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JUHANA AURA

INTERACTION OF MUSCARINIC ACETYLCHOLINE AND *N*-METHYL-D-ASPARTATE -TYPE GLUTAMATE RECEPTORS IN THE REGULATION OF SPATIAL LEARNING AND MEMORY

Doctoral dissertation

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Department of Neuroscience and Neurology, University of Kuopio
Department of Neurology, Kuopio University Hospital

Distributor: Department of Neurology
University of Kuopio
P.O. Box 1627,
FIN-70211 Kuopio,
FINLAND
Tel. +358 17 162 689
Fax +358 17 162 048

Author's address: Department of Neuroscience and Neurology
University of Kuopio
P.O. Box 1627,
FIN-70211 Kuopio
FINLAND
Tel. +358 17 162 014,
Fax. +358 17 162 048

Supervisors: Docent Heikki Tanila, M.D., Ph.D.
Department of Neuroscience and Neurology
University of Kuopio
Kuopio University Hospital

Dr. Sirja Ruotsalainen, Ph.D.
Department of Social Pharmacy
University of Kuopio

Reviewers: Professor Esa R. Korpi
Institute of Biomedicine
Pharmacology
University of Helsinki
FINLAND

Professor John K. Robinson, Ph.D.
Department of Psychology
State University of New York at Stony Brook
N.Y., USA.

Opponent: Dr. Paul Dudchenko, Ph.D.
Department of Psychology
University of Stirling
SCOTLAND, UK

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ABSTRACT

Degeneration of cholinergic cells in the basal forebrain correlates with the cognitive impairment seen in Alzheimer's disease (AD), but so far the cognitive improvement of AD patients receiving cholinergic therapy has only been modest at best. In addition, degeneration of glutamate-containing cells has been observed in AD, thus suggesting that dysfunction of glutamatergic receptors may contribute to the cognitive decline of AD patients. The aim of this study was to investigate the role and possible interaction of the cholinergic system and N-methyl-D-aspartate (NMDA) glutamate receptors in the regulation of working memory and spatial navigation in rats. The role of muscarinic and NMDA receptors in the regulation of spatial working memory was studied using the delayed non-matching to position (DNMTP) task and intracerebroventricular administration of the muscarinic receptor antagonists scopolamine, pirenzepine and methoctramine, as well as the NMDA receptor antagonist CPP. The contribution of cholinergic system and NMDA receptors to age-related deficit in spatial navigation was assessed using the Morris water maze task and intraperitoneal administration of D-cycloserine and tetrahydroaminoacridine. The main results were the following. 1) Blockade of muscarinic receptors in the central nervous system with scopolamine and pirenzepine delay-independently disrupted DNMTP performance, suggesting a non-mnemonic effect on performance. Selective blockade of M_2 receptors with methoctramine delay-dependently improved performance in the DNMTP task, suggesting a specific, although modest improvement of working memory at long delays. 2) Blockade of central NMDA receptors with CPP resulted in a delay-independent defect, while combined administration of subthreshold doses of CPP and scopolamine disrupted only non-mnemonic task parameters (motor activity, attention or motivation). Combined CPP and pirenzepine administration at subthreshold doses had no effect on DNMTP task performance. These results suggest that combined blockade of central non-M_1 receptors and NMDA receptors disrupts non-mnemonic aspects of DNMTP performance. 3) In the prefrontal cortex, CPP dose-dependently disrupted maintenance of working memory, whereas scopolamine disrupted only non-mnemonic parameters. 4) D-cycloserine and tetrahydroaminoacridine alleviated age-related deficit in spatial navigation when administered either separately or in combination with subthreshold doses. 5) However, the alleviating effect of both D-cycloserine and tetrahydroaminoacridine (separately or in combination) disappeared after pre-training under different conditions, including pre-training for non-spatial escape strategies. In contrast, the age-related spatial navigation defect still remained after the pre-training procedures. These results suggest that tetrahydroaminoacridine and D-cycloserine do not themselves alleviate age-related spatial memory deficit, but may enhance procedural aspects of water maze learning in aged rats. CONCLUSION: Although the conjoint modulation of muscarinic and NMDA receptors most likely has no direct effect on memory functions, the indirect stimulating effects on attention, arousal and motivation might help alleviate cognitive impairment in AD patients.

National Library of Medicine Classification: WT155, WT104, QV77, QV126, WM173.7

Medical Subject Heading: Alzheimer disease; aged; cholinergic agents; N-methylaspartate; receptors, cholinergic; muscarinic antagonists; learning; memory; memory, short-term; drug therapy; rats; cognition; drug interactions; spatial behavior

This book is dedicated to Annamari and Konrad

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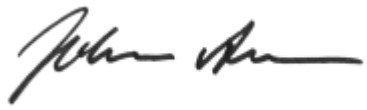


Juhana Aura

ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
ANOVA	analysis of variance
ChAT	choline acetyltransferase
CNS	central nervous system
CPP	(+/-)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid
DCD	delayed conditional discrimination task
DCS	D-cycloserine
dmPFC	dorsomedial prefrontal cortex
dlFC	dorsolateral frontal cortex
DMTP	delayed matching to position
DNMTP	delayed non-matching to position
HC	hippocampus
i.c.	intracerebral
i.c.v.	intracerebroventricular
LTP	long-term potentiation
M_{1-5}	muscarinic acetylcholine receptor subtypes 1-5
MS	medial septal nucleus
NMDA	*N*-methyl-D-aspartate
NBM	nucleus basalis of Meynert
NR1-2	NMDA receptor subunits 1-2
PFC	prefrontal cortex
SPSS/PC+	statistical package for social sciences/personal computer
THA	tetrahydroaminoacridine (tacrine)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-V**.89

I Aura J, Sirviö J, Riekkinen P Jr. Methoctramine moderately improves memory but pirenzepine disrupts performance in delayed non-matching to position test. *Eur. J. Pharmacol, 333:129-134, 1997.*

II Aura J, Riekkinen P Jr. Blockade of N-methyl-D-aspartate and muscarinic receptors jointly disrupts performance in delayed non-matching to position task. Submitted.

III Aura J, Riekkinen P Jr. Blockade of NMDA receptors located at the dorsomedial prefrontal cortex impairs spatial working memory in rats. *Neuroreport, 10:243-248, 1999.*

IV Aura J, Riekkinen M, Riekkinen P Jr. Tetrahydroaminoacridine and D-cycloserine stimulate acquisition of water-maze spatial navigation in rats. *Eur. J. Pharmacol, 342:15-20, 1998.*

V Aura J and Riekkinen P Jr. Pre-training blocks the improving effect of tetrahydroaminoacridine and D-cycloserine on spatial navigation performance in aged rats. *Eur J Pharmacol. 390: 313, 2000.*

CONTENTS

1. INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia, accounting for approximately 50% of all dementias in western countries (Francis et al., 1999). The first symptoms of AD include difficulties in learning new information and impairment of recent memory (Kaye, 1998). Typical pathological changes in AD are beta amyloid plaques, neurofibrillary tangle formation and loss of cholinergic cells in the basal forebrain. Cholinergic cell death has been the basis for the cholinergic hypothesis of AD and the development of the first AD drugs, the acetylcholinesterase (AChE) inhibitors (Bartus et al., 1982). AChE inhibitors decrease the cleavage of acetylcholine (ACh) in the synaptic cleft, thus enhancing the compromised cholinergic function in AD. During the design of the studies presented in this thesis, the AChE inhibitor tetrahydroaminoacridine (tacrine) was the only drug available for relieving the symptoms of AD. Today, three other AChE inhibitors, donepezil, rivastigmine and galantamine, are in clinical use. These new AChE inhibitors are well tolerated and have proved to be safer than tacrine, which is liver toxic in humans. However, the cognition enhancing effect of even the new AChE inhibitors is modest at best, clearly underscoring the urgent need for developing new treatments for AD.

The pathogenesis of AD is complex and involves several different biochemical pathways. In addition to disrupted cholinergic function, a loss of glutamatergic terminals in neocortical areas and the hippocampus is seen in AD (Francis et al., 1999). Moreover, the number of NMDA receptors decreases over the course of the disease, suggesting that decreased NMDA receptor function may be related to the symptoms of AD (Wang et al., 2000; Young, 1987). On the other hand, it has been suggested that that NMDA receptor-mediated Ca^{2+} toxicity may contribute to nerve cell degeneration in the cortex and hippocampus, thus implicating *increased* NMDA receptor activity as one possible cause for neuronal cell death in AD (Maragos et al., 1987). It appears that the NMDA receptor is a Janus-faced mediator with complex mechanisms involved in AD pathology and symptoms.

As in AD, defects in NMDA receptor-mediated neurotransmission as well as dysfunction of the brain cholinergic system occur during aging, especially in laboratory rodents (Amenta et al., 1995; Michalek et al., 1990; Mitchell and Anderson, 1998; Tamaru et al., 1991). In addition, aged rats show a spatial learning deficit in the Morris water maze task which has been linked to dysfunction of the hippocampus and the septo-hippocampal pathway

(Gallagher and Nicolle, 1993; Nilsson and Gage, 1993). Because of this link between dysfunctional hippocampus and spatial learning deficit, aged rats have been used to model the memory impairment occurring in AD (Riekkinen et al., 1998). In rodents, performance in operant-delayed working memory tasks has also been linked to proper functioning of the hippocampus, a brain structure that is damaged early in AD (Aggleton, 1992; Dunnett, 1985; Dunnett et al., 1990; Maruki et al., 2001). In addition to the hippocampus, the performance of rodents in delayed tasks depends on the function of the prefrontal cortex, a structure known to be important for the regulation of working memory and attentional control of behaviour (Dunnett, 1990; Dunnett et al., 1990).

Recently, a new drug has been introduced for the treatment of AD. The NMDA antagonist memantine has been used for over ten years in Germany as a neuroprotective agent, and is now gaining acceptance throughout the world. Thus far, the beneficial effects have been modest, as with the cholinesterace inhibitors; however, it is only a matter of time until studies with combined administration begin to bear fruit. Although the motivation for treating AD symptoms with memantine is based on an approach different from our model, possible interactions between the cholinergic system and the NMDA receptors are more interesting today than ever before. A possible benefit resulting from combined medication is the use of smaller drug doses with probably fewer side effects. For future drug development, a growing amount of studies are needed aimed at examining the interaction of NMDA receptors and the cholinergic system in animal models.

In the present series of studies, we aimed to examine the interaction between cholinergic and NMDA receptor medication. Firstly, we studied whether separate or combined blockade of brain NMDA and muscarinic receptors induces working memory deficit in the delayed non-matching to position task. This task is also capable of monitoring whether non-mnemonic aspects of performance, such as sensorimotor disturbance or attentional dysfunction, contribute to behavioral changes. We focused this study on the prefrontal cortex, which is the brain structure most closely implicated in spatial working memory. Secondly, we studied whether conjoint stimulation of cholinergic system and NMDA receptors attenuates an age-related spatial navigation deficit in rats undergoing the Morris water maze task. We also used a modification of the task, in which pre-training of the rats helped them to acquire the basic skills needed in this task, thus enhancing the task specificity for testing the spatial aspects of

navigation behaviour.

2. REVIEW OF THE LITERATURE

2.1 Classification of memory

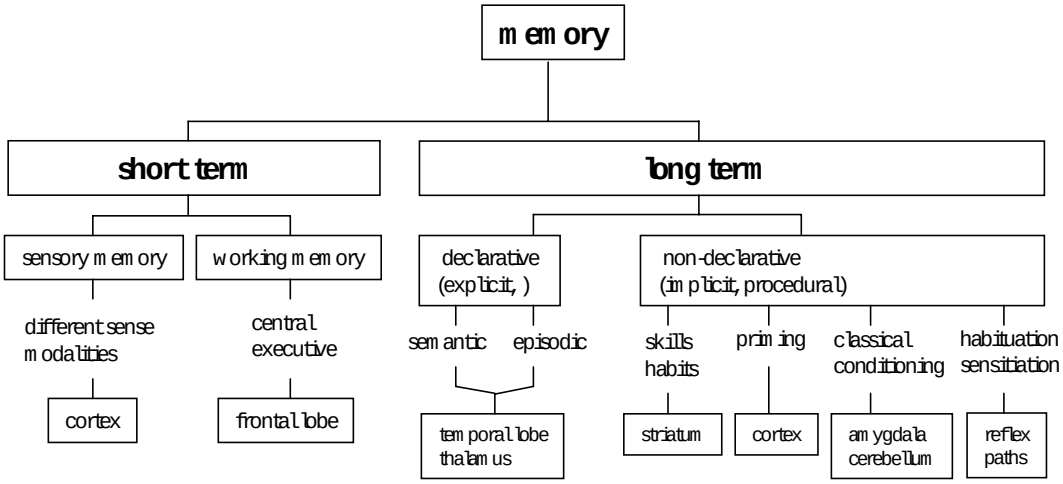


Fig. 1. Classification of memory (adapted from (Squire et al., 1993)).

Memory can be defined as the process by which we can retain newly acquired information over time. The formation of memory "engram", a representation of memory in the brain, is a process of time-dependent consolidation (McGaugh, 2000). Based on the time period that information can be sustained, memory can be divided into three basic types or stages: sensory, short-term and long-term memory (Woolf, 1997). The incoming information is first held in very brief sensory storage, either in visual sensory memory (iconic memory) or auditory sensory memory (echoic memory). Most of the information in sensory memory is lost within a fraction of second, thus enabling only a small portion of information to be transmitted into short-term memory. The processing of incoming sensory information takes place in the cortical areas that are responsible for the initial perception of sensory stimuli, i.e. in the primary auditory and visual cortices (Woolf, 1997).

The second stage, short-term memory, is responsible for acquisition and conscious processing of the information to be later transmitted into either long-term memory storage or subsequently forgotten (Squire et al., 1993; Willingham, 1997). The time period during which short-term memory is responsible for maintaining information is elastic, varying from seconds

to 10 - 20 minutes, depending on the type of information and task used to assess memory (Glassman, 1999). In animal research, the term *working memory* is often used as a synonym for short-term memory. The definition of working memory varies depending on the animal species, task used and corresponding researcher. For example, professor Patricia Goldman-Rakic has used delayed response tasks for monkeys that “tap working memory processes because the animal must retain the memory of the location of the stimulus during the period of a delay” (Goldman-Rakic, 1992). In delayed response tasks, the delay is normally from 5 to 60 s. Professor David Olton, the developer of the radial arm maze task for rats, defines working memory as a procedure where “stimulus information is useful for one trial of an experiment, but not for subsequent trials” (Olton et al., 1979). In Oltons task, the delay is from 30 s to 2 h. Since the definitions vary, it is very important to know which definition of working memory and which task is used for a particular study; otherwise the reader’s interpretation of the results may be misleading (table 1).

In human psychology, short-term memory can also been seen as a system that provides temporary storage, maintenance and controlled manipulation of the information necessary for complex cognitive tasks, such as language comprehension, reasoning and spatial ability (Baddeley, 1992; Wagner, 1999). This concept often uses the term working memory instead of short-term memory. Human working memory is not considered to be a single unitary system but consists of three independent subsystems. The central executive system is concerned with the attentional control of behaviour and coordinating the function of two slave systems: the phonological loop (verbal tasks) and the visuospatial "sketch pad" (visual tasks) (Baddeley, 1992). Typical features of human working memory are the capability to keep information “on-line” over a delay period (Willingham, 1997), and attention span (digit span), i.e. the capacity to handle the average of seven individual items simultaneously in working memory (Glassman, 1999; Miller, 1956).

Table 1. Widely used rodent tasks to evaluate short-term/working memory and long-term memory.

SHORT-TERM MEMORY

	memory span	reinforcement
D(N)MTP		
- operant box	1-30 s	positive
- T-maze, Y-maze	10 - 120 s	positive
DELAYED ALTERNATION		
- T-maze, Y-maze	10 - 120 s	positive
SPONTANEOUS ALTERNATION		
- T-maze, Y-maze	1 - 120 s	no (= spontaneous)
RADIAL ARM MAZE (all arms baited)	1 - 30 min	positive
WATER MAZE (working memory version)	1 - 5 min	negative
3-PANEL RUNWAY	2 min	positive
HOLE BOARD	1 – 5 min	no

LONG-TERM MEMORY

	memory span	reinforcement
PASSIVE AVOIDANCE	24h - days	negative
CONTEXT CONDITIONING	24h - days	negative
WATER MAZE	1min – days	negative
RADIAL ARM MAZE (3-4/ 8 arms baited)	1min – days	positive
HOLE BOARD	1min – days	positive
POSITION DISCRIMINATION (T-maze)	1min – days	positive/negative

The third stage of memory, long-term memory, can be divided into two forms: explicit (declarative, relational) and implicit (non-declarative, procedural) memory (Squire et al., 1993; Willingham, 1997). Explicit memory allows us to consciously remember facts and events from the past, and to learn new information of *when* and *where* something has happened, and *what* that something is. When people are talking about memory in general, they usually mean explicit memory that is related to everyday experiences and associated with things that we are aware of. Explicit memory can be further divided into episodic and semantic memory (Willingham, 1997). Episodic memory allows us to learn new information of *when* and *where* something has happened, i.e. the memories of experiences associated with a particular time and place. Semantic memory, on the other hand, allows us to learn *what* something is, i.e. the memories that are associated with general knowledge of things and facts. The other form of long-term memory, implicit memory, is associated with the learning of skills and habits (Squire et al., 1993; Willingham, 1997). Implicit memories cannot be brought to mind as conscious awareness of what is being remembered. Typically, an implicit memory is formed through repeated practice, and once the memory is acquired, it is resistant to forgetting. The classification of implicit memory also includes classical conditioning, emotional conditioning, and a phenomenon called priming (Willingham, 1997). Priming can be defined as "an improved facility for detecting or identifying perceptual stimuli on recent experience with them" (Squire et al., 1993).

The division between explicit and implicit memory is based on findings from patients with damage in the medial temporal lobe structures, particularly the hippocampus (HC), entorhinal and perirhinal cortex, and the diencephalon (Willingham, 1997). One of these patients known by his initials, H.M., is probably the most famous patient in the history of neuroscience. These patients fail to learn new information and to recall or recognise it even a few minutes later. In addition to anterograde amnesia, these patients also have a retrograde amnesia for up to 10 years. Since these patients have intact working memory and implicit memory (conditioning, priming and learning of motor skills), these types of memory have to be processed in other brain areas than the medial temporal lobe (Willingham, 1997). The medial temporal lobe structures are also responsible for a special kind of memory processing in animals. (Since the terms explicit or declarative represent a human type of behaviour, it has been suggested that the term *relational memory* would be more appropriate for animal

research. Similarly, the term *procedural memory* is more suitable for animal research, instead of implicit or non-declarative memory.) Studies with monkeys and rats have shown that the HC and adjacent cortical structures are important for delayed recognition tasks and spatial learning (relational memory), but not for learning stimulus-reward associations and motor skills (procedural memory) (Eichenbaum et al., 1996). Because spatial learning and memory can be investigated with behavioural tasks in man, monkey and rat, spatial behaviour provides a link for investigating relational memory processes across species.

