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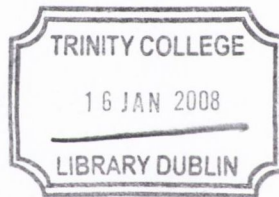
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# Neurotransmitter Interactions In The Control of Spatial Memory And Exploratory Activity In The Rat

By

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A dissertation submitted for the degree of Doctor of Philosophy of the University of Dublin, Trinity College, Dublin 2, Ireland. The research was conducted in the department of Pharmacology and Therapeutics, Faculty of Health Sciences, under the supervision of Prof. Michael Rowan.
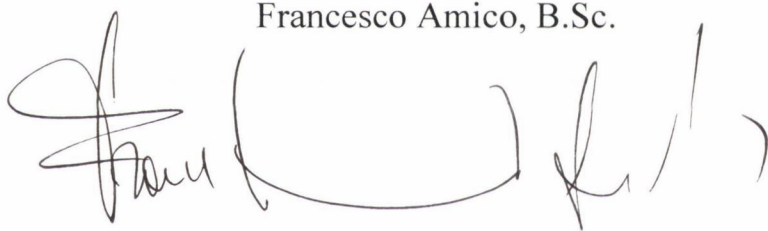
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## Declaration

I declare that the material contained in this thesis is entirely my own work. This thesis has not been submitted as an exercise for a degree at any other university. The library of the University of Dublin may lend  or copy this thesis upon request.

Francesco Amico, B.Sc.

# Acknowlegements

Finally, thanks to the messy Boris and Lara for always giving me the warmest welcome home every time I was back to Sicily.

# Abbreviations

| | |
|---|---|
| **ACh** | Acetylcholine |
| **AChE** | Acetylcholinesterase |
| **AD** | Alzheimer's Disease |
| **AFDX 116** | 11- {2-[(Diethylamino)methyl]- 1-piperidinyl} acetyl-5,1 l-dihydro-6H pyrido(2,3b)-(1,4)benzodiazepine-6-one |
| **AFDX 384** | 5,11-dihydro-11-[[(2-[2-[(dipropylamino) methyl]-1-piperidinyl] ethyl) amino] carbonyl]-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one |
| **A 68930** | (1*R*,3*S*)-1-aminomethyl-5,6-dihydroxy-3-phenylisochroman HCl |
| **ANOVA** | Analysis of Variance |
| **AP5** | 2-amino-5-phosphonopentanoate |
| **BNF** | British National Formulary |
| **BIBN-99** | 5,11-dihydro-8-chloro-11-[[4-[3-[(2,2-dimethyl-1-oxopentyl)ethylamino]propyl]-1-piperidinyl]acetyl]-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one |
| **cAMP** | Cyclic Adenosin(e) Mono Phosphate |

| | |
|---|---|
| $Ca^{2+}$ | Calcium ion |
| **CNS** | Central Nervous System |
| **CO** | Carbon Monoxide |
| **G right** | Goal arm right |
| **G left** | Goal arm left |
| $D_1$ | Dopamine Type 1 |
| $D_2$ | Dopamine Type 2 |
| $D_3$ | Dopamine Type 3 |
| $D_4$ | Dopamine Type 4 |
| $D_5$ | Dopamine Type 5 |
| **DR** | Dorsal Raphe |
| **DRN** | Dorsal Raphe Nucleus |
| **5-HT** | 5-hydroxy-tryptamine (serotonin) |
| $5\text{-}HT_{1A}$ | 5-hydroxy-tryptamine type 1A |
| **5,7-DHT** | 5, 7 dihydroxytryptamine |
| **DA** | Dopamine |
| **DLB** | Dementia with Lewy Bodies |
| **DR** | Dorsal Raphe Nucleus |
| **EAA** | Excitatory Amino Acid |
| **Exp.** | Experiment |
| **F** | F value (ratio of the Model Mean Square to the Error Mean Square) |
| **FDA** | Food and Drug Administration |
| **GABA** | Gamma Amino Butyric Acid |

| | |
|---|---|
| **GPS** | General Problem Solver |
| **Hd** | Head dips |
| **[$^3$H] AFDX 384** | Tritiated AFDX 384 |
| **i.p.** | Intraperitoneal |
| **Ipsa** | Ipsapirone |
| **hDBB** | Horizontal Limb of the Diagonal Band of Broca |
| **Lc** | Lines Crossed |
| **LTDg** | Latero-DorsalTegmental Nucleus |
| **LTP** | Long term potentiation |
| **M$_1$** | Muscarinic type 1 receptor |
| **M$_2$** | Muscarinic type 2 receptor |
| **M$_3$** | Muscarinic type 3 receptor |
| **M$_4$** | Muscarinic type 4 receptor |
| **M$_5$** | Muscarinic type 1 receptor |
| **MAO** | Monoamine Oxidase |
| **MDL 73005** | 8-[2-(2,3-dihydro-1,4-benzodioxin-2-ylmethylamino) ethyl]-8-azaspiro[4,5] decane-7,9-dione methyl sulphonate |
| **(+) MK-801** | (+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine |
| **MR** | Medial Raphe |
| **MRN** | Medial Raphe Nucleus |
| **MS** | Medial Septum |

| | |
|---|---|
| **MS** | Medial Septum |
| **NMDA** | N-methyl-D-Aspartate |
| **NBM** | Nucleus Basalis Magnocellularis |
| **NIK-247** | (9-amino-2,3,5,6,7,8-hexahydro-1 H-cyclopenta(b)-quinoline mono-hydrate hydrochloride) |
| **NS** | Not significant |
| **6-OHDA** | 6-Hydroxydopamine |
| **8-OH-DPAT** | Hydroxy-2-(di-n-propylamino) tetralin |
| **P** | Probability |
| **PCPA** | p-Chlorophenylalanine |
| **PKA** | Protein kinase A |
| **PD** | Parkinson's Disease |
| **ppm** | Parts per million |
| **PPTg** | Pedunculopontine Tegmental Nucleus |
| **Ro 04-6790** | 4-amino-*N* (2,6bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide |
| **Rp-cAMPS** | Rp-adenosine 3,5-cyclic monophosphorothioate triethylamine |
| **Re** | Rearing (Rears) |
| **REM** | Rapid eye movement |
| **S** | Start arm |
| **SCH 57790** | 4-cyclohexyl--[4-[[4- |

|          |                                                                                           |
|----------|-------------------------------------------------------------------------------------------|
|          | methoxyphenyl]sulfmyl]-phenyl]-1-piperazineacetonitrile                                    |
| **s.c.** | Sub Cutaneous                                                                              |
| **Sal**  | Saline (0.9% sodium chloride sterile water solution)                                       |
| **SEM**  | Standard Error Mean                                                                        |
| **Scop** | Scopolamine                                                                                |
| **SCH 23390** | 4-cyclohexyl--[4-[[4-methoxyphenyl]sulfmyl]-phenyl]-1-piperazineacetonitrile          |
| **SKF 38393** | (±)-phenyl-2,3,4,5-tetrahydro- $1H$ -3-benzazepine-7,8-diol                           |
| **TBI**  | Traumatic Brain Injury                                                                     |
| **T-CAT** | T-maze continuous alternation task                                                        |
| **vDBB** | Vertical Limb of the Diagonal Band of Broca                                                |
| **U-50488H** | Trans-(±)-3,4-dichloro-$N$-methyl-$N$-(2-[1-pyrrolidinyl]cyclohexylbenzeneacetamide methane-sulfonate salt |
| **Veh**  | Vehicle (sterile distilled water)                                                          |
| **vs.**  | Versus                                                                                     |
| **VTA**  | Ventral Tegmental Area                                                                     |
| **WAY 100135** | $N$- 2- 4- 2-methoxyphenyl -1-piperazinyl– $N$- 2-pyridinyl cyclohexanecarboxamide   |

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# Summary

The role of the central cholinergic system in learning and memory is well established, with many investigations reporting that central cholinergic hypofunction is correlated with impaired performance on various behavioural tasks, whereas improved performance is often observed with elevated cholinergic function. Attempts to enhance cholinergic activity have generally taken one of the following approaches: precursor manipulation, elevation of endogenous acetylcholine (ACh) levels via inhibition of acetylcholinesterase (AChE), and activation of postsynaptic receptors using cholinergic agonists. These strategies have met with limited clinical success.

An alternate method to elevate cholinergic neurotransmission involves manipulation of the presynaptic receptor. Data from pharmachological and physiological investigations have demonstrated elevated ACh levels following administration of muscarinic ACh type-2 ($M_2$) receptor antagonists such as AFDX 116, AFDX 384, BIBN 99, AQ-RA71, SCH 57590, SCH 7278.

In particular, AFDX 384 and BIBN 99 have been found to improve learning in a variety of "models" of memory disruption and in a wide selection of behavioural tasks. The goal of the studies described in Chapter 3 and 4 was to evaluate and compare the efficacy of AFDX 384 or BIBN 99 at attenuating the effects of a dose of the muscarinic antagonist scopolamine which, given alone, induced learning deficits in a watermaze and in a T-maze, and disruption of exploratory motor activity in a novel arena (hole board). In previous studies, scopolamine has been shown to disrupt normal performance in several behavioural tasks such as the watermaze, object recognition, passive avoidance, radial-arm maze, and to increase exploratory motor activity.

In Chapter 5, the effects of the $D_1$-like ($D_1$) receptor partial agonist SKF 38393 and full agonist A 68930, and the $D_1$ receptor antagonist SCH 23390 were studied in similar models. These experiments were designed on the basis

of a growing amount of data suggesting that activation of $D_1$ receptors located on hippocampal cholinergic nerve terminals increases the release of ACh and that the manipulation of this dopaminergic receptor subclass differentially affects memory and exploratory activity.

In Chapter 6, the effects of the serotonin type-1A (5-HT$_{1A}$) receptor partial agonist ipsapirone combined with a low dose of scopolamine were investigated in order to evaluate the interaction between the cholinergic and the serotonergic neurotransmitter systems in spatial memory. Previously, the combination of sub-amnesic doses of scopolamine, given systemically, and intrahippocampal administration of a 5-HT$_{1A}$ full agonist has produced impairments in acquisition of the watermaze. Conversely, stimulation of 5-HT$_{1A}$ receptors in the dorsal raphe has been shown to alleviate learning and memory impairments caused by systemic administration of scopolamine. The present experiments, evaluated whether or not a combination of sub-amnesic doses of scopolamine and ipsapirone, given systemically, can affect watermaze learning, locomotor, and exploratory behaviour.

Finally, in Chapter 7, the effects of a low-level exposure to carbon monoxide (CO) gas on cognition were evaluated by measuring behavioural responsiveness to a scopolamine challenge. It is well documented that exposure to CO can produce disruption of cognitive functions. In this study, rats were tested in a watermaze and in a hole board after exposure to CO combined with the administration of a sub-amnesic dose of scopolamine. This approach was designed in order to study the possible synergistic interaction between the partial loss of muscarinic receptor activation, induced by a low dose of scopolamine, and exposure to a potentially cytotoxic level of CO.

Performance in the behavioural tasks was not affected by the administration of either AFDX 384 (5 mg/kg, i.p.) or BIBN 99 (0.5 mg/kg, s.c.) alone, although they appeared to improve watermaze navigation strategy. BIBN

99 attenuated the disruption of watermaze performance induced by scopolamine (improving only probe test performance but not acquisition), whereas AFDX 384 had no effect. In the T-maze task, both AFDX 384 and BIBN 99 fully reversed the scopolamine-induced deficit, whereas neither drug affected scopolamine induced hyperactivity in the hole board.

The $D_1$ receptor ligands gave more complex results. Watermaze performance was not significantly affected by the administration of SKF 38393 (6 mg/kg, i.p.). A 68930 (6 mg/kg, i.p.) did not affect watermaze acquisition but had disruptive effects on probe test performance. In the same task, SCH 23390 (0.05 mg/kg, i.p.) induced motor slowing and impaired both acquisition and probe test. In both the hole board and T-maze tasks, SKF 38393, given alone, did not affect performance, whereas A 68930 induced motor slowing (hole board and T-maze) and spontaneous alternation deficit (T-maze). SCH 23390 induced catalepsy. Neither SKF 38393 nor A 68930 affected scopolamine-induced disruption of watermaze or holeboard performance. In the T-maze task, SKF 38393 fully normalized the scopolamine-induced performance disruption. Given the effects that A 68930 had on its own, the effects of this drug combined with scopolamine were not investigated in the T-maze task.

The results reported in Chapter 6 show that, in the watermaze task, the administration of a dose of 0.2 mg/kg (i.p.) scopolamine had no effect on performance, whereas ipsapirone (5 mg/kg, i.p.), given alone or in combination with scopolamine impaired search strategy. In the hole board, injection of ipsapirone alone partially reduced habituation. The combination of scopolamine with ipsapirone significantly reduced normal locomotion and rearing and disrupted habituation.

Finally, in the animals exposed to CO (2400±240ppm/1hr), watermaze performance was not impaired, but swim speed was increased as was exploratory activity in the hole board task. In the animals that received an

injection of a sub-effective dose of scopolamine (0.2 mg/kg, i.p.) after having been exposed to CO, watermaze target quadrant bias was disrupted whereas normal hole board exploratory activity was not affected.

Overall, this research suggests that cholinergic modulation of cognitive functions can be influenced by activation or inhibition of a wide range of neurotransmitter receptors, including muscarinic $M_2$, dopamine $D_1$, and $5\text{-HT}_{1A}$ receptors. These studies also help provide further insight into possible mechanism(s) of reference and working memory and the potential clinical usefulness of the agents that were investigated.

# CHAPTER 1

## 1.1 General Introduction

### 1.1.1 Learning, memory, and evolution

Learning is a skill that enables humans to acquire knowledge about the environment they live in. In human cultures, as soon as a new individual is born, he or she is trained to learn sets of skills, behavioural norms, and facts, beliefs that are considered useful to successfully survive and reproduce in a given environment. Humans are not the only organisms capable of learning. However, while primitive creatures are capable of learning, the human capacity for learning is unmatched by that of any other living organism.

Species survival depends on the adaptation of physical, physiological and behavioural features to the environment. In particular, behaviour, allows individuals to relate not just to the environment but also to other members of the species. Thus, within a given group of individuals, behaviour patterns that become important for interaction with the environment and social interaction will be passed from generation to generation. Some behaviours eventually become innate, a direct expression of the genetic endowment of an individual, and others will need to be developed through learning. Although the process of behavioural learning requires considerable commitment by an individual, it increases its likelihood of survival in environments that are not stable or predictable enough to enable behaviours to be shaped through an evolutionary process. Thus, within a given culture, behavioural patterns can be continuously shaped according to environmental pressures.

How people learn, remember and know is a question much older than organized psychology. Philosophers and biologists have wondered and

mental chemistry approach of combining existing knowledge to produce new ideas is similar to Descartes' rationalism, except that the source of the initial differs in the two philosophies. The philosophy of Descartes and Locke certainly influenced the first psychologists, and, in particular, empiricism inspired the first research efforts aimed at investigating the role of environmental experiences in the acquisition of knowledge.

In 1859, **Darwin** published *"On the Origin of Species"*. In this work Darwin suggests for the very first time that organisms change over generations in order to better adapt to environmental chances. He also noted that, within a given species, there were physical differences among individuals. This was the key point of his evolutionary theory, according to which only the individuals that best adapted to the environment could survive and reproduce. In this view, the ability to learn could be considered as an adaptative specialization that increases the likelihood of an animal to survive environmental pressures. In addition, the belief that different species were related through a common evolutionary history suggested there was a continuity of mind across species. Thus, animals other than humans could be studied, with generalizations proceeding in either direction along the phylogenetic scale.

## 1.1.3 History of learning and memory research

The first scientific report on the topic of learning and memory was published by **Hermann Ebbinghaus** (1885) in the eighteenth century. Using lists of nonsense syllables, Ebbinghaus carried out a series of studies on retention. He found that, after having memorized a list, forgetting would occur very rapidly within an hour, but would decrease by about 30% for delays of up to two days. After this time window, forgetting would still occur but at a lower

rate. Ebbinghaus was the first researcher who introduced the curves of learning and forgetting as an experimental methodology to study learning and memory.

In 1890, **William James** published a research in which he suggested that human memory could be subdivided into two subclasses. He called the first subclass *primary memory,* or what we now call short-term memory. This memory is short lived as it lasts only seconds and leaves no trace in consciousness. James called the second memory subclass *secondary memory.* This memory would correspond to what today is called long-term memory. The information stored in long-term memory is held indefinitely and even though it does not reside in consciousness, it is available to bring to consciousness if desired.

However, most of the early studies on learning and memory were based on experiments carried out on animals. The three most important researchers that used this approach were: Thorndike, Pavlov and Skinner.

**Edward L. Thorndike** investigated learning in cats that were required to learn a specific sequence of behavious in order to escape from a box.

This research (1898) led Thorndike to formulate two laws of learning:

1. *The law of exercise:* according to this law, an association or a neural connection is strengthened proportionally to the frequency of a specific behaviour.

2. *The law of effect:* a neural connection is strengthened when an association induces a behaviour that is useful to successfully complete a task.

Thorndike is also known for his study on *transfer of training.* He disproved the general belief that studying difficult subjects "strengthens" the mind, as he found that only if two subjects were similar to each other, the study of one would help the study of the other.

**Ivan Pavlov**, a Russian physiologist, is remembered for his studies on classical conditioning carried out on dogs. In 1904, these studies earned him the

Nobel Prize. Pavlov discovered that a stimulus that is biologically neutral (conditioned stimulus), can be paired to a stimulus that normally elicits a physiological response (unconditioned stimulus) in a dog. After a number of such pairings, the conditioned stimuls acquires the ability to evoke the response by itself. Subsequently, Pavlov determined that the cerebral cortex played a major role in the process of learning through classical conditioning, as he proved that conditioned responses could not be induced after removal of the dog's cerebral cortex (Pavlov, 1927).

In 1925, **John B. Watson** published a book that he called "Behaviorism". The revised edition, published in 1930, was the final statement of his position. According to Watson, behaviour is determined by the association of stimulus-response. By means of conditioning, we can acquire information about the world. He also denied the existence of any human instincts, personal talents, and temperaments. This radical environmentalism is reflected in what is perhaps his best known quote: "Give me a dozen healthy infants, well-formed, and my own specified world to bring them up in, and I'll guarantee to take any one at random and train him to become any type of specialist I might select - doctor, lawyer, artist, merchant-chief and, yes, even beggar-man and thief, regardless of his talents, penchants, tendencies, abilities, vocations, and race of his ancestors" (in Behaviorism, 1930).

Influenced by Pavlov's findings, **Frederic Skinner** studied stimulus-response reactions in humans, also experimenting with pigeons and rats to develop his theories. He built a box that contained levers which an animal could press, a number of stimulus lights and places in which rewards like food could be delivered. A starved rat was placed in the box. The rat had to learn that every time it pressed a lever a small pellet of food was dropped onto a tray. In this experiment the lever pressing behaviour is reinforced by food. Further, the animal had to learn that pressing the lever is reinforced (the rat gets food) when

a light is on but not when it is off. Skinner showed that after a number of trials, the rat could discriminate between light and dark (Skinner, 1938). In this experiment, Skinner demonstrated the ideas of "operant conditioning" and "shaping behaviour". Unlike Pavlov's "classical conditioning," where an existing behaviour (salivating for food) can be associated to a new stimulus (e.g., ringing of a bell), operant conditioning can be described as the rewarding of an act that approaches a new desired behaviour.

In "The Child's Conception of the World" (1926), **Jean Piaget**, while recognizing the contribution of the environment, thought that perception was filtered by the "internal cognitive structure" of the individual, emphasizing that the behaviour of an adult is markedly influenced by his experiences as a child.

The work of **Karl S. Lashley** and his "theory of memory trace" are today considered a very important contribution to the understanding of learning processes. According to Lashley's theory, during learning, new connections of neurons (*engrams*) are formed in the cerebral cortex. In 1929, Lashley published his famous "Brain mechanisms and intelligence". In this publication, he showed the results of experiments in which learning had been asssesed after removal of portions (varying from 10-50%) of a rat's cerebral cortex. These studies brought about two important principles. According to the first principle (*principle of mass action*), learning is disrupted proportionally to the amount of cortex removed. The second principle, that he called *principle of equipotentiality*, states that all areas of the cortex are functionally important to learning (Lashley, 1931). According to Lashley's view, behaviour is the result of changes in the nervous system and because the nervous system is a physical-chemical system, changes in behaviour following learning must induce physical-chemical alterations. Therefore, learning must involve an interaction between the central nervous system's input and output (Lashley, 1950-1968). However, Lashley was never able to prove the existence of engrams and

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therefore concluded: "the necessary conclusion is that learning just is not possible" (Lashley, 1968).

