure was not taken into consideration.

The neuro-physiologic eye movement parameter saccadic peak velocity was the only test where an age related augmented sensitivity to scopolamine was found, a finding not earlier reported. Age was correlated with a decline in the $EC_{50}$ in the Peak Velocity of the horizontal Saccadic Eye Movements test with a decrease mainly observed above ~45 years. Pons and midbrain, both located in the brainstem, are particularly rich in muscarinic acetylcholine receptors (Kobayashi *et al*, 1978) which are an important binding site for anti-muscarinic compounds as demonstrated *in vivo* with imaging techniques that measured enrichment of radiolabeled scopolamine in these brain areas (Frey *et al*, 1992). The horizontal Saccadic Movements test evaluates the coordinated action of brain areas that involve the occipital lobe for acquisition and brain stem structures such as the pons and midbrain for execution, explaining the high sensitivity of this test in detecting scopolamine effects.

Previous studies have reported significant differences in sensitivity of older subjects compared to younger subjects after scopolamine administration, only while carrying out cognitive tests involved in short term acquisition or verbal and numeric working memory (Molchan *et al*, 1992; Ray *et al*, 1992; Zemishlany and Thorne, 1991) or visuo-spatial praxis(Flicker *et al*, 1992). The N-back test was the most sensitive experiment performed in our battery of memory tests specifically evaluating verbal acquisition, processing and execution. Even though in all three paradigms (0-, 1- and 2-back) a small percentage of the subjects in the older subjects group scored lower even when receiving a lower dose compared to the younger group, the great majority did not have a larger decrease in the performance. The above-mentioned outliers in the older subjects group scored consistently poorer in the three tests as reflected in the individual $E_{max}$ estimates graphed per age (Figure 2.2). A larger number of older subjects scored lower compared to the younger group where subjects with an estimated $E_{max}$ above the sample's upper 95% CI were above 65 years, even when receiving a lower dose, at the population level there was no significant difference between the higher decrease in the score or baseline and age. This might provide evidence that, while in general older subjects perform similarly to younger adults after scopolamine administration in tests evaluating verbal working memory and execution, the number of subjects who perform worse during the test increases with a higher age; suggesting that although age as such is not correlated with a worse performance during scopolamine administration, the number of subjects with a higher sensitivity to the anti-cholinergic compound increases with age. Future research should be directed to longitudinally study whether these subjects are prone to develop Mild Cognitive Impairment.

Contrary to previous literature, we suggest that scopolamine administration does not lead to an increase in sensitivity of cognitive function tests (e.g.: decrease in performance) in healthy older subjects compared to healthy young. This finding suggests that increased sensitivity to scopolamine in healthy older subjects may not be due to incipient cholinergic neuronal degeneration in the majority of older subjects. Healthy subjects of advanced age perform similar to younger subjects after scopolamine administration in tests evaluating verbal working memory and execution. This finding seems to be





in line with previous experiments that could not demonstrate a decline in the availability of nicotinic acetylcholine receptors with increasing age in healthy human older subjects with a MMSE score above 27 points, a similar population in the current study (Ellis *et al*, 2009). Prescription of scopolamine in healthy older subjects should be in general safe and well tolerated, however caution is advised when co-administered with CYP3A4 inhibitors or in subjects with a low weight since the apparent peripheral volume of distribution was influenced by total body weight. Further studies should be performed in subjects above 78 years and in subjects with cognitive impairment.

Regarding the reaction time measured during the N-back test at baseline, older subjects were slower in responding than younger subjects. Nevertheless once scopolamine was administered, the increase in the reaction time was similar in both groups. According to the estimations in our model, on the average population estimate, a subject would increase his/her reaction time with a rate of 4.46% every 10 years (Figure 2.4), similar to a decrease previously reported (Ratcliff *et al*, 2001).

Scopolamine effects on the resting state EEG performed with eyes closed included a decrease in $\alpha$ and an increase in $\delta$ and $\vartheta$ frequencies of both Fz-Cz and Pz-Oz leads. In addition, no difference in sensitivity was found between older and younger subjects after scopolamine administration. These findings are consistent with previous literature; intramuscular administration of 0.25 mg of scopolamine in subjects between 24 and 31 years produced a significant increase in absolute $\delta$ power and in relative $\delta$ and $\vartheta$ power in subjects with eyes closed, mainly over central and parieto-occipital regions, and a decrease in absolute and relative $\alpha$ power, widespreaded but mainly over frontal regions (Kikuchi *et al*, 1999). Higher intramuscular doses of scopolamine (0.5 and 0.75 mg) induced a dose-related increase of relative power in low- and high-frequencies and a decrease in the $\alpha$ range in the posterior leads (Sannita *et al*, 1987). A previously published PK-PD model that described the EEG effects of an intravenous and intramuscular administration of 0.5 mg of scopolamine reported a decrease in the $\alpha$ power over the frontal, central, and occipital brain areas compared to placebo, explained by a sigmoidal $E_{max}$ model. Even though non-significant, the appreciable changes in the $\delta$, $\vartheta$ and $\beta$ frequencies were previously reported (Ebert *et al*, 2001).

In the present study, an age related difference in the EEG baseline was detected in the low frequency ranges (δ and ϑ) but not in the *α* frequency. Older subjects had a decreased absolute power in the δ frequency and ϑ frequency. These findings are consistent with reported literature where an age dependent decrease in EEG low frequency activity was identified (Leirer *et al*, 2011).

Due to teratogenicity risks related to other studied drugs rather than scopolamine, no women were allowed to participate in the first three studies, however no sex differences are to be expected based on neuroimaging morphology (Raz *et al*, 2005) and longitudinal assessments in cognitive function (Aartsen *et al*, 2004).

This study provides evidence that plasma scopolamine concentrations in the healthy older subjects are influenced not only by body weight but also by age and once this difference in exposure is corrected for, most age-related increases in anticholinergic sensitivity disappear. The peak velocity of the saccadic eye movements was the only test where sensitivity increased with age.





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**TABLE 2.1 Subject demographics.**

Mean ± Standard Deviation. Age is presented as Mean (minimum – maximum).

| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | All |
|---|---|---|---|---|---|
| Subjects (n) | 43 | 43 | 13 | 36* | 135 |
| Age (years) | 29.3 (18 - 55) | 27.7 (18 – 55) | 25.4 (19 – 36) | 69.0 (65 - 78) | 39.0 (18 - 78) |
| Weight (kg) | 78.6 ± 7.54 | 78.6 ± 7.74 | 81.1 ± 9.76 | 77.8 ± 11.82 | 78.6 ± 9.08 |
| BMI ($kg \cdot m^{-2}$) | 23.6 ± 2.30 | 23.3 ± 2.69 | 24.6 ± 2.82 | 25.9 ± 2.51 | 24.2 ± 2.73 |
| Scopolamine dose (mg) | 0.5 | 0.5 | 0.5 | 0.3 | NA |

** 13 female subjects. NA: not applicable.*

**TABLE 2.2 Population estimates for pharmacokinetic and pharmacodynamic models for scopolamine.**

Parameters are reported as population estimate ± standard error. IIV: Inter-individual Variability expressed as Coefficient of Variation and shrinkage between parenthesis.

| | CL [α] (L · min$^{-1}$) | IIV (shrinkage) | VC (L) | IIV (shrinkage) | $V_p$ [β] (L) | IIV (shrinkage) | Q (min$^{-1}$) | CAC | CWC | CWV | Error ($\sigma^2$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Scopolamine pharmacokinetics | 1.09 ± 0.096[AB] | 10.3% (9.7) | 2.66 ± 1.050 | 74.1% (49.3) | 62.10 ± 10.100 [B] | 9.5% (25.6) | 1.01 ± 0.247 | -0.12 ± 0.019 | 0.56 ± 0.097 | 0.38 ± 0.120 | 0.045 (prop) |
| | EC50 [ε] (pg·ml$^{-1}$) | IIV (shrinkage) | $E_{max}$ (%) | IIV (shrinkage) | $K_{in}$ [λ] | IIV (shrinkage) | $K_{out}$ | CAB or CAE | γ [c] | OC | Error ($\sigma_2$) |
| 0-back RT (msec) | 1300 ± 363 | 99.8% (33.2) | 0.837 ± 0.118 | NA | 3.42 ± 0.361[A] | 10.5% (8.13) | 0.00974 ± 0.00099 | 229 ± 40.9 | NA | NA | 0.00999(prop) |
| Saccadic Inaccuracy (%) | 55.1 ± 29.8 | 13.7% (36.9)[e] | 0.388 ± 0.079 | 49.9% (36.2)[e] | 22.8 ± 0.731 | 13.2% (21.86) | 3.33 ± 0.0398 | NA | NA | -0.06 ± 0.157 | 0.0493 (prop) |
| Saccadic Peak Velocity (deg · sec$^{-1}$) | 2530 ± 213[A] | 277.0% (20.9) | 0.232 ± 0.00113 | NA | 1280 ± 21 | 9.9% (3.1) | 2.73 ± 0.0468 | 34.0 ± 1.39 | NA | NA | 768.0 (prop) |
| Adaptive Tracker (%) | 386 ± 22.6 | 85.5% (10.50) | 1 | NA | 0.636 ± 0.0413 | 22.4% (4.1) | 0.0294 ± 0.0018 | NA | 1.10 ± 0.063 | NA | 8.64 (add) |
| EEG alpha Fz-Cz (μV) | 2.55 ± 1.06 | 1530.0% (48.4) | 0.232 ± 0.0258 | 95.4% (14.9) | 0.206 ± 0.0344 | 46.6% (1.1) | 0.0699 ± 0.0113 | NA | NA | NA | 0.0256 (prop) |
| EEG alpha Pz-Oz (μV) | 14.7 ± 0.168 | 1309.3% (27.9) | 0.443 ± 0.00389 | 37.6% (18.0) | 3.05 ± 0.135 | 57.9% (2.1) | 0.553 ± 0.0055 | NA | NA | NA | 0.0429 (prop) |
| EEG delta Fz-Cz (μV) | 469 ± 70.8 | 154.5% (35.5)[e] | 0.419 ± 0.0515 | 325.4% (19.2)[e] | 0.0354 ± 0.00369[A] | 28.9% (2.1)[e] | 0.0166 ± 0.00161 | 161 ± 30 | NA | 1.410 ± 0.722 (EC50 vs. $E_{max}$); -0.153 ± 0.0494 ($E_{max}$ vs. $k_{in}$) | 0.0338 (prop) |
| EEG delta Pz-Oz (μV) | 1230 ± 368 | 1166.2% (29.6) | 0.537 ± 0.0601 | 55.8% (48.0) | 0.726 ± 0.186 [A] | 30.3% (1.7) | 0.348 ± 0.084 | 210 ± 63.7 | NA | NA | 0.0304 (prop) |
| EEG theta Fz-Cz (μV) | 4110 ± 1410 | 2162.7% (31.1) | 0.594 ± 0.106 | NA | 0.863 ± 0.0422[A] | 28.4% (1.8) | 0.594 ± 0.106 | 142 ± 24.7 | NA | NA | 0.0297 (prop) |
| EEG theta Pz-Oz (μV) | 8240 ± 6270 | 1778.6% (27.9) | 1.15 ± 0.179 | NA | 13 ± 8.79 | 33.4% (0.5) | 6.08 ± 4.23 | NA | NA | NA | 0.0379 (prop) |
| | EC50 (pg·ml$^{-1}$) | $K_{in}$ | IIV (shrinkage) | $K_{out}$ | $E_{max}$ (%) | IIV (shrinkage) | $E_0$ | IIV (shrinkage) | NA | OC | Error ($\sigma^2$) |
| 0-back ratio (% correct answers)[f] | 2140 ± 1720 | 0.0354 ± 0.00244[d] | NA | 0.036 ± 0.0061 | 1 | 631.3% (42.1)[e] | 3.77 ± 0.274 | 37.8% (20.1)[e] | NA | 0.40 ± 0.141 | 0.0039 (add) |
| 1-back ratio (% correct answers)[f] | 0.624 ± 0.0053 | 1.10 ± 0.0175 | 578.5% (47.3) | 221 ± 4.35 | 0.961 ± 0.00511 | 121.6% (30.0) | 3.33 ± 0.0293 | NA | NA | NA | 0.0059 (add) |
| 2-back ratio (% correct answers)[f] | 212 ± 72.4 | 0.54 ± 0.103 | NA | 0.317 ± 0.0704 | 1.09 ± 0.248 | 109.8% (31.9)[e] | 2.84 ± 0.164 | 35.9% (21.5)[e] | NA | 0.23 ± 0.107 | 0.0085 (add) |

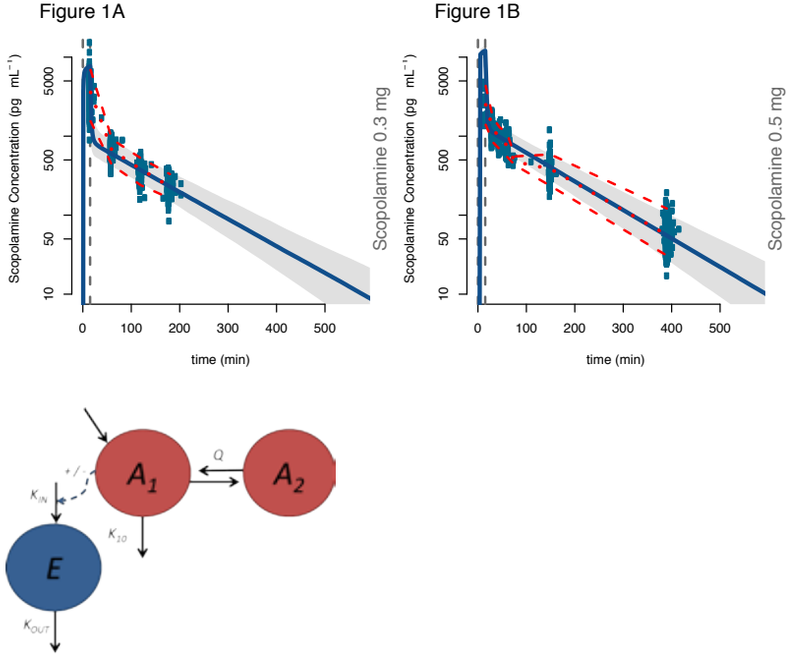
*Prop: proportional error. CAC: coefficient relating age and clearance. CWC: coefficient relating age and weight. CWV: coefficient relating apparent peripheral volume of distribution and weight.*

Baseline is calculated as the ratio of $K_{in}$ and $K_{out}$. **A**. Age used as a covariate (ε and λ in Equation 2.1). **B**. Weight used as covariate. **C**. Exponent in $E_{max}$ (γ). **D**. 4 buffer compartments. **E**. Omega block structure. **F**. Parameters reported as natural log odds.

*CAB: coefficient relating age and $K_{in}$. CAE: coefficient relating age and EC50. OC: correlation between Omegas. Eo: baseline. Add: additive error. NA: not applicable.*

**FIGURE 2.1 Scopolamine pharmacokinetics and pharmacodynamic effects.**

The column on the left represents the scopolamine 0.3 mg group, the middle column the scopolamine 0.5 mg group and the column on the right the placebo group. Figure 2.1A and 2.1B represent the plasma scopolamine concentrations. Figure 2.1C is the schematic representation of the model where the **light grey** dots represent the central ($A_1$) and peripheral ($A_2$) PK compartments and the **dark grey** circle (E) the effect (PD) compartment. Figure 2.1D, 2.1E and 2.1F display the Adaptive Tracker test, Figure 2.1G, 2.1H and 2.1I the Peak Velocity of the Saccadic Eye Movements test, 2.1J, 2.1K, 2.1L the Reaction Time of the 0-Back test. The continuous **dark grey** line represents the model population predicted values per group and the **grey** area represents the 95% prediction interval. Dots represent the observations. The middle discontinuous line represents the median of the observations with the upper and lower 95% confidence interval. Dotted, vertical **grey** lines represent the start and stop time of the scopolamine infusion. The differences in baseline-estimated values are indicated by a dotted line and the population value (when applicable). Simulations were performed with 1000 subjects.



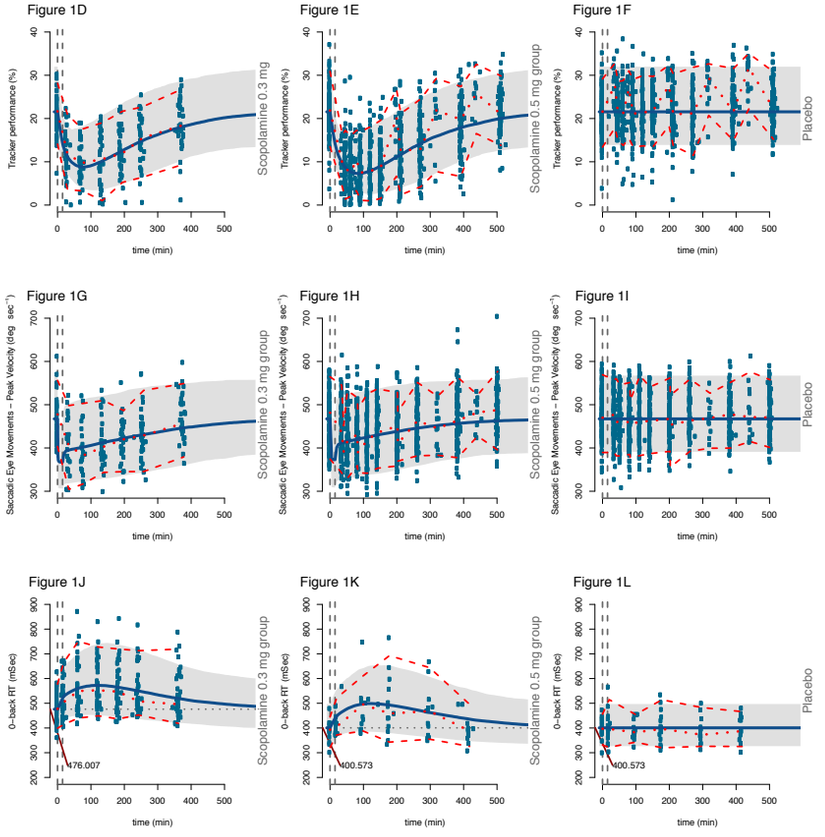



**FIGURE 2.2 Scopolamine pharmacodynamic effects in the N-back (number of correct answers).**

The row on the left represents the scopolamine 0.3 mg group (Figures 2.2A, 2.2D and 2.2G), the middle row the scopolamine 0.5 mg group (Figures 2.2B, 2.2E and 2.2H) and the row on the right the estimated $E_{max}$ (Figure 2.2C, 2.2F and 2.2I). The first row represents the 0-back, the second row the 1-back and the last row the 2-back percentage of correct answers in the paradigm. The continuous **dark grey** line represents the model population predicted values per group and the **grey** area represents the 95% prediction interval. Points represent the actual observations. Dotted, vertical gray lines represent the start and stop time of the scopolamine infusion. Simulations were performed with 1000 subjects.

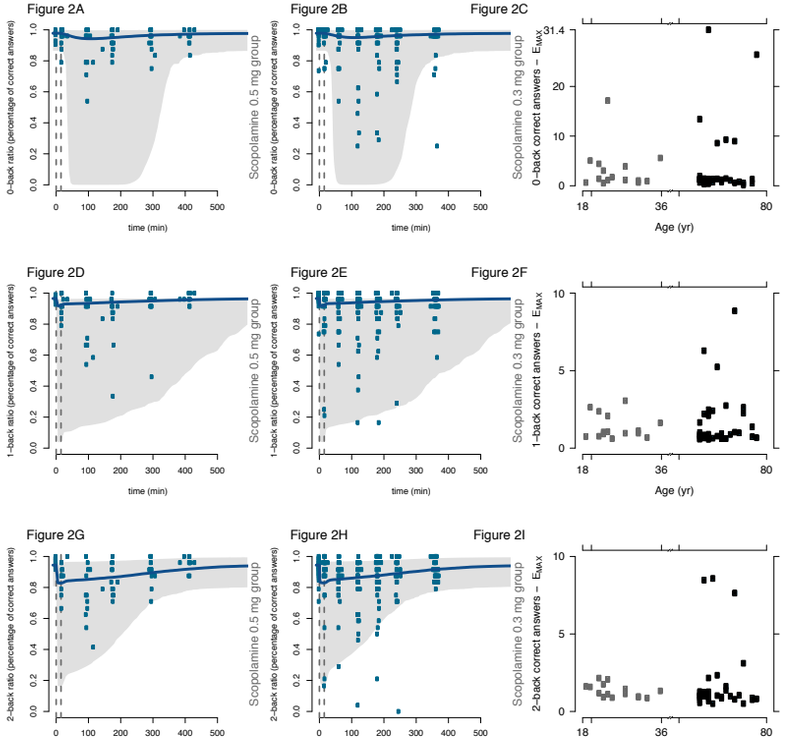







**FIGURE 2.3 Relationship between $EC_{50}$ and age in the Saccadic Eyes Movement Peak Velocity.**

The estimated $EC_{50}$ value is represented by the **black** (younger adults) and **grey** (older adults) points plotted against age. The continuous line represents the function with the population values for $EC_{50}$.

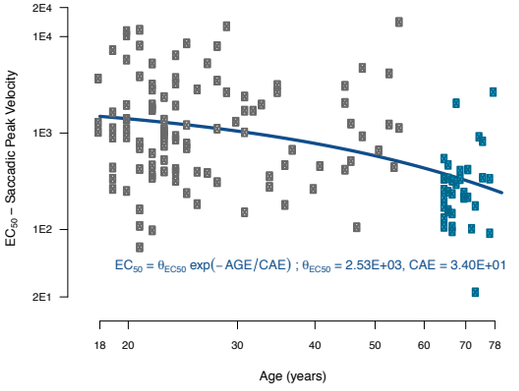
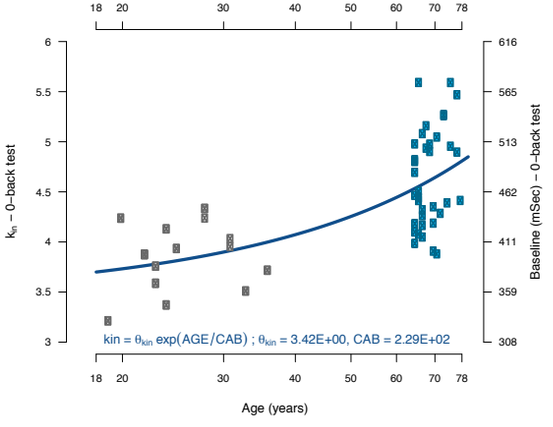



**FIGURE 2.4 Relationship between $\kappa_{in}$, baseline and age in the Reaction Time of the 0-back test.**

The estimated $\kappa_{in}$ value is represented by the **black** (younger adults) and **grey** (older adults) points plotted against age. The right column represents the baseline value in milliseconds. The continuous line represents the function with the population values for $\kappa_{in}$.



# AN ANTI-NICOTINIC COGNITIVE CHALLENGE MODEL USING MECAMYLAMINE IN COMPARISON WITH THE ANTI-MUSCARINIC COGNITIVE CHALLENGE USING SCOPOLAMINE

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## ABSTRACT

The muscarinic acetylcholine receptor antagonist scopolamine is often used for proof-of-pharmacology studies with pro-cognitive compounds. From a pharmacological point of view, it would seem more rational to use a nicotinic rather than a muscarinic anticholinergic challenge to prove pharmacology of a nicotinic acetylcholine receptor agonist. This study aims to characterize a nicotinic anticholinergic challenge model using mecamylamine and to compare it to the scopolamine model. In this double blind, placebo controlled, four way cross-over trial 12 healthy male subjects received oral mecamylamine 10 and 20 mg, intravenous scopolamine hydrobromide 0.5 mg and placebo. Pharmacokinetics were explored using non-compartmental analysis. Pharmacodynamic effects were measured with a multidimensional test battery that includes neurophysiological, subjective, (visuo)motor and cognitive measurements. All treatments were safe and well tolerated. Mecamylamine had a $T_{max}$ of 2.5 hours and a $C_{max}$ of 64.5 ng·ml$^{-1}$ for the 20 mg dose. Mecamylamine had a dose dependent effect which decreased the adaptive tracking performance, VAS alertness, finger tapping time and performance in the visual verbal learning task. No effects were seen on the simple reaction time test or saccadic peak velocity. Scopolamine significantly affected almost all pharmacodynamic tests. This study demonstrated that mecamylamine causes nicotinic receptor specific temporary decline in cognitive functioning. Compared with the scopolamine model, pharmacodynamic effects were less pronounced at the dose levels tested, but mecamylamine caused less sedation. The cognitive effects of scopolamine might at least partly be caused by sedation. Whether the mecamylamine model can be used for proof-of-pharmacology of nicotinic acetylcholine receptor agonists remains to be established.


## INTRODUCTION

Alzheimer's Disease (AD) is the most common form of dementia, with a prevalence of 3–7% in the Western European population (Takizawa *et al*, 2015). AD causes significant burden for the patients and their caregivers and high health care costs for society. Even though many research groups aim to unravel the pathophysiology and many pharmaceutical companies are searching for pharmacological targets for a curative treatment, no new drugs have been registered for this indication since 2003. The only approved therapy for mild to moderate AD is symptomatic treatment with cholinesterase inhibitors (CEIs), increasing the acetylcholine level in the synaptic cleft of cholinergic neurons. The cholinergic system is hypothesized to play an important role in several cognitive processes such as attention and memory (Drachman and Leavitt, 1974). Also, pathology studies have shown decreased levels of acetylcholine levels in the brains of patients with AD. Nevertheless, treatment with CEIs is only effective in about 14–36% of the AD patients and the dose is limited by peripheral side effects such as nausea, vomiting and diarrhoea (Birks, 2006; Birks *et al*, 2009; Olin and Schneider, 2002; Rösler *et al*, 1999; Tariot *et al*, 2000). CEIs inhibit esterases peripherally and in the central nervous system (CNS) so they will not only enhance functioning of cholinergic neuronal system, but will also induce peripheral cholinergic side effects, mainly via autonomic parasympathetic neurons. These peripheral side effects could be avoided with agonists that are more selective for AChRs with a higher presence in the CNS than peripherally, such as the $\alpha_7$ and $\alpha_4\beta_2$ nicotinic acetylcholine receptor (nAChR). nAChR are mainly located in the hippocampus, thalamus, amygdala, striatum, entorhinal, frontal and pre-frontal cortex. Based on the localization of nAChR in the human brain, nicotinergic blockade could be expected to result in an impairment of cognitive functions such as acquisition, processing and recall of information (Paterson and Nordberg, 2000). Accumulating evidence suggests that $\alpha_7$ nAChRs play an important role in the pathophysiology of neuropsychiatric diseases, including schizophrenia and AD. Hence, a number of pharmaceutical industries have developed selective and high affinity $\alpha_7$ nAChR agonists as therapeutic drugs for these neuropsychiatric diseases

(Toyohara and Hashimoto, 2010). Therefore, specific agonists targeting nAChR are currently being developed.

Proof-of-pharmacology studies with cholinergic compounds are often performed in healthy subjects after administration of scopolamine (Blin *et al*, 2009; Buccafusco, 2009; Cho *et al*, 2011; Deiana *et al*, 2009; Lee *et al*, 2009; Liem-Moolenaar *et al*, 2010; van Ruitenbeek *et al*, 2008; Snyder *et al*, 2005). Scopolamine is a competitive muscarinic acetylcholine receptor (mAChR) antagonist with similar binding to all five known muscarinic receptor subtypes. From a pharmacological point of view, it seems more rational to use a nicotinic rather than a muscarinic anticholinergic challenge in a proof of pharmacology study of a nicotinic acetylcholine receptor agonist.

Mecamylamine is a nAChR antagonist that has been used for the treatment of severe hypertension since the 1950s. In 2009 it was withdrawn from the market because of its unfavourable risk-benefit profile compared with many other available antihypertensives. Mecamylamine's antihypertensive effects are mediated through nAChR in peripheral autonomic ganglia. However, it also binds to nAChR present in the CNS (Stone *et al*, 1956). Previous studies have confirmed that mecamylamine, temporarily and reversibly, perturbs the above-mentioned cognitive processes in healthy volunteers (Little *et al*, 1998; Newhouse *et al*, 1992, 1994; Thompson *et al*, 2000; Voss *et al*, 2010).

With this study we aimed to better characterize the pharmacodynamic and pharmacokinetic effects of mecamylamine compared to scopolamine in order to improve the knowledge about a nAChR specific anti-cholinergic challenge and to develop a challenge model that may be suitable for proof-of-pharmacology studies with nAChR agonists.

## METHODS

### TRIAL DESIGN AND SUBJECTS

This double blind, double dummy, placebo controlled, four-way cross-over study was performed in healthy, non-smoker, young male subjects. On four different occasions with a wash-out of 7 days in between, all subjects received an oral dose of mecamylamine 10 mg with intravenous placebo, an oral dose of mecamylamine 20 mg with intravenous placebo, an intravenous dose of scopolamine hydrobromide 0.5 mg with oral placebo and both oral and intravenous placebo. The expected $T_{max}$ of scopolamine was 15 minutes after the start of the infusion, while the expected $T_{max}$ of mecamylamine was 3 hours after oral administration(Liem-Moolenaar *et al*, 2011; Young *et al*, 2001). Therefore, the intravenous dose of scopolamine or placebo was given 2.45 hours after administration of mecamylamine or placebo with infusion duration of 15 minutes in order to have a $T_{max}$ of both drugs at approximately the same time point. All subjects gave written informed consent for participation in the study. The ethics committee of the Leiden University Medical Center (The Netherlands) approved the study.

### DOSING RATIONALE

For the treatment of hypertension, the approved starting dose of mecamylamine was 25 mg per day and in various cognitive studies, a maximum of 20 mg orally produced few adverse effects, other than mild hypotension (Dumas *et al*, 2006, 2008, 2010; Ellis *et al*, 2006; Erskine *et al*, 2004; Ford *et al*, 1956; Green *et al*, 2005; Little *et al*, 1998; Newhouse *et al*, 1992, 1994; Thienel *et al*, 2009; Thompson *et al*, 2000; Voss *et al*, 2010; Young *et al*, 2001). Cognitive impairments are observed at dose levels of 15 mg and higher (Little *et al*, 1998; Newhouse *et al*, 1992, 1994; Thompson *et al*, 2000). For the pharmacological challenge in this study a lower (10 mg) and higher (20 mg) dose were chosen in order to better determine concentration-effect relationships. Mecamylamine uptake is characterized by complete absorption from the gastrointestinal tract (Young *et al*, 2001).

Scopolamine has been validated and frequently used as a pharmacological challenge in previously published studies with minimal adverse effects and demonstrable cognitive impairments at 0.5 mg scopolamine intravenously dosed (Liem-Moolenaar *et al*, 2011).

### PHARMACOKINETICS

Venous blood samples were obtained via an indwelling catheter before administration of mecamylamine or placebo and at 0.5, 1.0, 2.0, 3.0, 3.25, 4.0,

6.0, 8.0, 10.0 and 22.0 hours after drug administration. Plasma concentrations of mecamylamine and scopolamine were determined at the department of Clinical Pharmacology and Pharmacy at the VU University Medical Centre (Amsterdam, The Netherlands) by a validated method using high performance liquid chromatography coupled to tandem-mass spectrometry (LC-MS/MS).

The LC-MS/MS consisted of a Waters Alliance 2795 separation module and a Quattro Micro tandem mass spectrometer from Waters (Watford, UK). System control, data acquisition and data processing were performed using MassLynx v4.1. Chromatography was performed on a Kinetex C18 analytical column from Phenomenex. The particle size was 2.6 µM, column length was 150 mm and column diameter was 3.0 mm. The mobile phase ratio of 70% mobile phase A and 30% mobile phase B was run with a flow of 0.5 $mL \cdot min^{-1}$. Both mobile phases contained 0.05 % (v/v) trifluoretic acid and 5 mM ammoniumformate, whereas mobile phase A was prepared in purified water and mobile phase B was prepared in methanol. Ionization of the drugs was achieved in the positive electrospray modus. The respective MRM transitions were 168.1 > 137.1 m/z for mecamylamine, 304.2 > 138.1 m/z for scopolamine, 171.2 > 137.1 m/z for mecamylamine-D3 and 307.1 > 141.1 m/z for scopolamine-D3. For sample preparation, 100 µL of an aqueous solution containing 1 M zinc sulphate was added to 40 µL plasma and short vortexed. Hereafter 100 µL of the internal standard was added containing 100 $\mu g \cdot L^{-1}$ of mecamylamine-D3 and scopolamine-D3 in methanol. After vortexing for 3 minutes the samples were centrifuged at 10900 g for 3 minutes. The clear supernatant was transferred to vials and 25 µL was injected on the LC-MS/MS.

### PHARMACODYNAMIC ASSESSMENTS

To determine the pharmacodynamic effects of mecamylamine, a battery of tests (NeuroCart®) with a previously shown sensitivity to drug effects on a wide range of CNS domains was used (Liem-Moolenaar *et al*, 2011; van Steveninck *et al*, 1991, 1999; de Visser *et al*, 2003). All tests were performed twice at baseline, and repeated at 1.0, 2.0, 3.25, 4.0, 6.0, 8.0 and 10.0 hours after administration of mecamylamine or placebo. The only exception was the visual verbal learning test, which was performed 3.5 hours after dosing (immediate recall) and 5 hours after dosing (delayed recall and recognition). Measurements were performed in a quiet room with ambient illumination with only one subject per session in the same room.

**FINGER TAPPING** ✳ This test evaluates motor activation and fluency and has been adapted from the Halstead Reitan Test Battery (Andrew, 1977). The volunteer was instructed to tap as quickly as possible with the index finger of the dominant hand. Each session contained 5 performances of 10 seconds. Feedback on performance was given by a counter in the centre of the screen, while the amount of taps of each 10 second trial was shown on the screen in between the trials. The mean tapping rate of five trials per time point was used for statistical analysis.

**N-BACK** ✳ This test evaluates the working memory and requires buffering and updating consonants, matching, encoding and responding. The N-back test consists of three conditions, with increased working memory load. Letters were presented consecutively on the screen with a speed of 30 letters per minute. In the first condition subjects had to indicate whether the letter on the screen was an 'X'. In the second condition, subjects indicated whether the letter seen was identical to the previous letter. In the third condition, subjects were asked to indicate whether the letter was identical to two letters before the letter seen (Lim *et al*, 2008; Rombouts *et al*, 2002; Sweet *et al*, 2006).

**ADAPTIVE TRACKING** ✳ Adaptive tracking is a pursuit-tracking task, measuring attention and eye-hand coordination. A circle moves pseudo-randomly about a screen. The subject must try to keep a dot inside the moving circle by operating a joystick. If this effort is successful, the speed of the moving circle increases. Conversely, the velocity is reduced if the test subject cannot maintain the dot inside the circle. The average performance scores over a three-minute period was used for analysis. Before study participation, subjects performed three training sessions and at each occasion two baseline measurements were done (Gijsman *et al*, 1998; van Steveninck *et al*, 1991, 1993, 1999).

**SACCADIC PEAK VELOCITY** ✳ Saccadic peak velocity (SPV) is one of the most sensitive parameters for sedation. The use of a computer for measurement of saccadic eye movements has been described elsewhere (Baloh *et al*, 1975; van Steveninck *et al*, 1991, 1999). Average values of latency (reaction time), saccadic peak velocity of all correct saccades and inaccuracy of all saccades were used as parameters. Saccadic inaccuracy was calculated as the absolute value of the difference between the stimulus angle and the corresponding saccade, expressed as a percentage of the stimulus angle.

**SMOOTH PURSUIT EYE MOVEMENTS** ✳ The same system as used for saccadic eye movements was also used for measurement of smooth pursuit. For smooth pursuit eye movements, the target moves at a frequency ranging from 0.3 to 1.1 Hz, by steps of 0.1 Hz. The amplitude of target displacement corresponds to 22.5 degrees eyeball rotation to both sides. Four cycles are recorded for each stimulus frequency. The time in which the eyes were in smooth pursuit of the target was calculated for each frequency and expressed as a percentage of stimulus duration. The average percentage of smooth pursuit for all stimulus frequencies was used as parameter (Baloh *et al*, 1975; Bittencourt *et al*, 1983).

**PHARMACO-ELECTROENCEPHALOGRAPHY** ✳ Pharmacoelectroencephalography (p-EEG) was used to monitor any drug effects, which can be interpreted as evidence of penetration and activity in the brain (Cohen *et al*, 1985; Van Steveninck *et al*, 1993). EEG recordings were made using gold electrodes, fixed with EC2 paste (Astromed) at Fz, Cz, Pz and Oz, with the same common ground electrode as for the eye movement registration (international 10/20 system). The electrode resistances were kept below 5 KOHM. EEG signals were obtained from leads Fz-Cz and Pz-Oz and a separate channel to record eye movements (for artefacts). The signals were amplified by use of a Grass 15LT series Amplifier Systems with a time constant of 0.3 seconds and a low pass filter at 100 Hz. Data collection and analysis were performed using customized CED and Spike2 for Windows software (Cambridge Electronics Design, Cambridge, UK). Per session eight consecutive blocks of eight seconds were recorded. The signal was AD-converted using a CED 1401 Power (Cambridge Electronics Design, Cambridge, UK). Data blocks containing artefacts were identified and these were excluded from analysis. For each lead, fast Fourier transform analysis was performed to obtain the sum of amplitudes in the very low (0.5–2 Hz), δ (2–4 Hz), ϑ (4–7.5 Hz), α (7.5–13.5 Hz), β (13.5–35 Hz), and γ (35–48.9 Hz) frequency ranges. The duration of EEG measurements was 64 seconds per session.