The final storage of lifelong memories takes place within the cortex, in the same regions that are involved in the perception and analysis of the items to be remembered. Memories are stored in component parts, geographically distributed across separate regions of the brain that are needed to process the perception into a "memory engram" (Squire et al., 1993). The explicit memory system needs interaction between the neocortex and limbic/diencephalic brain systems, and is related to conscious recollections. The implicit memory system, which provides for non-conscious responses to the world, needs the participation of those brain areas responsible for the learning processes. Skill and habit memory requires participation of the striatum, classical conditioning depends on the cerebellum, emotional conditioning depends on the amygdala and priming takes place in the cortical areas responsible for perception (Squire et al., 1993; Willingham, 1997).

In addition to the medial temporal lobe, another central brain area for memory is the prefrontal cortex, which is considered to be important for working memory functions (D'esposito and Grossman, 1996; Wagner, 1999). In the primate brain, the cortex of the anterior pole of the frontal lobe excluding the motor and premotor cortex is commonly designated the prefrontal cortex. Patients with damage to the prefrontal cortex have a deficit in tasks assessing working memory, and modern studies with functional neuroimaging have provided evidence of prefrontal cortex activation during working memory tasks (D'esposito and Grossman, 1996). Similarly, monkeys with prefrontal lesions, specifically the principal sulcus region of the dorsolateral prefrontal cortex, have disrupted performance in visuospatial delayed response tasks, where the animal has to "keep in mind" the location of visual stimulus over a delay (Rosenkilde et al., 1981). In addition, the prefrontal cortex of the monkey brain shows increased metabolic activity during spatial delayed response tasks (Friedman and Goldman Rakic, 1994). Furthermore, some of the prefrontal neurons have

been shown to be active specifically during the delay period, which provides electrophysiological evidence for the role of the prefrontal cortex in the maintenance of working memory (Funahashi et al., 1989; Fuster, 1973). In rodents, the prefrontal cortex is also important in the regulation of spatial working memory (Dunnett, 1990; Poucet, 1990; Rogers et al., 1992; van Haaren et al., 1988). It is possible to compare the prefrontal cortex in the rat, monkey and man using physiological and neural connectivity for defining the prefrontal cortex. Across species, PFC can be defined as the "cortical areas for which the reciprocal connections with the mediodorsal nucleus of the thalamus are stronger than are the connections with other thalamic nuclei" (Uylings and van Eden, 1990). However, it should be noted that the anatomical and functional characteristics of prefrontal cortex are much less specific in rats than in primates, including humans. For example, in rats the rostral part of frontal area 2 and the anterior cingulate area have primate premotor cortex characteristics, but cannot be segregated from the prefrontal cortical areas (Uylings and van Eden, 1990). In addition, different sub-areas of the prefrontal cortex have distinct roles in the regulation of primate memory (Bachevalier and Mishkin, 1986; Dias et al., 1996; Wilson et al., 1993). For example, the dorsolateral prefrontal cortex of primates is selectively involved with spatial working memory, whereas the ventromedial part of the prefrontal cortex is necessary for object recognition but not spatial working memory (Bachevalier and Mishkin, 1986). In rodents, the area thought to be responsible for the regulation of spatial working memory is the medial area in the upper edge and medial surface of the hemisphere. Prefrontal sub-areas can also be found in the rat (Broersen et al., 1995; Dunnett, 1990; Eichenbaum et al., 1983; Kolb, 1984; Mogensen and Holm, 1994), although exact definitions of such sub-areas as functional parts of rodent prefrontal cortex are still lacking.

In addition to working memory, the prefrontal cortex is important for the regulation of attentional control of behaviour (Muir et al., 1996; Sarter et al., 2001). Attention is a complex process and consists of several distinct mechanisms. Attention can be divided into sustained attention (vigilance), divided attention and selective attention (Muir, 1996). Sustained attention refers to a subject's ability to detect rarely and unpredictably occurring signals over prolonged periods of (Sarter et al., 2001). Divided attention refers to a subject's ability to attend to and process simultaneously more than one stimuli presented together, and selective attention requires focusing of resources to a restricted number of sensory channels, typically under some form of distraction (Muir, 1996). In rodents, attention can be further divided into

five categories: orienting, expectancy, stimulus differentiation, sustained attention, and parallel processing (Bushnell, 1998). However, it is often difficult to distinguish behaviourally between these processes, since overlapping attentional processes often contribute to the behavioural outcome in attentional tasks (see (Bushnell, 1998) for a detailed review).

Attentional processes in the mPFC are regulated by the ascending cholinergic fibers originating from the NBM, specifically if demands on attentional processing are increased by a distractor (Sarter et al., 2001). The NBM cholinergic cells are regulated by descending glutamatergic projections from the prefrontal cortex, which provides a feedback mechanism for attentional processing (Sarter et al., 2001). Behaviourally, it has been reported that infusion of APV, an NMDA receptor antagonist, into the basal forebrain produces similar effects on sustained attention as those resulting from cholinergic lesions. It is therefore likely that stimulation of attentional processes in the cortex by ascending cholinergic fibers from the NBM is regulated by NMDA receptors (Sarter et al., 2001).

2.2 Muscarinic receptors

2.2.1 Introduction: Brain cholinergic system

Acetylcholine. ACh is a neurotransmitter that is synthesized in cholinergic cells from choline and acetyl-coenzyme A by the enzyme choline acetyltransferase (ChAT). The effects of ACh are mediated by two groups of receptors: muscarinic and nicotinic receptors. Once released from the pre-synaptic terminal, ACh is rapidly inactivated by hydrolysis into acetate and choline by the enzyme AChE (Wilson et al., 1950). In the brain, cholinergic pathways arise from small nuclei that contain cholinergic neurons intermingled with other, non-cholinergic neurons (Mesulam, 1995). The highest density of cholinergic axons in the brain occurs in core limbic structures, such as the HC and amygdala (Mesulam et al., 1992). Basically, cholinergic cells form two systems, one in the basal forebrain and the other in the brainstem.

Basal forebrain. Cholinergic cells in the basal forebrain provide diffuse neural projections that innervate the entire cerebral cortex. The density of cholinergic axons is highest in the superficial layers of the cerebral cortex (Mesulam, 1995). The Ch1–Ch4 nomenclature

designates the cholinergic cells within different nuclei (Mesulam, 1995; Mesulam et al., 1983). Ch1 refers to cholinergic cells within the medial septal nucleus (MS), and Ch2 to those within the vertical nucleus of the diagonal band. The main targets of Ch1 and Ch2 cholinergic cells are the hippocampal formation, cingulate cortex, olfactory bulb and hypothalamus. Ch3 cells within the horizontal limb of the diagonal band nucleus (HDB) provide the major cholinergic innervation for the olfactory bulb. Ch4 contains the cholinergic cells within the nucleus basalis of Meynert (NBM) that provide cholinergic innervation for the rest of the cerebral cortex and the amygdala (Mesulam, 1995; Mesulam et al., 1983).

Brainstem. The brainstem cholinergic system consists of four cholinergic cell groups, Ch5–Ch8 (Mesulam, 1995; Mesulam et al., 1983). Ch5 designates cholinergic cells in the pedunculopontine nucleus, and Ch6 cells in the laterodorsal tegmental nucleus and rostral brainstem. The Ch5 and Ch6 cells project to the thalamus. Ch7 cells in the medial habenula innervate the interpeduncular nucleus, and Ch8 cells in the parabigeminal nucleus project to the superior colliculus. In addition to Ch1–Ch8 cells, there are intrinsic cholinergic interneurons for example in the striatum.

2.2.2 Signal transduction and localization

Acetylcholine receptors can be divided into two major classes, nicotinic and muscarinic receptors. Whereas nicotinic receptors are all ion channel receptors, muscarinic receptors are linked to G- proteins. Nicotinic receptor is a pentamer composed of five homologous membrane-spanning subunits (ABGD E=G) around a central ion channel (Paterson and Nordberg, 2000). Several variants of each subunit have been characterized. Neuronal nicotinic receptors generally comprise two a and three b subunits, the most common of which is a beta2/alpha4 composition. In addition, a common neuronal nicotinic receptor consists of five alpha7 subunits (Paterson and Nordberg, 2000). In general, the ion channel of the nicotinic receptor is primarily permeable to Na^+ and K^+ ions, but the alpha7 receptor subtype is also Ca^{2+} permeable (Girod et al., 2000). Opening of the channel results in an inward flux of Na^+ producing a local depolarisation of the membrane. In addition to postsynaptic sites, there also exist pre-, peri- and extra synaptic nicotinic sites that may modulate neuronal function through a variety of actions (Paterson and Nordberg, 2000).

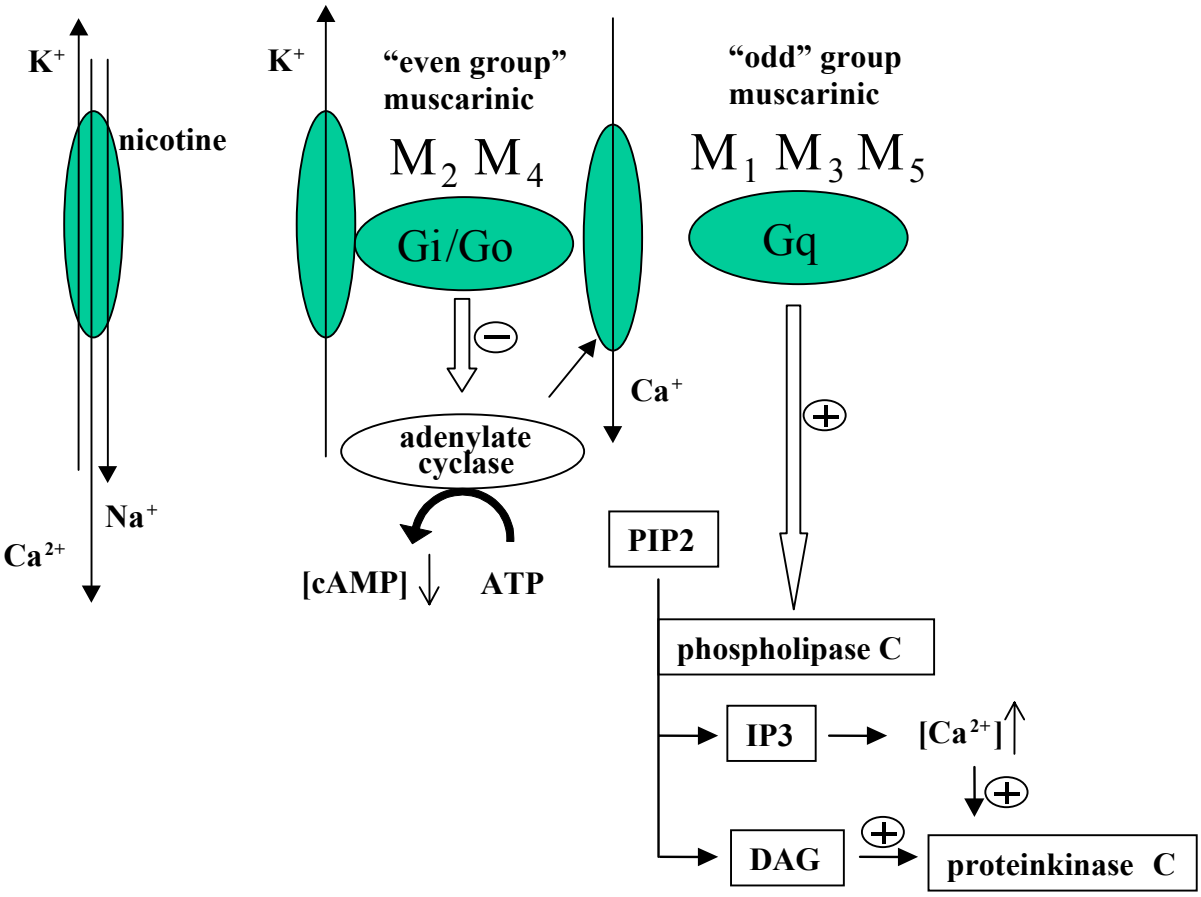


Fig. 2. Signal transduction of acetylcholine receptors.
Opening of the nicotinic receptor channel results in an inward flux of Na^+, producing local depolarisation of the membrane. In addition, the alpha7 receptor is also ca^{2+} permeable. Activation of "odd" group receptors results in phospholipase Cβ activation that leads to stimulation of phosphoinositide hydrolysis. Activation of "even" group G-protein leads to inhibition of adenylate cyclase. Finally, activation of the M_1 M_2 and M_3 receptors leads to regulation of protein functions by phosphorylation, whereas activation of the M_2 and M_4 receptors results in a decreased cytosolic cAMP level.

This review focuses on muscarinic receptors, which will be described in greater detail in the next chapter.

Subtypes. Muscarinic receptors belong to the superfamily of G-protein-coupled receptors (Bonner et al., 1987). Typically, these receptors have seven transmembrane segments that form a barrel-like structure having a central pore. ACh and other muscarinic ligands bind at a

site inside this pore, which leads to activation of a G-protein (Hulme et al., 1990). Today five muscarinic receptor subtypes, M_1, M_2, M_3, M_4 and M_5 receptors, have been identified using both pharmacological and molecular biological techniques (Caulfield and Birdsall, 1998). The subtypes can be divided into two main groups that activate different G-proteins: an "odd-numbered" group (M_1, M_3 and M_5 receptors) and an "even-numbered" group (M_2 and M_4 receptors). At the cellular level, both groups of muscarinic receptors regulate the basal activity of neurons. Activation of the "odd" group G-protein results in phospholipase Cβ activation, which leads to the stimulation of phosphoinositide hydrolysis. Activation of the "even" group G-protein leads to inhibition of adenylate cyclase (Caulfield and Birdsall, 1998). Finally, the activation of M_1 M_2 and M_3 receptors leads to regulation of protein functions by phosphorylation, whereas the activation of M_2 and M_4 receptors results in a decrease in the cytosolic cAMP level (Caulfield and Birdsall, 1998).

Distribution. In general, the absolute density of muscarinic receptors is highest in the various regions of the forebrain, but declines in more caudal regions of the brain (Ehlert et al., 1995). The M_1, M_2 and M_3 receptors represent the majority of muscarinic receptors in various brain regions (table 2). The relative abundance of M_1 receptors is greatest in the cerebral cortex (34 %), HC (47 %) and striatum (29 %) (Yasuda et al., 1993). The M_2 receptor represents 75 % of the total muscarinic receptors in the cerebellum, whereas in the cortex and HC the expression is around 20 %. However, the absolute density of the M_2 receptor is relatively uniform throughout the brain (Li et al., 1991). The M_2 receptor may act as a presynaptic autoreceptor, a presynaptic heteroreceptor, or a postsynaptic receptor in the septo-hippocampal pathway (Rouse et al., 1997). The M_3 receptor has minor expression in the brain. The relative expression of M_3 receptor mRNA is greatest in the cerebral cortex and HC (11 %), but lower in the more caudal regions of the brain (Yasuda et al., 1993). The M_4 receptors have highest expression in the striatum (46 %) and cortex (24 %). In the HC, the expression of M_4 receptors is equal to that of M_2 receptors (19 %) (Yasuda et al., 1993). The characterization of the M_5 receptor is still incomplete (Caulfield and Birdsall, 1998). Small amounts of the M_5 receptor can be found in the substantia nigra, but the M_5 subtype represents only 2% of the total amount of muscarinic receptors in the brain (Caulfield and Birdsall, 1998; Yasuda et al., 1993).

Table 2. The relative expression of muscarinic receptor subtypes in major brain structures.

Brain area	M_1	M_2	M_3	M_4	M_5
Cortex	34 %	20 %	11 %	24 %	< 2 %
Hippocampus	47 %	19 %	11 %	19 %	< 2 %
Striatum	29 %	12 %	8 %	46 %	< 2 %
Thalamus	16 %	43 %	6 %	20 %	< 2 %
Cerebellum	2 %	75 %	5 %	3 %	< 2 %

2.2.3 Cognitive functions mediated by muscarinic receptors

Central. Muscarinic receptors in the central nervous system (CNS) are involved in the regulation of learning and memory functions. Originally, Drachman and Leavitt (Drachman and Leavitt, 1974) found that the muscarinic antagonist, scopolamine, produced amnesia in young volunteers similar to that observed in aged non-demented people (Bartus et al., 1982). In rodents, scopolamine is known to disrupt performance in spatial learning and memory tasks (Andrews et al., 1994; Buresova et al., 1986; Riekkinen et al., 1990; Sirviö et al., 1992). However, the cognitive effects mediated by muscarinic receptors are not specific to learning and memory, since muscarinic receptors also mediate processes that are needed in the regulation of attention and arousal (Broks et al., 1988; Callahan et al., 1993; Parrott, 1986; Phillips et al., 2000; Ruotsalainen et al., 2000). It is possible, that disruption of attentional and other non-cognitive processes in the CNS is the actual cause of the learning and memory deficits caused by muscarinic antagonists (Blokland, 1996; Ebert and Kirch, 1998).

Classically, muscarinic receptors have been divided into M_1 and M_2 receptors, with high and low affinities to muscarinic receptor antagonist pirenzepine, respectively. Muscarinic receptor subtypes mediate different aspects of behaviour, and there are reports indicating that post-synaptic M_1 receptors mediate the performance-disrupting effects of scopolamine. For example, in the water maze and radial arm maze tasks, pirenzepine, a selective M_1 antagonist, causes performance disturbance similar to that with scopolamine or atropine (Hagan et al., 1987; Hunter and Roberts, 1987; Sala et al., 1991). In addition, pirenzepine has been reported to cause more specific effects on spatial short-term memory performance than scopolamine in

the delayed non-matching to position task (DNMTP) (Andrews et al., 1994). In this study, the result of pirenzepine administration was a delay-dependent disruption of performance, whereas scopolamine induced a non-specific and delay-independent disruption of all task parameters, including motivation and motor performance (Andrews et al., 1994). In the same study, an M_2 receptor antagonist (AFDX 116) had no effect on DNMTP performance, suggesting that the M_2 receptors do not mediate the disrupting effect of muscarinic antagonists on spatial short-term memory. In theory, it is possible that blockade of presynaptic M_2 receptors enhances the function of ACh system by increasing the release of ACh, which may lead to beneficial effects on cognitive behaviour (Quirion, 1993; Quirion et al., 1995).