Learning theories such as Watson's, and Thorndike's were not without their critics. The most influential critic of the time was **Edward C. Tolman**. He thought that the simple association of stimulus-response is a study at too "molecular" a level and that behaviour should be studied at "molar level". Tolman suggested that rats had a purposeful behaviour, that is a goal-directed behaviour, and that they used prior experience to test and form new hypotheses. Tolman was interested in what he called "mentalistic" processes or what today we would call *cognitive processes*. He suggested that while an animal is exploring an environment, it develops a *cognitive map* of the environment (Tolman et al., 1946-1947). The process is called *latent learning*, which is learning in the absence of rewards or punishments. In this learning model, the animal develops *expectancies* (hypotheses) which can be *confirmed* or not by further experience. Rewards and punishments can influence learning only as motivators for performance, but are not the causes of learning itself (Tolman and Honzik, 1930b). Tolman is today considered one of the precursors of the cognitive movement (cognitivism).

In 1949, **Donald O. Hebb** published his "Theory of Cell Assemblies", a theory based on the concept of engram introduced by Lashley. This theory supports Lashley's view that learning induces changes in the physiology of neurons affecting the way they communicate with one another.

In 1953, the result of a surgery carried out on the patient H.M. by the neurosurgeon **Willliam B. Scoville** added important information on memory processes. Dr. Scoville, in an effort to stop the epileptic seizures of H.M., performed a bilateral medial temporal resection, removing also the amygdala and hippocampus. When tested on memory tasks, H.M. showed severe memory impairment, even though his overall IQ remained above average. Even though

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his perceptual functioning and his memory for events that occurred prior to his surgery had not been disrupted, H.M. would often forget what happened hou

rs, or in some cases, minutes before (Milner, 1970). The case of patient H.M., which became subject of investigation for more than 35 years, suggested for the first time that the study of learning should aim at understanding the process of storing memories rather than just at locating the areas of the brain where learning induces physiological changes. Further, the case of patient H.M suggested that that there are different classes of memory (H.M. had intact long term memory), which may be more independent than originally believed.

According to many psychologists the behaviourist view was too reductionistic. For them, behaviourism focused too much on the description of single perceptions or images, which should be considered as a whole rather than as a sum of the component parts. Such a view profoundly influenced the idea of learning across psychologists. Behaviourists thought that environment shapes behaviour whereas, according to this group of researchers (who called themselves Gestalt psychologists), the individual's mental processes have more importance. In other words, they were concerned with cognition, the act or process of knowing.

In 1968, **Richard Atkinson and Richard Shiffrin** proposed a "stage model" of human memory that influenced the modern view of human memory. According to their model, the brain processes information from the time of the input to the time of storage in long-term memory. There are three main stages involved in the process of acquiring knowledge: the *sensory registers, short-term* memory and *long-term* memory. The sensory registers store representations of external stimuli from the environment. They suggested that there are different sensory registers for each sense. The information stored in the registers has a short life unless it is transferred into short-term memory. According to Atkinson and Shiffrin, short-term memory can be thought of as

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conscious memory because, the information stored in this stage can be manipulated, interpreted and transformed. Finally, short-term memory can be transferred to the long-term memory stage. Once the information is stored in long-term memory, it stays.

The work of the all researchers mentioned above suggested that learning consists of the formation of associations between stimuli and responses, and that remembering consists of producing the response appropriate for a particular stimulus. These ideas dominated until the 1950's, when new views were introduced by scientists working in the world of communication and computing sciences. The concept of *information processing* began to emerge. According to this view, learning occurs as the result of a series of events that take place in a specific sequence. Thus, memory research began to focus on encoding, storage and retrieval processes, on the architecture of memory systems, and on the way in which information is represented when it is stored in memory. **Allen Newell** and **Herbert Simon**, whose main interest was artificial intelligence, introduced this research approach. They incorporated many ideas from artificial intelligence into their theories of human cognition, at the same time incorporating ideas from their theories of cognition into their work on artificial intelligence. The centerpiece of Newell and Simon's work was the General Problem Solver, or GPS (Newell and Simon, 1972). GPS was a computer simulation that used a way of developing knowledge in problem solving called *means-ends analysis.* The basic steps in applying means-ends analysis are the following:

1) Identify the major difference between the current situation and the goal: that is, focus on the end.

2) Select some action that is relevant to eliminating that difference; that is, select some means relevant to that end.

3) If the operator can be applied, apply it. If not, make the goal to enable the operator and start over again at step 1.

Although GPS was not specifically concerned with learning, it's clear how to consider learning according to this model. Learning is involved in acquiring the operators on which the theory is based. For example, when a rat explores a maze, it acquires knowledge about it. In the absence of any goal this knowledge remains dormant and latent. But if the rat realizes that choosing a specific location of the maze can earn it a reward, it will have a goal and it can treat the knowledge acquired about the maze as operators relevant to that goal. Each turn in the maze can be treated as an operator that gets them closer or further away from the goal. Thus, GPS offers a model of transition from knowledge to behaviour.

## Table 1.1

Some historical breakthroughs in the understanding of learning and memory

**1885**  Ebbinghaus publishes the first scientific report on the topic of learning and memory. Ebbinghaus was the first researcher who introduced the curves of learning and forgetting as an experimental methodology to study learning and memory.

**1890**  James suggests that human memory can be subdivided into two sub-classes: primary and secondary memory.

**1898**  Thorndike formulates two laws of learning: the *law of exercise* and the

*law of effect*. He is also remembered for his studies on "transfer of training".

**1904** Pavlov is awarded with the Nobel Prize for his studies on classical conditioning carried out in the 1890s.

**1926** Piaget publishes "The Child's Conception of the World", in which he emphasizes the role played in perception by the "internal cognitive structure" of the individual.

**1927** Pavlov publishes a report proving that the cerebral cortex plays a major role in the process of learning through classical conditioning.

**1929** Lashley publishes "Brain mechanisms and intelligence" in which he suggests that learning must induce physical-chemical alterations in the nervous system.

**1930** Watson publishes the revised edition of "Behaviorism" in which he states that, by means of conditioning, we can acquire information about the world.

**1938** Skinner introduces the concept of "operant conditioning" and "shaping behaviour".

**1949** Hebb publishes his "Theory of Cell Assemblies" in which he supports Lashley's view that learning induces changes in the physiology of neurons affecting the way they communicate with one another.

**1953** The neurosurgeon Scoville carries out surgery on patient H.M.

**1968**     Atkinson and Shiffrin propose a "stage model" of human memory. According to their model, the brain processes the information from the time of the input to the time of storage in long-term memory.

**1970**     Milner publishes the results of his experiments on patient H.M.

**1972**     Newell and Simon introduce the concept of GPS, a model of transition from knowledge to behaviour.


## *1.1.4 Current research influences on the study of learning and memory*


According to most contemporary theories, memory can be divided into multiple systems related to different neural substrates that contribute to performance in a relatively independent manner. Recent data suggest that, during performance of a given memory task, complex interactions among different systems occur (Kim and Baxter, 2001). Modern views, typically describe learning as a process occurring through information acquisition, storage and retrieval (Squire, 1987; Kandel and Pittenger, 1999). For many, learning different tasks involves different types of memory. For example, the memory used to learn to ride a bicycle is qualitatively different from that involved in learning to solve mathematical equations. The concept that memory can be subdivided in different components, was suggested by the discovery of some preserved learning and memory capabilities in the amnesic patient H.M. (Milner et al., 1968; Squire, 1987) and has received widespread acceptance in spite of the continuing discussion of how memory systems should be categorized (Atkinson and Shriffrin, 1968; Weldon, 1999). The study of memory systems is typically carried out using tasks that require the use of

individual systems in relative isolation. A task is chosen for its suitability to a system of interest, which is then investigated at multiple levels of analysis (e.g., behavioural, neuropsychological, functional imaging, physiological and genetic), an approach that has considerably broadened our understanding of memory systems (Morris et al., 1990; Milner et al., 1998).

## 1.1.5 Separate memory systems associated with separate brain structures

Evidence linking particular brain structures with apparently different kinds of memory has been gathered in several laboratories. For instance, Mishkin and his collegues have considered two possible memory systems in monkeys (Mishkin and Appenzeller 1987; Mishkin, Malamut and Bachevalier, 1984). One is linked with simple multi-trial discrimination learning and is referred to as a **habit memory system**. This system, based on associations between specific stimuli and responses, is controlled by the neostriatum, which serves as a target of cortico-striatal pathways. The other system is called **recognition memory system**. This system is viewed as an example of higher order organization or representational memory. It has been suggested that the recognition memory system is regulated by higher order sensory areas of the cortex, including a circuit that involves the limbic system, thalamus and cortex (Mishkin, Malamut and Bachevalier, 1984).

Two other distinguished hypothetical systems are **reference memory** and **working memory**. We can call reference memory, that memory containing general information about rules and procedures that are applicable to many different instances of the same class of events (Olton, 1983; Cassel et al., 1998). This memory does not require that the current instance of the class be distinguished from any other instance of the same class. A second class of

memories is much more specific, and does require that one instance be distinguished from other instances. This is what is called working memory (Olton, 1983; Cassel et al., 1998). These two memory systems involve different psychological processes, and reflect different functions. At behavioural level, testing procedures vary in the extent to which they require working memory or reference memory for their solution, so that performance in these tests can be used to measure the accuracy of these memory processes.

The repetitive rules and procedures are coded by reference memory, the current item by working memory. In all cases, the important point has to do with whether or not information about the temporal/personal context of an event is necessary to behave appropriately. Working memory emphasizes this temporal/personal context and separates one instance from another. Reference memory ignores this context, and emphasizes information that is applicable to a large number of similar instances.

**Procedural memory** and **declarative memory** provide another example of classification. Procedural memory refers to the learning of skills and habits. This type of memory is developed slowly but is very reliable. It is inflexible in that the information is not readily expressed by response systems that were not involved in the original learning. Procedural memory is a heterogeneous collection of separate abilities, which depend on brain systems outside of the medial temporal lobe and diencephalon (Squire, 1993). Declarative memory can be defined as recollections of facts and events (Cohen and Squire 1980; Squire 1987). Learning is fast (one can either remember the fact or one does not) but not always reliable in that the memory may be imperfectly remembered. This form of memory is flexible in that it is accessible to multiple response systems. Declarative memory is believed by Squire and his colleagues to be controlled by a circuit that involves the medial temporal cortex, including hippocampus and related anatomical structures such as the enthorinal, perirhinal and

parahippocampal cortices and also the diencephalic areas of the brain (Squire, 1993).

Kesner (1991) proposed a relatively extensive theory of the multiple memory systems by discrete neural structures. Kesner's approach is marked by careful attention to the literature of both human and animal memory, the creation of innovative tests of animal memory to simulate basic features of human memory, and the view that a memory consists of a variety of attributes. He proposes that an acquired memory includes attributes to represent each of the five critical features of an episode: temporal, spatial, affect, sensory-perceptual, and response attributes. Kesner considers how memory attributes interact for higher levels of organization that include contextual control of retrieval and the development of cognitive maps. Analogous to several broad-based theories of memory, a distinction is made between *a data-based memory system* (the relatively recent facts at hand) and an *expectancy-based system* (acquired associations that generate expectancies in the face of particular stimuli). A large number of experiments have indicated that particular brain areas are linked with separate aspects of Kesner's memory system. Included are apparent relationships between the hippocampus and attributes for external context, between the amygdala and attributes for internal context, between the caudate nucleus and hippocampus for spatial attributes, and among areas of the motor cortex, brain stem, and cerebellum for the control of skills resulting from the interaction between sensory-perceptual and response attributes.

A good example of the reach of Kesner's theory can be taken from tests of his hypothesis about memories involving temporal attributes. He proposes that such memories are mediated within the expectancy-based memory system by the dorsolateral or medial prefrontal cortex. The evidence to support this begins with the familiar observation that humans with damage to the frontal cortex have difficulty with short-term retention for the temporal order in which

events occur. Although these patients may remember which events were presented, they have difficulty remembering the order in which events occurred, or which is the more or less recent event. In other words, although retention of item information is relatively unaffected, retention of order information is drastically impaired. Monkeys with lesions in the dorsolateral prefrontal cortex seem to show a similar deficit in terms of tasks such as delayed matching-to-sample (Goldman and Rosvold, 1970; Goldman et al., 1971). It is possible to solve the delayed matching problem, especially when only a few alternative samples are used, by simply discriminating the most recent event from the less recent event. Rats with lesions in the medial prefrontal cortex (which probably serves the same function as the dorsolateral prefrontal cortex in monkeys and humans) also are deficient in memory for temporal order. Some studies have suggested this in terms of retention of specific response sequences or in delayed alternation. Kesner and Holbrook (1987) devised a more convincing test for dissociating item and order information in the rat. Rats were trained until they learned that in an eight-arm radial maze, once an arm had been visited and the reinforcer consumed, no further reward would be found in that alternative. They then were trained to remember the particular sequence with which they had visited four of the arms in the radial maze. This was assessed with a test in which two of the arms were made available for entry (say, items that were second and third in the previous sequence) and the rat's response was reinforced only if it chose the arm (item) that had occurred earlier in the sequence. Item memory was tested by allowing the animal entry into two alleys, one of which previously had been visited and one of which had not, with reward present only in the latter. After the rats had become expert at remembering the sequential information and the item information, some of them were given lesions of the medial prefrontal cortex. These animals subsequently showed drastic impairment in remembering the sequence in which they had previously visited

the alleys (order information), although they were relatively effective in remembering whether or not they had previously visited a particular alley (item information). Such a result lends significant support to the notion that this area of the prefrontal cortex is selective in its control over retention: retention of order information (as represented by a spatial attribute) is spared. In this sense, one aspect of memory is dissociated from another in terms of its control by a particular brain structure.

This sort of dissociation experiment has been used to great advantage by Kesner and others. Kesner has completed several experiments that dissociate in both directions such that one area (e.g., the hippocampus) controls memories involving spatial attributes but not those involving temporal attributes, whereas another (e.g., prefrontal cortex) controls memories involving temporal attributes but not involving spatial attributes (for another example, see Packard and McGaugh, 1992). Such dissociation experiments are difficult to accomplish, but they provide a necessary condition for treating memory systems as if they were separate and perhaps independent.

## 1.1.6 Relationship between learning and retention

If equal learning always led to equal, complete retention, the concepts of retention and forgetting would be unnecessary. Equally learned episodes may, however, be forgotten at different rates. Learning and retention are independent concepts. Degree of original learning, nevertheless, can determine degree of retention. Retention generally is more accurate and more enduring with higher degrees of learning (Schwartz and Reisberg, 1991).

In tasks requiring multiple trials, we consider that retention, rather than learning, is measured whenever the interval after the last training trial exceeds

the interval used between the training trials. That is, a retention interval is defined as any duration longer than the inter-trial interval. In tasks acquired in only a single trial, time between that trial and a test trial is the retention interval.

Thus, retention refers to the expression of previously acquired information at some point after an organism is removed from the physical presence of the information. The implication is that active processing of episode by the subject has been interrupted. Conversely, testing before the interruption would assess learning.

Another important distinction between learning and retention is functional: some variables affect learning and retention in quite different ways. The literature on human verbal learning (Keppel 1964; Wickelgren, 1972) provides clear examples of this:

4)    learning is faster when a target list of verbal items is high rather than low in 'meaningfulness," as one might expect. But once the list is learned, meaningfulness has little influence on retention.

5)    Some people learn very rapidly and others more slowly. This individual difference in rate of learning does not predict ability to retain the information. Once slow and fast learners have mastered the task to an equivalent degree, no differences are found in rate of forgetting.

6)    In paired associate tasks, the distribution of practice (spacing of trials) has little effect on learning the response. However, under certain conditions greater distribution of practice can markedly facilitate retention.

Similarly with animals, there are cases of functional differences between learning and retention. For example, there are a variety of tasks that may be learned equally rapidly by immature and mature animals but which, after long retention intervals, are forgotten more rapidly by animals that learned during immaturity (Campbell and Spear, 1972). Another variable that influences

learning but not retention is the intensity of shock in avoidance learning (Feigley and Spear, 1970).

A variety of processes have been suggested to affect retention quite independently of the circumstances of learning. Such processes include: interference from previous or subsequent learning; hypothetical decay of the physiological underpinnings of memory; a consolidation or elaboration process occurring subsequent to learning to facilitate storage of the memory; changes in stimuli (whether subtle or obvious and whether external or internal to the organism) from those that had been present during original learning; and special cues just prior to testing that may facilitate retrieval. These are some of the features of retention and forgetting that require us to consider this topic separately from learning. Retention is evident when information previously acquired by an organism comes to influence its present behaviour. When this influence is absent despite adequate perception and motivation, we say that forgetting has occurred.

## 1.2 Amnesia

### 1.2.1 Symptoms of amnesia and brain structures involved

Perhaps the most dramatic evidence that learning and memory depend on the brain is organic amnesia, which occurs when damage to the brain erases memories and makes it difficult or impossible to form new ones. Different symptoms of amnesia have been characterized:

The first is **anterograde amnesia,** the difficulty or inability to form new memories; the second is **retrograde amnesia,** the loss of some memories prior to the event causing the brain damage responsible for the amnesia. The retrograde amnesia is graded so that the more remote a memory is from the traumatic event the more likely it is to be remembered. The third symptom is

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**confabulation**: When asked to recall an event lost to memory, amnesics may make up a story instead. Finally, amnesics' difficulties are restricted to memory, for they possess normal intelligence.

The most famous and most researched case of amnesic syndrome is the patient H.M (Milner, 1970; Cotman and McGaugh, 1980; Squire, 1987). H. M. suffered from epilepsy and when the symptoms became uncontrollable by medication, surgery was performed in an effort to relieve the patient's seizures. The operation was successful in controlling the epilepsy but, accidentally and unfortunately, it caused total anterograde amnesia. Surgery involved the removal of structures from the limbic system (medio-temporal areas of the brain): two thirds of the hippocampus, the parahippocampal gyrus, the uncus, and the amygdala. H.M.'s intelligence, personality and working memory were intact (he had a normal digit span of 6 to 7 items) but he could not form new memories. Even after 25 presentations of a long word list he could not recall more than the 6 items stored in his working memory, and he showed no primacy effect. As H.M. said: "Every day is alone in itself...everything looks clear to me, but what happened just before?...It's like waking from a dream. I just don't remember" (quoted by Cotman and McGaugh, 1980, p. 334). H.M. could not even recognize a current photograph of himself, and needed constant custodial care.

Cases of amnesia have proven valuable in distinguishing between various forms and processes of memory. The distinction between working memory and long-term memory (LTM) is supported by the amnesic syndrome. H.M. is typical among amnesics in having a normal digit span, and thus intact working memory, while being unable to lay down memories in LTM. Amnesia also shows that we must distinguish between the site of memory storage and the sites of the processes and mechanisms that create memories. H.M. could recall remote events, so we know that his permanent memory-storage sites were not

damaged by surgery, but he could not form new memories, so we know that the structures responsible for memory creation were damaged.

Moreover, the graded nature of retrograde amnesia suggests that memory consolidation is a drawn out but time-limited process in which some structures of the brain participate in laying down memories in some other part of the brain with which contact is eventually broken off (Squire, 1987). One remarkable finding with H.M., and confirmed in other cases of amnesia, supports important distinctions between types of long-term memory. H.M. shows essentially normal rates of learning for a variety of motor skills, such as mirror drawing, even though each time he performs the task he says he has never done it before.

Amnesics also show normal acquisition of simple Pavlovian responses without memory of the apparatus, although complex Pavlovian phenomena such as latent inhibition and reversal learning are impaired. Amnesics lack declarative memory, conscious recall of past events, but retain procedural memory, the ability to learn new behaviours. Mirror drawing remained ever new to H.M. even though he got better and better at it.