**PUPIL SIZE** ✳ Pupil diameter was determined using a digital camera (Canon powershot A620) and a flash. The subject was instructed to look into the lens. A sharp picture of the eyes was taken using a camera with flash. All pictures were stored digitally. The diameters of the pupil and the iris were determined in the number of pixels used horizontally. For each eye, these values were recorded on data collection forms, and the pupil / iris ratio was subsequently calculated as a measure of pupil size.

**BODY SWAY** ✳ The body sway meter allows measurement of body movements in a single plane, providing a measure of postural stability. Body sway was measured with a pot string meter (celesco) based on the Wright ataxia meter (Wright, 1971). This method has been used to demonstrate effects of sleep deprivation (van Steveninck *et al*, 1999), alcohol (van Steveninck *et al*, 1993) and benzodiazepines (van Steveninck *et al*, 1993; Van Steveninck *et al*, 1997). With a string attached to the waist, all body movements over a period of time were integrated and expressed as mm sway. The total period of body-sway measurement was two minutes.

**STROOP** ✳ The Stroop test mainly investigates inhibition, interference and controlled versus automatic processing. A two trial version of the colour-word Stroop task was presented to the subjects. In the first trial, six coloured items in green, red or blue were presented at random and subjects indicated which colour they saw. In the second trial, 34 colour and word pairs were presented randomly to the subject, forming either congruent or incongruent matches. The subjects were asked to indicate the colour of the word (for example: if the word blue was written in red, the correct answer was 'red') (Laeng *et al*, 2005).

**SIMPLE REACTION TIME TASK** ✳ The Simple Reaction Time Task (SRTT) measures the attention and speed of information processing of the participant. In this task, participants view a black computer screen. At random intervals (0.5–1.5 seconds), a white circle appears in the centre of the computer screen. Participants were instructed to press the space bar with the index finger of their dominant hand each time the circle appears. They were instructed to respond as quickly as possible after appearance of the circle. A total of 40 circles were presented, and the duration of the task was approximately 1 minute. The outcome of the task is the time between stimulus display and response. It has been shown to respond to several classes of sedative drugs (Wezenberg *et al*, 2007).

**VISUAL ANALOGUE SCALE** ✳ Changes in subjective conditions are important aspects of drug effects, and a visual analogue scale (VAS) is one of the most commonly used ways to assess subjective states. It is a psychometric response scale, which is particularly suited to repeatedly quantify present subjective states. In the VAS according to Bond & Lader, the 'directions' of different scales on a form were alternated, to avoid 'habitual scoring' by subjects. Composite scores were derived for alertness, mood and calmness (Norris, 1971).

**VISUAL VERBAL LEARNING TEST** ✳ The Visual Verbal Learning Test (VVLT) contains three different subtests that cover almost the whole scope of learning behaviour (i.e., acquisition, consolidation, storage and retrieval) (de Haas *et al*, 2009). Subjects were presented 30 words in three consecutive word trials. Each trial ended with a free recall of the presented words (Immediate Recall). Approximately thirty minutes after start of the first trial, the volunteers were asked to recall as many words as possible (Delayed Recall). Immediately thereafter, the volunteers underwent memory recognition test, which consisted of 15 presented words and 15 'distractors' (Recognition).





### SAFETY ASSESSMENTS

All subjects underwent medical screening, including medical history, physical examination, vital signs measurement in supine and standing position, 12-lead electrocardiogram (ECG), urinalysis, drug screen and safety chemistry and haematology blood sampling. During study periods, safety was assessed using monitoring of adverse events, vital signs, ECG and safety chemistry and haematology blood sampling.

### PHARMACOKINETIC AND STATISTICAL ANALYSIS

The graphs and the pharmacokinetic parameters for mecamylamine were calculated by non-compartmental analysis in R (R Core Team, 2013). Primary pharmacokinetic endpoints were: maximum plasma concentration ($C_{max}$), time of maximum plasma concentration ($T_{max}$), area under the plasma concentration vs. time curve ($AUC_{0\text{-}last}$), area under the plasma concentration vs. time curve extrapolated to infinity ($AUC_{0\text{-}\infty}$), apparent terminal half-life, apparent clearance (CL/F) and apparent volume of distribution (Vd/F).

A mixed model analysis of covariance using SAS 9.1.3 for Windows (SAS Institute Inc., Cary, NC, USA) was used for analyses of pharmacodynamic effects, with subject, subject by treatment and subject by time as random effects; treatment, study period and by treatment by time as fixed effects; and the average baseline value as covariate. VVLT was analysed using a mixed model analysis of variance with fixed factors treatment and period, random factor subject and, if available, the (average) baseline. As this was an exploratory study, no formal adjustment for multiple testing was used. A $p$ value below 0.05 was considered statistically significant. In order to properly compare scopolamine and mecamylamine effects, two timepoints before scopolamine administration (1 and 2 hours after mecamylamine administration) were not included in the LSM graphs.

## RESULTS

A total of 15 healthy male subjects participated in the trial. During execution of the study, three subjects stopped prematurely, due to personal circumstances

(1), difficulties in blood sampling (1) and because of adverse events (nausea; 1). A total of 14 subjects completed at least one study period with treatment of mecamylamine and 12 subjects completed all study occasions. Subjects had a mean age of 25.9 (range 19–36) years, weight of 80.9 (range 59.9–90.0) kg and BMI of 24.4 (range 18.6–30.3) $kg \cdot m^{-2}$.

### SAFETY

All subjects reported at least one treatment emergent adverse event. Most frequent occurring adverse events were somnolence, dizziness, fatigue, nausea, dry mouth and headache (table 3.1). Adverse effects were mild and occasionally moderate and all disappeared spontaneously within a few hours. 3 of 14 subjects reported postural dizziness at the 20 mg mecamylamine dose. This coincided in all cases with measurable orthostatic hypotension.

The difference between standing and supine blood pressure significantly increased on the 20 mg mecamylamine dose, compared to placebo, while heart rate was significantly higher (table 3.2). Also, the difference in blood pressure between supine and standing position was significantly higher on the 20 mg mecamylamine dose, compared to placebo. On the 10 mg dose of mecamylamine, only the increase in supine and standing heart rate was statistically significant compared to placebo. There were no other consistent changes in ECG or laboratory safety parameters.

### PHARMACOKINETICS

The mean $T_{max}$ of mecamylamine was 2.1 hours (range 1–3.3) with a $C_{max}$ of 33.9 $ng \cdot ml^{-1}$ (range 23.4–44.1) for the 10 mg dose and 2.5 hours (range 0.5–6) with a $C_{max}$ of 64.5 $ng \cdot ml^{-1}$ (range 45.9–80.1) for the 20 mg dose (table 3.3). When analysing the individual plots. The terminal half-life was estimated to be 8.5 hours for 10 mg and 11.7 hours for 20 mg mecamylamine. This difference was not statistically significant. Other pharmacokinetic parameters were estimated as follows: CL/F = 17.9 $L \cdot h^{-1}$ (range 15.1–20.7) and Vd/F = 283 L (range 260–307).





Scopolamine pharmacokinetics could not be described in detail due to the low sample frequency after administration of scopolamine. The mean $C_{max}$ of scopolamine was 2549 $pg \cdot ml^{-1}$ (range 1349–4835) measured 15 minutes after the start of scopolamine infusion in all subjects. This is consistent with a previously published PK model of scopolamine (Liem-Moolenaar *et al*, 2011).

### PHARMACODYNAMICS

The main outcome parameters of the pharmacodynamic effects are summarized in table 3.4 and figure 3.1; more detailed information is reported in the supplementary material. Both administration of scopolamine and the 20 mg dose of mecamylamine led to a significant decrease compared to placebo in performance on adaptive tracking, the second and third trial of the immediate recall and the delayed recall of the visual verbal learning test (figure 3.2), finger tapping, body sway and VAS alertness. The effects of scopolamine were significantly stronger than those of mecamylamine on all these parameters, except for finger tapping and body sway. In contrast to mecamylamine, scopolamine administration resulted in an increase in reaction time and an increased score on the VAS for calmness compared to placebo. Scopolamine also induced a decrease in performance on all N-back parameters, a decrease in alpha and beta power on the p-EEG, and a decreased performance on the first immediate recall and the delayed recognition of the VVLT, the SRT and saccadic peak velocity and accuracy and smooth pursuit eye movements, while mecamylamine administration did not affect these tests. On the Stroop test, mecamylamine administration led to a decrease in reaction time compared to placebo, while scopolamine led to an increase in performance. Saccadic reaction time only increased after administration mecamylamine. No consistent differences between mecamylamine and placebo could be observed for N-back, SRT, p-EEG, saccadic inaccuracy, saccadic peak velocity, smooth pursuit eye movements and VAS Calmness. Reaction time on the VVLT recognition, pupil size and VAS mood were not affected by either scopolamine or mecamylamine compared to placebo.

## DISCUSSION

In this study, we investigated the pharmacodynamic and pharmacokinetic profile over time of mecamylamine using an extensive CNS test battery that included cognitive as well as visuomotor and neurophysiological measures. Two oral doses of mecamylamine were compared to intravenously administered scopolamine and placebo in order to determine the profile of a nAChR specific anti-cholinergic pharmacological challenge model. All treatments administered were considered safe and well tolerated, since all adverse events were transient and mild to moderate in severity. Pharmacokinetics of scopolamine are in line with previously described results (Liem-Moolenaar *et al*, 2011). The plasma concentrations of mecamylamine almost doubled with the doubling of the dose, which suggests dose-proportionality, as has been described before (Young *et al*, 2001).

Mecamylamine showed a dose dependent decrease in performance on several tests that represent different cognitive domains. The decline in performance on adaptive tracking and reduced VAS alertness reflected a deficiency in sustained attention. The decrease on the third trial of the immediate and the delayed recall of the VVLT represents a reduction in learning ability and memory retrieval. This mecamylamine induced impairment in acquisition and recall of information was expected, based on the localisation of nAChRs the brain (Paterson and Nordberg, 2000). These effects last up to 10 hours after drug administration. Mecamylamine did not have any significant effects on measures for sedation (SRTT and saccadic peak velocity).

The cognitive effects of mecamylamine found in this study are consistent with previous research, where mecamylamine was administered at doses of 5, 10 and 20 mg to healthy young and elderly volunteers (Newhouse *et al*, 1992, 1994). In these studies, the effects on cognition were studied one and two hours after dosing. A dose-dependent decrease in learning ability and reaction time was reported, which was more pronounced in elderly volunteers. There was no effect on subjective scales for drowsiness. Another study reported significant decrease in learning ability and semantic memory after administration of 15 mg mecamylamine (Little *et al*, 1998) and also a decrease in inspection time after administration of 20 mg of mecamylamine was reported (Thompson *et al*, 2000). Cognitive testing was done at one (Little *et al*, 1998; Thompson *et al*, 2000) or two (Newhouse *et al*, 1992, 1994) time points after dosing and tests for sustained attention were not performed in these studies. In none of the previously mentioned studies plasma mecamylamine concentrations were measured.

Conversely, several other studies found no effects of mecamylamine on various cognitive tests (Dumas *et al*, 2008; Ellis *et al*, 2006; Erskine *et al*, 2004; Green *et al*, 2005; Thienel *et al*, 2009; Voss *et al*, 2010). However, these studies all used a dose of 15 mg and investigated the cognitive effects at only one time point after dosing. With this relatively low dose and measurements at only one time point, modest effects may have been missed. This is supported by the finding that the attentional network measured with fMRI was down regulated after administration of the same dose of mecamylamine, while cognitive tests were not influenced (Dumas *et al*, 2010; Thienel *et al*, 2009). The slightly higher dose of mecamylamine and the frequency and sensitivity of our test may have attributed to the positive results of our study.

The second aim of this study was to compare the mecamylamine model with the anti-muscarinic scopolamine model. Several previous studies attempted to do this before, but none of these studies found significant cognitive effects of mecamylamine to compare with, probably due to low doses and few measurements (Dumas *et al*, 2008; Ellis *et al*, 2006; Erskine *et al*, 2004; Green *et al*, 2005; Little *et al*, 1998; Voss *et al*, 2010). In this study, scopolamine had a significant effect on all cognitive domains measured, including inhibition and working memory, as has been described before (Broks *et al*, 1988; Ellis *et al*, 2006; Green *et al*, 2005; Liem-Moolenaar *et al*, 2011; Little *et al*, 1998). The increase in reaction time and decrease in saccadic peak velocity, which was not observed after mecamylamine administration, and the larger reduction of VAS alertness, suggest that scopolamine has a strong sedative effect. These sedative effects of scopolamine have been previously reported (Kamboj and Curran, 2006; Koller *et al*, 2003; Pergolizzi *et al*, 2012). It is unlikely that this is related to relative dose differences between the doses of mecamylamine and scopolamine given in this study, since sedation is also reported after lower doses of scopolamine (Koller *et al*, 2003) and mecamylamine has been given as antihypertensive in doses up

to 80 mg in the past without any relevant sedation. The brainstem and basal brain areas controlling arousal and wakefulness contain more mAChR than nAChR (Brown *et al*, 2012), which is a likely explanation for the difference in sedative effects between mecamylamine and scopolamine. The scopolamine induced sedation may contribute to the cognitive effects of scopolamine in this study which are more pronounced than those of mecamylamine (Ford *et al*, 1956; MCQUEEN and SMIRK, 1957). The larger magnitude of the effects of scopolamine may seem attractive, but smaller, though still relevant effects of a new compound might get lost in the margins of variability or get overshadowed by the sedation caused by scopolamine. Due to the absence of sedation, the mecamylamine challenge may not only be more suitable for proof of pharmacology studies with a nAChR agonist, but also for other procognitive compounds.

We can conclude from this study that the nicotinic anticholinergic pharmacological challenge with mecamylamine results in measurable cognitive deficits with a nAChR specific profile, which is clearly distinguishable from the profile of the mAChR antagonist scopolamine. The mecamylamine challenge could therefore be suitable for proof of pharmacology studies with nAChR agonists. Furthermore, the relevant lack of sedation is an advantage of the mecamylamine challenge, compared with the scopolamine challenge.

A PK-PD-model of mecamylamine would be helpful in designing studies with the mecamylamine challenge. However, with the results of this study, PK-PD-modelling of the neurophysiological endpoints was not possible due to the narrow range of difference in pharmacodynamic effects between the mecamylamine lower and higher dose.

In conclusion, this study demonstrated that mecamylamine causes nicotinic receptor specific temporary decline in cognitive functioning and affects different CNS domains. Compared with the scopolamine model, pharmacodynamic effects were less pronounced at the dose levels tested and caused less sedation. Whether the mecamylamine model can be used for proof-of-pharmacology of nicotinic acetylcholine receptor agonists remains to be established.



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**TABLE 3.1 Most frequent treatment emergent adverse events.**

Number of adverse events and percentage from the subjects experiencing the adverse events.

| | Placebo n=14 | Mecamylamine 10 mg n=12 | Mecamylamine 20 mg n=14 | Scopolamine 0.5 mg n=13 |
|---|---|---|---|---|
| Subjects with at least 1 AE | 7 (50.0%) | 8 (66.7%) | 12 (85.7%) | 13 (100%) |
| Number of different AEs | 8 | 9 | 33 | 19 |
| Somnolence | 2 (14.3%) | 6 (50.0%) | 9 (64.3%) | 7 (53.8%) |
| Dizziness | - | 2 (16.7%) | 4 (28.6%) | 10 (76.9%) |
| Fatigue | 2 (14.3%) | 2 (16.7%) | 5 (35.7%) | 4 (30.8%) |
| Nausea | 2 (14.3%) | 1 (8.3%) | 5 (35.7%) | 3 (23.1%) |
| Dry mouth | 1 (7.1%) | - | 1 (7.1%) | 5 (38.5 %) |
| Headache | 2 (14.3%) | 2 (16.7%) | 1 (7.1%) | 2 (15.4%) |
| Disturbance in attention | - | 1 (8.3%) | 2 (14.3%) | 1 (7.7%) |
| Dysgeusia | 1 (7.1%) | - | 2 (14.3%) | 1 (7.7%) |
| Diplopia | - | - | 1 (7.1%) | 2 (15.4%) |
| Dizziness postural | - | - | 3 (21.4%) | - |





**TABLE 3.2 Vital signs per treatment group.**

Per group the difference estimate and in parenthesis the confidence interval is presented.

| | Treatment effect | Mecamylamine 10 mg n=12 | Mecamylamine 20 mg n=14 | Scopolamine 0.5 mg n=13 |
|---|---|---|---|---|
| Diastolic BP (supine) (mmHg) | p = 0.1372 | 1.5 (-1.2, 4.2) p=0.2674 | -0.6 (-3.1, 2.0) p=0.6652 | -1.7 (-4.3, 1.0) p=0.2067 |
| Diastolic BP (standing) (mmHg) | p = 0.0021 | 0.1 (-3.4, 3.5) p=0.9682 | -6.2 (-9.5,-2.8) p=0.0007 | -2.2 (-5.7, 1.2) p=0.1995 |
| Diastolic BP (standing-supine) (mmHg) | p = 0.0028 | -1.0 (-4.3, 2.3) p=0.5428 | -5.5 (-8.6,-2.5) p=0.0009 | -0.3 (-3.4, 2.9) p=0.8698 |
| Systolic BP (supine) (mmHg) | p = 0.0379 | -0.4 (-4.0, 3.3) p=0.8436 | -4.5 (-8.0,-0.9) p=0.0149 | -3.4 (-7.0, 0.2) p=0.0632 |
| Systolic BP (standing) (mmHg) | p = 0.0030 | -1.7 (-6.0, 2.6) p=0.4277 | -7.8 (-12.0,-3.7) p=0.0005 | -1.6 (-5.9, 2.7) p=0.4507 |
| Systolic BP (standing-supine) (mmHg) | p = 0.0129 | -1.7 (-5.3, 1.9) p=0.3445 | -4.9 (-8.4,-1.3) p=0.0090 | 0.8 (-2.8, 4.5) p=0.6441 |
| Heart rate (supine)(bpm) | p < 0.0001 | 6.9 (3.4,10.3) p=0.0003 | 9.4 (6.3,12.6) p<0.0001 | -4.5 (-7.8,-1.2) p=0.0099 |
| Heart rate (standing) (bpm) | p < 0.0001 | 8.7 (2.9,14.5) p=0.0042 | 16.0 (10.4,21.5) p<0.0001 | -4.4 (-10.3, 1.5) p=0.1390 |

**TABLE 3.3 Summary of mecamylamine pharmacokinetic parameters.**

| | Mecamylamine 10 mg (n=12) | | | | Mecamylamine 20 mg (n=14) | | | |
|---|---|---|---|---|---|---|---|---|
| **Characteristic** | **Mean** | **SD** | **Min** | **Max** | **Mean** | **SD** | **Min** | **Max** |
| $C_{max}$ (ng/ml) | 33.9 | 5.96 | 23.4 | 44.1 | 64.5 | 10.9 | 45.9 | 80.1 |
| $T_{max}$ (hr) | 2.05 | 0.92 | 1 | 3.28 | 2.57 | 1.61 | 0.5 | 6 |
| Terminal half life (hr) | 8.48 | 1.47 | 5.44 | 11.22 | 11.66 | 5.41 | 6.16 | 23.9 |
| $AUC_{0\text{-inf}}$ | 503.8 | 126.3 | 332.9 | 746.1 | 1346.1 | 564.7 | 672.3 | 2621.8 |
| $AUC_{0\text{-last}}$ | 410.1 | 90.0 | 277.7 | 607.0 | 913.8 | 187.3 | 603.5 | 1260.6 |

**TABLE 3.4 Pharmacodynamic effects on cognitive tests.**

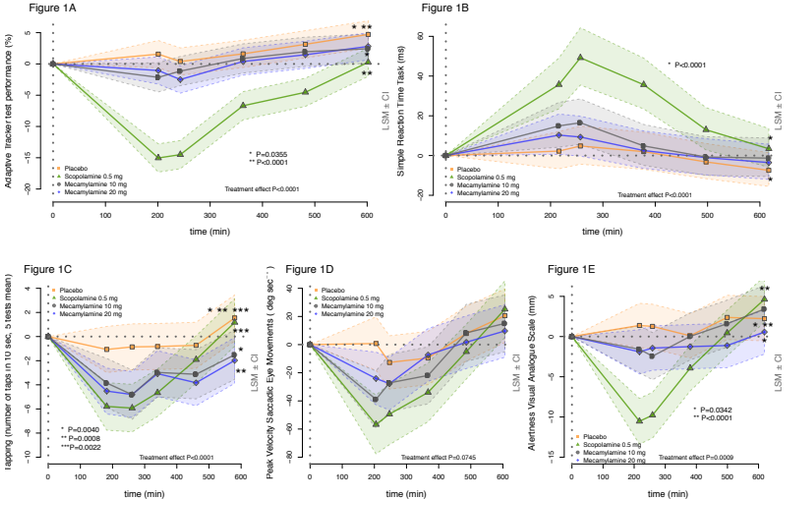
Per group the difference estimate and in parenthesis the confidence interval is presented.

| | Treatment effect | Mecamylamine 10 mg n=12 | Mecamylamine 20 mg n=14 | Scopolamine 0.5 mg n=13 |
|---|---|---|---|---|
| Adaptive tracking (%) | p < 0.0001 | -1.89 (-3.90, 0.12) p=0.0647 | -2.06 (-3.97,-0.15) p=0.0355 | -10.4 (-12.4,-8.39) p<0.0001 |
| VAS alertness (mm) | p = 0.0009 | -1.3 (-3.7, 1.2) p=0.2962 | -2.5 (-4.8,-0.2) p=0.0342 | -5.3 (-7.7, -2.9) p<0.0001 |
| Finger tapping (taps in 10 sec) | p = 0.0025 | -2.87 (-4.75,-0.99) p=0.0040 | -3.25 (-5.05,-1.46) p=0.0008 | -3.04 (-4.89,-1.18) p=0.0022 |
| VVLT 3rd recall (number of words) | p < 0.0001 | -2.7 (-5.1, -0.3) p=0.0286 | -3.6 (-5.9,-1.4) p=0.0025 | -7.7 (-10.1, -5.4) p<0.0001 |
| VVLT delayed recall (number of words) | p < 0.0001 | -3.1 (-5.8, -0.4) p=0.0259 | -3.8 (-6.4,-1.2) p=0.0051 | -7.1 (-9.8, -4.5) p<0.0001 |
| Simple reaction time task (% change) | p < 0.0001 | 7.0% (-0.8%, 15.5%) p=0.0786 | 3.8% (-3.5%, 11.7%) p=0.3080 | 26.8% (17.6%, 36.8%) p<0.0001 |
| Saccadic peak velocity (deg·sec-1) | p = 0.0745 | -14.3 (-33.5, 4.8) p=0.1367 | -10.9 (-29.0, 7.1) p=0.2232 | -25.4 (-44.2, -6.6) p=0.0098 |



**FIGURE 3.1 Effect on Tests Evaluating Fine Coordination, Reaction Time, Alertness, Motor Fluency and Eye Movements.**

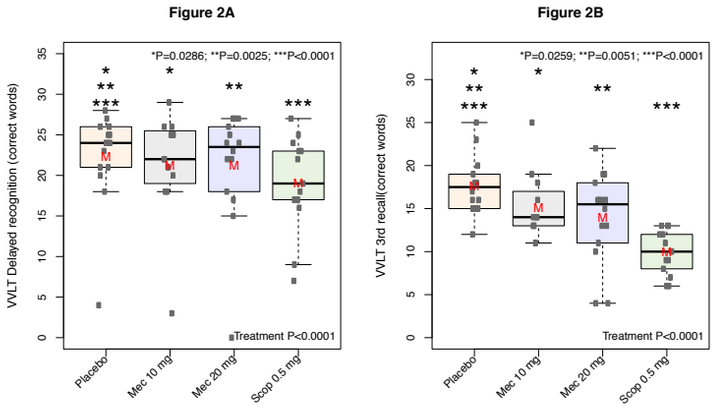
Mecamylamine 10 mg, mecamylamine 20 mg, scopolamine 0.5 mg or placebo effect versus time during the Adaptive Tracking test, Simple Reaction Time Task, Tapping, Peak Velocity of the Saccadic Eye Movements and the Visual Analogue Scale evaluating Alertness.




*Symbols represent the least square means per treatment group and the polygon (shaded area around the mean) the predicted confidence interval. Asterisks represent significance between groups (p value is mentioned per overall treatment effect and per group, when applicable). Vertical discontinuous line represents time point zero and the horizontal line represents zero.*

**FIGURE 3.2 Effect on Tests Evaluating Retrieval.**

Mecamylamine 10 mg, mecamylamine 20 mg, scopolamine 0.5 mg or placebo effect versus time during the Delayed Word Recognition and the number of correct answers during the third Recall condition of the Verbal Visual Learning Test. The box plots represent the first and third quartile, the middle line the group mean and the 'M' represents the median. The vertical lines the confidence interval. Individual observations are plotted as well.








# 4

# REVERSAL OF MECAMYLAMINE-INDUCED EFFECTS IN HEALTHY SUBJECTS BY NICOTINE RECEPTOR AGONISTS: COGNITIVE AND (ELECTRO)PHYSIOLOGICAL RESPONSES

SUBMITTED TO:
Psychopharmacology

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## ABSTRACT

Establishing a pharmacologic challenge model could be an important tool to understand the complex nicotinic cholinergic system role in cognition and to develop novel compounds acting on the nicotine acetylcholine receptor. We examined not only the effects of the nicotinic antagonist mecamylamine on a battery of cognitive and neurophysiologic test, but also the effect of nicotine or galantamine co-administration in reversing the cognitive impairment caused by mecamylamine. We conducted a randomized, double-blind, double-dummy, placebo-controlled, four way cross-over study in 33 healthy subjects receiving a single oral dose of 30 mg of mecamylamine (or placebo) in combination with either 16 mg of oral galantamine or 21 mg of transdermal nicotine (or its double-dummy). Mecamylamine 30 mg induced significant disturbances of cognitive functions. Attention and execution of visual - (fine) motor tasks was decreased, short- and long-term memory was impaired and the reaction velocity during the test was slower when compared to placebo. Mecamylamine 30 mg produced a decrease in posterior $\alpha$ and $\beta$ power in the surface EEG, effects that were reversed by nicotine co-administration. Memory and motor coordination tests could be partially reversed by the co-administration of nicotine. Mecamylamine administration induced slowing of the EEG and produced decrease in performance of tests evaluating motor coordination and short and long-term memory. These effects could be partially reversed by the co-administration of nicotine, and to a lesser extent by galantamine.


## INTRODUCTION

The nicotinic cholinergic system plays an important role in key cognitive processes such as attention and working- and associative-memory, and is therefore essential for learning (Jones *et al*, 1999; Levin *et al*, 2006). Cholinergic dysfunction is recognized to be involved in the pathophysiology of neurodegenerative diseases (e.g. dementia) and psychiatric conditions (e.g. schizophrenia) and is, therefore, considered a promising therapeutic target (Court *et al*, 2000; Parri *et al*, 2011).

Scopolamine is the most frequently used challenge drug to induce temporary, reversible, cognitive disturbances resembling those of Alzheimer's disease (AD) in healthy subjects (Ebert and Kirch, 1998). Scopolamine is a selective and competitive muscarinic acetylcholine receptor antagonist, binding to all muscarinic receptor types (Ali-Melkkilä *et al*, 1993). With several nicotinic receptor agonists in the clinical phase of drug development (Beinat *et al*, 2015; Vallés *et al*, 2014), the interest in nicotinic acetylcholine receptor (nAChR) pharmacology is rising. The use of muscarinic receptor antagonist scopolamine to investigate the pharmacology of nicotinic receptor agonists would seem less direct and therefore we aimed to develop a pharmacological challenge model targeting the nicotinic cholinergic system.

Mecamylamine is a selective non-competitive nAChR antagonist (Webster *et al*, 1999). Mecamylamine 20 mg produced impairments in learning and retrieval (Newhouse *et al*, 1994), acquisition, increased reaction time and errors (Newhouse *et al*, 1992) and an increased inspection time during a visual discrimination test (Thompson *et al*, 2000). In order to be able to use mecamylamine as a challenge model to prove pharmacological effects of nicotinic compounds, it is necessary to demonstrate reversal of its temporary negative effects on cognition. In animals, successful reversal of mecamylamine-induced disturbances was demonstrated with nicotine co-administration (Brucato *et al*, 1994; Woodruff-Pak, 2003). To our knowledge, only one study in humans described partial reversal of increased inspection time induced by 20 mg of mecamylamine, when 5 mg of donepezil, an acetylcholinesterase inhibitor, was co-administered (Thompson *et al*, 2000).

In an previous exploratory study we confirmed that administration of 10 and 20 mg of mecamylamine in healthy subjects led to a temporary, dose-dependent

disturbance of several cognitive functions including fine motor coordination and fluency, short- and long-term memory, attention and concentration (Baakman *et al*, 2016). In this confirmatory study we further investigated the dose-effect relationship of mecamylamine with a higher dose of 30 mg. Furthermore, we aimed to further validate mecamylamine as a nicotinic anticholinergic challenge model by investigating the potential reversal of the observed effects of mecamylamine on cognition by co-administering galantamine (a cholinesterase inhibitor) and nicotine (a nAChR agonist).

## MATERIALS AND METHODS

### STUDY DESIGN

This was a randomized, double-blind, double-dummy, placebo-controlled, four-way cross-over study of a single oral dose of mecamylamine (or placebo) in combination with either galantamine or nicotine. The treatment arms were: mecamylamine plus placebo, mecamylamine plus nicotine, mecamylamine plus galantamine and (double)placebo. A minimal wash-out period of one week was utilized.

Oral medication was administered with water at time point zero of every visit. Five minutes thereafter, a nicotine or placebo patch was placed on the skin at the shoulder blade region. Subjects were discharged 32 hours post-dose after monitoring of vital signs was performed and if subjects were asymptomatic.



### SUBJECT SELECTION

A medical ethics committee approved the study protocol. After giving written informed consent, all subjects were medically screened prior to study participation. Healthy male incidental smokers (age between 18 and 45 years and Body Mass Index (BMI) between 18 and 32 $kg \cdot m^{-2}$; both inclusive) were included in the study. Incidental smokers, defined as subjects smoking at least once a month, but no more than 5 cigarettes per day within the past 3 months, were included in the study due to the fact that non-smokers might have experienced more severe side effects derived from the nicotine and galantamine administration. Main exclusion criteria included any relevant medical abnormalities including conditions causing cognitive impairment, orthostatic hypotension (Kaufmann, 1996) or hypertension (>140/90 mmHg). Use of agents or drugs known to influence CNS performance were not allowed during study participation.

### MEDICINAL PRODUCTS AND DOSING RATIONAL

Drug accountability of all medicinal products was managed by the Leiden University Medical Centre Clinical Trials pharmacy.

Mecamylamine 30 mg capsules (Euticals SpA, Milan, Italy) containing 36.6 mg mecamylamine HCl and microcrystalline cellulose as filling agent (used also in the placebo capsules) were administered orally. Based on an interim PK-PD modeling of the concentration-effect relationship of mecamylamine 10 and 20 mg on the blood pressure investigated in the exploratory study (data not presented), a single oral dose of 30 mg was considered safe. Moreover, the dose was expected not to exceed $E_{max}$ (still allowing reversal) and not to cause functionally limiting hypotension.

Transdermal patches containing 21 mg nicotine (NiQuitin®, Glaxo-Smithkline, Bolton, UK), with blinding covering were administered to reverse mecamylamine effects. Blinded vaseline patches were used as placebo. Nicotine 21 mg patches are the highest commercially available dose that is well tolerated without significant adverse events in smokers (DeVeaugh-Geiss *et al*, 2010).

Galantamine hydrobromide 4 mg over encapsulated capsules (Reminyl®, Janssen-Cilag SpA, Latina, Italy) or matching placebo capsules were orally administered. Four Galantamine 4 mg capsules or placebo were administered for a total dose of 16 mg. Doses up to 15 mg without titration have been safely administered in healthy subjects (Riemann *et al*, 1994) and in our center, galantamine 16 mg was previously administered in healthy elderly subjects (unpublished data). Galantamine was chosen as it exerts an allosteric nicotinic modulatory activity that donepezil lacks (Coyle and Kershaw, 2001; Maelicke *et al*, 2001; Maelicke and Albuquerque, 2000).

### SAMPLE SIZE DETERMINATION

Sample size calculations were performed using the data obtained from the Visual Verbal Learning test, performed in the previous study with

mecamylamine 10 and 20 mg. The sample size was calculated using 80% power in a paired t-test with a two-sided 0.05 significance level.

### COGNITIVE AND NEUROPHYSIOLOGY MEASUREMENTS

The NeuroCart® is a computerized test-battery of sensitive tests used to evaluate a wide range of central nervous system (CNS) effects of neuro- and psychoactive drugs. A practice session of all tests was performed at screening. At each study visit, baseline training was performed twice to ensure stable performance and minimize learning effects. The NeuroCart® test battery was subsequently performed at time points 30, 80, 130, 180, 230, 280, 360 and 480 minutes post-dose, except for the Visual Verbal Learning Test (VVLT) which was only performed once per occasion and the Milner Maze test (MMT) which was not performed at 130 and 230 minutes.

**N-BACK TEST** ✳ Subjects were asked to remember and correlate a sequence of letters presented in a random order (Lim *et al*, 2008) thereby allowing evaluation of (short-term) working memory. Performance is expressed as the ratio of correct and incorrect answers ([correct – incorrect]·total$^{-1}$) and reaction time on the 0-, 1- and 2-back conditions.

**ADAPTIVE TRACKING TEST** ✳ The test was performed as previously described (Borland and Nicholson, 1984; van Steveninck *et al*, 1991). The test mainly evaluates vigilance and arousal and visuo-motor coordination.

**ELECTRO-ENCEPHALOGRAM (EEG)** ✳ Resting state eyes-closed EEG recordings were obtained for 64 seconds per time point using four cranial superficial gold electrodes (Fz, Cz, Pz, Oz), placed following the 10-20 system and fixed with EC2 paste with the same common ground and eye movement registration. Electrode resistance was kept below 5 kΩ. Grass 15LT series Amplifier Systems was used for signal amplification with a time constant of 0.3 seconds and a low pass filter at 100 Hz. The signal was AD-converted using CED 1401 Power (Cambridge Electronics Design, Cambridge, UK). Fast Fourier transformed absolute power (μV) was calculated from the raw measurements in the $\alpha$ [7.5 – 13.5 Hz], $\beta$ [13.5 – 35 Hz], $\delta$ [2 – 4 Hz], $\vartheta$ [4 – 7.5 Hz] and $\gamma$ [>35 Hz] frequency ranges in two bipolar leads: Fz-Cz and Pz-Oz.



**FINGER TAPPING** ✳ Dominant hand finger tapping test was performed to evaluate motor activation and fluency (Andrew, 1977; Liem-Moolenaar *et al*, 2010).

**SIMPLE REACTION TIME TEST (SRT)** ✳ Subjects were instructed to react as soon as possible after a visual stimulus was presented.

**VISUAL VERBAL LEARNING TEST (VVLT)** ✳ This test evaluates the different aspects of learning (i.e. acquisition, consolidation, storage, retrieval) and was performed as previously described (Liem-Moolenaar *et al*, 2010; Schmitt *et al*, 2006; Zuurman *et al*, 2010).

**MILNER MAZE TEST (MMT)** ✳ The MMT is a visuo-spatial working memory test (Milner, 1965). The computerized version has an immediate, a delayed and a reverse trial where the same maze has to be completed in the reverse order. Outcome measures are time to complete (milliseconds) and accuracy (number correct and incorrect steps).

**VISUAL ANALOGUE SCALES (VAS)** ✳ The VAS is a frequently used scale to measure subjective feelings of drug effects, as previously described (Bond and Lader, 1974). From these measurements, three main factors are the calculated as described by the authors: alertness (from nine scores), contentedness (often called mood; from five scores), and calmness (from two scores). A VAS was added evaluating nausea.

**PUPIL DIAMETER MEASUREMENTS** ✳ The pupil/iris ratio was measured as previously described (Liem-Moolenaar *et al*, 2010; Twa *et al*, 2004).

### PHYSIOLOGIC MEASURES

Safety assessments, including registration of adverse events, electrocardiogram (ECG), body temperature, blood pressure and heart rate were performed at predefined times throughout the study. Hematology, biochemistry, urinalysis, alcohol and drugs test were performed at medical screening, pre-dose per visit and at follow-up.

