AGE DEPENDENT PUPILLARY AND COGNITIVE RESPONSES TO
CHOLINERGIC DRUGS AS A MODEL FOR IDENTIFICATION OF FUTURE
BIOLOGICAL MARKERS FOR ALZHEIMER'S DISEASE

by

Jeanne Fourie

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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Master of Science Degree 1999
Jeanne Fourie
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ABSTRACT

Background. An increased pharmacological response to cholinergic drugs has been proposed as a predictor of deficits in cholinergic neurotransmission in Alzheimer's disease. This thesis assessed the effect of normal aging on a) pupillary response to tropicamide and pilocarpine b) scopolamine effect on memory in healthy elderly and young volunteers.

Methods. Elderly (n=10, age: 70 ± 2) (mean ± SD) and young (n=9, age: 33 ± 2) volunteers were tested over four sessions following a randomized double-blind design. In one session, tropicamide (20μl, 0.01%) was administered to one eye, and placebo to the other. In another session, tropicamide (20μl, 0.01%) was administered to both eyes followed at 25 minutes post-tropicamide by application of pilocarpine (20μl, 0.1%) to one eye and placebo to the other. In both sessions, pupil diameter was assessed using a computerized infrared pupillometer over a period of 4 hours. On two separate sessions, a single dose of scopolamine (0.5 mg, iv) or placebo was administered and effects on word-recall were measured for 2 hours.

Results. Aging did not significantly affect pupillary response to tropicamide (p >0.05). The placebo-corrected pilocarpine effect on pupil size was greater in the elderly at 85, 125, 165 and 215 minute time points compared to the young (p <0.05). For example, at 215 minutes post-pilocarpine administration, the decrease in pupil diameter was 23.9% ± 2.4% (mean ± SEM; % of placebo effect) in the elderly and 8.8% ± 4.9% in the young (p <0.05). Scopolamine effect on word-recall was greater in the elderly at 60 (41.7% ± 10.8, % of baseline), 90 (43.1% ± 12.5) and 120 (47.1% ± 12.7) minute time points compared to young subjects (58.7% ± 9.0, 60.6% ± 7.8, 103.0% ± 7.6, respectively) (p <0.05).

Conclusions. Scopolamine effect on cognitive function is increased during normal aging, in agreement with previous studies showing an age-related decline in central cholinergic function. There is an age-related increase in pupil response to pilocarpine whereas the effect of tropicamide on pupil size is not changed with aging. The findings with pilocarpine warrant further experiments to evaluate the pupillary response to pilocarpine in Alzheimer's disease.
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<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>ACh</td>
<td>Acetylcholine</td>
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<tr>
<td>AChE</td>
<td>AcetylCholine esterase</td>
</tr>
<tr>
<td>ChAT</td>
<td>Choline acetyltransferase</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>nAChRs</td>
<td>Nicotinic acetylcholine receptors</td>
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<td>MAChR⁺</td>
<td>Muscarinic acetylcholine receptors</td>
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Chapter I

The Normal Physiology of the Acetylcholinergic System
INTRODUCTION

Acetylcholinergic synapses control several physiologically important functions such as cardiovascular function and neuromuscular control as well as higher complex functions such as memory, learning and cognition (Felder 1995). The central cholinergic system is mainly made up of three cholinergic nuclei located in the basal forebrain. These are:

- the medial septum,
- the diagonal band of Broca,
- the nucleus basalis magnocellularis (nucleus basalis of Meynert).

The medial septum and the diagonal band of Broca both project through the fimbria (which contains the septo-hippocampal tract) to the hippocampus in the temporal lobe, whereas the cholinergic axons originating from the nucleus basalis of Meynert project to the cerebral cortex (Mesulam et al. 1983).

Cholinergic neurotransmission is mediated by two main classes of receptors. These are the muscarinic and the nicotinic cholinergic receptors. This classification of the cholinergic receptors is based on ability of the alkaloid muscarine (derived from the mushroom Amanita muscaria) to mimic the effects of the endogenous ligand of the cholinergic system, acetylcholine (ACh), on the muscarinic class of receptors, and the ability of nicotine to mediate the effects of ACh on the nicotinic cholinergic receptors. This chapter will review the neurochemical organization of the central cholinergic synapse and the cholinergic (muscarinic and nicotinic) receptors with an emphasis on the muscarinic receptors under normal physiological conditions since this background information will be important to understand the role of the acetylcholinergic neurotransmission in the development of biological markers and new treatment strategies for AD.
Neurochemical Organization and Regulation of the Cholinergic Synapse

ACh is synthesized within the cytoplasm of pre-synaptic cholinergic nerve terminals by the synthesizing enzyme choline acetyltransferase (ChAT) from co-substrates choline and acetyl coenzyme A (AcCoA). The ChAT enzyme is a selective and specific neurochemical marker of the cholinergic neurons. The newly synthesized ACh is actively transported and concentrated in the synaptic vesicles inside the pre-synaptic cholinergic terminals. The membrane of these synaptic vesicles contain an electrogenic H⁺-ATPase which creates a chemical (acidic inside the vesicle) and electrical (positive inside) proton gradient across the vesicular membrane (Usdin et al. 1995). This allows the transport of ACh and ATP into the synaptic vesicle coupled to the outflow of H⁺ down the potential gradient.

Upon electrical depolarization of the nerve terminal, ACh is released with ATP by exocytosis into the synaptic cleft where ACh binds to cholinergic receptors on the pre- and post-synaptic membrane. The action of the released ACh is then terminated through hydrolysis by the enzyme acetylcholinesterase (AChE) into choline and acetate. Subsequently, choline is recycled back into the pre-synaptic cholinergic nerve terminal via the sodium-coupled high-affinity plasma-membrane choline transporter. This transporter is dependent on a Na⁺ gradient created by a (Na⁺/K⁺)-ATPase (Usdin et al. 1995). It is thought that the supply of choline is the rate-limiting step in the synthesis of ACh, with the activity of choline uptake closely linked to the release and the demand for resynthesis of ACh (Ducis 1988, Jope 1979). The neurochemical organization of the cholinergic synapse is summarized in Figure 1.
Figure 1. Neurochemical organization of the cholinergic synapse
**Nicotinic Cholinergic Receptors in the brain**

Nicotinic cholinergic receptors (nAChRs) have a pentameric protein structure and are composed of α, β, γ, δ and ε subunits. These subunits form a cation ion channel which is opened after binding of nicotine or acetylcholine to the nicotinic receptors. Although the existence of nicotinic receptors have been known in the peripheral nervous system and the neuromuscular junction, identification of neuronal nAChRs in the brain was made possible only recently.

Animal and human studies using autoradiography and immunohistochemistry techniques have indicated the presence of nAChRs in the cerebral cortex, which show a high binding affinity for nicotine (Clarke et al. 1985, Whitehouse et al. 1986, Schröder et al. 1989). Presynaptic neuronal nAChRs are involved in the stimulation of the release of several neurotransmitters including ACh, GABA, norepinephrine, aspartate and glutamate, and may play a neuromodulatory role in the central nervous system (Vidal 1996). For example, in the rat prefrontal cortex, a recent study has shown that nicotine selectively enhances the amplitude of excitatory synaptic potentials mediated by glutamatergic neurons (Vidal 1996). Since glutamate is implicated in synaptic plasticity and learning, the enhancement of glutamatergic neurotransmission by nAChRs may explain some of the cognitive enhancing effects of nicotine in animal models and in patients with AD (Voytko et al. 1994).

Animal studies using microinjection of nicotinic antagonists have also shown that nAChRs may play a physiologically important role in working memory and attentional processes (Granon et al. 1995). In aged humans, a decrease in cortical nAChRs was found but this change appears to occur independent of changes in ChAT activity (Court et al. 1992). Furthermore, in studies with
neocortical brain specimens obtained from AD patients, number of nicotinic binding sites were decreased compared to healthy controls (Vidal 1996). Due to the association between nicotinic and glutamatergic neurons, changes in nAChRs may also cause deficits in glutamatergic neurotransmission.

Muscarinic receptor subtypes and distribution in the mammalian CNS

The muscarinic receptors are composed of a single protein without a subunit structure and is included in the G-protein-coupled receptor superfamily. The muscarinic receptors in the central nervous system (CNS) play an important role in memory and cognitive function (Fibiger 1991, Müller et al. 1991). Dysfunction in the central cholinergic system and changes in muscarinic receptors are also some of the most consistent findings in Alzheimer's disease (AD), and these changes are thought to be important in the development of cognitive decline and memory loss characteristic of this disease.

Muscarinic receptor subtypes were initially grouped pharmacologically based on their affinity for the muscarinic receptor antagonist pirenzepine. Those muscarinic receptors with a high affinity for pirenzepine were classified as M1 while those with a low affinity were named as M2 (Felder 1995). Recent molecular cloning studies have identified five mammalian subtypes of muscarinic acetylcholine receptors (mAChRs) as part of the G-protein-coupled receptor superfamily. These cloned muscarinic receptors are designated with lowercase letters (m1, m2, m3, m4 and m5) according to the order of their discovery, and refer to the five genes for the receptors and their mRNA and protein products (Bonner 1989). Quantitative immunoprecipitation studies with rabbit antibodies raised against the third intracytoplasmic loop of the solubilized m1-m5 muscarinic receptors indicated that the m1, m2 and m4 subtypes are the most abundant in the
forebrain region of the human brain (Flynn et al. 1995) (Table 1). The m1 mAChR is the major postsynaptic muscarinic cholinergic receptor and accounts for approximately 35 to 60% of the total [N-methyl-3H]-scopolamine labeled muscarinic receptor population in all cortex areas (frontal, parietal, temporal and occipital cortex) and in the hippocampus (Levey 1996, Flynn et al. 1995, Levey et al. 1991, Buckley et al. 1988). The m1 subtype appears in lower levels in the nucleus basalis of Meynert, constituting about 15-20% of the total muscarinic receptors.

Considering the hippocampus and the cerebral cortex, the m2 and m4 receptors each account for approximately 15 to 25% of the total number of mAChRs (Flynn et al. 1995). On the other hand, the m2 receptor is enriched in the occipital cortex (36%) and the nucleus basalis of Meynert (41%) in the forebrain, whereas the m4 receptor is the predominant subtype in the caudate and putamen (50%) (Levey 1996, Flynn et al. 1995). Cortical immunostaining techniques provide evidence that the m2 receptor may function as a pre-synaptic autoreceptor where it may serve to inhibit the further release of ACh after an action potential, and also as a post-synaptic receptor (Levey et al. 1991). The m3 and m5 receptors appear to be expressed at lower levels than the m1, m2 and m3 receptors in the brain (Flynn et al. 1995, Levey et al. 1991).
Table 1. Localization of the mAChR subtypes in the mammalian brain

<table>
<thead>
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<th>Regional abundance</th>
<th>Cellular localization</th>
<th>Synaptic localization</th>
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<tr>
<td>m1</td>
<td>Abundant in the forebrain (neocortex, hippocampus, neostriatum)</td>
<td>Pyramidal neurons</td>
<td>Postsynaptic &gt;&gt; Presynaptic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Striatal spiny neurons</td>
<td></td>
</tr>
<tr>
<td>m2</td>
<td>Moderately abundant throughout the brain</td>
<td>Cholinergic neurons, nonpyramidal neurons in cortex and hippocampus</td>
<td>Presynaptic &gt;&gt; Postsynaptic</td>
</tr>
<tr>
<td>m3</td>
<td>Low levels throughout the brain</td>
<td>Neuronal</td>
<td>Unknown</td>
</tr>
<tr>
<td>m4</td>
<td>Abundant in the neostriatum, moderate levels in the hippocampus and the cortex</td>
<td>Striatal spiny neurons</td>
<td>Presynaptic and postsynaptic</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Associational and commissural hippocampus projections</td>
<td></td>
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<tr>
<td>m5</td>
<td>Low levels in the hippocampus and the substantia nigra</td>
<td>Pyramidal neurons, substantia nigra pars compacta, microglia</td>
<td>Unknown</td>
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Reproduced from Levey 1996, with permission.
Muscarinic receptor structure

mAChRs belong to the G-protein-coupled receptors (Wess 1993, Hulme et al. 1990) and as a characteristic of this family of receptors, they are composed of seven α-helically arranged hydrophobic transmembrane domains (TMI-TMVII), which are connected via three extracellular (o1-o3) and three intracellular loops (i1-i3). The NH2 terminal of the muscarinic receptors resides on the extracellular side of the plasma membrane while the COOH terminal is located on the cytoplasmic surface. The amino acid sequences of the transmembrane domains of the five muscarinic receptor subtypes show at least 90% homology (Bonner 1989). However, the extracellular and cytoplasmic tails and loops significantly differ in their amino acid sequence among m1 to m5.

Evidence from mutagenesis studies and low-resolution electron microscopic imaging suggests that the seven TM helices are arranged in a ring-like formation, forming a tight helical bundle, with residues that are known to be involved in the binding of acetylcholine located on the inner surfaces of the helices (Baldwin 1994, Schwartz 1994, Strader et al. 1994, Baldwin 1993, Wess 1993). Furthermore, studies using hybrid m2/m3 muscarinic receptors, have shown that the N-terminal 16 to 21 amino acids of the i3 loop (i.e. the third intracytoplasmic loop) are integral in the determination of the G protein coupling profile of a given muscarinic receptor subtype (Wess et al. 1990, Lechleiter et al. 1990, Wess et al. 1989).
Muscarinic Receptors and Signal Transduction

The activation of the muscarinic receptors by the endogenous ligand ACh results in multiple signal transduction events mediated by the effectors of G protein-coupled receptors (GPRs). The type of GPR (e.g. m1-m5), G-protein and the associated effector (e.g. adenylate cyclase versus phospholipase C) determine the physiological outcome and different effects of ACh in different tissues (Caulfield 1993). Specifically, the m1, m3 and m5 receptors couple with the α-subunit of the Gq or G11, while m2 and m4 subtypes activate the inhibitory Gi or Go α-subunits and/or βγ-dimers (Offermanns et al. 1994).