As a result of these and other findings, Daniel Schachter (1985, 1987) has distinguished between explicit and implicit long-term memory. **Explicit memory** requires conscious awareness of a previous event, and is lost in amnesia; **implicit memory** does not require conscious awareness and is spared in amnesia. While cases of amnesia may suggest which structures of the brain are involved in learning, they cannot answer more precise questions about how various structures operate and interact while forming, storing, and retrieving memories. Amnesic's brains may be damaged in ways not always apparent in autopsy, and many amnesics such as H.M. (who had suffered from epilepsy and had lost his capacity of forming new explicit memory after a surgical procedure) and those acquiring amnesia from disease, such as Korsakoff's syndrome (this

is caused by a damage of the diencephalic midline of the brain, including the thalamus, hypotalamus, and mammillary bodies), may have incurred other physiological damage, making identification of memory structures tentative.

Squire (1992) reviewed attempts to replicate the human amnesic syndrome by lesioning, in rats and monkeys, the various parts of the brain found to be implicated in human amnesic syndrome. He concluded that the key structures involved in the formation of long-term memory are the hippocampus and associated cortical tissue (especially the perirhinal cortex and the parahippocampal gyrus).

## 1.3 The Biology of long-term memory

### 1.3.1 Multiple memory systems

Psychologists have drawn many distinctions between forms of long term-memory (Kesner, 1991), such as episodic-semantic or explicit-implicit. A perennial question has been whether these different psychological memory systems could prove to be neurologically distinct as well. Squire et al. (1992, 1994, 1995) have proposed a classification of memory systems based on extensive findings on the neurophysiology of learning and memory.

### 1.3.2 The critical role of hippocampus

The most studied neurological memory structure is the hippocampus and its related cortical structures, which many psychologists believe is uniquely involved in forming declarative memories. Squire (1992) proposes that the essential psychological function of the hippocampus is to rapidly establish links between ordinarily unrelated patterns of events by establishing flexible

representations of mutiple stimuli, which then become available to multiple response systems. The hippocampus is thus involved in general purpose learning, while the learning systems spared in amnesia are special purpose, reflexive, rigid, and slow. Recent research on the hippocampus has begun to focus on the different roles played in learning and memory by different hippocampal structures, especially the parahippocampal gyrus and the hippocampus proper (Eichenbaum et al., 1994; Eichenbaum and Bunsey, 1995; Gluck and Myers, 1995). Figure (1.1) shows a simplified structural flow diagram of how the structures of the hippocampus relate to the brain.

Highly processed information
from all sensory modalities
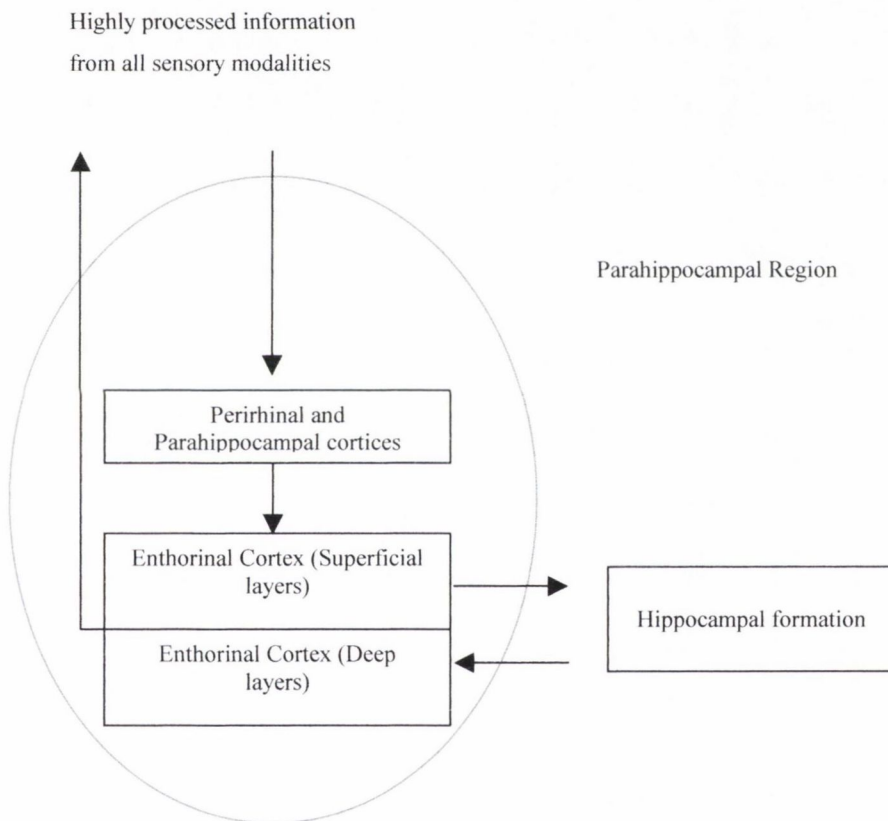


**Figure 1.1**

Simplified block diagram showing information flows between the hippocampus, its related structures in the parahippocampal gyrus, and the rest of the brain. (from "Representation and Association in Memory: A Neurocomputational View of Hippocampal function" by M. A. Gluck and C.E. Myers).

The hippocampus proper is self-contained and connects with the rest of the brain through the parahippocampal structures. Early psychologists distinguished two ways in which ideas might be bound together by learning and stored in memory. One way was called **fusion.** In fusion, the ideas blend together so perfectly that they can no longer be separated. An example is our perception of a harmonious chord played on the piano. Played alone, we can distinguish two or three notes, but if they are played together as a chord we experience them so blended that we no longer can hear each note separately. The other form of ideas binding was called **association**. In association each idea retains its identity, and representation in memory, but a link is formed between them. For example, in an old-fashioned paired associate learning task one might learn to say "BOK" when he or she sees "DIV" presented on memory drum, but the nonsense syllables do not blend together in an inextricable tangle the way the notes in a chord do.

While their theories are somewhat different, Eichenbaum and his colleagues (1995) propose that the hippocampus proper forms associations while the parahippocampal gyrus forms fusions. For example, Eichenbaum and Bunsey (1995) report a series of experiments with rats using a variation on the old paired-associate learning paradigm. The rats were either normal or had their parahippocampus lesioned (thus cutting off the hippocampus from the brain), or had their hippocampi proper lesioned. The task involved learning to bar-press for reinforcement, only when certain pairs of odours were presented in a sniff tube. Thus pairs such as A-B, or C-D, or D-C were followed by reward, while other pairings were not. Non-reinforced pairs were of two types, mispairings or non-relational sequences. Mispairings were pairs of odours that occurred in the list of paired associates but which were not paired with each other, such as A-D. Non-relational sequences were pairings of completely new odours.

Normal rats learned to distinguish between paired associates, mispairings and non-relational sequences. Rats with parahippocampal lesions (which also rendered the hippocampus inoperative) never learned to distinguish paired associates from mispairings but they did learn to distinguish paired associates from non-relational sequences. Thus, it appears that rats without a functional hippocampus can tell novelty when they see it (and thus reject non-relational sequences, which always involve at least one new odour), but cannot distinguish between pairs of familiar odours. Also, rats with hippocampal lesions learned to ignore non-relational sequences, and were better at distinguishing paired associates from mispairs than normal rats were. From these findings, Eichenbaum concluded that hippocampal structures form different sorts of associations. The hippocampus forms associations, while the parahippocampal gyrus forms fusions. Rats with lesions to the parahippocampal gyrus, that is with no effective hippocampus at all, cannot learn any association at all, but can recognize novelty. Rats with lesions to the hippocampus can form fusions, and thus can readily distinguish pairs from mispairs, because the fusions of, for example, A-B and A-C are recognizably different. Interestingly, human amnesics can, with effort, learn rigidly defined questions about baseball, but only if the question asked is repeated in exactly the same way on each occasion. Eichenbaum suggests that they form fusions, not associations, between the ideas in the question and answer. If even one part of the fused idea is changed, it cannot be retrieved from memory.

Although the hippocampus is involved in forming memories, it is believed that it is not the site of engrams [the area(s) in the brain where a physical/physiological alteration occurs in response to a stimulus in order to form a memory trace]. Amnesics can remember their past despite losing their hippocampal function. Thus the role of hippocampus in laying down memories is time-limited (Squire, 1992). The hippocampus thus seems to play a central

role in memory consolidation as revealed in temporary amnesia. Indeed, in temporary amnesia, memory formation is disrupted as long as hippocampal function is impaired (Squire, 1992). McClelland et al. (1995) have proposed that the hippocampus is responsible for consolidation of memory by repeatedly presenting connectionist theory-like "training pairs" to the cortex. Roughly speaking, as the cortex forms the cell assemblies that will eventually store a given memory, it tests them against a record stored temporarily in the hippocampus, and corrects itself by connectionist weigh-modification methods until it correctly represents the memory. Eventually, the hippocampal memory vanishes, leaving only the cortical representation. Thus amnesia is temporally graded, as we have seen. Old memories are intact because the hippocampus had finished training the cortex; memories just before and after trauma are lost because the hippocampus was completely out of operation; and memories for events in between are partially lost because the hippocampus was active for a while and then damaged, only partially training the cell assemblies for the cortex.

## 1.4 Anatomy of the hippocampal formation

### 1.4.1 Definition of hippocampal formation

The locations of the various fields of the hippocampal formation are indicated in Fig. 1.2 (A, B). Despite extensive study, there is still substantial confusion concerning the terminology of the various components of the hippocampal formation, and which components should be included under this term. However, according to the most accepted definition, the term hippocampal formation comprises six cytoarchitectonically distinct regions, including the **dentate gyrus**, **hippocampus** (or *hippocampus proper*, subdivided into three

fields: CA3, CA2 and CA1), **subiculum**, **presubiculum**, **parasubiculum** and **enthorinal cortex.** The subiculum, presubiculum and parasubiculum are sometimes grouped together as *subicular complex*. The main justification for including these regions under the rubric hippocampal formation is that they are linked, one to the next, by unique and largely unidirectional excitatory aminoacid (EAA) mediated projections (Fig. 1.3). The enthorinal cortex provides the dentate gyrus with its major input via the so-called **perforant pathway.** The enthorinal to dentate gyrus projection is not reciprocated, however, because none of the cells in the dentate gyrus project back to the enthorinal cortex. Rather than projecting to the enthorinal cortex, the dentate granule cells project, via their distinctive mossy fibers, to the CA3 field of the hippocampus. Although some CA3 cells contribute axon collaterals to the deep or polymorphic layer of the dentate gyrus, these axons do not innervate granule cells. A similar unidirectional pattern holds for the other major intrinsic connections (CA3$\Rightarrow$CA1; CA1$\Rightarrow$subiculum) of the hippocampal formation.

Whereas the main principle of the neocortex is to arrange the cooperating element in radially oriented columns or bands, the main principle of organization in the hippocampal cortex is tangential. That is to say, the afferent fibres run parallel to the cortical surface and will cross the main axis at right angles (Ramon y Cajal, 1911).
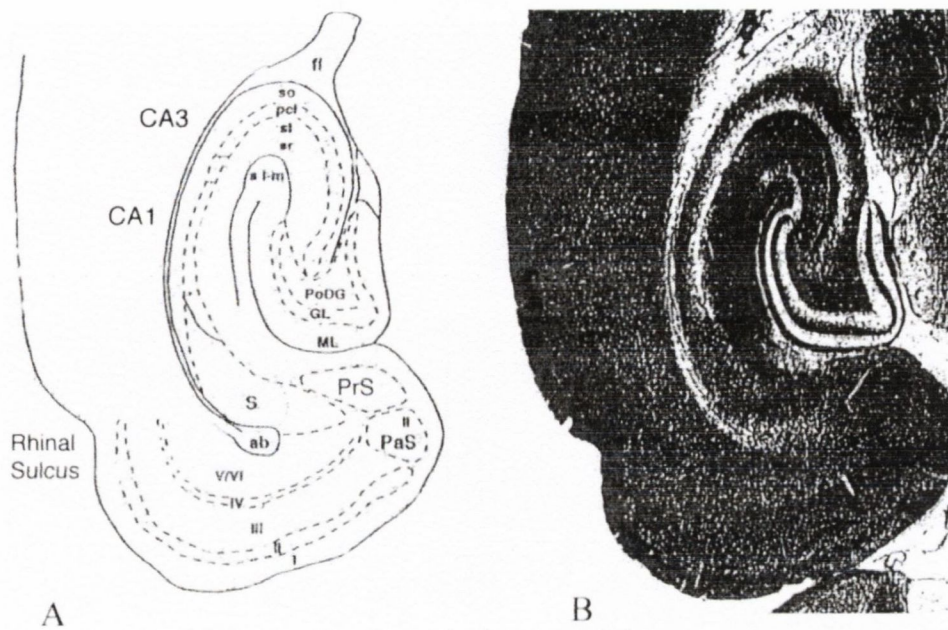
**Fig 1.2 (A, B)**

Fig 1.2 A indicates various regions, layers, and fiber pathways of the rat hippocampal formation. Fig 1.2 B shows a Nissl (A)-and Timm's sulfide silver-stained horizontal section. Abbreviations in Fig. A: ab, angular bundle; PaS: parasubiculum; PrS: presubiculum; ML, GL, and PoDG: molecular, granule cell, and polymorphic layers, respectively, of the dentate gyrus; so: stratum oriens; pcl: pyramidal cell layer; sl: stratum lucidum; sr: stratum radiatum; sl-m: stratum lacunosum-moleculare (from David G. Amaral, Menno P. Witter, chap. 21, 1995, second edition).

## Subcortical Inputs

Amygdala
Claustrum
Septal Nuclei
Supramammillary Nucleus
Lateral Hypothalamus
Anterior Thalamus
Midline Thalamus
Ventral Tegmental Area
Raphe nuclei
Locus Coeruleus

## Subcortical Outputs

Olfactory Regions
Amygdala
Septal Nuclei
Nucleus Accumbens
Anterior Thalamus
Midline Thalamus
Hypothalamus
Mammillary Nuclei

## Cortical Interconnections

Perirhinal Cortex
Retrosplenial Cortex
Medial Frontal Cortex
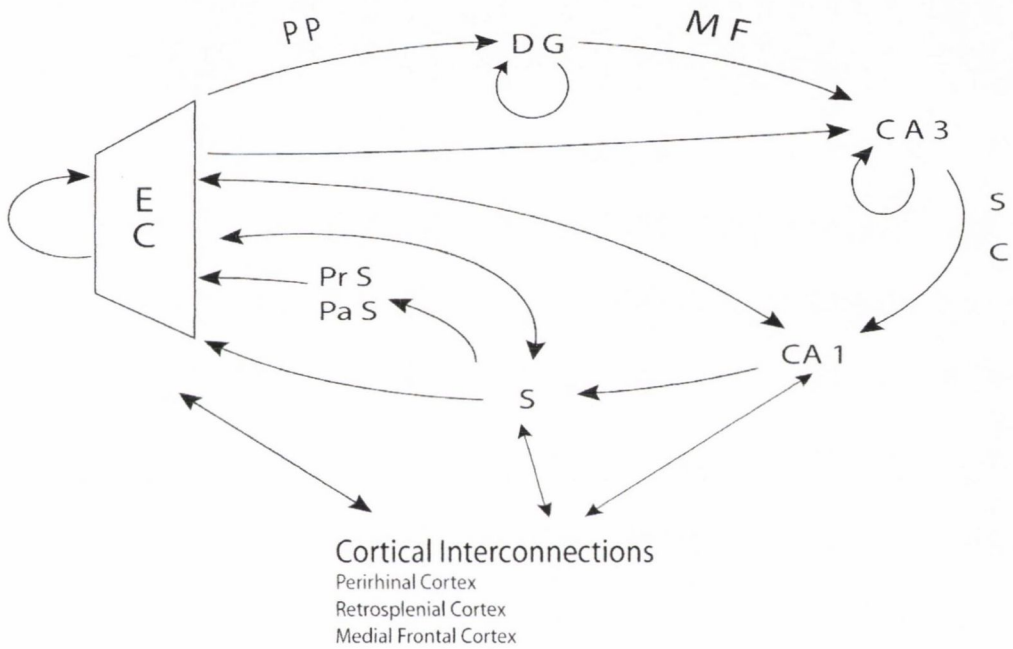
**Fig 1. 3**

Schematic illustration of the major intrinsic connections of the rat hippocampal formation. Many of the named pathways (perforant pathway, mossy fibers, and Shaffer collaterals) are unidirectional. Several of the major cortical and subcortical inputs and outputs of the hippocampal formation are also listed. Abbreviations: EC, entorhinal cortex; DG, dentate gyrus; S, subiculum; PrS, presubiculum; PaS, parasubiculum; MF, Mossy Fibers, SC, Schaffer Collaterals, PP, Perforant Path, (from David G. Amaral, Menno P. Witter, chap. 21, 1995, second edition).

## 1.5 The Hippocampus and memory related eletrophysiological processes

The evidence showing that there is a relationship between the electrical activity of specific cells in the hippocampus and spatial navigation has been provided by studies proving that specific cells in the CA1 region fire selectively in a complex spike pattern when an animal is in a specific environment. These data suggested that these cells (named "place cells") play a role in the formation of a "cognitive map" of the environment. For this reason the function of the hippocampus has been associated with spatial memory (O'Keefe and Nadel, 1978). Other studies (Vanderwolf, 1988) have shown that, in the hippocampus, electroencephalogram exhibits two main waveforms: a "large irregular activity" (LIA) and a more regular pattern called "rhythmical slow activity" (RSA) or "theta activity". While LIA can be observed during automatic behaviours (e.g., standing immobile, gnawing, shivering), RSA is present during voluntary behaviours (e.g., moving and changing posture).

The evidence that the hippocampal formation plays an important role in memory has suggested that its synaptic connections must be "plastic" that is able to change the strength of their interactions. Although it's now clear that memory storage itself does not occur in the hippocampus, this was not understood when work on hippocampal cell physiology first began. The studies published by Bliss and Lomo in 1973 showed for the first time that the stimulation of axons of the perforant pathway of the rabbit induced a long-term increase in the magnitude of excitatory postsynaptic potentials (EPSPs). That is, the stimulation applied led to greater synaptic strength in the perforant pathway such that later stimulation induced larger postsynaptic responses in the granule cells of the dentate gyrus. This phenomenon, which they called long-term potentiation (LTP) could last hours in isolated slices of hippocampal tissue. The

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discovery of LTP confirmed physiologically Hebb's law (1949), according to which if a weak and a strong stimulation act on a cell at the same time, the weak synapse becomes stronger. It has been shown that LTP generation in the hippocampus can be explained by the properties of the ionotropic glutamate receptor class N-methyl-D-aspartate (NMDA) located on the dentritic spines of postsynaptic neurons that show LTP (Nicoll and Malenka, 1995). Glutamate is the major excitatory transmitter in the hippocampus (see Malenka and Nicoll, 1999 for a review) and it can bind with NMDA and non-NMDA receptor. When 2-amino-5phosphonopentanoate (AP5) is injected locally in the CA1 area, NMDA receptors are chemically blocked and LTP induction is prevented. But the AP5 treatment does not produce any effect on previously established LTP in these cells. Therefore, NMDA receptors are thought to be crucial to producing LTP but not maintaining it. There is evidence that maintenance of LTP may depend on the non-NMDA receptors (Nicoll and Malenka, 1995; also see Malenka and Nicoll, 1999 for a review on LTP). Barnes (1988) showed that LTP decay rate is significantly greater in older rats than in young ones and that older rats display spatial learning deficits. Other evidence based on behavioural studies suggests that LTP has a role in memory. It has been shown that AP5, at a dose that blocked hippocampal LTP, induced the same learning deficits observed following specific hippocampal lesions (Morris, 1993). Other evidence suggests that hippocampal metabotropic receptor activity is also important in the generation and expression of LTP. For example, Naie and Manahan-Vaughan (2004) have shown that inhibition of the receptor mGluR5 in the dentate gyrus leads to inhibition of both LTP induction and expression, and to a marked impairment of both working and reference memory in vivo. Recently, another study from the same authors has shown that the effects of mGluR1 receptor antagonism were distinctly less potent albeit more selective. Thus, in association with an impairment of LTP expression, only reference

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memory but not working memory performance was impaired (Naie and Manahan-Vaughan, 2005). However, although there is a general agreement that the physical substrate of memory in the mammalian brain resides in alterations of synaptic efficacy the role of LTP is still being unraveled. There is great debate over whether the maintenance of LTP is necessary for spatial memory. Two points of agreement are that LTP does exist at cellular level and that NMDA receptors play a crucial role in LTP-induction in many pathways of the brain. Because LTP is also in brain areas outside the hippocampal system, the possibility that LTP forms the basis for long-term modification within synaptic networks remains promising (Malenka and Nicoll, 1999).