### STATISTICAL ANALYSIS

All variables were summarized by treatment and time. Repeated measured data were analyzed with a mixed model analysis of variance with fixed factors treatment, period, time and treatment-by-time and as random factors subject, subject-by-treatment and subject-by-time and the average pre-dose values as covariate. Single measured pharmacodynamic data were compared with a mixed model analysis of variance with fixed factors treatment, period, random factors subject and the average pre-dose values as covariate. The analysis was performed by an independent statistician using SAS software for windows v9.4 (SAS Institute, Inc., Cary, NC, USA). Graphs were created using R v2.14.1 (R Foundation for Statistical Computing, Vienna, Austria).

## RESULTS

### SUBJECT DEMOGRAPHICS

Fifty-one healthy male subjects underwent medical screening and thirty-three subjects were included in the study. The mean age was 23.3 years (range 19–35), average body weight was 74.5 ± 8.3 kg (range 60.25–91.25) and BMI was 22.6 ± 2.4 $kg \cdot m^{-2}$ (range 19.4–27.7). Twenty-six subjects completed all four-study visits. Five subjects cancelled their participation after the first visit due to the side effects. One subject was withdrawn from the study because it was not possible to place an intravenous catheter and one subject stopped his participation for personal reasons.



### COGNITIVE AND NEUROPHYSIOLOGICAL MEASUREMENTS

The SRT and pupil diameter test were not significantly influenced by mecamylamine, nicotine or galantamine.

**ADAPTIVE TRACKING TEST** ✳ The mean performance on the Adaptive Tracking test was significantly influenced by mecamylamine administration (overall treatment effect $p<0.0001$), as shown in Table 4.1. As expected, mecamylamine alone produced a significant impairment in the mean performance of -3.3 % (-4.6 – -2.0, $p<0.0001$) in the Adaptive Tracking. Nicotine co-administration caused a significant improvement of 1.5 % (0.2 – 2. 8, $p<0.05$) in comparison to mecamylamine alone. Galantamine co-administration did not significantly reverse the effects of mecamylamine (mean group difference 0.2 %; Figure 4.1).

**N-BACK TEST** ✳ Examination of the mean correct - incorrect ratio by time in the 0-back condition showed a significant overall treatment effect, producing in average a decrease of -0.023 (-0.044 – -0.003, $p<0.05$) in the ratio after administration of mecamylamine (Figure 4.2, Table 4.1). Mecamylamine administration also produced a non-significant reduction in the ratio of correct-incorrect answers 1-back (-0.015) and 2-back (-0.018) condition. Nicotine co-administration non-significantly reversed mecamylamine effects during the 0-back (group mean 0.007), 1-back (0.015) and 2-back (0.018) conditions. Co-administration of galantamine produced a non-significant worsening of mecamylamine effects during the 0- (group mean -0.006), 1- (-0.013) and 2-back (-0.016) conditions, when compared to the mecamylamine group.

Regarding the reaction time (RT) during the N-back test, the only paradigm where a significant overall treatment effect ($p=0.0432$) was observed was the 2-back condition, the most difficult one. Mecamylamine administration produced a mean increase of 28.3 ms (2.0 – 54.6, $p<0.05$) in the 2-back RT (Figure 4.1). The increase in the RT due to mecamylamine administration was significantly reversed by the co-administration of both nicotine (mean difference -36.0 ms (-62.2 – -9.7, $p<0.01$)) and galantamine (mean difference -27.2 ms (-53.3 – -0.8, $p<0.05$). Mecamylamine administration increased non-significantly the RT in the 0-back (8.0 ms) and 1-back (6.2 ms). Nicotine non-significantly reversed mecamylamine effects during the 0-back (-9.6 ms) and 1-back (-4.5 ms) conditions. Galantamine reversed non-significantly mecamylamine effects during the 0-back (-4.0 ms) and further increased the 1-back (0.9 ms) condition.

**ELECTRO-ENCEPHALOGRAM** ✳ As shown in Figure 4.3, the mean *a* power over Pz-Oz by time showed a significant overall treatment effect ($p=0.0132$), however, the only significant contrast was an increase of 14.9% (6.0 – 24.6, $p<0.005$) when nicotine was co-administrated compared to

mecamylamine alone (Table 4.1). Administration of mecamylamine decreased non-significantly the *α* power over the Pz-Oz by -6.2% when compared to placebo and galantamine non-significantly reversed this effect (6.7%) when compared to placebo. Mecamylamine also decreased to a lesser extent and non-significantly the mean *α* power over Fz-Cz compared to placebo (-0.6%), effect that was non-significantly reversed by the co-administration of nicotine (3.0%) and galantamine (3.9%).

Mecamylamine showed a significant overall treatment effect on *β* power in the Pz-Oz lead. Mecamylamine administration reduced the *β* power by -7.1% (-13.7 - -0.1%, $p<0.05$) when compared to placebo. Nicotine co-administration reversed mecamylamine effects by 10.7% (2,9 – 19.1, $p<0.01$). Galantamine co-administration also appeared to reverse mecamylamine effects (4,5%), but the difference was not significant (Figure 4.3). Mecamylamine administration reduced also the *β* power Fz-Cz lead non-significantly (-2.6%).

No significant effects of mecamylamine were detected on the EEG in the γ, ϑ and δ frequency power at the Pz-Oz and Fz-Cz leads.

**FINGER TAPPING** ✳ Mecamylamine significantly decreased the mean number of taps recorded during the Finger Tapping tests by -5.3 taps (-6.8 – -3.8, $p<0.0001$). Mecamylamine plus nicotine or galantamine caused small non-significant decreases in the mean number of taps (-0.158 and -0.586, respectively).

**VISUAL VERBAL LEARNING TEST** ✳ The only parameter from the VVLT conditions where mecamylamine had a significant overall treatment effect was on the number of correct answers during the delayed word recognition ($p=0.0284$). Mecamylamine administration caused more mistakes than placebo (-1.87 correct answers; -3.46 – -0.28; $p=0.02$). Treatment with nicotine, appeared to reverse mecamylamine effects by 0.29 words, but this effect was not significant (Figure 4.2, Table 4.1).

**MILNER MAZE TEST** ✳ Mecamylamine administration produced a non-significant mean increase of 2195.1 ms in the exploration time during the Immediate condition (p=0.0167). Unexpectedly, mecamylamine caused a decrease of -1108 ms in the Delayed condition of the MMT when compared to placebo (p=0.0388). Contrary to what was observed in all other tests, galantamine co-administration produced a significant slowing (increase) in the mean exploratory time when compared to mecamylamine; the mean Exploratory Time in the group with galantamine co-administration was 5604.0 ms (429.1 – 10779, p<0.05) during the Immediate condition and 1740.3 ms (304.1 – 3176.6, p<0.05) during the Delayed condition. Nicotine co-administration slowed the mean Exploratory Time in the Delayed condition by 1976.8 ms (505.9 – 3447.6, p<0.01) when compared to the mecamylamine group.

**VISUAL ANALOGUE SCALES** ✳ Mecamylamine induced no significant differences compared to placebo on the mean VAS evaluating calmness and mood. A significant overall treatment effect was detected on the mean VAS alertness (overall treatment effect $p<0.05$) and nausea ($p<0.0001$). Mecamylamine administration produced a significant decrease in the mean subjective feeling of alertness by -1.82 mm (-3.61 – -0.02, $p<0.05$) on the VAS scale (Figure 4.1). This was not significantly reversed by either galantamine or nicotine.

Mecamylamine plus galantamine increased the mean VAS nausea measurement 90% (47% – 146%, $p<0.0001$; back-transformed), and the combination with nicotine caused an increase of 53% (19% – 98%, $p<0.005$; back-transformed) compared to mecamylamine alone.

### PHYSIOLOGIC MEASURES

**VITAL SIGNS** ✳ Examination of the mean standing systolic blood pressure (SBP) by time showed a significant overall treatment effect ($p<0.005$). While mecamylamine non-significantly decreased the mean standing SBP by -5.3 mmHg, nicotine co-administration produced an additional decrease of -8.8 mmHg (-16.1 – -1.6, $p<0.05$) when compared to mecamylamine alone (Table 4.1). A significant overall treatment effect in standing and supine position ($p<0.0001$) was observed in both position measurement of the heart rate. Mecamylamine administration produced an increase in heart rate in supine (mean 12.3 bpm [9.7 – 14.9], $p<0.0001$) and standing (mean 26.7 bpm [19.7 – 33.8], $p<0.0001$) positions. Co-administration of nicotine and galantamine did not influence the heart rate significantly. There were no changes in the body temperature in any of the groups compared to placebo.

There were no clinically significant changes in values for hematology, chemistry and urinalysis parameters.

**ADVERSE EVENTS (AE)** ✳ AE were less frequently reported in the placebo group (46.4%), followed by the galantamine (89.3%), nicotine (89.7%) and finally the mecamylamine (93.1%) group had the highest incidence of AEs in the trial. Table 4.2 displays the most incident AEs per treatment group. No severe or serious AEs were reported.

## DISCUSSION

A consistent pattern was observed after mecamylamine was administered: healthy subjects performed worse compared to placebo across cognitive and neurophysiological tests evaluating attention, motor fluency, visual (fine) motor coordination, short- and long-term memory and reaction time. Mecamylamine *in vitro* non-competitively antagonizes the most important central nicotinic receptors, $\alpha_3\beta_4$, $\alpha_7$ and $\alpha_4\beta_2$, related to cognitive functions (Papke *et al*, 2001). These receptors are situated principally in the prefrontal, motor and entorhinal cortex, and with lower density, in the cingular and temporal cortex, in the thalamus (principally the dorsomedial and ventrolateral nuclei) and basal ganglia in the human brain (Paterson and Nordberg, 2000). The afore-mentioned structures are associated with visuo-spatial and declarative memory, decision-making processes, integration of acquired stimuli, fine motor skills and learning, correlating with the measured mecamylamine induced effects observed as a result of nAChR blockade in this study.

Reversal of mecamylamine effects by a nAChR agonist has not been previously demonstrated in humans, probably because lower doses were used in previous experiments. In this study we provided evidence that nicotine partially reversed the effects produced after mecamylamine administration. Nicotine 21 mg administered transdermally over a period of 8 hours, significantly but not completely, reversed mecamylamine effects on the tests evaluating visual (fine) motor coordination, short- and long-term memory and reaction time. Co-administration of nicotine also appeared to reverse mecamylamine effects in tests evaluating alertness and visuo-spatial memory, but these effects were not significant. *In vivo* reversal by nicotine of the cognitive effects resulting from mecamylamine administration indicates that both drugs affect the same system, namely the nicotinic cholinergic central neuronal system. Mecamylamine is a nicotinic non-competitive antagonist that *in vitro* completely blocks the effect of nicotine on several nAChRs (Albuquerque *et al*, 2009). In order to determine the competitive effect-concentration relationship between nicotine and mecamylamine, a range of nicotine doses should be explored to better elucidate this relationship *in vivo* and determine if the partial nature of the reversal can be complete. Co-administration of a nicotinic agonist with different activity, i.e. selective $\alpha_3\beta_4$, $\alpha_7$ and $\alpha_4\beta_2$ agonist, should produce different profiles in the different cognitive areas and may help better characterize the drug *in vivo,* a reason for nicotine to reverse almost all test where mecamylamine had an effect, except for tests evaluating motor fluency and verbal short- and long-term memory. Different cognitive profiles with different nicotinic agonist might provide a functional challenge model with an interesting proof-of-pharmacology profile.

While galantamine appeared to reverse mecamylamine induced cognitive effects, the differences with placebo were not significant in any of the mecamylamine-induced cognitive or neuro-physiological effects except for the reaction time during the 2-back test. Galantamine has been reported to reverse electroencephalographic and sedative disturbances produced by scopolamine. One possible explanation might be that in the scopolamine study in which partial reversal by galantamine was shown, a galantamine dose of 0.5 mg · kg$^{-1}$ was used (Baraka and Harik, 1977), while in the current study the dose was on average 0.21 mg · kg$^{-1}$. We expected that the 'direct' reversal of a nicotinic antagonist by a nicotinic would require a lower concentration range than 'indirect' reversal of a muscarinic antagonist. Still, we cannot exclude that higher galantamine doses would have produced a more extensive reversal of mecamylamine-induced cognitive effects. Even though a higher galantamine dose in this study was considered, the expected side effects (severe nausea and vomiting) in healthy subjects after an acute administration of galantamine was an important argument not to administer higher doses of galantamine. In

retrospect, this was the right decision, as in this study there was already a high incidence of adverse events related to the mechanism of action of the drug (see Table 4.2).

Mecamylamine produced in the EEG a decrease in $\beta$ frequency power in the posterior bipolar leads of the surface EEG, and also led to a non-significant decrease in $\alpha$ power and an increase in $\vartheta$ power, which corresponds to reports from previous studies with mecamylamine (Pickworth *et al*, 1997). A decrease in posterior $\alpha$ power and an increase in frontal and posterior $\theta$ power has also been observed in patients with Alzheimer's disease (van Straaten *et al*, 2014). Nicotine significantly diminished the decrease in $\alpha$ and $\beta$ power induced by mecamylamine in the posterior leads of the EEG, mainly at the last time points (>300 minutes), producing an even greater increase when compared to placebo. The $T_{max}$ during transdermal nicotine patch administration is reported at 6 hours (360 minutes), consistent with the time where the maximum effect was observed in the EEG (DeVeaugh-Geiss *et al*, 2010). The increase of the $\beta$ power at the end of the trial observed in the EEG could be explained by a difference in the $T_{max}$, of mecamylamine and nicotine.

Administration of a single dose of 30mg of mecamylamine was safe, and generally tolerated well enough for a challenge model involving cognitive testing. The most common AEs in the active groups were known symptoms related to gastrointestinal and central nervous system AChR agonists administration. Nausea and vomiting were the most frequently reported adverse events on occasions where nicotine and galantamine were co-administered. It could be postulated that the mechanism for the nausea and vomiting is related to the high density of $\alpha_3$, $\alpha_4$, $\beta_2$ and to a less extent $\alpha_5$ and $\beta_4$ nAChRs in the area postrema (Léna and Changeux, 1997). Although we deliberately enrolled sporadic smokers in the study to avoid nausea due to administration of nicotine 21 mg (the approved starting dose for patients willing to abstain from smoking) a high incidence of nausea and vomiting was still observed. Mecamylamine decreased the BP in supine and standing positions, only significantly different compared to the placebo group in standing position. Blockage of the sympathetic system by mecamylamine and its effects on the BP has been extensively studied and described before in patients with hypertension, however not in healthy subjects (Ford *et al*, 1956). Mecamylamine effect on BP in healthy subjects mainly impaired the compensatory mechanisms of orthostatic hypotension.

Scopolamine 0.5 mg induced in previous studies in healthy subjects a higher incidence of somnolence (ranging from 24.0 to 58.3%; unpublished data) and dizziness (ranging from 48.0 to 76.9%; unpublished data) when compared to mecamylamine 30 mg (dizziness 17.2 and somnolence 34.5%) as shown in this study in Table 4.2. The decrease in attention after mecamylamine administration might suggest that this is not due to sedation (as with muscarinic antagonists) but to impairment of attention/concentration due to mecamylamine, suggesting that mecamylamine as challenge drug might be preferred to induce cognitive impairment with fewer sedative effects. Donepezil 5 mg has been reported as the only drug that partially reversed the effects induced by mecamylamine 20 mg in healthy subjects, which consisted of slowing of the inspection time during visual discrimination (Thompson *et al*, 2000). Similar to our study in humans, mecamylamine-induced cognitive effects were significantly reversed by nicotine in mice. In this animal study, however, nicotine did not reverse scopolamine induced effects (Levin *et al*, 1997). While numerous groups have been able to demonstrate reversal of scopolamine effects by co-administration of compounds with nAChR agonist activity in animal models, none of these results were ever reproduced in humans with the mecamylamine challenge model. The proposed mecamylamine model therefore seems superior to the scopolamine challenge model to use in translational and early phase clinical drug studies investigating novel nicotinic agonists.

In conclusion, we have confirmed in humans that a single dose of mecamylamine 30 mg induces a significant disturbance in cognitive functions such as visual (fine) motor coordination, short- and long-term memory, reaction time and changes in the EEG (decrease in $\alpha$ and increase in $\vartheta$ power), and that these effects could be partially reversed by the co-administration of nicotine. This suggests that the mecamylamine challenge model can be used for proof-of-pharmacology studies nAChR agonists in humans, providing a useful tool in drug development of cognition enhancing compounds currently being developed to treat Alzheimer's disease and schizophrenia, between other diseases.

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**TABLE 4.1 Mean differences (contrasts) and Least Squared (LS) means per treatment group on the neuro- and physiological parameters.**

| | LS Means | | | | | Contrasts | | |
|---|---|---|---|---|---|---|---|---|
| Parameter | Placebo | Mecamylamine | Mecamylamine + Galantamine | Mecamylamine + Nicotine | Treatment effect p-value | Mecamylamine vs. placebo | Mecamylamine + Galantamine vs Mecamylamine | Mecamylamine + Nicotine vs. Mecamylamine |
| Adaptive tracking (%) | 31.05 | 27.78 | 28.00 | 29.24 | $p$ <0.0001 | -3.27 (-4.58--1.97) $p$<0.0001 | 0.223 (-1.09-1.537) $p$=0.7355 | 1.467 (0.153-2.780) $p$=0.0292 |
| Taps: Mean of 5 trials (n/10 sec) | 64.30 | 59.00 | 58.41 | 58.84 | $p$ <0.0001 | -5.30 (-6.80--3.80) $p$<0.0001 | -.586 (-2.08-0.909) $p$=0.4367 | -.158 (-1.66-1.341) $p$=0.8343 |
| Simple reaction time task (sec) | 288.13 | 293.41 | 294.92 | 298.54 | $p$ =0.4640 | 1.8% (-2.6%-6.4%) $p$=0.4166 | 0.5% (-3.9%-5.1%) $p$=0.8182 | 1.7% (-2.7%-6.4%) $p$=0.4429 |
| N-back corr-incorr/total 0 | 0.94 | 0.92 | 0.92 | 0.93 | $p$ =0.0410 | -.023 (-.044--.003) $p$=0.0270 | -.006 (-.027-0.015) $p$=0.5784 | 0.007 (-.013-0.028) $p$=0.4839 |
| N-back mean RT 0 back (msec) | 447 | 455 | 451 | 445 | $p$ =0.7043 | 8.0 (-9.8-25.8) $p$=0.3721 | -4.0 (-21.8-13.9) $p$=0.6588 | -9.6 (-27.4-8.2) $p$=0.2836 |
| N-back corr-incorr/total 1 | 0.90 | 0.89 | 0.87 | 0.90 | $p$ =0.0602 | -.015 (-.038-0.008) $p$=0.2021 | -.013 (-.036-0.010) $p$=0.2634 | 0.015 (-.009-0.038) $p$=0.2184 |
| N-back mean RT 1 back (msec) | 497 | 503 | 504 | 498 | $p$ =0.7850 | 6.2 (-10.0-22.4) $p$=0.4485 | 0.9 (-15.3-17.2) $p$=0.9076 | -4.5 (-20.8-11.9) $p$=0.5873 |
| N-back corr-incorr/total 2 | 0.84 | 0.82 | 0.80 | 0.84 | $p$ =0.1079 | -.018 (-.049-0.014) $p$=0.2664 | -.016 (-.048-0.015) $p$=0.3043 | 0.018 (-.014-0.049) $p$=0.2689 |
| N-back mean RT 2 back (msec) | 575 | 603 | 576 | 567 | $p$ =0.0432 | 28.3 (2.0-54.6) $p$=0.0356 | -27.2 (-53.5--0.8) $p$=0.0437 | -36.0 (-62.2--9.7) $p$=0.0079 |
| Word recall correct 1 | 9.4 | 8.4 | 8.2 | 8.1 | $p$ =0.2031 | -1.00 (-2.32-0.32) $p$=0.1344 | -0.19 (-1.52-1.15) $p$=0.7806 | -0.30 (-1.64-1.05) $p$=0.6601 |
| Word recall correct 2 | 14.3 | 12.5 | 12.4 | 12.9 | $p$ =0.0790 | -1.79 (-3.39--0.20) $p$=0.0281 | -0.10 (-1.71-1.52) $p$=0.9040 | 0.37 (-1.26-2.00) $p$=0.6534 |
| Word recall correct 3 | 16.9 | 15.6 | 15.0 | 15.5 | $p$ =0.1835 | -1.29 (-2.97-0.39) $p$=0.1314 | -0.53 (-2.23-1.17) $p$=0.5365 | -0.04 (-1.76-1.68) $p$=0.9664 |
| Delayed word recall correct | 12.1 | 10.9 | 9.7 | 11.0 | $p$ =0.0470 | -1.22 (-2.89-0.45) $p$=0.1502 | -1.24 (-2.93-0.45) $p$=0.1484 | 0.08 (-1.63-1.79) $p$=0.9275 |
| Delayed word recognition correct | 25.1 | 23.2 | 22.7 | 23.5 | $p$ =0.0284 | -1.87 (-3.46--0.28) $p$=0.0220 | -0.49 (-2.10-1.12) $p$=0.5440 | 0.29 (-1.33-1.91) $p$=0.7200 |
| Delayed word recog RT corr (msec) | 903.1 | 925.1 | 912.3 | 941.1 | $p$ =0.4593 | 21.98 (-26.4-70.34) $p$=0.3681 | -12.8 (-61.7-36.12) $p$=0.6039 | 15.99 (-33.4-65.40) $p$=0.5213 |
| EEG α Fz-Cz (uV) | 3.07 | 3.05 | 3.17 | 3.14 | $p$ =0.7186 | -0.6% (-7.9%-7.3%) $p$=0.8760 | 3.9% (-3.8%-12.2%) $p$=0.3262 | 3.0% (-4.7%-11.2%) $p$=0.4518 |
| EEG α Pz-Oz (uV) | 5.48 | 5.14 | 5.48 | 5.91 | $p$ =0.0132 | -6.2% (-13.4%-1.6%) $p$=0.1149 | 6.7% (-1.6%-15.6%) $p$=0.1124 | 14.9% (6.0%-24.6%) $p$=0.0012 |
| EEG β Fz-Cz (uV) | 2.08 | 2.03 | 2.00 | 2.13 | $p$ =0.1292 | -2.6% (-7.9%-3.1%) $p$=0.3628 | -1.4% (-6.8%-4.5%) $p$=0.6362 | 5.3% (-0.6%-11.5%) $p$=0.0778 |
| EEG β Pz-Oz (uV) | 2.42 | 2.25 | 2.35 | 2.49 | $p$ =0.0439 | -7.1% (-13.7%--0.1%) $p$=0.0474 | 4.5% (-2.8%-12.5%) $p$=0.2315 | 10.7% (2.9%-19.1%) $p$=0.0068 |
| EEG δ Fz-Cz (uV) | 2.06 | 2.04 | 1.99 | 1.99 | $p$ =0.4487 | -1.0% (-6.3%-4.6%) $p$=0.7281 | -2.7% (-8.0%-2.9%) $p$=0.3282 | -2.6% (-7.8%-3.0%)$p$=0.3563 |
| EEG δ Pz-Oz (uV) | 2.04 | 2.08 | 2.05 | 2.02 | $p$ =0.9445 | 1.8% (-6.8%-11.3%) $p$=0.6822 | -1.1% (-9.5%-8.1%) $p$=0.8062 | -2.6% (-11.0%-6.5%) $p$=0.5571 |
| EEG γ Fz-Cz (uV) | 0.66 | 0.65 | 0.66 | 0.68 | $p$ =0.3345 | -0.6% (-6.1%-5.3%) $p$=0.8386 | 0.8% (-4.9%-6.8%) $p$=0.7872 | 5.0% (-0.9%-11.2%) $p$=0.0987 |
| EEG γ Pz-Oz (uV) | 0.64 | 0.63 | 0.61 | 0.67 | $p$ =0.3225 | -1.4% (-10.8%-9.1%) $p$=0.7825 | -2.2% (-11.6%-8.2%) $p$=0.6580 | 7.3% (-3.0%-18.7%) $p$=0.1708 |
| EEG ϑ Fz-Cz (uV) | 2.37 | 2.51 | 2.51 | 2.48 | $p$ =0.2088 | 5.7% (-0.5%-12.3%) $p$=0.0735 | 0.2% (-5.8%-6.6%) $p$=0.9437 | -0.9% (-6.8%-5.4%) $p$=0.7755 |
| EEG ϑ Pz-Oz (uV) | 2.55 | 2.77 | 2.79 | 2.66 | $p$ =0.1770 | 8.8% (-0.8%-19.2%) $p$=0.0716 | 0.9% (-7.9%-10.6%) $p$=0.8385 | -3.8% (-12.1%-5.4%) $p$=0.4066 |
| VAS Alertness (mm) | 48.9 | 47.1 | 45.8 | 47.2 | $p$ =0.0124 | -1.82 (-3.61--0.02) $p$=0.0470 | -1.30 (-3.13-0.53) $p$=0.1610 | 0.17 (-1.63-1.97) $p$=0.8539 |
| VAS Calmness (mm) | 52.9 | 53.8 | 53.7 | 53.2 | $p$ =0.6272 | 0.85 (-0.66-2.35) $p$=0.2641 | -0.04 (-1.55-1.47) $p$=0.9533 | -0.54 (-2.10-1.02) $p$=0.4906 |
| VAS Mood (mm) | 52.1 | 52.5 | 51.8 | 51.8 | $p$ =0.5487 | 0.44 (-0.73-1.60) $p$=0.4567 | -0.74 (-1.91-0.42) $p$=0.2060 | -0.73 (-1.91-0.45) $p$=0.2199 |
| VAS Nausea log(mm) | 0.331 | 0.365 | 0.644 | 0.551 | $p$ <0.0001 | .0337 (-.079-.1462) $p$=0.5526 | .2787 (.1670-.3903) $p$<0.0001 | .1860 (.0746-.2974) $p$=0.0013 |
| Left Pupil/Iris ratio | 0.4807 | 0.5003 | 0.5060 | 0.4879 | $p$ =0.1729 | 0.01960 (-0.00506-0.04426) $p$=0.1177 | 0.00574 (-0.01896-0.03044) $p$=0.6449 | -0.01235 (-0.03702-0.01231) $p$=0.3220 |
| Right Pupil/Iris ratio | 0.4944 | 0.5066 | 0.5101 | 0.4886 | $p$ =0.1946 | 0.01217 (-0.01007-0.03441) $p$=0.2795 | 0.00353 (-0.01870-0.02576) $p$=0.7529 | -0.01797 (-0.04023-0.00430) $p$=0.1123 |
| MMT_Imm: Exploratory Moves | 192 | 192 | 194 | 194 | $p$ =0.6157 | -0.3 (-4.4-3.7) $p$=0.8725 | 2.0 (-2.0-6.1) $p$=0.3250 | 2.2 (-1.9-6.3) $p$=0.2930 |
| MMT_Imm: Exploratory Errors | 23 | 23 | 24 | 24 | $p$ =0.6191 | -0.2 (-2.2-1.8) $p$=0.8226 | 1.0 (-1.0-3.0) $p$=0.3366 | 1.1 (-0.9-3.2) $p$=0.2688 |
| MMT_Imm: Exploratory Time (msec) | 74337 | 76533 | 82137 | 79947 | $p$ =0.0167 | 2195 (-3024-7414) $p$=0.4042 | 5604 (429.1-10779) $p$=0.0342 | 3415 (-1731-8560) $p$=0.1899 |
| MMT_Rev: Exploratory Moves | 38 | 38 | 38 | 37 | $p$ =0.3082 | 0.3 (-0.9-1.5) $p$=0.6378 | -0.3 (-1.5-1.0) $p$=0.6823 | -1.1 (-2.4-0.1) $p$=0.0749 |
| MMT_Rev: Exploratory Errors | 4 | 4 | 4 | 4 | $p$ =0.4044 | 0.1 (-0.5-0.7) $p$=0.7542 | -0.1 (-0.7-0.5) $p$=0.6960 | -0.5 (-1.1-0.1) $p$=0.1128 |
| MMT_Rev: Exploratory Time (msec) | 16826 | 17439 | 17016 | 17286 | $p$ =0.9342 | 612.6 (-1422-2648) $p$=0.5501 | -423 (-2488-1643) $p$=0.6845 | -153 (-2196-1889) $p$=0.8815 |
| MMT_Del: Exploratory Moves | 37 | 37 | 36 | 36 | $p$ =0.5768 | -0.1 (-1.3-1.0) $p$=0.7952 | -0.6 (-1.7-0.5) $p$=0.2993 | -0.3 (-1.4-0.9) $p$=0.6292 |
| MMT_Del: Exploratory Errors | 4 | 4 | 3 | 4 | $p$ =0.7074 | -0.1 (-0.6-0.5) $p$=0.8285 | -0.2 (-0.8-0.3) $p$=0.3747 | -0.1 (-0.6-0.5) $p$=0.7600 |
| MMT_Del: Exploratory Time (msec) | 15770 | 14662 | 16402 | 16639 | $p$ =0.0388 | -1108 (-2541-325.2) $p$=0.1277 | 1740 (304.1-3177) $p$=0.0182 | 1977 (505.9-3448) $p$=0.0091 |
| Diastolic BP standing (mmHg) | 70 | 66 | 65 | 61 | $p$ =0.0088 | -4.3 (-9.3-0.7) $p$=0.0880 | -0.9 (-5.6-3.8) $p$=0.7078 | -4.3 (-9.2-0.5) $p$=0.0769 |
| Diastolic BP supine (mmHg) | 62 | 63 | 63 | 62 | $p$ =0.6500 | 0.6 (-1.3-2.6) $p$=0.5105 | 0.3 (-1.6-2.2) $p$=0.7535 | -0.8 (-2.7-1.2) $p$=0.4372 |
| Systolic BP standing (mmHg) | 121 | 116 | 112 | 107 | $p$ =0.0027 | -5.3 (-12.8-2.2) $p$=0.1623 | -3.4 (-10.5-3.7) $p$=0.3487 | -8.8 (-16.1--1.6) $p$=0.0177 |
| Systolic BP supine (mmHg) | 116 | 114 | 115 | 115 | $p$ =0.3579 | -1.7 (-3.8-0.4) $p$=0.1099 | 0.2 (-1.9-2.3) $p$=0.8358 | 0.3 (-1.8-2.4) $p$=0.7886 |
| Heart rate standing (bpm) | 65 | 92 | 89 | 90 | $p$ <0.0001 | 26.7 (19.7-33.8) $p$<0.0001 | -3.3 (-10.2-3.6) $p$=0.3383 | -2.1 (-8.9-4.8) $p$=0.5555 |
| Heart rate supine (bpm) | 57 | 69 | 69 | 71 | $p$ <0.0001 | 12.3 (9.7-14.9) $p$<0.0001 | -0.5 (-3.1-2.2) $p$=0.7224 | 1.3 (-1.3-3.9) $p$=0.3227 |

**TABLE 4.2 Summary of number of subjects with an adverse event and number of adverse events with the highest incidence in descending order of incidence.**

| | Mecamylamine (n=29) | | Mecamylamine + Galantamine (n=28) | | Mecamylamine + Nicotine (n=29) | | Placebo (n=28) | |
|---|---|---|---|---|---|---|---|---|
| **Adverse event** | **Nr of events** | **Nr of subjects (%)** | **Nr of events** | **Nr of subjects (%)** | **Nr of events** | **Nr of subjects (%)** | **Nr of events** | **Nr of subjects (%)** |
| All Events | 76 | 27 (93.1) | 101 | 25 (89.3) | 108 | 26 (89.7) | 26 | 13 (46.4) |
| Nausea | 3 | 3 (10.3) | 15 | 14 (50.0) | 12 | 12 (41.4) | - | - |
| Somnolence | 14 | 10 (34.5) | 12 | 12 (42.9) | 11 | 10 (34.5) | 1 | 1 ( 3.6) |
| Dizziness | 5 | 5 (17.2) | 13 | 11 (39.3) | 13 | 11 (37.9) | 1 | 1 ( 3.6) |
| Fatigue | 8 | 7 (24.1) | 8 | 8 (28.6) | 6 | 6 (20.7) | 4 | 4 (14.3) |
| Orthostatic hypotension | 8 | 8 (27.6) | 4 | 4 (14.3) | 5 | 5 (17.2) | 6 | 4 (14.3) |
| Application site pruritus | 1 | 1 ( 3.4) | - | - | 7 | 6 (20.7) | 1 | 1 ( 3.6) |
| Ocular hyperemia | 3 | 2 ( 6.9) | 2 | 2 ( 7.1) | 6 | 6 (20.7) | - | - |
| Vision blurred | 6 | 5 (17.2) | 1 | 1 ( 3.6) | 4 | 4 (13.8) | - | - |
| Constipation | 5 | 4 (13.8) | 2 | 2 ( 7.1) | 5 | 5 (17.2) | - | - |
| Vomiting | 1 | 1 ( 3.4) | 3 | 3 (10.7) | 4 | 4 (13.8) | - | - |
| Headache | 3 | 3 (10.3) | 3 | 3 (10.7) | 6 | 6 (20.7) | 4 | 2 ( 7.1) |
| Dizziness postural | 2 | 1 ( 3.4) | 1 | 1 ( 3.6) | 3 | 3 (10.3) | - | - |
| Abdominal pain | 3 | 3 (10.3) | 3 | 3 (10.7) | 2 | 2 ( 6.9) | - | - |
| Feeling abnormal* | 1 | 1 ( 3.4) | 3 | 3 (10.7) | - | - | 2 | 2 ( 7.1) |
| Abdominal distension | 1 | 1 ( 3.4) | 3 | 3 (10.7) | - | - | - | - |

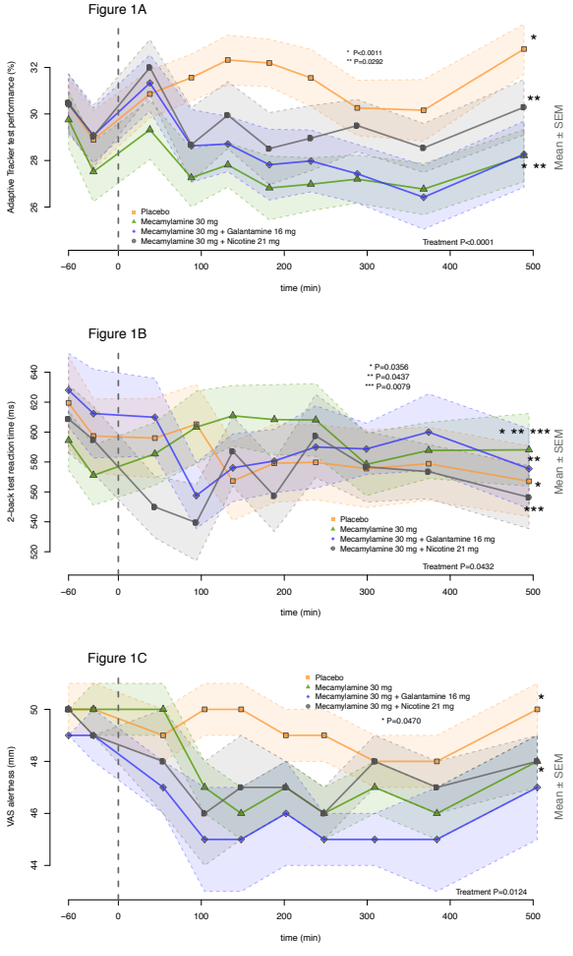
** Feeling abnormal was used by the research physician when no other symptom could describe the feeling the subject was experiencing.*





**FIGURE 4.1 Effect on Tests Evaluating Fine Coordination, Reaction Time, Attention and Alertness.**

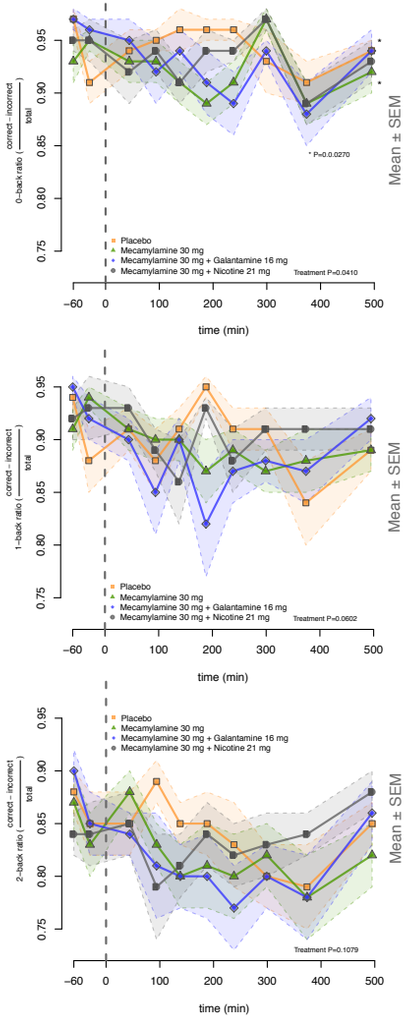
Mecamylamine, nicotine and galantamine effect versus time during the Adaptive Tracking test, Reaction Time during the 2-back condition and Visual Analogue Scale evaluating Alertness.