Genetic receptor inactivation. A novel approach to defining the role of muscarinic receptors in the CNS is the use of receptor knockout mice that lack a specific subtype of an mACh receptor. Knockout studies support the role of M_1 receptors as the primary mediator of postsynaptic muscarinic receptor signalling in the cortex and hippocampus (Porter et al., 2002). It has also been reported that M_1 receptor knockout mice show impaired spatial water maze learning (Hamilton et al., 2001), but this may not be a memory-specific effect due to pronounced non-specific hyperactivity caused by the gene deletion (Miyakawa et al., 2001). Thus far, knockout studies have revealed that the M_3 subtype plays a key role in salivary secretion, pupillary constriction, and bladder detrusor contractions (Matsui et al., 2000), whereas the M_3 receptors have no significant role in ACh-induced G-protein signalling in the the cortex and hippocampus (Porter et al., 2002). Knockout studies also support the role of M_2 receptors as the main presynaptic autoreceptor in the cortex and hippocampus, whereas in the striatum the autoinhibition is predominantly mediated by the M_4 receptors (Zhang et al., 2002). Central M_2 receptors are critically involved in mediating tremor, hypothermia and analgesia, whereas the M_4 receptor knockout mice have increased basal motor activity (Felder et al., 2001; Gomeza et al., 2001). M_4 receptors are also involved in the regulation of prepulse inhibition of the startle reflex, a measure of attention (Felder et al., 2001). The M_5 muscarinic receptor knockout mice have been reported to be intact in various behavioural tests, but ACh is not able to dilate cerebral arteries and arterioles in these knockout mice, suggesting that the receptor is physiologically relevant in the regulation of cerebral blood flow (Yamada et al., 2001).

What is problematic about the use of gene-manipulated mice is that compensatory mechanisms during embryonal development may alter the normal physiological balance, thus making it difficult to determine the actual role of receptor subtypes in the regulation of on-line behaviour. One way to limit developmental abnormalities is to use inducible gene manipulation affecting only one specific brain area at a specific time. Thus far, such studies have provided interesting data on NMDA receptors, though there is still a clear lack of studies focusing on muscarinic ACh receptors.

Peripheral. Muscarinic receptors also mediate peripheral responses, especially responses of the autonomic nervous system. Muscarinic antagonists induce peripheral side effects that may contribute to the behavioural effects of these drugs. For example, scopolamine causes blurred vision by reducing accommodation, decreases resting heart rate, causes urinary retention and reduces salivation which may decrease appetite (Stromberg et al., 1991) (Parrott, 1989) (Parrott, 1987). Some of the peripheral effects can be used for treatment purposes. For example, scopolamine blocks the auriculoemetic reflex and reduces intestinal secretion (Honkavaara and Pyykko, 1999; Muir and von Gunten, 2000), which can be used for protection of motion sickness and perioperational nausea (Parrott, 1989; Stromberg et al., 1991). Still, if one is interested in investigating brain memory processing, even these peripheral effects may not be desired and should be minimised.

AChE inhibitors. Inhibition of AChE leads to an increase in ACh concentration within the synaptic cleft, thus prolonging and increasing the binding of ACh into the receptors. In rodents, scopolamine-induced spatial learning and memory deficit in the water maze and DNMTP tasks can be alleviated by AChE inhibitors, such as tetrahydroaminoacridine (THA), metrifonate and rivastigmine (Bejar et al., 1999; Murray et al., 1991; Riekkinen et al., 1991; Riekkinen et al., 1996). In addition, THA is able to improve water maze performance in aged and MS lesioned rats (Riekkinen et al., 1991; Riekkinen et al., 1996). According to the cholinergic hypothesis of AD, stimulation of the brain cholinergic system should alleviate AD symptoms (Bartus et al., 1982). In AD patients, AChE inhibitors, such as THA, donepezil and rivastigmine, provide relief of AD symptoms, but the cognitive effects are modest at best (Emilien et al., 2000; Francis et al., 1999). Moreover, the AChE inhibitors are more efficient in relieving hallucinations and defects in attentional and arousal processes, than memory impairment *per se* (Francis et al., 1999).

Cholinergic lesion. Recently, the development of a selective cholinergic neurotoxin, IgG-saporin, has provided new information on the role of the brain cholinergic system in cognition (McGaughy et al., 2000). IgG-saporin is a neurotoxin that binds to low-affinity nerve growth factor receptor p75, inducing apoptotic cell death in cholinergic cells. Only the projections from Ch5 and Ch6 to the thalamus, and the projection from Ch4 to the amygdala, are not sensitive to IgG-saporin (McGaughy et al., 2000). Studies using i.c.v. administration of IgG-saporin have shown that damage of the basal forebrain cholinergic cells disrupts acquisition of water maze tasks only if there is additional damage to cerebellar Purkinje cells (McGaughy et al., 2000; Waite et al., 1999). More selective lesions of MS and/or NBM cells have no effect on the acquisition of water maze tasks, suggesting that neither the septo-hippocampal nor cortical cholinergic innervation is needed for spatial learning (Baxter et al., 1996). However, selective lesioning of cholinergic cells in MS is able to produce delay-dependent impairment in the delayed matching to position task (DMTP), suggesting that the septo-hippocampal pathway is important for the regulation of spatial short-term memory (Torres et al., 1994). Nevertheless, even selective cholinergic lesions of basal forebrain induce non-specific behavioural disturbance, such as hyperactivity (Torres et al., 1994). Correspondingly, disturbance of attention or arousal is likely to contribute to the disruption of cognitive functions by cholinergic lesioning, as reflected by disruption of performance in sustained attention and serial conditioning tasks (McGaughy et al., 2000).

2.2.4 Muscarinic receptors in aging and AD

Aged rats. Spatial learning deficit in aged rats has been used as a model for the cognitive decline related to aging and AD (Barnes, 1994). The anatomical and physiological changes occurring in the aged rodent brain resemble those occurring in AD. For example, the decrease in the ACh synthesizing enzyme ChAT during aging may reflect degeneration of cholinergic cells in the basal forebrain, although the evidence supporting a ChAT decrease in the rodent brain is somewhat contradictory for both the cortex and HC (table 3). In addition, AChE activity is decreased in both AD (Namba et al., 2002) and rodent aging (table 3). Aged rats, like young rats with hippocampal damage, show greater impairment in water maze learning than do young controls (Gage et al., 1989; Gallagher et al., 1994). Those aged rats that are

most severely impaired in spatial tasks also have the greatest amount of degeneration in cholinergic cells in the basal forebrain, which further supports the view that the degeneration of the septohippocampal cholinergic system is linked to age-related learning impairment (Gallagher et al., 1994; Rapp and Amaral, 1992). Furthermore, the function of muscarinic receptors may be disrupted over the course of aging, since both memory-impaired and memory-intact aged rats are more sensitive to the disrupting effects of scopolamine than young rats (Gallagher et al., 1994). The spatial learning deficit of aged rats can be used for investigating the efficacy of drugs in improving memory function, and the results from such studies may help in finding new treatments for the cognitive decline seen in AD.

At the receptor level, there seems to be a decline in the total density of muscarinic receptors in the cortex and HC of aged rats, when compared to young controls (table 3). In the cortex, both muscarinic M_1 and M_2 receptor subtypes decrease during aging; whereas, in the HC, sparing of both M_1 an M_2 subtypes has been reported (table 3). In different rat strains the cholinergic system may be differentially changed during aging (Michalek et al., 1990). In Wistar rats, a decrease in both cortical and hippocampal muscarinic receptors and AChE activity has been reported during aging in Wistar rats that were used for our experiments (table 3).

Table 3. Changes in AChE and ChAT activity and muscarinic receptor density during rodent aging.

	ChAT act	AChE activity	All MR:s	M_1	M_2
Cortex	↓ 3,6,7 O 7,7,**11**	↓ 7,7,7,**11**,	↓ 4,5,7,**12** O 7,7	↓ 1,5,8 O 6	↓ 1,6,8
HC	↓ 3 O 5,7,7,7,**11**	↓ 7,7,7,**11**,	↓ 4,7,7,**9** O 5,14	↓ 1,**2**,13 O 5,6,8,**10**,14	↓ 1,6 O **2**,8,14
Striatum	↓ 3,7,7,**7** O 5	↓ 7,7,7,**11**	↓ 4,7,7 O 5	↓ 1,8 O 5,6	↓ 1,6,8

1) Fisher 344 (Narang, 1995)
2) **Wistar** (Amenta et al., 1995)
3) Fisher 344 (Ogawa et al., 1994)
4) Rats (Blake et al., 1991)
5) Fisher 344 (Schwarz et al., 1990)
6) Rats (Araujo et al., 1990)
7) Fisher 344, *Sprague Dawley* and **Wistar** (Michalek et al., 1990)
8) Rats (Biegon et al., 1989)
9) **Wistar** (Kadar et al., 1994)
10) **Wistar** (Sirviö et al., 1988)
11) **Wistar** (Sirvio et al., 1989)
12) **Wistar** (Gilad et al., 1987)
13) Fisher 344 (Tayebati et al., 2002)
14) Long-Evans (Chouinard et al., 1995)

Table 4. Changes in AChE and ChAT activity and muscarinic receptor density in Alzheimer's disease.

	ChAT activity	AChE activity	All MR:s	M_1	M_2	M_3	M_4
Cortex	↓ 1,2,7	↓ 7,12	↓ 2,4 O 9 ↑ 7,8	↓ 3*,4 O 1,2,3 ↑ 5	↓ 1,2,3,3* O 6 ↑ 5	O 4	↑ 3,3* O 4
HC	↓ 1,2,7	↓ 7,12	↓ 2,4,10	↓ 4,11 O 2	↓ 1,2,4,6	↓ 4	O 4
Striatum	↓ 7		↓ 10 ↑ 7	↑ 1	O 1 ↑ 4	O 4	O 4

1) (Aubert et al., 1992)
2) (Quirion et al., 1989)
3) (Flynn et al., 1995)
4) (Rodriguez-Puertas et al., 1997)
5) (Nordberg et al., 1992)
6) (Rinne et al., 1989)
7) (Danielsson et al., 1988)
8) (Nordberg and Winblad, 1986)
9) (Perry et al., 1990)
10) (Rinne et al., 1985)
11) (Shiozaki et al., 2001)
12) (Namba et al., 2002)

* = immunoreactivity vs radioligand binding

Aging and Alzheimer's disease. AD is the most common cause of memory loss and dementia in elderly people. The cholinergic hypothesis by Raymond Bartus posits that degeneration of the brain cholinergic system is linked to the cognitive symptoms of AD (Bartus et al., 1982). In the course of the disease, cholinergic cells in the NBM and other parts of the basal forebrain die, resulting in decreased cholinergic innervation particularly in the temporal lobe (Geula and Mesulam, 1996). The degeneration of the brain cholinergic system is strongly correlated with the degree of cognitive impairment in AD patients, and occurs early in the course of the disease (Bartus, 2000; Francis et al., 1999). In AD, cholinergic cell death is reflected by a reduction in presynaptic markers of the cholinergic system, i.e. by a reduction in cortical ChAT activity and ACh synthesis (Francis et al., 1999).

At the receptor level, there is little or no difference between normal aged people and AD patients in the overall expression of cortical muscarinic receptors (Perry et al., 1990; Schroder et al., 1991) (table 4). The M_1 receptor subtype in the cortex has generally been reported to be preserved in AD (table 4). However, coupling of M_1 receptors to their G-proteins may be disrupted in AD brains (Flynn et al., 1995; Warpman et al., 1993). The M_1 receptors in the HC are suggested to be either preserved or decreased (table 4). Cortical M_2 receptors appear to be decreased in AD, though preservation and even an increase have been reported (table 4). Several different studies agree that a decrease in hippocampal M_2 receptors occurs in AD, which is thought to reflect pre-synaptic degeneration of cholinergic fibres in the HC (table 4). The M_3 receptors have been reported to be preserved in the cortex, but decreased in the HC. The M_4 receptors are either preserved or increased in the cortex and preserved in the HC (table4). The expression of the M_5 receptor subtype in AD has been reported to remain unchanged (Flynn et al., 1995).

2.3 *N*-methyl-D-aspartate (NMDA) receptors

2.3.1 Introduction: glutamatergic receptors

Glutamate is a nonessential amino acid that serves as the predominant excitatory neurotransmitter in the mammalian brain. It is a neurotransmitter having numerous clinically important pathways, as well as a crucial role in cortical and hippocampal cognitive function, motor function, and sensory function (Greenamyre and Porter, 1994). Glutamate activates

several classes of receptors, each of which has a distinct pharmacology, biochemistry and physiology. Glutamate receptors are classified into two major categories termed ionotropic and metabotropic receptors. Ionotropic receptors are linked to ion channels that are permeable to Na^+ or Ca^{2+} cations. They have been classified into three major subtypes that have different relative permeability to Na^+ or Ca^{2+}: NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and kainate receptors (Dunah et al., 1999). The subtypes are named according to the agonists that are able to elicit a specific physiological response. The structure and function of the *NMDA receptors* are described in detail in the next chapter. The *AMPA receptors* mediate the majority of the fast excitatory neurotransmission in the CNS. Generally, activation of the AMPA receptor results in a Na^+ influx, though some subtypes are also permeable to Ca^{2+} (Greenamyre and Porter, 1994). Kainate receptors are also mainly permeable to Na^+, although the physiological and pharmacological properties vary depending upon the subunit composition of the individual receptor (Greenamyre and Porter, 1994). The other category, *metabotropic glutamate receptors*, couples to G-proteins and cytoplasmic enzymes. Depending on the receptor subtype, these receptors mediate such processes as inositol phosphate metabolism, the release of arachidonic acid or changes in cyclic adenosine monophosphate levels (Greenamyre and Porter, 1994).

2.3.2 NMDA receptor structure and signal transduction

The NMDA receptor is the best defined of all glutamate receptors, among which it has unique characteristics. The receptor itself (Fig. 3.) has binding sites for two "co-agonists", glutamate and glycine, both of which are needed for full receptor activation. The NMDA receptor is gated both by ligands and by voltage, thus requiring depolarization of the postsynaptic membrane before the binding of ligands results in a cation influx (Meldrum, 2000; Mori and Mishina, 1995). This voltage dependence is due to a Mg^{2+} block within the ion channel, which is removed by membrane depolarization (Mori and Mishina, 1995). Usually, the depolarization is caused by activation of the AMPA receptors that have faster kinetics than the NMDA receptors. Electrophysiologically, activation of the AMPA receptor can be seen as the fast initial component of excitatory postsynaptic potential (EPSP), whereas the EPSP mediated by the NMDA receptor displays a slower and prolonged time course (Mori and Mishina, 1995). NMDA receptor activation is also modulated by polyamines, such as

spermine and spermidine. Polyamines are not needed for receptor activation, but they increase the ability of glutamate and glycine to open the receptor ion channel (Greenamyre and Porter, 1994). Unlike the other ionotropic receptors, the NMDA receptors are highly permeable to Ca^{2+}. The result of the Ca^{2+} influx is an activation of several Ca^{2+} dependent enzymes, such as protein kinase C, phospholipase C, Ca^{2+} protein kinase II, nitric oxide synthase and various proteases and endonucleases (Greenamyre and Porter, 1994). Some of the Ca^{2+} dependent enzymes phosphorylate either AMPA or NMDA receptors, resulting in increased activity of the receptors. The activation of Ca^{2+} dependent enzymes may also lead to transcription of several genes, which in turn leads to prolonged changes in the receptor structure and activity, i.e. synaptic plasticity (Michaelis, 1997).

The NMDA receptors consist of several subunits. In the rat, these subunits can be divided into two families, NMDAR1 (NR1) and NMDAR2 (NR2). The NR2 family has four members, NR2A - D (Mori and Mishina, 1995). Both families also have numerous splice variants. Functional receptors express the subunit NR1 combined with NR1 or with one of the members of the NR2 family. In the mouse, NR1 and NR2A-D are named GluRzeta and GluRepsilon1-4, respectively. The NMDA receptor subtypes have different distributions in the brain. For example, NR1 is the predominant subunit type in the HC and cerebral cortex, while NR2A is also highly expressed in the septum, the caudate putamen, the olfactory bulb and the thalamus. In addition, the NMDA receptor subtypes have different gating properties and sensitivities to Mg^{2+} and Ca^{2+} (Mori and Mishina, 1995)

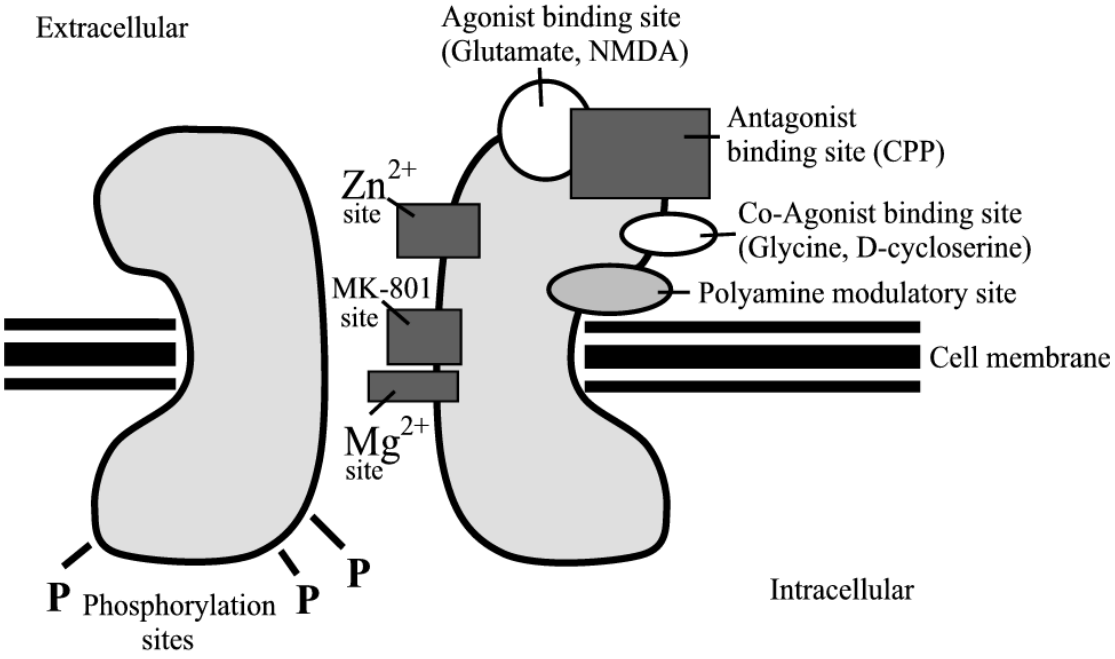


Fig. 3.**.** Schematic representation of the NMDA receptor and the binding sites of major agonists, antagonists and allosteric modulators.