G-protein stimulation by activated mAChRs produces effects on various effectors. The precise relationship between the receptor subtypes and effectors is not exclusive, (Burford et al. 1995, Caulfield 1993) however, some relationships between specific mAChR subtypes and effectors have been identified. For example, the coupling of m2 and m4 to G1 or Go α-subunits produces inhibition of adenylate cyclase activity which leads to a decrease in the formation of cyclic AMP in a pertussis toxin-sensitive manner (Felder 1995). Subsequently, protein kinase A-dependent phosphorylation of various proteins such as Ca\(^{2+}\) channels is reduced (Löffelholz 1996). On the other hand, phospholipases C and D are preferentially activated by m1, m3 and m5 subtypes of receptors. For example, the phosphoinositide-specific phospholipase C produces inositol phosphates which mobilize intracellular stores of Ca\(^{2+}\) and also produces diacylglycerol which activates the protein kinase C (Exton 1994). Phospholipase A2 hydrolyzes the membrane phospholipids...
and produces arachidonic acid which is then converted to several biologically active compounds such as prostaglandins, thromboxanes and leukotrienes. The m1, m3 and m5 subtypes have also been shown to mediate phospholipase A2 activation.

**Muscarinic receptor regulation by desensitization**

Although the mechanisms are not completely elucidated, signaling through the activated GPRs such as the muscarinic receptors is thought to be terminated by multiple intracellular events which include the desensitization of activated GPRs and removal of the ligands responsible for the activation of the receptors (Liggett & Lefkowitz 1994).

The process of desensitization of GPRs includes a collection of events which may be independently regulated. Desensitization may include uncoupling of the receptors from the G-proteins and/or loss of the high affinity agonist binding. The event of phosphorylation mediated uncoupling appears to be controlled by specific protein kinases known as G protein-coupled receptor kinases (GRKs). The GRKs have been shown to terminate signaling in experiments with sensory receptor systems (Hargrave & Hamm 1994, Dawson et al. 1993, Schleicher et al. 1993). Also involved in the desensitization is the process is sequestration (i.e. internalization of the receptors) and down-regulation (loss of receptors from the cell) which usually occur only upon prolonged stimulation of the receptors. These last two processes require protein synthesis for recovery. Desensitization may also be homologous or heterologous. In homologous desensitization only activated receptors are desensitized, whereas heterologous desensitization refers to
the process in which activation of one receptor system may produce signals that feed back on other receptor systems.

Mechanisms of phosphorylation and homologous desensitization of mAChRs by GRKs have been investigated in vitro (Richardson et al. 1993) and in vivo (Kong et al. 1994). For example, Richardson and colleagues (1993) reconstituted purified recombinant human m2 mAChRs into phospholipid vesicles, and the ability of expressed and purified recombinant GRKs to phosphorylate the m2 mAChR was tested in vitro. In this study, agonist-dependent phosphorylation of the receptors with GRK2 and GRK3 was found. This suggested that the GRKs may be important in the phosphorylation and desensitization of mAChRs. To further complicate the picture of desensitization, recent evidence suggests that GRKs may themselves be regulated by complex mechanisms involving lipids and G-proteins (Shi et al. 1995, Haga et al. 1994, Kameyama et al. 1994, Stoffel et al. 1994, Chen et al. 1993, Palczewski et al. 1991).

In a recent review by Hosey et al. (1996), a working model for the regulation and inactivation of the mAChRs was described in detail (Figure 2). In brief, during the resting state, the G protein is thought to be in its inactivated heterotrimeric form, while the receptor is unligated. During this first stage, the GRKs may be partly membrane-bound, or cytosolic. In stage two, the muscarinic receptor is activated by ACh, causing the dissociation of the G protein into the βγ and α subunits which activate the effectors. During this stage, the GRK may be activated by the βγ subunit and undergoes
translocation from the plasma membrane. During the third stage, the agonist dependent receptor phosphorylation by activated GRK occurs. In the last stage, arrestin-type proteins may then bind to the phosphorylated receptor which would cause receptor-G protein uncoupling and a loss of the high affinity agonist binding (Liggett and Lefkowitz 1994, Lohse et al. 1990, Lohse et al. 1992). In order for the receptor to return to the resting state, dissociation of the arrestin, and dephosphorylation is required (Hosey et al. 1996).

Recent studies have provided a better understanding of the molecular basis of cholinergic neurotransmission and its regulation. Insight has also been gained into the differential expression of mAChR subtypes at various synapses in memory circuits. The pathophysiological importance of the cholinergic neurotransmission and the individual muscarinic receptor subtypes in disease states such as AD are reviewed in chapter 2.
Figure 2. Model of desensitization of MACHRs (Reproduced from Hosey et al. 1996, with permission)
Chapter II

The Acetylcholinergic System in Alzheimer's Disease
INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of the central nervous system and is the most common cause of memory loss and dementia among the elderly population. In the United States, AD is estimated to affect 2% of those between the ages of 65 and 69, and with advancing age, the prevalence increases to 30 to 45% in those over the age of 85 (Wernicke & Reischies 1994, Evans et al. 1989, Jorm et al. 1987). AD is a major financial and social burden on society with annual direct and indirect costs amounting to $40 and $70 billion, respectively, in the United States (Ernst & Hay 1994, Huang et al. 1988, Hay & Ernst 1987). It is estimated that the number of people over the age of 65 in the United States will increase by 105% in the years ranging from 1985 to 2025, and therefore, the impact of this disease is becoming increasingly important (International Population Reports 1987).

Although the exact molecular and pathological events involved in the etiology and progression of AD still remain elusive, the changes in the central cholinergic system are one of the most important findings and frequently proposed explanations for the cognitive deficits associated with AD. Therefore, the knowledge of the cholinergic dysfunction in AD may provide a basis for the development of future pharmacological treatments and biological markers for this disease. The present chapter will provide a brief background on the cholinergic hypothesis and pathobiology of AD.

Cholinergic hypothesis of AD

Several lines of evidence indicate that the central cholinergic neurons play a critical role in learning, memory and the cognitive deficits associated with AD:

The decrease in ChAT activity and the loss of cholinergic neurons in AD correlate with the cognitive deficits before death (Bierer et al. 1995, Lehericy et al. 1993, Doucette et al. 1986, Perry et al. 1978).

In animal studies, lesions of the cholinergic nuclei (e.g. nucleus basalis of Meynert) and muscarinic receptor antagonists (e.g. scopolamine) cause an impairment in tasks involving learning and memory (Berger-Sweeney et al. 1994, Wenk et al. 1994, Dunnett 1993, Squire et al. 1993, Wenk & Olton 1987, Bartus et al. 1985, Dunnett 1985, Flicker et al. 1985).

The memory deficits produced by lesions of the nucleus basalis of Meynert and the cholinergic denervation of the cortex can be restored by cortical surgical grafts of cells which are genetically engineered to produce ACh (Winkler et al. 1995, Nilsson et al. 1992).

- Treatments that increase cholinergic neurotransmission such as AChE inhibitors and muscarinic receptor agonists cause an improvement in cognitive deficits in AD (Giacobini 1998, Bodick et al. 1997, Knapp et al. 1994).

These studies indicate that central cholinergic neurons may serve as a target for understanding the pathophysiology of AD and dementia. Recent research has also focused on obtaining insight into the changes in expression and function of individual muscarinic receptor subtypes in AD. An understanding of how these receptors are altered by disease processes would allow for the development of new treatment strategies that could target viable muscarinic receptors in memory-related forebrain circuits, and help elucidate how the muscarinic receptor system may be implicated in the pathological mechanisms of AD.

In a recent study by Flynn et al. (1995), quantitative immunoprecipitation techniques were used with a panel of muscarinic receptor subtype-specific antibodies raised against the third intracellular loop (i3 loop) of the m1 to m5 receptors to determine the level of expression of the m1 to m5 receptor protein in brain regions of normal elderly control subjects and age-matched patients with AD. In this study, the levels of the immunoprecipitated m1 receptor were significantly decreased in the brain tissue of patients with AD, versus that of normal elderly controls. Specifically, m1 levels were decreased by 53% in the hippocampus, by 34% in the frontal and temporal cortex and by 27% in the parietal cortex, compared to controls. On the other hand, the level of m1 in brains from AD patients (123.7 ± 12.8) (mean ± SEM, \(B_{max}\) values expressed as fmol/10 mg of tissue) was not significantly different than controls (117.7 ± 7.9), when measured pharmacologically using \(^{3}H\)-pirenzepine binding (Flynn et al. 1995). Previous
studies have also indicated that m1 binding remains the same in AD patients but its ability to couple with its G-protein and activate second messengers is dysfunctional in AD (Ferrari-DeLeo et al. 1995, Ferrari-DiLeao & Flynn 1993, Flynn et al. 1991,). Furthermore, the altered m1 - G protein interaction in AD is not as a result of decreased levels of G protein (Ferrari-DeLeo et al. 1995). However, the decreased m1 immunoreactivity observed by Flynn et al. (1995) suggests an altered form of the m1 receptor protein at the third intracytoplasmic loop (i3) in AD, since the antibodies used in that study were raised against that region of the m1 protein. The i3 loops are critical in the muscarinic receptor-G protein interaction, receptor mediated functional responses, agonist selectivities, and receptor internalization and phosphorylation (Brann et al. 1993). Therefore, a dysfunction at the i3 loop may explain normal ligand binding, a decreased immunoreactivity and inefficiency of m1 in coupling with the G-proteins in AD. Therefore, treatment of AD with m1 selective agonists may have a limited efficacy due to dysfunction in signalling mechanisms (Flynn et al. 1995).

The levels of immunoprecipitated m2 receptor protein have been found to be decreased in AD to (43%) and (69%) of values measured in elderly controls, in the frontal cortex and hippocampus, respectively, whereas m3 and m5 immunoprecipitates were not changed significantly from control values in AD (Flynn et al. 1995). The m2 levels in the nucleus basalis of Meynert, however, do not appear to change significantly in AD (Mufson et al. 1998). In contrast to the decreased levels of m1 and m2 immunoprecipitates in AD, increases in the levels of immunoprecipitated m4 receptor proteins have been reported in the frontal, temporal and parietal cortices, with values at 138%, 135% and 129% of control values in normal elderly, respectively (Flynn et al. 1995). Taken together, these data indicate a differential modulation of the muscarinic receptor subtypes in AD, and that specific receptor subtypes (e.g. m4) may be
valuable targets for therapeutic replacement treatments directed against muscarinic receptor binding sites.

**Histopathological lesions in AD**

Pathological lesions which characterize AD are amyloid deposition in senile and diffuse plaques, and neurofibrillary tangles. In addition, there is extensive neuronal cell death that targets the cholinergic system neurons (Giacobini 1990, Bartus et al. 1985).

Senile plaques are defined as extracellular, focal, spherical collections of tortuous neuritic processes which are surrounded by an amyloid core. The amyloid deposits consist of extracellular accumulations of aggregated and nonaggregated forms of amyloid β-peptides (Aβ). These Aβ peptides are a group of approximately 4-kd peptides that are 39 to 43 amino acid residues in length (Vigo-Pelfrey et al. 1993, Haass et al. 1992b, Shoji et al. 1992). They are derived from the proteolytic cleavage of a large ubiquitous transmembrane precursor protein, amyloid β-protein precursor (APP) coded for on chromosome 21 (Tanzi et al. 1987). APP consists of a single-membrane spanning domain, a large N-terminal ectodomain and a short cytoplasmic C-terminal tail (Kang, et al. 1987). There are two proteolytic pathways through which APP can be processed normally. In the first pathway, APP is cleaved within the Aβ sequence between residues 16 and 17 by a plasma membrane-associated endoprotease, α-secretase protease, this is followed by the extracellular secretion of soluble APP ectodomain (APPs) (Sisodia 1992). In this pathway, the Aβ can not be formed, and there is no release of Aβ-protein (Sisodia et al. 1990).
On the other hand, in the second pathway, the full length APP is reinternalized from the surface, and cleavage occurs in an acidic intracellular compartment on either side of the Aβ amino acid sequence by the β- and γ-secretases (Haass et al. 1992a, Haass et al. 1992b, Shoji et al. 1992). This results in the production and secretion of the Aβ peptide (Haass et al. 1992b). Excess accumulation of Aβ peptides adversely affects cholinergic cell function and may be involved in mediating neurotoxic processes involving induction of apoptotic neuronal cell death, free radical generation, oxidation, destabilization and microglial cell activation (Cummings & Mega 1996, Itoh et al. 1996, Kelly et al. 1996, El Khoury et al. 1996, Yan et al. 1996, Behl et al. 1994).

The second histopathological hallmark of AD is neurofibrillary tangles. Neurofibrillary tangles are composed of paired helical filaments located in neuronal cytoplasm. The filaments are composed of abnormally phosphorylated forms of the axonal microtubule-associated protein (tau), which normally functions by facilitating microtubule assembly (Lee et al. 1991). The amount of neurofibrillary tangle accumulation appears to be in close correlation with the presence or severity of cognitive impairments in AD (Goedert 1993). It has been suggested that the presence of nonfunctional tau proteins in the brains of patients with AD may have pathological consequences for cytoskeletal structure and for the function of neuronal cells (Iqbal et al. 1986). However, the exact mechanisms by which the neurofibrillary tangles are involved in the pathogenesis of AD are still under investigation.

Modulation of the amyloid precursor protein (APP) processing by muscarinic receptors

Currently the causes for the development of the most common form of AD (nonfamilial form) and the mechanisms by which amyloid deposition and neurofibrillary tangles form in this disease remain elusive. There is, however, evidence suggesting links between cholinergic system
degeneration and amyloid deposition and cognitive deterioration in AD. Therefore an understanding of the cholinergic system may provide answers to the pathological development of the nonfamilial form of AD.

In addition to the disease associated changes in muscarinic receptor subtype expression and signal transduction, muscarinic receptors have also been implicated in the pathogenesis of AD. Specifically, muscarinic receptors are implicated in the processing of amyloid precursor protein (APP). APP processing is important in the pathobiology of AD, as it is responsible for the production of one of the major histopathological lesions found in AD (i.e. amyloid deposits in senile plaques).