## 1.6 Neurotransmitter systems involved in the modulation of cognitive functions

### 1.6.1 Acetylcholine

It is over 40 years since cholinergic transmission in the brain was originally correlated with consciousness and shown to increase during waking and dreaming compared with non-dreaming sleep. Since then, different cholinergic pathways have been characterized, and their involvement in neurodegenerative diseases that affect cognition and consciousness have been reported (see Perry and Perry, 1999-2004 for a review). Disruption of the cholinergic system has been consistently identified in cognitively impairing disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and Dementia with Lewy bodies (DLB) (Perry, and Perry, 1995). In AD, loss of explicit memory (more than implicit) occurs due to early degeneration in the medial-temporal lobe memory systems, which are primarily, if not exclusively, involved in declarative memory. Implicit memory for novel patterns and cognitive or motor-skill learning have, however, been reported to be unimpaired (Hirono et al., 1997). In AD, different pathological manifestations, such as cortical and subcortical β-amyloidosis (which results in plaque formation), abnormal "tau" (which results in the development of tangles and dystrophic neurites), neuronal and synapse loss, and various transmitter deficits, leave clinical-neuropathological correlations open to a variety of interpretations. Deficits in cholinergic neurotransmission are unlikely to account for the full spectrum of cognitive and non-cognitive symptoms. The situation is less complex in PD and DLB, in which cortical neurofibrillary tangles are less usual or absent, and β-amyloid plaques are not invariant. In these disorders, neocortical deficits in ACh-mediated neurotransmission are generally greater than in AD and yet cognitive impairments are not so severe. Patients with DLB,

and to a lesser extent, those with PD experience hallucinations that are primarily visual and frequently persistent (McKeith et al., 1996). In DLB, neocortical ACh-related activities (especially in the temporal and parietal cortex) are lower in patients with hallucinations (Perry et al., 1993). While this identifies a cortical "cholinergic correlate" of hallucinogenesis in DLB, abnormalities in REM sleep reported in this disorder could also implicate the pathology of pedunculopontine neurons, with intrusions of REM into the waking state. It is likely that the disruption of muscarinic cholinergic receptors might be involved in the induction of hallucinations, as there is evidence showing that the integrated visual images of people or animals that are encountered by hallucinating patients are similar to those experienced following ingestion of muscarinic-receptor antagonists, such as scopolamine or atropine, in ritualistic, recreational or medical situations (Perry and Perry, 1995). In both PD with dementia and AD, hallucinations are attenuated by the cholinesterase inhibitors physostigmine, tacrine or metrifonate (Cummings et al., 1993; Morris, et al., 1998). Another feature of DLB that is distinct from AD is the prominent fluctuation in symptoms, which include the level of consciousness with episodes of reduced awareness of surroundings (McKeith et al., 1996). Patients, while not unconscious or asleep, can, for seconds, minutes or hours, cease to respond to external stimuli. These absence episodes are not epileptic in origin, nor are they obviously the result of cardiovascular deficiencies. Reductions in the number of cholinergic projections to the thalamic reticular nucleus have been identified in DLB that do not occur to the same extent as in AD or PD (Perry, et al., 1998), despite the loss of pedunculopontine neurons in the latter. Whether dysfunction of the cholinergic system accounts for these changes in the level of conscious awareness remains to be established.

As drugs emerge for the treatment of AD and ligands for imaging the cholinergic system *in vivo* proliferate, new opportunities arise that allow the

examination of the role of cholinergic systems in the human brain. Beyond objective measures of cognition, memory and behaviour, it will be valuable to explore subjective experiences that involve conscious awareness, including such components as hallucinogenesis, levels of consciousness, and REM sleep or dreaming. Although the physiological, pharmacological and pathological data mentioned above support the concept that the action of ACh in the cortex and thalamus is essential for the maintenance of the normal experience of conscious awareness, "no behaviour is a one-transmitter affair, yet, frequently the cholinergic system constitutes the significant correlate" (Karczmar, 1993). The way in which ACh might contribute to generating the integrated, coherent experience of conscious awareness remains to be established.

1.6.1.1 Relevant cholinergic pathways

The cholinergic system consists of a variety of different nuclei with extensive projections. Cholinergic projections from the basal forebrain to the cortex and thalamus are considered to be essential for controlling selective attention, and the fact that 90% of brainstem projections to the thalamus are cholinergic (Bentivoglio and Steriade, 1990) is of particular interest in view of the importance of the thalamus in conscious awareness.

According to Mesulam (1995) the extent of nucleus-basalis cholinergic projections to the human cortex indicates that, "this pathway is likely to constitute the single most substantial regulatory afferent system of the cerebral cortex". On the basis of the observation that cortical activation is maintained during REM sleep in minimal monoaminergic-neuron activity (for example, noradrenergic and serotonergic), Buzsaki et al. (1988) concluded that the ascending cholinergic system alone is capable of keeping the neocortex in its operative mode. However, the excitatory glutamatergic input to the nucleus basalis that arises in the brainstem, together with the major thalamo-cortical

glutamatergic projections, indicate that the combined actions of ACh and glutamate are essential in this respect.

The consensus view on the role of cortical cholinergic projections is that they control selective attention. According to Delacour et al. (1995), selective attention and consciousness overlap, and as Baars et al. (1998) pointed out, the two processes might share a common neural basis. While attention processes can occur at the non conscious-implicit level, conscious awareness, which represents only a fraction of cerebral activity at any time, clearly involves a selection process. Current theories of the role of cortical ACh include the possibility that it affects discriminatory processes, increases signal-noise ratios, modulates the efficiency of cortical processing of sensory and association information, controls the reception and evaluation of stimuli for their level of significance, modifies cortical responsiveness in terms of the relevance of novelty; and confines the contents of the conscious stream (Perry, and Perry, 1995). Basal-forebrain cholinergic neurons project not only to all cortical areas but also to specific thalamic nuclei, including the reticular nucleus (Mitrofanis, and Guillery, 1993), which has been implicated in selective attention.

Combined retrograde labelling and choline acelyltransferase immunohistochemical studies have established that 85-95% of brainstem afferents to most thalamic nuclei, which include specific relay, non-specific and reticular nuclei, originate in the rostral brainstem where pedunculopontine cholinergic nuclei and lateral dorsal tegmental nuclei are maximally developed (Bentivoglio, and Steriade, 1990). These inputs are excitatory and exert their effects both directly, via fast nicotinic receptor-mediated and slower muscarinic receptor-mediated depolarization, and also indirectly, via hyperpolarization of GABAergic (inhibitory) reticular neurons (McCormic et al., 1990). Co-activation of brainstem and basal-forebrain cholinergic neurons that project rostrally (occurring in both wakefulness and REM sleep) provides the thalamus

and cortex with a role in integrative modulation of distant neurons (synchronization) that could represent a component mechanism of conscious awareness.

### 1.6.1.2 Basic pharmacology of acetylcholine and clinical use of cholinomimetic agents

Release of ACh from a presynaptic terminal is dependent on an influx of calcium ions. After activating the postsynaptic receptor, ACh is enzymatically hydrolyzed by acetylcholinesterase (AChE) in the synaptic cleft into choline and acetate, terminating the action of the transmitter. Choline is then actively transported back into the presynaptic cell. Mitochondria near the terminal, through the action of acetyltransferase, are capable of recycling ACh at a rapid rate (Pappano, 1998; Dumitru and Gitter, 2002).

Indirect-acting cholinomimetic agents act by either irreversibly or reversibly inhibiting the action of AChE, increasing the concentration of endogenous ACh. Among the most commonly clinically used of AChE inhibitors are tacrine, donepezil, rivastigmine and galantamine. Unfortunately, these agents have also shown to have prominent effects on the cardiovascular and gastrointestinal systems, and the neuromuscular junction. Action of these agents on the cardiovascular system results in a modest bradycardia, fall in cardiac output, and vomiting. Dizziness, salivation, sweating, bloating, and fatigue are also reported. Longer-term side effects, such as depression and sleep disturbances, are also possible. Sudden withdrawal of the agent could lead to tardive dyskinesia (Mysiw and Clinchot, 1997; Drugdex, 2001-2002).

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*1.6.1.2.1 Tacrine*

In 1995, tacrine was approved in the United Stated for the treatment of Alzheimer's disease (Kurtz, 1998) and currently it is marketed under the name of Cognex® (FDA, 2005). Tacrine, an acridine derivative, is an indirect cholinomimetic agent that functions as a competitive, reversible, long-acting inhibitor of cholinesterase. Not only does tacrine act as a cholinesterase inhibitor, but it also blocks butyrylcholinesterase, may inhibit monoamine oxidase (MAO) and increase serotonin, dopamine, and gamma-aminobutyricacid (GABA) release (Soares and Gershon, 1995). Double-blind placebo-controlled clinical trials have yielded mixed results in regard to the efficacy of tacrine in improving cognition and slowing of disease progression in Alzheimer's dementia (Soares and Gershon, 1995). Two of the largest studies did reveal cognitive benefits in the tacrine treatment groups. However, a significant percentage of patients, especially those receiving higher doses of tacrine, withdrew because of intolerable hepatotoxicity and cholinergic side effects (Farlow et al., 1992; Knapp et al., 1994). Recently, new tacrine derivatives have been synthesized. It has been shown that these compounds inhibit AChE but also interfere with neuronal calcium overloading preventing apoptosis. It has been suggested that the neuroprotecting properties of some of these compounds could be useful in the treatment of neurodegenerative and ischaemic brain diseases (Villarroya et al., 2004).

*1.6.1.2.2 Donepezil*

Donepezil is currently marketed in the United States and in the United Kingdom mostly under the name of Aricept® (FDA, 2005; BNF, 2005) Donezepil, a piperidine derivative, is a central-acting, noncompetitive, reversible AChE inhibitor. Its mechanism of action, like other agents of its

class, is to enhance the central cholinergic activity by increasing the amount of endogenous ACh at the synaptic sites. Although the exact morphological sites of action are unknown, donepezil has been shown to have Anti-ChE activity in the cerebral cortex, hippocampus, striatum, and thalamus of the rat brain (Kasa et al., 2000).

Donepezil seems to lack the hepato-toxicity of other agents and displays greater specificity for brain tissue. Treatment with donepezil has been demonstrated to improve cognition in patients with moderate to severe Alzheimer's disease (e.g., Onder et al., 2005; Forchetti, 2005).

*1.6.1.2.3 Rivastigmine*

Rivastigmine is FDA approved for adults with cognitive disorders secondary to Alzheimer's-type dementia (Rosler et al., 1999). It is currently marketed in the United States and Great Britain under the name of Exelon[®] (FDA, 2005; BNF 2005). Rivastigmine is a reversible, carbamate AChE inhibitor unique for its specificity to the hippocampus and cerebral cortex (Enz et al., 1993). Its mechanism of action is similar to other agents in its class. Rivastigmine is unique, however, because of its preference for the G1 form of the AChE enzyme, which is found in high levels in the CNS of Alzheimer's disease patients (Drudgex, 2000-2001). The interaction of rivastigmine with the G1 surface receptor on AChE inhibits the enzyme's ability to hydrolyze ACh, thereby increasing ACh concentration in the CNS (Bourne, 1998). Rivastigmine has been shown in clinical trials to significantly inhibit both AChE and butyrylcholinesterase in the CNS, with minimal peripheral nervous system activity (Kennedy et al., 1999). In 1999, Rosler et al. published results of a large multicenter randomized, double-blind, placebo-controlled clinical trial of rivastigmine in mild to moderate Alzheimer's disease population. The study

followed patients for 26 weeks. Initial clinical response was found to take as long as 12 weeks to observe and commonly required doses of 6 mg/day or more. The trial demonstrated delay in progression of disease, improvement on cognition, global function, and participation in activities of daily living. Again, gastrointestinal side effects were the most common reason for patient withdrawal from these trials (Drudgex, 2000-2001). Acetylcholinesterase and butyrylcholinesterase inhibition occurs within 12 hours of dose administration.

### 1.6.1.2.4 Galantamine

Galantamine (or Galanthamine) is currently marketed as Razadyne® in the United States and as Reminyl® in the United Kingdom (FDA, 2005, BNF, 2005). Galantamine is a tertiary alkaloid originally derived from bulbs of the *Amaryllidaceae,* family of flowering plants that include daffodils and the common snowdrop (*Galanthea woronowii*). Galantamine reversibly and specifically binds to the active site of AChE, thereby inhibiting the enzyme that hydrolyzes ACh into acetate and choline (Thomsen and Kewitz, 1990).

Although the AChE inhibitor activity of galantamine appears weaker than that of donepezil or rivastigmine (Thomsen et al., 1991), its therapeutic effects on AD cognitive functions are at least comparable to the effects of these other agents (see Wilkinson et al., 2004 for a review). These observations suggest that the therapeutic effects of galantamine in AD are mediated by an additional mechanism. Such a potential other mechanism is the allosteric modulation of nicotinic cholinergic receptor (nAChR) activity (Albuquerque et al., 1997). An allosteric modulator interacts with a receptor at a binding site distinct from that which recognizes the natural agonist. Galantamine binds to an allosteric site on the nAChR and acts synergistically with acetylcholine to facilitate nAChR activation (Maelicke and Albuquerque, 2000). Because the concentration of nAChR is reduced in AD (Whitehouse et al., 1986; Perry et al, 1995) and short-

term nicotinic agonist administration improves cognition in AD (Sahakian and Jones, 1991; White and Levin 1999), the allosteric ligand potentiating effect of galantamine at nAChR is potentially therapeutically relevant. Given that an allosteric modulator is much less likely than a direct agonist to down-regulate nAChR, long-lasting therapeutic effects are possible.

### 1.6.1.3 Muscarinic agonists and antagonists in the treatment of the cholinergic dysfunction

A different cholinergic approach to AD has been the development of ligands endowed with an affinity for postsynaptic $M_1$ receptors (Avery et al., 1997). Stimulation of these receptors has been shown to have cognitively enhancing effects in animals. Although a substantial effort has been devoted to the development of these compounds, the promise of this approach has not been fully realized, in part because many of these drugs have shown modest selectivity for the $M_1$ receptor. This results in a variety of side effects, due to activation of $M_3$ receptors in the intestines, bladder and lung. Also, non-selective interactions with $M_4$ and $M_5$ receptors in the CNS have been reported. Moreover, it has been shown that poorly selective $M_1$ agonists also activate presynaptic $M_2$ autoreceptors, thereby reducing release of ACh (see Clader and Wang, 2005 for a review). Many of these compounds are derivatives of the natural product arecoline. Of these, xanomeline has comparable affinity for each of the muscarinic receptor subtypes but has been shown to be functionally selective for the $M_1$ receptor. Unfortunately, this did not translate into an acceptable safety margin in patients. In clinical trials, xanomeline improved cognition but was accompanied by significant dose-related cholinergic side effects (e.g., nausea and diarrhea) (Graul et al., 1996). One positive outcome from the clinical studies was the observation that xanomeline dose dependently

improved the behavioural disturbances and hallucinations often associated with AD (Mirza et al., 2003). This has led to the speculation that $M_1$ agonists might be useful as antipsychotic agents (Andersen et al., 2003). The advent of more sub-type selective agents now suggests that the antipsychotic effects may be due to antidopaminergic effects mediated via the $M_4$ receptor (Bohme et al., 2002).

Another compound called CI-1017 has shown comparable affinity for all five muscarinic receptors but functional selectivity for the $M_1$ receptor. Although selectivity for the $M_1$ receptor versus the $M_3$ receptor is still modest, CI-1017 shows virtually no agonist activity towards the $M_2$ receptor. In animal models, CI-1017 improved spatial memory after oral administration. Peripheral side effects such as salivation, presumably mediated via peripheral muscarinic receptors, were only reported at higher doses (Buckley, 2000). CI-1017 was scheduled to enter Phase II trials in 2000 (Tecle et al., 2000) but no development has been reported so far (Clader and Wang, 2005).

Talsaclidine (WAY 2014 FU) is another functionally selective $M_1$ agonist that has been studied both preclinically and clinically (Wienrich et al., 2002). Talsaclidine has shown comparable affinity for the $M_1$ and $M_3$ receptors but has 3-4 times less affinity for the $M_2$ receptor. In clinical trials, Talsaclidine induced dose-limiting side effects (salivation and sweating). However, its effects on cognition were not statistically significant and its development has been discontinued.

WAY-132983 has shown an affinity for $M_1$ receptors comparable to that of xanomeline, but it is 6-fold more potent. WAY-132983 has beneficial effects on learning and memory in both rodent and primate models but like xanomeline, it induces dose dependent side effects, especially salivation (Bartolomeo et al., 2000). No clinical development has been reported for this compound (Clader and Wang, 2005).

Cevimeline, also known as AF102B (Fisher et al., 1989), has shown to improve cognition in a variety of animal models but this promising profile was not followed by promising results in clinical trials (Fisher, 1996). There continue to be reports of new $M_1$ agonists (e.g., Spalding et al., 2002; see also Messer, 2002 for a review) but altogether the treatment of AD with $M_1$ agonists has shown to be of little use in clinical trials (Clader and Wang, 2005).

A third, so far, relatively less-popular cholinergic approach to AD has been the development of antagonists of the presynaptic $M_2$ autoreceptors (Doods 1995, Clader, 1999). Studies on animals have demonstrated that the blockade of these receptors at presynaptic terminals results in increased levels of ACh, and performance improvements in several behavioural tasks (Vannucchi et al., 1997; Quiron et al., 1995; Carey et al., 2001). Although a number of potent $M_2$ antagonists have been reported (Clader, 1999), only a few have shown selectivity versus other muscarinic receptor subtypes. The most interesting of these are AF-DX 116, AF-DX 384, BIBN 99 and SCH 57790 which produce release of ACh and improve learning ability in aged and cognitively-impaired rats after administration (Stillmann et al., 1996; Vannucchi et al., 1997; Quirion et al., 1995; Pike and Hammm, 1995; Doods, 1995; Carey, 2001).

However, recent sudies have shown that the complete blockade of $M_2$ receptors may interfere with hippocampal plasticity and certain cognitive tasks (Seeger et al., 2004). These authors reported that in $M_2$ receptor knock-out mice ($M_2^{-/-}$), normal acquisition, but not retention, in a spatial memory task was disrupted, suggesting that $M_2$ receptors are not essential for spatial memory retention. This finding is consistent with previous observations showing that scopolamine, a non-subtype-selective muscarinic antagonist, primarily interferes with the acquisition of new information rather than with memory retention in spatial memory tasks (Hagan et al., 1986; Decker et al., 1990;

Anagnostaras et al., 1995). Also, $M_2^{-/-}$ mice showed performance impairments in a version of T-maze and a disruption of neuronal plasticity (short-term potentiation, STP, and LTP) at the Schaffer collateral-CA1 synapse. The same group (Seeger et al., 2004) also found that perfusion of $M_2^{-/-}$ hippocampal slices with $GABA_A$ receptor antagonist bicuculline fully restored STP and significantly increased LTP. They suggested that a likely explanation for this finding is that presynaptic $M_2$ receptors mediate suppression of GABAergic ($GABA_A$ receptor mediated) inhibition of CA1 neurons in the normal ($M_2^{+/+}$) hippocampus. This concept is supported by previous observations indicating that $M_2$ receptors are present on GABA-containing nerve terminals throughout the hippocampus (Rouse et al., 1997; Hajos et al., 1998) and that activation of hippocampal muscarinic receptors inhibits GABA release from hippocampal interneurons (Ben Ari, et al., 1981; Beherends and ten Bruggencate, 1993).

## 1.6.2 Dopamine

The last decade has seen a large increase in the experimental evidence for a role of DA in memory processes. The cloning of five DA receptors and the development of more specific agonists and antagonists of the different DA receptors has helped in characterizing the role played by DA in cognition. In particular, a large number of different behavioural paradigms have been used to examine the role of DA in memory in mostly three brain regions: the striatum, the hippocampus and the prefrontal cortex (see Jay, 2003 for a review).