*Symbols represent the mean per treatment group and the polygon (shaded area around the mean) the standard error. Asterisks represent significance between groups (p value is mentioned per overall treatment effect and per group, when applicable). Vertical discontinuous line represents time point zero.*

**FIGURE 4.2 Effect on Tests Evaluating Short and Long Term Retrieval.**

Mecamylamine, nicotine and galantamine effect versus time during the o-back condition Ratio of Correct-Incorrect answers. Symbols represent the mean per treatment group and the polygon (shaded area around the mean) the standard error. Asterisks represent significance between groups (*p* value is mentioned per overall treatment effect and per group, when applicable). Vertical discontinuous line represents time point zero.








Asterisks represent significance between groups (p value is mentioned per treatment and per group, when applicable). Mecamylamine, nicotine and galantamine effect versus time during the Delayed Word Recognition condition of the Verbal Visual Learning Test number of correct answers. The box plots represent the first and third quartile, the middle line the group mean and the vertical lines the confidence interval. Individual observations are plotted as well.

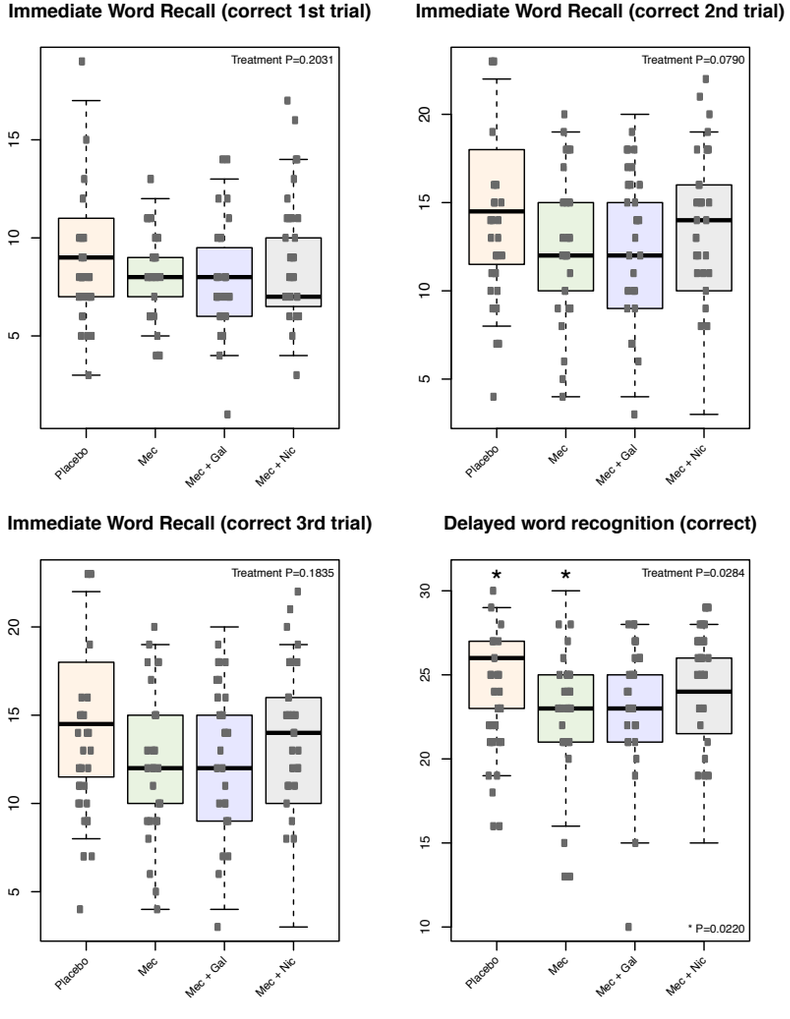
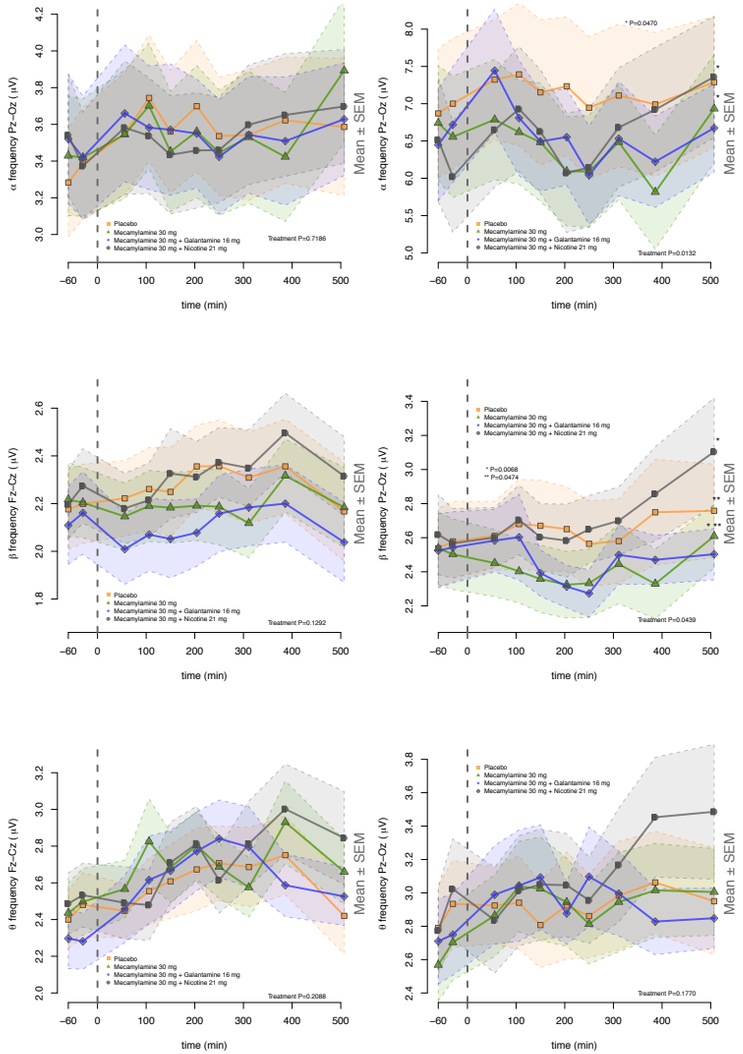


**FIGURE 4.3 Effect on the Electro-Encephalogram.**

Mecamylamine, nicotine and galantamine effect versus time for the EEG *a*, *β* and *ϑ* frequency.




*Symbols represent the mean per treatment group and the polygon the standard error around the mean. Asterisks represent significance between groups (p value is mentioned per treatment and per group, when applicable). The vertical discontinuous line represents time point zero*



# PHARMACOKINETICS AND PHARMACODYNAMICS OF ORAL MECAMYLAMINE – DEVELOPMENT OF A NICOTINIC ACETYLCHOLINE RECEPTOR ANTAGONIST COGNITIVE CHALLENGE TEST USING MODELLING AND SIMULATION

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## ABSTRACT

A pharmacologic challenge model with a nicotinic antagonist could be an important tool not only to understand the complex role of the nicotinic cholinergic system in cognition, but also to develop novel compounds acting on the nicotinic acetylcholine receptor. The objective was to develop a PK-PD model using non-linear mixed effects (NLME) methods to quantitate the pharmacokinetics of three oral mecamylamine doses (10, 20 and 30 mg) and correlate the plasma concentrations to the pharmacodynamic effects on a cognitive and neurophysiologic battery of tests in healthy subjects. A one-compartment linear kinetic model best described the plasma concentrations of mecamylamine. Mecamylamine's estimated clearance was 0.28 ± 0.015 $L \cdot min^{-1}$. The peripheral volume of distribution (291±5.15L) was directly related to total body weight. Mecamylamine impaired the accuracy and increased the reaction time in tests evaluating short term working memory with a steep increase in the concentration-effect relationship at plasma concentrations below 100 $\mu g \cdot L^{-1}$. On the other hand, mecamylamine induced a decrease in performance of tests evaluating visual and fine motor coordination at higher plasma concentrations ($EC_{50}$ 97 $\mu g \cdot L^{-1}$). Systolic and diastolic blood pressure decreased exponentially after a plasma mecamylamine concentration of 80 $\mu g \cdot L^{-1}$, a known effect previously poorly studied in healthy subjects. The developed mecamylamine PK-PD model was used to quantify the effects of nicotinic blockade in a set of neuro-physiologic tests in humans with the goal to provide insight into the physiology and pharmacology of the nicotinic system in humans and the possibility to optimize future trials that use mecamylamine as a pharmacological challenge.


## INTRODUCTION

Integrity of the cholinergic system is essential for maintaining adequate cognitive functions. Impairment of the system is seen in both neurodegenerative and psychiatric conditions such as Alzheimer's disease (AD) and schizophrenia and has become an important therapeutic target.

Scopolamine, a selective competitive muscarinic antagonist, has been widely used as a challenge drug to induce temporary disturbances resembling those of Alzheimer's disease (AD) (Ebert and Kirch, 1998). Scopolamine administration induces mainly disturbances in visuo-spatial memory and orientation, short-term verbal, numeric and episodic memory, attention and acquisition (Flicker *et al*, 1992; Molchan *et al*, 1992; Ray *et al*, 1992; Snyder *et al*, 2005; Zemishlany and Thorne, 1991). These cognitive effects were also confirmed by different methods in which scopolamine also induced a diminished hippocampal activation in the MRI (Sperling *et al*, 2002), increased slow frequency waves on EEG (Ebert and Kirch, 1998) and magnetoencephalographic band specific functional brain connectivity disturbances observed in young healthy subjects, similar to those of patients with Alzheimer's disease (Bajo *et al*, 2015). However in the last decade, interest has increased towards understanding the nicotinic acetylcholine receptor (nAChR) and its role in different cognitive functions (Levin, 2002), consequences of functional abnormalities (Court *et al*, 2000) and possible uses as therapeutic target (Hurst *et al*, 2013). The use of a muscarinic agonist as scopolamine would seem less appropriate to investigate cognitive functions involved with nicotinergic agonists and compounds with activity on the nicotinergic system in general.

Mecamylamine (a selective nicotinic acetylcholine receptor antagonist) has slowly regained attention amongst neuroscientists after being an almost obsolete and forgotten drug to treat hypertension (Shytle *et al*, 2002). In the past two decades several studies have explored the neuro-physiological effects of mecamylamine in healthy subjects. Mecamylamine 10 mg induced significant impairment in learning in healthy elderly (Newhouse *et al*, 1994a). In younger subjects, however, mecamylamine doses below 20 mg generally do not produce significant cognitive deterioration (Ellis *et al*, 2009; Little

*et al*, 1998; Newhouse *et al*, 1994a; Voss *et al*, 2010). Mecamylamine 20 mg in younger healthy subjects cause significant increases in the number of errors in a learning and retrieval task, and an increase in the inspection time during a visual discrimination test, effect that was partially reversed by 5 mg of donepezil (a cholinesterase inhibitor) (Thompson *et al*, 2000). Several authors have suggested that co-administration of scopolamine and mecamylamine would better resemble cognitive impairment observed in AD patients (Ellis *et al*, 2006; Little *et al*, 1998). For a proper characterisation of nicotinic-muscarinic interactions, it is important to first quantify the neuro-physiological effects induced of either compound alone. We have previously described the concentration-effect relationships of scopolamine (Alvarez-Jimenez *et al*, 2016; Liem-Moolenaar *et al*, 2011); and we have now examined the concentrations and effects mecamylamine alone in healthy subjects, in order to determine the plasma concentration-effects (PK-PD) relationship of mecamylamine. PK-PD modelling is a widely used technique that integrates the exposure (measured using the plasma concentrations) and effects in a semi-mechanistic model approach in order to better interpret and understand experimental results and trial outcomes. The technique has gain popularity since it provides a more mechanistic explanation of the studied system and offers the possibility to create hypothetical scenarios by simulating outcomes in different situations offering a confirmatory rather than exploratory approach to clinical trials (Danhof *et al*, 2007).

A PK-PD model of mecamylamine-induced neurophysiological and cognitive effects may be used to optimise pharmacological challenge tests of this compound, to explore the effects of antagonism of the nicotinergic system and possible reversal by selective agonists.

In the current experiments, three mecamylamine doses (10, 20 and 30 mg compared to placebo) were administered to healthy subjects to further correlate the plasma mecamylamine concentrations with the effects while concentrations and effects were frequently measured. We utilized non-linear mixed effects (NLME) methods to quantitatively correlate the pharmacokinetic plasma mecamylamine concentrations to the pharmacodynamic cognitive and neurophysiologic effects in healthy subjects based on two related clinical studies.



## METHODS

### STUDY POPULATION

Forty-four healthy male subjects between 18 and 45 years of age (under and upper limits included) participated in two clinical studies performed at the Centre of Human Drug Research (Leiden, the Netherlands). Information on demographics and dose levels administered can be found in Table 5.1. A medical ethics committee approved the study protocols. After giving written informed consent, all subjects were medically screened prior to study participation. Exclusion criteria included the use of agents or drugs known to influence cognitive performance and evidence of relevant medical abnormalities including conditions that could cause any kind of cognitive impairment.

### STUDY DESIGN

Data for this analysis were obtained from two related clinical studies. The first was an exploratory study intended to describe the cognitive effects of mecamylamine10 and 20 mg to those of scopolamine. In the second study, after performing an interim analysis to determine a safe dose increase, the dose range was expanded to 30 mg, and the effects of two cholinergic agonists (nicotine and galantamine) were examined. Galantamine was chosen as it exerts an allosteric modulatory activity on the nAChR, which other cholinesterase inhibitors lack (Coyle and Kershaw, 2001; Maelicke *et al*, 2001; Maelicke and Albuquerque, 2000). Both studies are in preparation for publication. However, since neither of the manuscripts would allow an integrated description of concentration-effect relationships for mecamylamine across the full (10-30 mg) dose range, we decided to perform a separate dedicated PK-PD analysis that is described in this article.

In both studies mecamylamine was administered orally in fasting conditions. Subjects were fasting for at least 4 hours and administration occurred with water. Mecamylamine capsules (Euticals SpA, Milan, Italy) containing mecamylamine HCl and microcrystalline cellulose as filling agent (used also in the placebo capsules) were administered

orally in blinded conditions. Plasma mecamylamine concentrations were determined using a validated, selective and sensitive liquid chromatography coupled to tandem-mass spectrometry (LC-MS/MS) method (Lower Limit of Quantification for the first trial was 1.54 $\mu g \cdot L^{-1}$ and for the second trial lowered to 0.5 $ng \cdot L^{-1}$).

The NeuroCart battery of tests evaluating different neurophysiological, psychomotor and cognitive tests was performed to quantify mecamylamine pharmacodynamic effects on different domains. The battery of tests has been previously extensively used in clinical drug development, and detailed descriptions can be found in other publications on a range of different compounds (de Haas *et al*, 2008; Liem-Moolenaar *et al*, 2010a, 2010b, 2011; van Steveninck *et al*, 1999; Strougo *et al*, 2008), including anticholinergic challenge tests (Liem-Moolenaar *et al*, 2010a, 2010b, 2011). On each study day, all pharmacodynamic tests were performed frequently at different time points per occasion in a quiet room with ambient illumination with only one subject in the same room (and a research assistant) per session. During the first trial, the NeuroCart test battery was subsequently performed at time points 30, 60, 120, 180, 195, 240, 480, 600 and 1320 minutes and for the second trial at time points 30, 80, 130, 180, 230, 280, 360 and 480 minutes post-dose. Washout periods between occasions were at least one week in both studies.

All subjects were thoroughly trained and familiarized with the psychometric tests within 21 days preceding study start to minimize inter-individual variability at baseline and to make sure subjects were able to understand and perform the tests. Each baseline assessment (pre-dose battery of tests) was performed twice at the beginning of each occasion. The mean of the two pre-dose measurements was used as baseline. A combination of tests evaluating neurophysiological and cognitive variables was analysed. Tests were included in the PK-PD analysis if they showed a statistically significant effect at 30 mg when compared to placebo. The blood pressure was modelled as a secondary measure since it was also used to determine the maximum tolerable dose for the second study, to predict the tolerability of mecamylamine in healthy subjects.





### ADAPTIVE TRACKER TEST

The test evaluates attention and executive skills such as visuo-motor coordination (Borland and Nicholson, 1984; van Steveninck *et al*, 1991). Subjects were asked to use a joystick to keep a randomly moving target on the screen inside a circle. The percentage of time that the target was kept in the circle was calculated. Even though attention is a cognitive process involved in numerous functional areas and therefore can be indirectly measured via many cognitive tests, the adaptive tracker is a more specific test for attention (arousal, vigilance) as the complexity of the test resides in sustained attention since it is very simple to perform from a psychomotor performance point of view. We have shown earlier that the adaptive tracker test was very sensitive to subtle disturbances in attention caused by ethanol, sleep deprivation, and benzodiazepines, and also to subtle enhancements by e.g. caffeine and donepezil in healthy subjects (van Steveninck *et al*, 1991, 1999; de Visser *et al*, 2003).

### N-BACK TEST

Subjects were instructed to remember and correlate a sequence of letters presented in a random order, thereby allowing evaluation of (short-term) working memory (Lim *et al*, 2008). Performance was expressed as the percentage of correct answers on the 0-back paradigm, and as reaction time of all answers on the 2-back paradigm. The fraction of correct answers was logit-transformed prior to model fitting.

Based on exploratory data analysis, the following NeuroCart tests were not considered in the model, because no significant effect of 30 mg of mecamylamine compared to placebo was observed: Visual Analogue Scales (evaluating alertness mood and calmness), Finger Tapping (evaluating motor fluency), Visual Verbal Learning Test (evaluating verbal working memory), Milner Maze Test (evaluating visuo-spatial working memory) and electroencephalogram (EEG). The electroencephalogram was measured tasks free during one minute with eyes closed.

### SOFTWARE

Pharmacokinetics and pharmacodynamics analyses were performed using non-linear mixed-effect (NLME) modelling in NONMEM v7.2 and v7.3 (Beal *et al*, 2009). The database and all graphs were created using R v2.13.1 (R Core Team, 2013). Statistical analysis and calculations were performed using SAS software for windows v9.4 (SAS Institute, Inc., Cary, NC, USA).

### MODEL DEVELOPMENT AND EVALUATION

Plasma mecamylamine concentration-time dependent data were analysed using a consecutive NLME modelling approach; once the best pharmacokinetic model was obtained, the individual pharmacokinetic parameter estimates were fixed to develop the pharmacodynamic models. The first order conditional estimation method with interaction (FOCE-I) was used. Several compartment models were explored for the pharmacokinetic model. Weight, height, age, body mass index and body surface area (calculated using DuBois's formula) were tested as potential covariates for parameters on which inter-individual variability (IIV) could be identified and were incorporated in the model as covariates if needed.

For the pharmacodynamic endpoints, several structures including direct and indirect (using an effect compartment) sigmoidal, truncated, linear, exponential and $E_{max}$ model structures were tested. Delay compartments were taken into consideration for the pharmacodynamics models only when an indirect model was chosen.

For all models, once the structural model was defined additive, proportional, exponential or combined error models were tested. IIV was tested in each parameter estimate and correlations between post-hoc Bayesian parameter estimates and between post-hoc Bayesian parameter estimates and potential covariates were explored using coefficient of determination ($r^2$). Correlations with an $r^2 \geq 0.4$ that were considered clinically relevant were taken forward in formal testing of omega block structures and covariate analysis (weight, age and height). Competing models were compared based on their Goodness of Fit (GOF) plots, decrease of the objective function value (OFV), plausibility of parameter estimates, residual error, parameter precision (in terms of residual standard error; RSE), shrinkage and parameter distribution. The OFV is a goodness of fit statistic defined as minus two times the logarithm of the likelihood and it is provided in each model's output file provided by NONMEM. A decrease in the OFV of at least 3.84 units ($p<0.05$) was considered statistically significant. GOF plots included observations vs. population and individual predictions, conditional weighted residuals with interaction (CWRESI) vs. time and CWRESI vs. observations and IIV frequency distribution, boxplots and QQ graphs. The VPCs were obtained by simulating 1000 subjects, using the population parameter estimates and the full variance-covariance matrix. Covariates were sampled from the observed population distribution.

## RESULTS

### MODEL DEVELOPMENT – PLASMA MECAMYLAMINE CONCENTRATIONS

Shortly after oral mecamylamine administration, plasma mecamylamine concentrations increased rapidly and, once they reached the equilibration phase, plasma mecamylamine concentrations decreased gradually (Figure 5.1). A one-compartment (consisting of a dose and a central compartment) linear pharmacokinetic model structure best described the plasma mecamylamine pharmacokinetic data. A two-compartment linear model resulted in a negligible inter-compartmental clearance estimate (0.000022) with a gradient that approached zero and therefore the model was abandoned. Non-linear (Michaelis-Menten) kinetics was also tested. This provided no improvement in the fit or OFV and produced an estimated $K_m$ above the measured concentrations (158 $\mu g \cdot L^{-1}$) and was therefore rejected. Inter-individual variability could be identified on the lag time related to the oral administration (ALAG time), absorption rate constant ($K_{12}$) and clearance (CL).

The estimation of the elimination rate ($K_{20}$) was dependent on the clearance and the central apparent volume of distribution as showed in Equation 5.1.Body weight was identified as covariate on the central volume of distribution (V) ($r^2$=0.66, $p<0.01$) and incorporated as mean body weight-

normalised covariate (ΔOFV = -27 points; Equation 5.2), which completely explained the inter-individual variability (IIV) on this parameter. Equation 5.3 and 5.4 show the one-compartment model differential equations and the way the lag time (ALAG) was incorporated. The rate of absorption ($K_{12}$) was negatively correlated with the lag time or ALAG ($r^2$ = 0.53, $p$<0.01) and an omega block structure (variance-covariance structure) was used, reducing the IIV of the ALAG (from 0.276 to 0.099) without influencing the OFV. Pharmacokinetic model graphical result estimates can be found in Table 5.2.

$$k_{20} = \frac{CL}{V_C} \quad (5.1)$$

$$\frac{V_C}{F} = e^{\left\{\left[\log(\vartheta_{V_C})\right] + \left[CWC \cdot \log\left(\frac{WGT}{77.698}\right)\right]\right\}} \quad (5.2)$$

$$\frac{dA_A}{dt} = -k_{12} \cdot A_A \cdot (t > ALAG1) \quad (5.3)$$

$$dA_C = k_{12} \cdot A_A \cdot (t > ALAG1) - k_{20} \cdot A_C \quad (5.4)$$



## MODEL DEVELOPMENT – MECAMYLAMINE EFFECTS

**PERCENTAGE OF ACCURACY OF THE ADAPTIVE TRACKER TEST** ✳ Figure 5.2 shows the effect over time of mecamylamine administration on adaptive tracker performance (%-point accuracy). At baseline, subjects consistently scored a mean of 29 ± 0.82 %. Mecamylamine, compared to placebo, produced a decrease in performance of -1.89 %-point (confidence interval: -3.90 – 0.12; $p$=0.0647) after administration of 10 mg of mecamylamine, -2.06 % (-3.97 – -0.15; $p$=0.0355) after 20 mg of mecamylamine and -3.27 (-4.58 – -1.97; $p$<0.0001) after 30 mg of mecamylamine. The effect was observed promptly at the first time point after mecamylamine administration. In accordance, during PK model development, a direct $E_{max}$ model proved similar when compared to an indirect model structure (ΔOFV = 0.6 points) and the direct model structure was therefore chosen. Equation 5.5 depicts the equation used to relate plasma mecamylamine concentrations (C) with the effect. The right side of the equation has as a consequence a reduction in the baseline (BL) or pre-dose value. Addition of a learning or practice effect linear and exponential function to describe the placebo data was unsuccessful since estimated OFV decreased by 12 points. Moreover it gave negligible improvement in the fit and caused difficulties estimating the learning function (parameter with the highest gradient and covariate step aborted) and was therefore abandoned.

Adding an exponent ($\gamma$) to the $E_{max}$ model function provided a non-significant decrease in the OFV (1 point), however it improved the shrinkage, the uncertainty of the parameters and the fit of the model and therefore was accepted. In the best model, IIV was identified for BL (ΔOFV = -809 points) and $EC_{50}$ (ΔOFV = -152 points). An omega block was required between BL and $EC_{50}$.

$$Tracker = BL \cdot \left\{1 - \left[\frac{E_{MAX} \cdot C^{\gamma}}{EC_{50}^{\gamma} + C^{\gamma}}\right]\right\} \quad (5.5)$$

**PERCENTAGE OF CORRECT ANSWERS IN THE 0-BACK PARADIGM OF THE N-BACK TEST** ✳ Following mecamylamine administration, the number of correct answers decreased significantly with the highest dose when compared to placebo (Figure 5.3). Administration of mecamylamine 10 mg produced an average decrease in the 0-back ratio of correct answers of -0.03 % of correct answers (-0.08 – 0.01; $p$=0.1348), 20 mg -0.02 (-0.06 – 0.03; $p$=0.4714) and 30 mg produced a significant reduction of -0.023 (-.044 – -.003; $p$=0.0270). Compared to an indirect model, a direct model performed best (Equation 5.6). An $E_{max}$ model proved superior compared to linear, truncated and exponential model structures (ΔOFV = 5157). A sensitivity analysis was performed to investigate the impact of one extreme outlier (Subject 6) on parameter estimation and uncertainty. Excluding this subject resulted in near identical parameter estimates and the SEM of the $EC_{50}$ decreased from 17.1 to 4.8 $\mu g \cdot L^{-1}$, indicating that this data point has no substantial influence of model performance. Subject 6 was included in the final model run.

$$0\ back\ correct\ answers = BL \cdot \left\{\frac{E_{MAX} \cdot C}{EC_{50} + C}\right\} \quad (5.6)$$

**REACTION TIME IN THE 2-BACK PARADIGM OF THE N-BACK TEST** ✳ Figure 5.4 presents how mecamylamine administration increased the reaction time of the majority of the subjects during the 2-back test. Administration of mecamylamine 10 mg produced a non-significant increase of 7 milliseconds (-37–51; $p$=0.7503), 20 mg -1 milliseconds (-43 – 41; $p$=0.9677) and 30 mg produced a significant increase of 28.3 milliseconds (2.0 – 54.6; $p$=0.0356) in the 2-back reaction time when compared to placebo. Addition of intra-occasion variability at baseline occurred at an early stage of model development since it was observed when fitting the data and, once implemented, resulted in a significant drop in OFV of 165 points and improved the fit of the data. The best model structure proved to be a direct model. An exponential model provided a better fit and results when compared to an $E_{max}$ model (exponential model decreased the OFV by 22 points). The parameter estimates provided by the $E_{max}$ model were also above the measured mecamylamine concentrations and therefore this model was rejected. Variability (inter-occasion variability) was identified only at baseline and this was sufficient to describe the data correctly. One equation was needed to correctly describe the learning or practice effect without the influence of mecamylamine ($E_0$), where a time-dependent function described an ascending trend seen in all subjects (Equation 5.7). Afterwards, this function was used in Equation 5.8 to characterize the effect mecamylamine exerted in the *reaction time of the 2-bask test*. Again, the concentrations ($C$) in the exponent multiplied by a constant ($\lambda$) related the concentrations with the effect on the test.

$$E_0(t) = BL - t^{\gamma} \quad (5.7)$$

$$2\ back\ RT = E_0 \cdot \{e^{(C \cdot \lambda)}\} \quad (5.8)$$

**SYSTOLIC AND DIASTOLIC BLOOD PRESSURE** ✳ The blood pressure decrease effect of mecamylamine was the limiting factor for the dose increase in the studies and therefore was also modelled. Figure 5.7 presents the time dependent graphs per mecamylamine dose. The systolic and diastolic blood pressure (SBP and DBP, respectively) were modelled simultaneously since they are intimately correlated. Rhythmic oscillations around an identity (base) line were observed in the data from the placebo group. In order to describe the baseline circadian variability, a one-cosine function was used (Van Rijn-Bikker *et al*, 2013). Shortly after mecamylamine was administered, both the SBP and DBP decreased in a dose-dependent manner. A direct truncated effect model performed better than both an $E_{max}$ ($\Delta$OFV = -22 points) and a linear model ($\Delta$OFV = -12 points). A direct model structure was chosen. IIV best described the data when placed at baseline. SBP and DBP baselines were highly correlated ($r^2$=0.37) and physiologically plausible, therefore an omega block was placed, reducing the IIV of both parameters. The Body Mass Index (BMI) was also highly correlated to the baseline SBP ($r^2$ = 0.49, $p$<0.01), adding it as a covariate produced a OFV decrease of 13 points and provided a better fit to the data. *Systolic and Diastolic Blood Pressure* were calculated with a cosine function of time (Equation 5.9), which was correlated to the plasma mecamylamine concentrations with an exponent (Equation 5.10). The amplitude (AMP) of the oscillations, the frequency (FREQ) and the point in the daytime that it starts (PHS) were estimated parameters. Only for the systolic blood pressure, the body mass index (BMI) was divided by the population BMI value and a constant (CBMIB) was used as correction factor to calculate the baseline as shown in Equation 5.11.

$$E_0(t) = BL + AMP \cdot \cos\left\{2 \cdot \Pi \cdot \left[\frac{t\text{-}PHS}{FREQ}\right]\right\} \quad (5.9)$$

$$BP = E_0 - (BASE \cdot e^{C}) \quad (5.10)$$

$$BLS = e^{\left\{\left[log(\vartheta_{BLS})\right] + \left[CBMIB \cdot \log\left(\frac{BMI}{23.25}\right)\right]\right\}} \quad (5.11)$$

### MODEL EVALUATION

The GOF plots for all models indicate that the central and individual trend of the data is well described, and that no bias occurs over time or observations. The shrinkage was acceptable in all models except for the $E_{max}$ estimated

in the 0-back percentage of correct answers (41.3 %) and the baseline of the 2-back reaction time (31.2 %). The VPCS indicate that the variability for these parameters is well described as 95% of the data appears lie within the 95% prediction interval.

## DISCUSSION

This is the first time that neurocognitive and neurophysiological effects of mecamylamine have been quantified using an exposure-effect (pharmacokinetic and pharmacodynamic) relationship approach.

Mecamylamine is a highly lipophilic secondary amine that acts by binding non-competitively and non-selectively to the nicotinic acetylcholine receptor as an antagonist to the voltage gated function of the ion channel (Varanda *et al*, 1985). Due to its chemical properties, mecamylamine distributes profusely in the body including the Blood Brain Barrier and therefore exerts its effect in the Central Nervous System without delay or use of an effect compartment in the model. Bioavailability of mecamylamine is unknown. Even though mecamylamine has been administered intravenously in the past, plasma concentrations have not been determined; probably due to the fact that the drug was developed more than 70 years ago when plasma concentration methods were not available (Allanby and Trounce, 1957). It has been previously reported in literature that mecamylamine bioavailability is complete, however it was determined after comparing the reduction of the systolic and diastolic blood pressure after oral and intramuscular administration in healthy subjects, without measuring plasma mecamylamine concentrations (Ford *et al*, 1956). The reported one-compartment linear-kinetic model and the estimates obtained for mecamylamine are comparable to a model developed for dexmecamylamine. Dexmecamylamine (TC-5214) is a compound with similar chemical structure, when compared to mecamylamine. The compound is currently in clinical development to treat hyperactive bladder symptoms (Xu *et al*, 2014). The authors also reported that the corrected body weight was an important covariate directly correlated to the apparent central volume of distribution, as corroborated in our model. Non-linear kinetics proved not better than zero-order clearance of plasma mecamylamine in our model. While in our model Michaelis-Menten kinetics were tested, the value estimated for the $K_M$, concentration at which the reaction rate is half of $v_{max}$, was above the measured plasma concentrations, not excluding that at higher concentrations saturation of the system may be present.

Based on previously reported work (Ellis *et al*, 2006; Little *et al*, 1998; Voss *et al*, 2010), effects induced by mecamylamine doses below 20 mg have been previously difficult to quantify in healthy subjects. In this study, we performed PK-PD modelling on statistically significant effects of mecamylamine in a dose range of 10-30 mg. This demonstrated consistent effects on all evaluated neuro-physiologic tests even at dose levels as low as 10 mg. We were able to characterize the effects of administration of mecamylamine in a set of tests that were not earlier reported such as attention, vigilance and visuo-motor coordination (Adaptive Tracker) and confirm the effects previously reported in literature: impairment of learning and retrieval or working memory (N-back percentage of correct answers) and increase in reaction time (N-back reaction time).

Subjects receiving mecamylamine were more prone to commit mistakes during the 0-back paradigm compared to those in the placebo group. The estimated $EC_{50}$ and $E_{max}$ were low (8.7 $\mu g \cdot L^{-1}$ and 30%, respectively) resulting in long-lasting effects (higher possibilities of making mistakes) even at low plasma concentrations. Previously reported cognitive effects after administration of 20 mg of mecamylamine (and even 10 mg of mecamylamine in elderly) include an increase in working memory errors and reaction time, compared to the placebo group (Newhouse *et al*, 1992), consistent with our findings. Nicotinic blockade in humans produces impairment in the recall and integrative brain pathways (both needed to respond correctly in the N-back paradigms), probably secondary to nAChR inactivation in the basal forebrain structures where the receptor density is high (Zoli *et al*, 2015). Despite the fact that only 18 of the total 491 (4%) plasma mecamylamine concentrations were above the $EC_{50}$ in the Adaptive Tracker model, the $E_{max}$ model structure described the data substantially better when compared to more simple models and was therefore accepted as most appropriate model structure. As a result, the predictive value of $E_{max}$ for higher doses should be careful considered.

In order to use mecamylamine as a cognitive challenge model drug to explore the nicotinic central activity, and manipulate the system with drugs that exert their mechanism of action through the same nAChR receptor, it is useful to first analyse the concentration-effect relationships. The choice of the optimal mecamylamine dose should depend on the balance between desired and unwanted effects, including central and peripheral effects. Mecamylamine exerts its action in a dose-dependent manner in different brain areas, translated in an individual dose-effect relationship per cognitive area. The different evaluated effect-concentration relationships per test are shown in Figure 5.5. Compared with other functions, accuracy and reaction time in N-back test of working memory are relatively sensitive to mecamylamine. The decrease of the accuracy and increase in the reaction time observed in the N-back test occurs at low concentrations and reaches a steady maximum around 100 $\mu g \cdot L^{-1}$. Above this concentration other less sensitive but potentially undesirable effects will be observed without a further clinically significant decrease in the performance on working memory. On the other hand, performance in the Adaptive Tracker (a tests evaluating attention and executive skills as visuo-motor coordination) may still decrease with higher doses of mecamylamine, since the estimated $EC_{50}$ concentration (97.2 $\mu g \cdot L^{-1}$) was barely surpassed with the administration of mecamylamine 30 mg. Mecamylamine has been used in the past as a drug to treat moderately severe- to severe-hypertension due to its parasympathetic ganglionic effect. Mecamylamine effects only caused an average decrease in systolic blood pressure of 5 mmHg in our healthy subject population. This was less pronounced than the reduction of approximately 20 mmHg in hypertensive patients with hypertension, after oral administration of 20 mg of mecamylamine (Ford *et al*, 1956). Our findings further suggest, as has previously been assumed(Shytle *et al*, 2002), that one third or even less of the usual dose used to treat hypertension is enough to produce measurable central effects and higher doses than 30 mg of mecamylamine would not provide a greater decrease in tests evaluating working memory but would further decrease the blood pressure in an exponential way. Higher doses should only be considered after a careful hemodynamic risk assessment has been performed and if other cognitive areas rather than working memory are the main outcome.





Using the currently developed model could help simulate new scenarios with different mecamylamine doses based on the cognitive area of interest. A dose of 20 mg seems reasonable to induce disturbance in memory with minimum changes in the SBP as shown in Figure 5.5. On the other hand, the previous dose would seem insufficient to induce a decrease in visuo-spatial coordination where as shown in Figure 5.6, however higher doses should provide a greater decrease in performance with as consequence a more sensitive inflection point with small dose changes or co-administration of nicotinergic agonists, showed a significant effect.

It has been proposed that elderly subjects and patients with mild cognitive impairment are more sensitive to mecamylamine effects (Newhouse *et al*, 1994a, 1994b). The developed models may be helpful to further quantitate these differences by using age as covariate in the different estimates, e.g.: the estimated $EC_{50}$, Hill exponent, $E_{max}$, depending on the structural model used. Other applications of the PK-PD-models could be the translational integration of pre-clinical and clinical study results, to further understand the implications of manipulation of the nicotinic cholinergic neuronal system.

A learning effect secondary to consecutive testing, measured as a slight improvement in performance after several repetitions during the course of the occasion, was identified for the reaction time of the N-back as has been previously described (Bartels *et al*, 2010; Collie *et al*, 2003; Goldberg *et al*, 2015). This learning effect was successfully incorporated in both models using time-dependent functions (Ito *et al*, 2010; Samtani *et al*, 2015). Mecamylamine also induced a reduction of the practice or learning curve resulting of repetition during the reaction time of the 2-back test. Even though mecamylamine 10 mg administration by itself did not produced a statistically significant effect in this test, modelling showed that corresponding low levels did decrease the ability of subjects to learn (or perform better after practicing) in a quantifiable way.