The m1 muscarinic receptor subtype is the predominant muscarinic receptor subtype expressed in the brain (Flynn et al. 1995). A recent study (Nitsch et al. 1992) in which human embryonic kidney cell lines were transfected with the gene for the human brain m1 receptor provides support for the role of the m1 receptor in the regulation of APP processing. Following transfection in this study, the m1 receptors were stimulated with carbachol which resulted in an increased basal release of soluble APP ectodomain which is a characteristic of nonamyloidogenic APP processing (Nitsch et al. 1992). Similarly, in studies with transfected cells and brain slices, the activation of m1 receptor stimulates α-secretase processing of APP which is associated with decreased formation of Aβ (Farber et al. 1995, Wolf et al. 1995, Buxbaum et al. 1994, Dyrks et al. 1994, Gabuzda et al. 1993, Hung et al. 1993). For example, in a study using both transfected human astrocytoma cell lines and tissue slices from rat brain, the m1 selective agonist talsaclidine increased the release of APP ectodomain (Müller et al. 1997). The talsaclidine induced increase
in APP ectodomain release was concentration-dependent, and was blocked by atropine (Müller et al. 1997).

Other mechanisms by which the functional integrity of the muscarinic receptor subtypes may be important in the pathobiology of AD are through signal transduction pathways. Muscarinic receptor activation regulates neuronal protein kinase C activity and Ca\(^{2+}\) levels, and PKC activation has been shown to inhibit cellular production of Aβ protein (Gabuzda et al. 1993). Therefore, possible changes in signal transduction mechanisms in AD such as the decreased interaction between m1 receptors and G-proteins described earlier may disrupt neuronal Ca\(^{2+}\) homeostasis, and lead to increased sensitivity to oxidative stress, cell injury and amyloidogenic processing of APP (Disterhoft et al. 1993, Mattson et al. 1993).

Overall, changes in the cholinergic system including the loss of cortical cholinergic innervation and changes in muscarinic receptor expression and regulation occur early in the course of AD and are thought to play an important role in the pathogenesis of AD (Geula & Mesulam 1994). The m1 receptors may play a neuroprotective role by increasing the non-amyloidogenic processing of the amyloid precursor protein. Therefore, changes in muscarinic receptor expression and cell signaling that are characteristic of AD may predispose or lead to formation of pathological lesions such as amyloid deposition. Biological markers that can measure the evolution of cholinergic system changes in AD, and their influence on cognitive function would be important tools in the clinical treatment and the understanding of the impact of the cholinergic system on the disease mechanisms involved in the development of AD. Such markers may for example assess the function of specific muscarinic receptor subtypes, or the global deterioration of the cholinergic system. This may allow for the early use of pharmacological treatments such
as m1 receptor agonists which may potentially prevent the early development of pathological lesions such as amyloid production which appears to be regulated by muscarinic receptors.

Risk factors in the development of AD

Alzheimer's disease is likely to have a heterogeneous etiology, combining interactions with genetic and non-genetic factors. Some examples for the possible non-genetic risk factors for AD as suggested by epidemiological studies include low educational level, and head trauma (Katzman 1993, Heyman et al. 1984). Age is an important factor which modifies the risk of AD. The prevalence of AD is estimated to double every 5 years beyond age 65, such that at ages 65 to 70 years, the prevalence is 1% to 4%, and at ages of 85 to 90 years, it becomes >22% (Hofman et al. 1991).

The non-familial or the sporadic form of AD is the most common type of AD, and causes for its development are unknown. Recent research has focused on establishing the causes of early onset familial AD (family mean age at onset < 60 years). AD which is caused by known genetic mutations is defined as autosomal dominant familial AD, and it is clinically and neuropathologically similar to the common sporadic and nonfamilial type of AD (Martin et al. 1991, Bird et al. 1989, Nee et al. 1983, Goudsmit et al. 1981, Cook et al. 1979). Studies in large multi-generational kindreds with familial AD, have led to the identification of 'causal genes' in which a mutation in the gene is linked to the early-onset familial type of AD which is estimated to represent 1 to 2 % of all AD cases in the general population. Currently three causative genes for the early-onset familial AD have been identified however, the roles of the gene products in the development of AD are still unclear. The three genes are located on chromosomes 21, 14 and 1,
and show autosomal dominant inheritance, where lifetime risk for the development of AD in first-degree relatives approaches 50% (Farrar et al. 1995, Mohs et al. 1987).

Early onset familial AD: Mutations on chromosome 21

Mutations on chromosome 21 occur within the APP gene, and are associated with the early-onset familial AD (Fidani et al. 1992, Karlinsky et al. 1992, Goate et al. 1991, Naruse et al. 1991). These APP mutations flank or are within the Aβ sequence which is encoded by parts of exons 16 and 17 in the APP gene (Levy-Lahad & Bird 1996). Currently there are four APP mutations known to cause early-onset familial AD. The most common of these mutations occurs in codon 717 in exon 17, which is three residues beyond the carboxyl terminus of Aβ, and involves a valine/isoleucine substitution (Val717Ile) (Fidani et al. 1992, Karlinsky et al. 1992, Naruse et al. 1991). The mutations in the APP gene may result in alternative APP processing which could be implicated in the pathogenesis of AD (Levy-Lahad & Bird 1996). Recent studies provide evidence for such alternative processing mechanisms that may produce a shift in APP processing towards the β- and γ-secretases pathway leading to an increased synthesis or accumulation of Aβ peptide (Haass et al. 1994, Cai et al. 1993, Citron et al. 1992). It has also been shown that the APP mutations are associated with a higher ratio of long Aβ peptides, which are more prone to forming amyloid fibrils than the shorter forms of the peptide, and are thought to be more pathogenic (Suzuki et al. 1994, Burdick et al. 1992). In support of this, in vitro studies in which cells are transfected with APP constructs containing the Val717Ile mutation found in familial AD pedigrees, show a relative increase in the production of “long” (42aa) versus “short” (40aa) Aβ (Suzuki et al. 1994, Tamaoka et al. 1994). Therefore, genetic mutations may be one mechanism by which “long” Aβ peptides accumulate in AD, leading to the production of amyloid deposits and neuronal destruction (Jarrett & Lansbury 1993). However,
as these genetic mutations on chromosome 21 are not present in all forms of AD, there are possibly non-genetic causes for a shift in processing of APP to the β- and γ-secretases pathways leading to the accumulation of Aβ (Mattson et al. 1993). Such causes may be related to altered cholinergic system function.

**Early onset familial AD: Mutations on chromosome 14**

The S182/PS-1 gene is located on chromosome 14 and mutations in this gene are associated with very early onset of familial AD. Pedigree studies show that mutations in this gene may cause AD with a range of onset from 35 to 55 years (Schellenberg et al. 1992, St. George-Hyslop et al. 1992). This gene encodes a 467-aa protein (presenilin1) with 7 to 10 hydrophobic transmembrane domains, however its function is unknown (Sherrington et al. 1995). Twenty-five mutations have been identified, twenty-four of which are missense mutations (single aa substitutions) which may result in a change in this protein’s function (Wasco et al. 1995). The remaining mutation results in destruction of the splice acceptor site of exon 9, leading to a loss of exon 9 which encodes part of a hydrophilic loop of the protein (Perez-Tur et al. 1995b).

**Early onset familial AD: Mutations on chromosome 1**

The third identified causative gene for familial AD is located on chromosome 1 (STM-2/PS-2 (presenilin 2) gene, and two missense mutations have been identified on this gene (Levy-Lahad et al. 1995a, Levy-Lahad et al. 1995b, Rogaev et al. 1995). This gene is predicted to encode a 448-aa protein of unknown function that is 67% identical to the presenilin 1 protein (Levey-Lahad et al. 1995a, Levy-Lahad et al. 1995b).
Apolipoprotein E genotype and risk for development of AD

In addition to the three causative genes located on chromosomes 1, 14 and 21, the apolipoprotein E (ApoE) gene located on chromosome 19 is associated with an increased risk or susceptibility for the development of familial and non familial AD (Levy-Lahad & Bird 1996, Pericak-Vance et al. 1991). The ApoE e2/e3/e4 polymorphism consists of single base-pair changes at codons 112 and 158 of the ApoE gene. These base-pair changes produce three allelic variants of the gene, which in turn code for three isoforms of the ApoE lipid binding protein. The ApoE e3 allele is the most common isoform and represents about 78% of all alleles in the European and American white populations, while the ApoE e4 and e2 allele frequencies in these populations are at 16% and 7% respectively (Plassman & Breitner 1996). Parallel to increased risk of AD which is related to ApoE genotype, age at onset of this disease also decreases with increasing e4 dose (Lucotte et al. 1995, Corder et al. 1993, Mayeux et al. 1993, Stritmatter et al. 1993). The inheritance of each e4 allele has been shown to lower the age at onset by 7 to 9 years in late onset familial AD, and by 3 to 7 years in sporadic AD (Poirier et al. 1993).

Late onset sporadic and familial AD is characterized with an mean age of onset > 60 years. In general, case-control and autopsy studies of late onset AD patients have shown the ApoE e4 allele frequencies to be higher than the frequencies of control populations, with a range of 30% to 50% in both sporadic and familial AD (Mayeux et al. 1993, Rebeck et al. 1993, Saunders et al. 1993, Schmechel et al. 1993, Broussseau et al. 1994). Similarly, the association between ApoE genotype and the risk of AD is also implicated in early-onset familial AD (Perez-Tur et al. 1995a, Van Duijn et al. 1994).
Overall, the exact biological role of ApoE in AD is still under investigation, and several mechanisms for its involvement in the pathogenesis of AD have been reported. The ApoE e4 protein shows enhanced binding to Aβ in vitro, and is associated with increased deposition of Aβ in autopsied AD brain tissue (Strittmatter et al. 1993, Schmechel et al. 1993). In another study it was observed that ApoE e4 binds with reduced affinity to the microtubule-associated protein tau in comparison with the e3 and e2 isoforms (Strittmatter et al. 1993). From this finding it was hypothesized that the reduced binding affinity of the e4 isoform of ApoE to tau protein allows for early phosphorylation events to occur which may initiate the formation of paired helical filaments and neurofibrillary tangles (Strittmatter et al. 1994).

Cholinergic system and treatment strategies in AD

Current pharmacological treatment of AD is based on improving symptoms such as cognitive deficits. For example, the mechanism by which acetylcholine esterase inhibitors improve cognitive function is by increasing the amount of ACh at the cholinergic synapse to improve cholinergic neurotransmission. Differential regulation and expression of muscarinic receptors in the central nervous system in AD may also allow for drug treatments that would target specific viable receptor subtypes. Parallel to the development of these treatments which alleviate the symptoms of the disease by targeting dysfunctional neurotransmitter systems, newer treatment strategies are also being developed based on slowing down or preventing the evolution of dementia. Such treatments would target the mechanisms involved in the development of the disease, and may act through retardation of the formation of pathological lesions and the development of neuronal degeneration (Shvaloff et al. 1996).
Difficulties in the development of medications for AD have mainly been due to the lack of biological markers which could detect AD in its early stages, and incomplete understanding of the etiology and pathological evolution of the disease. The changes in the central cholinergic system may have important implications in understanding the pathology of AD, and in elucidating the mechanisms of cognitive and memory dysfunction associated with AD. The central cholinergic system may therefore be an important target for the development of biological markers for AD.
Chapter III

Age-related Differences in Pupillary Response to Pharmacological Probes of the Central and Peripheral Cholinergic Systems
INTRODUCTION

The percentage of the elderly population is expected to increase significantly in the coming decades and AD will continue to be one of the main health problems in that age group. Therefore, the development of biological markers for the diagnosis, treatment and monitoring of this disease would be very important.

The definitive diagnosis of AD is made by histopathological examination of brain specimens to identify lesions such as senile plaques and neurofibrillary tangles (Jellinger & Bancher 1997). Since brain biopsy is very invasive and is not acceptable for most patients and their families, definitive diagnosis can only be made post-mortem. The clinical diagnosis of probable AD relies on the identification of dementia which is characterized by severe memory loss and dysfunction in at least two cognitive areas such as orientation, attention, language, praxis and visuospatial skills, onset between ages 40 and 90, no disturbance of consciousness and progressive worsening of memory and cognitive functions over time (American Psychiatric Association 1994, McKhann et al. 1984).

Clinical assessments include repeated cognitive examination and neuropsychological tests using the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) guidelines (McKhann et al. 1984). The diagnosis of AD requires the exclusion of all secondary causes for cognitive decline. Some patients with advanced AD show increased slow-wave activity in electroencephalograms but this finding may also be observed in other dementias. Computerized tomography may be used to differentiate other causes of dementia secondary to strokes, brain
tumors, hydrocephalus, Parkinson's disease, subdural hematomas, Huntington's disease, Creutzfeldt-Jakob disease and alcoholic dementia (Black 1994, Holman & Devous 1992). Magnetic resonance imaging, positron emission tomography, and regional cerebral blood flow measurements may differentiate AD and dementia associated with cerebrovascular disease or demyelinating disorders. The analysis of cerebrospinal fluid may be helpful patients with chronic infections such as cryptococcal meningitis and syphilis. However, neuropsychological testing and neuroimaging may not be sensitive enough to distinguish between these confounding conditions and the AD early in the course of the disease. The extensive diagnostic work-up involved to achieve accurate clinical diagnosis may often not be practical in non-research clinical settings. Such factors contribute to the finding that the clinical diagnosis of AD may be inaccurate in 20% of the cases (Wade et al. 1987). Currently, there are no established practical, noninvasive and clinically relevant biological markers for AD. Such neurobiological markers or probes may have clinical use in identification of individuals who are predisposed to develop AD prior to its clinical manifestation or to monitor the progressive decline in cholinergic neurotransmission as the disease progresses.

The concentration of the microtubule-associated protein (tau) or the specific domains of beta-amyloid precursor proteins in the cerebrospinal fluid was proposed as a marker of AD (Arai et al. 1995, Nitsch, et al. 1995, Hock et al. 1995, Wagner et al. 1994, Vandermeeren et al. 1993). For example, AD patients show an increase in abnormally phosphorylated form of tau in the cerebrospinal fluid which can be detected by monoclonal tau antibodies (Blennow et al. 1995). However, significant overlap in tau concentration is often present between patients with AD, vascular dementia, frontal lobe dementia and controls, limiting the clinical diagnostic value of this marker (Blennow et al. 1995). Determination of tau concentration in the cerebrospinal fluid
requires lumbar puncture and this invasive procedure is another caveat which prevents its clinical applicability. Therefore, there is a clear need for the development of practical biological markers for AD that would aid in improving the sensitivity and accuracy of clinical diagnosis and prevent the need for post-mortem confirmation of diagnosis.

