Challenging the cholinergic system : ageing, cognition & inflammation

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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 $\alpha_4\beta_2$, α_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 α frequency in the posterior brain regions. In our studies, scopolamine (but not mecamylamine) increased global ϑ frequency and mecamylamine (but not scopolamine) decreased the β frequency in the posterior regions (Chapter 2, 3, 4 and 5). Both, the decrease of α and of β 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 β 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 Aβ(1-42), led to an inflammatory response of which choline inhibited mainly IL-1β and IL-6, with only negligible inhibition of TNF-α. 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, β- and γ-secretase inhibitors (including BACE-1 inhibitors) and α-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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