2.3.3 Behavioural functions mediated by NMDA receptors

Antagonists. The psychopharmacological profile of the NMDA receptor antagonists is complex. NMDA antagonists have anxiolytic effects and potent anticonvulsant properties, are muscle relaxants and may even act as behavioural stimulants (Cotman and Iversen, 1987). In humans, the cognitive effects of NMDA antagonists are not limited to learning and memory functions. For example, NMDA antagonists are able to induce different types of memory impairments, but are also known for their capability to induce hallucinations and other schizophrenia-like symptoms including disturbed attention (Hetem et al., 2000; Malhotra et al., 1996; Newcomer et al., 1999; Oranje et al., 2000). In rodents, NMDA antagonists disrupt performance in various memory tasks, including tasks that assess spatial learning and spatial short-term memory (Cole et al., 1993; Davis et al., 1992; DeNoble et al., 1990; Ward et al., 1990), and have also been shown to have non-cognitive effects (Cole et al., 1993; Doyle et al., 1998; Parada-Turska and Turski, 1990; Pontecorvo et al., 1991). Therefore, it is likely that the sensorimotor disturbance caused by NMDA antagonists may contribute to spatial learning and memory deficits (Cain, 1998; Doyle et al., 1998; Saucier et al., 1996). The effect profiles

of different NMDA antagonists vary, with non-competitive antagonists appearing to have more robust behavioural effects than competitive antagonists (Cole et al., 1993).

D-cycloserine. Use of NMDA agonists in cognitive research is problematic, since NMDA receptor activation may lead to calcium-mediated cell death. NMDA itself can be used to make lesions in the brain (Myhrer, 1993), and one theory even suspects NMDA receptor mediated exicotoxicity to be involved in cell death occurring in AD (Maragos et al., 1987). However, positive modulation of NMDA receptors, instead of direct agonism, may provide beneficial effects on cognition without cell degeneration. D-cycloserine (DCS) is a positive modulator of the strychnine insensitive glycine site in the NMDA receptor, i.e. binding of DCS to its binding site enhances the natural function of the NMDA receptor. It is found as an endogenous ligand in both the rodent and human brain. In humans, DCS alleviates scopolamine induced cognitive decrements (Jones et al., 1991) and has beneficial effects for cognition in AD patients (Schwartz et al., 1996; Tsai et al., 1999). In rodents, DCS is able to alleviate the age-related (Popik and Rygielska, 1999) and scopolamine-induced spatial learning deficit (Pitkänen et al., 1995; Puumala et al., 1998).

Genetic receptor inactivation. Studies with genetically engineered mice have revealed that the NMDA receptors have a vital role in normal development. Homozygous knockout mice totally lacking the NR1 subunit, and thus functional NMDA receptors, have neurodevelopmental abnormalities and die soon after birth from respiratory failure (Forrest et al., 1994; Li et al., 1994). Knockout mice having reduced levels of NMDA receptors exhibit behavioural disturbances varying from impaired synaptic plasticity, learning and memory (Sakimura et al., 1995) to hyperlocomotion, increased stereotypy and abnormalities in social and sexual interactions (Mohn et al., 1999).

Mice that lack the NR1 subtype specifically in the CA1 field of the hippocampus have provided a new approach for investigating the brain NMDA receptors. These knockout mice grow into adulthood normally but have impaired spatial memory and long-term potentiation, supporting the idea that hippocampal synaptic plasticity mediated by the NMDA receptors is needed in the regulation of spatial memory (Tsien et al., 1996). In addition, studies in CA1 specific NR1 knockout mice suggest that hippocampal NMDA receptors are also needed for the encoding and flexible expression of stimulus relations in *non-spatial* memory (Huerta et

al., 2000; Rondi-Reig et al., 2001).

Functional glycine binding sites are essential for normal NMDA receptor function, since homozygous animals with severely dysfunctional glycine sites do not survive (Kew et al., 2000). However, a milder reduction in the agonist-binding capacity of functional glycine sites in mice with targeted point-mutations results in an impairment of spatial learning and hippocampal LTP. Reduced levels of glycine sites are also known to cause alterations in anxiety-related behaviour (Kew et al., 2000).

2.3.4 NMDA receptors in aging and AD

Table 5. Changes in NMDA receptor density during aging in rats.

Brain area	NMDAR:s
Cortex	↓ [1,2,5,6,10**6*,7*,9*** O [3,7]
HC	↓ [2,5,6,10 **6*,7*, 9*** O [3,4, 7,**8***
Striatum	↓ [1,2,4,5,10]

1) Fisher 344 (Mitchell and Anderson, 1998)
2) Female wistar (Wardas et al., 1997)
3) Fisher 344 (Shimada et al., 1997)
4) Long evans (Nicolle et al., 1996)
5) Fisher 344 (Castornia et al., 1994)
6) Fisher 344 (Tamaru et al., 1991) *
7) Fisher 344 (Kito et al., 1990) *
8) Fisher 344 (Bonhaus et al., 1990) *
9) Fisher 344 (Miyoshi et al., 1990) *
10) Wistar (Serra et al., 1994)

* = glycine site studied.

Aged rats. The number of NMDA receptors in the cortex and HC declines during aging, although preservation in both areas has also been reported (table 5). However, the number of NMDA receptors containing the strychnine insensitive glycine binding site has consistently been reported to decrease during aging (Kito et al., 1990; Miyoshi et al., 1990; Tamaru et al., 1991) (table 5). A similar trend is also evident in the striatum, where the number of NMDA receptors declines during aging (table 5)

Alzheimer's disease. The number of NMDA receptors is reduced in the cortex and HC in AD, although sparing has also been reported (table 6). NMDA receptors in different cortical areas may be differentially affected in AD (Scheuer et al., 1996). By contrast, the NMDA receptors seem to be spared in the striatum (table 6).

Table 6. Changes in NMDA receptor density in Alzheimer's disese

Brain area	NMDAR:s
Cortex	↓ [2,4,7*,9*,10,12] O [3,5,8]
HC	↓ [2,6,12] O [1,3,5,11]
Striatum	O [3,4]

1) (Geddes and Cotman, 1986)
2) (Young, 1987)
3) (Cowburn et al., 1988)
4) (Simpson et al., 1988)
5) (Mouradian et al., 1988)
6) (Penney et al., 1990)
7) (Del Bel and Slater, 1991) *
8) (Porter et al., 1992)
9) (Carlson et al., 1993) *
10) (Scheuer et al., 1996)
11) (Thorns et al., 1997)
12) (Wang et al., 2000)
* = glycine site studied.

2.4 Interaction between muscarinic and NMDA receptors in learning and memory functions

Receptors. Muscarinic and NMDA receptors may interact at the synaptic level to modulate neuronal activity. For example, cholinergic synaptosomes from the cerebral cortex are able to co-release glutamate, indicating the coexistence of ACh and glutamate in the same cells (Docherty et al., 1987). Moreover, within the cortex, ACh can increase the release of glycine, a potent positive modulator of NMDA receptors (Russo et al., 1993), and both glutamate and NMDA increase the release of ACh from rat cortical and striatal slices (Ulus et al., 1992). Furthermore, electrophysiological data suggests that the induction of long-term potentiation (LTP) is stimulated by both muscarinic and NMDA receptors (Hirotsu et al., 1989) which can thus modulate the neuronal processing required for memory functions in the cortex and HC.

Behaviour. Preliminary evidence suggesting that NMDA and muscarinic receptors interact in

the regulation of memory processes comes from primate research, indicating that MK-801 (a non-competitive NMDA antagonist) and scopolamine, given in subthreshold doses, jointly disrupt memory performance in visual recognition tasks. In rodents, both NMDA antagonists and scopolamine induce performance deficits in spatial learning tasks, but the effects may not be specific to memory (Cain, 1998; Saucier et al., 1996). Findings showing that combined scopolamine and MK-801 administration at subthreshold doses produces short-term amnesia in the elevated plus maze task and impairs inhibitory avoidance learning suggest that there is an interaction between muscarinic and NMDA receptors in the regulation of basic memory processes. Interaction in the regulation of spatial learning and memory is supported by studies showing that DCS reverses the acquisition deficit induced by scopolamine in the water maze (Pitkänen et al., 1995; Puumala et al., 1998; Sirviö et al., 1992), T-maze (Fishkin et al., 1993) and passive avoidance tasks (Zajaczkowski and Danysz, 1997). In the radial arm maze task, NMDA antagonists have been shown to potentiate the scopolamine induced amnesic effect (Li et al., 1997). Furthermore, our laboratory has found that subthreshold doses of scopolamine and DCS in the dorsal HC induce an acquisition deficit in the water maze task, suggesting that muscarinic and NMDA receptors jointly modulate spatial navigation at the hippocampal level (Riekkinen and Riekkinen, 1997). Interaction at the hippocampal level is further supported by the finding that DCS reduces the scopolamine-induced deficit of working memory, as assessed in a three-panel runway task (Ohno and Watanabe, 1996). Interestingly, physostigmine does not reverse working memory deficit induced by microinjections into the HC of the competitive NMDA receptor antagonist ((+/-)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP), suggesting that disrupted NMDA receptor mediated mechanisms regulating working memory in this task cannot be restored with muscarinic receptor stimulation in the HC (Ohno et al., 1996). Pharmacological evidence also favors the importance of colinergic and NMDA receptor dysfunction in the cognitive decline of aged rats, as indicated by the finding that THA and DCS dose dependently alleviated failures in water maze spatial navigation induced by aging in rats (Baxter et al., 1996; Riekkinen et al., 1996).

3. AIMS OF THE STUDY

The purpose of this study was to investigate the role of cholinergic system and muscarinic ACh receptors, as well as their possible interactions with glutamatergic NMDA receptor mediated mechanisms in the modulation of working memory and the age-related spatial navigation deficit of rats. The specific aims were as follows.

1. To study whether blockade of central NMDA or muscarinic receptors, especially the M_1 and M_2 subtypes, affects working memory by investigating the effects of the NMDA antagonist CPP or the muscarinic receptor antagonists scopolamine, pirenzepine and methoctramine on the performance of rats in the delayed non-matching to position (DNMTP) task (I and II).

2. To examine whether central muscarinic and NMDA receptor mediated mechanisms interact in the modulation of working memory by comparing the effects of combined and separate blockade of these receptors on DNMTP task performance (II).

3. To investigate whether blockade of muscarinic or NMDA receptors in the dorsomedial prefrontal or dorsolateral frontal cortex affects working memory by determining the effects of intracerebral injections of scopolamine and CPP on DNMTP task performance (III).

4. To study the interaction between cholinergic (THA) and NMDA receptor (DCS) stimulation in alleviating the spatial navigation deficit of aged rats by using either separate or combined administration of these drugs and several variants of the water maze task (IV and V).

4. MATERIALS AND METHODS

4.1 ANIMALS

Male Han:Wistar rats were used in this study. The animals were singly housed in stainless steel shoe-box cages (44 x 27 x 15 cm; l x w x h) in a controlled environment (National Laboratory Animal Centre, Kuopio, Finland; temperature 20°C, humidity 50-60%, lights on from 07.00 to 19.00 hours) with water and food freely available during water maze testing (IV and V). During training and testing of the DNMTP task, the rats were deprived of food 12-14 h before daily training or testing (I - III). After daily behavioural training or testing in the DNMTP task, the rats received 10-12 g of food pellets (Special diet service, England), thus enabling the animals to be maintained at 80 – 85 % of their free-feeding weight. Water was freely available except in the test apparatus. The studies were conducted according to EC guidelines and were approved by the State Provincial Office of Kuopio.

4.2 IMPLANTATION OF INFUSION CANNULAS

The rats were anaesthetised and placed in a stereotaxic frame (I - III). The guide cannulae were implanted unilaterally in the lateral ventricle (I and II) or bilaterally in the dmPFC (Fig. 4.) or dlFC (III). The implantation of infusion cannulae is described in more detail in publications I - III.

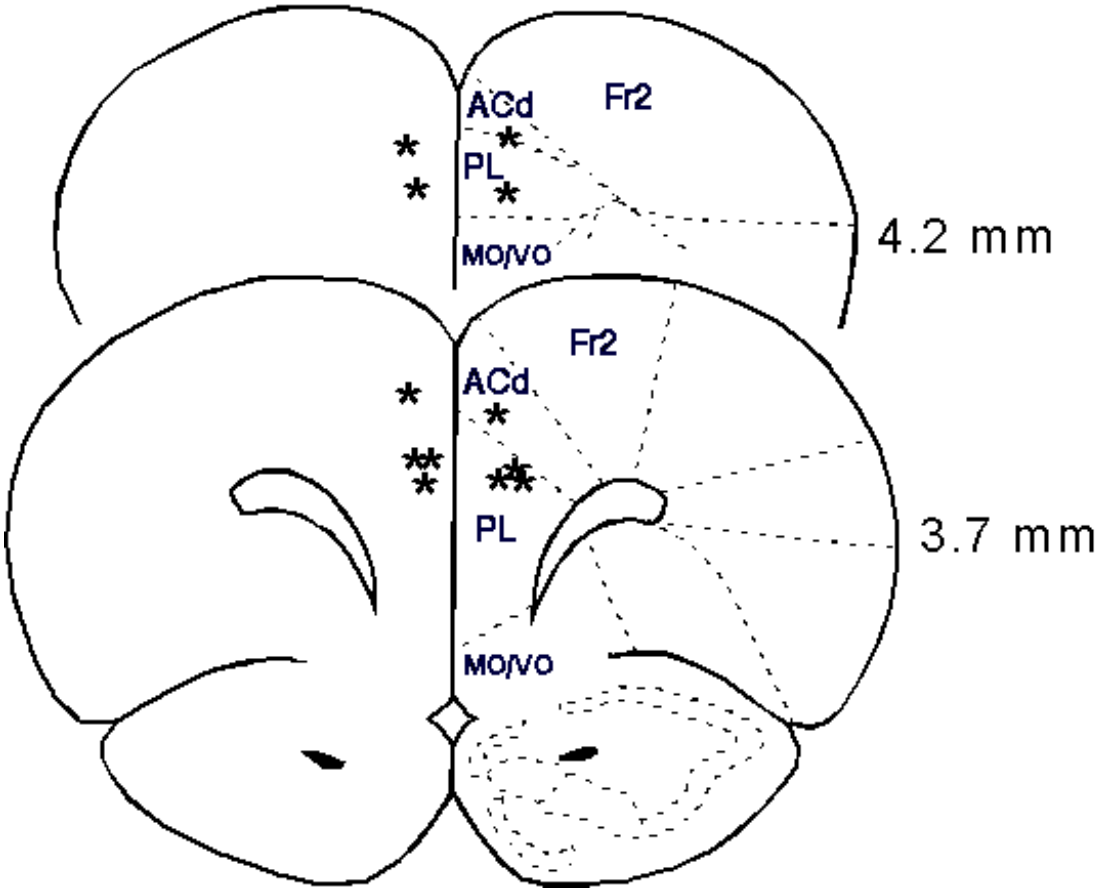


Fig. 4. The location of cannulae tips in the dmPFC (marked by asterisks). The numbers represent millimetres anterior to the bregma (adapted from Paxinos and Watson, 1986). Abbreviations (according to (Krettek and Price, 1977)): Fr2 = frontal area 2; ACd = dorsal anterior cingulate area; PL = prelimbic area; MO/VO = medial / ventral orbital area.

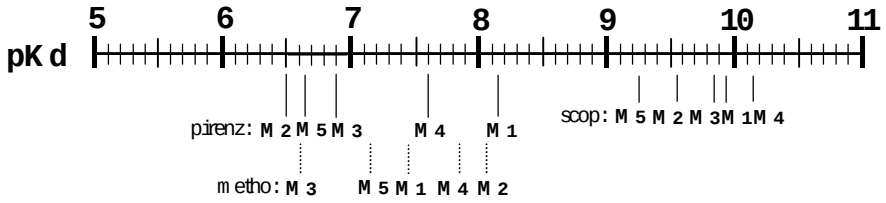
4.3 PHARMACOLOGICAL AGENTS

The following pharmacological agents were used in the experiments: a non-selective muscarinic antagonist, scopolamine (Sigma Chemical Company, St. Louis, MO, USA); a selective muscarinic M_1 receptor antagonist, pirenzepine (Research Biochemicals International (RBI), Natick, MA, USA); a selective muscarinic M_2 receptor antagonist, methoctramine (RBI); a cholinesterase inhibitor, tetrahydroaminoacridine (THA) (RBI); a partial agonist of the glycine-B -site of the NMDA receptor, D-cycloserine (DCS) (RBI); and

a selective NMDA receptor antagonist, CPP ((+/-)-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (RBI)).

Table 7. Antagonist affinities of muscarinic receptor subtypes in numeric and scale form. (adapted from (Girdlestone, 1998).

	M_1	M_2	M_3	M_4	M_5
Scopolamine (Kd)	80-150	200-400	150-250	50-100	500-700
Scopolamine (pKd)	9.8-10.1	9.4-9.7	9.6-10.1	10-10.3	9.2-9.3
Pirenzepine(pK_d or pK_B)	7.8-8.5	6.3-6.7	6.7-7.1	7.1-8.1	6.2-7.1
Methoctramine (pK_d or pK_B)	7.1-7.8	7.8-8.3	6.3-6.9	7.4-8.1	6.9-7.2



Publication I addressed the effects of i.c.v. infusions of scopolamine (3 and 10 μg), pirenzepine (10 and 30 μg) and methoctramine (2 - 20 μg) on DNMTP task performance.

Publication II addressed the effects of i.c.v. infusions of scopolamine and pirenzepine given in combination with CPP at sub-threshold doses on DNMTP task performance.