1. **Cholinergic deficit in AD:**

   **Potential as a target for the development of biological markers**

   The pathological changes in the central cholinergic system are one of the most well-characterized findings in AD (see chapter 2). Recent pharmacological research on AD has focused on the development of cholinergic treatments that would increase central cholinergic neurotransmission and improve clinical symptoms of the disease such as deficits in cognition (Black 1994). Acetylcholinesterase (AChE) inhibitors such as tacrine and donepezil prevent the hydrolysis of ACh in cholinergic synapses, which prolongs its duration of action and interactions with the cholinergic receptors. This treatment approach does not reverse the underlying neuropathology but provides symptomatic relief and delays the progression of the cognitive decline in mild to moderate AD (Rogers & Friedhoff 1996, Knapp et al. 1994). An important clinical issue with the AChE inhibitors is that their efficacy would be dependent on the presence of endogenously available ACh. For example, in patients with a severe cholinergic denervation and reduction in ChAT activity and ACh synthesis, inhibition of the AChE enzyme would not be expected to increase the efficiency of cholinergic neurotransmission. Therefore, the development of markers to estimate the level of central cholinergic dysfunction would be advantageous for pharmacological treatment of AD with AChE inhibitors.
Future pharmacological treatment strategies for AD may involve slowing down or preventing the evolution of dementia by targeting the mechanisms involved in the cholinergic neuronal cell death and development of the disease (e.g. prevention of amyloid deposition) (Shvaloff et al. 1996). These future treatments may prevent the development of severe dementia and increase the number of patients with only mild to moderate illness who are eligible for treatment with the AChE inhibitors. Therefore, markers for the efficiency of the central cholinergic system function will continue to remain as an important tool for the clinical monitoring of response to AChE inhibitors.

Taken together, the cholinergic changes in AD may have important implications in understanding the pathology of this disease, and as a potential target for the development of pharmacological treatments and biological markers. Such cholinergic markers would potentially aid in

- improvement of the specificity of current clinical diagnosis,
- establishment of early diagnosis before clinical symptoms manifest,
- assessment of cholinergic system integrity for the improvement of pharmacological treatment strategies during clinical stages of the disease.

2. Increased pharmacological response to scopolamine:

A clinical measure of decreased central cholinergic neurotransmission

Altered pharmacological response to drugs acting on the cholinergic system was previously used as a clinical measure of the functional status of central cholinergic neurotransmission in AD and normal aging (Scinto et al. 1994, Flicker et al. 1992, Sunderland et al. 1987). A challenge with a cholinergic drug may elicit cholinergic deficits that may not otherwise be clinically apparent, early in the course of the disease or during the pre-symptomatic
stage. Therefore, pharmacological challenges may be advantageous to develop biological markers of AD.

Scopolamine, a nonselective muscarinic cholinergic antagonist, is a well known centrally-acting cholinergic probe which causes an impairment in recent memory and new learning (Sunderland et al. 1987, Beatty et al. 1986). The affinity of scopolamine and some other centrally acting muscarinic receptor antagonists for the five previously cloned human muscarinic receptor subtypes expressed in Chinese hamster ovary cells is shown in Table 2 (Bolden et al. 1992).

<table>
<thead>
<tr>
<th>Muscarinic antagonist</th>
<th>m1</th>
<th>m2</th>
<th>M3</th>
<th>m4</th>
<th>m5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine</td>
<td>0.50</td>
<td>0.90</td>
<td>1.10</td>
<td>0.60</td>
<td>1.70</td>
</tr>
<tr>
<td>Benztropine</td>
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<td>1.40</td>
<td>1.10</td>
<td>1.10</td>
<td>2.80</td>
</tr>
<tr>
<td>Biperiden</td>
<td>0.48</td>
<td>6.30</td>
<td>3.90</td>
<td>2.40</td>
<td>6.30</td>
</tr>
<tr>
<td>Diphenhydramine</td>
<td>100.0</td>
<td>120.0</td>
<td>229.0</td>
<td>112.0</td>
<td>260.0</td>
</tr>
<tr>
<td>Quinuclidinyl benzilate</td>
<td>0.035</td>
<td>0.027</td>
<td>0.088</td>
<td>0.034</td>
<td>0.043</td>
</tr>
<tr>
<td>(QNB)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8</td>
<td>270</td>
<td>150</td>
<td>28</td>
<td>170</td>
</tr>
<tr>
<td>Scopolamine</td>
<td>1.1</td>
<td>2.00</td>
<td>0.44</td>
<td>0.80</td>
<td>2.07</td>
</tr>
</tbody>
</table>

Kd is the equilibrium dissociation constant for the receptor and ligand interaction and is inversely related to affinity of the antagonists for the receptors.
Scopolamine challenge has been used as a pharmacological tool to show dysfunction in cholinergic neurotransmission in normal aging and in AD. For example, deficits in memory and cognitive function after scopolamine administration were found to be greater in patients with AD compared to normal elderly (Sunderland et al. 1987). In addition, scopolamine also causes increased cognitive impairment in healthy elderly subjects compared to young adults (Flicker et al. 1992, Molchan et al. 1992). Although it is uncertain whether age-related pharmacokinetic changes may be responsible for the differential effects of scopolamine in AD, limited data from a single time point assessment of scopolamine plasma concentrations at 2 hours post intramuscular injection of three different doses (0.002, 0.004, 0.007 mg/kg) suggest that scopolamine kinetics may be similar in young and the elderly (Ray et al. 1992). Sunderland et al. (1987) found that cognitive tests of new learning and semantic knowledge show a significant impairment at a lower dose (0.25 mg/subject) in patients with AD compared to age- and sex-matched healthy elderly control subjects (0.50 mg/subject). This last study by Sunderland et al. (1987) suggests that the increased cognitive effects of scopolamine in AD can be explained by increased pharmacodynamic sensitivity, possibly due to degenerative changes in central cholinergic neurons in AD. However, more systematic studies with multiple time point analysis of plasma concentrations of scopolamine in healthy elderly subjects need to be conducted to rule out age-related pharmacokinetic changes for the increased scopolamine effects in AD. Overall, studies with scopolamine suggest that changes in response to scopolamine may be able to differentiate patients with AD from elderly controls based on magnitude of the pharmacological effect produced. However, the age-related increase in sensitivity cholinergic antagonists and side effects (e.g. delirium, confusion, sedation and psychomotor impairment) may be an important limitation for development of scopolamine as a clinical diagnostic tool for AD. The clinical value
of the scopolamine challenge in AD diagnosis and especially in the prediction of the efficacy of AChE inhibitors remain to be known.

3. **Assessment of the central cholinergic system in AD with pharmacological response to peripherally acting cholinergic probes**

AD affects cognition and other higher brain functions and therefore, deficits in cholinergic neurotransmission in this disease have been studied mainly in the brain. Recently, some evidence has accumulated suggesting that changes in peripheral cholinergic function may accompany the changes in central cholinergic system (Scinto et al. 1994, Appleyard & McDonald 1991, Trick et al. 1989). For example, adults with Down’s syndrome over the age of 30 develop memory impairment, dementia and neuropathological lesions (e.g. neurofibrillary tangles) characteristic of AD (Wisniewski et al. 1985). In these patients, the cholinergic antagonist atropine causes a greater increase in heart rate (Harris & Goodman 1968), while tropicamide (a non-selective muscarinic antagonist) and atropine cause increased pupillary dilatation (Sacks & Smith 1989, Berg et al. 1959). A decreased parasympathetic cardiac response to postural changes and a reduction of AChE activity in the adrenal gland in patients with AD were also reported (Aharon-Peretz et al. 1992, Appleyard & McDonald 1991).

As an alternative to centrally acting cholinergic drugs such as scopolamine, peripherally acting pharmacological probes may also be developed to assess central cholinergic system function. Recently, increased sensitivity in pupillary response to peripherally acting cholinergic receptor antagonists (e.g. tropicamide) and agonists (e.g. pilocarpine) was shown in patients with AD (Kaneyuki et al. 1998, Idiazuez et al. 1994, Scinto et al. 1994) (see Table 6 and 7 for literature review). Scinto and colleagues (1994) reported that dilute tropicamide (0.01%) might be used as
a diagnostic marker for AD as these patients had a significantly increased pupillary dilatation response to tropicamide (>13 % increase from baseline) when compared to age matched controls (<13 % increase from baseline) at 30 minutes post-tropicamide. However, subsequent studies with tropicamide in patients with AD failed to replicate this finding (FitzSimon et al. 1997, Graff-Radford et al. 1997, Growdon et al. 1997, Higuchi et al. 1997, Kálmán et al. 1997, Kurz et al. 1997, Arai et al. 1996, Fridh et al. 1996, Gomez-Tortosa et al. 1996, Litvan & FitzGibbon 1996, Loupe et al. 1996, Marx et al. 1995, Treloar et al. 1995). Nevertheless, these findings suggested that changes in cholinergic neurons outside the brain (e.g. in the eye) may also occur in AD and that they may be used to develop peripheral pharmacological probes to identify changes in central cholinergic neurons. Peripheral cholinergic probes acting on the eye may be advantageous because they would be noninvasive, practical and, unlike scopolamine, would not cause central cholinergic side effects. The affinity of tropicamide and pilocarpine for the muscarinic receptor subtypes is shown in Table 3 (Dong et al. 1995).

<table>
<thead>
<tr>
<th></th>
<th>m1</th>
<th>m2</th>
<th>m3</th>
<th>m4</th>
<th>m5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropicamide</td>
<td>6.98</td>
<td>7.62</td>
<td>6.95</td>
<td>7.55</td>
<td>7.24</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>5.74</td>
<td>5.78</td>
<td>5.28</td>
<td>5.39</td>
<td>6.16</td>
</tr>
</tbody>
</table>

pKi is the negative logarithm of the inhibitory constant (Ki) for each ligand. A high pKi value (i.e. a low Ki) is an indication of a high affinity of the ligand for the receptor.

Parasympathetic innervation of the muscles of the iris

There are two muscles in the iris which control the pupillary aperture, namely the sphincter iridis and the dilator pupillae. These muscles are controlled by the Edinger-Westphal nucleus, which is
part of the oculomotor nucleus in the midbrain. The efferent arm of the light reflex arc constitutes the parasympathetic innervation of the iris sphincter muscle. Efferent fibers which innervate the muscles of the iris travel from the Edinger-Westphal nucleus and leave the midbrain through the third cranial nerve. Pre-ganglionic fibers synapse on the ciliary ganglion located deep in the muscle cone of the orbit, and post-ganglionic fibers synapse onto the sphincter muscle of the iris. Upon arrival of an action potential, release of ACh occurs from the postganglionic cholinergic nerve terminals, leading to constriction of the pupil as the ACh binds to muscarinic receptors on the sphincter muscle (Figure 3).

Muscarinic receptor subtypes in the human ocular tissues

The cholinergic muscarinic receptor system mediates various physiological effects in the eye. Stimulation of muscarinic receptors by ACh mediates the constriction of pupillary aperture through contraction of the sphincter muscle of the iris. This muscarinic receptor system is also involved in the accommodation response of the lens which is mediated by contraction of the ciliary muscle and secretion of aqueous humor from the ciliary process. The anatomical structure of the eye is shown in Figure 4.
Figure 4. Anatomical structure of the eye (Adapted from Warwick RB: Eugene Wolff's anatomy of the eye and orbit, Philadelphia:WB Saunders;1976.p.30.)
Recently, the muscarinic receptor subtypes were quantified in human ocular tissues using immunoprecipitation (Gil et al. 1997). Specifically, $[^{3}H]$-quinuclidinyl benzilate (QNB) bound receptors solubilized from the human ciliary muscle, the iris sphincter muscle and the ciliary process were incubated with antisera against the five identified muscarinic receptor subtypes (Gil et al. 1997). Scintillation counting of the $[^{3}H]$-QNB-labeled receptors immunoprecipitated by each antisera and the $[^{3}H]$-QNB-labeled receptors not recognized by the specific antisera allowed for quantitation of the various receptor subtypes in these different ocular tissues (Gil et al. 1997). All five human receptor subtypes were detected in the iris sphincter muscle, and the m3 subtype was the predominant muscarinic receptor in all three ocular tissues. Specific quantities of the muscarinic receptors subtypes in the iris, the ciliary muscle and the ciliary process (expressed as percent immunoprecipitated by the subtype-selective antisera) are shown in Table 4 (Gil et al. 1997).

Table 4. Percent immunoprecipitation of mAChR subtypes by subtype selective antisera

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>m1</th>
<th>m2±SD</th>
<th>m3±SD</th>
<th>m4±SD</th>
<th>m5±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iris sphincter muscle</td>
<td>7.4</td>
<td>7.8±5.5</td>
<td>59.1±7.8</td>
<td>11.4±4.1</td>
<td>5.4±3.1</td>
</tr>
<tr>
<td>Ciliary muscle</td>
<td>0.8±1.3</td>
<td>5.4±2.6</td>
<td>73.5±0.9</td>
<td>4.9±4.7</td>
<td>2.4±2.1</td>
</tr>
<tr>
<td>Ciliary process</td>
<td>6.6±4.1</td>
<td>4.9±2.4</td>
<td>57.6±5.5</td>
<td>4.6±3.5</td>
<td>2.0±1.6</td>
</tr>
</tbody>
</table>

Values are mean ± SD from two to four independent experiments and represent the percentage of radiolabel that is immunoprecipitated out of the total $[^{3}H]$-QNB-bound receptor.
Rationale and framework for the development of peripheral pharmacological probes

The development of pharmacological probes for cholinergic system is based on the rationale that altered pharmacological response to cholinergic drugs may serve as a measure of changes in cholinergic system integrity. In addition to AD, normal aging is also associated with impaired central cholinergic function such as decreases in high affinity choline uptake and number of muscarinic receptor binding sites (Sherman & Friedman 1990). Therefore, normal aging may serve as a model for identification of future pharmacological markers for AD.

As with the development of central cholinergic probes (e.g. scopolamine), measurement of changes in pharmacological response to peripherally acting cholinergic agonists/antagonists in
1. healthy elderly versus young adults and
2. in patients with AD versus healthy elderly,
may provide a two-step framework for the development of peripheral cholinergic probes.