Scopolamine induces more sedative effects (Robbins *et al*, 1997) when compared to mecamylamine, and it is possible that scopolamine-induced cognitive deficits are at least partly related to sedation rather than direct disturbances of muscarinic brain cortical and basal areas involved in cognitive processing. The most sensitive tests to measure sedation (induced

by sleep deprivation or pharmacological agents), namely the adaptive tracker and saccadic eyes movement tests (de Haas *et al*, 2008; van Steveninck *et al*, 1991, 1999) were less affected by mecamylamine when compared to scopolamine. The fact that scopolamine produced more sedative effects than mecamylamine is in accordance with the fact that muscarinic receptors populate more densely the brain stem (including the ascending reticular ascending system), which regulates arousal (Flynn *et al*, 1997).

The mecamylamine pharmacological challenge model is useful to investigate the role of nAChR in neuro-physiological functions and to support clinical research. The better understanding of the relationship between the plasma concentrations of mecamylamine and its pharmacodynamic effects that this model has yielded, will aid to quantify the more subtle differences in performance that with other statistical methods are not discovered. This is of particular importance when trying to show cognitive improvement due to drugs that are being developed, as detrimental effects of psychoactive compounds on cognition are already difficult to demonstrate, but reversal or improvement of cognitive functions has rarely been reported (Buccafusco, 2009). Using a pharmacokinetic and pharmacodynamic model we provide a better insight into the complexity of the mechanism of action of central nicotine receptor blockade in healthy subjects. Antagonism of the nicotinic cholinergic system using mecamylamine resulted mainly in impairment of cognitive functions such as acquisition, processing and execution. The mecamylamine model in humans could be useful as a proof of pharmacology tool in drug development of novel nicotinic agents.





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**TABLE 5.1 Subject demographics.**

Mean ± Standard Deviation. ξ Mean (minimum-maximum).

| | Exploratory (first) trial | Confirmatory (second) trial | All |
|---|---|---|---|
| Subjects (n) | 14 | 30 | 44 |
| Age ξ (years) | 25.4 (19 - 36) | 23.5 (19 – 35) | 24.3 (19 - 36) |
| Weight (kg) | 80.3 ± 9.14 | 75.2 ± 8.47 | 77.3 ± 9.06 |
| Height (cm) | 182.5 ± 6.22 | 181.6 ± 6.16 | 182.0 ± 6.17 |
| BMI ($kg \cdot m^{-2}$) | 24.0 ± 2.18 | 22.8 ± 2.44 | 23.3 ± 2.41 |
| Mecamylamine doses (mg) | Placebo, 10 and 20 mg | Placebo and 30 mg | NA |

*NA: not applicable.*

**TABLE 5.2 Population estimates for pharmacokinetic and pharmacodynamic models for mecamylamine.**

Parameters are reported as population estimate.

| | Parameter | Units | Parameter estimate | SEM | IIV | Shrinkage | Observations |
|---|---|---|---|---|---|---|---|
| Mecamylamine pharmacokinetics | $ALAG_1$ | min | 26.8 | 1.04 | 0.096* | 19.10 | Correlation -0.294 |
| | $K_{12}$ | $min^{-1}$ | 0.0335 | 0.0088 | 2.115 * | 4.59 | |
| | CL | $L \cdot min^{-1}$ | 0.279 | 0.0154 | 0.326 | 7.63 | |
| | $\frac{V_C}{F}$ | L | 291 † | 5.15 | - | - | |
| | CWV | - | 0.794 | 0.132 | - | - | |
| | $\sigma^2$ | - | 0.0328 | 0.00446 | - | - | Proportional |
| Adaptive Tracker (percentage of accuracy) | $EC_{50}$ | $\mu g \cdot L^{-1}$ | 97.2 | 28.1 | 1.0399 * | 26.7 | Correlation 0.0451 |
| | BL | - | 29.0 | 0.82 | 0.182 * | 0.66 | |
| | $E_{max}$ | % | 0.27 | 0.053 | - | - | |
| | $\gamma$ | - | 1.58 | 0.548 | - | - | |
| | $\sigma^2$ | - | 11.5 | 1.2 | - | - | Additive |
| Correct Answers of the o-back § (percentage of correct answers) | $EC_{50}$ | $\mu g \cdot L^{-1}$ | 8.74 | 17.1 | - | - | |
| | $E_{max}$ | % | 0.377 | 0.163 | 0.885 | 41.3 | |
| | BL | % correct answers | 3.66 | 0.125 | 0.150 | 22.9 | |
| | $\sigma^2$ | - | 0.00174 | 0.000322 | - | - | Additive |
| Reaction Time of the 2-back paradigm (milliseconds) | BL | ms | 561 | 12.5 | 0.0446¶ | 31.2 | |
| | $\gamma_{bl}$ | - | 0.568 | 0.0345 | - | - | |
| | $\gamma_{eff}$ | - | 0.000763 | 0.000219 | - | - | |
| | $\sigma^2$ | - | 0.0132 | 0.00119 | - | - | Proportional |
| Systolic and Diastolic Blood Pressure (mmHg) | $BL_D$ | mmHg | 70.2 | 0.931 | 0.080 * | 6.91 | Correlation 0.055 |
| | $BL_S$ ‡ | mmHg | 121 | 1.26 | 0.064 * | 6.24 | |
| | $AMP_D$ | mmHg | 1.81 | 0.438 | - | - | |
| | $AMP_S$ | mmHg | 1.12 | 0.422 | - | - | |
| | $PHS_D$ | min | 8e-04 | - | - | - | |
| | $PHS_S$ | min | 802 | 74.5 | - | - | |
| | $FREQ_D$ | min-1 | 676 | - | - | - | |
| | $FREQ_S$ | $min^{-1}$ | 833 | - | - | - | |
| | $BASE_D$ | - | 0.0227 | 0.00329 | - | - | |
| | $BASE_S$ | - | 0.0284 | 0.00318 | - | - | |
| | CBMIB | - | 0.229 | 0.107 | - | - | |
| | σ2D | - | 0.0109 | 0.001 | - | - | Proportional |
| | $\sigma^2_S$ | - | 0.00615 | 0.000598 | - | - | Proportional |

*IIV: Inter-individual Variability expressed as Coefficient of Variation. ‡ BMI used as a covariate.*
*†Weight used as covariate. *Omega block structure. γ Exponent. 4 buffer compartments.*
*§ Parameters reported as natural log odds. ¶Highest inter-occasion variability. F: oral bioavailability.*

**FIGURE 5.1 Mecamylamine pharmacokinetics.**

Plasma mecamylamine concentrations visual predictive check graphs versus time after mecamylamine administration (time point zero) per dose. The solid line represents the model population prediction and the **grey** area the 95% predicted interval. Dots represent the observations. **Black** lines represent the 95% confidence interval and the dotted line in between the median of the observations.

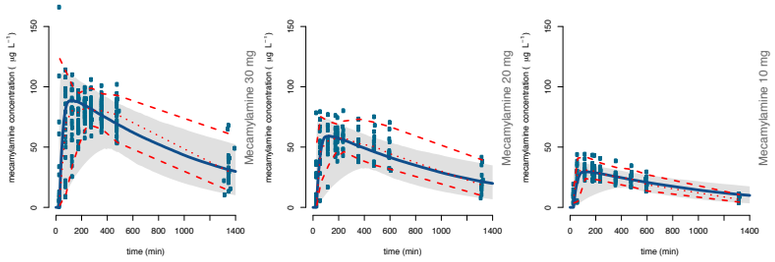







**FIGURE 5.2 Mecamylamine effects on the Adaptive Tracker.**

Performance during the Adaptive Tracker test versus time after oral mecamylamine administration (time point zero) per dose. The solid line represents the model population prediction and the **grey** area the 95% predicted interval. The **black** lines represent the individual predictions. Dots represent the observations.

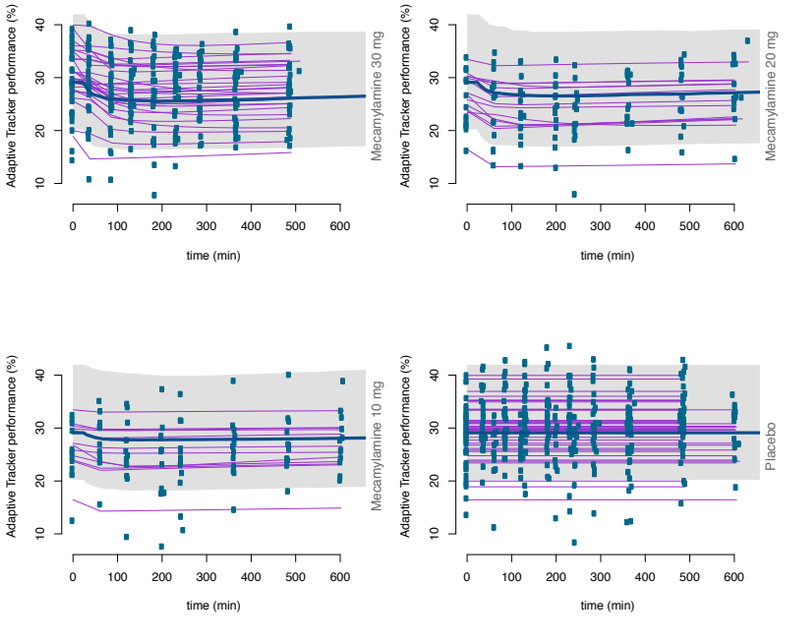


**FIGURE 5.3 Mecamylamine effects on the ratio of correct answers of the 0-back paradigm.**

Ratio of correct answers during the 0-back paradigm versus time after oral mecamylamine administration (time point zero) per dose. The solid line represents the model population prediction and the **grey** area the 95% predicted interval. The **black** lines represent the individual predictions. Dots represent the observations.

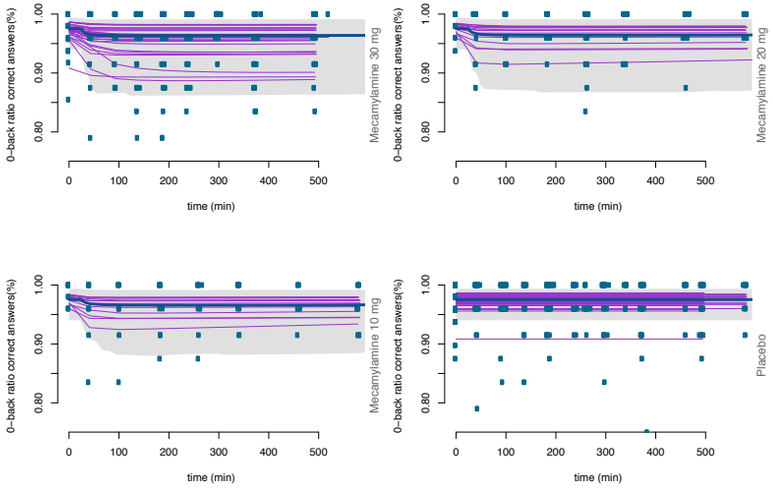







**FIGURE 5.4 Mecamylamine effects on the reaction time of the 2-back paradigm.**

Reaction time during the 2-back paradigm versus time after oral mecamylamine administration (time point zero) per dose. The solid line represents the model population prediction and the **grey** area the 95% predicted interval. The **black** lines represent the individual predictions. Dots represent the observations.

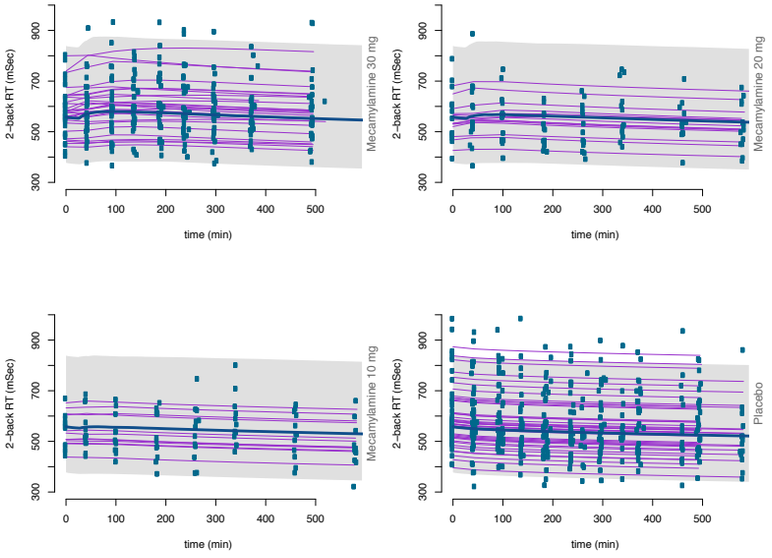


**FIGURE 5.5 Mecamylamine concentration-effect relationships.**

Plasma mecamylamine concentrations versus the effect per (neuro-) physiological test (N-back percentage of correct answers and reaction time, Adaptive Tracker and Systolic and Diastolic Blood Pressure). The solid line represents the model population prediction. The dots represent the individual predictions.

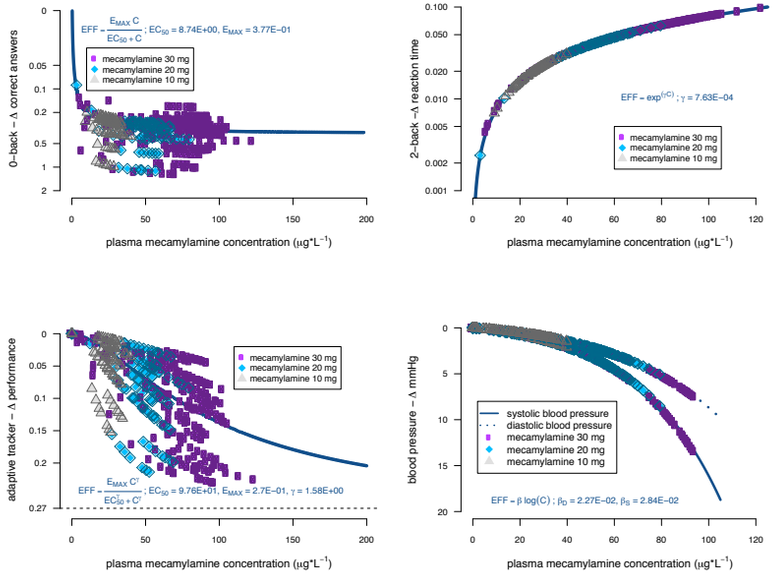







**FIGURE 5.6 Mecamylamine simulation of different doses.**

Plasma mecamylamine concentrations and resulting effects in the different physiologic and neurologic tests versus time. The simulations were performed using a normalized weight of 70 kg.

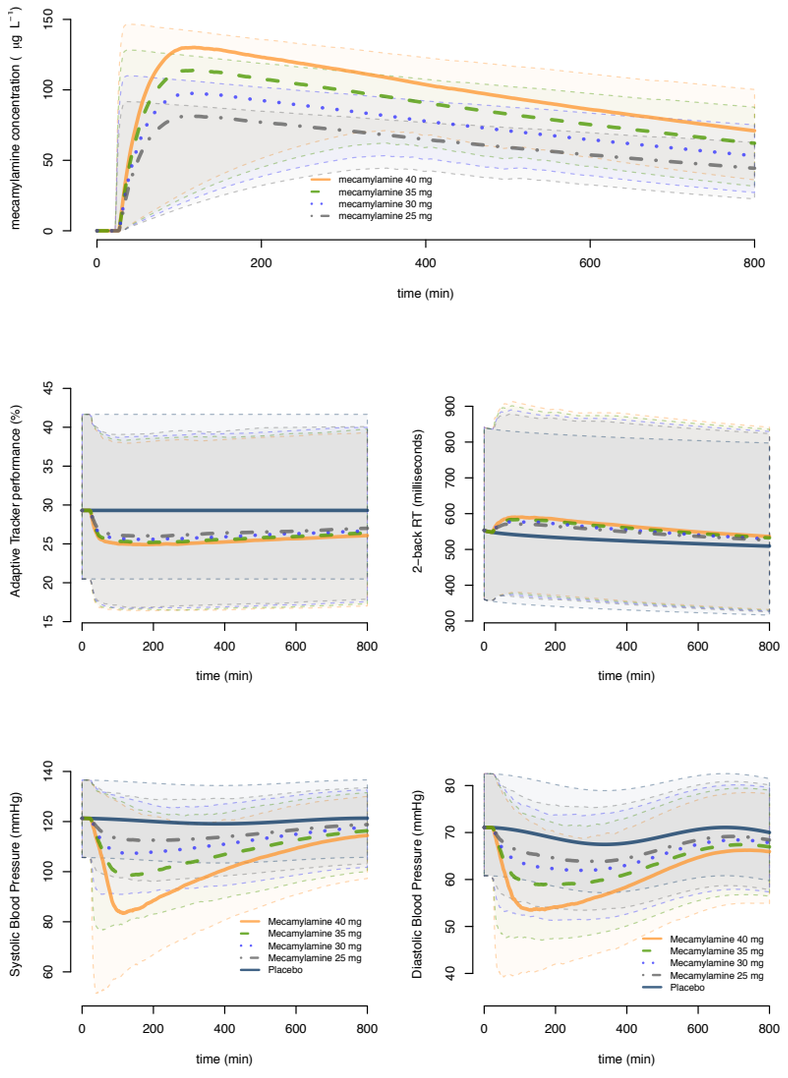
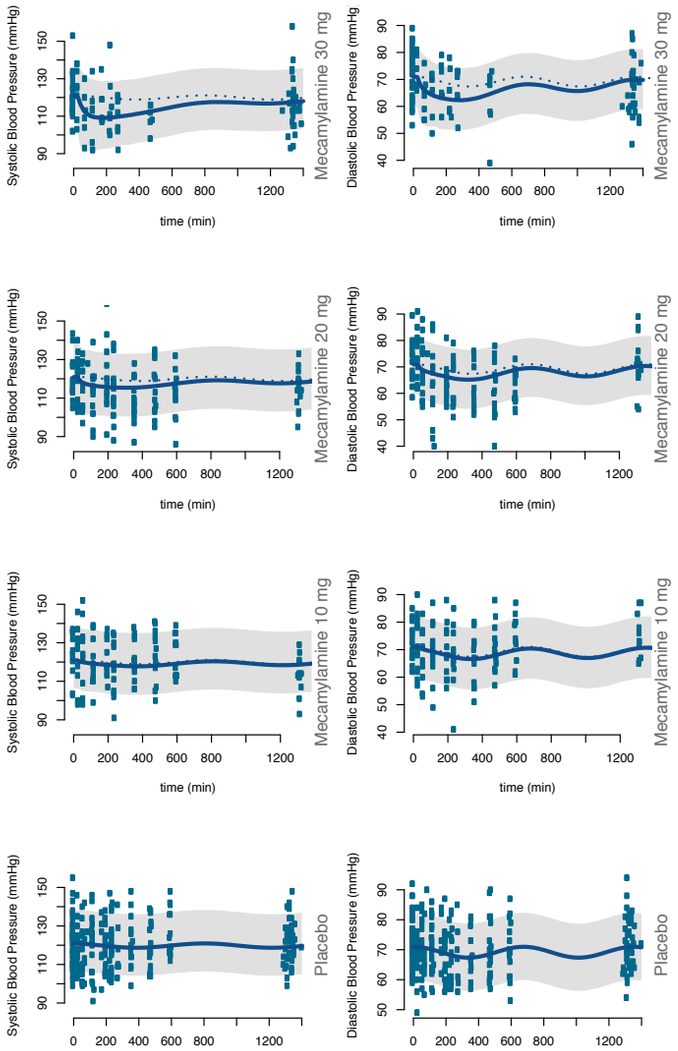


**FIGURE 5.7 Mecamylamine effects on the blood pressure.**

Blood pressure versus time after oral mecamylamine administration (time point zero) per dose. The solid line represents the model population prediction and the **grey** area the 95% predicted interval. The **black** lines represent the individual predictions. Dots represent the observations.








# APPROACHES TO EVALUATE THE CHOLINERGIC ANTI-INFLAMMATORY REFLEX IN HUMAN CLINICAL TRIALS

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## ABSTRACT

The cholinergic anti-inflammatory reflex has been well documented in murine models of endotoxin-induced inflammation, demonstrating the interconnected nature of neural-immune systems. Examining this connection in humans has proved challenging; however no non-invasive interventions have demonstrated consistent or significant results as with direct stimulation of the vagal nerve in humans. As the cholinergic neuronal system emerges as a drug target for CNS diseases such as Alzheimer's disease and schizophrenia, an opportunity to evaluate the cholinergic reflex in humans may become possible by pharmacological interventions. Linking changes in peripheral immune responses to alterations in CNS signaling due to drug interventions also offers a novel approach to indirectly explore the immunologic effects of nicotinic drugs during early phase clinical trials. In this report we developed standardized methods to explore inflammatory responses in healthy subjects. Using in vitro whole blood obtained from healthy subjects we optimized several features of our inflammatory challenge, including the inflammatory stimulus, the time of incubation, and the panel of pro-inflammatory cytokines utilized, in order to measure the most dynamic response to cholinergic signaling. We demonstrated that choline mediates inflammasome-driven IL-1$\beta$- and IL-6-release. In contrast, we observed far less inhibition of TNF-$\alpha$ release. Lastly we repeatedly measured ex vivo inflammatory responses in 25 healthy subjects to assess intra- and inter- subject variability to provide guidance when applying these measures in clinical studies. Notably, diurnal changes to ex vivo inflammatory challenge were observed; IL-1$\beta$ declined on average 18% and IL-6 30% while both IL-8 and TNF-$\alpha$ exhibited an average decline of 20%, during a 6 hour interval between morning and afternoon.


## INTRODUCTION

Highly conserved mechanisms control the balance ruling the innate immune response to injurious stimuli (Ashley *et al*, 2012). Initial experiments provided evidence that *in vitro* stimulation of T-lymphocytes with cholinergic agonists reduced the cytotoxic response to alloantigens (Strom *et al*, 1972). A couple of decades after, electric vagal nerve stimulation and also acetylcholine administration were found to decrease the immunologic response of rats, reducing the development of shock after exposure to lethal doses of lipopolysaccharide (LPS). Therewith defining the cholinergic anti-inflammatory reflex (Borovikova *et al*, 2000). Vagus nerve stimulation in patients with epilepsy has also proven effective to decrease peripheral blood levels of Tumor Necrosis Factor (TNF), IL-1$\beta$ and IL-6 and was also effective to reduce TNF blood levels and symptomatology in patients with rheumatoid arthritis (Koopman *et al*, 2016).

Both acetylcholine and nicotine are agonists of the $a_7$ nicotinic acetylcholine receptor (nAChR), which is indispensable for dampening immune responses via the cholinergic anti-inflammatory reflex (Wang *et al*, 2003). Several selective $a_7$ nAChR agonists were efficacious in inhibiting inflammatory effects in pre-clinical experiments. Choline has shown anti-inflammatory efficacy in murine endotoxemia models decreasing Nuclear Factor κB (NFκB) activity and Tumor Necrosis Factor (TNF) and High Mobility Group Box 1 (HMGB1) expression (Parrish *et al*, 2008). Pre-treatment of RAW 264.7 cells and human whole blood *in vitro* with GTS-21 and A-833834 (both selective $a_7$ nAChR agonists) decreased cytokine release in response to LPS stimulation and GTS-21 also inhibited endotoxemia-induced NFκB activation in an animal model (Li *et al*, 2011; Pavlov *et al*, 2007). GTS-21 protected neurons *in vitro* against damage induced by amyloid peptides, suggesting that $a_7$ nAChRs may have a immuno-modulating neuroprotective role (Shimohama, 2009). Besides GTS-21 and A-833834, other compounds with nicotinic agonist activity such as 4OHGTS, ARR17779, CAP55 and PNU-282987 are currently in development as well for their immune-mediatory functions (de Jonge and Ulloa, 2007).

Recently, the inflammasome, specifically nucleotide binding and oligomerization domain (NOD) and leucine rich-repeat-containing, pyrin domain containing 3 (NLRP3), has been linked to the cholinergic anti-inflammatory reflex (Lu *et al*, 2014). Canonical inflammasome activation occurs upon sensing two different danger or damage stimuli. The first signal, via a Toll like receptor, activates NFKB, which transcribes amongst others pro-interleukin (IL)-1$\beta$ and pro-IL-18 (Bauernfeind *et al*, 2009). A second signal is necessary to support NOD-like receptor or interferon-inducible protein Absent In Melanoma 2 (AIM2) oligomerization, which results in a caspase-1–activating scaffold. Active caspase-1 subsequently cleaves the pro-forms of IL-1$\beta$ and IL-18 into their bioactive forms (Latz *et al*, 2013). These second signals are generally related to cellular damage, such as exposure to particulates or aggregates, or increased extracellular adenosine 5'-triphosphate (eATP) in part released from neighboring, dying cells (Gombault *et al*, 2012). Aluminium hydroxide, the most widely used vaccine adjuvant, also exerts its activity in part by activating the NLRP3 inflammasome pathway (Eisenbarth *et al*, 2008; Hornung *et al*, 2008; Marrack *et al*, 2009; Vigano *et al*, 2015). While the classical two-signal inflammasome model has demonstrated additional checkpoints in the control of inflammatory cascades, it was shown recently that LPS alone is capable of inducing mature IL-1$\beta$ secretion, but not IL-18, by the non-canonical inflammasome pathway acting via caspase 4 and/or 5 (Vigano *et al*, 2015).

It has been suggested that anomalous inflammasome regulation plays either a causative or contributing role in several auto-inflammatory and autoimmune diseases, exacerbating a pathologic response to host-derived factors (Strowig *et al*, 2012). This also appears to be a contributing factor in several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD) (Masters and O'Neill, 2011). Misfolded proteins (SOD1 in ALS and *a*-synuclein in PD) activate microglia cells to produce pro-inflammatory mediators, which induce proteolytic processing of caspase-1. This suggests the inflammasome is activated in both PD and ALS, possibly via different pathways (Codolo *et al*, 2013; Li *et al*, 2000; Pasinelli *et al*, 1998). Amyloid-$\beta$ (A$\beta$) peptide deposition activates microglia and induces inflammasome processes in mouse models as well (Halle *et al*, 2008; Heneka *et al*, 2013) and post-mortem evaluation of patients with Alzheimer's disease (AD) demonstrates active caspase-1 in the hippocampus and cortex regions (Heneka *et al*, 2013). Specifically oligomeric species of A$\beta$ peptide have been demonstrated to activate LPS-primed monocytes, enhancing IL-1$\beta$ responses (Lorton *et al*, 2000). Interestingly, A$\beta$ has also been shown to bind to $\alpha_7$ nAChR with high affinity, thereby inactivating the receptor and inhibiting the anti-inflammatory effect the nAChR might have in microglia (Wang *et al*, 2000).

Further data suggest that nAChR could be a critical link between inflammation and neuro-degeneration in AD (Conejero-Goldberg *et al*, 2008; Oz *et al*, 2013). In mice, $\alpha_7$ and $\alpha_4\beta_2$ nAChR expression is decreased as a result of LPS exposure and also after administration of an $\alpha_7$ nAChR-specific antibody, $\alpha_7$ (1-208), leading to increased A$\beta$ accumulation and memory impairment. In the same experiments, the $\alpha_7$ (1-208) antibody also caused inflammation within the brain resulting in symptoms similar to Alzheimer's disease (Lykhmus *et al*, 2015). Several lines of investigation have demonstrated that oligomeric and fibrillary forms of A$\beta$ bind to $\alpha_7$ nAChR in brain extracts and neuronal cell lines (Lilja *et al*, 2011; Wang *et al*, 2000). Depending on the form of A$\beta$ and the concentration, its binding to $\alpha_7$ nAChR can have an activating or antagonizing effect, perturbing synaptic and probably inflammatory-mediating functions. Contributing to the multi-factorial complexity of Alzheimer's disease, modulation of nicotinic receptors is also associated with increased phosphorylation of tau (Buckingham *et al*, 2009).

Acetylcholine nicotinic receptor stimulation *in vivo* improves cognitive performance and might decrease the progression of AD and schizophrenia (Vallés *et al*, 2014). Several $\alpha_7$ nicotinic acetylcholine receptor ($\alpha_7$ nAChR) agonists (e.g. DMXB-A, EVP-6124, TC-5619, SSR180711) are currently being developed. These examples of promising early phase compounds have provided positive results as possible therapeutic options to treat neurodegenerative diseases with a nicotinic substrate (Toyohara and Hashimoto, 2010).

In order to measure the potential influence of $\alpha_7$ nAChR agonists on peripheral inflammatory processes in human clinical trials, we explored the effect of choline on induced inflammatory challenges in human whole

blood. We studied the effects of choline in two known inflammasome activation challenge models: LPS plus aluminium hydroxide and LPS plus extracellular ATP (eATP). Although monitoring stimulation of inflammasome responses is of direct interest for development of drugs targeting Alzheimer's disease, we sought to develop a bioassay that would be easily scalable for clinical studies and have broader application to inflammasome pathways for multiple neurodegenerative disorders. Such a test could also be applied as a (*ex vivo*) pharmacodynamic measure in future clinical pharmacology studies. Therefore we studied the effect of choline on LPS/aluminium hydroxide-induced cytokine release from human whole blood cultures to assess the intra- and inter-subject variability in these responses in healthy elderly.



## MATERIALS AND METHODS

### HUMAN SUBJECTS

A medical ethics committee approved the study protocols. After all subjects gave written informed consent, venous blood was drawn from a peripheral vein. Heparinized blood was used from healthy subjects. Initial, exploratory tests to determine the best experimental conditions were performed using blood from 3-6 donors. In order to validate the exploratory findings, further experiments included healthy elderly subjects (between 65 and 80 years of age) as representative of the target population with increased risk of neurodegenerative diseases, such as Alzheimer's Disease. All subjects were medically screened prior to study inclusion. Exclusion criteria included the use of agents or drugs known to influence CNS performance (including smoking and drug or alcohol abuse), consuming more than five cups of caffeine-containing drinks per day and evidence of relevant medical abnormalities.

### WHOLE BLOOD CULTURES

Choline chloride (Sigma-Aldrich, St Louis, MO, USA) was prepared in phosphate buffered saline (PBS) as a selective full-agonist binding with high affinity ($EC_{50}$ of 1.6 mM) to the $\alpha_7$ nAChR, with no activity on the $\alpha_4\beta_2$ receptor on hippocampal neurons and a partial agonist activity on the $\alpha_3\beta_4$ receptor on PC12 cells (Alkondon *et al*, 1997). 50 mM choline was previously demonstrated to exert the greatest decrease in TNF-$\alpha$ concentrations after stimulation in similar whole blood experiments (Parrish *et al*, 2008). Venous blood was drawn into sodium heparin tubes (Becton Dickinson, San Jose, CA, USA). Whole blood was incubated with choline chloride at the indicated concentrations for 10-15 minutes prior to stimulation with lipopolysaccharide at $2ng \cdot ml^{-1}$ (LPS, *Escherichia coli* serotype 0111:B4, Sigma-Aldrich), and $100\mu g \cdot ml^{-1}$ aluminium hydroxide (Alhydrogel 85, Brenntag, Frederikssund, Denmark) or LPS and 5mm adenosine 5'-triphosphate (ATP, Sigma-Aldrich) for the last 30 minutes of incubation. Cultures were performed in an endotoxin free manner, with minimal dilution and incubated at 37°C, 5% $CO_2$ for 3 hours.(Alkondon *et al*, 1997). Hereafter, culture supernatant was collected for multiplex cytokine analysis. Culture conditions were performed in single with cytokine measures performed in duplicate.

In order to apply this bioassay in an *ex vivo* manner, we evaluated potential circadian fluctuations in this response after six hours. To address this, blood was collected from 25 healthy elderly subjects at approximately 10:00 hrs and immediately cultured with LPS plus aluminium hydroxide for three hours, after which time supernatant was collected for later analysis of cytokines. This was then repeated at approximately 16:00 hrs on the same day. Culture supernatants were evaluated for IL-1$\beta$, IL-6, IL-8 and TNF-$\alpha$ secretion.

### CYTOKINE MEASUREMENTS

Culture and plasma supernatants were analyzed for cytokine secretion using Meso Scale Discovery electrochemiluminescence multiplex panels, human ultra-sensitive inflammatory cytokine 9-plex or V-plex human pro-inflammatory cytokine 4-plex (Mesoscale Discovery, Rockville, MD, USA) or IL-1$\beta$ Quantikine ELISA (R&D Systems, Minneapolis, USA) as indicated in figure legends. IL-18 was measured by ELISA (MBL, Japan). All cytokine measures were performed in duplicate.

### STATISTICAL ANALYSIS

Values are expressed as the mean ± standard deviation (SD). Comparison of inflammatory cytokine responses in conditions of LPS plus aluminium hydroxide with and without choline were analyzed by paired T-test using GraphPad Prism v6.00 for Windows (GraphPad Software, La Jolla CA, USA, www.graphpad.com). Differences at $p < 0.05$ were considered statistically significant.

## RESULTS

**CHOLINE EFFECT ON LPS-STIMULATED PRIMARY HUMAN BLOOD CELLS** ✳ Based on the conditions of human whole blood experiments (Wang *et al*, 2003), we attempted to standardize a LPS whole blood challenge that would be suitable for *ex vivo* application to human clinical trials. We assessed *in vitro* TNF-*α* secretion from LPS stimulated whole blood cultures from healthy volunteers, with or without 50 mM choline chloride exposure for 10 minutes prior to 3-hours LPS stimulation. Although we consistently found decreased TNF-*α* secretion as a result of choline exposure, individual responses ranged from only 18-33% less TNF-*α* than observed without choline incubation (n=6; mean response 27% ±5% decrease in TNF-*α*). Despite the robust decline in TNF-*α* demonstrated with vagal nerve stimulation in murine models of LPS challenge (Borovikova *et al*, 2000; Li *et al*, 2011; Parrish *et al*, 2008) we observed a relatively narrow dynamic range of TNF-*α* inhibition by choline in human whole blood cultures, suggesting this model would not be suitable to monitor the effects of cholinergic signaling on the peripheral inflammatory response for human clinical trials.

Further investigation by cytokine profiling of LPS-treated whole blood cultures from one individual, with and without incubation with 50 mM choline chloride, revealed IL-1*β* and IL-6 as strongly down regulated in response to choline (Figure 6.1). In this exploratory cytokine profiling, choline chloride decreased IL-1*β* release by 69% and IL-6 by 49%, while TNF-*α* secretion was only decreased by 28% in comparison to the parallel LPS stimulated condition. In addition, the release of GM-CSF was inhibited by 47%, while no inhibition of IL-8 release was observed.



**CHOLINE EFFECT ON PURINERGIC RECEPTOR- AND ALUMINIUM HYDROXIDE- INDUCED INFLAMMASOME ACTIVATION IN WHOLE BLOOD CULTURES** ✳ Our preliminary evidence from cytokine profiling indicating that IL-1*β* secretion by LPS exposed primary human blood cells was very sensitive to choline pre-treatment suggested that the effect of cholinergic signaling could be better observed in the context of IL-1*β* enhanced inflammatory models. To address this hypothesis, we studied the effect of choline on cytokine release in two inflammasome stimulating models; LPS and eATP or LPS and aluminium hydroxide. Importantly, both of these models can be applied to primary human blood cultures, making them feasible for clinical trials. Inflammasome activation with eATP following LPS priming resulted in a large increase in IL-1*β* secretion (Figure 6.2) (Gombault *et al*, 2012). Additionally, exposure to eATP is required to also process and secrete IL-18, resulting in a 4- to 5-fold increase in IL-18 secretion (Figure 6.2). Choline chloride reduced IL-1*β* secretion in response to eATP in a dose dependent manner. Addition of 10mM choline reduced IL-1*β* responses by 38% ± 5% (Figure 6.2). In contrast, choline chloride did not affect IL-18 secretion in the eATP inflammasome model (Figure 6.2). Whole blood cultures simultaneously exposed to LPS and aluminum hydroxide also enhanced IL-1*β* secretion after 3 hours (Figure 6.2). Addition of choline chloride also decreased IL-1*β* secretion in a dose dependent manner (Figure 6.3). 10 mM choline chloride decreased IL-1*β* levels by approximately half (45% ± 8%; Figure 6.3). IL-18 was also increased by 10-20% after LPS and aluminium hydroxide incubation, but this response was not sufficient to demonstrate a clear effect of addition of choline chloride (data not shown).

**CHOLINE EFFECT ON LPS AND ALUMINIUM HYDROXIDE CHALLENGE OF WHOLE BLOOD CULTURES FROM HEALTHY ELDERLY SUBJECTS** ✳ In order to validate the *in vitro* model in healthy subjects, we performed LPS and aluminium hydroxide stimulations of whole blood obtained from 16 healthy elderly volunteers. As depicted in Figure 6.3, secretion of both IL-1*β* and IL-6 in response to LPS plus aluminium hydroxide challenge, were significantly down-regulated by choline. TNF-*α* secretion was also decreased by choline, however to a lesser extent, yet remained

statistically significant (Figure 6.3). There was no change observed in IL-8 secretion in the same conditions (Figure 6.3).