Studies on the striatum have been reviewed by Lovinger and Tyler in 1996 and an increasing amount of data have been published since then. The cognitive impairment shown by PD patients (e.g., Owen, 2004) demonstrates the interference of DA depletion in the striatum with both motor activity and learning processes. Searching for models to study memory disabilities related to

PD, some studies have investigated the effects of specific DA loss in the dorsal striatum. Studies in monkeys have shown that the disruption of dorsal striatal dopaminergic transmission induced performance deficits in delay-matching-to-sample, delayed alternation tasks and in a classical conditioning task (Roeltgen and Scneider, 1994; Aosaki et al., 1994). Attentional deficits, task impersistence and impairment in the cognitive component of object retrieval were also described in these animals (Schneider and Pope-Coleman, 1995). Mura and Feldon (2003) have shown that bilateral lesions of the nigro-striatal dopaminergic system disrupt the ability to select and maintain watermaze spatial navigation strategies learned preoperatively. Also, it has been shown that in the rat, the nigro-striatal dopaminergic system plays a crucial role in spatial/relational memory processes, working independently of the hippocampal memory system (Da Cunha et al., 2003). Recently, a study carried out on patients ranging from 34 to 81 years of age showed a disruption in striatal dopaminergic activity associated with loss of episodic memory (word and figure recall, face recognition) and executive functions (visual working memory, verbal fluency) in older individuals (Erixon et al., 2005).

A study carried out by Gasbarri et al. (1996) in the rat showed that neurotoxic lesions of mesohippocampal dopaminergic neurons, induced by bilateral injection of 6-OHDA (6-hydroxydopamine) in the dorsal and ventral hippocampus, impair retention in a watermaze task but not in a classical inhibitory avoidance test. Additional data coming from pharmacological manipulation of hippocampal DA receptors, showed that intrahippocampal injection of $D_1$ and $D_2$ agonists improves spatial working memory in the win-shift eight-arm radial maze (Packard and White, 1991). Since most hippocampal DA systems are located in the ventral part of the hippocampus (Verney et al., 1985; Gasbarri et al., 1994), Wilkerson and Levin (1999) have investigated the effects of $D_1$ and $D_2$ agonists and antagonists on a working memory task when

these compounds were injected in this region. While no significant effects of the $D_1$ agonist and antagonists were observed, treatment with the $D_2$ agonist quinpirole improved performance, whereas memory impairment was observed following treatment with the $D_2$ antagonist raclopride. More recently, performance disruption mediated by $D_2$ receptors in the ventral hippocampus has also been observed in an aversive motivated T-maze (Umegaki et al., 2001). An in vivo microdialysis study has shown that the subcutaneous injection of nicotine induces an increase of dopamine and its metabolites in the ventral and dorsal hippocampus, and in the prefrontal and medial temporal cortex. This effect was inhibited by the local administration of the nicotinic antagonist mecamylamine, the muscarinic antagonist atropine or the $D_1$ antagonist SCH 23390 (Rossi et al., 2005). This study gives evidence for a cholinergic regulation of the hippocampal dopaminergic system also suggesting the involvement of $D_1$ receptor in the dopaminergic transmission within the hippocampus. Recent studies on rodents have shown that bilateral intraprefrontal cortical infusions of the $D_1$ agonist SKF 81297 improves visual attention, and affects short-term working memory performance (Chudasama and Robbins, 2004). Also, Hotte et al., (2005) have suggested that there might be a general modulatory effect of $D_1$ activation on the retrieval of a previously acquired information whether the nature of the information is spatial, non-spatial or of temporal order. They proposed that the control of retrieval by $D_1$ receptors takes place in the prefrontal cortex since previous studies have shown that dopamine $D_1$ receptor activity within the prefrontal cortex can influence long-term memory storage in declarative memory tasks (Floresco and Phillips, 2001; Runyan and Dash, 2004). There is also evidence that the stimulation of $D_1$ receptor activity increases cholinergic transmission in the frontal cortex in the rat. In contrast, the blockade of $D_1$ receptor induces a decrease in ACh extracellular levels and reduces the facilitatory influence of $D_1$ receptor

stimulation (Gobert et al., 2003).

Together, this experimental evidence suggests that dopaminergic transmission in the brain plays a major role in the control of memory processes and that the activity of different dopaminergic receptor subclasses selectively affects learning. This information is crucial to develop specific pharmacological treatments of models of memory disruption.

## 1.6.3 Serotonin

Accumulating evidence suggests that serotonin may modulate cholinergic function in several regions of the mammalian brain and that these serotonergic/cholinergic interactions influence cognition. There is evidence that serotonergic neurotransmission plays an important role in cognition. For example, the $5\text{-}HT_3$ antagonist ondansteron has beneficial effects on several types of behavioural disturbances and in age associated memory impairment (Crook et al., 1991; Costall and Naylor, 1992) and Ro 04-6790, a $5\text{-}HT_6$ receptor antagonist, reverses scopolamine-induced cholinergic deficits in rodent models of recognition memory (Wooley et al., 2003). Moreover, serotonergic neurons degenerate in disorders associated with dementia (Curcio et al., 1984) and animals with serotonergic lesions show cognitive deficits in behavioural paradigms (Richardson et al., 1991). Moreover, a large number of studies have reported the presence of serotonergic terminals or serotonergic receptors in brain regions involved in cholinergic function.

With regard to the receptors that might be involved in the cholinergic and serotonergic modulation of learning and memory, a fair amount of attention has focused on muscarinic cholinergic and $5\text{-}HT_{1A}$ serotonergic subtypes, which are highly concentrated in limbic areas such as the neocortex and the hippocampus, but also in the raphe nuclei (Hoyer, et al., 1986; Pazos et al.,

1988). One often-used approach to studying these receptor subtypes is to determine the effects that selective pharmacological agents have on cognition.

In this regard, the muscarinic antagonist scopolamine and the $5\text{-HT}_{1A}$ full agonist 8-OH-DPAT have been extensively studied. Reikkinen and colleagues have demonstrated that administration of the combination of sub-amnesic (=not impairing memory) doses of scopolamine and 8-OH-DPAT, prior to training, produced impairments on acquisition in the Morris watermaze (Reikkinen et al., 1995) and on retention in passive avoidance (Riekkinen, 1994). Conversely, stimulation of $5\text{-HT}_{1A}$ receptors in the dorsal raphe has been shown to alleviate learning and memory impairments caused by intrahippocampal (Carli et al., 1998-2000) or systemic (Cole et al., 1994; Meneses and Hong, 1999) administration of scopolamine.

Evidence for the importance of serotonergic modulation of cholinergic activity also derives from other studies, showing that 8-OH-DPAT and the partial $5\text{-HT}_{1A}$ agonists buspirone, ipsapirone and MDL 73005 also increase cortical and/or hippocampal ACh release in freely moving animals (Bianchi et al., 1989; Siniscalchi et al., 1991, 1994; Wilkinson et al., 1996). In an "operant delayed matching to position" task, Cole et al. (1994) have shown that both a low dose of 8-OH-DPAT and the partial agonist ipsapirone (which enhanced performance when given alone) antagonized the effect of scopolamine. They suggested that this effect is due to a primary action at $5\text{-HT}_{1A}$ autoreceptors. Conversely, Barrett and Rowan (1992) observed that the co-administration of the muscarinic antagonist, atropine and the $5\text{-HT}_{1A}$ agonist ipsapirone (with no effect if given alone) impaired rat spatial orientation in the Morris watermaze.

As stated by Decker and McGaugh (1991), "While our understanding of the cholinergic system and its role in learning and memory is far from complete, we have progressed to the point where it is necessary to address the importance of the interplay of neurotransmitter systems in the modulation of memory

processes. These interactions are potential concerns for histological, electrophysiological, neurochemical, pharmacological and behavioural approaches".

## 1.7 Animal models in the study of cognitive functions

A great deal of invaluable information on memory functions has been provided by studies on animals whose normal ability to learn was impaired following the specific disruption of brain functions. In general, the goal of such research is to develop animal models of human memory and amnesia with the final goal of finding the relations between specific memory functions and brain structures. Several animal species, ranging from invertebrates to monkeys, have been used for investigation and comparison to human memory and functional neuroanatomy. However, even though phylogenetically close to humans, animals such as rodents, dogs and monkeys display significant differences in the organization of their nervous systems. Thus, the studies using animal models must be always linked to experimental investigation on humans in order to be valuable. Over the years, memory research has developed a large amount of animal models in order to investigate the link between specific symptoms of amnesia and memory systems in the brain. For example, it has been reported that patients with medial temporal lobe damage show anterograde amnesia (Corkin, 1984; Scoville and Milner, 1957). Research has sought to define the components of the medial temporal lobe that contribute to the amnesic syndrome. A large amount of studies in this area of research have been carried out using a recognition memory task performed by rhesus monkeys (Squire and Zola-Morgan, 1991). In a task called "delayed match-to sample task", monkeys are presented with an object on an information trial. After a variable delay, the original object is presented with a novel object, and a selection of the novel

object is rewarded. It has been shown that damage to cortical regions of the medial temporal lobe (perirhinal, enthorhinal, parahippocampal cortex) produces a significant delay-dependent deficit in this task (Meunier et al., 1993; Suzuki et al., 1993). However, it is clear that damage to the hippocampus alone produces a less severe impairment than the damage restricted to the cortical regions of the medial temporal lobe (Murray, 1996). Similar findings have been reported in rodents in a version of the same task. Damage to cortical structures associated with the hippocampal formation produces delay-dependent impairments that are not observed after selective damage to the hippocampus (Otto and Eichenbaum, 1992). The delayed match-to sample task is also used to assess recognition memory in aged monkeys (Moss et al., 1988; Presty, et al., 1987; Rapp and Amaral, 1989). Monkeys approximately 25 years or older show memory impairments with increasing delays (Rapp and Amaral, 1989). Also, monkeys with such deficits perform more poorly on rapidly learned two-object discrimination problems (Rapp et al., 1993). In rodents, ageing has shown to impair performance in spatial tasks (e.g., Rowe et al., 2003). After a preliminary screening in a behavioural task such as the watermaze task, animals that show learning deficits can be used to investigate the effects of a specific treatment (e.g., Quirion et al., 1995; Rowe et al., 1998; Rowe et al., 2003).

Another model of memory disruption is the Traumatic Brain injury method (TBI). The injury is induced by the injection of a small volume of saline in the cranial cavity. The injection produces a brief displacement and deformation of the brain (Dixon et al., 1987). Cognitive dysfunction can last from months to years following mild to moderate TBI (Brooks et al., 1987; Capruso and Levin, 1992; Oddy et al., 1995). Long-term cognitive impairment post-TBI ranging from days to 1 year has also been observed (Hamm et al., 1993; Hamm et al., 1992; Pierce et al., 1994; Lyeth et al., 1990). There is evidence suggesting that loss of central cholinergic tone may play a role in the

long-term cognitive deficits following brain injury. For instance a time-dependent decrease in central choline acetyl-transferase activity (Grady et al., 1992; Leonard et al., 1994) as well as decreased hippocampal acetylcholine esterase terminal density has been observed after lateral fluid percussion brain injury (Grady et al., 1992).

An approach to the study of memory dysfuntions due to disruption of cholinergic transmission in the brain is the use of transgenic animals. Anagnostaras et al. (2003) have shown that in mice with a null mutation of the $M_1$ muscarinic receptor subclass, LTP generation in response to theta burst stimulation in the hippocampus was reduced. They also found that these animals had working memory and memory consolidation deficits in a non-matching-to-sample behavioural task. Reduction of neuronal plasticity at the Schaffer collateral-CA1 synapse and performance impairments in hippocampus dependent spatial memory tasks have also been observed in $M_2$ muscarinic receptor knock-out mice (Seeger et al., 2004).

Selective memory impairments can be induced by pharmacological manipulation of neurotransmitter systems. The inactivation of a specific receptor subclass gives the opportunity to investigate its effects on neuronal activity and on learning in behavioural tasks. The most widely used model is based on the evidence that scopolamine, a muscarinic receptor antagonist, induces amnesia in young healthy subjects, comparable with that of old, untreated subjects (Drachman, and Leavitt, 1974). Scopolamine is a relatively non-selective cholinergic muscarinic receptor antagonist with ten times higher affinity for postsynaptic than for presynaptic receptors (Szerb et al., 1977). Acting at postsynaptic receptors, scopolamine blocks the neurotransmission of Ach at those receptors (Szerb et al., 1977). At presynaptic autoreceptors, scopolamine blocks feedback inhibition of Ach release (e.g., Szerb et al., 1977; Sethy and Francis, 1988). As a consequence of increased ACh release,

scopolamine (and more generally muscarinic antagonists) induces a decrease of intracellular Ach levels (Polack and Meeus. 1996; Sethy and Van Woert, 1973; Bymaster et al, 1985) and an increase of Ach levels in extracellular fluid, as measured by microdialysis (Damsma et al., 1987). An *in vitro* study by Billard et al. (1995) showed that scopolamine, although similar in affinity for $M_1$, $M_3$, and $M_4$ sites, has a 10-fold lesser affinity for $M_2$ receptors which, in the CNS appear predominantly located on presynaptic terminals of acetylcholine containing neurons (Levey, et al., 1995) (Table 1.2a). These data are confirmed by a study that compared the behavioural and *ex vivo* neurochemical effects of scopolamine (Bymaster et al., 1993). The results of this study suggest that scopolamine induces a decrease of hippocampal Ach levels and also memory deficits acting at both $M_1$ and $M_2$ receptors but binding more potently to $M_1$ sites (Table 1.2b). This evidence confirms the results of those behavioural studies according to which alterations in memory and learning are mediated primarily via receptors of the muscarinic $M_1$ subtype (Hunter and Roberts, 1988; Messer et al., 1990).

In the rat, *in vivo* intraperitoneal administration of scopolamine has been shown to disrupt cholinergic transmission in the brain up to 3 hours from administration (Dimpfel, 2005). Moreover, it has been shown that, after sub-cutaneous injection, scopolamine distributes freely into the brain and, after 30 minutes, the percent of the administered dose measured is 2.7%. These data were obtained from an *ex vivo* analysis of scopolamine concentration in the brain of rats that showed memory deficits in a spatial task, tested 30 minutes after injection of the drug for a maximum of 60 minutes (Bymaster et al., 1993).

**a**

| Receptor subclass | $K_i$ (nM±SEM) |
|---|---|
| $M_1$ | 0.085±0.02 |
| $M_2$ | 0.088±0.07 |
| $M_3$ | 0.063±0.004 |
| $M_4$ | 0.1±0.01 |

**b**

| PZ $ED_{50}$ (mg/Kg) | QNB $ED_{50}$ (mg/Kg) | Behaviour MED (mg/Kg) | Brain Penetr. (%) | Ach $ED_{30}$ (mg/Kg) |
|---|---|---|---|---|
| 0.05 | 0.02 | 0.03 | 2.7 | 0.04 |

**Table 1.2 (a-b)**

a) *In vitro* binding affinities of scopolamine for muscarinic receptor subtypes in the rat. $Ki=IC_{50}/(1L/K_d)$, where $IC_{50}$ is the concentration causing 50% inhibition of a specific radioligand binding, L is the concentration of the radioligand used and $K_d$ is the dissociation constant of the radioligand-receptor complex (modified from Billard et al., 1995);

b) Comparison of *ex vivo* binding to $M_1$ and $M_2$ receptors and the disruption of a spatial task performance induced by scopolamine in the rat brain. Scopolamine penetration into the brain and its binding potency with $M_1$ and $M_2$ receptors, are expressed as its ability to inhibit binding of the muscarinic antagonists pirenzepine (PZ) and 3-quinuclidinyl benzilate (QNB) to $M_1$ receptors in the cortex and to $M_2$ receptors in the brain stem, respectively. Moreover, the ability of scopolamine to induce a reduction of Ach levels in the hippocampus is given. Abbreviations: PZ $ED_{50}$=dose of scopolamine required to inhibit pirenzepine binding by 50%; QNB $ED_{50}$=dose of scopolamine required to inhibit QNB binding by 50%; MED= minimal effective dose required to disrupt performance in a spatial task. ACh $ED_{30}$ = dose of scopolamine required to induce a decrease of Ach levels in the hippocampus by 30% (modified after Bymaster et al., 1993).

It has been shown that *in vivo* scopolamine administration inhibits LTP induction in the hippocampus (Ovsepian, 2004) and disrupts normal performance in several behavioural tasks such as the watermaze, object recognition, passive avoidance and radial-arm maze (eg., Whishaw et al., 1985, 1988; Levin et al., 1989; Paylor et al., 1990; Quiron et al., 1995; Vannucchi et al., 1997; Levin and Rose, 1991; Steele et al., 1997; Buckton et al., 2001) also increasing open field exploratory activity (e.g., Abood and Biel, 1962; Bushnell, 1987).

Finally, it has been proposed that exposure to carbon monoxide (CO) can provide an amnesic model for the investigation of memory deterioration, especially for progressive memory dysfunction (Hiramatsu et al., 1996). In humans, CO has been reported to cause memory deficits developing insidiously over the days following recovery from CO intoxication (Ginsberg et al., 1979). In mice, delayed neuronal damage (Ishimaru et al., 1991) can also be produced after CO exposure and deficiencies in learning and memory occur if exposure to CO takes place before training (Hiramatsu et al., 1994; Nabeshima et al, 1990-1991). This memory deficiency develops in a delayed manner, more than 3 days after CO exposure (delayed amnesia) (Hiramatsu et al., 1996; Nabeshima et al, 1991). Hiramatsu and Inoue (2000) showed that mice exposed to CO exhibit spontaneous alternation performance and passive avoidance deficits due to degeneration of cholinergic neurons of the frontal cortex, striatum and hippocampus. The association between the cholinergic system disruption and the memory deficits induced by exposure to CO finds support in those studies showing that CO-induced amnesia is improved after administration of nicotine, an acetylcholine receptor agonist (Hiramatsu et al., 1992-1994; Nabeshima et al., 1991). Similar results have been observed after administration of nootropics such as nefiracetam and NIK-247, which have been reported to facilitate cholinergic neuronal systems (Sarter, 1991). Furthermore, Hiramatsu et al.,

(1996) suggested that the disruption of the cholinergic system, induced by exposure to CO, might be mediated by the κ-opioidergic system. They showed that U-5048H, a selective κ-opioid receptor agonist, improved CO-induced delayed amnesia in mice, but its effects were antagonized by a low dose of scopolamine. These considerations are also supported by those studies showing that the κ-opioid agonist dynorphin A-(1-13) improved the scopolamine-induced impairment in spontaneous alternation performance (Ithoh et al., 1993) and its effects were antagonized by nor-binaltrophine, a κ-opioid receptor antagonist (Hiramatsu et al., 1995; Itoh et al., 1993). These experimental data suggest that learning and memory disruption following exposure to CO could provide a useful model to investigate the role of cholinergic receptors in the modulation of cognitive functions.

## 1.8 Aims

The goal of the present research was to investigate the role of cholinergic, dopaminergic and serotonergic systems in the modulation of cognitive functions. In particular, on the basis of the data showing that cholinergic muscarinic type-2 ($M_2$) antagonism can facilitate cholinergic transmission in the brain and improve cognition (e.g., Vannucchi et al., 1997; Quirion, 1995), the effects of two $M_2$ antagonists, AFDX 384 and BIBN 99, were evaluated in a variety of behavioural paradigms. Also, it was of interest to investigate the efficacy of these drugs in attenuating the effects of the non-selective muscarinic antagonist scopolamine in the same behaviourals tasks.

As several studies suggest that the dopaminergic system plays a role in the modulation of cognitive functions (e.g., Levin and Rose, 1991; Steele et al., 1997), attention was drawn to the $D_1$ receptor agonists, SKF 38393 and A 68930, and the $D_1$ antagonist SCH 23390. The partial agonist properties of SKF 38393 (Lovenberg et al., 1989; Mottola, et al., 1992) and the full $D_1$ agonism of A 68930 (De Ninno et al., 1991) were of interest in order to provide a more complete description of the effects of $D_1$ receptor stimulation on memory and exploration. In particular, given the evidence that dopamine ligands induce the release of acetylcholine in the brain (Steele et al., 1997) the effects of SKF 38393 and A 68930 were evaluated when these drugs were given alone or in combination with scopolamine.

Cholinergic functions have also been reported to be influenced by the activity of the serotonergic system (see Steckler and Sahgal, 1995 for review). In particular, there is evidence that $5-HT_{1A}$ receptors play a crucial role in the modulation of cholinergic transmission in the brain and of cognitive functions (Bianchi et al., 1989; Siniscalchi et al., 1991, 1994; Wilkinson et al., 1996). For this reason, a sub-amnesic (with no effect on learning and memory in the tasks

used) dose of the 5-HT$_{1A}$ receptor partial agonist ipsapirone was administred in combination with a sub-amnesic dose of scopolamine in order to evaluate the possible synergistic effects of these two drugs on spatial navigation and exploratory activity.