Previous studies investigating functional differences in response to peripheral probes of the cholinergic system (e.g. tropicamide) were limited to comparisons between healthy elderly versus patients with AD. Because quantitative changes due to normal aging have not been studied in the peripheral cholinergic system, it is uncertain what contribution normal ageing and AD itself may have to pharmacological responses with peripheral cholinergic probes in patients with AD. If a difference due to normal aging is found in response to a specific probe, it would also warrant further testing in patients to determine or quantify the changes that are due to cholinergic dysfunction associated with AD.
HYPOTHESES

1. Pupillary response to pilocarpine and tropicamide will be altered in healthy elderly compared to younger controls.

2. Scopolamine effect on cognitive function will be greater in normal elderly volunteers compared to young adult subjects.

OBJECTIVE

The objective of this study was to test the effect of normal ageing on a) tropicamide-induced increase in pupil diameter, b) pilocarpine-reversal of the tropicamide effect on pupil size and c) scopolamine effect on cognitive function in healthy elderly and young volunteers.

METHODS

_Subjects_. Elderly (range: 59 to 79 yr; n=10) and young (range: 18 to 40 yr; n=9) healthy volunteers were recruited by advertisements in local newspapers (Table 5). All subjects were Caucasian except subjects 2 (elderly), 6 (young) and 8 (young) who had a West-Indian, Korean and Afghan nationality, respectively. Subjects had good to excellent health as assessed by the General Health Questionnaire (Ware & Sherbourne 1992) and underwent a general ocular examination that included slit-lamp biomicroscopy by an ophthalmologist. An electrocardiogram was performed on the elderly subjects and read by a cardiologist prior to the study. Exclusion criteria were contraindication to any of the study drugs (tropicamide, pilocarpine or scopolamine), including a shallow anterior ocular chamber, history of intraocular surgery, cloudy cornea, glaucoma, any sign of cataracts, significant cardiac abnormality. Concomitant medications remained unchanged during the study and were assessed by interviews with the
subjects and their physicians when necessary. Cognitive status was assessed with the Mini Mental State Exam (MMSE) (Folstein et al. 1975) prior to the study, and scores for all subjects were within the category of having no cognitive impairment (24-30 out of a total score of 30) (Tombaugh & McIntyre 1992). Subjects refrained from alcohol, smoking, and caffeine containing food or beverages for at least 12 hours before and during the test sessions, and were advised to get a good night of rest before each session.

**Study medications.**

*Tropicamide* is a synthetic tertiary amine, non-selective competitive muscarinic antagonist (Dei et al. 1996) (Figure 5). Tropicamide binds to the human muscarinic receptor subtypes with similar affinity patterns (Table 3). The ophthalmic tropicamide solution is an aqueous solution (tropicamide hydrochloride) with pH 4 to 5.8 (The Merck Index 1989). Tropicamide produces pupillary dilatation (mydriasis) through blocking the action of ACh at the muscarinic receptors on the sphincter muscle of the iris. This results in relaxation of the iris sphincter muscle and pupil dilatation (Adler’s Physiology of the Eye 1992). The maximum tropicamide induced mydriatic effect occurs at approximately 20 to 40 minutes after ocular administration, and this effect may last for 6-7 hours with clinical doses. Tropicamide is used clinically prior to the examination of structures in the eye such as the fundus, at doses of 1 to 2 drops of 0.5% to 1% solution (AHFS Drug Information 1984).

*Pilocarpine* is an alkaloid obtained from *Pilocarpus microphyllus*, and is a non-selective cholinergic muscarinic receptor agonist (The Merck Index 1989) (Figure 5). Upon binding to the muscarinic receptors of the iris sphincter muscle, it produces contraction of this muscle and pupillary constriction (miosis) (Adler’s Physiology of the Eye 1992). This drug is used in the
treatment of open-angle glaucoma and the usual clinical dose is two drops of a 1% solution (AHFS Drug Information 1984).

**Scopolamine** is an alkaloid, non-selective muscarinic cholinergic antagonist, derived from the shrub *Hyoscyamus niger* (The Merck Index 1989)(Figure 6). Scopolamine readily crosses the blood-brain barrier. In therapeutic doses, scopolamine causes CNS depression which manifests as drowsiness, and anticholinergic effects include decreases in the function of recent memory and acquisition of new information. The drug metabolism pathways of scopolamine are currently unknown, and it is not known whether there are age related changes in the pharmacokinetics of scopolamine. There is however one study which showed that less than 6% of an oral dose of scopolamine is eliminated unchanged in the urine over a 24 hour time frame, suggesting its metabolism in the body (Putcha et al. 1989). The elimination half-life of scopolamine after an intravenous dose is 4.5 h ± 1.7 (mean ± SD) (Putcha et al. 1989).
Table 5. Description of the subjects

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Iris colour</th>
<th>Concomitant medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elderly subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>71</td>
<td>Male</td>
<td>Blue</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>Female</td>
<td>Blue</td>
<td>Conjugated Estrogens 0.625 mg/5days per week</td>
</tr>
</tbody>
</table>
| 3           | 79       | Male   | Brown       | Glibenclamide 25 mg/day  
Metformin HCl 1,500 mg/day  
Diltiazem HCl 180 mg/day |
| 4           | 72       | Male   | Brown       | None                    |
| 5           | 63       | Male   | Blue        | None                    |
| 6           | 70       | Male   | Blue        | Cilazapril 2.5 mg/day   |
| 7           | 59       | Female | Blue        | Cilazapril 2.5 mg/day   
Glibenclamide 2.5 mg/day  
Acetylsalicylic acid 325 mg/day |
| 8           | 68       | Male   | Blue        | None                    |
| 9           | 67       | Male   | Brown       | None                    |
| 10          | 74       | Male   | Blue        | Paroxetine 20 mg/day    |

Mean ± SEM

70 ± 2
**Table 5. Description of the subjects**

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>Iris colour</th>
<th>Concomitant medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38</td>
<td>Male</td>
<td>Green</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>Male</td>
<td>Green</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>Female</td>
<td>Blue</td>
<td>Levonorgestrel-Ethinyl Estradiol</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>Male</td>
<td>Brown</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>Male</td>
<td>Brown</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>Male</td>
<td>Brown</td>
<td>None</td>
</tr>
<tr>
<td>7</td>
<td>38</td>
<td>Female</td>
<td>Blue</td>
<td>Thyroxine 0.1 mg/day</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>Male</td>
<td>Brown</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>37</td>
<td>Male</td>
<td>Brown</td>
<td>None</td>
</tr>
</tbody>
</table>

*Mean ± SEM*  

33 ± 2
Figure 5. Structures of tropicamide and pilocarpine
Scopolamine

Figure 6. Structure of scopolamine
**Study design.** For all sessions, subjects arrived at the Human Psychopharmacology Laboratory at 10 AM. The laboratory light conditions were standardized to a luminance of 600 LUX. Sessions followed a balanced and randomized double-blind study design and were separated by a minimum of 4 days. During one session, subjects were administered tropicamide (20μl eye drop, 0.01%) (Alcon Canada Inc) to one eye, and placebo (Sodium Chloride 0.9%) (20μl eye drop) to the other eye. Pupil diameter was measured twice at baseline and at 17 time points over a period of 240 minutes by a computerized infra-red pupillometer (Kaplan 1995). In another session, tropicamide (20μl, 0.01%) was administered to both eyes followed at 25 minutes post-tropicamide by application of pilocarpine (20μl eye drop, 0.1%) (Alcon Canada Inc) to one eye and placebo to the other eye. In this session, pupil diameter was measured at 13 time points over a 215 minute period after pilocarpine administration. Eye drops were administered using a precision micro-pipette into the lower conjunctival cul de sac with the subject in the supine position. Subsequently, digital pressure was applied to the naso-lacrimal duct for one minute by the investigator.

In two separate test sessions, an indwelling catheter was inserted into the cephalic vein of the less-dominant forearm. After baseline assessment, an intravenous dose of scopolamine hydrobromide (0.5 mg) (Abbott Laboratories Ltd, Montreal Canada) or placebo was administered via slow injection over two minutes. Cognitive function was measured at baseline and at 0.5, 1, 1.5 and 2 hr after drug administration. At these assessment points, pupil diameter was also measured.
Assessment of scopolamine effect. Scopolamine induced memory and learning impairment was assessed by using a modified Buschke Selective Reminding test which has been used in previous studies with scopolamine by other investigators (Molchan et al. 1992, Sunderland et al. 1987, Broks et al. 1988, Buschke 1973). This test has been found to be sensitive to detect the cognitive impairment that is induced by scopolamine in both young and elderly individuals (Molchan et al. 1992, Broks et al. 1988, Sunderland et al. 1987, Sunderland et al. 1986).

Buschke Selective Reminding Test. The test was performed at each of the five assessment time points and consisted of a 12-word list in which the words had the same frequency of use in the English language and were categorically unrelated (Buschke 1973, Thorndike & Lorge 1944). Equivalent versions of the word lists were constructed and a different set was used at each time point after scopolamine or placebo administration. All 12 words were read to subjects (one word every three seconds) and the subjects were asked to recall as many words as possible during a 55 second time frame. This was followed by a 30 second pause interval, after which the investigator only read the words that were not recalled by the subject during the previous recall period. The subject was again asked to recall as many of the 12 word list as possible. This procedure was repeated seven times (total of 8 recall attempts). However, if the subject recalled all 12 words in two successive trials, the test was discontinued and maximum score (12) was assigned for each of the remaining recall trials. The total number of words recalled in eight trials was used as a measure of memory/learning at each time point.

Visuospatial praxis. Assessment was made using a block-construction subtest of the Wechsler Adult Intelligence Scale (WAIS) (Wechsler 1955). The test was administered at baseline and at 1.5 hours post scopolamine or placebo administration. The blocks used in each test were
identical, with two red sides, two white sides and two sides that are red and white. The subject was asked to make specific designs with the blocks during a specified limited time interval.

Subject-rated sedation and Digit Symbol Substitution Test. Subject-rated sedation was assessed at each time point using a 20 cm visual analogue scale that ranged from “not at all” at the left end to “extremely” at the right (Sellers et al. 1980). The digit symbol substitution subtest (DSST) of the WAIS was also performed at each time point to assess psychomotor function (Wechsler 1955). For the DSST, subjects were presented with a set of symbols and matching numbers and were asked to draw the correct symbol below each number during a 90 second interval.

Pupillometry. A previously validated infrared pupillometer connected to a personal computer was used for measurement of the pupil diameter (Kaplan 1995). The apparatus consisted of four infrared diodes that illuminated the eye, an infrared-sensitive video camera and a small computer-controlled display television for the eye. Each captured video image was processed to determine the edge of the pupil, and the maximal diameter value out of 40 computer-generated diameter measurements was recorded. During the pupillometry, the subject was seated in an armchair, with the chin placed on an adjustable chin-rest connected to the camera stand while the eyes were fixated onto a target at a standardized distance.

Data analysis. Data were analyzed using the Statistical Analysis System (SAS), version 6.11. The data on increase in pupil size after challenge with tropicamide and placebo were expressed at each time point as \([\text{pupil diameter after tropicamide} - \text{the corresponding pupil diameter after placebo}] \times 100/\text{pupil diameter after placebo at that time point}\). For the analysis of the pilocarpine data, the decrease in pupil size was expressed as \([\text{pupil diameter after pilocarpine} - \text{the corresponding pupil diameter...}]]
minus the corresponding pupil diameter after placebo] x 100/ [pupil diameter after placebo at that time point]. The tropicamide and pilocarpine data were tested for significant differences by two-way repeated measures ANOVA. The two variables in the ANOVA were age and time, where time was the repeated-measures variable. The scopolamine data were analyzed using a three-way repeated measures ANOVA, with three-way interaction. The variables in this ANOVA were age, treatment and time, with time as the repeated-measures variable. When ANOVA was significant (p <0.05), further multiple comparisons were performed at each time point using a t-test with Bonferroni correction, which maintained the significance level at p < 0.05. All data were presented as mean ± SEM.

RESULTS

There was no significant difference in MMSE scores between young (30.0 ± 0.3) and the elderly subjects (29.0 ± 0.2) (p >0.05). The level of education was also comparable in young and the elderly (17 y ± 1 versus 14 y ± 1) (p >0.05), and there were no significant differences in weight between the young (73 kg ± 5) and the elderly (75 kg ± 5) (p >0.05).

Tropicamide effect on pupil size. The time course of changes in pupil size after tropicamide administration in younger and older subjects is presented in Figure 7. The analysis of variance found a significant time effect (degrees of freedom=16, F=39, p<0.05) on pupil size but there was no significant effect of age, or age and time (degrees of freedom=16, F=1.34, p>0.05) on pupillary response to tropicamide. The mean peak increase in pupil diameter was observed at 50-minutes after tropicamide and was not significantly different between the two age groups (p >0.05). However, upon visual inspection of the data, it was noted that the tropicamide effect at
the 50-minute time point in the young group appeared as an outlying point (Figure 7). Two group comparative analysis (elderly versus young) were performed using the t-test on the descending limb of the tropicamide effect curve. This exploratory analysis found a significant difference at the 150 minute time point (p=0.02) (Figure 7).

**Pilocarpine effect on pupil size.** The time course of pilocarpine effect on pupil, subsequent to pharmacologically-induced increase in pupil size by tropicamide, is presented in Figure 8. The time of administration of pilocarpine was presented as the zero minute time point. The pilocarpine induced decrease in pupil size was faster and larger in the older group compared to the young group (Figure 8). The analysis of variance found a significant time (degrees of freedom=13, F=19.01, p<0.05) as well as age and time (degrees of freedom=13, F=2.51, p<0.05) effect on pupil response to pilocarpine. The peak placebo-corrected pilocarpine effect on pupil size was observed at 85 minutes after drug administration and the older subjects had a significantly greater mean decrease in pupil size at the 85, 125, 165 and 215-minute time points (34.0% ± 2.1, 30.6% ± 3.0, 28.9% ± 2.9, 23.9% ± 2.4, respectively) (% of placebo), compared to younger subjects (20.3% ± 5.2, 15.8% ± 5.4, 14.9% ± 3.0, 8.8% ± 4.9, respectively) (p <0.05). There was no significant difference in pupil size between the two groups at baseline (p >0.05). Gender, iris color and ethnicity did not affect pilocarpine effect on pupil size (p > 0.05).