Monocyte numbers fluctuate in circulation over the course of 24 hours, in response to diurnal influences (Petrovsky *et al*, 2003), resulting in changes to inflammatory stimuli, particularly LPS. In order to describe the variation expected for *ex vivo* inflammasome stimulation, we measured cytokine secretion from whole blood cultures taken in the morning and again in the afternoon, for 25 healthy elderly subjects. Figure 6.4 shows the changes in cytokine secretion per subject, for the two time points. Although individual's responses vary within the population, in general there was a decline for all the inflammatory cytokine responses evaluated in this period. When this change in cytokine secretion is expressed as a ratio of the afternoon response compared to the morning response (Table 6.1), we observe IL-1$\beta$ responses declined on average by 18% and IL-6 responses declined 30%; both IL-8 and TNF-$\alpha$ exhibited an average decline of 20%.



## DISCUSSION

The cholinergic reflex remains an incompletely understood mechanism of inflammatory regulation. Most recently, the cholinergic reflex has been utilized to diminish inflammatory activity in Rheumatoid Arthritis patients, by means of direct vagal nerve stimulation, and was associated with diminished cytokine release in whole blood cultured with LPS (Koopman *et al*, 2016). Nicotinic compounds in development represent potential pharmacological intervention of this anti-inflammatory pathway. By evaluating changes to peripheral inflammatory responses, clinical pharmacologists could assess *in vivo* activity of novel nicotinic compounds. In the present set of experiments, we optimized the inflammatory challenge model for whole blood to achieve a maximum window of cholinergic effect, as measured by modulation of IL-1$\beta$ secretion and demonstrated its suitability for clinical trials. Derived from the crescent interest in the immune mediatory activity of the nAChR, in specific the $\alpha_7$ nAChR (Conejero-Goldberg *et al*, 2008; Vallés *et al*, 2014), we have provided a reliable non-invasive clinical experimental model to measure the inflammatory effect of systemic administration of nicotinic agonists in humans. The current set of experiments provided evidence in humans that LPS plus aluminium and LPS plus ATP exert their action activating the inflammasome pathway and this effect can be diminished by stimulation with choline.

While we observed consistent decrease after TNF-$\alpha$ secretion in whole blood cultures stimulated with LPS in the presence of choline, we found the magnitude of this response rather negligible, as compared to IL-1$\beta$ or IL-6 secretion. This prompted us to explore more direct models of NLRP3 inflammasome activation that are associated with enhanced IL-1$\beta$ secretion. As shown in Figure 6.2, LPS plus aluminium and LPS plus ATP stimulation increased IL-1$\beta$ secretion from whole blood cultures as compared to LPS stimulation alone. Choline, as expected, consistently and dose dependently decreased IL-1$\beta$ in both models. Interestingly, we did not observe any changes to IL-18 in response to choline treatment, despite also being strongly regulated by the NLRP3 inflammasome. This suggests that choline signaling may induce its effects mainly via the non-canonical NLRP3 inflammasome pathway; utilizing caspase-4 and caspase-5 instead of caspase-1 (Latz *et al*, 2013; Vigano *et al*, 2015). Previous experiments demonstrated nicotinic agonists, acetylcholine and nicotine, decrease mitochondrial DNA damage, which is an activating ligand for the NLRP3 inflammasome, demonstrating a link between $\alpha_7$ nAChR signaling and regulation of the inflammasome (Lu *et al*, 2014). Future experiments should further explore the relationship between mitochondrial damage and inflammasome activation pathway after LPS stimulation in humans, particularly with respect to caspase-4 and caspase-5. Modulation of these specific pathways by nAChR agonists might further provide mechanistic insight into this tightly regulated response.

Although both whole blood inflammasome models we tested demonstrated a dose dependent decrease in IL-1$\beta$ secretion with addition of choline, we chose to continue with the LPS plus aluminium hydroxide model for two reasons. Aluminium hydroxide is thought to engage the inflammasome via the endosomal-lysomal pathways, similar to other particulates, such as pathogenic amyloid beta aggregates (Hornung *et al*, 2008). In addition, the chemical stability of aluminum hydroxide, and the one-step addition of reagents to blood cultures, allows this model to be readily adapted to clinical trials.

One challenge in implementing this test for drug trials is the response changes due to circadian rhythmicity, as monocyte levels in circulation generally decline during the course of the day (Hermann *et al*, 2006; Petrovsky *et al*, 2003) We also observed this effect as a decline in cytokine secretion, with a mean ratio decrease of 0.3 for IL-6, 0.2 for IL-8 and TNF-*a*, and 0.18 for IL-1$\beta$ (Table 6.1). As expected, gender did not influence the day variation. One possibility to overcome this diurnal pattern is to consider responses in relation to monocyte numbers at the moment of sampling, as these are the primary source of cytokines in this model. In any case these data can help design future clinical studies, taking into account circadian influences relative to the anticipated pharmacological activity of a test drug.



In addition to providing a stable model with improved dynamic range for monitoring cholinergic influence on inflammation, the LPS and aluminium hydroxide challenge of whole blood cultures provides a useful tool with several applications. First, this model provides a suitable readout to study the *in vitro* effects of nicotinic receptor occupancy with primary human blood cells. Additionally, the contribution of vagus nerve signaling may also be assessed by using a whole blood challenge to monitor changes in inflammatory responses before and after drug exposure to humans, in an *ex vivo* manner. Comparisons of *in vitro* effects of compounds influencing $a_7$ nAChR signaling versus *ex vivo* effects might shed light on complex pharmacokinetics contributing to some inter- and intra-individual variability.

In the current set of experiments we utilized choline chloride as a selective $a_7$ nicotinic agonist at concentrations of 50 mM and lower, to demonstrate the modulation to inflammatory stimuli in human whole blood cultures. While we still observed decreases in IL-1$\beta$ secretion at the lowest concentration of choline tested (100 µM, Figure 6.2), this was approximately 10 times higher than circulating plasma levels of choline in healthy subjects (Adamczyk *et al*, 2006). Despite this apparent discrepancy, it remains challenging to determine the concentrations of cholinergic agonists in a natural cellular microenvironment. For example, the anti-inflammatory effect of acetylcholine on LPS stimulated murine macrophages was only evident in the presence of acetylcholinesterase inhibitors (Wang *et al*, 2003). Given the tight regulation governing cholinergic signaling, our LPS aluminium whole blood challenge can be used to model the concentrations of nicotinic compounds applied *in vitro* and extrapolate it to the *in vivo* effects of a compound based on the *ex vivo* changes to cytokine secretion. Also when administering modulators of cholinergic signaling directly in humans, this model would provide a more feasible assessment compared to animal and *in vitro* models where bioavailability may not be complete or phylogenetic differences among species could be a problem.

Using choline as a selective $a_7$ nicotinic agonist, we have demonstrated a significant and consistent decrease in IL-1$\beta$ secretion following LPS and aluminium hydroxide exposure in human whole blood cultures. In addition we describe the variability of cytokine secretion in whole blood cultures from healthy elderly subjects, also in relation to circadian changes.

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**TABLE 6.1 Summary of healthy elderly participants and changes from the morning to the afternoon in ex vivo responses to LPS plus aluminium hydroxide challenge.**

| Demographics | | Total (n=25) | Male (n=16) | Female (n=9) |
|---|---|---|---|---|
| | Mean Age (range; years) | 69 (65-78) | 70 (65-78) | 69 (65-74) |
| | Mean BMI (range; $kg \cdot m^{-2}$) | 24.7 (19.9-32.4) | 24.8 (20.0-29.6) | 24.6 (19.9-32.4) |
| LPS + alum ex vivo response: Change from morning baseline | | | | |
| | Mean IL-1β ratio (± SD) | 0.82 (± 0.25) | 0.80 (± 0.22) | 0.87 (± 0.29) |
| | Mean IL-6 ratio (± SD) | 0.70 (± 0.16) | 0.66 (± 0.14) | 0.78 (± 0.16) |
| | Mean IL-8 ratio (± SD) | 0.80 (± 0.29) | 0.82 (± 0.23) | 0.88 (± 0.37) |
| | Mean TNFα ratio (± SD) | 0.80 (± 0.21) | 0.79 (± 0.21) | 0.72 (± 0.20) |





**FIGURE 6.1 Human whole blood cultures were stimulated with LPS for 3 hours, with or without choline chloride addition for 10 minutes at the indicated concentration.**

Culture supernatant was profiled for inflammatory cytokines by multiplex analysis (Meso Scale Discovery Human inflammatory cytokine panel) or by ELISA.

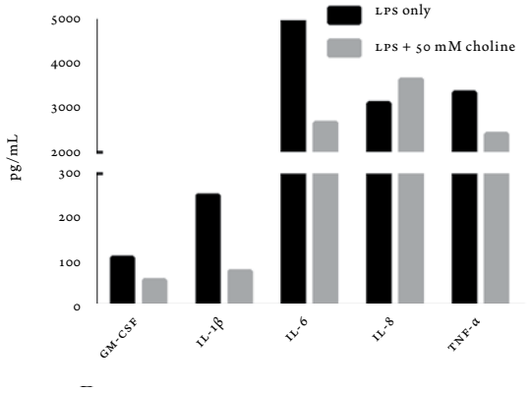


**FIGURE 6.2 Heparinized whole blood cultures were prepared from three healthy volunteers, and subjected to the indicated conditions.**

For LPS + eATP inflammasome activation, cultures were exposed to choline chloride for 15 minutes, following by LPS for 3 hours, with ATP added in the last 30 minutes of the incubation. For LPS + aluminium hydroxide, cultures were exposed to choline for 15 minutes following by simultaneous stimulation with LPS and aluminium hydroxide. Following the three-hour culture incubation, plasma supernatants were collected and secreted IL-1$\beta$ and IL-18 were measured by ELISA. Data are normalized to the individual's maximum cytokine release (100% was defined as the inflammasome response without any choline addition) and depicted as the mean of three individuals, including the standard deviation.

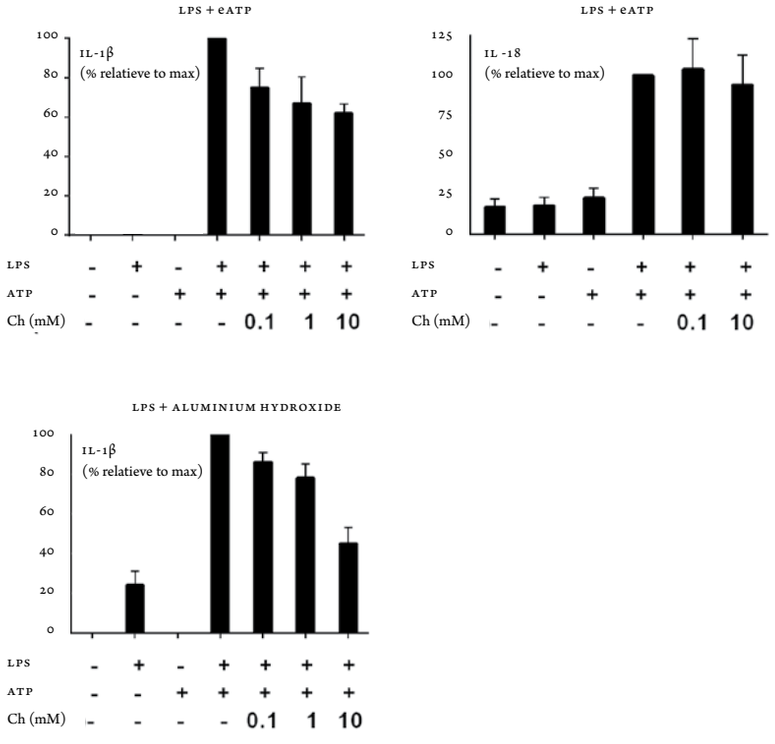







**FIGURE 6.3 Choline addition reduced select inflammatory cytokine secretion in response to LPS plus aluminium hydroxide.**

Heparinized whole blood cultures were prepared from healthy elderly volunteers (n=16; 10 male, 6 female). Blood was stimulated with LPS plus aluminium hydroxide, with or without 50 mM choline chloride addition (10 minutes). Following three hours culture, inflammatory cytokines were measured using Meso Scale Discovery human V-plex panel for IL-1$\beta$, IL-6, IL-8 and TNF$\alpha$. ****, ***, and * denotes p values <0.0001, <0.001, and <0.05, respectively. N.S. denotes not significant.

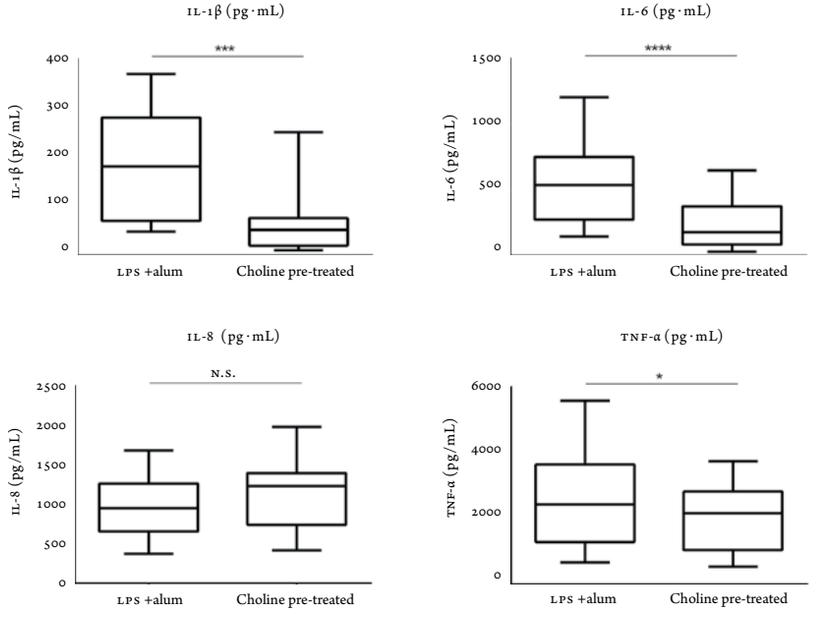
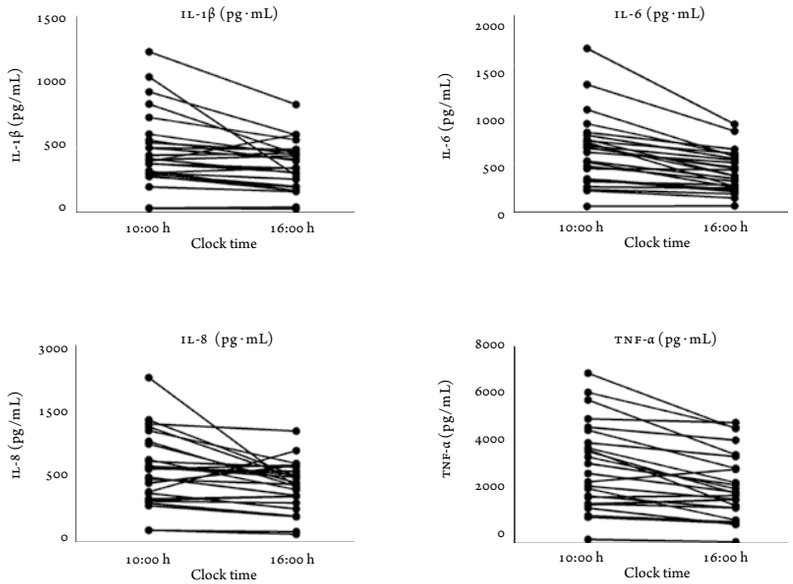


**FIGURE 6.4 Changes in response to LPS plus aluminium hydroxide challenge (daytime sampling).**

Blood cultures were prepared with LPS plus aluminium hydroxide at approximately 10:00h and 16:00h on the same day from 25 healthy elderly subjects. Cultures were incubated for 3 hours and then supernatants collected. Inflammatory cytokines were measured by multiplex Meso Scale Discovery panel as described in Figure 6.3.






7

# EEG MACHINE LEARNING FOR ACCURATE DETECTION OF CHOLINERGIC INTERVENTION AND ALZHEIMER'S DISEASE

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## ABSTRACT

Monitoring effects of disease or therapeutic intervention on brain function is increasingly important for clinical trials, albeit hampered by inter-individual variability and subtle effects. Here, we apply complementary biomarker algorithms to electroencephalography (EEG) recordings to capture the brain's multi-faceted signature of disease or pharmacological intervention and use machine learning to improve classification performance. Using data from healthy subjects receiving scopolamine we developed an index of the muscarinic acetylcholine receptor antagonist (mAChR) consisting of 14 EEG biomarkers. This mAChR index yielded higher classification performance than any individual EEG biomarker with cross-validated accuracy, sensitivity, specificity and precision ranging from 88–92%. The mAChR index also discriminated healthy elderly from patients with Alzheimer's disease (AD); however, an index optimized for AD pathophysiology provided a better classification. We conclude that integrating multiple EEG biomarkers can enhance the accuracy of identifying disease or drug interventions, which is essential for clinical trials.


## INTRODUCTION

An increasing number of drug candidates are being tested for their ability to modify disease or alleviate symptoms of brain disorders (Van der Schyf and Geldenhuys, 2011); however, to test these new pharmacological interventions and improve monitoring of the therapeutic response, informative and robust endpoints are urgently needed (Frank and Hargreaves, 2003; Golde, 2016; Oertel and Schulz, 2016). Clinical trials in central nervous system (CNS) drug development focus on behavioral and cognitive performance outcome measures of drug efficacy; however, quantitative electroencephalography (EEG) is gaining recognition in the field as a source of surrogate endpoints in early-phase studies (van Straaten *et al*, 2014). EEG offers insight into the mode of action of the pharmacological intervention, because of the high temporal resolution of electrophysiological measures (Leiser *et al*, 2011; Lopes da Silva, 2013). Still, it remains an important challenge to advance EEG biomarker analysis for enhanced prediction of therapeutic effects in clinical trials.

Scopolamine is the most extensively used pharmacological model of cognitive impairment (Klinkenberg and Blokland, 2010). As a selective competitive muscarinic receptor (mAChR) antagonist, it induces temporary deficits in cognitive functions that depend on the cholinergic system (Ebert *et al*, 1998). Scopolamine has a high affinity for all five muscarinic receptor subtypes ($M_1$–$M_5$) and a negligible affinity for histaminergic and dopaminergic receptors (Ali-Melkkilä *et al*, 1993). Muscarinic receptors are widely present in brain areas involved in attention and memory, and intravenous administration of scopolamine indeed causes impairments to these brain functions (Broks *et al*, 1988; Liem-Moolenaar *et al*, 2011). The scopolamine challenge test has been used in drug development to demonstrate the pharmacological activity of putatively cognition-enhancing compounds by reversal of scopolamine-induced cognitive deficits in healthy volunteers (Cho *et al*, 2011; Jones *et al*, 1991; Liem-Moolenaar *et al*, 2010a, 2010b; Preda *et al*, 1993; Prohovnik *et al*, 1997; Siegfried, 1993; Snyder *et al*, 2005).

EEG biomarkers have the potential to objectively determine whether reversal of scopolamine effects by a cholinergic compound is successful. In humans, scopolamine administration increases the power of delta and

theta activity, while alpha- and beta-frequency activity is reduced (Alvarez-Jimenez *et al*, 2016; Ebert *et al*, 1998; Liem-Moolenaar *et al*, 2011). It has been hypothesized that deficits of cholinergic signaling contribute to the EEG slowing in Alzheimer's disease (Agnoli *et al*, 1983; Blennow *et al*, 2006), which is also supported by the reversal of EEG slowing by cholinergic drugs (Citron, 2010; Jeong, 2004). Unfortunately, current biomarkers lack the desired accuracy for monitoring disease status or therapeutic response, because of large inter-individual variability compared to the often subtle drug-related changes. Most commonly, the functional state of the brain is assessed merely using one type of biomarker (Babiloni *et al*, 2004; Osipova *et al*, 2005; Sankari *et al*, 2012; Stam *et al*, 2003); however, pathophysiology is often expressed as changes to multiple properties of neuronal oscillations. Consequently, different biomarker algorithms may quantify distinct aspects of the brain's functional state. Combining these may increase accuracy of disease diagnosis and assessment of drug interventions (Dauwels *et al*, 2010b; Khodayari-Rostamabad *et al*, 2010; Lehmann *et al*, 2007; Montez *et al*, 2009; Poil *et al*, 2013). Here, we use machine learning to show that the complementary information of different EEG biomarkers can indeed be combined into an accurate index for better decision-making in clinical trials.

## METHODS

### SUBJECTS

Data were obtained from four separate trials conducted at the Centre of Human Drug Research (Leiden, the Netherlands) and approved by a medical ethics committee. All subjects signed a written informed consent prior to participation in the study and were medically screened.

Trial 1 and 2 evaluated the effect of investigational glycinergic compounds during a cognitive impairment scopolamine challenge test. A detailed description of the neurophysiologic tests has been reported previously (Liem-Moolenaar *et al*, 2010a, 2010b). In the two trials, a total of 83 male healthy subjects aged 18–55 years were recruited. Scopolamine (0.5 mg) or placebo was administered as a 15-minute intravenous infusion. Only the data where subjects received placebo or scopolamine (alone) was used in the analysis. Study periods were separated by a washout period of at least 1 week. The sampling and measurement schedules for the scopolamine challenges were identical for both studies. The measurements were performed during 36 hours treatments periods with 11 measurement time-points from baseline (pre-dose) to 8.5 hrs after scopolamine (or placebo) administration.

Trial 3 evaluated the effect of a novel $a_7$ nicotinic acetylcholine receptor agonist ($a_7$ nAChR) during a scopolamine challenge test. The study recruited 35 subjects between 65 and 85 years. All subjects received 0.3 mg scopolamine (IV) in 15 minutes. Neurophysiological tests were measured with 8 measurement times from twice at baseline (-1 day) to 6 h after scopolamine administration (open-label). A detailed description of the neurophysiologic tests can be found elsewhere (Alvarez-Jimenez *et al*, 2016; Liem-Moolenaar *et al*, 2011).

Trial 4 consisted of 40 mild to moderate AD patients who were recently diagnosed with 'probable AD' (according to NINCDS-ADRDA), mild to moderate severity of dementia (according to Clinical Dementia Rating Score, CDR of 0.5-2) and a Mini-Mental State Exam score of 18–26. Here, we used eyes-closed rest EEG recordings obtained in the baseline before the administration of an investigational drug.

### EEG RECORDINGS AND PRE-PROCESSING

EEG recordings were made using silver chloride electrodes fixed at Fz, Cz, Pz and Oz positions, with the same common reference electrode as for the eye movement registration (according to the international 10/20 system). Electrode resistances were kept below 5 kΩ. EEG signals were obtained from leads Fz-Cz and Pz-Oz and a separate channel to record eye movements (for artefacts). The signals were amplified by use of a Grass telefector (F-15EB/B1) and a 15LT series Amplifier Systems (Grass-Telefactor) with a time constant of 0.3 s and a low-pass filter at 100 Hz. The duration of the recordings was 64 s (Zuurman *et al*, 2008). Sampling frequency was 64768 Hz, afterwards down-sampled to 1012 Hz for the analysis. The ongoing EEG was visually inspected in windows of 10 s and sharp transient artefacts were cut out, as well as eye movement and muscle artefacts. Noisy channels were excluded from the subsequent analysis.

## EEG ANALYSIS

For the EEG analysis, the Neurophysiological Biomarker Toolbox (NBT) (http://www.nbtwiki.net/) (Hardstone *et al*, 2012) was used to calculate the biomarkers and custom made scripts were integrated with the NBT analysis pipeline for advanced statistics, employing data mining algorithms to combine the information from multiple biomarkers. We employed biomarker algorithms in order to extract both temporal and spectral information from the EEG signals in the classical frequency bands: δ (1–4 Hz), ϑ (4–8 Hz), *α* (8–13 Hz), and *β* (13–30 Hz). The power in these frequency bands was computed using the Welch method with a 4096-point Hamming window and a frequency resolution of 0.25 Hz. The relative power was calculated by dividing the absolute power in each frequency band with the integrated power in the range 1–45 Hz. The central frequency, *fc*, and bandwidth, *fσ* (Vural and Yildiz, 2010), were computed according to the formulas:

$$f_c = \frac{\sum_{f=f_L}^{f_H} fP(f)}{\sum_{f=f_L}^{f_H} fP(f)} \qquad f_\sigma = \sqrt{\frac{\sum_{f=f_L}^{f_H} (f-f_c)^2 P(f)}{\sum_{f=f_L}^{f_H} fP(f)}}$$

where *fL* and *fH* represent the lowest and highest frequency that defines a given frequency band, and *P(f)* denotes the power at frequency *f*. Thus, the central frequency biomarker provides information about where the power is concentrated in a given frequency band, whereas the bandwidth provides information about how much the power is spread out around the central frequency.

The amplitude envelope was extracted using the Hilbert transform and analyzed for long-range temporal correlations of the power-law form using detrended fluctuation analysis (DFA) (Hardstone *et al*, 2012; Linkenkaer-Hansen *et al*, 2001; Peng *et al*, 1995). If a sequence of events has a non-random temporal structure with slowly decaying autocorrelations, DFA can quantify how slowly these correlations decay as indexed by the DFA power-law exponent. Signals were filtered using a FIR filter with a Hamming window with a length corresponding to two $f_1$ Hz cycles for frequency band $[f_1, f_2]$. To





minimize temporal correlations introduced by the FIR filter, DFA was fitted in the interval from 4 to 20 seconds for δ and ϑ band, from 2 to 20 seconds for *α* and 1 to 20 seconds for the *β* band. The oscillation burst lifetime was used to quantify differences in amplitude dynamics of oscillations on short to intermediate time scales (< 1 s)(Montez *et al*, 2009; Poil *et al*, 2011). We used a threshold at the median of the amplitude envelope and defined the beginning and the end of an oscillation burst as the time points of crossing this threshold. The duration of oscillation bursts was calculated by taking the 95TH percentile of all durations measured within each channel, which we refer to as the 'oscillation burst lifetime' biomarker. In total, 20 biomarkers were extracted from each EEG signal. Each of the biomarkers was computed over two bipolar channels (Fz-Cz and Pz-Oz) resulting in a total of 40 features for classification analysis.

## STATISTICAL ANALYSIS

Machine learning techniques were used to find the biomarkers that best distinguished the peak effect of scopolamine from the baseline recording or that best distinguished AD patients from healthy controls. From time-dependent curves of EEG biomarkers (Figure 7.2), we evaluated 1.5 h after administration of scopolamine as the peak for most EEG biomarkers – in agreement with the peak drug effect ($T_{max}$) time point according to the cognitive measurements (Alvarez-Jimenez *et al*, 2016; Liem-Moolenaar *et al*, 2011); therefore, we performed classification on the EEG recorded at baseline and 1.5 h after administration of scopolamine for the development of the mAChR index. For the AD index, we used pre-intervention baseline recordings of AD patients and healthy controls.

A feature matrix was built from the EEG biomarkers – in the form #features × #samples – with the aim of identifying sets of biomarkers that were more discriminative between the two groups than each individual biomarker. Feature selection and classification were performed via the classical machine learning procedure steps: training and testing. In the training phase, the index was developed by applying the feature-selection algorithm to training data and in the test phase, the index was applied to predict the class membership

on the test data. The features used for machine learning were z-scored EEG biomarker values. To avoid introducing future information into the classifier, we normalized both the training and the test data by subtracting the mean and dividing by the standard deviation of biomarker values from the training data only.

Indices were identified by applying the classification algorithm to the whole dataset (Trials 1 and 2 for mAChR; Trials 3 and 4 for the AD index); however, cross-validation was used to evaluate the stability of the result, i.e., classification with 100 different splits of the data into training and test sets were performed to obtain the mean and standard deviation of the classification performance, which provides an estimate of the classification performance on an 'unknown' sample (Witten *et al*, 2011). To this end, we used the cross-validation with 70/30% random splitting, i.e., from a random permutation of the subjects, 70% were used for training and 30% for testing. The training set consisted of 115 EEG recordings, tested on 48 recordings for the mAChR index (Trial 1 and 2). The total number of recordings is twice the number of subjects: per subject, the baseline EEG recording was used as the first sample and the peak drug effect recording as the second sample. For the AD index, the training and test set consisted of 53 and 22 recordings (and subjects), respectively (Trial 3 and 4).

### ELASTIC NET LOGISTIC REGRESSION

Because of the correlation between some of the features, we performed feature selection and classification using the elastic net (Zou and Hastie, 2005), with sparsity and grouping of correlated features as properties. Elastic net is a feature selection and classification method based on regularized logistic regression that bridges the gap between lasso (Tibshirani, 2011) and ridge regression (Hoerl and Kennard, 1970) by combining their penalties and optimizing the number of features included in the integrated index through minimizing the function:

$$L(\lambda_1, \lambda_2, \beta) = y - X\beta^2 + \lambda_1\beta_1 + \lambda_2\beta_2{}^2$$





where X is the feature matrix, $y$ is the response vector (the labels) $\beta$ the weights, and $\lambda_1$ and $\lambda_2$ coefficients determining the influence of the $L_1$ and $L_2$ norm penalties, respectively. The first term is similar to logistic regression while the second and third terms form the elastic net penalty function. If we denote: $\alpha=\lambda_2\lambda_1+\lambda_2$, then the elastic net penalty can be rewritten as $(1-\alpha)\beta_1+\alpha\beta_{22}$, where $\alpha$ acts as the balancing term between the L1 and L2 norm penalties. We optimized $\alpha$ in random-splitting cross-validation procedure and found the best classification performance with $\alpha = 0.5$ (results not shown).

By minimizing the L-function, we obtain the set of $n$ selected features corresponding to the ones with highest $\beta$ values. If $p$ is the probability that an EEG recording belongs to the peak scopolamine condition, then the odds ratio is $p(1-p)$, which is the ratio of the probability of peak scopolamine to the probability of baseline recording. Logistic regression models the log odds ratio as a linear combination of the independent variables, via this equation:

$$\ln\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 f_1 + \ldots + \beta_n f_n$$

where $f_1$ are the features and $\beta_1$ the associated weights. The log odds can be transformed back to probabilities as:

$$p(t) = \frac{1}{1 + exp(-t)} \qquad t = \beta_0 + \beta_1 f_1 + \ldots + \beta_n f_n$$

The size of the final set of selected features is estimated as the one that gives the maximum classification performance on the training set, while keeping the feature set as small as possible. To obtain this set, we compared the accuracy of classifiers using the $k$ best $\beta$-s, with $k$ ranging from 1 to the number of features $n$ and selected the smallest feature set with optimal performance.

### CLASSIFICATION OUTCOME EVALUATION

Elastic net logistic regression algorithm was used for developing two integrated indices: 1) The mAChR index, which is classifying whether an EEG was recorded during the baseline or when scopolamine has been administrated; 2) The AD index, which is classifying whether an EEG was recorded from a healthy elderly or an AD patient. To evaluate the classification

performance of the indices we used four different measures. In the case of the mAChR index, they are defined as:

- Accuracy (ACC): (number of correctly classified peak scopolamine and baseline recordings)/(total number of recordings).
- Sensitivity (SE): (number of correctly classified scopolamine recordings)/(number of scopolamine recordings).
- Specificity (SP): (number of correctly classified baseline recordings)/(number of baseline recordings).
- Precision (PPV): (number of correctly classified scopolamine recordings)/(number of recordings classified as scopolamine).
- Area Under Curve (AUC): area under the Receiver Operating Characteristic (ROC) curve, which plots the true positive rate (SE) against the true negative rate (1-SP) as the discrimination threshold of the classifier is varied. A higher AUC means better classification performance.
- Analogous definitions apply for the classification performance of the AD index.

## RESULTS

### SCOPOLAMINE AFFECTS BOTH SPECTRAL AND TEMPORAL DYNAMICS OF THE EEG

To gain a comprehensive understanding of the effects of scopolamine on the EEG, we employed biomarker algorithms characterizing spectral content as well as temporal dynamics of neuronal oscillations. The spectral content was estimated using power spectrum analysis of the broadband EEG signals (Figure 7.1). The short-time scale temporal structure of narrow-band oscillations was quantified by extracting the amplitude envelope and applying oscillation-bursts lifetime analysis (Figure 7.1), whereas temporal dynamics on longer time scales was quantified using DFA (Figure 7.1).

To examine the effects of scopolamine administration compared to placebo, we quantified these differences systematically at 11 time points from 30 minutes before to 8.5 hours after scopolamine and placebo administration. In Figure 7.2, we display the results as time-dependent biomarker curves of relative power, central frequency, bandwidth, oscillation-burst lifetime, and DFA in the columns and the frequency bands in the rows. A significant effect of scopolamine compared to placebo was observed for several biomarkers (Wilcoxon rank sum test at 1.5 h after administration, Bonferroni corrected). Despite all of these robust effects, if biomarkers carry complementary information about scopolamine-induced EEG changes, then it may be possible to combine this information into a more sensitive measure of the anticholinergic effect compared to using any of the individual biomarkers.





### INTEGRATING BIOMARKERS ENHANCES CLASSIFICATION

We used machine-learning techniques to find the biomarkers that best distinguish the baseline from the peak scopolamine condition. In order to do this, we performed classification on the baseline recording and the EEG recorded 1.5 h after administration of scopolamine (Figure 7.2). The baseline was used as opposed to the placebo condition to eliminate variation between days.

An initial integrated index was developed using elastic net on the data from healthy subjects ($n$ = 83 males, Trial 1 and 2, see Methods) that received scopolamine, while allowing a fraction – determined by the algorithm – of the 40 available biomarkers to be included. Subsequently, to simplify the composition of the index, biomarkers with non-zero weights were sorted by decreasing absolute weight and added incrementally in that order (i.e., starting from an empty set, we added the biomarker with the largest absolute weight etc.) to evaluate the gain of including each subsequent biomarker to the classifier. Accuracy and AUC increased with the number of features included in the index up until a maximum performance was reached (Figure 7.3). We defined the optimal index to be the one with the smallest number of features for which the average of all performance measures had saturated. We estimated this number to be 14 (Figure 7.3) according to the 'elbow' method (Ketchen and Shook, 1996); together, this set of 14 biomarkers and their associated weights make up the integrated mAChR index (Figure 7.3).

The mAChR index had excellent performance when training and testing on the same data (accuracy 95%, sensitivity 96%, specificity 93%, precision

93% and area under curve 0.98), and much higher than the single-best biomarker, which was relative delta power (Figure 7.3). Accordingly, the difference between the baseline predicted group and the peak scopolamine predicted group (Figure 7.3) was much more pronounced for the mAChR index ($p = 2*10^{-26}$, Wilcoxon rank sum test) than for relative delta ($p = 6*10^{-16}$). To obtain a more accurate estimate of the classification performance, we used cross-validation. The difference in performance per cross-validation was due to different subsets of subjects used for training and testing in each iteration, resulting in slightly different biomarker selections and weights. Cross-validation on these two datasets (Figure 7.3) resulted in an accuracy of 90 ± 2%, sensitivity of 92 ± 4%, specificity of 88 ± 4% and precision of 88 ± 3%, which is still very high and significantly higher than using just relative delta: accuracy of 79 ± 2%, sensitivity of 79 ± 4%, specificity of 83 ± 4% and precision of 81 ± 3% ($p = 9*10^{-29}$ for accuracy, Wilcoxon rank sum test). Interestingly, the difference between the baseline predicted and scopolamine predicted groups was also more significant for the mAChR index ($p = 9*10^{-10}$, Wilcoxon rank sum test) than for relative delta ($p = 0.02$) when tested at washout – 8.5 h after scopolamine administration (Figure 7.3).

### THE MACHR INDEX IS ROBUST AND GENERALIZABLE

Test-retest stability is an important quality of a biomarker. We therefore compared the mAChR index scores of baseline recordings from two separate days in 75 subjects (Trial 1 and 2) and observed a strong correlation of 0.64 (Spearman correlation, $p = 2.5*10^{-10}$, Figure 7.3). To further demonstrate the generalizability of the mAChR index, we applied it to an independent cohort of healthy elderly subjects (Trial 3, see Methods) receiving a similar scopolamine intervention. Interestingly, in spite of the difference between the age groups in Trials 1–2 and 3, we observed an index performance very close to the cross-validation on the adult cohort (Figure 7.4; accuracy 87%, sensitivity 83%, specificity 91% and precision 91%). Importantly, the index also generalized to the other measurement time points both for Trials 1, 2 and 3 (Figure 7.4).





### SCOPOLAMINE IS A VALID MODEL OF AD PATHOPHYSIOLOGY

To test the validity of scopolamine as a model for the cholinergic dysfunction that occurs in AD, we next developed an AD index. Using the patients with mild to moderate AD (Trial 4) and the age-matched healthy elderly subjects (Trial 3), we derived an AD index consisting of 12 biomarkers (Figure 7.5), of which 5 are the same as those in the mAChR index. The AD index performed with an accuracy of 92%, sensitivity 87%, specificity 97% and precision 97% when training and testing on the same data (Figure 7.5). Cross-validated, the respective performances were 73 ± 6%, 73 ± 9%, 70 ± 10% and 75 ± 7%. Next, we investigated the relation of the AD index to the mAChR index, comparing their abilities to discriminate healthy elderly subjects from patients with mild to moderate AD, or discriminate baseline from peak scopolamine. Applying the mAChR index on healthy elderly and AD patients, we observed that it was able to discriminate them, with an accuracy of 62%, sensitivity 35%, specificity 91% and precision 81% (Figure 7.5). The separation was better when applying the AD index on subjects before and after scopolamine intervention, with accuracy 72%, sensitivity 89%, specificity 54% and precision 66%. Taken together our results show that the mAChR index captures cholinergic dysfunction that occurs in AD. This is reflected by several biomarkers shared between the mAChR and the AD index, as well as the mutual ability to distinguish subjects with AD or subjects given scopolamine, respectively. Also, the good performance of the AD index in discriminating healthy elderly from AD patients further demonstrates the value of multi-biomarker classification schemes.