Finally, given the experimental data suggesting that carbon monoxide gas (CO) exposure can provide a useful model to investigate the role of cholinergic receptors in the modulation of cognitive functions (Hiramatsu et al., 1992-1995-1996; Itoh et al., 1993; Nabeshima et al., 1991), the effects of exposure to a low level of CO, combined with a sub-amnesic dose of scopolamine, were investigated.

# CHAPTER 2

## 2.1 Materials and Methods

### *2.1.1 Drugs*

**Scopolamine hydrobromide** (TOCRIS, UK), dissolved in water for injection.

**AFDX 384** (5,11-dihydro-11-[[(2-[2-[(dipropylamino) methyl]-1-piperidinyl] ethyl) amino] carbonyl]-6H-pyrido(2,3-b)(1,4)-benzodiazepine-6-one), Boeringer Ingelheim (Germany), dissolved in water for injection.

**A 68930** [(1*R*,3*S*)-1-aminomethyl-5,6-dihydroxy-3 phenylisochroman HCl], (TOCRIS, UK), dissolved in saline.

**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}, Boeringer Ingelheim (Germany), dissolved in saline.

**CO** (Carbon Monoxide), (BOC, special gases, UK) given pure.

**Ipsapirone** (TOCRIS, UK), dissolved in water for injection.

**SCH 23390**{4-cyclohexyl-[4-[[4-methoxyphenyl]sulfmyl]-phenyl]-1-piperazineacetonitrile}, (TOCRIS, UK), dissolved in saline.

**SKF 38393** [(±)-phenyl-2, 3, 4, 5-tetrahydro- 1*H* -3-benzazepine-7,8-diol], (TOCRIS, UK), dissolved in saline.

## 2.1.2 Animals

Male Wistar rats (Bioresources Unit, Trinity College Dublin), 6 to 7 weeks old and weighing 200-250 g at the beginning of the experiments, were used.

When not being tested, the animals were housed in plastic cages (2 animals/cage; cage dimensions: 15x40x25cm), located in a regulated environment (20±2 °C, 45-50% humidity, 50 mmHg pressure), under a 12 hr light/dark cycle (lights on at 8:00 a.m.), with free access to laboratory chow and water. Experiments were carried out between 9:00 am and 6:00 p m, in a sound-attenuated and air-regulated experimental room. To minimize inter-group differences due to different times of testing within the experimental time window, treatment groups and controls were tested in all behavioural tasks in a random order.

## 2.2 Behavioural tasks

### 2.2.1 Morris watermaze

#### 2.2.1.1 Introduction

Since the beginning of the 20th century, the study of learning and memory has been carried out using behavioural tasks in which the animals must escape from water in order to complete trials (Glaser, 1910; Wever, 1932). These tasks have been employed over the years in the investigation of the effects of aging, experimental lesions, and drug treatments, especially in rodents.

In particular, for two decades, the Morris watermaze (Morris, 1984) has been the task most extensively used and accepted by behavioural psychologists and pharmacologists (a cursory search on PubMed revealed 3415 "hits", from 1984 to 2006, when the keywords "watermaze" and "memory" were entered

together). The spatial version of the watermaze is one of the most widely used learning and memory tests for rodents (see McDonald et al., 2004 for a review). In this version, an animal must localize and use extramaze visual cues in order to successfully navigate in a tank filled with water. The memorization of the position of the visual cues is crucial for the animal in order to find a platform hidden under the water (Morris, 1984; McNamara and Skelton, 1993). Several studies have shown the usefulness of this behavioural task as a method of investigation in the study of neurodegenerative diseases, such as AD, which feature cognitive decline. In particular, it has been found that:

1) Performance of the watermaze is impaired following the disruption of forebrain cholinergic systems activity. This disruption correlates well with the degree of dementia in AD (Perry et al., 1999; Francis et al., 1999).

2) The hippocampus is an essential structure for place learning (McDonald and White, 1995) and in patients with AD, incidentally, the hippocampus is atrophic (Terry and Katzman, 1983).

3) Anticholinergic agents (e.g., scopolamine) that are commonly used to impair performance in the watermaze also disrupt memory in healthy humans and worsen dementia in patients with AD (Sunderland et al., 1988; Ebert and Kirch, 1998).

4) Finally, in the watermaze, navigation is impaired if an animal cannot memorize the location of extramaze cues. Similarly, AD patients show a poor ability to recognize an environment on the basis of its visual features. Visuospatial, visuoperceptual deficits and topographic disorientation are detectable very early in the course of AD and become more pronounced as the disease progresses (Eslinger and Benton, 1983; Morris et al., 1991). The common observation of spatial and visual agnosia in AD patients also indicates the disruption of complex processes, which involve both visual pathways and mnemonic processing (Henderson et al., 1989; Mendez et

60

al., 1990). Further, when compared with other behavioural paradigms, the use of the watermaze offers a number of advantages:

1) Animals need no pre-training period and can learn the task in a short period of time. For example, normal (unimpaired) rats can learn the task in 10 to 20 trials, after just a few days of testing.

2) At the end of the last day of training it is possible to run probe tests (also called transfer trials), in order to test learning as a retrieval process (spatial bias).

3) Olfactory trails or cues are eliminated.

4) Through the use of video tracking devices and the measure of swim speed, non-mnemonic behaviours or strategies (i.e., taxon, praxis, thygmotaxis) can be delineated and motoric or motivational deficits identified.

5) It is possible to run visible platform tests in order to identify motor-visual deficits following treatments.

6) By changing the platform location, both learning and re-learning experiments can be accomplished.

7) While immersion into water may be somewhat unpleasant, more aversive procedures such as food deprivation or exposure to electric shock are circumvented.

8) The use of curtains, partitions etc., reduces distractions that might affect behaviour.

9) Finally, the watermaze is quite easy to set up in a relatively small laboratory, is comparatively less expensive to operate than many other types of behavioural tasks, and is easy to master by research and technical personnel.

The use of the watermaze task has been found to be quite useful in drug development studies for screening compounds for potential cognitive

enhancing effects (Terry et al., 1999), as well as investigating deleterious effects of neurotoxicants on cognition (Pendergast, 1997).

### 2.2.1.2 *Experimental procedure*

In this test the animals were required to find a submerged (1 cm) perspex platform (14x10x1.5 cm; height: 20 cm) in a black pool (diameter: 200 cm, height: 50 cm, Fig. 2.1) filled with water (water level: 21 cm), within 120s. If an animal found and climbed on the platform, it was left standing on it for 10s and then placed in a bucket for approximately 1min. Rats were given 4 trials a day for five consecutive days (in this thesis, these trials are referred to as *acquisition trials* or simply *acquisition*). During this period, the time taken to find the platform *(escape latency)* was recorded and used to plot a learning curve. If a rat did not find a platform within 120s this value was assigned as latency.

On the fifth day, the animals were run on probe tests after 3 hrs from the last acquisition trial. The interval between the last acquisition trial and the probe test was not meant to assess the acute effect of treatments on learning but to evaluate their effects on consolidation memory. In probe tests, the platform was removed and the animals were allowed to swim for a 60s. A latency of 60s was assigned to rats that did not reach the platform location within the trial duration. The experiments with A 68930 and SCH 23390 (shown in Chapter 5) were part of a study carried out separately from the experiments with SKF 38393 (also shown in Chapter 5), in collaboration with an other experimenter who had assessed watermaze control learning running acquisition trials lasting 90s instead of the usual 120s, and probe tests immediately after the last trial of the 5th day of training (for this reason, the statistical analyses of this part of the

study were carried out separately from those of the experiments with SKF 38393).

Values measured were: *"escape latency"* (time to get to the location where the platform had been positioned during acquisition trials, *"percentage of total time in spent in the target quadrant"*, *"percentage of total time in spent in the quadrant opposite to the target quadrant"* (Abbr. *"opposite"*), *"percentage of total time spent in the periphery of the tank"* (this measure appears only in the studies shown in Chapters 3, 4, 5, as it was introduced after completion of the studies shown in Chapters 6 and 7), *"quadrant bias"* (percentage of total trial time spent in the target quadrant vs percentage of total trial time time spent in the opposite quadrant), *"mean swim speed"*, and *"total distance travelled"*. The latter was used as a measure of efficiency of search strategy (Spowart-Manning and van der Staay, 2005)

The room (width: 270cm; length: 350cm; height: 310cm was lit with 4 fluorescent bulbs (length: 123cm) located on two opposite walls at approximately 2.5 m from the floor. The apparatus used to carry out recordings consisted of a computer (located outside the room) connected to a camera placed on the ceiling above the pool (distance from the surface of the water: 218 cm). The software package used to track the animals and to record behavioural activity and to analyze data was *Ethovision*, Version 3.0, Noldus. Recording started automatically as soon the animal was placed in the pool and stopped 3s after the rat reached and climbed on the platform. During acquisition trials, escape latencies were calculated by *Ethovision* by subtracting 3s from the total time spent by the rat to reach the platform. If an animal did not reach the platform and dwell on it for at least 3s before the end of trial, *Ethovision* automatically stopped recording as soon as the trial duration time elapsed. In probe tests, as the platform was removed, *Ethovision* calculated the time spent

by the rat to reach the platform location and the time spent in each selected area. Recording stopped automatically after 60s.

### 2.2.1.3 Analysis of data and statistics

A "Repeated Measures Analysis of Variance Between Groups" test was carried out on acquisition mean escape latency values from all of the groups in a given study. The data was log-transformed ($\log_{10}$) to ensure a normal distribution (Winer, 1971). Also, the distribution of the residuals of each log-transformed value (residuals are defined as "observable estimates of the unobservable errors" for each measure of the sample) was analysed using a "Shapiro –Wilk test for normality". A normal distribution of residual ensured that the log transformation of mean escape latency values was fully effective. It was found that, in all studies, residuals were always distributed normally. Also a t-test was carried out in order to evaluate inter-group differences. A day by group comparison (day/group) was also carried out in order to reveal day/group interactions (difference between groups changing with day). For each single treatment group, learning during acquisition was evaluated through a "Repeated Measures Analysis of Variance Within Groups" test, carried out on acquisition escape latency values averaged across trials for each day.

The preference for the target quadrant during probe tests (target quadrant bias) was evaluated using a within group "paired t-test", which compared the amount of time spent in the target quadrant with the amount of time spent in the opposite quadrant. In Chapter 5, probe test latency values with no standard error of the mean (in probe tests none of the animals of group Scop+SKF 38393 found the platform within 60 seconds) were compared to other latency values using a Wilcoxon/Kruskall-Wallis test.
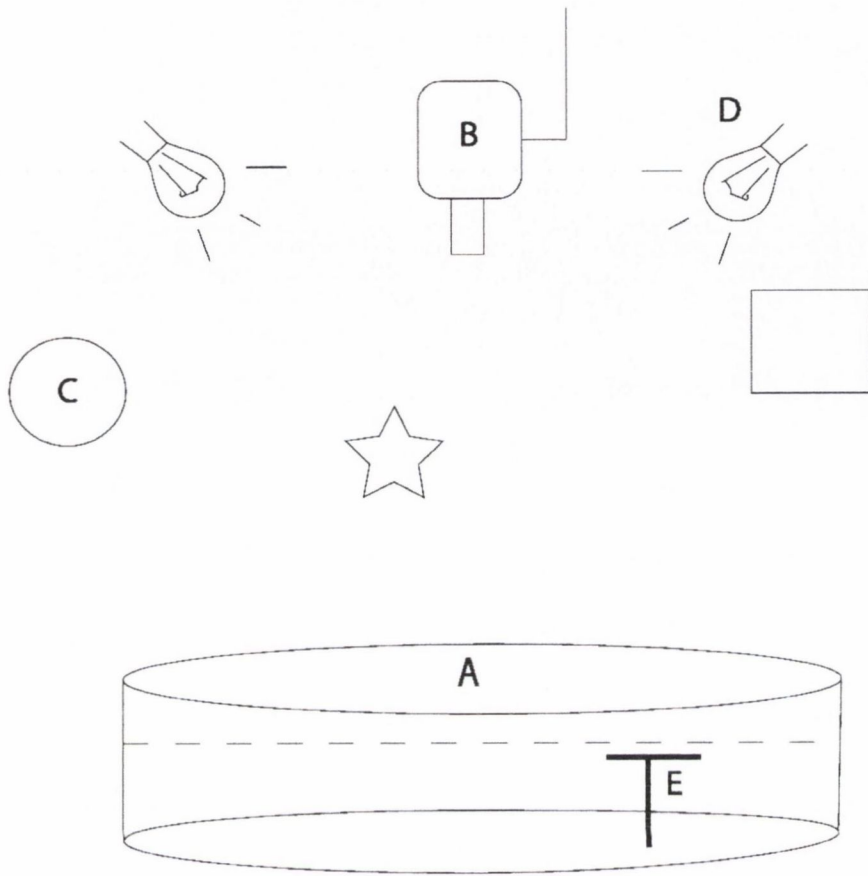
**Fig. 2.1** Diagrammatic illustration of the Morris watermaze testing room and apparatus. A) tank; B) camera; C) visual cue; D) light; E) submerged platform.

## 2.2.2 Hole board test

### 2.2.2.1 Introduction: neurotransmitter modulation of locomotor and exploratory activity in the open field

It is well established that complete lesions of the hippocampal formation produce a marked increase in spontaneous locomotor activity (Bannerman et al., 1999; Douglas et al., 1964). It has been suggested (Bender et al., 1968; Bannerman et al., 1999) that the dorsal hippocampus could underlie much, if not all, of the hyperactivity induced by complete lesions of the hippocampus in the open field. This may in turn reflect an effect of the lesion on exploration rather than emotionality, and may be related to the spatial learning deficits observed with dorsal hippocampus damage (Moser et al., 1995). Also, several lines of evidence suggest that cholinergic disruption may produce hyperactivity through direct or indirect interactions with dopaminergic systems. For example, interactions between the dopaminergic and cholinergic systems in the striatum are well documented. Nigro-striatal dopaminergic cells synapse onto striatal cholinergic interneurons, and dopamine decreases both the firing rate of cholinergic interneurons and the release of acetylcholine (e.g., Stoof et al., 1979; Hertting, 1980).

The repeated exposure of an animal to the open field allows one to evaluate habituation to an environment, which is believed to be one of the most elementary forms of learning both in animals and humans. Habituation is defined as a response decrement with repeated or continous presentation of indifferent stimuli, which is not dependent on muscle fatigue or receptor adaptation (Thompson and Spencer, 1966). In rodents, habituation is often analysed in terms of exploration of an environment. This leads to pronounced behavioural activation when the animal is exposed to a novel environment, and to decreased behavioural activity when the environment becomes familiar (Cerbone, 1994; Sadile 1979). Although methodologically rather simple to

evaluate, habituation of exploratory behaviour is a complex process. On the one hand, it involves responses to novelty, including arousal, emotionality, and stress-related factors, and, on the other hand, a diminished response due to familiarity, which requires learning-related processes and recognition or recall (Dai et al., 1995; Gerhardt et al., 1993). Open field exploration and its habituation are closely related to the hippocampus and its cholinergic input (Carlton 1968; Gray and McNaughton, 1983), which is provided by neurons located in the medial septum. It has been shown that exploratory behaviour is associated with hippocampal theta activity (Wishaw and Vanderwolf, 1973), which, in turn, is dependent on the cholinergic input.

It has been proposed that specific cells located in the hippocampus, termed "place cells", contribute to create a spatial map of an environment (O'Keefe and Nadel, 1978) selectively firing when an animal is in a specific location (O'Keefe and Dostrovsky, 1971). This has suggested that place cells code spatial locations in the environment, giving anatomical support to the hypothesis that the hippocampus is crucial for spatial learning (e.g., Anderson and Jeffery, 2003; Hayman, et al., 2003; Mizumori et al., 1999; Nadel and Wilner, 1980). Also, exposure to an open field leads to pronounced expression of immediate early gene products in hippocampal neurons (Papa, 1993). On the other hand, lesions of the hippocampus or pharmacological blockade of its cholinergic input strongly affect exploratory behaviour and its habituation (Poucet, 1989).

## 2.2.2.2 *Experimental procedure*

The apparatus design (Fig. 2.2), validated by File (1975), was derived from the hole board introduced by Boissier and Simon (1962). This apparatus can be thought as an open field with some modifications aimed at increasing the motivation of an animal to explore. The hole board consisted of a wooden box 60x60x43 cm, in which the inside walls and floor were painted white. The

bottom of the box had 4 circular holes, each 4 cm in diameter, whose centre was located 18 cm away from each of two adjacent walls. A grid was also drawn on the floor. The experiments were carried out in a dimly lit room (width: 270cm; length 350; height: 230) with only one bulb light pointing at the wall opposite the maze location. Rats were kept in the room for at least 30 mins before the first trial.

The behavioural measurements recorded during experimental sessions were: "*lines crossed*", *head dips*"(head dip= nose poking at the holes) and "*rears*". All measurements were carried out using manual clickers. Each rat was placed in one of the corners facing the wall. The experiment consisted of two halves, each one lasting 3 min, run at an interval of 3 min from each other. The surface of the hole board was cleaned with tap water every time a new rat was placed in the apparatus but not between the first and the second half of the experiment. The analysis of the activity displayed by the animals during each of the two halves of the experiment was used for inter-group comparisons, whereas differences between the first and the second half were used to evaluate the level of habituation within the same treatment group. The hole board experiments associated with the watermaze studies were carried out three days after the watermaze study was completed, to allow the animals to recover from testing. This procedure was also designed to allow a washout time for the drug that had been administered during the 5 days of testing in the watermaze task, thus increasing the likelihood that any changes in behaviour in the hole board were due to an acute treatment effect (treatments were always administered 30 min before starting the first half of the experiment).
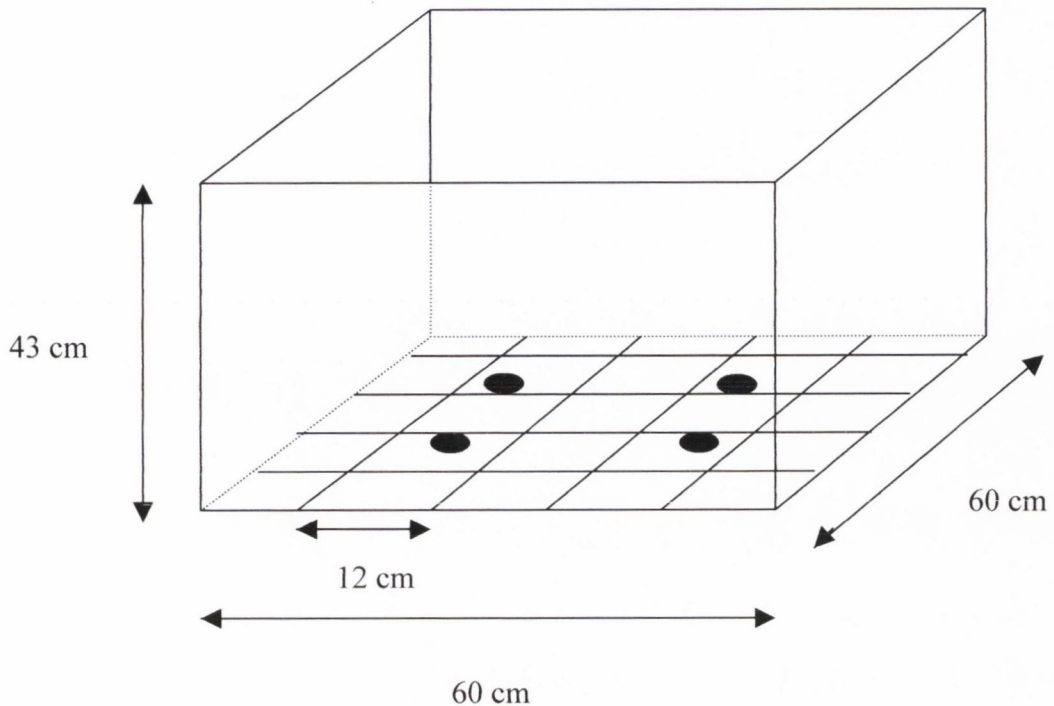
**Fig. 2.2** The hole board apparatus

## 2.2.2.3 Analysis of data and statistics

An Analysis of Variance (ANOVA) was carried out to compare the activity of all treatment groups during both the first and the second half of the experiment. Subsequently, a t-test was used to carry out inter-group comparisons. A within-group Wilcoxon signed-rank test was used to compare the activity of the first half of the experiment to the activity of the second half. The Wilcoxon signed-rank test is a conservative method that is robust if the distribution of the values is not normal. It was chosen as it was assumed that the measurements carried out in two separate halves of the experiment did not necessarily have the same distribution. However, the P values obtained using the Wilcoxon signed-rank test always agreed with the P values obtained using a

t-test. A significant reduction in activity, during the second half of the experiment, was interpreted as habituation to the environment.