**Scopolamine effect on cognitive function.** The time course of scopolamine induced decrease in word-recall for the Buschke Selective Reminding Test is illustrated in Figure 9. The analysis of variance found a significant time (degrees of freedom=3, F=7.18, p<0.05) as well as age, time and treatment effect on word-recall after scopolamine (degrees of freedom=3, F=3.06, p<0.05).
The impairment in word-recall was greater in the elderly at 60 (41.7% ± 10.8% of baseline), 90 (43.1% ± 12.5) and 120 (47.1% ± 12.7) minute time points compared to young subjects (58.7% ± 9.0, 60.6% ± 7.8, 103.0% ± 7.6, respectively) (p <0.05). Scopolamine effect on visuospatial praxis was not significantly affected by aging (p >0.05). Gender and ethnicity did not significantly influence scopolamine effect on cognitive function (p >0.05). The peak scopolamine induced increase in pupil size from baseline was similar in the young (35% ± 8) and the elderly group (32% ± 8) (p >0.05). Also, there was no significant difference in DSST scores and subject rated sedation between the young and older subjects (p >0.05).
Figure 7. Time course of tropicamide effect on pupil diameter in the young (Y) and elderly (E) subjects.
Figure 8. Time course of pilocarpine effect on pupil diameter following tropicamide-induced increase in pupil size, in young [Y] and elderly [E] volunteers.
Figure 9. Time course of changes in word recall after scopolamine administration in young [Y] and elderly [E] volunteers.
DISCUSSION

The present study is the first report on the comparative time course of pupil response to tropicamide in normal young and elderly subjects. We measured the tropicamide effect on pupil diameter using a computerized infra-red pupillometer and over a considerably long time frame to quantify the tropicamide effect in more detail. Our results suggest that tropicamide may not elicit possible functional changes in cholinergic system in the iris due to normal aging. The previous reports on tropicamide effects in patients with AD are reviewed in Table 6. The sample size in our study (10 elderly and 9 young subjects) had 80% power to show at least 25% difference in tropicamide effect at the 240 minute time point, when alpha was chosen as 0.05 (two-tailed test).

Tropicamide was initially suggested as a probe to differentiate patients with Down’s syndrome from normal young control subjects (Sacks & Smith 1989). Due to the similarities in the neuropathological lesions in AD and during the later stages of the Down’s syndrome, several studies were conducted in which response to tropicamide was tested as a potential biological diagnostic marker for AD.

The first study in which pupillary response to tropicamide was assessed in patients with AD concluded that tropicamide may be a valid diagnostic marker of AD (Scinto et al. 1994). Specifically, Scinto et al. (1994) administered a single drop of tropicamide (0.01%) to the conjunctival sac of patients diagnosed with probable AD based on standard clinical criteria (n=14), and to healthy age-matched controls (n=32). This was followed by pupil diameter assessment over a one-hour time frame, and at 29 minutes post drug administration, the patients with AD had a statistically significant increased response to tropicamide (23.4% ± 3.8%; change
from baseline) compared to the control group (5% ± 1.7%), with very small overlap in pupillary response between the AD and the control group (Figure 10). From their results, this group suggested that patients could be distinguished from normal controls based on a significantly increased pupillary dilatation response to tropicamide (>13% increase from baseline) when compared to age matched controls (<13% increase from baseline) at 29 minutes post-tropicamide administration. However, results of the subsequent studies were in variance with that reported by Scinto and colleagues (Table 6). A significant example of the tropicamide studies with the largest sample size (n=101) is the investigation by Growdon et al. (1997), in which response to tropicamide was compared between three groups consisting of 13 patients with mild dementia, 20 with moderate dementia and 17 with severe dementia, and with a control group of 51 healthy elderly subjects. Growdon et al. (1997) and the majority of the studies reported after Scinto et al. (1994) found no difference in response to tropicamide in patients with AD compared to control groups (Table 6). Few studies found an increased response to tropicamide in AD patients versus control subjects, or versus patients with extrapyramidal syndromes, and other dementias such as vascular dementia (Kálmán et al. 1997, Arai et al. 1996, Gomez-Tortosa et al. 1996). However, those studies also showed a large overlap in pupillary response to tropicamide between the AD and the controls (Kálmán et al. 1997, Arai et al. 1996, Gomez-Tortosa et al. 1996). In addition, the cut-off score (i.e. > 13% increase in pupil diameter from baseline post 0.01% tropicamide solution) proposed by Scinto et al. (1994) to differentiate AD patients could not be replicated. Taken together, the majority of the subsequent studies (Table 6) was unable to replicate the increased pupillary response to tropicamide in AD reported by Scinto et al. (1994).
FIGURE 10. The time course of tropicamide effect in patients with Alzheimer's disease (solid circles) and healthy elderly volunteers (open circles). Scinto et al. 1994, with permission.
<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Tropicamide dose</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sacks &amp; Smith 1989</td>
<td>Down's syndrome</td>
<td>13</td>
<td>(12-50)</td>
<td>Not reported</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at 3 time points over 60 minutes; by photograph</td>
<td>Greater increase in pupil diameter in Down's syndrome group versus the younger group:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20 min = 1.91 fold higher</td>
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<td>30-35 min = 2.54 fold higher</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45-60 min = 2.75 fold higher</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(p &lt; 0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p-value refers to difference from control group response</td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
<td>12</td>
<td>(14-58)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scinto et al. 1994</td>
<td>AD</td>
<td>14</td>
<td>74 ± 7</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and 7 time points over 51 minutes; by video pupillometer</td>
<td>Increase from baseline at 29 minutes:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>23.4% ± 3.8 (SEM)</td>
</tr>
<tr>
<td></td>
<td>Age-matched healthy controls</td>
<td>32</td>
<td>72 ± 6</td>
<td>Neuropsychological screen</td>
<td></td>
<td></td>
<td>5.0% ± 1.7 (SEM) (p = 0.009)</td>
</tr>
<tr>
<td>Study Id</td>
<td>Subjects</td>
<td>Sample size</td>
<td>Age (yr)</td>
<td>Diagnostic criteria</td>
<td>Tropicamide dose</td>
<td>Frequency of pupil size measurement and technique</td>
<td>Results</td>
</tr>
<tr>
<td>----------</td>
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</tr>
<tr>
<td>Arai et al. 1996</td>
<td>AD</td>
<td>25</td>
<td>71</td>
<td>NINCDS-ADRDA criteria; MMSE; Clinical Dementia Rating</td>
<td>0.01% solution (2 drops)</td>
<td>Pupil area measured at baseline and at 29 minutes; by video pupillometer</td>
<td>Increase from baseline at 29 minutes: AD group: 25.6 % ± 14.9</td>
</tr>
</tbody>
</table>

In the AD group, tropicamide effect at 29-minute time point was dependent on the ApoE ε4 allele: 4/4 43.1 % ± 12.7 4/3 23.2 % ± 3.8 (p <0.05) 3/3 20.0 % ± 14.1 (p <0.05) p-value refers to difference in pupil response in comparison to the subgroup homozygous for the ApoE ε4 allele

Non-AD Dementia: 13 69.3

Parkinson's (n=10) 16.7 % ± 9.7% (p <0.05) p-value refers to significance of difference from the AD group.

Frontal Lobe Dementia (n=2)

Progressive Supranuclear Palsy (n=1)

Healthy controls 11 75.2

Control group: 15.6 % ± 10.3 (p <0.05) p-value refers to significance of difference from the AD group.
<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Tropicamide dose</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fridh et al. 1996</td>
<td>AD</td>
<td>17</td>
<td>71.9</td>
<td>DSM-III-R and NINCDS-ADRDA criteria; MMSE score: 0-9 (n=5) 12-21 (n=10) 23 (n=2)</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and 30 minutes; by Flash photograph</td>
<td>Increase from baseline at 30 minutes: 19.7 % ± 11</td>
</tr>
<tr>
<td>Gomez-Tortosoa et al. 1996</td>
<td>AD</td>
<td>20</td>
<td>70.3</td>
<td>MMSE scores: 28-30</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and 6 time points over 40 minutes; by photographs and a ruler</td>
<td>19.1 % ± 9 (p &gt;0.05)</td>
</tr>
<tr>
<td>Non AD Dementia Vascular (n=5)</td>
<td>AD</td>
<td>7</td>
<td>(56-78)</td>
<td>Clinical and neuropsychological examinations, MRI and SPECT neuroimaging</td>
<td></td>
<td></td>
<td>17.4 % (p &gt;0.05)</td>
</tr>
<tr>
<td>Frontal lobe (n=2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extrapyramidal Disorders</td>
<td></td>
<td>13</td>
<td>(50-73)</td>
<td>Clinical and neuropsychological examinations</td>
<td></td>
<td></td>
<td>15.8 % (p &lt;0.05)</td>
</tr>
<tr>
<td>Parkinson's disease (n=9)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Supranuclear palsy (n=3)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Corticobasal degeneration (n=1)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age-matched healthy controls</td>
<td></td>
<td>30</td>
<td>(50-78)</td>
<td>MMSE score: 28-30</td>
<td></td>
<td></td>
<td>14.3 % (p &lt;0.001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p-values refer to significance of difference from the AD group</td>
<td></td>
</tr>
<tr>
<td>Study Id</td>
<td>Subjects</td>
<td>Sample size</td>
<td>Age (yr)</td>
<td>Diagnostic criteria</td>
<td>Tropicamide dose</td>
<td>Frequency of pupil size measurement and technique</td>
<td>Results</td>
</tr>
<tr>
<td>---------------</td>
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<td>-----------------------------------------------</td>
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<td>----------------------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>Litvan &amp; FitzGibbon 1996</td>
<td>Progressive Supranuclear palsy (PSP)</td>
<td>14</td>
<td>69 ± 2</td>
<td>Clinical criteria for probable PSP</td>
<td>0.01% solution</td>
<td>Pupil diameter measured at baseline and at 7 time points over 51 minutes; by video pupillometer</td>
<td>Peak increase from baseline: 39% ± 5 (p &gt;0.05)</td>
</tr>
<tr>
<td></td>
<td>Age-matched healthy controls</td>
<td>14</td>
<td>69 ± 2</td>
<td>MMSE score: 30 ± 1</td>
<td></td>
<td></td>
<td>27% ± 4</td>
</tr>
<tr>
<td>Loupe et al. 1996</td>
<td>AD not taking acetylcholine esterase inhibitors</td>
<td>9</td>
<td>75.6 (+ ± 5.2)</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.01% solution</td>
<td>Pupil diameter measured at 4 time points over 40 minutes (no baseline measure); by video pupillometer</td>
<td>Mean change in drug treated pupil diameter from untreated eye at 20 minutes (mm) 0.76 ± 0.39 mm</td>
</tr>
<tr>
<td></td>
<td>AD taking Acetyl Choline Esterase Inhibitor</td>
<td>10</td>
<td>70.4 (+ ± 10.3)</td>
<td>NINCDS-ADRDA criteria</td>
<td></td>
<td></td>
<td>0.95 ± 0.40 mm</td>
</tr>
<tr>
<td></td>
<td>Healthy controls</td>
<td>11</td>
<td>69.5 (+ ± 6.0)</td>
<td>Mattis Dementia Rating Scale; Boston Naming Test</td>
<td></td>
<td></td>
<td>0.52 ± 0.50 mm</td>
</tr>
<tr>
<td></td>
<td>Young healthy controls</td>
<td>10</td>
<td>36.3 (+ ± 11.4)</td>
<td>MMSE score: 29-30</td>
<td></td>
<td></td>
<td>0.51 ± 0.76 mm (p=0.11) p-value refers to significance of repeated measures analysis of variance</td>
</tr>
</tbody>
</table>
Table 6. Summary of Tropicamide Challenge Studies