## DISCUSSION

Resting-state EEG signals are complex and information rich (Linkenkaer-Hansen *et al*, 2001). A variety of spectral, spatial and temporal biomarker algorithms have been used to uncover brain electrophysiological changes in disease or with pharmacological intervention (Arns and Olbrich, 2014; Montez *et al*, 2009; Mucci *et al*, 2006); however, they all have too low sensitivity and specificity to become standard tools in hospitals and

clinical trials (Ommundsen *et al*, 2011; van Straaten *et al*, 2014). To address this problem, we tested whether extensive characterization of EEG using multiple biomarkers and subsequent application of machine learning could improve the accuracy of classifying disease state or drug intervention. We developed a mAChR index with superior sensitivity and specificity to the complex structure of the EEG changes induced by scopolamine intervention compared to any single biomarker. The enhanced accuracy could be of great value in evaluating the efficacy of drugs that aim to induce effects opposite to scopolamine, e.g., for the treatment of AD and schizophrenia-related cognitive impairment. We believe our methodological approach could prove invaluable in a wide range of challenge tests used in CNS drug development.

## SCOPOLAMINE AFFECTS VARIOUS PROPERTIES OF NEURONAL OSCILLATIONS

Scopolamine is known to decrease alpha power and increase relative delta and theta power, mainly in posterior regions (Kikuchi *et al*, 1999; Liem-Moolenaar *et al*, 2011; Sannita *et al*, 1987). These EEG changes are hallmarks of cognitive impairment associated with AD (Bennys *et al*, 2001; Dauwels *et al*, 2010a; Jeong, 2004) and also observed in our healthy subjects after scopolamine administration (Figure 7.2). Other biomarkers affected by scopolamine included the oscillation burst lifetime biomarker, which decreased in the alpha band (Figure 7.1) and increased in the theta band (Figure 7.2) as observed also in early-stage AD (Montez *et al*, 2009). Scopolamine produced an increase in DFA in all frequency bands, albeit this effect was only sufficiently strong in the beta band for inclusion in the mAChR index (Figure 7.1and Figure 7.2). The mAChR index also comprised the central frequency effects of a decrease in the theta band and an increase in the alpha and beta bands (Figure 7.2). Changes in the central frequency and bandwidth were correlated, decreasing in the theta band and increasing in alpha and beta bands. Larger bandwidth could be associated with less frequency stability of alpha and beta oscillations, which has previously been linked to a less efficient working memory (Kopell et al, 2011).





## MACHINE LEARNING FOR MORE ACCURATE MEASURES OF PATHOPHYSIOLOGY

A similar analysis using machine learning for classifying scopolamine effects on the EEG has been developed in the past ( Johannsson et al, 2015); however, with different features and analysis methods used and without reporting the classification performance of the index; therefore, it cannot readily be compared with our findings. Our cross-validation (training and testing on different data) resulted in a remarkable performance (Figure 7.3). Importantly, the test-retest reliability of the mAChR index across two baseline recordings was very high (Figure 7.3), and validation on an independent set of data confirmed that the index generalizes to new cohorts (Figure 7.4). Interestingly, when classifying on the washout period, classification with the mAChR index was highly significant, whereas that of the single-best biomarker was only marginally significant (Figure 7.3), suggesting that clinical situations with many more subtle drug effects will gain substantially from the proposed machine learning and data-integration approach. This is particularly useful in view of the fact that acute pharmacodynamics effects of pro-cognitive, cholinergic compounds are often difficult to measure in healthy subjects or patients with AD (Balsters *et al*, 2011; Beglinger *et al*, 2004, 2005; Nathan *et al*, 2001).

To examine the validity of scopolamine as a model of AD pathophysiology, we applied the mAChR index to healthy elderly controls and patients with AD. We also derived an AD index to test whether scopolamine-induced EEG changes resemble those of AD. Applying the mAChR index to AD patients and controls we observed that it indeed showed an effect (Figure 7.5); however, it discriminated less accurately than the AD index and with a shift in the classification threshold. According to the mAChR index, some AD patients were misclassified as healthy elderly, presumably because the EEG is affected more strongly by scopolamine than mild (or moderate) AD. Nonetheless, because the differences were in the same direction, applying the AD index to the subjects on scopolamine resulted in a much better separation (Figure 7.5). The misclassification here was opposite: a few baseline recordings were predicted as scopolamine, because the differences between healthy elderly and AD are weaker and, therefore, the separation threshold for the AD index is

lower. Together, this suggests that scopolamine is a good cognitive impairment model for AD, mimicking the changes seen in AD patients; however, with a difference in the magnitude of effects. Nicotinic blockade added to the muscarinic anticholinergic effects might better resemble changes reflected in both indices and might further explain the difference observed between AD and scopolamine peak effects (Ellis *et al*, 2006; Erskine *et al*, 2004; Gitelman and Prohovnik, 1992; Little *et al*, 1998).

## mAChR index – potential as a clinical EEG tool and future developments

Integrating information from multiple EEG biomarkers has an advantage over the standard power spectrum, because of the often subtle changes from baseline and the considerable inter-individual variability at baseline for EEG and cognitive tests. This approach also reduces the multiple-comparisons problem when analyzing several EEG biomarkers in clinical trials. A specific mAChR index may help to quantify effects of pro-cognitive cholinergic compounds, and muscarinic agonists in particular. Reversal of detrimental effects induced by scopolamine on cognitive performance has been demonstrated in humans with donepezil (Snyder *et al*, 2005; Thomas *et al*, 2008) and galantamine (Baraka and Harik, 1977) – two cholinesterase inhibitors that increase acetylcholine in the synaptic cleft and prescribed for the symptomatic treatment of patients with AD. Further research to develop a nicotinic cholinergic index would also be an important tool in drug development as nicotinic reversal has also been successfully reported (Woodruff-Pak *et al*, 2003), therefore an index for nicotinic antagonists could provide a useful non-invasive method to monitor the effects of an important class of drugs.

Furthermore, while improvement of cognitive functions is difficult to quantify in healthy subjects (Beglinger *et al*, 2004, 2005), administration of the agonists may induce changes in the mAChR index that might not be quantifiable with other cognitive tests without the use of a pharmacologic challenge test (Cohen, 2010). Therefore, a more accurate measure of the EEG effects of scopolamine and of cholinergic compounds may result in superior





detection of pharmacological (scopolamine reversing) effects. This is very important for drug development, both in terms of proof-of-pharmacology and dose finding. Showing reversal of scopolamine effects by cholinergic compounds (even those proven to be effective in the clinic) is difficult, but this method holds potential: it improves detection of muscarinic anticholinergic EEG effects, so we can expect it to be beneficial at showing the reversal of those effects as well. Moreover, this method may also help to detect cholinergic effects in healthy subjects (or AD patients) who have not been given the scopolamine challenge.

In conclusion, scopolamine effects on the EEG are clearly present and the spectral ones are well known; however, the mAChR index also accommodates the temporal dynamics to provide deeper insight into the brain's cholinergic electrophysiology. The index serves as a sensitive biomarker to detect the effect of scopolamine in a dose-dependent manner as well as provide evidence for drug penetration and, therefore, holds potential for being used in experimental pharmacology.

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**FIGURE 7.1-1 Spectral and temporal correlation biomarkers exhibit sensitivity to scopolamine administration.**

**A**. EEG of a subject in the baseline (**dark grey**) and scopolamine (**light grey**) condition. **B**. Grand average normalized power spectra indicate large effects of scopolamine, most notably a reduction of power in the alpha and beta bands, and an increase of delta and theta power.

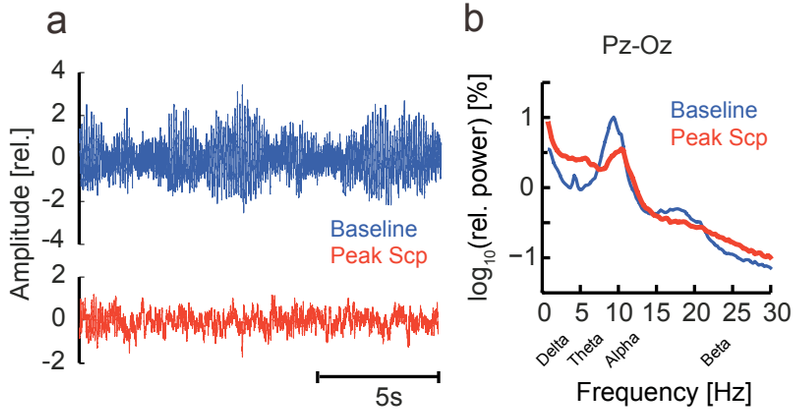


**FIGURE 7.1-2 Spectral and temporal correlation biomarkers exhibit sensitivity to scopolamine administration.**

**C**. Oscillation dynamics were studied by extracting the amplitude envelope from band-pass filtered data (e.g., the alpha band, **black**) using the Hilbert transform (**dark grey, light grey**) and a median-amplitude threshold to determine the onset and offset of a burst. **D**. A cumulative probability distribution of all oscillation bursts revealed a tendency towards longer alpha bursts in the baseline condition. **E**. Amplitude envelopes of beta oscillations (13–30 Hz) suggest a more complex temporal structure in the peak scopolamine (**light grey**) than in the baseline (**dark grey**) condition on time scales of seconds to tens of seconds. **F**. The long-time scale differences in beta oscillations are reflected in the grand average DFA showing larger scaling exponents for peak scopolamine (**light grey**) than for the baseline recording (**dark grey**). All figures were based on the Pz-Oz channel.

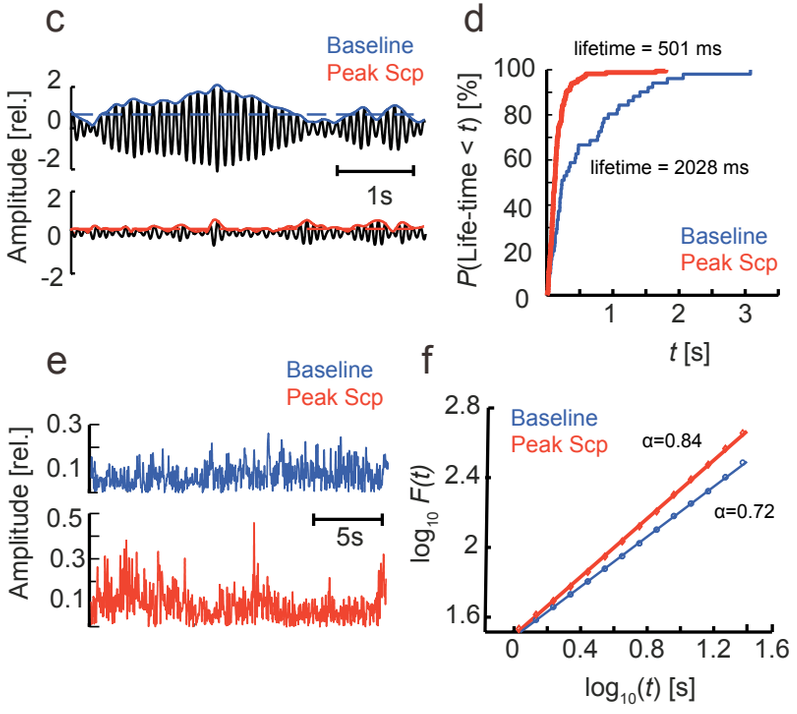
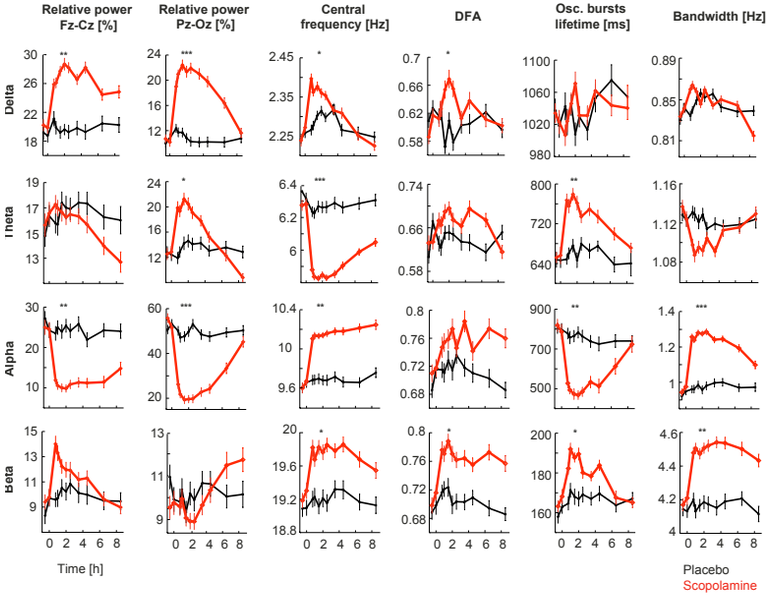







**FIGURE 7.2 Scopolamine affects many characteristics of the EEG.**

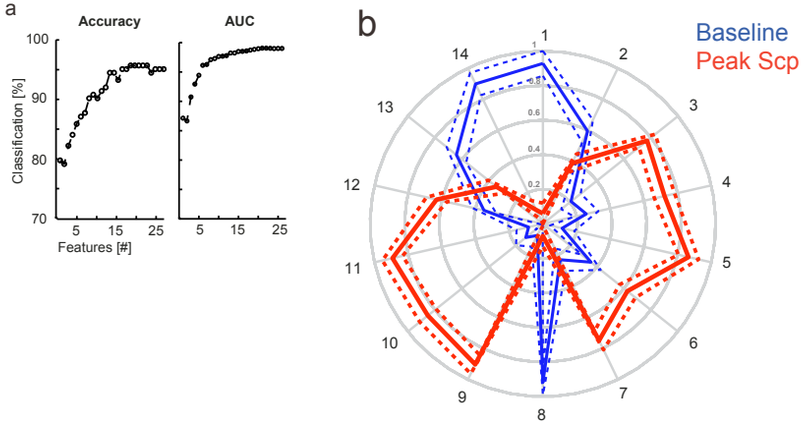
Time dependence of different EEG biomarkers (columns) and frequency bands (rows) for placebo (black) and scopolamine (**light grey**). All biomarkers are shown as averages over the 2 channels, except for relative power, for which the Fz-Cz and Pz-Oz channels are shown separately, because the effects in the delta and beta bands were opposite for the two derivatives. Sixteen biomarkers were significantly affected by scopolamine, with the peak effect occurring 1.5 h after administration.




*Significance levels: * denotes $p < 0.05$, ** $p < 10^{-5}$, *** $p < 10^{-10}$ Bonferroni corrected for multiple comparisons.*

**FIGURE 7.3-1 Enhanced detection of scopolamine-induced EEG changes using machine learning.**

**A**. Classification performance increased with the number of features included in the integrated index. **B**. All of the biomarkers selected by elastic net logistic regression for inclusion in the integrated mAChR index differed significantly between baseline and peak scopolamine. Biomarkers are ordered by their absolute weights, decreasing clockwise from the top. Weights ($\beta$) are listed next to each biomarker in the legend (PO denotes Pz-Oz and FC denotes Fz-Cz). The values plotted on the spider plot are the z-score group means and standard error of the mean, normalized to [0, 1] by subtracting the minimum across all biomarkers and dividing with the largest range present (i.e., the difference between the minimum and maximum value found for the biomarkers with the largest difference).



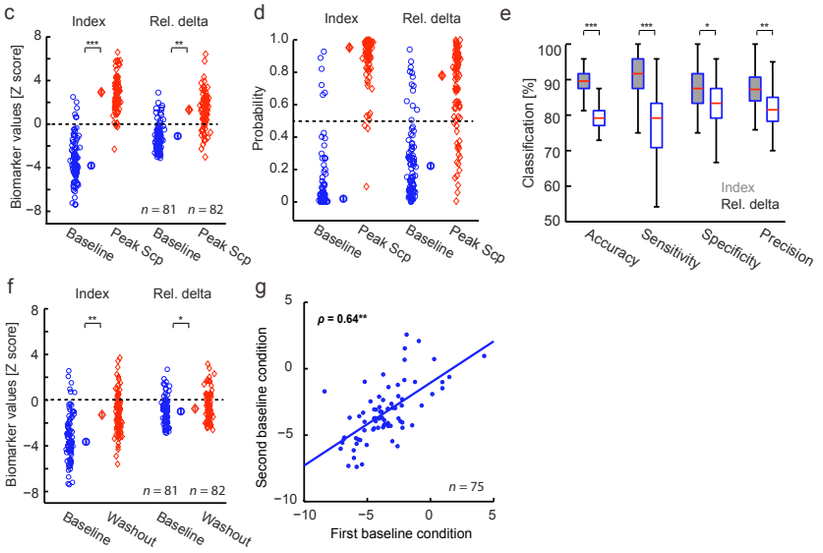

1. Rel. power (alpha, PO), $\beta_1$ = -0.89
2. Rel. power (beta, PO), $\beta_2$ = -0.79
3. DFA (beta, PO), $\beta_3$ = 0.75
4. Central freq. (beta, PO), $\beta_4$ = 0.73
5. Lifetime (theta, PO), $\beta_5$ = 0.67
6. Rel. power (beta, FC), $\beta_6$ = 0.5
7. Central freq. (delta, FC), $\beta_7$ = 0.45
8. Central freq. (theta, PO), $\beta_8$ = -0.42
9. Rel. power (delta, PO), $\beta_9$ = 0.42
10. Central freq. (alpha, FC), $\beta_{10}$ = 0.41
11. Bandwidth (beta, PO), $\beta_{11}$ = 0.37
12. Lifetime (beta, PO), $\beta_{12}$ = 0.36
13. Bandwidth (theta, FC), $\beta_{13}$ = 0.36
14. Central freq. (theta, FC), $\beta_{14}$ = -0.35





**FIGURE 7.3-2**

**C**. The mAChR index was more sensitive to the scopolamine (SCP) intervention than relative delta power. The plot shows z-scored biomarker values per subject recording. Singled-out symbols represent median values per group with standard error bars. The dashed line indicates the threshold of the classifier to predict the recordings as a baseline (below) or a peak scopolamine (above) recording. **D**. Same as C. but instead of z-scored biomarker values, predictive probabilities obtained from the classifier are shown. **E**. Classification performance of baseline vs scopolamine at the peak drug effect using 100 cross-validations is significantly higher for the mAChR index (**grey** boxplots) than the relative delta power (white boxplots). **F**. The superiority of the integrated index was also pronounced at washout. Relative delta power in the scopolamine condition was almost back to normal at 8.5 h after administration, whereas the mAChR index produced a highly significant effect. **G**. The integrated index has high test-retest reliability across weeks.




*Significance levels legend for this figure: * denotes $p < 0.05$, ** $p < 10^{-10}$, *** $p < 10^{-20}$, using Wilcoxon rank sum test.*

**FIGURE 7.4 The mAChR index generalizes to a new cohort of subjects.**

**A**. The mAChR index also generalizes to a cohort of healthy elderly subjects receiving the same scopolamine intervention (Trial 3), with validation accuracy 87%, sensitivity 83%, specificity 91% and precision 91%. **B**. Time dependence curves demonstrate the generalizability of the index at all the time points. The mAChR index for placebo (**black**) and scopolamine (**light grey**) is shown for Trials 1 and 2 used for developing the index and for independent data from Trial 3 (in the latter there was no placebo condition). The values plotted are group means and standard errors of the mean computed for the within-subject design (Franz and Loftus, 2012).

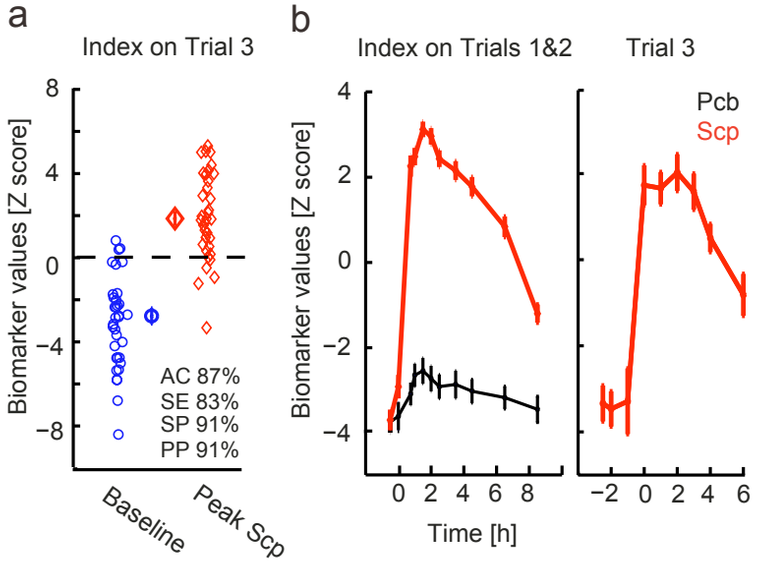
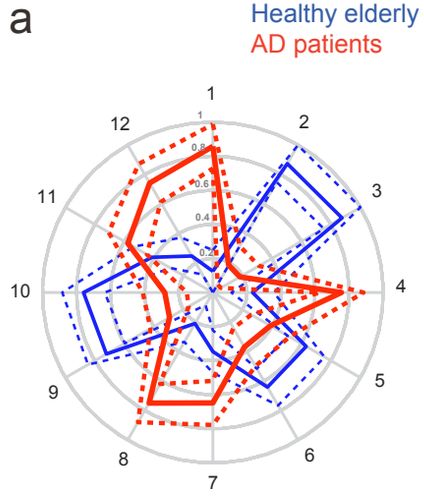







**FIGURE 7.5-1 The integrated AD index captures scopolamine-induced effects and validates scopolamine as a model of AD pathophysiology.**

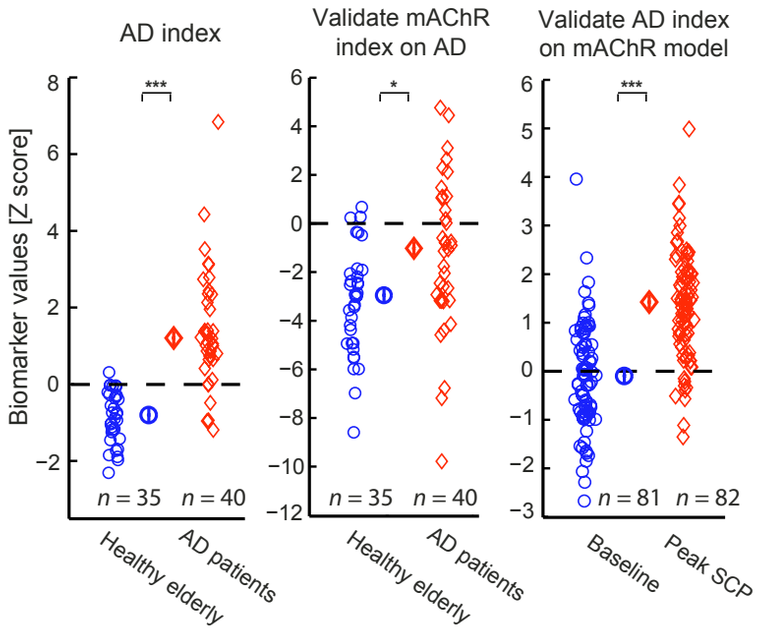
**A**. Illustration of the twelve biomarkers composing the integrated Alzheimer's index. Several of these biomarkers also compose the scopolamine index, with the same directionality of change in both the scopolamine-induced cognitive impairment and Alzheimer's disease. The values plotted are as explained in Figure 7.3B. **B**. Integrated AD index separates healthy elderly from Alzheimer's disease patients with high precision. **C**. The mAChR index discriminates the healthy elderly and AD patients albeit less accurately than the AD index. **D**. Validating the AD index on the scopolamine data gives much better discrimination.




1. Rel. power (theta, FC), $\beta_1$ = 0.83
2. Rel. power (beta, PO), $\beta_2$ = -0.54
3. Rel. power (alpha, PO), $\beta_3$ = -0.45
4. Lifetime (delta, FC), $\beta_4$ = 0.37
5. Bandwidth (delta, PO), $\beta_5$ = -0.21
6. Bandwidth (delta, FC), $\beta_6$ = -0.2
7. Cen. freq. (delta, FC), $\beta_7$ = 0.19
8. Lifetime (theta, PO), $\beta_8$ = 0.17
9. Lifetime (alpha, FC), $\beta_9$ = -0.15
10. Lifetime (beta, PO), $\beta_{10}$ = -0.15
11. Cen. freq. (alpha, PO), $\beta_{11}$ = 0.09
12. Rel. power (delta, FC), $\beta_{12}$ = 0.09

**FIGURE 7.5-2**

**B**. Integrated AD index separates healthy elderly from Alzheimer's disease patients with high precision. **C**. The mAChR index discriminates the healthy elderly and AD patients albeit less accurately than the AD index. **D**. Validating the AD index on the scopolamine data gives much better discrimination.






# 8

# DISCUSSION AND FINAL CONCLUSION

## DISCUSSION

As discussed in the introduction (Chapter 1), the cholinergic system controls the most crucial physiological functions in most species (Karczmar, 2007). In humans, it not only controls parasympathic vital functions such as vascular tone, heart chrono- and inotropism, gastrointestinal motility and gland secretion, (McCorry, 2007), and is involved in voluntary movement of skeletal muscles (Sine, 2012), it also controls cognitive functions such as learning and consciousness (Woolf and Butcher, 2011). Most of the current knowledge on the role of the cholinergic system in cognition is a result of diseases affecting the cholinergic neuronal system and of known side effects of drugs that antagonize acetylcholine receptors. Many *in vivo* pharmacological tests with the muscarinic acetylcholine receptor antagonist scopolamine have provided evidence that acetylcholine is an indispensable neurotransmitter involved principally in cognitive functions including attention, learning, visuo-spatial orientation and working memory (Broks *et al*, 1988; Liem-Moolenaar *et al*, 2011; Robbins *et al*, 1997; Thomas *et al*, 2008; Woodruff-Pak and Hinchliffe, 1997). Blockade of the nicotinic receptors mainly impairs attention, learning and working memory(Ellis *et al*, 2006; Newhouse *et al*, 1992; Rasch *et al*, 2006). Conversely, cholinergic agonists are known to improve cognitive performance (Newhouse *et al*, 2004) or reduce the cognitive effects of cholinergic blockade (Snyder *et al*, 2005; Wesnes and Warburton, 1984). Since more than three decades, Alzheimer's disease (AD) has been one of the most studied diseases in which cholinergic dysfunction plays an etiological role (Coyle *et al*, 1983). Acetylcholine inhibitors are currently approved as a symptomatic treatment for AD. The mechanism of action is to increase acetylcholine in the synaptic cleft of cholinergic neurons. However the non-selective nature of this cholinergic stimulation leads to numerous undesired, mainly peripheral nervous system mediated, effects, (Colović *et al*, 2013). More specific AChR agonists and allosteric modulators are currently being developed and have shown promising results (Fisher, 2008a; Foster *et al*, 2014; Lombardo and Maskos, 2015; Toyohara and Hashimoto, 2010; Vallés *et al*, 2014).







The main loss in cognitive functions observed in healthy ageing generally involves memory, attention and perception (Glisky, 2007). Many authors have proposed that increasing age is related to a cholinergic deficiency related to increased age based on observations that older adults are more sensitive to anticholinergic drugs (such as scopolamine) when compared to younger controls (Ellis *et al*, 2009; Flicker *et al*, 1992; Molchan *et al*, 1992; Newhouse *et al*, 1994; Ratcliff *et al*, 2001; Ray *et al*, 1992; Zemishlany and Thorne, 1991). Developing a pharmacokinetic and pharmacodynamic (PK-PD) model (Chapter 2) helped not only to further quantify the effects of scopolamine on a battery of CNS tests in healthy subjects, but also to compare the effects within age groups even when a different dose was used. This comparison also took the exposure to scopolamine into consideration, which had not been done before. Our results suggest that a cholinergic neuronal dysfunction is not the cause of increased sensitivity of elderly to scopolamine, since most of the differences from young subjects disappeared when the effects where corrected for scopolamine plasma concentrations. The only test where an age-related difference was observed was in the peak velocity during the saccadic eye movement test where older healthy adults had a slower peak velocity when scopolamine was administered. The voluntary eye movements are the result of a meticulous coordination between several brain areas (i.e. brainstem, nucleus basalis and cortex). Such a complex system with multiple indispensable sub-components might be more susceptible to dysfunction when compared to younger subjects. On the other hand, the fact that only the peak velocity of the saccadic eye movements was affected after scopolamine administration by age provides evidence that the model is sensible enough to detect accurately age differences in performance. Age was not associated with worse performance on the cognitive tests, however it was evident that a greater number of older subjects scored worse compared to younger subjects. On average (comparing the population estimates) there were no significant differences between both groups (Figure 2.2). It would be interesting to try to find out if the increased sensitivity of elderly to other drugs with known anti-cholinergic side effects, such as e.g. tricyclic antidepressants, are also

to a larger extent caused by pharmacokinetic differences instead of reduced cholinergic neuronal reserve. Scopolamine is a muscarinic challenge test, whereas increasing age is known to be associated with diminishing central nAChRs (Tohgi *et al*, 1998). Determining age differences among subjects challenged with a nicotinic acetylcholine receptor antagonist (i.e. mecamylamine) will be a necessary next step to study if the nicotinic system also remains unchanged with age.

### CENTRAL NICOTINIC AND MUSCARINIC EFFECTS

A pharmacological challenge model should be able to provide evidence of the pharmacological mechanism of action of a drug (i.e. provide 'proof-of-pharmacology') and should also be able to show dose dependency. More importantly, the test should be safe and it should be possible to reverse the effects by an agonist acting on the same system (van Gerven, 2005). A pharmacological disease model of the cholinergic system measures the effects with a cognitive and neurophysiological test battery, which mimic the diagnostic symptoms of early Alzheimer's disease that are due to dysfunction of the cholinergic system. Central muscarinic effects have been extensively studied using scopolamine in healthy subjects as a model to induce temporary cognitive deficits related to non-selective muscarinic blockade (Liem-Moolenaar *et al*, 2011). The effects of central nicotinic blockade, however, were not yet as extensively studied, and e.g. effects over time and plasma concentration-effect relationship have not previously been described. In order to quantify the effects of central nicotinic blockade, mecamylamine in different doses was administered to healthy subjects and the effects were compared to those of scopolamine and placebo (Chapter 3). Mecamylamine, even at the highest dose given, produced more modest effects on most of the Central Nervous System (CNS) tests when compared to the scopolamine, but also had a distinct profile of CNS effects. Mecamylamine induced a decrease in performance in tests evaluating memory (VVLT and N-back tests), standing body balance (body sway) and fine motor coordination (adaptive tracker).

Nicotinic $\alpha_4\beta_2$, $\alpha_7$ and $\alpha_3\beta_2$ and muscarinic $M_1$ receptors are often co-localized in cortical and subcortical brain areas of the brain and may be





responsible for the overlapping effects on cognitive functions (Albuquerque *et al*, 2009; Flynn *et al*, 1997). Administration of scopolamine, but not mecamylamine, induced significant disturbances in tests evaluating the conjugated eye movements (peak velocity, smooth pursuit and inaccuracy). Muscarinic blockade with scopolamine had a relatively large influence on the eye movements, probably because of the sole presence of muscarinic ($M_2$ and $M_4$) receptors in the pons and midbrain which are important nuclei controlling the eye movements (Sparks, 2002). Scopolamine also induced a greater decrease in subjective alertness than mecamylamine, and interestingly an increase in the calmness feeling, contrary to mecamylamine that decreased it. Finally, both scopolamine and mecamylamine induced a similar deficit in tests evaluating motor fluency (tapping test). Even though it is difficult to differentiate sedative effects from effects on attention, the fact that subjects reported to be more drowsy and somnolent after scopolamine administration may be related to scopolamine inducing attention deficits through sedation. This could be explained by the presence of $M_2$ receptors in the brainstem with a strong influence on the pontine reticular formation (Coleman *et al*, 2004), while instead mecamylamine lacks nAChR in the brainstem and more likely acts on a cortical level to influence alertness (Gotti *et al*, 1997). Mecamylamine effects on blood pressure were a limiting factor to increase the dose and therefore were also quantified in our study. Mecamylamine effects on the blood pressure are well known since it has been used for more than half a century as an autonomic ganglion blocking antihypertensive (Ford *et al*, 1956). Based on the PK-PD model that was developed in Chapter 5, mecamylamine oral doses higher than 30 mg would have led to only a limited increase in CNS effects but would have caused a significant and likely clinically relevant decrease in blood pressure in healthy subjects. In this way, the development of a PK-PD-model contributed significantly to the validation and optimisation of mecamylamine as a nicotinic anticholinergic challenge test.

An important application of pharmacological challenge tests is the investigation of (potential) drugs with an opposite or modulating pharmacological effect. Reversal of scopolamine has previously been demonstrated with a number of muscarinic and nicotinic agonists (Baraka

and Harik, 1977; de Bruin and Pouzet, 2006; Dawson and Iversen, 1993; Preston *et al*, 1988; Snyder *et al*, 2005; Warburton, 2002; Wesnes and Warburton, 1984). It is remarkable however that the scopolamine challenge model has been more often used as the standard test to induce temporary cognitive deficits, even when most novel cholinergic cognitive enhancers are nicotinic compounds. It would be more reasonable to use mecamylamine as a challenge model when testing nicotinic compounds rather than scopolamine. However, there is only limited experience with mecamylamine in humans. In one previous study nicotine partially reversed mecamylamine-induced changes in the EEG (Pickworth *et al*, 1988). As a further step in the validation of mecamylamine as a challenge model, we considered it necessary to reverse the effects induced by mecamylamine using a wider range of tests that evaluate nicotinic functions and using different compounds with nicotinic activity. Since it is essentially unknown which CNS tests most accurately reflect nicotinic functions in the CNS, the NeuroCart was used to profile the effects of nicotinic agonists on the mecamyline challenge. The NeuroCart consist of a large number of standardized drug sensitive tests (de Haas *et al*, 2008, 2009, Liem-Moolenaar *et al*, 2010a, 2010b, 2010c; Van Der Post *et al*, 2005; van Steveninck *et al*, 1999; de Visser *et al*, 2001; Zuurman *et al*, 2010). In Chapter 4, nicotine was chosen as pure agonist to reverse mecamylamine effects. Nicotine administration partially reversed the effects of 30 mg of mecamylamine in tests evaluating motor coordination (adaptive tracker) and numerical working memory (N-back reaction time), but not in tests evaluating verbal working memory (VVLT) or motor fluency (tapping), nevertheless in the VVLT a clear trend was observed where the nicotine and mecamylamine treatment group performed superior when compared to the mecamylamine alone group (Figure 4.3). As expected, nicotine successfully reversed cognitive tests, however even though motor fluency was affected by mecamylamine, nicotine did not reverse these effects. Galantamine was also administered to counteract mecamylamine effects. Galantamine is a tertiary alkaloid with mainly cholinesterase inhibitor activity, nevertheless it also acts as an allosteric modulator of the nAChR and therefore was chosen rather than a more selective cholinesterase inhibitor such as donepezil (Harvey, 1995). Co-administration of galantamine only partially reversed mecamylamine effects on the reaction time of the most difficult working memory test, namely the 2-back test. Therapeutic effects of galantamine effects are observed after a longer period of administration when compared to other cholinesterase inhibitors and galantamine had lower concentrations in the brain of the experimental animals (Geerts *et al*, 2005). Therefore, the acute pharmacological effects of galantamine might not have been sufficient to reverse those of mecamylamine after a single administration.





### CHOLINERGIC EFFECTS ON THE ELECTROENCEPHALOGRAM

Although the experiments with nicotinic agonists and antagonists showed effects on several NeuroCart tests, none of these showed unequivocal relationships to the concentrations or pharmacological activities of the nicotinic compounds. The lack of a clear drug-related effect (or profile of effects) is an important shortcoming for a pharmacological challenge test. The electroencephalogram (EEG) has been widely used to study anticholinergic effects (Ebert and Kirch, 1998; Kikuchi *et al*, 1999; Pickworth *et al*, 1988, 1997; Sannita *et al*, 1987). Administration of both, scopolamine and mecamylamine shifted the eyes-closed resting state surface EEG to the lower frequencies, producing in general a decrease in the $\alpha$ frequency in the posterior brain regions. In our studies, scopolamine (but not mecamylamine) increased global $\vartheta$ frequency and mecamylamine (but not scopolamine) decreased the $\beta$ frequency in the posterior regions (Chapter 2, 3, 4 and 5). Both, the decrease of $\alpha$ and of $\beta$ activity after mecamylamine administration were reversed when nicotine was co-administered. Interestingly, patients with Alzheimer's disease also have a shift of EEG activity to the low frequency regions with both loss of alpha activity in the posterior regions and increase in theta activity with subtle decrease in the $\beta$ frequencies (Babiloni *et al*, 2004; Coben *et al*, 1983; Jeong, 2004). The combination of both scopolamine and nicotine effects on the EEG resembles changes found in patients with AD better than each alone. Also, based on the clinical findings in the CNS tests, it seems that both, nicotinic and muscarinic dysfunction are involved in the aetiology of AD, rather than an isolated dysfunction of one of the two central cholinergic system as other authors have suggested (Little *et al*, 1998; Sunderland *et al*, 1997).