### 2.2.3 T-maze

#### 2.2.3.1 Introduction

Spontaneous alternation is a measure of exploratory behaviour, most often evaluated in rodents (Tolman, 1925). Gerlai (Gerlai, 1998, 2001) developed a T-maze continuous alternation task (T-CAT) to assess spatial exploratory performance in mice. Spontaneous alternation is defined as a visit to a different goal arm of the T-maze (Fig. 2.3) to that visited in the previous trial.

In order to alternate or avoid a revisit, an animal must remember the goal arm chosen in the previous trial. The information about the previous visit is stored in the spatial working memory (Olton et al., 1976). The T-CAT has been used to assess the effects of putative cognition enhancing and cognition impairing experimental manipulations. In a series of experiments, Gerlai showed that this task appears to depend upon normal functioning of the hippocampus, is guided by extra-maze cues, and is sensitive to strain differences (Gerlai, 1998).

Drugs that have been shown to decrease cholinergic transmission have been used to investigate the role of acetylcholine in regulating spontaneous alternation. For example, the alternation rate is decreased following scopolamine administration to rats (Egger 1973; Meyers and Domino, 1964) and mice (Anisman, 1975). Impairment in spontaneous alternation is also observed with the administration of pirenzepine, an $M_1$ muscarinic cholinergic receptor antagonist (Ukai et al., 1995). Contrary to the effects of cholinergic receptor antagonists, physostigmine, a cholinesterase inhibitor, has been shown to increase spontaneous alternation rates (Egger, 1973).

## 2.2.3.2 *Experimental procedure(only for the experiments shown in Chapters 3, 4 and 5)*

The T-maze apparatus (modified after Gerlai, 1998 and Spowart-Manning and Staay, 2004) consisted of three arms made of transparent perspex (start arm: length=60 cm, width=16.5 cm; goal arms: length: 50 cm, width=16.5 cm, maze height 30 cm). The maze was equipped with three black guillotine doors and had sawdust on the floor. In order to reduce olfactory cues that could distract rats from exploring the environment, urine and faeces were removed after each session and the sawdust was mixed every time a new rat was placed in the apparatus but not between trials. Also, the experiments were carried out in the same room where the hole board experiments took place (see section 2.2.2.2 for details on the room dimensions and lighting conditions). Some visual cues (objects) were positioned at approximately 30-40 cm from the maze in order to add variety to the environment and stimulate exploratory behaviour. As this apparatus was designed to measure spontaneous alternation, no additional cue was included. The evaluation of the effects of treatments on T-maze spontaneous alternation was introduced only after completion of the studies shown in Chapter 6 and 7. For this reason, in these chapters, no experimental data relative to the behaviour of animals in the T-maze task have been reported.
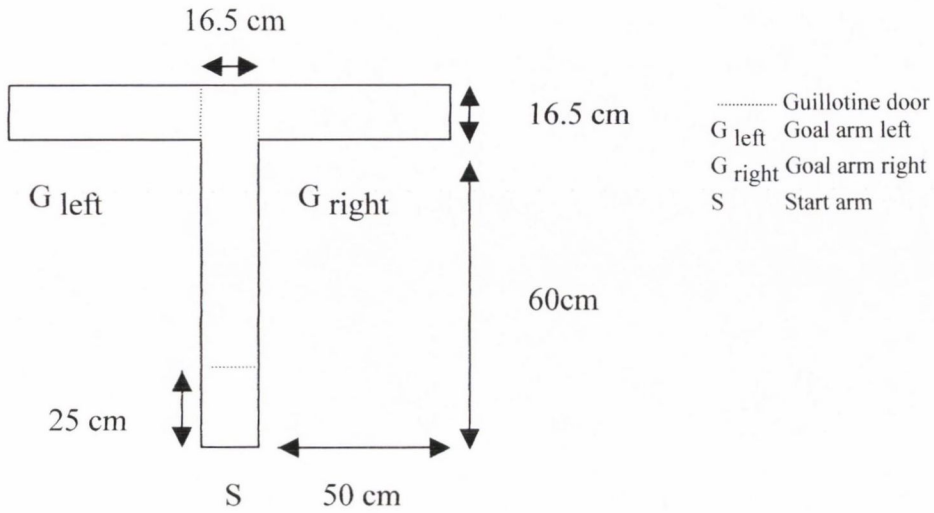
**Fig. 2.3.** The T-maze apparatus

Training of a rat consisted of one single session, which started with one forced-choice trial, followed by 14 free-choice trials.

Forced-choice trial: in the first, trial, the "forced trial", lowering the guillotine door blocked the left or right goal arm. After the animal was released from the start arm, it would negotiate the maze, eventually entering the open goal arm, and returning to the start position. Once there, the animal was confined for 5 seconds by lowering the guillotine door of the start arm.

Free choice trials: during the 14 "free-choice" trials, the animal could choose freely between the left and right goal arm. After opening the guillotine door of the start arm, the animal was free to choose between both goal arms (all guillotine-doors were open). As soon as the rat entered one goal arm, the other goal arm was closed. The animal eventually returned to the start arm, and the next free-choice trial started after a 5-second confinement in the start arm. A session was terminated and the animal was removed from the maze as soon as

14-free choice trials had been performed or 30 minutes had elapsed, whatever event occurred first. During the session, the experimenter never handled the animals.

### 2.2.3.3 *Analysis of data and statistics*

The data involving animals that completed less than 8 free-choice trials within 30 minutes was excluded from further analyses. The percent alternation, out of 14 trials, was calculated with the formula: (alternation/14)*100. An analysis of variance (ANOVA) was carried out on the alternation percentage of all treatment groups to evaluate statistical differences. Subsequently, a t-test was used to evaluate statistical differences for inter-group comparisons.

## *2.3* **Exposure to Carbon Monoxide**

### 2.3.1 *Exposure chamber*

The exposure chamber was a hermetically sealed cabinet connected to a pipe feeding into it (Table 2.1 and Fig.2.4). A cylinder equipped with a pressure regulator (BOC Special Gases, UK), supplied Carbon Monoxide (CO) to the chamber. A gas detector (Polytron 2, Dräger, UK) was connected to a sensor located inside the chamber. Also, a portable detector (microPac Plus, Dräger, UK) was used to monitor CO concentration outside the exposure chamber and in the experimental room. An air extractor (whose speed could be doubled in case of an overflow of gas into the exposure chamber) provided a continuous airflow from the inside of the exposure chamber to a filter located above.

Reading the display of the gas detector connected to the sensor located inside the chamber, the experimenter monitored gas concentration and regulated the gas flow through adjustments of the knob located in the regulator. At the beginning of the experiment, the concentration of CO inside the exposure chamber was 0ppm and the composition of air was uniform to that of the experimental room (in which the oxygen percent ratio was approximately 20% of total gas composition). Through slow adjustments of the regulator, CO was fed into the exposure chamber, until its concentration reached 2400ppm. If CO concentration inside the chamber increased beyond 2640ppm, the experimenter activated the air extractor provided, until the gas concentration fell within range (0 to2640ppm).

The outer unit, which contained the exposure chamber, was equipped with a special filter/catalyst (Hopkalite) to remove CO from the air promoting the oxidation of CO into $CO_2$. $CO_2$ was exhausted out of the laboratory through a pipe connected to the filter. The exposure chamber, the air extractor and the outer unit were provided by Euro Aire®, Spain.

| | Width (cm) | Height (cm) | Depth (cm) |
|---|---|---|---|
| **Outer unit** | 80 | 100 | 70 |
| **Exposure chamber** | 76 | 56 | 61.5 |

Table 2.1 Outer unit and exposure chamber dimensions
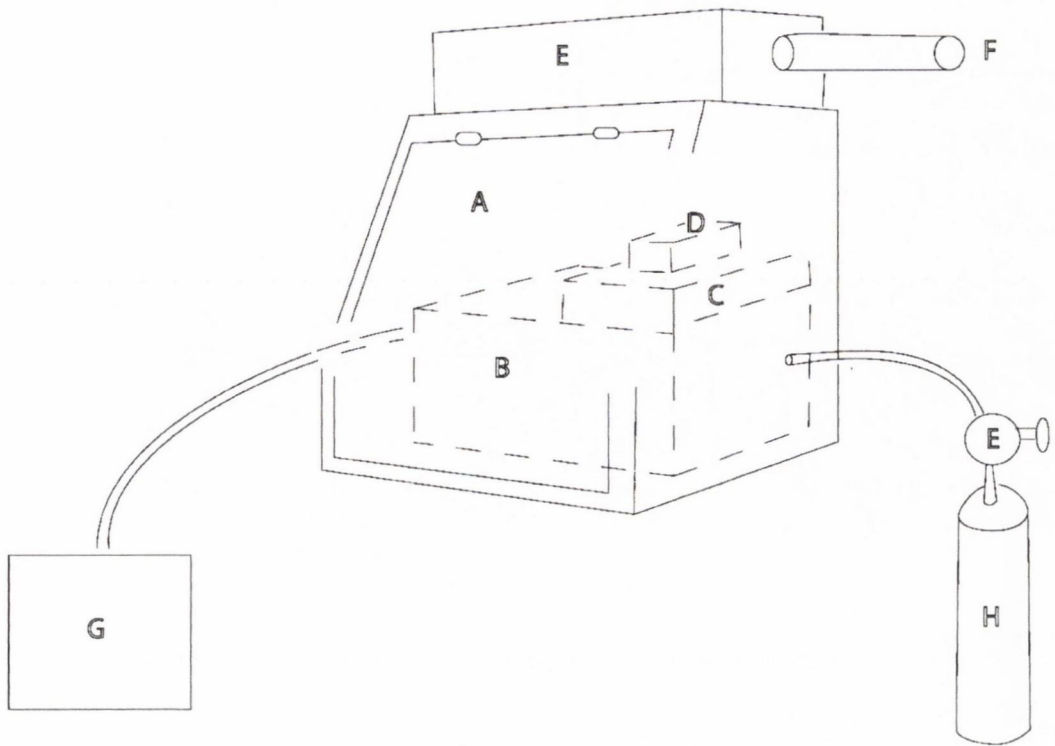
**Fig 2.4**
Exposure chamber: A) outer unit; B) exposure chamber; C) exposure chamber filter; D) gas extractor; E) outer unit extractor and filter; F) exhaust pipe; G) gas detector; H) gas cylinder; E) gas pressure regulator.

## 2.3.2 Protocol of exposure

Rats were placed in the chamber and exposed to pure CO (2400±240ppm) for 1 hr. The concentration of CO was monitored by the experimenter and kept as stable as possible. After exposure, the animals were kept in a heated environment to maintain body temperature at 37-38 °C in order to prevent the body hypothermia being induced by exposure to the gas.

Control animals ("Air" group) were exposed to air in the same chamber used for the exposures to CO, for the same amount of time.

## 2.4    Timelines of treatments relative to testing

Experiments started after two days from the delivery of animals to the experimental facilities. Animals were randomly assigned to treatment groups and injected 30 minutes before trials in all tests. In the watermaze task, treatments were administered every day for 5 days, before the 1$^{st}$ acquisition trial. In the studies described in Chapters 3, 4, 5, and 7, two or three days (day 8 or 9) after completion of the test in the watermaze task rats were tested in the hole board. In the studies described in Chapters 3, 4 and 5, animals were tested also in the T-maze on days 10, 11 or 12. In the study shown in Chapter 7, animals were exposed to CO, two days before the start of the experiment in the watermaze. Only groups CO+Veh and CO+Scop but not groups Air and CO received injections before tests (Fig.2.5b). Moreover, in the study described in this chapter, groups Veh+Veh and Scop+Veh were the same as those in the study described in Chapter 6.

**a**

Week 1: watermaze

| Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
|-------|-------|-------|-------|-------|

↑    ↑    ↑    ↑    ↑

Injections

Week 2: hole board and T-maze

| Day 8 or 9 | Day 10, 11 or 12 |
|------------|------------------|

↑    Hole board    ↑    T-maze

Injections

**b**

Week 1: watermaze (starting two days after exposure)

| Day 1–Day2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |
|---|---|---|---|---|---|

Exposure to CO
or Air
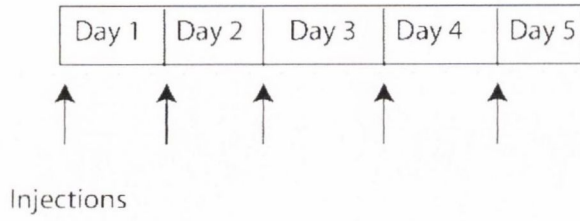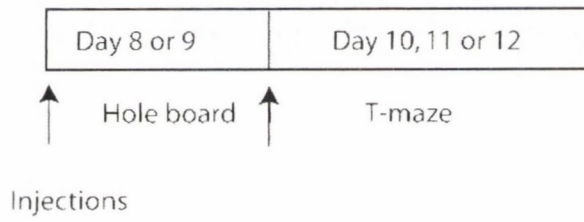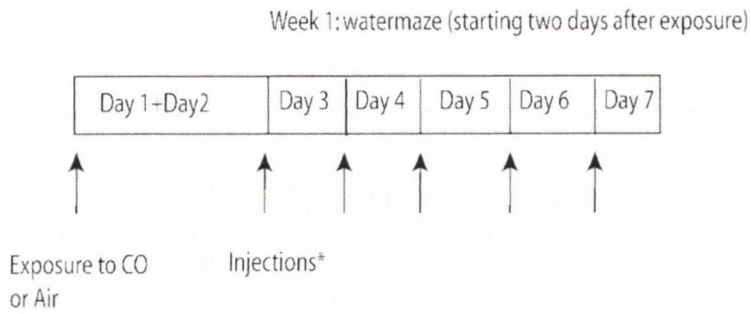
Injections*

Week 2 (after 2 days from completion of watermaze test): hole board

| Day 8 or 9 |
|---|

Hole board
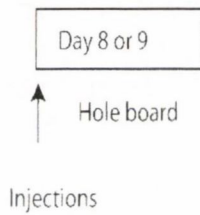
Injections

*Injections were given only to groups CO+Veh and CO+Scop

**Fig.2.5**

Timelines of treatments relative to tests in behavioural tasks for the studies shown in: Chapters 3, 4, 5 and 9 (a); Chapter 7 (b). Rats in chapter 6 did not undergo the test in the T-maze task.

# CHAPTER 3

## Effects of the $M_2$ antagonist AFDX 384 on scopolamine-induced cognitive impairment

### 3.1 Introduction

Acetylcholine (ACh) concentration in the synaptic cleft is influenced by the activation of nicotinic and muscarinic autoreceptors (Araujio et al., 1990; Quirion et al., 1994). Their interaction with nicotinic or muscarinic agonists increases or decreases, respectively, ACh release from cholinergic terminals.

It has been shown that, in the cortex, cholinergic transmission is modulated by three different muscarinic receptor subtypes, namely $M_1$, $M_2$ and $M_4$, while, in the rat hippocampus, it seems to depend mostly on $M_1$ and $M_4$ receptor subtypes (Vannucchi et al., 1995). Further investigation has demonstrated that the $M_2$ subtype is primarily responsible for the modulation of cholinergic transmission in the cerebral cortex (Quirion et al., 1994) and in the striatum (Billard et al., 1995).

AFDX 384 is a muscarinic antagonist with a high affinity for the $M_2$ and $M_4$ receptor subtypes (Dorje et al., 1991). Piggot et al. (2002) have shown that, in humans, [$^3$H]AFDX 384 binding is moderate to high in the striatum, the cortex (especially the visual cortex), the thalamus, and the cerebellum and that its binding pattern is similar to the distribution reported in the rat (Quirion et al., 1993; Aubert et al., 1992; Gattu et al., 1997). In particular, the highest binding was observed in the outer layers of the temporal and occipital cortex, especially in the $CA_1$ subregion of the hippocampus and in the visual cortex (Piggot et al., 2002).

Using *in vivo* microdialysis techniques, Vannucchi et al. (1997) observed that the concentration of ACh was increased in the cortex and the hippocampus of freely moving rats after perfusion of these structures with AFDX 384. Also, in an object recognition and a passive avoidance task, they found that a dose of 5 mg/kg, intraperitoneally administered 20-30 minutes before the training trial, was capable of bringing performance to control levels in aged cognitively impaired rats and of fully reversing a scopolamine-induced performance deficit. Up to date, this is the only study in which the effects of AFDX 384 on memory deficits were evaluated using behavioural tasks.

These findings suggest that there may be a close relationship between cognitive functions and the activity of cortical/hippocampal cholinergic systems and also that $M_2/M_4$ antagonist AFDX 384 may have a potential role in the therapy of cognitively disrupting diseases. However, more research must be addressed to better clarify the therapeutic value of AFDX 384 in the disruption of different types of memory, caused by a decreased cholinergic transmission within the brain.

This study was designed to investigate whether the same dose and method of administration of AFDX 384 used by Vannucchi et al. (5mg/kg, i.p.) was capable of improving a scopolamine-induced performance impairment in the watermaze and in the T-maze, two tests that measure reference and working memory, respectively (Morris, 1984; Glaser, 1910; Wever, 1932; Olton et al., 1976). Also, in order to investigate the effects of this treatment on general locomotor activity, exploratory behaviour and its habituation, rats were tested in the hole board

## 3.2 Methods

Male Wistar rats weighing 200-250 g at the beginning of the experiment were tested in the Morris watermaze, hole board and T-maze.

The experiment was carried out in three phases. In the initial phase of this study, a comparison was made between two groups of rats, one injected with water for injection (Veh+Veh) and another one with vehicle and AFDX 384 (Veh+AFDX 384). Subsequently, a comparison was made between two groups of animals, one injected with 1.5 mg/kg scopolamine (a dose that was just above the threshold to disrupt normal performance in all behavioural tasks) and water for injection (Scop+Veh) and the other one with just water for injection (Veh+Veh). In the third phase of the study, a group of rats injected with 1.5 mg/kg scopolamine and 5 mg/kg AFDX 384 (Scop+AFDX 384) was compared to a group of rats that received injections of the same dose of scopolamine and just water for injection (Scop+Veh). In each experiment, a maximum of 16 rats (8 rats/group) was tested. Rats were tested in all tasks 30 minutes after injections (see section 2.4 for details on randomization of treatments and Fig. 2.5a for the timelines of injections relative to behavioural tests). The time windows between injections and end of testing (45min in the watermaze, 40 min in the hole board and 60 min in the T-maze) were chosen on the basis of the evidence showing that AFDX 384, injected i.p. 20-30 minutes before test was able to reverse a scopolamine-induced object recognition memory disruption in a test lasting 70 minutes (Vannucchi et al., 1997). Also, similar results were obtained in a passive avoidance task, in which acquisition and retention trials started about 30 minutes and 24 hrs, respectively, after injection of AFDX 384 (Vannucchi et al., 1997). Both scopolamine and AFDX 384 were dissolved in water for injection and injections were performed intraperitoneally (i.p.). Each rat received one injection/treatment. AFDX 384 was a gift of Boeringer Ingelheim (Germany).

## 3.2.1 Treatment groups

At the beginning of every experiment rats were randomly assigned to a treatment group. In each leg of the experiment only two treatments were used. Statistical comparisons were carried out combining the same treatment groups from different experiments. Thus, since in every experiment only two treatment groups (8 animals/group) were tested, the total number of animals/group differed across groups. Thus, treatment groups were:

1) Water for injection (Veh+Veh; watermaze, n=32; hole board, n=24; T-maze, n=16).

2) Water for injection+AFDX 384 (Veh+AFDX 384; watermaze, n=8; hole board, n=8; T-maze, n=8).

3) Scopolamine+water for injection (Scop+Veh; watermaze, n=32; hole board, n=24; T-maze, n=24).

4) Scopolamine+AFDX 384 (Scop+AFDX 384; watermaze, n=16; hole board, n=16; T-maze, n=16).