<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Tropicamide dose</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>FitzSimon et al. 1997</td>
<td>AD</td>
<td>20</td>
<td>82.0 (72-89)</td>
<td>Diagnostic and Statistical Manual of Mental Disorders, 3rd edition, NINCDS-ADRDA criteria, MMSE score: ≤ 24</td>
<td>0.01% solution (40 μL)</td>
<td>Pupil diameter measured at baseline and 8 time points over 40 minutes; by Binocular Infrared pupillometer</td>
<td>Increase from baseline at 30 minutes: 14.5% ± 15.1</td>
</tr>
<tr>
<td>Age-matched healthy controls</td>
<td>20</td>
<td>81.5 (72-88)</td>
<td>MMSE score: ≥ 27</td>
<td>0.01% solution (50 μL)</td>
<td>Pupil diameter measured at baseline and 7 time points over 30 minutes; by video pupillometer</td>
<td>Increase from baseline at 30 minutes: 19.5% ± 17.0 (p &gt;0.05)</td>
<td></td>
</tr>
<tr>
<td>Graff-Radford et al. 1997</td>
<td>AD</td>
<td>23</td>
<td>71.6 (59-85)</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.01% solution (50 μL)</td>
<td>Pupil diameter measured at baseline and 7 time points over 30 minutes; by video pupillometer</td>
<td>Increase from baseline at 30 minutes: 4.72 % ± 15.10 (p &gt;0.05)</td>
</tr>
<tr>
<td>Non AD Dementia</td>
<td>6</td>
<td>67.8 (56-73)</td>
<td>Neuropsychological tests such as Dementia Rating Scale, Boston Naming Test</td>
<td>0.01% solution (50 μL)</td>
<td>Pupil diameter measured at baseline and 7 time points over 30 minutes; by video pupillometer</td>
<td>21.89 % ± 23.18 (p &gt;0.05)</td>
<td></td>
</tr>
<tr>
<td>Isolated memory difficulty</td>
<td>4</td>
<td>73.2 (68-78)</td>
<td>Neuropsychological tests such as Dementia Rating Scale, Boston Naming Test</td>
<td>0.01% solution (50 μL)</td>
<td>Pupil diameter measured at baseline and 7 time points over 30 minutes; by video pupillometer</td>
<td>1.66 % ± 6.87 (p &gt;0.05)</td>
<td></td>
</tr>
<tr>
<td>Age-matched healthy controls</td>
<td>22</td>
<td>68.7 (52-83)</td>
<td>Neuropsychological tests such as Dementia Rating Scale, Boston Naming Test</td>
<td>0.01% solution (50 μL)</td>
<td>Pupil diameter measured at baseline and 7 time points over 30 minutes; by video pupillometer</td>
<td>8.49 % ± 19.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p-values refer to significance of difference from the control group</td>
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<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Dose of tropicamide</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higuchi et al. 1997</td>
<td>Healthy subjects with ApoE ε 4 allele</td>
<td>16</td>
<td>56 ± 12</td>
<td>MMSE ≥ 28</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil area measured at baseline and 3 time points over 50 minutes; by pupillometer</td>
<td>Increase from baseline at 50 minutes for different ε 4 allele genotypes: 4/4 = 36.2% 3/4 = 23.6% (p &lt; 0.0001)</td>
</tr>
<tr>
<td></td>
<td>Healthy subjects without ApoE ε 4 allele</td>
<td>28</td>
<td>56 ± 9</td>
<td>MMSE ≥ 28</td>
<td></td>
<td></td>
<td>3/3 = 12.2% 2/3 = 7.7%, p-value refers to significance of difference in response to group with no ApoE ε 4 allele</td>
</tr>
<tr>
<td>Growdon et al. 1997</td>
<td>AD Mild Dementia (n=13) Moderate Dementia (n=20) Severe Dementia (n=17)</td>
<td>50</td>
<td>mean age greater than control subjects (p&lt;0.001)</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and 4 time points over 30 minutes; by pupil and corneal reflection tracking system</td>
<td>Increase from baseline at 30 min: 1 mm ± 1.1 1.1 mm ± 0.9 (p &gt; 0.05) p-value refers to significance of difference in response from AD group</td>
</tr>
<tr>
<td></td>
<td>Healthy elderly controls</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study Id</td>
<td>Subjects</td>
<td>Sample size</td>
<td>Age (yr)</td>
<td>Diagnostic criteria</td>
<td>Dose of tropicamide</td>
<td>Frequency of pupil size measurement and technique</td>
<td>Results</td>
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</tr>
<tr>
<td>Kálmán et al. 1997</td>
<td>AD</td>
<td>67</td>
<td>82.9 ± 6.9</td>
<td>DSM-IV and the NINCDS-ADRDA criteria</td>
<td>0.01% solution (1 drop)</td>
<td>Ratio of the left/right pupil (tropicamide/placebo) diameter measured at 30 minutes; by photograph</td>
<td>Ratio of tropicamide/placebo response 68.2 % ± 27.3 (p&lt; 0.001)</td>
</tr>
<tr>
<td></td>
<td>Vascular dementia</td>
<td>43</td>
<td>72.7 ± 10.8</td>
<td>MMSE score: 18.3 ± 7.3</td>
<td></td>
<td></td>
<td>47.1 % ± 23.8 (p &gt;0.05)</td>
</tr>
<tr>
<td></td>
<td>Age-matched healthy controls</td>
<td>37</td>
<td>78.3 ± 7.2</td>
<td>MMSE score: 28.2 ± 1.1</td>
<td></td>
<td></td>
<td>40.5 % ± 33.1 (p value refers to significance of difference from control group response)</td>
</tr>
<tr>
<td>Kurz et al. 1997</td>
<td>AD</td>
<td>20</td>
<td>71.3 (54-91)</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and at 6 time points over 30 minutes; by slit lamp</td>
<td>Peak increase in pupil diameter from baseline: 42% (mean)</td>
</tr>
<tr>
<td></td>
<td>Age-matched healthy controls</td>
<td>20</td>
<td>69.8 (52-92)</td>
<td>MMSE score: &gt; 27</td>
<td></td>
<td></td>
<td>36.5 % (p &gt;0.05) p-value refers to significance of difference in response from AD group</td>
</tr>
<tr>
<td>Reitner et al. 1997</td>
<td>AD</td>
<td>23</td>
<td>69 ± 11</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.01% solution (volume not reported)</td>
<td>Pupil diameter measured at baseline and every 15 minutes for 90 minutes; by infrared video pupillometer</td>
<td>Increase from baseline at 45 minutes post administration 18.6 % ± 11.0</td>
</tr>
<tr>
<td></td>
<td>Healthy controls</td>
<td>31</td>
<td>54 ± 14.2</td>
<td>MMSE score: = 30</td>
<td></td>
<td></td>
<td>No significant difference between response in control group and AD group</td>
</tr>
</tbody>
</table>
### Table 6. Summary of Tropicamide Challenge Studies

<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Dose of tropicamide</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaneyuki et al. 1998</td>
<td>AD</td>
<td>10</td>
<td>73.0 ± 3.7</td>
<td>NINCDS-ADRDA criteria MMSE score: 15.6 ± 3.7</td>
<td>0.01% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and 5, 10, 20, 30 and 45 minutes; by video pupillometer</td>
<td>Percent increase from baseline No difference between two groups (p = 0.54)</td>
</tr>
<tr>
<td></td>
<td>Healthy controls</td>
<td>10</td>
<td>71.9 ± 3.4</td>
<td>MMSE score: 29.0 ± 0.5</td>
<td></td>
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</tr>
</tbody>
</table>

AD - Alzheimer's disease; (MMSE) - Mini Mental State Examination; (NINCDS-ADRDA) - National Institute of Neurological, Communicative Disorders and Stroke and Alzheimer’s Disease and Related Disorders Association; (MRI) - Magnetic Resonance Imaging; (SPECT) - Single Photon Emission Tomography. The data are presented as mean ± SD (when SD was available), and/or range. Age-matching between the patients and control subjects was reported when it was clearly stated in the reviewed studies.
Pilocarpine is another peripherally acting pharmacological probe proposed to differentiate AD patients from healthy elderly. We found that the pilocarpine induced decrease in pupil diameter was faster and larger in the elderly compared to young controls. Our results are in agreement with the limited number of previous studies which compared pilocarpine induced decrease in pupil diameter (miosis) in healthy elderly controls versus cognitively impaired subjects with dementia of unspecified origin (Sitaram & Pomara 1981) or in patients with AD (Kaneyuki et al. 1998, Idiaques et al. 1994) (Table 7).

For example, Kaneyuki et al. (1998) measured pupil diameter at baseline and over a short 45 minute time frame following administration of two drops of pilocarpine (0.0625%) in patients with AD and elderly controls. The AD group had a significantly greater decrease in pupil diameter compared the control group. At 45 minutes post drug, a cut-off point of 23% decrease in diameter from baseline was obtained, with 85.7% of patients with AD having a decrease >23% from baseline and 90% of controls with a decrease in diameter <23%. Sitaram and Pomara (1981) also reported an increased miotic response to dilute pilocarpine (0.125%) in cognitively impaired elderly subjects versus age-matched controls. However, the results from that study (Sitaram & Pomara 1981) were not specific for AD, as the diagnosis of AD was not established, and the experimental group was diagnosed with dementia of unknown origin. Idiaquez et al. (1994) found an increased pupillary miotic response to pilocarpine (0.0625%) in AD. However, the methodology in that study was limited because the pupil diameter was assessed at a single time point, and without the use of a computerized pupillometer or a placebo control.
### Table 7. Summary of Pilocarpine Challenge Studies

<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Pilocarpine dose</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staram &amp; Pomara 1981</td>
<td>Cognitively impaired patients with unspecified dementia</td>
<td>15</td>
<td>74.6 ± 6.0</td>
<td>Global Deterioration Scale ≥ 3.5</td>
<td>0.125% solution (1 drop)</td>
<td>Pupil diameter measured at baseline and at 6 time points over 1 hour; by video pupillometer</td>
<td>Peak decrease from baseline: 46.3 % ± 12.0</td>
</tr>
<tr>
<td></td>
<td>Age matched healthy controls</td>
<td>11</td>
<td>70 ± 4.5</td>
<td>Global Deterioration Scale &lt; 3</td>
<td></td>
<td></td>
<td>30.7 % ± 13 (p &lt;0.01) p-value refers to significance of difference between cognitively impaired and control subjects</td>
</tr>
<tr>
<td>Idiaques et al. 1994</td>
<td>AD</td>
<td>26</td>
<td>72.3 (56-83)</td>
<td>MMSE score: 11.1 ± 1</td>
<td>0.0625% solution (2 drops)</td>
<td>Pupil diameter measured at baseline and at 20 minutes; by ruler and photograph</td>
<td>Decrease from baseline at 20 minutes: 1.28 mm ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Healthy controls</td>
<td>23</td>
<td>72.8 (60-83)</td>
<td>MMSE score: 28.1 ± 1</td>
<td></td>
<td></td>
<td>0.28 mm ± 0.6 (p &lt; 0.0001)</td>
</tr>
</tbody>
</table>
Table 7. Summary of Pilocarpine Challenge Studies

<table>
<thead>
<tr>
<th>Study Id</th>
<th>Subjects</th>
<th>Sample size</th>
<th>Age (yr)</th>
<th>Diagnostic criteria</th>
<th>Pilocarpine dose</th>
<th>Frequency of pupil size measurement and technique</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaneyuki et al. 1998</td>
<td>AD</td>
<td>14</td>
<td>72.0 ± 4.8</td>
<td>NINCDS-ADRDA criteria</td>
<td>0.0625% solution (2 drop)</td>
<td>Pupil diameter measured at baseline and 5, 10, 20, 30 and 45 minutes; by video pupillometer</td>
<td>Percent decrease from baseline at 45 minutes ≥ 23% in 85.7 % of AD patients</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>10</td>
<td>71.9 ± 3.4</td>
<td>MMSE score: 29.0 ± 0.5</td>
<td></td>
<td></td>
<td>&lt; 23% in 90% of control subjects (p &lt; 0.001) p value refers to a significant difference in response from AD group</td>
<td></td>
</tr>
</tbody>
</table>

AD - Alzheimer's disease; (MMSE) - Mini Mental State Examination; (NINCDS-ADRDA) - National Institute of Neurological, Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association; (MRI) - Magnetic Resonance Imaging; (SPECT) - Single Photon Emission Tomography. The data are presented as mean ± SD (when SD was reported), and/or range. Age-matching between patients and control subjects was reported when it was clearly indicated in the reviewed studies.
Overall, the studies reviewed in Table 7 suggest that pilocarpine may detect possible disease associated changes in the peripheral cholinergic system of the iris. Ideally, in studies with pilocarpine, baseline pupil diameter should be relatively large before challenge with pilocarpine, in order to optimize the detection of the pupillary constriction effects of pilocarpine. The induction of a large pupil diameter at baseline (pharmacologically or by adjustment of the ambient light intensity) may allow for better resolution of the pilocarpine effect on pupil diameter.

Our study was different in comparison to the previous studies with pilocarpine as it was placebo controlled, and the pupillary measurements were made very frequently over a longer time frame using a computerized pupillometer. In the present study, we also used a pharmacologically induced pupil dilatation by tropicamide at baseline, before challenge with pilocarpine, in order to optimize the detection of the pupillary constriction effects of pilocarpine.

In the interpretation of our results, it should be acknowledged that the differences in pilocarpine effect on the pupil between young and the elderly subjects may not be solely due to a dysfunction in the peripheral cholinergic system of the iris. An alternative explanation may be that there can be age related increase in corneal permeability to pilocarpine due to, for example, changes in lacrimal flow and ocular microcirculation. Differences in corneal permeability to pilocarpine between the two groups may lead to an increased amount of pilocarpine at the site of action in the elderly which may be responsible for the difference in response observed between young and elderly individuals. Nevertheless, our results are the first to provide evidence that pilocarpine may detect changes in the peripheral cholinergic system due to normal aging. These findings
warrant further experiments in patients with AD to quantify disease effects and to observe how they may compare to effects due to normal aging.

The elderly have been shown to be more sensitive to the cognitive effects of scopolamine than young individuals, and it has been suggested that this may be due to central cholinergic system deficit associated with aging (Flicker et al. 1992). In this study we assessed the effects of scopolamine on memory in the young and older subjects to study whether the older individuals have an increased sensitivity to scopolamine. Therefore, scopolamine acted as a positive control, which suggested that the elderly had an impaired central cholinergic system function, and that we were investigating possible changes in the peripheral cholinergic system in a model where parallel central cholinergic changes have also occurred. One limitation of this model is that it is uncertain whether differences in sensitivity to the cognitive effects induced by scopolamine are due to increased plasma concentrations of scopolamine in the elderly. Limited data from a single time point assessment of scopolamine plasma concentrations at 2 hours post intramuscular injection of three different doses of scopolamine (0.002, 0.004, and 0.007 mg/kg) suggest that there are no differences in kinetics of scopolamine due to aging (Ray et al. 1992). However, further systematic studies with multiple time point analysis of plasma concentrations of scopolamine in the elderly need to be conducted in the young and in the elderly to rule out age-related pharmacokinetic causes for the increased scopolamine effects. Overall, our results were in agreement with previous studies showing sensitivity in the elderly to scopolamine effects on memory (Molchan et al. 1992, Broks et al. 1988).
In conclusion, our results suggest that dilute tropicamide (0.01%) may not be able to detect potential age-related changes in the peripheral cholinergic system of the iris. This is the first study to indicate that pilocarpine may detect changes in the peripheral cholinergic system due to normal aging. The results with pilocarpine warrant further studies to assess the ability of pilocarpine to detect potentially larger changes in the peripheral cholinergic system due to AD.
Chapter IV

Future Research and Recommendations
INTRODUCTION

Central cholinergic dysfunction in AD presents with a decreased synthesis of ACh and atrophy of the main projections from the cholinergic nuclei. If similar neuropathological changes also occur in the peripheral cholinergic system of the iris, then these changes may serve as a basis to elucidate the mechanism of an increased response in the eye to topically applied cholinergic and anti-cholinergic drugs. For example, denervation/degeneration of the post-ganglionic cholinergic fibers in the iris could produce a decreased release of ACh at the receptor sites. Upon challenge with a muscarinic cholinergic antagonist such as tropicamide, there would be less competition by the endogenous ligand ACh which may lead to an increased pupillary dilatation response to tropicamide. Similarly, decreased release and synthesis of ACh due to atrophy of cholinergic neurons or changes in ACh biosynthesis may produce an increased response to cholinergic agonists such as pilocarpine, due to compensatory muscarinic cholinergic receptor upregulation. A possible limitation to clinical value of ocular probes in AD is that muscarinic receptors in the eye mainly consist of the m3 subtype. Since there appears to be no significant change in this receptor subtype in AD, experiments performed using the ocular cholinergic system model may be limited in terms of their applicability to changes in the central cholinergic system. Further studies are needed to assess biochemical and/or anatomical changes in the peripheral cholinergic system that may be responsible for such functional observations in AD and during normal aging.