Publication III addressed the effects of i.c. infusions of scopolamine (10 μg) and CPP (0.01 – 0.1 μg) into the dmPFC and scopolamine (10 μg), and CPP (0.1 and 0.3 μg) into the dlFC on DNMTP task performance.

Publication IV addressed the effects of THA (3 mg / kg) and DCS (10 mg / kg), either separately or in combination, on the spatial navigation of aged rats in the water maze task.

Publication V addressed the effects of THA (3 mg / kg) and DCS (10 mg / kg), either separately or in combination, on the spatial navigation of the aged rats after pre-training.

4.4 BEHAVIOUR

4.4.1 Delayed non-matching to position task (DNMTP) (publications I - III)

Apparatus: DNMTP testing was conducted in four operant chambers, each equipped with two retractable levers and a food pellet dispenser (Campden Instruments, London, UK). The operant chambers were under the online control of microprocessors (Paul Fray Ltd., Cambridge, UK) programmed using Spider (Paul Fray Ltd., Cambridge, UK) (Sirviö et al., 1991). The food pellet dispenser was located between the levers and delivered 45 mg of dustless precision pellets (Campden Instruments, UK).

Training: Prior to training for the DNMTP task, 35 rats at the age of three months were first deprived of food overnight, and 5 rats were kept on a free feeding regimen as age-matched weight controls. The weights were kept at approximately 85 % of their free feeding weight by giving them a daily 10 – 12 g dose of food pellets (Special Diet Service, UK). During the first week, the weights of the rats were controlled daily; during the second two weeks, twice a week; and during the rest of the time, once a week.

Before the actual training started, the rats were first habituated to the chamber for 10 min. The perspex door of the food pellet dispenser was open and the magazine was filled with reward pellets, thus assuring that the rats would learn to associate the magazine with the reward pellets. The actual training schedule was adapted from Sirviö et al. (1991).

In the first phase of training, the rats were trained to collect food pellets and to associate the click of the dispenser plus illumination of the panel light with pellet delivery. A pellet was delivered every time a rat made a nose poke into an illuminated pellet magazine. If a rat did not respond within 45 s, the magazine light was turned off for 5 s. The rats were trained 20 minutes/day until they had learned to obtain at least two pellets/minute. All the rats learned to collect pellets within 3 days.

In the second phase, the rats learned to associate the lever press with the delivery of a food pellet. Both levers were inserted and, every time a rat pressed a lever, a food pellet was delivered into the magazine, which was illuminated. If a rat did not react within 45 s, the

levers were retracted for 5 s. The rats needed one or two training periods in phase two to learn the task (more than one response/ minute).

In phase three, rats learned to press a lever (either right or left) when it was inserted into the chamber in order to get a food pellet. The right or left lever was inserted randomly, and if the rat pressed the lever, a food pellet was delivered and the magazine was illuminated. Then the lever was retracted, and after a 5-s period, one of the levers was inserted once again. If a rat did not press the lever within 45 s, the lever was retracted and the house light was turned off for 5 s. All the rats tested in phase three acquired the task in one session (more than one response/minute).

In phase four, the rats were trained for the non-matching to position task (0-delay). A right or left lever, which was selected randomly, was inserted into an operant chamber (sample phase, fig. 5, part A). When the rat pressed the lever, it was retracted and a magazine was illuminated, but no food pellet was delivered. When the rat made a nose poke into the magazine (fig. 5, part B), the magazine light was turned off and both levers were inserted (choice phase). Pressing of the non-sample lever was reinforced with the delivery of a food pellet into the illuminated magazine (fig. 5, part C, left). If the rat pressed the sample lever, the house light was turned off for 5 s (fig. 5, part C, right). After a 5-s period, a new cue lever was inserted. If a rat did not press a cue lever (omission 1) or one of the choice levers (omission 2), the house light was turned off for 5 s and a new sample lever was inserted after a 5-s interval. After the rats had been trained for 10 days in the task (> 85 % correct responses), delays (0, 1, 2, 4, 8 and 16 s) before inserting the choice levers were included. All delays were used in each session of the DNMTP task in a random order (about10 trials/delay). The training (20-30 minutes/day; 2-3 days/week) continued for four months (36 training days) before the first group was operated.

Testing: Detailed testing paradigms can be found in publications I - III. The variables used in the data analysis were the percentage of overall correct responses (correct responses / (correct + incorrect responses)* 100 %), the percentage of correct responses for each delay, the percentage of omissions (omitted trials / all trials)* 100 %), and the latencies for sample press, nose pokes and food collection (table 8).

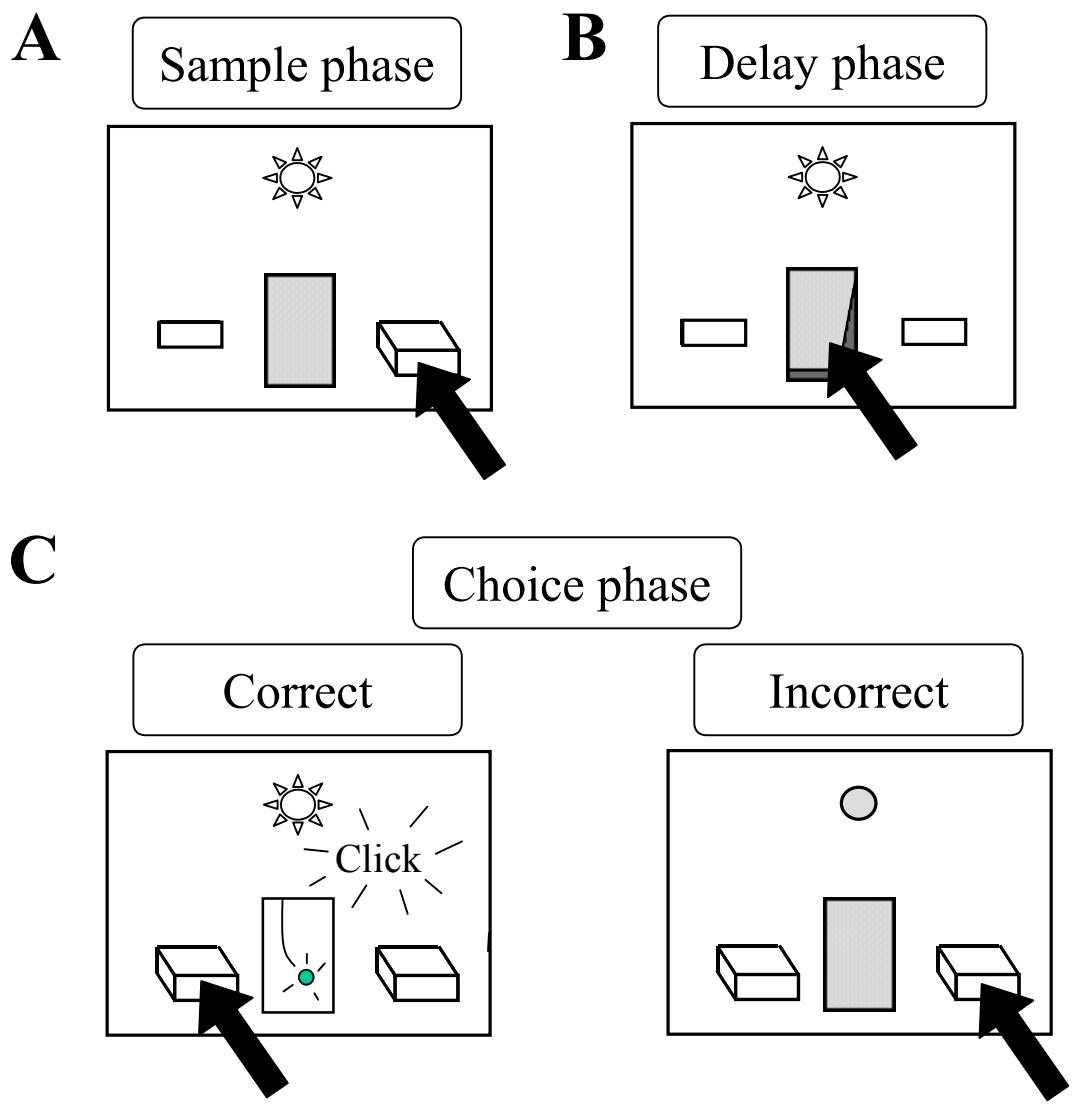


Fig. 5. The delayed non-matching to position task. Part A represents the first phase of one trial of the DNMTP task. During the *sample* phase, one of the levers is presented into the chamber, and a rat pushes it to start the next phase. Part B represents the second phase, the *delay* phase, during which the rats are actively poking the magazine door. During this phase, the rat is supposed to "keep in mind" the next choice. Part C represents the last phase, the *choice* phase, which begins after the first nose poke into the magazine door after the delay (0, 1, 2, 4, 8 or 16 s in a random order). Both levers are presented into the chamber, and the rat has to choose the lever it did not push before in order to get a reward pellet. If it makes a correct choice and pushes the choice lever, the magazine light is turned on, and the dispenser provides a reward pellet with a typical clicking sound. The rat has 5 s to eat his reward before the next trial begins. However, if it chooses the sample lever again, no reward is provided and the chamber light is turned of for another 5 s as a mark for incorrect response. A new sample phase begins automatically after 5 s.

Table 8. DNMTP task parameters with explanations.

Measured parameter	Estimated behavioral variable
Percentage of correct responses	Overall accuracy
Delay dependency	Decay of memory
Omissions	Motivation, attention, motor activity
Latency of sample presses	Motivation, attention, motor activity
Latency of nose pokes	Motivation, attention, activity during delays
Latency of food collection	Appetite, motivation, motor activity

4.4.2 Morris water maze (publications IV and V)

The Morris water maze (pool diameter 150 cm) was used to study spatial learning (Fig. 6, A). A computer connected to an image analyser (HVS Image, Hampton, UK) monitored the swim pattern (Fig. 6, B, C and D). The timing of the latency to find the submerged platform (10 x 10 cm) was started and ended by the experimenter. The following variables were used in the data analysis: the daily escape distance values (IV and V), the number of counter crossings during the spatial bias test and the number of rats that escaped to the platform during the first trial of platform training (IV) (table 9).

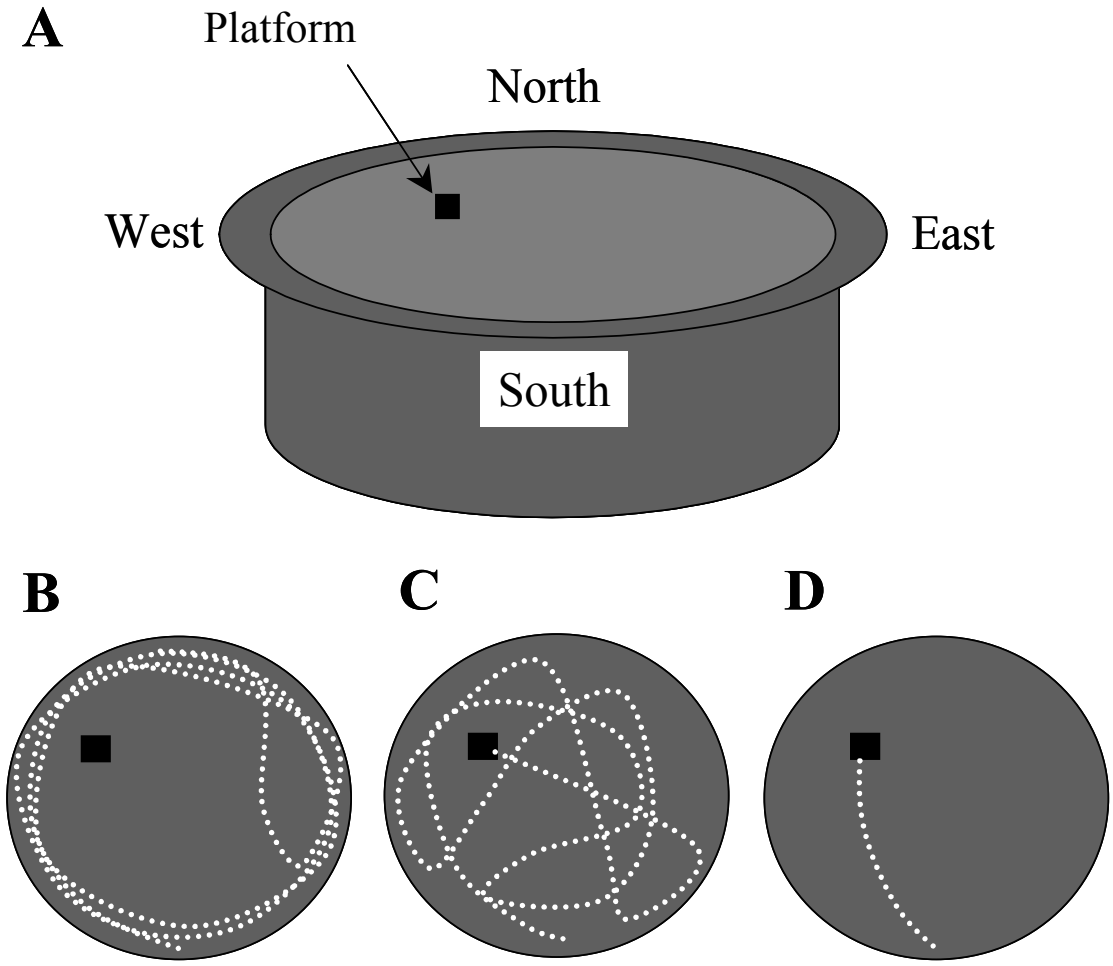


Fig. 6. Schematic drawing of the water maze pool. Part A: The cardinal points (North, East, South, West) represent the starting locations. The starting points were selected in a semi-random order (never from the same point as the previous start). Parts B C and D: Typical swimming paths as seen on a computer monitor during the trials. Part B represents a typical path in the beginning of the training (thigmotaxis i.e. swimming close to the wall). Part C represents a typical search path of a rat that knows how to search for, but does not know/remember the location of the hidden platform. Part D represents a typical search path of a rat that is well aware of the rescue location.

Before testing, the rats were physically examined for external signs of disease (tumours, infection, ulcers etc.). The rats were also tested for disabilities in swimming by introducing one probe trial without platform in the pool. The starting locations on the pool rim (labelled north, south, east, and west) were chosen arbitrarily (Fig. 6, A). The pool was divided into 4 quadrants (Southwest (SW), Southeast, Northwest, and Northeast (NE)) and 3 concentric annuli of equal surface area. The water temperature was 22 ± 1.5 °C. The rats were placed in the water with their nose pointing towards the wall, at one of the starting points. Those rats that did not find the platform before the cut off time of 70 s were placed on the platform by the experimenter. The rats were allowed to stay on the platform for 5 s. Every experiment consisted of three daily trials, with a 30-s interval between trials. After the last training session the rats were gently dried with a towel and placed back in their cages.

The water maze experiments are schematically described in Table 10. The training paradigms and testing systems are described in detail in publications IV and V.

Table 9. Water maze task parameters with explanations.

Measured parameter	Estimated behavioural variable
Random navigation (first trial of a test)	Initial navigation strategy
Swimming speed	Motor function
Escape latency	Navigation accuracy + swimming speed
Escape distance	Navigation accuracy + strategy
Search bias (annulus crossings)	Navigation accuracy

Table 10. The testing schemes of the water maze studies. In publication IV (part A), the testing took place in only one water maze room. The escape performance of aged rats receiving drugs was compared to that of the aged and young control groups receiving NaCl. In publication V (part B), the rats were first pre-trained with either hidden or visible platforms, and then tested in the same or different water maze room. Again, the escape performance of aged rats receiving drugs was compared to that of the aged and young control groups receiving NaCl. Abbreviations: Exp = experiment, D = the stage has both drug groups and NaCl groups, Hidden = platform is hidden below the water surface, N = the stage has only NaCl groups, Visible = platform has a clearly visible sign.

A. (Publication IV)

ROOM 1		
	Testing	
Exp 1	Hidden	D
Exp 2	Hidden	D
Exp 3	Hidden	D
Exp 4	Hidden	D

B. (Publication V)

	ROOM 1				ROOM 2	
	Pre-training		Testing		Testing	
Exp 1	Hidden	N	Hidden	D		
Exp 2	Hidden	N	Hidden	D		
Exp 3	Visible	N	Hidden	D		
Exp 4	Hidden	N			Hidden	D
Exp 5	Hidden	D			Hidden	N

4.5 HISTOLOGY

In publications I and II, the rats were decapitated one week after the completion of testing. The brains were removed and the brain area containing the infusion site was placed into 4 % formalin in 0.1 M phosphate-buffered saline for 24 h. In publication III, the rats were euthanized with an overdose of chloral hydrate and perfused through the heart with 0.9 % saline followed by 4 % formalin in 0.1 M phosphate-buffered saline. The brains were removed and immersed in 4 % formalin in 0.1 M phosphate-buffered saline until sectioning.

After fixation, serial coronal sections (60 μm) were cut and stained with cresyl violet to determine whether the cannulae had been correctly placed into the lateral ventricle (I and II) or the dlFC and dmPFC (III)

4.6 STATISTICS

Statistical analyses were made using SPSS/PC+ software. Analysis of variance (ANOVA) for repeated measures and Student's t-test were used to analyse the behavioural parameters of the DNMTP task (I-III). Before the ANOVA, the distribution of the data was normalized. The data for the percentage of correct responses and of omissions were transformed using the arcsin transformation, and the data for latencies were transformed using logarithmic transformation. A oneway-ANOVA followed by Duncan's post hoc multiple group comparisons was used to measure the effects of the drug treatments on water maze acquisition (IV-V). A twoway-ANOVA was used to assess the interactions of THA and DCS treatments on water maze escape distance in the aged rats. The proportion of the rats that found / failed to find the escape platform on the first trial was analyzed with a chi-square test. Values of $P < 0.05$ were considered significant. Methods for statistical analyses are described in detail in publications I-V.

5. RESULTS

5.1 DNMTP TASKS

Table 11. The effects of muscarinic antagonists and CPP (i.c.v.) on DNMTP task performance. Abbreviations: METHO = methoctramine, PIRENZ = pirenzepine, SCOP = scopolamine, 0 = data not available, – = no effect (P > 0.05), + = significant effect, ↑ = increasing effect, ↓ = decreasing effect, * = P < 0.05, ** P < 0.005.