More recently, newer analysis techniques have offered more reliable methods to detect subtle changes in the EEG in order to quantify cholinergic activity. Encoding and retention of information has been associated with temporal EEG correlations. Measuring the temporal EEG correlations may provide a diagnostic tool to help differentiate healthy subjects and subjects with Alzheimer disease (Montez *et al*, 2009) and even patients with Mild Cognitive Impairment (MCI) at risk to progressing to AD (Poil *et al*, 2013). Applying analyses of power, central frequency, bandwidth as biomarkers and correlating these using machine learning algorithms made it possible to develop an index with high sensitivity and specificity for scopolamine as indirect measure of muscarinic antagonism (Chapter 7). Many of the biomarkers used to conform the muscarinic index in the EEG were also found to be present in abnormal EEGs of patients with MCI of AD supporting the hypothesis that muscarinic dysfunction is part of the aetiology causing AD. A more accurate detection of the mAChR antagonism in the EEG may help detect subtler effects of new compounds with cholinergic activity and better understand the neurophysiological changes in health and disease. Next steps include the development of a nicotinic index using EEG data after mecamylamine administration.

### THE CHOLINERGIC SYSTEM AS A LINK BETWEEN THE BRAIN AND THE IMMUNE SYSTEM

It is well established that activation of the cholinergic receptors modulates the inflammatory response to different noxious stimuli (Borovikova *et al*, 2000; Lu *et al*, 2014; Wang *et al*, 2003). Chapter 6 was dedicated to experiments providing evidence that nicotinergic stimulation *in vitro*, inhibits the inflammatory response. Stimulation of white blood cells using LPS in combination with aluminium hydroxide, eATP or A$\beta$(1-42), led to an inflammatory response of which choline inhibited mainly IL-1$\beta$ and IL-6, with only negligible inhibition of TNF-$\alpha$. This corroborates that the canonical pathway of the inflammasome might be responsible for the inhibitory effect of choline. It's possible that the cholinergic neuronal dysfunction in AD is related to or exacerbates the inflammatory state in different areas in the brain





that is observed in AD (Boess *et al*, 2013; Egea *et al*, 2015; de Jonge and Ulloa, 2007; Thomsen and Mikkelsen, 2012), however further analysis should provide more evidence to support this hypothesis. It is, however, attractive to hypothesize that nicotinic agonists may not only improve cognition, but also positively modify neuro-inflammation and therefore disease progression in AD. Nicotinic modulation of inflammation may also offer possibilities for other inflammatory diseases (Parrish *et al*, 2006; Pavlov *et al*, 2007; Wang *et al*, 2004; Wu *et al*, 2014).

### CHALLENGE MODELS TO TEST NEW COMPOUNDS

Use of challenge models to test novel compounds can provide important information on the mechanism of action and possible interactions. The obtained information can be used for further indications and development strategies (Cohen, 2010; Heuberger *et al*, 2015; Kleinloog *et al*, 2015; Liem-Moolenaar *et al*, 2010a; Paul *et al*, 2010). Until now, different therapeutic strategies in AD including the use of cholinesterase inhibitors, active (vaccines) and passive (monoclonal antibodies) immunization, $\beta$- and $\gamma$-secretase inhibitors (including BACE-1 inhibitors) and $\alpha$-secretase potentiators have, until now, not proven to be efficacious as disease modifying treatments, nevertheless recently, compounds in early phase such as aducanumab seem promising disease modifying drugs (Sevigny *et al*, 2016). It is well possible that in the next coming 5-10 years a drug that slows disease progression of Alzheimer's Disease will be developed. However, this will only lead to more patients who will remain in a disease stage where symptomatic (cholinergic) treatment is needed. Compounds with cholinergic activity will therefore still represent an important therapeutic option. This is even more enhanced by the fact that the number of patients with AD will increase as life expectancy increases. Therefore, optimization of compounds with cholinergic effect is essential.

The methodological work proposed in this thesis may have applications beyond AD. It could be expected that cholinergic compounds change current treatment of neurodegenerative diseases like Parkinson's Disease and Lewy body disease and of Schizophrenia (Beinat *et al*, 2015; Fisher, 2008b; Foster

*et al*, 2014; Levey, 1996; Lombardo and Maskos, 2015; Perez-Lloret and Barrantes, 2016; Pérez and Quik, 2011; Toyohara and Hashimoto, 2010; Woodruff-Pak, 2002; Xiang *et al*, 2012). Exciting times are to come as the scientific community impatiently works further to elucidate the aetiology of these neurodegenerative diseases and to find effective therapeutic options for them.

## CONCLUSION AND FUTURE DIRECTIONS

The complexity of neuronal dysfunction that constitutes dementia can of course not be simulated using a pharmacological challenge model. However, the scopolamine and mecamylamine models do provide valuable tools to study drugs that enhance the cholinergic system, which are being developed for the symptomatic treatment of dementia. This thesis expands the body of knowledge on cholinergic challenge tests to provide insight into how a pharmacokinetic and pharmacodynamic model might be used to simulate and predict the effects of a pharmacological challenge. Cholinergic challenge tests can be used as models to provide proof-of-pharmacology for compounds enhancing the cholinergic system and as a tool to develop new compounds with cholinergic activity. For the non-selective muscarinic scopolamine challenge, sensitive biomarkers with accurate PK-PD models have already been identified in previous studies. However, most currently developed cholinergic drugs are targeted at muscarinic or nicotinic receptor subtypes. The effects of manipulations of these cholinergic subsystems proved to be difficult to measure, because the changes were subtle and dose-effect relationships were less clear.

In this thesis, the NeuroCart was used to measure functional effects related to muscarinic or nicotinic receptor subtypes. This CNS test battery is composed of tests that are sensitive to a wide range of pharmacological agents, which were selected based systematic reviews of the literature on drug effects in healthy subjects (Dumont *et al*, 2005; Dumont and Verkes, 2006; Rijnbeek *et al*, 2003; de Visser *et al*, 2001, 2003; Zoethout *et al*, 2011; Zuurman *et al*, 2009). In general, the NeuroCart has proven to be very sensitive to CNS active drugs, including scopolamine. The relatively modest





responses to the more selective cholinergic agonists in this thesis therefore came as some surprise. The greatest measurable effects were evident with the EEG. However, this became much more apparent when a muscarinic cholinergic index was developed, which combined different characteristics derived from the EEG that were related to subtle changes in the cholinergic system. Although this shows that innovative ways to analyse or combine measurements can lead to new informative functional biomarkers, there is certainly a need for more specific tests of cholinergic systems. The search for specific biomarkers may also contribute to a better understanding of the functional roles of cholinergic (sub)systems in health and disease. Pharmacological challenge tests based on subtype selective agonists and antagonists will provide essential tools to validate such new biomarkers. In this sense, PK-PD models are also important validation instruments. A clear concentration-effect relationship provides strong evidence that an effect of a challenge test is directly related to the pharmacology of the challenge agent. Moreover, the models can be used to simulate theoretical scenarios in order to optimize the outcome of future clinical studies. Further validation of the cholinergic pharmacological challenges with the use of cognitive enhancers to reverse the effects of mecamylamine and scopolamine should provide more information on the human cholinergic system and possibilities as new therapeutic options for diverse neurodegenerative diseases.

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# ENGLISH SUMMARY

## CHALLENGING THE CHOLINERGIC SYSTEM: AGEING, COGNITION & INFLAMMATION

The neurotransmitter acetylcholine (ACh) is produced and released by neurons in order to signal to other cells. Neurotransmitters bind to specific receptor proteins located at the plasma membrane of target cells (e.g., other neurons, muscle cells, etc.), allowing rapid cell-cell communication. The release of ACh increases its concentration in the synaptic cleft (the space between the axon of one neuron and the dendrite of the other neuron); however, ACh levels decrease rapidly via the activity of extracellular acetylcholinesterase, an enzyme that breaks down ACh in the synaptic cleft. Thus, ACh is available only at specific sites and only for a brief period of time. ACh plays important roles in several cognitive functions, including the learning and processing of new information, the recall of learned information (i.e., memory), attention, and the capacity to concentrate.

Dementia is a relatively broad term used to define nearly 50 distinct brain disorders that cause a gradual decrease in cognitive function. Among all types of dementia, Alzheimer's is arguably the most commonly known form of dementia. The cause – or etiology – of dementia is not completely understood. To date, drugs that inhibit acetylcholinesterase activity (thereby indirectly increasing synaptic ACh concentrations) are among the few approved treatments for Alzheimer's disease. Acetylcholinesterase inhibitors improve cognitive function in patients with Alzheimer's in the initial stages of the disease.

In both animal models and human subjects, symptoms that resemble dementia can be induced temporarily by administering drugs that block ACh binding to ACh receptors (AChRs). For example, scopolamine binds to muscarinic AChRs and therefore can induce some dementia-like symptoms. Scopolamine has been widely used to study the effects of new compounds that potentially act on the cholinergic system. To test the possible effect of new compounds that may affect the cholinergic system in humans, the test compound is co-administered with scopolamine, and cognitive function





is measured and compared with subjects who received scopolamine alone (without the test compound). Therefore, drugs that might improve cholinergic activity might also be used to treat patients with Alzheimer's disease. This so-called 'scopolamine challenge model' is often used because *i*) the effects of scopolamine are robust enough to be measured, *ii*) it is a widely published model, and *iii*) scopolamine is widely available at every pharmacy due to its clinical use in treating patients with motion sickness.

Cholinergic neurons are distributed widely throughout the central and peripheral nervous systems. In the peripheral nervous system, ACh triggers the contraction of skeletal muscles, serves as a signal for secretory glands responsible for transpiration, stimulates gastrointestinal (peristaltic) movement, and produces changes in heart rate and cardiac tone; these are just a few examples of the many functions of the cholinergic system. In healthy subjects, boosting cholinergic activity with cholinesterase inhibitors induces nausea and vomiting, excessive saliva production, headache, muscle and joint pain, tremors, and nervousness.

In addition to muscarinic AChRs, ACh can also bind and activate nicotinic AChRs. Nicotinic AChRs are not just located in brain areas related to cognitive function; indeed, they are distributed nearly as widely as muscarinic AChRs. Nicotine, a substance present in cigarettes, binds primarily to nicotinic AChRs (hence, the name). Recently, research has focused on drugs that specifically activate nicotinic AChRs. Given their high selectivity for nicotinic AChRs, these compounds might cause fewer adverse events compared to acetylcholinesterase inhibitors, which increase ACh levels non-selectively. Nicotinic drugs are being developed to treat symptoms related to dementia; in addition, these drugs are also being developed to treat cognitive symptoms secondary to psychiatric diseases such as schizophrenia. Patients with schizophrenia can develop so-called 'positive' symptoms (e.g., hallucinations), as well as 'negative' symptoms (e.g., a decline in cognitive function). Based in part on the observation that most schizophrenia patients smoke and those who smoke tend to experience an improvement in symptoms, it has been suggested that the

negative symptoms of schizophrenia are caused by impaired nicotinergic activity. Patients with Parkinson's disease might also benefit, as their motor and cognitive symptoms can improve following nicotinic stimulation. The question therefore arises, *Why not treat all of these patients with a nicotine patch or nicotine-based chewing gum?* The clear answer is that nicotine can cause various adverse events and – more importantly – can be highly addictive. The challenge is therefore to develop a more selective compound that can bind a specific subtype of nicotinic AChRs located in a specific brain area in order to improve cognitive function, but without causing addiction (which is mediated by a different brain area).

Recently, the first nicotinic cholinergic drugs developed to treat the cognitive symptoms of dementia and schizophrenia were administered to healthy subjects. In the early stages of drug development, these compounds are administered to healthy subjects during the scopolamine challenge model in order to measure whether the compound has the desired effects; after confirming the desired effects in healthy subjects, the compounds are then administered to patients. These so-called first-in-human studies provide a wealth of valuable information, including information used to determine the optimal dose for future studies with patients. The Centre for Human Drug Research (CHDR) in Leiden, where this thesis project was conducted, specializes in early-phase drug development research.
Similar to scopolamine, mecamylamine also binds AChRs; however, unlike scopolamine, mecamylamine binds selectively to nicotinic AChRs. Drugs that block nicotinic AChRs also produce a temporary decrease in cognitive performance in both animal models and human subjects; however, the symptoms induced by mecamylamine differ from those induced by scopolamine. Pharmacologically speaking, mecamylamine (an anti-nicotinic drug) would be more suitable than scopolamine (an anti-muscarinic drug) in a challenge model for testing nicotinic compounds. CHDR has performed several trials in order to *i*) determine the effects of scopolamine and mecamylamine in humans, *ii*) determine the use of mecamylamine as a suitable challenge model, and *iii*) obtain evidence that nicotinic drugs may decrease – or even reverse – the effects of mecamylamine.





The work described in this thesis is based on trials designed to further investigate the cholinergic system in humans by administering various drugs with cholinergic effects. The effects of these cholinergic drugs with respect to cognitive function were measured using various executive tests and by measuring various physiological parameters before, during, and after the administration of the cholinergic drug, thereby making it possible to objectively correlate drug concentration with the measured effects. The thesis contains eight chapters. In **Chapter 1**, we provide a general introduction and the theoretical frame of the research. In **Chapter 2**, we compare the effects of scopolamine between elderly subjects and young subjects. The prevailing hypothesis was that a functional defect in the cholinergic system underlies the higher sensitivity to scopolamine among elderly subjects. However, we found that scopolamine is eliminated (i.e., cleared) more slowly from elderly adults compared to young adults. This slower clearance of scopolamine leads to higher concentrations of scopolamine in the blood, for a longer period of time. Prior to receiving scopolamine, the elderly subjects had also slower brain wave activity (measured using an electroencephalogram) and had slower reaction times in cognitive testing compared to young subjects. On the other hand, although they were slower to answer, the elderly subjects had the same number of correct answers as the younger subjects. These findings suggest that future experiments should be performed using mecamylamine in order to analyze whether the number and/or functional availability of nicotinic AChRs changes with aging.

In **Chapter 3**, we compare the effects of scopolamine (an anti-muscarinic drug) with the effects of mecamylamine (an anti-nicotinic drug). Although the effects of scopolamine were more pronounced than those of mecamylamine, scopolamine induced relatively fewer severe adverse events, whereas mecamylamine produced a decrease in systemic blood pressure (hypotension), which limited the maximum dose of mecamylamine that could be tested. Scopolamine caused lower performance in nearly all cognitive tests; however, it also induced somnolence (drowsiness); therefore, it is difficult to determine whether the decrease in cognitive function was an indirect (i.e., secondary) effect of the somnolence or was

due to a direct decrease in concentration and/or memory. Both scopolamine and mecamylamine impaired the subjects' performance in terms of problem-solving capacity, memory, coordination, and reaction time. On the other hand, scopolamine – but not mecamylamine – caused diminished eye movements. Most of mecamylamine's effects were reversed by nicotine (discussed in **Chapter 4**). This is the first demonstration that the effects of mecamylamine can be reversed in humans, showing the robustness of using the mecamylamine challenge model to test nicotinergic compounds. The fact that mecamylamine's effects can be reversed provides indirect evidence that nicotine stimulates the nicotinic system, as mecamylamine blocks nicotinic AChRs. In future trials, nicotine can be replaced by other nicotinic compounds in order to test the effect of these compounds in humans.
To further capitalize on the value of using mecamylamine as a challenge model, the effects in healthy subjects can be quantified and related to blood concentrations using mathematical models (for example, see **Chapter 2**). These mathematical models can serve as a tool for predicting the effects of a given mecamylamine concentration and can provide important information regarding the effects of mecamylamine at low concentrations. The model can also be used to predict the relationship between mecamylamine concentration and the resulting decrease in blood pressure. The complete model and development process are provided in **Chapter 5**.

In recent years, researchers discovered that ACh can reduce inflammation; however the precise mechanism – and the extent of this effect – has remained poorly understood. In Alzheimer's disease, inflammation in the brain affects cholinergic neurons. One might therefore speculate that cholinergic stimulation with an acetylcholinesterase inhibitor (which increases synaptic ACh levels) might have a favorable effect in terms of slowing disease progression. In **Chapter 6**, we examined the effects of cholinergic stimulation using choline (which activates nicotinic AChRs) on inflammation. We drew blood samples from healthy subjects and stimulated these samples *in vitro* in order to induce inflammation. The inflammatory response was then analyzed in the presence and absence of choline by measuring inflammatory proteins (interleukins) in the plasma. We found





that choline decreased the response of a recently discovered multiprotein complex called inflammasome; interestingly, this complex is functionally impaired in patients with Alzheimer's disease. These findings suggest that selectively stimulating the nicotinergic system using novel compounds might help reduce brain inflammation in patients with Alzheimer's disease. Whether this reduction will be clinically significant, and whether disease progression can be slowed, requires further study.

In **Chapter 7**, we used electroencephalography (an objective measure of the brain's electrical activity) to analyze the effects of either activating or blocking cholinergic signaling. Although the effect of cholinergic drugs on electrical activity in the brain has been widely studied, we developed a new method for measuring cholinergic activity using computer-learning techniques, thereby allowing us to determine the most sensitive parameters in the electroencephalogram. As a result, we developed an extremely sensitive index comprised of multiple parameters derived from the electroencephalogram, and we can now use this index to measure the effect of muscarinic cholinergic drugs in healthy subjects or in patients with dementia.

Finally, **Chapter 8** provides a general discussion of these results, putting the findings in a broader scientific and clinical context.

In summary, this thesis describes several state-of-the-art methods used to study the cholinergic system by measuring the effects of compounds that selectively act upon muscarinic or nicotinic acetylcholine receptors. In addition, using highly sensitive tests to quantify anti-cholinergic effects, our results also revealed the complexity of the cholinergic system, its changes related to aging, its relationship with cognitive function and performance, and its role in inflammation.

# NEDERLANDSE SAMENVATTING

## CHALLENGING THE CHOLINERGIC SYSTEM: AGEING, COGNITION & INFLAMMATION

Acetylcholine is een belangrijke lichaamseigen stof die betrokken is bij de impulsoverdracht van zenuwcellen, een zogenaamde neurotransmitter. Neurotransmitters binden aan receptoren (eiwitten die neurotransmitters herkennen) aan de oppervlakte van zenuwcellen en hierdoor kunnen deze onderling communiceren. Als zenuwcellen communiceren door middel van acetylcholine dan komt er acetylcholine vrij in de ruimte tussen de zenuwcellen, de synapsspleet. Het enzym (actief eiwit) dat de acetylcholine uit de synapsspleet verwijdert heet acetylcholinesterase. Acetylcholine speelt een belangrijke rol bij het uitvoeren van veel cognitieve functies: het vermogen om kennis op te nemen en te verwerken, het geheugen, de aandacht en de concentratie.

Dementie is een verzamelnaam voor ruim vijftig ziektes die alle leiden tot teloorgang van cognitieve functies; de precieze oorzaak is meestal onbekend. De ziekte van Alzheimer is de bekendste vorm van dementie. Geneesmiddelen die het enzym acetylcholinesterase remmen behoren tot de weinige goedgekeurde en effectieve behandeling voor patiënten met de ziekte van Alzheimer. Deze middelen remmen het acetylcholinesterase en zorgen dus dat er meer acetylcholine beschikbaar is in de synapsspleet. Dit zorgt voor een vermindering van de cognitieve klachten van patiënten met de ziekte van Alzheimer in de beginfase van de ziekte.

In experimentele modellen kunnen symptomen van dementie tijdelijk geïnduceerd worden in dieren en gezonde proefpersonen door toediening van medicijnen die sommige acetylcholine receptoren blokkeren. Scopolamine is een dergelijk middel en kan enkele van de symptomen van dementie tijdelijk nabootsen. Scopolamine toediening aan gezonde proefpersonen wordt veel gebruikt in geneesmiddelenontwikkeling om effecten van geneesmiddelen die het functioneren van de zenuwcellen die voor hun communicatie gebruik maken van acetylcholine, het zogenaamde cholinerge systeem, te testen. Nieuwe (genees)middelen in ontwikkeling die de werking van het





cholinerge systeem positief kunnen beïnvloeden worden tegelijkertijd met scopolamine toegediend om te testen of de nadelige effecten van scopolamine op cognitieve functies zouden kunnen worden tegengehouden of omgekeerd. Als dat namelijk zo is, dan zou een dergelijk geneesmiddel misschien ook de cognitieve functies van patiënten met de ziekte van Alzheimer kunnen verbeteren. Dit 'scopolamine model' wordt vaak gebruikt omdat de effecten sterk zijn en dus goed meetbaar, omdat er over het scopolamine model veel literatuur is en omdat het middel gemakkelijk te verkrijgen is aangezien het ook wordt voorgeschreven als behandeling van misselijkheid ten gevolge van wagenziekte en dus verkrijgbaar is bij elke apotheek. Scopolamine blokkeert alleen een deel van het cholinerge systeem, namelijk het deel dat communiceert door middel van muscarine-type acetylcholine receptoren.
Cholinerge zenuwcellen bevinden zich niet alleen in de hersenen, maar overal in het lichaam. Acetylcholine wordt gebruikt door de zenuwcellen om de spieren te laten contraheren, om de zweetklieren te laten werken, en activatie van cholinerge zenuwcellen leidt ook tot maagontlediging en stimulatie van de peristaltiek van de darmen, en doet het hart langzamer kloppen, om slechts enkele voorbeelden te noemen. Gezonde mensen die cholinesteraseremmers toegediend krijgen, kunnen hierdoor onder andere, misselijk worden en zelfs braken, produceren meer speeksel, kunnen hoofdpijn krijgen, pijn in gewrichten en spieren, tintelingen, en kunnen zich nerveus voelen en trillen ontwikkelen.

Acetylcholine kan niet alleen aan muscarine-type, maar ook aan nicotine-type acetylcholine receptoren binden. Nicotinerge receptoren bevinden zich in andere delen van de hersenen en zijn ook betrokken bij cognitieve functies. Het genotsmiddel nicotine stimuleert ook nicotinerge acetylcholine receptoren, maar niet de muscarine-type receptoren, wat de reden is dat deze receptoren voor acetylcholine 'nicotinerg' worden genoemd. In de laatste jaren is er ook veel interesse in middelen die cognitieve functies zouden kunnen doen verbeteren door een selectieve binding aan het deel van het cholinerge systeem dat gebruik maakt van nicotinerge receptoren. Die middelen zouden namelijk niet leiden tot bijwerkingen elders in het lichaam zoals de cholinesterase remmers wel doen. Nicotinerge middelen worden

niet alleen ontwikkeld voor de behandeling van cognitieve symptomen bij dementie, maar ook voor cognitieve symptomen ten gevolge van psychiatrische aandoeningen zoals schizofrenie. Bij schizofrenie treden naast hallucinaties en wanen, (positieve symptomen) ook een achteruitgang van cognitieve functies op (negatieve symptomen). Gedacht wordt dat deze negatieve symptomen van schizofrenie te maken hebben met het niet goed functioneren van het nicotinerge deel van het cholinerge systeem. Dit zou de reden kunnen zijn waarom zo'n hoog percentage van de patiënten gediagnosticeerd met schizofrenie rookt en waarom sommige schizofrenie patiënten zo veel roken. Naast Alzheimer dementie en schizofrenie, zou ook bij de dementie die optreedt bij sommige patiënten met de ziekte van Parkinson het niet goed functioneren van het nicotinerge deel van het cholinerge systeem een rol kunnen spelen. Toediening van middelen die het nicotinerge cholinerge systeem stimuleren laat verbetering zien van de motorische en cognitieve symptomen in experimentele modellen van de ziekte van Parkinson. Ongetwijfeld vraagt u zich af: waarom geven we niet aan al die patiënten een nicotine pleister of nicotine kauwgum? Het antwoord is simpel, namelijk vanwege de bijwerkingen, maar ook en met name vanwege de sterk verslavende effecten van nicotine en de gezondheidsrisico's.

De huidige uitdaging voor de farmaceutische industrie is het om selectievere en specifiekere middelen te ontwikkelen die alleen aan bepaalde type nicotinerge receptoren werken, bijvoorbeeld alleen in bepaalde delen van de hersenen, maar niet aan andere, bijvoorbeeld degene die op spieren zitten, en met name niet aan de receptoren die te maken hebben met het verslavende effect van nicotine.

De eerste nicotine-type cholinerge geneesmiddelen die ontwikkeld worden voor de behandeling van cognitieve symptomen van dementie en schizofrenie zijn in de afgelopen jaren voor het eerst aan mensen gegeven. In de vroege fasen van het geneemsiddelonderzoek, nog voordat een nieuw middel aan patiënten gegeven wordt, wordt vaak eerst onderzocht of een geneesmiddel doet wat het hoort te doen in gezonde proefpersonen. Dat is het type onderzoek waarin het Centre for Human Drug Research (CHDR) in Leiden gespecialiseerd is. Wanneer de effecten van geneesmiddelen die mogelijk de cognitieve functies doen verbeteren worden onderzocht in gezonde proefpersonen wordt vaak het scopolamine model gebruikt om vast te stellen dat het middel inderdaad werkt en belangrijker, welke dosis de juiste is, voordat een grote studie in patiënten wordt uitgevoerd. Voor het aantonen van de effecten van nicotinerge middelen wordt ook het scopolamine model gebruikt, maar wij vonden dit niet logisch. Scopolamine blokkeert per slot van rekening de muscarine-type receptoren terwijl nicotine-type cholinerge geneesmiddelen de nicotine-type receptoren stimuleren.

Mecamylamine heeft een gelijksoortig werkingsmechanisme als scopolamine, echter het middel bindt aan nicotinerge receptoren (in plaats van aan muscarinerge receptoren zoals scopolamine). Nicotinerge blokkade leidt in gezonde vrijwilligers en dieren ook tot een tijdelijke verslechtering van de cognitieve functies, alleen is het profiel van cognitieve verstoring anders dan de cognitieve vestoring die ten gevolge van scopolamine optreedt. Vanuit farmacologisch oogpunt zou het gebruik van mecamylamine als model voor tijdelijke cognitieve symptomen om de effecten van geneesmiddelen die ontwikkeld worden voor de behandeling van dementie aan te tonen logischer zijn dan het gebruik van scopolamine. In de afgelopen jaren verrichtte het CHDR onderzoek naar het profiel van cognitieve symptomen ten gevolge van scopolamine en mecamylamine, naar het gebruik van mecamylamine als model voor tijdelijke cognitieve klachten en naar het proberen aan te tonen dat er ook geneesmiddelen zijn die de tijdelijke cognitieve symptomen ten gevolge van mecamylamine toediening positief kunnen beïnvloeden.

Het werk in dit proefschrift was erop gericht om het cholinerge systeem verder te onderzoeken door het aanwenden van middelen die invloed hebben op het cholinerge systeem. De effecten die de middelen hebben op cognitieve functies kunnen gemeten worden door cognitieve testen uit te voeren en hersenfuncties te meten voor, tijdens en na de geneesmiddel toediening. Hierdoor kunnen de effecten van verschillende concentraties van het middel op het functioneren van de hersenen geobjectiveerd worden. Dit proefschrift is verdeeld in 8 hoofdstukken: In het eerste hoofdstuk vindt u

een algemene introductie en theoretische achtergrond van de onderzoeken. Wij vergelijken daarna in hoofdstuk 2 de gevoeligheid van gezonde ouderen blootgesteld aan scopolamine met die van gezonde (jonge) volwassenen. Met de leeftijd treedt er een achteruitgang op van het functioneren van het cholinerge systeem in de hersenen en algemeen werd aangenomen dat de reden dat ouderen een verhoogde gevoeligheid lijken te hebben voor scopolamine gelegen is in dit minder goede functioneren van het cholinerge systeem. Uit onze analyses moest blijken of gezonde ouderen inderdaad gevoeliger zijn voor scopolamine dan jongeren. Interessant genoeg vonden wij een verschil in de snelheid waarmee het middel verwijderd wordt uit het lichaam van ouderen (klaring van het middel) ten opzichte van jongeren, waardoor de ouderen na toediening van een lagere dosis eenzelfde hoogte van bloedconcentraties hebben als jongeren die een hogere dosis kregen. Voor de huidige hypothese dat ouderen gevoeliger zouden zijn voor scopolamine, hebben wij geen aanwijzingen kunnen vinden. In andere woorden, wij hebben er aanwijzingen voor dat een verhoogde gevoeligheid voor scopolamine bij ouderen een consequentie is van hogere concentraties van het middel in bloed en niet van minder muscarine-type receptoren in de gebieden van de hersenen betrokken bij cognitieve functies. We hebben wel significante verschillen gevonden tussen beide groepen voordat scopolamine werd toegediend, dus onder normale omstandigheden. Een tragere elektrische activiteit gemeten met behulp van een elektro-encefalogram (die meet elektrische activiteit van de hersenen) was gerelateerd aan de leeftijd; dit was overigens al bekend. Ook neemt de reactietijd op cognitieve tests af met het toenemen van de leeftijd, al was er geen verschil in het aantal correcte antwoorden tussen ouderen en jongeren. In de toekomst zouden wij eenzelfde onderzoek willen doen naar de verschillen in gevoeligheid voor het anti-nicotinerge mecamylamine tussen jongere en oudere volwassenen. In het derde hoofdstuk werden de effecten van mecamylamine (als anti-nicotinerg middel) vergeleken met die van scopolamine (anti-muscarinerg middel). Scopolamine liet sterkere effecten zien op het congitieve functioneren dan mecamylamine. Het is echter belangrijk om hierbij te vermelden dat een vrij hoge dosis scopolamine toegediend kan worden met relatief weinig ernstige bijwerkingen, terwijl na toediening





van mecamylamine een ernstige daling van de bloeddruk kan optreden bij doses boven 30 mg. Dit beperkte de hoogte van de dosis mecamylamine die toegediend kon worden. Scopolamine veroorzaakte een sterkere mate van verminderd cognitief functioneren, maar ook duidelijk meer slaperigheid. Het is dus moeilijk te zeggen of de gemeten cognitieve effecten gevolg zijn van slaperigheid of van een 'pure' verminderde concentratie of geheugen. Beide middelen lieten een achteruitgang zien in testen die geheugen, het oplossen van problemen, reactietijd en motorische coördinatie meten. Scopolamine, maar niet mecamylamine, had invloed op de oogbewegingen. De meeste mecamylamine effecten konden, door midden van gelijktijdige toediening van nicotine, omgekeerd of tegen gegaan worden (zie hoofdstuk vier). Dit was de eerste keer dat het aangetoond is in mensen dat nicotine de effecten van mecamylamine kan omkeren. De validiteit van het gebruik van het 'mecamylamine model' om de werking van geneesmiddelen die het nicotinerge systeem positief beinvloeden kon hiermee dus aangetoond worden. Het omkeren van de effecten van mecamylamine bevestigt indirect dat mecamylamine de nicotinerge acetylcholine receptoren blokkeert en dat nicotine het systeem stimuleert. In de toekomst kan nicotine vervangen worden door nieuwe nicotinerge geneesmiddelen om de effectiviteit ervan te testen in mensen.

Om het hierboven genoemde 'mecamylamine challenge model' te kunnen gebruiken in de praktijk, moesten de effecten in gezonde proefpersonen eerst gekwantificeerd worden. Het vijfde hoofdstuk van het proefschrift probeert de gekwantificeerde effecten te relateren aan de mecamylamine concentraties die gemeten werden in het bloed. Door middel van wiskundige modellen kunnen de bloedconcentraties gerelateerd worden aan de geneesmiddeleffecten (net zoals in hoofdstuk 2). Met behulp van die wiskundige modellen kan ook berekend worden wat de effecten zullen zijn bij bloedconcentraties die niet daadwerkelijk gemeten zijn. Kennis over welke effecten verwacht kunnen worden bij bepaalde concentraties kan helpen bij het opzetten van nieuwe studies en bij de interpretatie van de effecten van nieuwe nicotinerge middelen die we van plan zijn te testen gebruikmakend van dit model. Ook geven deze modellen belangrijke informatie over de

gevoeligheid van de testen voor mecamylamine, zelfs bij lage doseringen. Nog belangrijker is dat we nu weten welke bloeddrukdaling we zouden kunnen verwachten wanneer we de dosering zouden willen verhogen.

De remmende invloed die acetylcholine zou kunnen hebben op ontsteking is de laatste jaren een 'hot topic' in de wetenschappelijke literatuur. Hoe het cholinerge systeem inflammatie zou kunnen remmen en de mate waarin, is echter nog grotendeels onbekend. Het is wel bekend dat cholinerge zenuwcellen als eerste getroffen worden bij de ziekte van Alzheimer en dat ontstekingsprocessen in de hersenen daarbij een rol spelen. Hiermee rijst ook de vraag of de huidige behandeling van symptomen van de ziekte van Alzheimer die het cholinerge systeem stimuleren ook een ontstekingsremmend effect hebben. In hoofdstuk zes hebben we onderzocht wat voor effecten choline, een andere stof die nicotine-type acetylcholine receptoren bindt en stimuleert, heeft op ontsteking (inflammatie). Eerst namen wij bloed af van gezonde proefpersonen en stimuleerden we het bloed ex vivo, dat wil zeggen buiten het lichaam, met verschillende middelen die ontsteking veroorzaken, in de aanwezigheid en afwezigheid van choline. Daarna werden de verschillende eiwitten gemeten (interleukinen) die afweercellen in de context van de inflammatoire reactie produceren. Choline remde enkele interleukinen die deel uitmaken van het inflammasoom, een recent ontdekt complex van eiwitten gerelateerd aan ontsteking dat ook in de hersenen van patiënten met de ziekte van Alzheimer wordt aangetroffen. Dit zou kunnen betekenen dat de nieuwe geneesmiddelen die nog specifieker en krachtiger het cholinerge systeem stimuleren, misschien ook in lichte mate de ontstekingsachtige processen in de hersenen kunnen verminderen en daarmee de progressie van de ziekte van Alzheimer enigszins kan vertragen. Het zevende hoofdstuk is gerelateerd aan een objectieve manier om effecten van cholinerge geneesmiddelen te meten door middel van het elektro-encefalogram (EEG). De effecten van cholinerge en anti-cholinerge middelen op de elektrische activiteit in de hersenen zijn al uitgebreid beschreven, maar wij deden hier onderzoek naar een nieuwe methode die gebruik maakt van computer-learning technieken om de gevoeligheid van het EEG voor het vaststellen van de invloed van nieuwe geneesmiddelen die ontwikkeld





worden voor de ziekte van Alzheimer door middel van beïnvloeding van het cholinerge systeem te doen toenemen. De resulterende EEG index is een gevoeliger maat voor geneesmiddelen met cholinerge activiteit die muscarine-type acetylcholine receptoren beïnvloeden dan de huidige bestaande EEG methode.

Samenvattend beschrijft dit proefschrift hoe met behulp van anti-cholinerge middelen de effecten van nieuwe geneesmiddelen die het cholinerge systeem stimuleren gemeten kunnen worden en hoe wij die tests verder hebben ontwikkeld. Ook beschreven wij hoe de effecten van geneesmiddelen met cholinerge en anti-cholinerge effecten beter gekwantificeerd kunnen worden. Het proefschrift geeft ook weer wat de complexiteit is van het cholinerge systeem en de invloed van veroudering erop, alsmede de samenhang met cognitieve functies en de invloed op ontsteking.

## CURRICULUM VITAE

Ricardo Alvarez-Jimenez was born on February 29, 1984 in Mexico City. During the last years of secondary education he discovered his fascination for medicine and later on for pharmacology while studying to become a paramedic. He worked in the pre-hospital service of the chaotic Mexico City for two years as a volunteer and combined it with his medical studies at the school of medicine of the Autonomous National University of Mexico (Universidad Nacional Autónoma de México) where his passion for pharmacology continued to hatch. He obtained his medical degree in 2010 and worked for one year as researcher. In 2011 he moved to the Netherlands to study the master in bio-pharmaceutical sciences at the University of Leiden. During the master he performed his internship at the Centre for Human Drug Research where he learned to apply non-linear mixed effects models to pharmacology. After graduating, prof. Cohen invited him to join the Centre for Human Drug Research's staff where in 2012 he began as PhD student and project leader where he performed most of the work described in this thesis. In the meanwhile he certified as a physician in the Netherlands and Spain, obtained his registry as clinical pharmacologist and worked as physician at the intensive care of the Leiden University Medical Center. He recently started the residency to become an anesthesiologist and presently works at the VU Medical Center. He currently lives in Amsterdam with his partner Pieter Vebruggen.

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## NOTES





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