Confounding factors which may cause differences in outcome of studies with tropicamide and pilocarpine

The inability to replicate the positive findings with tropicamide reported by Scinto and co-workers (1994) prompted attempts to explain the discrepancies in conclusions between the
studies. Several clinical, methodological and pharmacological factors may confound the outcome of studies with peripheral (ocular) cholinergic probes in AD, which may lead to false positive or negative results (Table 8).

1. **Uncertainty in diagnosis and variations in clinical stages of AD in subjects**

AD is likely to have a heterogeneous neurochemical pathogenesis leading to clinical symptoms of dementia. Therefore, selection of an experimental group based on clinical diagnostic criteria (due to a lack of established cholinergic biochemical criteria which can be tested ante-mortem) may introduce a large amount of variability in response to cholinergic drugs within the AD group. A related problematic issue is the difficulty in distinguishing AD from other types of dementia. Currently, it is also not clear what influence progression of AD or the severity of dementia may have on the functional response to peripherally acting pharmacological cholinergic markers. Care should therefore be taken to conduct the studies in patients who are in the same stage of the disease as established based on current standardized clinical diagnostic guidelines. The potential of a pharmacological probe may be better assessed when measurements are taken in a relatively homogeneous experimental group with small variance in age of onset of the disease, in clinical stage and form of AD (i.e. familial or sporadic). Ideally, the sample size should be sufficiently large to allow the comparison of results in such subgroups of patients with AD.

2. **Genetic factors**

Apolipoprotein E genotype is considered as a risk factor for the development of AD. In particular, the possession of the ε4 allele as opposed to the ε2 and ε3 alleles significantly increases the risk for AD and modifies the age of onset of the disease in a gene-dose dependent fashion (Lucotte et al. 1995, Corder et al. 1993, Mayeux et al. 1993, Stritmatter et al. 1993). It
was also suggested that genetic factors and the ApoE genotype may influence the pupillary response to tropicamide in patients with AD or in healthy controls. For instance, Arai et al. (1996) found a greater increase in pupil diameter after tropicamide challenge in patients with AD who are homozygous for the ε4 allele, compared with patients heterozygous for the ε4 allele and those without the ε4 allele. In a recent study, Higuchi et al. (1997), reported that an increased response to tropicamide could be elicited in healthy normal subjects carrying the ApoE ε4 allele. This could suggest that the increased response observed to tropicamide may not be specific to patients with AD, but may also occur in elderly control subjects with the ApoE ε4 allele. Alternatively, the increased response to tropicamide in this subgroup of the healthy population may represent an increased risk for the development of AD (as determined by the dose of the ε4 allele) and that these control subjects with no apparent cognitive deficit may develop AD in the future if longitudinally followed-up for a sufficient period of time.

Although it is unclear what the mechanism of such an interaction between Apo E genotype and response to tropicamide may be, it could potentially produce heterogeneity in response between subjects with different allelic variants of this gene within the AD group and the normal elderly controls. Such within-group variability may therefore cause some decrease in the probability of detecting differences between patients with AD and normal control subjects. However, these genetic studies overall suggest that even if genetic load of the ε4 allele may modulate tropicamide effect in the eye, the ocular tropicamide challenge test is not specific enough to aid in diagnosis of the AD.
3. Changes in cholinergic system due to normal aging

In addition to the AD, normal aging also causes changes in the central cholinergic system. As discussed in chapter 3, previous studies investigating functional differences in response to peripheral probes of the cholinergic system (e.g. tropicamide/pilocarpine) were limited to comparisons in between healthy elderly versus patients with AD. Some of the studies listed in Table 6 (Reitner et al. 1997, Growdon et al. 1996) did not match the subjects in the AD and the control groups for age which might have contributed to inconsistent results in studies with tropicamide. Currently, there have been no studies assessing the possible changes in the peripheral cholinergic system of the iris due to normal aging. Therefore, studies are needed to assess potential age related variability in response to drugs acting on the peripheral cholinergic system, and its effect size.

4. Concomitant medications, disease and baseline pupil diameter

The testing of pharmacological markers in elderly patients/controls may be problematic, as these subjects will often be on concomitant medications that may affect response to peripheral cholinergic probes. For example, concomitant medications with cholinergic actions such as tricyclic antidepressants are frequently prescribed in the elderly population, and may confound the results of such studies (Morgan et al. 1988). In addition, cognitive enhancing drugs such as AChE inhibitors when administered to patients with AD may produce effects on the peripheral cholinergic system and affect response to peripheral pharmacological markers. In cases where concomitant medications are not avoidable, an attempt should be made to keep the dosing regimen constant for the concurrently administered medications with cholinergic effects throughout the study period.
Patients should be screened for frequent ocular abnormalities in the elderly such as glaucoma and cataracts which would influence the pupillary response to cholinergic drugs. Physiological factors such as wakefulness of the subject may affect the pupil diameter measurements (Löwenstein & Loewenfeld 1962). It is known that just before sleep, the pupil diameter decreases, and therefore measurement of pupil diameter in a darkened room, for a prolonged time, may induce sleep in subjects and introduce experimental error.

Baseline pupil diameter is also an important factor to take into account, as the diameter at baseline may influence the size of the drug effect observed. If the pupil diameter is large at baseline, and a drug such as tropicamide is administered, the maximal drug effect (mydriasis) may not be measurable if the pupil is close to the maximal physiological diameter before drug administration. Therefore, drug effect may appear similar between elderly controls and patients with AD, simply due to the fact that in both groups maximal pupil diameter was reached before maximal drug effect could be assessed. Similarly, measurement of pilocarpine induced decrease in pupil diameter should not be limited by having a baseline pupil diameter that is close to the minimum physiological pupil diameter:

5. **Ocular pharmacokinetic factors**

The changes in response to cholinergic drugs may be affected by ageing or disease effects on ocular pharmacokinetics. Ocular drug absorption is the process by which the proportion of administered drug retained by the pre-corneal tear film is absorbed through the layers of the cornea including the corneal epithelial and endothelial layers. Subsequent to absorption, the drug enters the anterior chamber which contains the aqueous humor, and from there it may distribute
to its site of action at receptor sites on the muscles of the iris. The bioavailability of applied drugs (i.e. amount reaching the aqueous humor) may be contingent upon drug elimination prior to absorption, physical characteristics of the drug that influence its absorption through the layers of the cornea and potential age or disease associated pharmacokinetic changes in the eye. To optimize bioavailability and have reproducible results, these factors should be considered in the design of well controlled studies where pharmacological response is assessed in the eye as a measure of the cholinergic function.

The amount of drug that is absorbed through the layers of the cornea to the aqueous humor subsequent to instillation may be influenced by several factors. It has been determined that the human conjunctival cul-de-sac, in which eye drops are administered, normally holds a tear volume of 7 μl to 10 μl, and has a maximum capacity of approximately 30 μl (Mishima et al. 1966). Also, approximately 10% to 15% of this volume is replaced per minute under physiological conditions (Mishima et al. 1966).

Previous studies have investigated the impact of the volume of drug that is administered to the eye on drug bioavailability in aqueous humor. For example, it was shown that the fraction of the total dose absorbed increases as the volume of drug administered decreases (Patton 1977). In another study in rabbits, a reduced dose of pilocarpine delivered in a small drop volume of 5μl produced the same concentration of drug absorbed into the aqueous humor as a larger dose administered in an increased drop volume of 25 μl (Patton & Francoeur 1978). These data suggest that a smaller volume of drug administered to the eye results in more efficient drug absorption as the smaller volume of drug administered would have less probability of exceeding
the maximum capacity of the conjunctival cul-de-sac (30 μl), and therefore would not be spilled out of the eye.

In the majority of studies in which tropicamide challenge was assessed as a biological marker for AD, the precise volume of the drop administered was not reported (Reitner et al. 1997). The volume of drops from commercial eye drop dispensers ranges from 40 μl to 50 μl (Mishima et al. 1966), and therefore if these volumes are applied, a large fraction of the dose may be lost out of the cul-de-sac during drug administration. This may cause a smaller fraction of the applied dose to be absorbed, compared to studies which administered the same dilution of drug in smaller drop volumes less than 50 μl (FitzSimon et al. 1997). Also to be considered is the observation that the drainage of instilled drug into the nasolacrimal duct is a function of instilled volume which may affect ocular bioavailability (Chrai et al. 1973). Techniques such as digital pressure applied to the nasolacrimal duct has been demonstrated to increase bioavailability after drug administration (Duke-Elder 1962). Mechanistically this may be due to increased corneal contact time leading to greater intraocular penetration. Other factors that may affect the ability of the drug to reach its site of action are a rapid blinking rate, increased tearing, entrapment of solution underneath eye lids and the drug administration technique used, which are reviewed in more detail elsewhere (Fraunfelder 1976).

Differences in the physical characteristics can make some drugs more suitable as pharmacological markers. It is thought that the drug transfer from the corneal epithelium to aqueous humor is the rate-limiting step in the determination of aqueous humor drug concentrations (Mishima et al. 1966). This mechanism appears to be true for the transfer of pilocarpine and nonionized basic alkaloids which are rapidly taken up by the lipid containing epithelium where they are converted
to the ionized water soluble form and released into the anterior chamber (Sieg & Robinson 1976, Cogan & Hirsch 1944). On the other hand, watersoluble fluorescein is trapped in the corneal stroma which serves as a depot, and resistance due to the lipophilic corneal endothelium serves as the rate-limiting factor (Mishima et al. 1966). Therefore, differing physical characteristics of drugs may allow for sequestration in different ocular depots, and may lead to differential bioavailability between drugs, and differential potentials as biological markers of the cholinergic system function.

It has not been studied how aging or AD may affect the bioavailability of drugs to the iris. Changes in layers of the cornea may affect the ability of the drugs to reach the site of action and result in age or disease associated changes in bioavailability. In a study by FitzSimon and colleagues (1997), AD associated changes in corneal permeability were assessed to understand whether increased pupillary effects of cholinergic drugs in patients with AD may be due to altered kinetics of tropicamide. In that study, the bioavailability in the anterior chamber of topically applied fluorescein sodium was measured 2 hours post administration in patients with AD and healthy elderly control subjects. This study found no significant difference in the bioavailability of fluorescein in the anterior chamber between the two groups, suggesting that there may be no difference in corneal permeability to topically applied drugs in AD. Caution should be taken in generalizing from this study using fluorescein to bioavailability of tropicamide, pilocarpine or other drugs in AD or during normal aging, as the corneal pharmacokinetics of each drug may be differentially affected by these factors. Overall, further studies need to be conducted to assess possible disease or age-related changes in the corneal structure and ocular pharmacokinetics of cholinergic drugs. These confounding factors should be controlled where possible in order to assure the reproducibility of results (Table 8).
Table 8. Factors which may confound the outcome of ophthalmological studies with peripheral pharmacological cholinergic probes.

<table>
<thead>
<tr>
<th>Pharmacological confounders</th>
<th>Clinical and experimental confounders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Differences in corneal permeability between the AD patients, healthy elderly and young adults</td>
<td>Criteria and techniques used to diagnose AD</td>
</tr>
<tr>
<td>Differences in amount of the drug administered (e.g. lack of control for drop size between subjects)</td>
<td>Type and clinical stage of AD</td>
</tr>
<tr>
<td>Water/lipid solubility of the drugs, and drug concentration in the aqueous humor versus in the tear film on the cornea</td>
<td>Genetic make-up of the subjects (e.g. apo E genotype)</td>
</tr>
<tr>
<td>Technique for the application of the drug (e.g. loss through the nasolacrimal duct or excessive blinking)</td>
<td>Effect of normal aging on cholinergic response (e.g. using healthy elderly controls not matched for age)</td>
</tr>
<tr>
<td>Pharmacology and the cholinergic receptor affinity profile of the selected probe</td>
<td>Presence of ocular disease or concomitant medications which may alter ocular physiology or the mechanical properties of the iris</td>
</tr>
<tr>
<td></td>
<td>Light conditions during the measurement of pupil diameter at baseline and after drug administration</td>
</tr>
</tbody>
</table>
CONCLUSIONS

1. Scopolamine effect on cognitive function is increased during normal aging, in agreement with previous studies showing an age-related decline in central cholinergic function.

2. There is an age-related increase in pupil response to pilocarpine whereas the effect of tropicamide on pupil size does not appear to change with aging.

3. The findings with pilocarpine warrant further experiments to evaluate the pupillary response to pilocarpine in patients with AD.

RECOMMENDATIONS

Differences in ocular response to cholinergic drugs remain as a viable, noninvasive and practical strategy to develop peripheral pharmacological probes for the AD. Future studies with pilocarpine and other cholinergic drugs are needed to determine the within-subject and between-group (AD patients versus healthy elderly) reproducibility of cholinergic challenges to establish their clinical diagnostic value. Large sample sizes would be necessary to allow for comparisons in different patient populations with AD (e.g. familial versus sporadic forms of the disease). The specificity and reliability of an increased response to peripherally acting cholinergic agents in AD should be compared with responses in patients with dementia of non-AD origin. Confounding factors such as uncertainty in the clinical diagnosis and stages of the participating subjects with AD, techniques used to measure the pupil diameter (e.g. computerized pupillometry versus photographs), genetic factors (e.g. Apolipoprotein E genotype), changes in ocular pharmacokinetics due to aging or disease should be controlled carefully in future studies.
References


Burford NT, Tobin AB, Nahorski SR. Differential coupling of m1, m2 and m3 muscarinic receptor subtypes to inositol 1,4,5-trisphosphate and adenosine3',5' cyclic monophosphate accumulation in Chinese hamster ovary cells. J Pharmacol Exp Ther 1995;274:134-42.


Dunnett SB. Comparative effects of cholinergic drugs and lesions of nucleus basalis or fimbria-fornix on delayed matching in rats. Psychopharmacology 1985;87:357-63.


Mufson EJ, Jaffar S, Levey AI. m2 muscarinic acetylcholine receptor-immunoreactive neurons are not reduced within the nucleus basalis in Alzheimer’s disease: Relationship with cholinergic and galaninergic perikarya. J Comp Neurol 1998;392:313-29.


Sacks B, Smith S. People with Down’s syndrome can be distinguished on the basis of cholinergic dysfunction. J Neurol Neurosurg Psychiat 1989;52:1294-5.