	SCOP 3 μg	SCOP 10 μg
Choice accuracy	↓ *	–
Delay dependency	–	–
Omissions (%)	–	↑ *
Latency of sample press (s)	–	↑ *
Latency of nose poke (s)	–	–
Latency of food collection (s)	–	–

	PIRENZ 10 μg	PIRENZ 30 μg
Choice accuracy	–	↓ *
Delay dependency	–	–
Omissions (%)	–	–
Latency of sample press (s)	↑ *	↑ *
Latency of nose poke (s)	–	–
Latency of food collection (s)	–	–

	METHO 2 μg	METHO 5 μg	METHO 20 μg
Choice accuracy	↑(longest delays)	–	–
Delay dependence	(+) P = 0.058	–	–
Omissions (%)	–	–	–
Latency of sample press (s)	–	–	–
Latency of nose poke (s)	–	–	–
Latency of food collection (s)	–	–	–

	CPP 0.1μg	CPP 0.3 μg
Choice accuracy	–	↓ *
Delay dependence	–	–
Omissions (%)	–	↑ *
Latency of sample press (s)	–	↑ *
Latency of nose poke (s)	↓ *	0
Latency of food collection (s)	–	0

5.1.1 The effects of scopolamine, pirenzepine, methoctramine and CPP (i.c.v.) on DNMTP task performance (Publications I and II).

These tests were made to investigate whether the effects of central administration of muscarinic and NMDA receptor antagonists on task performanced is mnemonic (delay-dependent change in performance accuracy) or non-mnemonic (delay-independent change in performance accuracy or changes in other variables) in nature. Table 11 summarises the results.

Scopolamine 3 μg decreased choice accuracy, but the higher 10 μg dose did not significantly affect the choice accuracy (I: results p. 131 and Fig. 1, top left). Scopolamine 10 μg increased the percentage of omissions during the sample phase, but scopolamine 3 μg had no effect on the omissions. (I: results p. 131 and table 1). Scopolamine 3 or 10 μg had no effect on the sample press latency (I: results p. 131 and table 1) nose poke or food collection.

Pirenzepine 30 μg decreased choice accuracy, but the lower 10 μg dose had no effect on choice accuracy (I: results p. 131 and Fig. 1, top right). Pirenzepine did not significantly change the percentage of omissions but increased the latency to sample press (I: results p. 131, 132 and table 1).

Methoctramine, 2, 5 and 20 μg, had no significant treatment effect, but the delay by treatment interaction was almost significant (I: results p. 132, Fig. 1, bottom left and right). A comparison of the effects of methoctramine, 2, 5, and 20 μg, on choice accuracy at different delays revealed that the lowest dose improved accuracy at the longest delays, with the effect reaching significance at the 16-s delay (I: results p. 132). At the lowest dose, 2 μg, the variances did not differ significantly between the delays, thus speaking against a possible "bottom effect" behind the effect (I: results p. 132) ("bottom effect" means that statistic results may be biased, if the variance of the choice accuracy is lower in the longer than shorter delays). In addition, if the real effect of a drug is an impairment of choice accuracy in the DNMTP task, the result might not become statistically significant, because the performance has already reached its limit, i.e. performance is at a chance level. This separate analysis was added, because one of the referees suggested that the bottom effect might explain the result). Methoctramine, 5 and 20 μg, had no effect on choice accuracy at any

delays. Methoctramine, 2, 5 and 20 μg, did not affect omissions or latency to sample press (I: results p. 132, table 1).

CPP 0.1 had no effect on choice accuracy (II: results p. 10 and Fig. 1C), the percentage of omissions or the latency of sample press but decreased the latency of nosepokes (II: results p. 10 and table 1). CPP 0.3 μg markedly and delay-independently reduced the choice accuracy (II: results p. 10 and Fig. 1D). Furthermore, CPP 0.3 μg increased omissions and the latency of sample press (II: results p. 10 and table 1).

5.1.2 The effects of combined i.c.v. administration of scopolamine, pirenzepine and CPP on DNMTP task performance (Publications I and II).

Table 12. The effects of combined i.c.v. administration of muscarinic antagonists and CPP on DNMTP task performance. Abbreviations: METHO = methoctramine, PIRENZ = pirenzepine, SCOP = scopolamine, 0 = data not available, – = no effect ($P > 0.05$), + = significant effect, ↑ = increasing effect, ↓ = decreasing effect, * = $P < 0.05$, ** $P < 0.005$.

	CPP 0.03 μg	SCOP 1 μg	CPP 0.03 μg + SCOP 1 μg
Choice accuracy	–	–	–
Delay dependence	–	–	–
Omissions (%)	–	–	↑ **
Latency of sample press (s)	–	–	↑ **
Latency of nose poke (s)	–	–	↑ **
Latency of food collection (s)	–	–	-

	CPP 0.03 μg	CPP 0.03 μg + PIRENZ 10 μg
Choice accuracy	–	–
Delay dependence	–	–
Omissions (%)	↓ *	–
Latency of sample press (s)	–	–
Latency of nose poke (s)	–	–
Latency of food collection (s)	–	–

These experiments were conducted to investigate whether the effects of combined central administration of muscarinic and NMDA receptor antagonists at subthreshold doses were mnemonic (delay-dependent change in performance accuracy) or non-mnemonic (delay-independent change in performance accuracy or changes in other variables) in nature. Table 12 summarises the DNMTP results from i.c.v infusions of muscarinic antagonists and CPP.

Administration of CPP 0.03 μg or scopolamine 1μg alone had no effect on the choice accuracy (II: results p. 10 and Fig. 1F), the percentage of omissions or the latency of sample press (II: results p. 10 and table I). Simultaneous administration of these sub-threshold doses of CPP 0.03 μg and scopolamine 1μg had no effect on the choice accuracy (II: results p. 10 and Fig. 1F) but robustly increased the omissions, the latency of sample press and increased the latency of nose pokes (II: results p. 10 and table 1).

Simultaneous administration of CPP 0.03 μg and pirenzepine 10 μg had no effect on choice accuracy (II: results p. 11 and Fig 1G), the omissions or the latency of sample press. In this test, CPP 0.03 μg on its own slightly decreased the omissions (II: results p. 11 and table 1).

None of the treatments significantly affected the latency of food collection (II: table 1).

5.1.3 The effects of CPP and scopolamine (i.c.) on DNMTP task performance in dmPFC or dlFC (Publication III).

These tests were made to investigate whether administration of the NMDA receptor antagonist CPP directly into the dmPFC have mnemonic or non-mnemonic effects on performance. These drug effects were compared to the effects of scopolamine, which have been reported to cause a non-mnemonic performance deficit in the DNMTP task (Dunnett et al., 1990; Herremans et al., 1996). As a control area, we used the dorsolateral frontal cortex that according to the literature is not likely to be important in the regulation of WM (Dunnett, 1990; Wortwein et al., 1994).

Table 6 summarises the DNMTP results from i.c. infusions of CPP and scopolamine into the dmPFC or dlFC.

Table 6. The effects of CPP and scopolamine on DNMTP task performance in the dmPFC or DlFC. Abbreviations: METHO = methoctramine, PIRENZ = pirenzepine, SCOP = scopolamine, 0 = not tested, – = no effect (P > 0.05), + = significant effect, ↑ = increasing effect, ↓ = decreasing effect, * = P < 0.05, ** P < 0.005.

CPP 0.01 and CPP 0.03 μg	dmPFC	DlFC
Choice accuracy	–	0
Delay dependence	–	0
Omissions (%)	↑	0
Latency of sample press (s)	↑	0
Latency of nose poke (s)	↑ (CPP 0.01)	0

CPP 0.1 μg	dmPFC	DlFC
Choice accuracy	–	–
Delay dependence	+*	–
Omissions (%)	↑	–
Latency of sample press (s)	↑	–
Latency of nose poke (s)	↑	–

CPP 0.3 μg	dmPFC	DlFC
Choice accuracy	↓	–
Delay dependence	–	–
Omissions (%)	–	–
Latency of sample press (s)	↑	↑
Latency of nose poke (s)	↑	↑

Scopolamine 10 μg	dmPFC	DlFC
Choice accuracy	–	–
Delay dependence	–	–
Omissions (%)	–	–
Latency of sample press (s)	↑	–
Latency of nose poke (s)	↑	–

In the dmPFC, both CPP 0.01 and CPP 0.03 μg increased the percentage of omissions and the latency of sample press (III: results p. 244, 246 and table 1) but had no effect on choice accuracy (III: results p. 246 and Fig. 1A). CPP 0.01 also increased the latency of nose pokes (III: table 1)

In the dmPFC, the main effect of CPP 0.1 μg on choice accuracy was non-significant, though there was a significant drug by delay interaction. The decrease in choice accuracy was significant at 1 and 2 s delays, indicating a delay-dependent defect in performance (III: results p. 246 and Fig. 1B). The treatment also increased omissions and the latencies of sample press and nose poke (III: results p. 246 and table 1). The highest CPP dose 0.3 μg decreased the choice accuracy delay-independently (III: results p. 246 and Fig. 1C) and increased the omissions and the latencies of sample press and nose poke in the dmPFC (III: results p. 246 and table 1).

Scopolamine 10 μg in the dmPFC had no effect on choice accuracy or omissions (III: results p. 246, Fig 1C and table 1) but increased the latencies of sample press and nose poke (III: results p. 246 and table 1).

In the dlFC, both CPP and scopolamine had markedly different effect profiles when compared to the effects in the dmPFC. CPP 0.1 μg had no effect on the choice accuracy, omissions or latencies of sample press and nose poke in the dlFC (III: results p. 246, Fig 1D and table 1). CPP 0.3 μg had no effect on the choice accuracy or omissions (III: results p. 246, Fig 1D and table 1) but increased the latency of sample press and nose poke (III: results p. 246, Fig 1D and table 1).

Scopolamine 10 μg had no effect on the choice accuracy, omissions, sample press or nose pokes in the dlFC (III: results p. 246, Fig 1D and table 1).

5.1.3 Histology

Examination with light microscope revealed that all the cannulae of the rats included in the analysis had been correctly placed into the left or right ventricle (I and II). In addition, the cannulae targeted into the dmPFC and dlFC had also been correctly placed (III). All the dmPFC cannulae had been placed between the coordinates 3.7 mm and 4.2 mm anterior to the bregma (Paxinos and Watson, 1986), i.e. in the rostral part of the dmPFC (III).

5.2 WATERMAZE TASKS

5.2.1 The effects THA and DCS (i.p.) on spatial navigation of aged rats (IV).

This study was designed to elucidate whether combined treatment with a cholinesterase inhibitor, THA, and a positive modulator of the NMDA receptor, DCS, is more effective than treatment with either drug on its own in stimulating spatial navigation in aged rats. Table 14 presents the testing scheme, and Table 15 summarises the water maze results from i.p. infusions of THA and DCS.

Table 14.Schematic representation of the water maze testing procedure (IV).

WATER MAZE 1			
	Training days	**Platform**	**Administration**
Exp 1	5	hidden	drugs before training
Exp 2	5	hidden	drugs before training
Exp 3	5	hidden	drugs before training
Exp 4	4	hidden	drugs after training

Table 15. The effects of THA and DCS (i.p.) on the performance of aged rats in the water maze (compared to aged controls).

Drug groups (mg/kg)	Initial search strategy	Escape distance	Spatial bias after testing
THA 0.3	0	0	0
THA 1	0	0	0
THA 3	0	↓	0
DCS 1	0	0	0
DCS 3	0	0	0
DCS 10	0	↓	↑
THA 0.3 + DCS 1	0	0	0
THA 1 + DCS 3	0	↓	↑
THA 3 + DCS 10	0	↓	↑
THA 3 + DCS 10 (post training)	-	0	0

Abbreviations; 0 = no effect, ↓ = drug decreased escape distance, ↑ = drug increased number of annulus (platform area) crossings during probe trial after training, - = not tested.

In the first study, we used 5 groups of 8 rats: young and aged controls, aged THA 3 mg/kg, aged DCS 10 mg/kg and aged THA 3 mg/kg + DCS 10mg/kg. The proportion of rats that found the platform during the first trial of the first day did not differ between the groups, suggesting that the initial search strategies did not differ between the groups (IV: results p. 17 and table 1A). THA 3 mg/kg or DCS10 mg/kg alone were equally effective in decreasing the escape distance of aged rats (IV: results p. 17 and Fig. 1A). A combination of THA 3 mg/kg and DCS10 mg/kg was no more effective in decreasing the escape distance than single treatments alone (IV: Fig. 1A). Furthermore, we found no interaction between the two treatments (DCS10 mg/kg x THA 3 mg/kg; IV: results, p. 17). During the spatial probe trial, aged rats that were treated with DCS10 mg/kg or THA 3 mg/kg + DCS 10 mg/kg during the training phase made more correct platform crossings than did the other groups of aged rats (IV: results p. 17 and table 1B). Aged rats treated with either DCS 10 mg/kg alone or with the combination of DCS10 mg/kg and THA 3 mg/kg had as strong spatial bias towards the target quadrant as the young control rats. In contrast, THA 3 mg/kg alone had no effect on the spatial bias of aged rats (IV: results p. 17 and table 1B).

In the second study, we used 5 groups of 9 rats: young and aged controls, aged THA 1 mg/kg, aged DCS 3 mg/kg and aged THA 1 mg/kg + 3 mg/kg. Again, we found that the proportion of rats that found the platform during the first trial of the first day did not differ between the groups (IV: results p. 17 and table 1A). In aged rats, THA 1 mg/kg or DCS 3 mg/kg on their own had no effect on escape distance over days 1 to 5 (Fig 1B) or spatial bias in the probe test (table 1B). However, the ANOVA revealed a significant interaction between these treatments: aged rats treated with the combination of THA 1 mg/kg and DCS 3 mg/kg had the shortest escape distance when compared to the other aged groups (IV: results p. 18 and Fig. 1B). THA 1 mg/kg + DCS 3 mg/kg also interacted to increase the spatial search bias of aged rats (IV: results p. 18 and table 1B) in the final probe test.

In the third study, we used 4 groups of 9 aged rats: controls, THA 1 mg/kg, DCS 1 mg/kg and THA 0.3 mg/kg + DCS 1 mg/kg. The proportion of rats that found the platform during the first trial of the first day did not differ between the groups (IV: results p. 18 and table 1A). THA 0.3 mg/kg or DCS 1 mg/kg plus their combination had no effect on escape distance or spatial bias (IV: results p. 18, Fig 1C and table 1B).

In the fourth study that examined the effect of drugs administered after daily training sessions, we used 5 groups of 8 rats: young controls, aged controls, aged THA 3 mg/kg, aged DCS 10 mg/kg and aged THA 3 mg/kg + DCS 10 mg/kg. The proportion of rats that found the platform during the first trial of the first day did not differ between the groups (IV: results p. 18 and table 1A). The results revealed that all aged rats were worse than the young rats in finding the hidden platform during the training days 1-5 (IV: results p. 18 and Fig 1D). Administration of single or combined DCS 10 mg/kg or THA 3 mg/kg after daily training trials failed to decrease escape distance or increase spatial bias.

5.2.2 The effects of pre-training on the improved spatial navigation of aged rats induced by THA and DCS (V).

In this study, we examined the effects of pretraining on the previously observed improvement in spatial navigation of aged rats induced by THA and DCS. Pre-training took place under different conditions and included pre-training for a non-spatial escape strategy. We also investigated whether the pre-training might had any effect on the age-related spatial

navigation deficit itself.

Table 16. Schematic representation of the water maze testing procedure (IV).

	WATER MAZE 1							WATER MAZE 2
	Pre-training			**Pause**	**Testing**			-
Exp 1	5 days	hidden	placebo	30 days	5 days	hidden	drugs	-
Exp 2	5 days	hidden	placebo	30 days	5 days	hidden	drugs	-
Exp 3	3 days	visible	placebo	30 days	5 days	hidden	drugs	-

	WATER MAZE 1					WATER MAZE 2		
	Pre-training			**Pause**	**room change**	**Testing**		
Exp 4	8 days	hidden	placebo	2 days	"	6 days	hidden	drugs
Exp 5	6 days	hidden	drugs	2 days	"	5 days	hidden	placebo

Summary of the results. The alleviating effect of both D-cycloserine and tetrahydroaminoacridine (separately or in combination) on the age-related defect in escape performance disappeared after pre-training under different conditions, including pre-training for a non-spatial escape strategy. The spatial navigation defect in aged rats still remained after the pre-training procedures. (see V p. 315 and 316 for figures)

In experiment 1 (V: Fig. 1A), we used 5 groups of 10 rats: young and aged controls, aged THA 3 mg/kg, aged DCS 10 mg/kg and aged THA 3 mg/kg + DCS 10 mg/kg. During the first five-day training period, with no drug treatments, all the groups of aged rats were worse than young controls in finding the hidden platform in the SW quadrant (V: results p. 316 and Fig. 1A, left plot). In the second training period occurring after a break of 1 month, none of the single or combined treatments improved the performance of the aged rats in locating the hidden platform in the SW quadrant (V: results p. 316 and Fig. 1A, right plot).

In experiment 2 (V: Fig. 1B), we used 5 groups of 10 rats: young and aged controls, aged THA 3 mg/kg, aged DCS 10 mg/kg and aged THA 3 mg/kg + DCS 10 mg/kg. Again, during the first training period, with no drug treatments, all groups of aged rats were worse than young controls in finding the hidden platform in the SW quadrant (V: results p. 317 and Fig. 1B, left plot). In the second training period, drug treatments failed to alleviate the age-related

impairment of learning, as none of the single or combined treatments improved the aged rats to find the hidden platform in the NE quadrant (V: results p. 317 and Fig. 1B, right plot).

In experiment 3 (V: Fig. 1C), we used 5 groups of 10 rats: young and aged controls, aged THA 3 mg/kg, aged DCS 10 mg/kg and aged THA 3 mg/kg + DCS 10 mg/kg. In the first stage of training with a visible platform, no age-related defect was found (V: results p. 317 and Fig. 1C, left plot). In the second stage with a hidden platform, the aged rats were more impaired than the young controls. None of the single or combined drug treatments alleviated this impairment of the aged rats (V: results p. 317 and Fig. 1C, right plot).

In experiment 4 (V: Fig. 2 A), we used 4 groups of 12 rats: young and aged controls, aged THA 3 mg/kg and aged DCS 10 mg/kg. During the first stage (hidden platform), the aged rats were more impaired than the young controls. At this stage, the rats received no drug treatments, and no difference was observed in the escape distance among different groups of the aged rats (V: results p. 317 and Fig. 2 A, left plot). The second stage (hidden platform) was conducted in a novel testing room after a break of two days. Again, the aged rats were more impaired than the young controls. The drug treatments failed to alleviate the impaired performance of the aged rats (V: results p. 317 and Fig. 2 A, right plot).

In experiment 5 (V: Fig. 2 B), we used 3 groups of 12 aged rats: controls, aged THA 3 mg/kg and aged DCS 10 mg/kg. In the first stage of training with a hidden platform, the groups treated with THA and DCS had shorter escape distance values than the aged controls (V: results p. 317 and Fig. 2 B, left plot). During the second stage conducted in a novel testing room (hidden platform), the rats received no drug injections. Those groups that had been treated with THA and DCS during the first stage performed no better than those previously treated with the vehicle (V: results p. 317 and Fig. 2 B, right plot).

6. DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 DNMTP task.

Advantages. The strength of the DNMTP task is that the mnemonic specificity of the effects can be determined against a baseline forgetting curve. A mnemonic effect is reflected as a delay-dependent change in the memory decay curve, whereas a delay-independent effect suggests additional disturbance of non-mnemonic processes such as attentional, motivational or sensorimotor processes. The task also allows for measuring additional non-mnemonic performance parameters, such as motivation and motor activity, thus making this task suitable for examining the specificity of working memory effects, although attention and arousal processes are difficult to differentiate from motor activity. In addition, the DNMTP task is automated, which helps eliminate the introduction of any subjective component to the testing. Furthermore, each individual is compared to itself, which adds to the statistical power and allows for the use of fewer subjects.

Critique 1. Despite the advantages, the DNMTP task is not perfect. Today it is known that rats are able to adopt a biased response strategy that leads to high level of accuracy and high amount of omissions (Dunnett, 1993). In such a case, the rat only responds to one lever, and ignores those trials that begin by presenting the other lever. Our DNMTP analysis programme did not measure such bias. However, in the case of methoctramine, which improved performance accuracy in the longest delays, there was no increase in the omissions, thus speaking against any significant beneficial response bias. On the other hand, if pressing one of the levers during the choice phase is favoured and the responses during the sample phase are normal, the acquired bias leads to decreased accuracy. Although the accuracy disrupting bias could not be analysed with our programme, our DNMTP task measured other parameters that covered a variety of non-mnemonic performance deficits.

Critique 2. During statistical analysis, such phenomena as ceiling (roof) and floor (bottom)

effects can cause problems. Because the accuracy is measured on a percentage scale, the variance of performance accuracy may be lower in the shortest or longest delaysthan that in the medium-length delays. Because of this possibility, we used arcsin correction in the analysis, which converts the results from a percentage into a logarithmic scale, thus correcting the statistical problem. However, if the accuracy is "too high" across all the delays, it is more difficult to improve the performance accuracy than if the accuracy level is modest. Similarly, if the performance accuracy at the longest delays is already at the chance level, it is impossible to further decrease the accuracy. This behavioural aspect of floor and ceiling effects cannot be corrected by using logarithmic corrections in statistical analysis. In our studies, the behavioural, but not the statistical, bottom effect may have masked some of the accuracy deficits at the longest delays. Furthermore, variation in the basal performance accuracy, as well as strain differences and differences in task details, make it difficult to directly compare our results with that from other studies using operant D(N)MTP tasks.

Critique 3. Recently, serious criticism has been raised against the validity of operant D(N)MTP tasks as working memory tasks. Critical groups have shown that the rats are able to use several types of positional strategies (Chudasama and Muir, 1997; Herremans et al., 1996). Originally, it was suggested by Dunnett that active nose poking behaviour acts as a distracter and prevents the rats from using a positional (mediating) strategy during the delay phase (Chudasama and Muir, 1997; Dunnett, 1985). The critical groups suggest that rats do not use their working memory during the delay, but a strategy that is a reflection of motor rather than mnemonic performance. The use of positional strategies could not be monitored in our studies. However, although the use of positional strategies is well documented, there is one property in the DNMTP and DMTP tasks indicating that working memory processes, and not only mediating strategies, account for the performance accuracy. Namely, a phenomenon called proactive interference has an influence on the performance accuracy in delayed operant tasks (Dunnett et al., 1990; Hampson et al., 1998). Proactive interference means that the memory of the preceding trial has an effect on the performance accuracy in the ongoing trial. If the performance accuracy only reflects mediating strategies, then proactive interference should have no significant effect on it. Interestingly, two tasks that do not allow use of any mediating strategies have recently been developed: the delayed conditional discrimination task (DCD) developed by Herremans and Hizen (1997) and the discrete paired-trial T-maze task developed by Aultman and Moghaddam (2001). In the DCD task, memory decay occurs

within 20 s (log d of accuracy around 0.8 at the longest 20 s delay), and in the T-maze task the memory decays within 40 s (accuracy around 60% at the longest 40 s delay). In our DNMTP task the performance was near chance level within 16 s. This suggests that the accuracy in our task did not exceed the working memory capacity of rodents, which in turn increases the probability that the memory curve in our task reflects working memory processes. Therefore, it can be concluded that the benefit from using the mediating strategies was minimal, when compared to the use of "pure" working memory only. In addition, the delay-independent effect of scopolamine found in our study is similar to delay-independent effects reported in studies using both DCD and T-maze tasks (Aultman and Moghaddam, 2001; Herremans et al., 1997). These results indicate that our DNMTP task is able to provide reliable non-mnemonic effects with respect to the memory decay curve.

Attention and DNMTP task. A working memory task such as the DNMTP requires the rat to focus on "keeping in mind" the location of a hidden lever. Yet, if the rat does not focus on what it is supposed to do during the task, the monitored non-mnemonic performance deficit may be due to disruption of several aspects of behaviour that are not easily differentiated. For example, attentional deficits may cause similar changes in such task parameters as defects in motivation, sensorimotor processes, arousal or impulsivity/restlessness. Behaviourally, systemic administration of muscarinic and NMDA antagonists disrupt performance in tasks modelling sustained attention in rodents (Robbins, 2002; Sarter et al., 2001), thus suggesting that attentional problems may contribute to the performance deficit also observed in our study. However, because the reported drug effects have not been specific to attentional processes, it is likely that non-specific effects on sensorimotor or motivational processes may also contribute to the performance deficit in so-called attentional tasks (Robbins, 2002; Sarter et al., 2001). For example, scopolamine has been shown to both decrease choice performance accuracy in the 5-choice serial reaction time task for rodents, as well as disrupt overall task performance (Jäkälä et al., 1992; Ruotsalainen et al., 2000). In addition, systemic administration of dizocilpine, an NMDA receptor antagonist, not only impairs choice performance accuracy in this task but also induces additional performance deficits, such as increased premature responses (Grottick and Higgins, 2000). Furthermore, Both scopolamine and the NMDA antagonist MK-801 have been shown to disrupt overall performance in Spatial signal detection task (Presburger and Robinson, 1997).

It is of special relevance to our study using local prefrontal microinjections that lesioning of the mPFC impairs sustained attention as assessed in the 5-choice serial reaction time task, although additional performance deficits not specific to attention may also occur (Robbins, 2002; Sarter et al., 2001). In addition, scopolamine has been reported to induce attentional deficit when administered locally into the mPFC, although at the antero-dorsal part of the PFC, non-specific effects were also present (Robbins, 2002).

Our analysis programme lacked the ability to monitor performance changes as a function of test time. Therefore, possible changes in activity or choice performance accuracy during the course of the testing session, which could reflect changes in arousal or motivation, could not be detected. In future studies, more information could be gained if the analysis included such modifications as separate response latencies for correct vs incorrect choices, delay activity before correct vs incorrect responses, and measurement of response bias.

6.1.2 Water maze task

In order to learn how to complete a water maze task successfully, a rat must also acquire non-spatial components of learning such as swimming, searching for and climbing onto a hidden platform and "appreciating" that the platform represents rescue from the tank, and to abandon the natural first strategy to search for an escape in the pool wall (thigmotaxis). Consequently, if a drug facilitates acquisition of non-spatial components of learning, then these already acquired abilities are not likely to be further improved in the new environment. Earlier studies have shown that non-spatial pre-training in a different water maze alleviates or even prevents the learning deficit caused by the NMDA receptor antagonist, (+/-)-2-amino-5-phosphonopentanoic acid (AP-5) (Cain et al., 1996). Since it is possible that THA and DCS enhance the spatial navigation performance of aged rats by improving non-spatial components of learning, such as sensorimotor skills or motivation, we examined the influence of pre-training on the effects of THA and DCS on spatial navigation in the water maze. Our hypothesis was that if a drug primarily improves spatial memory and learning, its effects should still be significant after pre-training for a non-spatial cue strategy, spatial training in a

different environment or reversal of the relationship between the extra maze cues and the escape platform.

Pre-training of the rats tests more reliably than the original version of the water maze task whether non-mnemonic aspects of spatial navigation learning are affected. Still, the traditional version of water maze task also allows for controlling whether the rats have acquired a true place strategy, i.e. ability to use extra maze cues to navigate onto the escape platform. For example, impaired navigation onto a *visible* platform may be an indication of additional disturbance of non-mnemonic processes such as perception, motivation or sensorimotor coordination. In addition, using a probe trial at the end of an experiment reveals whether the rats have acquired a spatial bias, i.e. tendency to swim across and in close vicinity to the area that previously contained the hidden platform. Development of a spatial bias reveals that the rat has learned the location of the hidden platform correctly, uses place strategy for navigation and is motivated to search for it. The development of a spatial bias gives more specific information about navigation learning than latency to find the hidden platform, since swimming speed can be affected by drug treatments. The aged rats we used were large and over-weight, which made them slower swimmers than the young controls. In order to correct for the effect of group difference in the swimming speed, we used escape distance as the parameter for navigation learning, instead of escape latency. Nevertheless, it is possible that the greater weight leading to decreased swimming speed and overall clumsiness could have introduced a confounding factor, which may in part explain the parallel learning curves described in publication IV.

6.2 SPATIAL WORKING MEMORY

6.2.1 The effect of centrally administered muscarinic antagonists is not specific for working memory.

Effective doses of scopolamine and pirenzepine caused delay-independent deficits in the DNMTP task, suggesting that both drugs do not have a specific effect on working memory, but instead may cause attentional, motor or other non-specific behavioural disturbances. Scopolamine (10 μg) decreased motor activity, as reflected by the increased sample press

latency, and caused additional disturbances in attention or motivation by increasing omissions during the sample phase. Pirenzepine (10 and 30 µg) caused a milder effect than scopolamine, since it only decreased motor activity during the sample phase, though it had no additional effects on motivation. Neither scopolamine (3 and 10 µg) nor pirenzepine (10 and 30 µg) affected appetite or motor activity during delays, as reflected by food collection and nose poke latencies, respectively.

Scopolamine. The non-mnemonic effects of scopolamine in our study are in agreement with a number of studies using delayed response paradigms (Andrews et al., 1994; Chudasama and Muir, 1997; Han et al., 2000; Stanhope et al., 1995). It is likely that disruption of attentional and other non-mnemonic processes caused by scopolamine is reflected as delay-independent performance deficit in delayed tasks (Jones and Higgins, 1995; Jäkälä et al., 1992; Phillips et al., 2000; Ruotsalainen et al., 2000). Whether the behavioural effects of scopolamine are mediated by peripheral or central muscarinic receptors has usually been investigated by using methyl scopolamine, a compound that should not pass the blood-brain-barrier, as a comparison drug. Like scopolamine, methyl scopolamine decreases performance accuracy in both DMTP and DNMTP tasks, suggesting that the effects of scopolamine may be mediated through peripheral receptors (Andrews et al., 1994; Buxton et al., 1994). However, there is evidence that methyl scopolamine is able to enter the brain despite its highly polarised nature, and therefore its behavioural effects cannot be directly attributed to peripheral receptors (Andrews et al., 1994; Moore et al., 1992). Our results show that the i.c.v. administration of scopolamine decreases performance accuracy, thus linking the delay-independent disruption of choice accuracy in the DNMTP task directly to central receptors. In addition, non-selective blockade of central muscarinic receptors also disturbed motivation and motor activity, suggesting that centrally acting non-selective muscarinic antagonists produce a robust disruption of attentional, motor or other non-memory specific processes.

Pirenzepine. Our results suggest that the M_1 receptors (high affinity to pirenzepine) mediate the disrupting effects of scopolamine on performance accuracy, since the effect of 30 µg pirenzepine was similar to that with 10 µg scopolamine. In our study, the potency ratio of pirenzepine vs scopolamine was similar to the study of Hagan et al. (1987). In their study, 93 µg (209 nmoles) of pirenzepine disrupted learning in the Morris water-maze task as effectively as 28.9 µg (72 nmoles) of scopolamine, suggesting that pirenzepine is

approximately 3-fold weaker than scopolamine in disrupting spatial place navigation (Hagan et al., 1987). Although the study of Hagan et al (1987) as well as a study by Hunter and Roberts (1987) suggest that 30 µg of pirenzepine disrupts water-maze navigation without additional disruption in performance, our results are not in line with that interpretation. Instead, the present results suggest that pirenzepine may induce attentional or other non-mnemonic disturbances; thus, it can be assumed that the water maze task used in previous studies (Hagan et al., 1987; Hunter and Roberts, 1987) was probably not sufficiently sensitive. Our results disagree with Andrews et al. (1994) who reported that i.c.v. pirenzepine at doses of 3.2 – 32 µg disrupted DMTP performance delay-dependently, without additional behavioural disturbances. Notably, in their study the basal performance accuracy was much higher (75 % correct at 45 s delay) than in our study, making it possibly more resistant to disruption than in our study. However, such differences can also be explained by the fact that the DMTP and DNMTP are two different tasks that involve use of qualitatively different conditional decision rules (Dunnett,). Therefore, a direct comparison between the two tasks is difficult.

Methoctramine. Our data showing that methoctramine 2 µg modestly improved choice performance accuracy selectively at long delays agrees with previous evidence indicating that treatment with muscarinic M_2 receptor antagonists may enhance performance in tests used to assess learning and memory performance. For example, administration of BIBN 99 (5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl)ethylamino]propyl]-1-piperidinyl]acetyl]-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one), another muscarinic M_2 receptor antagonist, enhanced acquisition of the water maze task in young rats subjected to head trauma (Pike and Hamm, 1995) and in aged, cognitively impaired rats (Quirion et al., 1995). BIBN 99 has been shown to increase ACh release in the cortex of aged rats, suggesting that the effect of the drug on water-maze navigation is mediated via blockade of presynaptic muscarinic M_2 autoreceptors (Quirion et al., 1995). However, as explained above, it is difficult to interpret the beneficial effects of drugs administered prior to daily water maze testing simply in terms of improved learning and memory. Therefore, the present results are important, since they reveal a genuine delay-dependent, although modest, improvement, indicating an improvement in short-term memory *per se*. Interestingly, Packard et al. (1990) showed that the administration of AF-DX 116 ([R,S]-11-[[2-[diethylamino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro[2,3-b][1,4]-benzodiazepin-6-one), a muscarinic M_2 receptor

antagonist, stimulated memory consolidation in two different radial arm maze tasks. Therefore, present and previous (Packard et al., 1990) data suggest that an M_2 antagonist may improve maintenance of short-term memory and consolidation of long-term reference memory. The failure of higher doses of methoctramine to improve memory function may be attributed to the loss of selectivity to M_2 vs. muscarinic M_1 receptors. Indeed, a recent study by Ohno et al. (1995) described how only a dose of methoctramine high enough to block muscarinic M_1 receptors could impair three-panel runaway performance and that AF102B, a muscarinic M_1 receptor agonist, attenuated this impairment. Another potential explanation for the failure of higher doses to improve memory functions is that higher doses of methoctramine produce too robust an increase in synaptic ACh levels, thus disrupting the physiological, phasic variation in the cholinergic signal transmission and introducing 'noise' to the system processing spatial short-term memory.

6.2.2 Central administration of the NMDA receptor antagonist CPP is associated with several non-mnemonic effects.

An effective dose of CPP (0.3 μg) caused a delay-independent decrease in correct responses, suggesting that CPP induced disturbance of other processes than working memory *per se*. In addition, CPP 0.3 μg disrupted sensory, motor or attentional functions as evidenced by increasing omissions and latency to respond during the sample phase. CPP 0.1 μg increased motor activity during delays, but had no effect on correct responses, motivation (omissions) or motor activity during the sample phase. CPP (0.1 or 0.3 μg) did not significantly affect motor activity during food collection, suggesting that CPP did not decrease general motivation and appetite.

Our results are in agreement with previous studies reporting that CPP disrupts performance accuracy independent of the delay. For example, Tan et al. showed that CPP (10 mg/kg) caused a delay-independent impairment in a conditional discrimination task (Tan et al., 1989), and Pontecorvo et al. (1991) showed that CPP (5 mg/kg) caused a delay-independent impairment of performance accuracy in a non-spatial continuous non-matching to sample task. However, our results disagree with Cole et al. (1993), who reported that CPP (10 mg/kg) disrupted accuracy delay-dependently in Dunnet's DMTP task. Again, the apparent discrepancy could be explained by the difference in the non-matching vs matching

behavioural paradigms, but it should be noted that a delay-independent deficit has been reported with the DMTP task as well (Doyle et al., 1998). In addition, the study of Cole et al. (1993) showed that performance accuracy was higher over all delays, (over 75 % correct in 30 s delay). It has been shown that if the performance accuracy is high during the longest delays, the use of effective mediating strategies may also be higher, which makes it easier to gain delay-dependent effects (Herremans, 1996). Furthermore, the Cole et al. study also found additional disruption of motor activity, as reflected by the increased latency to respond and the number of nose pokes. Therefore, both previous and our own data suggest that CPP disrupts attentional and other non-memory-specific processes, thus causing secondary disruption of working memory.

6.2.3 Combined central administration of CPP and either scopolamine or pirenzepine has no specific effect on working memory.

Individual administration of CPP (0.03 µg) or scopolamine (0.1 µg) did not affect correct responses or any of the other measured behavioural parameters, suggesting that these parameters had no significant effect on motivation, motor activity or appetite. When these sub-threshold doses of CPP and scopolamine were combined, the consequence was a clear disruption of motivational processes and motor activity before and during the delays, though correct responses remained intact. On the other hand, the combination of pirenzepine (10 µg) with CPP (0.03 µg) did not affect any of the measured behavioural parameters, including correct responses.

The main finding of this study was that the NMDA and muscarinic receptors conjointly modulated non-mnemonic components of DNMTP task performance, but did not influence working memory. This finding was not unexpected since extensive anatomical distribution and the overall general nature of the two neurotransmitter systems in the brain suggest that non-mnemonic effects would be anticipated to occur when muscarinic and NMDA receptors are manipulated concurrently. In addition, antagonists of both the muscarinic and NMDA receptors induce sensorimotor, attentional and other non-specific behavioural dysfunction in animals and humans (Blokland, 1996; Broks et al., 1988; Cotman and Iversen, 1987; Dunne and Hartley, 1986; Ebert and Kirch, 1998; Oranje et al., 2000). Our second finding was that

pirenzepine was totally ineffective when combined with CPP, suggesting that the muscarinic M_1 receptors do not mediate the performance disrupting interaction with NMDA receptors. This was quite unexpected, since pirenzepine on its own disrupted performance in our DNMTP task delay-independently, suggesting that the M_1 receptors probably mediate the performance deficit caused by scopolamine. It is not likely that the doses of pirenzepine (10 μg) or CPP (0.03 μg) were too low, since pirenzepine (10 μg) on its own slightly increased the latency of sample presses, and CPP (0.03 μg) disrupted performance when administered with a subthreshold dose of scopolamine (0.1 μg). In addition, because we kept the rats drug-free for two weeks before the last experiment to avoid tolerance, it is not likely that the rats could have developed tolerance to the drug effect. Therefore, based on the antagonist affinity profiles (table 7), it can be concluded that additional blockade of the non-M_1 receptors, possibly excluding the M_5 and M_2 receptors, is needed for the synergistic effect with an NMDA receptor blockade.

Since the low doses of CPP and pirenzepine did not interact in regulating the performance of rats in the DNMTP task, it could be possible that low doses of these drugs mediate independent behavioural effects. In fact, low doses of NMDA antagonists are able to improve performance in short-term memory tasks (Mondadori and Weiskrantz, 1993; Sharma and Kulkarni, 1991). Moreover, NMDA antagonists have been reported to act as behavioural stimulants and to cause anxiolytic effects (Cotman and Iversen, 1987). It is therefore possible that there was no additive/synergistic effect by the combined blockade of NMDA and muscarinic receptors, because the low doses of pirenzepine and CPP mediate counteracting behavioural effects. However, a lack of effect with two sub-threshold doses is not sufficient evidence to prove the existence of some form of counteracting process, and therefore this explanation is only speculative.

In our experimental setup, it was possible to study the memory-specific effects separately from non-specific effects. Other studies have also examined the interaction of muscarinic and NMDA receptors in the regulation of learning and memory processes; however, these studies lacked the ability to differentiate the non-mnemonic performance deficits from attentional or sensorimotor effects. For example, in a study by Hlinak and Krejci sub-effective doses of scopolamine and MK-801 produced short-term amnesia in an elevated plus maze task that does not measure, for example, attentional aspects of performance (Hlinak and Krejci, 1998).

Similarly, Ohno and Watanabe reported that scopolamine and MK-801 at sub-threshold doses impair learning of inhibitory avoidance, which is a rather non-specific form of learning (Ohno and Watanabe, 1996). Our study suggests that non-mnemonic performance deficits were present in these studies, and probably significantly contributed to the results. Our study revealed only a performance deficit of a non-mnemonic type, suggesting that working memory is not the first process affected in the line of spatial information processing in the CNS.

We also found that the performance accuracy in our DNMTP task is, to a certain extent, resistant to attentional or motor performance deficit caused by combined treatment with scopolamine and CPP. However, whether this is caused by the resistance of spatial working memory processes to the disrupting effects of the two drugs, or resistance of other factors involved in the performance of the DNMTP task, cannot be answered based on our study alone. Further studies are still needed to reveal the exact co-operative role of muscarinic and NMDA receptors in the regulation of spatial working (short-term) memory performance in rodents.

6.2.4 Local microinfusion of the NMDA antagonist CPP in the PFC disrupted working memory dose-dependently.

The blockade of NMDA receptors in the dmPFC dose-dependently disrupted performance in the DNMTP task. Importantly, CPP 0.1 μg caused a delay-dependent decrease in the percentage of correct responses, indicating a deficit of working memory function. The performance was unaffected at the 0 s delay, but was clearly disrupted at the 1 s and 2 s delays. The delay-dependent effect was mild, since even after vehicle treatment, the performance of our Wistar rats decreased rapidly close to the chance level, leading to the loss of significant differences at the longest delays, propably due to the “floor effect”. In addition, there was also an increase in the percentage of omissions and the latencies of sample press and nose pokes, suggesting that CPP 0.1 μg caused a mixture of mnemonic and non-mnemonic (such as attentional) deficits. Earlier studies have shown that a lesion to the rostral part of the mPFC in rats results in a delay-dependent decrease in task performance, whereas a lesion in more caudal parts causes a delay-independent performance deficit in the DNMTP

task (Dunnett, 1990). Our findings support the hypothesis that the rostral part of the dmPFC has an important role in short-term memory regulation and further suggest the view that NMDA receptors in the rostral dmPFC are significantly involved in the working memory processing. Dunnett has suggested that disruption of the mPFC - nucleus accumbens - ventral pallidum - mediodorsal thalamic nucleus (MD) - mPFC circuit leads to a delay-dependent SWM deficit in rats (Dunnett, 1990). Since the HC projects both to the ventral pallidum and mPFC, it is possible that the disruption of this circuit leads to a disconnection between the HC and the mPFC, which may deteriorate SWM performance. Therefore, a potential mechanism for NMDA receptor mediated SWM disruption is the functional disturbance of glutamatergic pathways from the MD (Pirot et al., 1994) or the HC (Gigg et al., 1994) to the mPFC.

The highest CPP dose (0.3 µg) in the dmPFC caused an overall disruption of performance and a delay-independent decrease in task performance, which cannot be linked primarily to mnemonic functioning. Since a lesion of the rostral mPFC does not cause a corrsponding performance deficit, it is possible that the highest dose of CPP may have diffused to more caudal parts of the mPFC. Lesioning of this area causes a similar kind of delay-independent deficit. In contrast to our findings after infusing CPP 0.3 µg into the dmPFC, spontaneous motor activity measured in automated photocell cages was not disrupted after a mPFC lesion in the study of Dunnett (1990). However, it is difficult to make a direct comparison of the motor deficits between these two tasks, since they measure different aspects of motor behaviour. Moreover, the blockade of NMDA receptors specifically modulates only glutamatergic transmission, and thus its effects cannot be directly compared to the effects of lesioning.

6.2.5 Local administration of muscarinic antagonist scopolamine in the PFC disrupted non-mnemonic parametres in the DNMTP task.

In our study, the 10 µg dose of scopolamine was able to induce non-cognitive performance deficit by increasing the latencies of sample press and nose pokes, but it did not disrupt the memory performance. Similarly, Dunnett has found that scopolamine 12 µg in the mPFC caused an overall disruption of performance in the DNMTP task, though, in addition, the memory performance also decreased delay-independently (Dunnett et al., 1990). However,

because most of the infusion cannulae in that study were located in the *ventromedial* PFC, the effect of different scopolamine doses on DNMTP task performance may have varied in different subdivisions of the mPFC. In addition, it is also possible that differences between the strains of rats used may have affected the results, since Sprague-Dawley rats were used in that study, whereas we used Wistar rats. On the other hand, Herremans et al. have used Wistar rats and the placement of the cannulae was near the infusion area of our study (Herremans et al., 1996). They used the *DMTP* procedure and found that scopolamine 10 and 30 µg dose-dependently induced a delay-independent decrease in choice accuracy and disrupted non-cognitive measures of performance. The present and Herremans et al. study agree that infusion of scopolamine into the dorsal parts of the mPFC produces non-cognitive deficits. Therefore, the earlier and present data indicate that muscarinic blockade in the dorsal and ventral parts of the mPFC primarily results in non-cognitive performance deficits that cause a delay-independent decrease in task performance. Furthermore, our study showed a clear difference between the effects of scopolamine and CPP, suggesting that the NMDA and mACh receptors located in the rostral part of dmPFC mediate different aspects of behaviour in the DNMTP task.

Previous data suggest that the dlFC is not important for normal spatial working memory. First, a bilateral lesion of the dlFC causes no disruption of memory performance in the DNMTP task (Dunnett, 1990). Second, a T -maze study found that a total bilateral lesion of the dorsolateral isocortex caused only minor disruption of delayed alternation performance, when compared to the bilateral lesion of the mPFC (Wortwein et al., 1994). Our study did not show significant effects of scopolamine or CPP on choice accuracy in the DNMTP task when the drugs were infused into the dlFC. Therefore, our findings contribute to the body of evidence indicating that the mACh or NMDA receptors in the dlFC do not mediate functions that are needed for normal spatial working memory *per se,* as assessed in the DNMTP task. Moreover, infusion of scopolamine and CPP into the dlFC had different effects on non-cognitive measures of this task. Scopolamine did not affect non-cognitive performance measures, whereas CPP 0.3 µg increased the latencies of sample press and nose pokes, suggesting that a blockade of NMDA receptors, but not muscarinic receptors, in the dlFC interferes with non-cognitive functioning.

6.3 SPATIAL LEARNING AND MEMORY

6.3.1 Aging and spatial navigation

The present study showed that after repeated spatial navigation training periods conducted in the same or different environments, aged rats remained impaired in learning the spatial navigation task, and were still deficient in their ability to relearn the location of the escape platform. Furthermore, aged rats were as accurate as young rats in non-spatial navigation (visible platform), but were still grossly impaired in learning the location of the hidden platform after non-spatial pre-training in a familiar testing environment. These results indicate that learning the basic skills of water maze performance during the different pre-training conditions did not help the aged rats to learn the spatial relationships between the hidden escape platform and the cues in the testing environment. This is further support for the concept that the age-related water maze deficit is due to impaired functioning of the septo-hippocampal system.

Several studies have shown that hippocampal function may be compromised during aging. Barnes (1994) has reviewed evidence indicating that during normal aging there are regionally specific deficits in hippocampal synaptic transmission (Barnes, 1994). For example, the number of synapses from the medial entorhinal cortex to the middle third of the granule cell dendritic tree is reduced by approximately one-third in aged rats (geinisman et al., 1986) and this is paralleled by a reduction in the size of the presynaptic fibre potential at stimulus intensities above threshold (Barnes and McNaughton, 1980b). The aged brain appears to have a mechanism to compensate for this loss of synapses by making the synapses functionally more powerfully. The maintenance of synaptic plasticity, as assessed by LTP analysis, is also impaired in the dentate gyrus of aged rats (Barnes and McNaughton 1980a). Furthermore, the rate of decay of hippocampal LTP in aged and young rats is inversely correlated with their learning rates in a spatial memory test (Squire, 1992). Interestingly, previous behavioural studies have found that lesions of the septo-hippocampal system can also impair spatial navigation accuracy irrespective of the pre-training protocol. Therefore, our finding that pre-training did not reverse the spatial navigation deficit in aged rats is compatible with the notion of HC dysfunction in aged animals.

6.3.2 Combined administration of THA and DCS at sub-effective doses improves water maze learning in aged rats, but the improving effect disappears after pre-training.

Our results from the first set of water maze experiments (IV) support previous evidence that administration of THA and DCS before daily training sessions can improve acquisition of spatial navigation in aged rats (Riekkinen et al., 1996; Riekkinen and Riekkinen, 1997). Importantly, a combination of sub-threshold doses of THA and DCS produced a greater effect on water maze acquisition and spatial search bias than either of the treatments on their own. However, THA and DCS had no effect on memory consolidation, as treatment with the study drugs immediately after daily training did not improve spatial escape behaviour. The second set of experiments (V) revealed that any kind of pre-training, either non-spatial or spatial, was able to block the beneficial effects of THA and DCS, administered either separately or in combination. Furthermore, THA and DCS had no effect on age-related impairment of reversal learning. When compared to the young controls, the spatial learning deficit of aged rats still remained after either spatial or non-spatial pre-training.

In the first set of water maze experiments (IV), the number of rats that found the platform on the first trial of the first day was not affected by the drug treatments. This suggests that the compounds do not change the spontaneous exploration patterns of rats, and may also rule out effects on anxiety. However, the beneficial action of THA and DCS on water maze escape performance during the hidden platform trials cannot be interpreted solely in terms of enhanced spatial memory. It is possible that changes in arousal and attention may affect water maze acquisition if the drugs are administered before daily training sessions. For example, the learning curves of rats treated with THA and DCS were parallel, which may be a reflection of some non-mnemonic improvement in performance. In addition, THA alone had no effect on the spatial bias measure, suggesting that the encoding of spatial cues was not improved by THA. Importantly, in the second set of experiments (V), THA lost its navigation improving effect after non-spatial pre-training. Thus, it is likely that THA treatment stimulates acquisition of spatial navigation by modulating some process other than spatial memory per se. The NBM cholinergic system modulates the functioning of the frontal cortex and plays an important role in attention (Jäkälä et al., 1992; Muir et al., 1994; Muir et al., 1995). Lesions of the NBM induced by infusion of excitatory amino acid analogues or treatment with a mACh receptor antagonist impair behaviour in young rats in tests measuring attention (Jäkälä et al.,

1992; Muir et al., 1994; Muir et al., 1995). Therefore, it is possible that improved attention in the aged rats induced by THA treatment may contribute to the facilitated water maze acquisition.

We observed that DCS enhanced spatial bias during the memory retrieval trial, suggesting that the drug may stimulate spatial memory function. However, a study by Bannerman et al. suggests that NMDA receptors mediate functions other than spatial memory, such as the development of an escape strategy, that are important for water maze spatial navigation (Bannerman et al., 1995). Furthermore, we observed that the learning curves of aged rats treated with DCS or a placebo before daily training sessions were parallel, and DCS failed to stimulate memory consolidation. Therefore, it is possible that DCS does not specifically stimulate spatial memory in aged rats, but modulates other cognitive processes needed for effective spatial navigation (Bannerman et al., 1995). Findings from our second set of experiments support this since, as in the case of THA, the improving effect of DCS vanished, despite a variety of different types of (including non-spatial) pre-training (V). Taken together, the results from our experiments suggest that both DCS and THA facilitate other cognitive components of water maze learning than spatial memory per se.

Our studies showed that a cholinesterase inhibitor and a NMDA modulator jointly improved acquisition in the water maze task, since we found that a combination of THA and DCS at sub-threshold doses decreased escape distance and increased spatial bias. In contrast, a combination of active THA and DCS doses did not produce any greater effect on acquisition or retention of water maze behaviour than did either of the treatments alone. These results indicate that THA and DCS may act synergistically to enhance functioning of the brain systems necessary for water maze acquisition, but only DCS stimulated those mechanisms important for retrieval of spatial memories. Importantly, it has been shown that treatment with the cholinesterase inhibitor physostigmine can increase functioning of glutamate containing projection neurons (Dijk et al., 1995). These workers used ‘in vivo’ microdialysis to show that glutamate release from corticostriatal fibres was enhanced by cholinesterase inhibition. Thus, it is possible that the increase in ACh activity occurring after THA treatment can stimulate water maze performance by enhancing glutamate mediated functions. Therefore, sub-threshold doses of THA and DCS may produce a measurable effect on water maze navigation by stimulating glutamate release and NMDA receptor activity, respectively (Dijk

et al., 1995; Huettner, 1991).

An additional possible mechanism for increasing ACh activity to improve water maze navigation is the stimulation of nicotinic receptors. We have observed in our laboratory that subthreshold doses of nicotine and DCS dose-dependently improved spatial navigation in aged rats (Riekkinen and Riekkinen, 1997). We also found that DCS alleviated the water maze acquisition defect induced by a nicotinic antagonist, and a subthreshold dose of the NMDA antagonist CPP blocked the beneficial effect of nicotine on water maze acquisition (Riekkinen and Riekkinen, 1997). Therefore, it is possible that activation of nicotinic ACh receptors may stimulate water maze acquisition in aged rats via increased activity of NMDA receptors and also partially mediate the simulating effects of THA. Indeed, THA can activate niconinic receptors through an allosteric binding site (Paterson and Nordberg, 2000). However, further studies are still needed to specify the relationship between muscarinic and nicotinic receptor activation in the regulation of age-related cognitive decline in rodents.

The present results may have some relevance for the development of pharmacological treatments for cognitive defects observed in AD that are associated with impaired functioning of NMDA and basal forebrain cholinergic systems (Aigner, 1995; Francis et al., 1999; Whitehouse et al., 1982). It is possible that combined treatment with a cholinesterase inhibitor and a positive NMDA modulator may produce a clinical response with lower doses than either treatment alone, which would also decrease dose-dependent side effects. Furthermore, drugs acting via different neurochemical systems may enhance separate cognitive domains, such as memory or attention, although combined treatment with a cholinesterase inhibitor and a positive allosteric modulator of NMDA receptors probably does not directly stimulate memory functions. However, the drugs likely have indirect beneficial effects on cognition. Our results suggest that a combination of these drugs may interact in alleviating cognitive deficits and clinical dementia in AD. Unfortunately, DCS can only be considered an experimental molecule, suitable for evaluating the possible therapeutic actions of drugs acting via NMDA receptors, as efficacy in human studies has been dissapointing (Laake and Oeksengaard, 2002). Nevertheless, our data support the development of new compounds targeted to enhance NMDA receptor activity.

7. CONCLUSIONS

1. Antagonism of NMDA and muscarinic M_1 (+ M_3 + M_4) acetycholine receptors impaired performance in the DNMTP task, a typical working memory task.

   Because the effect was largely delay-independent and observed in non-mnemonic task parameters, it is likely to be mediated by non-mnemonic aspects of the task, and not by specific disruption of working memory processes. Instead, M_2 antagonists at a low dose may block presynaptic autoreceptors and thus stimulate cholinergic function.

2. The non-specific effects of muscarinic and NMDA receptor blockade were even more pronounced, when a combination of muscarinic ACh and NMDA receptor antagonists was administered.

3. At the prefrontal cortex level, the antagonism of NMDA receptors has different effect profile when compared to the muscarinic receptor antagonism. NMDA receptors mediate processes that are needed for working memory regulation, whereas muscarinic receptors regulate non-mnemonic aspects of the DNMTP task.

4. Stimulation of cholinergic system and NMDA receptors interact in enhancing procedural aspects of water maze navigation learning, which is reflected as an alleviation of the age-related spatial navigation deficit in rats.

5. Although the conjoint modulation of muscarinic and NMDA receptors probably does not directly enhance memory functions, the indirect effects on learning possibly due to stimulation of attentional or other non-mnemonic processes might help to treat the cognitive symptoms of patients suffering from age-related neurodegenerative diseases, such as AD.

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