## 3.3 Results

### 3.3.1 Watermaze

### 3.3.1.1 Acquisition

Fig.3.1 (a, b) shows the 5-day learning profiles for the groups tested. An overall analysis revealed that there was a significant difference across treatment groups [$F_{(3, 92)}=8.3$, $P< 0.0001$] and a significant difference across trials $F_{(4, 89)}=74$, $P< 0.0001$]. An individual group analysis showed that all treatment groups significantly learned the task [Veh+Veh: $F_{(4, 28)}=44.5$, $P< 0.0001$;

Scop+Veh: $F(4, 28)=22.6$, $P< 0.0001$; Veh+AFDX 384: $F(4, 12)=36.5$, $P< 0.0001$; Scop+AFDX 384: $F(4, 12)=7.3$, $P< 0.05$].

Fig.3.1a shows the learning profiles of groups Veh+Veh and Veh+AFDX 384 and Scop+Veh. Comparison between groups Veh+Veh and Veh+AFDX 384 revealed no inter-group difference and no significant day/treatment interaction, whereas there was a significant difference between groups Veh+Veh and Scop+Veh [$F(1, 62)=11$, $P< 0.05$] and a significant day/group interaction between their learning profiles [$F(4, 59)=6.3$, $P< 0.0001$]. Finally, the learning profiles of groups Scop+Veh and Scop+AFDX 384 were not significantly different and had no significant day/treatment interaction (Fig.3.1b). The outcomes of overall and inter-group comparisons are summarized in Table 3.1.

| Comparisons | df | F | P< |
|---|---|---|---|
| Overall (between groups) | 3, 92 | 8.3 | 0.0001 |
| Overall (between trials) | 4, 89 | 74 | 0.0001 |
| Veh+Veh vs Veh+AFDX 384 | 1, 46 | 0.005 | NS |
| Veh+Veh vs Scop+Veh | 1, 62 | 11 | 0.05 |
| Scop+Veh vs Scop+AFDX 384 | 1, 46 | 2 | NS |

**Table 3.1**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on acquisition of the watermaze task. Treatment groups: vehicle+vehicle (Veh+Veh, n=32/group), vehicle+AFDX 384 (Veh+AFDX 384, n=8), scopolamine+vehicle (Scop+Veh, n=32) and scopolamine+AFDX 384 (Scop+ AFDX 384, n=16). Table shows F scores and P values for inter-group comparisons.

**a**
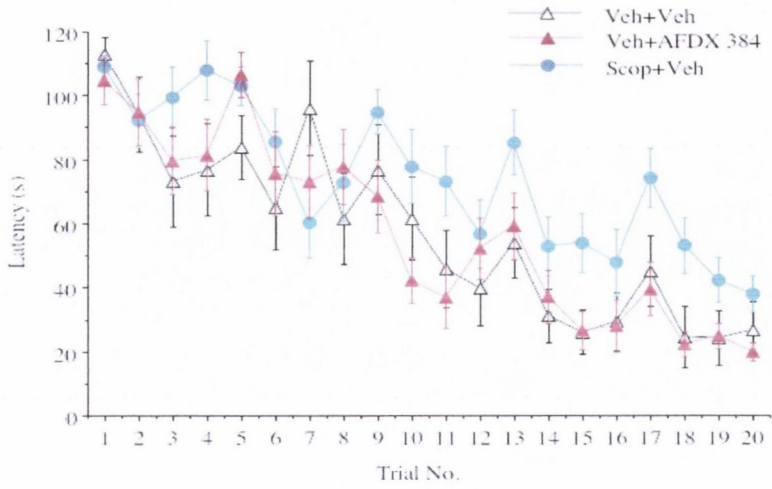


**b**
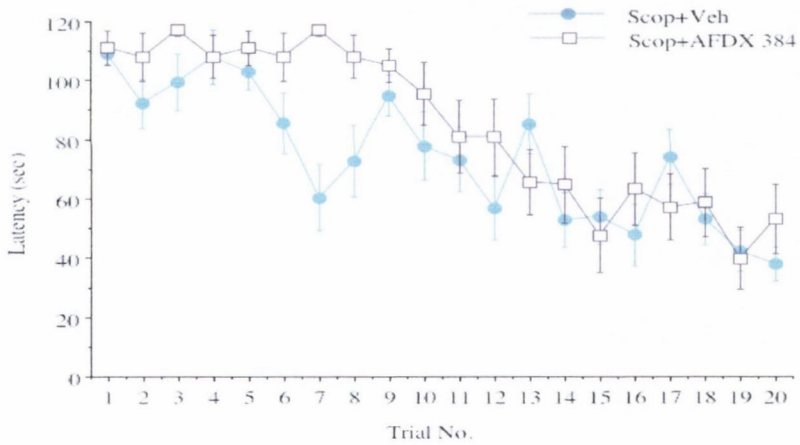


**Fig. 3.1 (a, b)**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on acquisition of the watermaze task. Treatment groups: vehicle+vehicle (Veh+Veh, n=32/group), vehicle+AFDX 384 (Veh+AFDX 384, n=8), scopolamine+vehicle (Scop+Veh, n=32) and scopolamine+AFDX 384 (Scop+ AFDX 384, n=16); a) Veh+Veh, Veh+AFDX 384, Scop+Veh; b) Scop+Veh and Scop+AFDX 384. Values are means±SEM.

## 3.3.1.2 Probe test

The analysis of probe test performance showed significant differences for all values measured (Table 3.2a).

Comparison between groups Veh+Veh and group Veh+AFDX 384 revealed that the animals of group Veh+AFDX 384 spent less time in the periphery of the tank. No other inter-group difference was found for this comparison. Group Scop+Veh group had significantly higher latency values, spent less time in the target quadrant, travelled a longer distance and had a faster swim speed than group Veh+Veh. There was no significant difference between groups Scop+Veh and Scop+AFDX 384. A within group analysis of quadrant bias showed that the animals of the Veh+Veh and Veh+AFDX 384 groups spent a significantly higher percentage of total trial time in the target quadrant relative to the opposite quadrant (Veh+Veh: $P< 0.0001$; Veh+AFDX 384: $P< 0.01$), whereas no target quadrant bias was shown by the animals of groups Scop+Veh and Scop+AFDX 384. The above results are shown in Fig. 3.2 (a-e) and Table 3.2 (a, b).

**a**
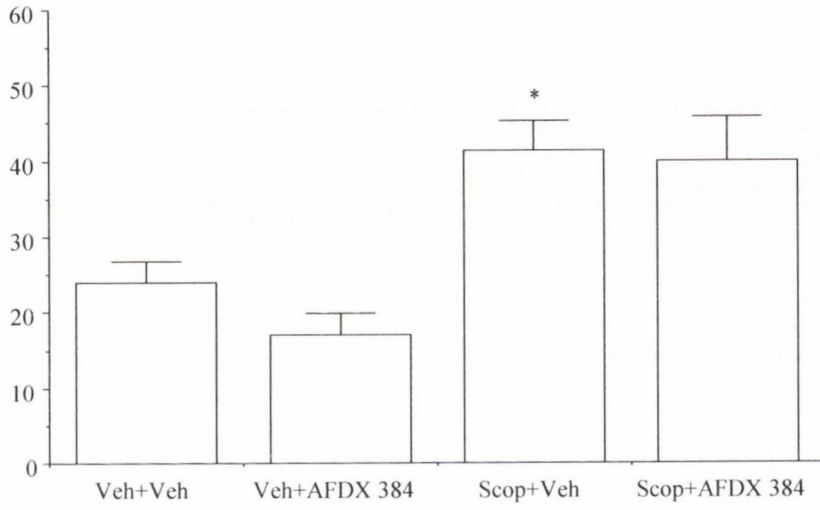


**b**
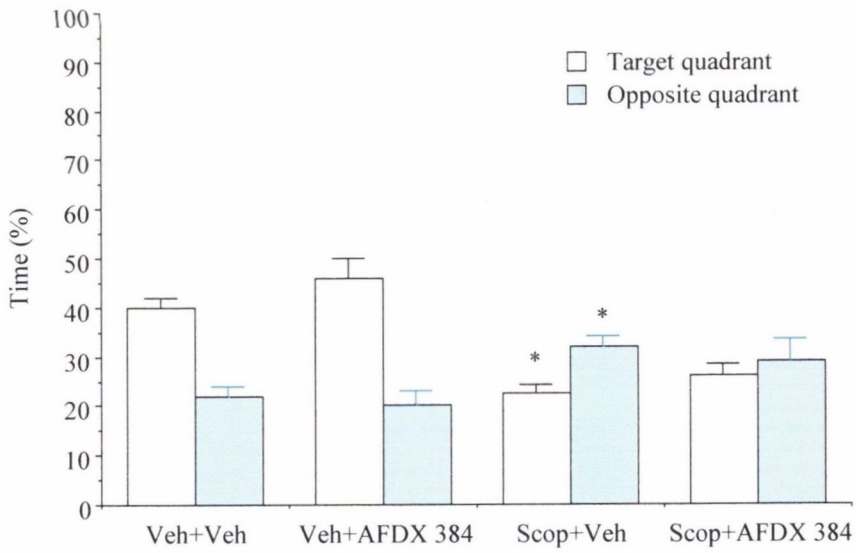
**c**
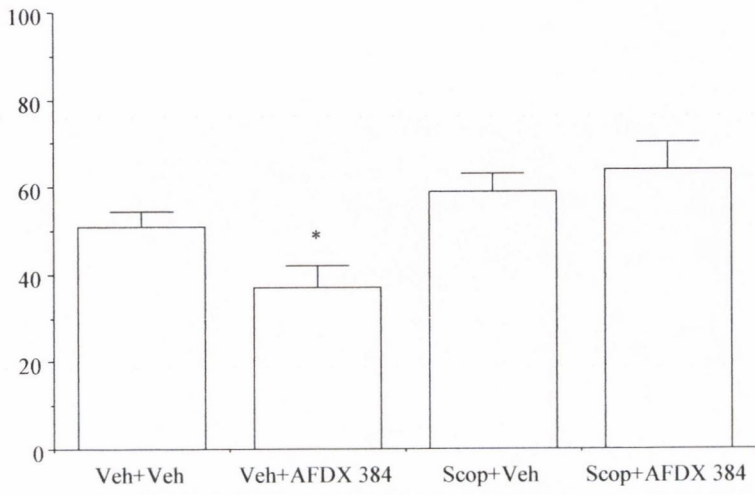


**d**
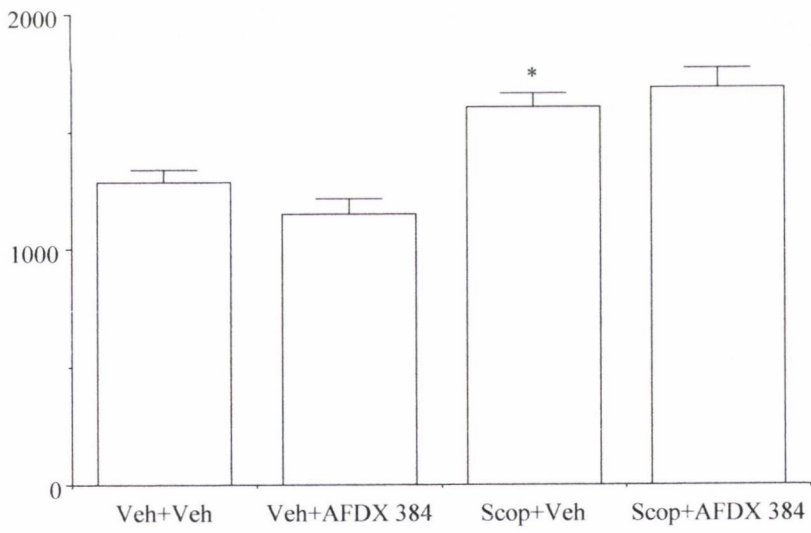
**e**
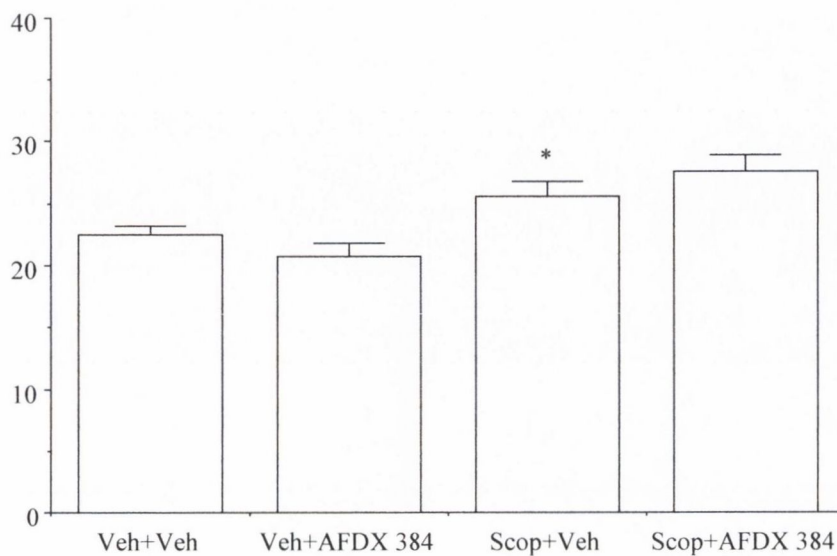


**Fig. 3.2 (a-e)**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on watermaze probe test performance. Treatment groups: a) vehicle+vehicle (Veh+Veh, n=32/group), Veh+AFDX 384, n=8), scopolamine+vehicle (Scop+Veh, n=32), scopolamine+AFDX 384 (Scop+AFDX 384, n=16); a) latency to the platform location; b) percent time spent in the target quadrant vs. percent time spent in the opposite quadrant; c) percent time spent in the periphery of the tank; d) total distance travelled; e) swim speed. Asterisk (*) indicates statistical difference to the Veh+Veh group. Values are mean±SEM.

| | Group | Latency to platform (s) | Time in tg.q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. (%) | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Veh+Veh | 24±3 | 40±2 | 22±2 | 18±3.5 | 2.5±0.3 | 1283±53 | 22±1 | 51±3.5 |
| 2 | Veh+AFDX 384 | 17±3 | 46±4 | 20±3 | -26±6 | 4±1 | 1146±68 | 21±1 | 37±5 |
| 3 | Scop+Veh | 41.5±4 | 22.4±2 | 32±4.4 | -9.5±4 | 1±0.1 | 1602±61 | 26±1 | 60±4 |
| 4 | Scop+AFDX 384 | 40.5±6 | 26±2.5 | 30±4.5 | -4±6 | 1.3±0.3 | 1686±81 | 27±1 | 64±6 |
| | Overall ANOVA (P <) | 0.0001 | 0.0001 | 0.05 | 0.0001 | 0.0001 | 0.0001 | 0.001 | 0.001 |

t-test P values (P<)

| | Latency to platform (s) | Time in tg.q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. (%) | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (%) |
|---|---|---|---|---|---|---|---|---|
| Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS | NS | 0.05 |
| Gr.1 Vs. Gr. 3 | 0.001 | 0.0001 | 0.01 | 0.0001 | 0.0001 | 0.001 | 0.05 | NS |
| Gr.3 Vs. Gr. 4 | NS | NS | NS | NS | NS | NS | NS | NS |

**Table 3.2**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on watermaze probe test performance. Treatment groups: vehicle+vehicle (Veh+Veh, n=32/group), vehicle+AFDX 384 (Veh+AFDX 384, n=8), scopolamine+vehicle (Scop+Veh, n=32), scopolamine+AFDX 384 (Scop+AFDX 384, n=16); Table shows mean±SEM values, P values for the overall comparison between groups (ANOVA) and t-test P values for inter-group comparisons.

### 3.3.2 Hole board

An overall analysis showed the only significant difference to be the number of lines crossed during the second half of the experiment (Table 3.3 a).

There was no significant difference between groups Veh+Veh and Veh+AFDX 384. Relative to group Veh+Veh, the number of head dips was significantly higher in the Scop+Veh group, during both the first and the second half of the experiment. Comparison between groups Scop+Veh and Scop+AFDX 384 revealed that the Scop+Veh group scored a significantly higher number of head dips during the first half of the experiment. During the second half of the experiment the Scop+AFDX 384 scored a significantly higher number of lines crossed relative to the Scop+Veh group.

A within group analysis (Wilcoxon signed-rank test) showed that, in the groups Veh+Veh and Veh+AFDX 384, all values were significantly reduced during the second half of the experiment. In the Scop+Veh group, the number of lines crossed was the only value significantly reduced during the second half of the experiment. In the Scop+AFDX 384 group all values except the number of head dips were reduced during the second half of the experiment. These results are shown in Fig. 3.3 and Table 3.3 (a, b, c).
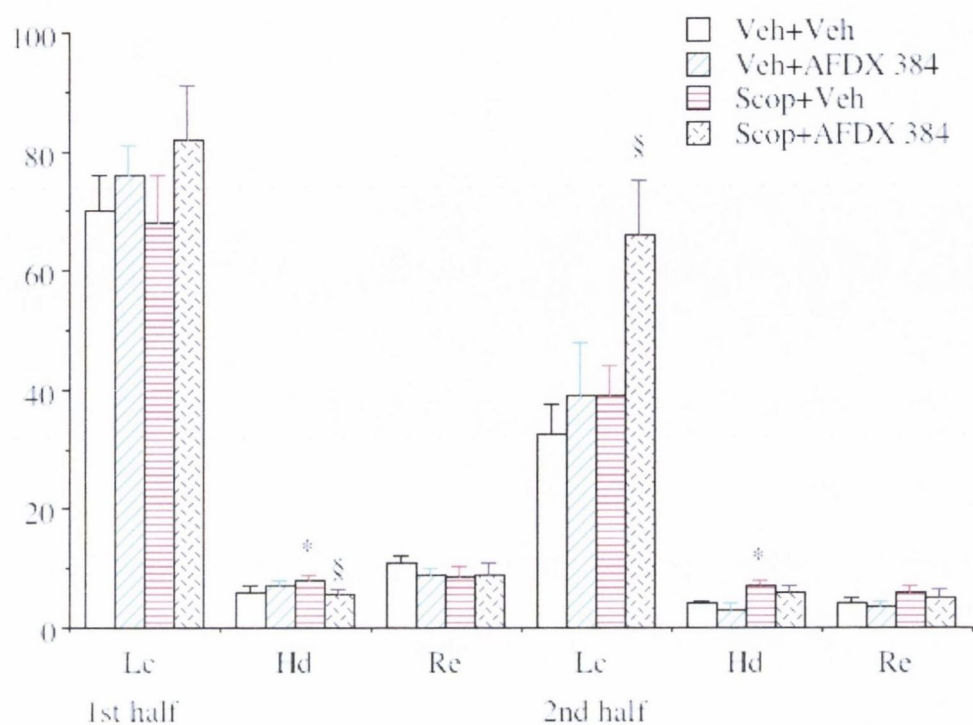
**Fig.3.3**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on the hole board activity. Treatment groups: vehicle+vehicle (Veh+Veh, n=24), vehicle+AFDX 384 (Veh+AFDX 384, n=8), scopolamine+Veh (Scop+Veh, n=24) and scopolamine+AFDX 384 (Scop+AFDX 384, n=16). Asterisk (*) and symbol "§" show statistically significant difference from the Veh+Veh and Scop+Veh groups, respectively. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing. Values are means±SEM

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| a | Group | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|---|
| | | 1st half | 2st half | 1st half | 2nd half | 1nd half | 2nd half |
| 1 | Veh+Veh | 70±6 | 32.5±5 | 6±1 | 4±0.5 | 11±1 | 4±1 |
| 2 | Veh+AFDX 384 | 76±5 | 40±9 | 7±1 | 3±1 | 9±1 | 3.5±1 |
| 3 | Scop+Veh | 68±8 | 39.5±5 | 8±1 | 7±1 | 8.5±2 | 6±1 |
| 4 | Scop+ AFDX 384 | 82±9 | 66±9 | 5.5±1 | 6±1 | 9±2 | 5±1.5 |
| | Overall ANOVA P value (P<) | NS | 0.01 | NS | 0.05 | NS | NS |
| t-test P values (P<) | | | | | | | |
| | Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS |
| | Gr.1 Vs. Gr. 3 | NS | NS | 0.05 | 0.01 | NS | NS |
| | Gr.3 Vs. Gr. 4 | NS | 0.05 | 0.05 | NS | NS | NS |

| b | Groups | P Values (P<) | | |
|---|---|---|---|---|
| | | lc2-lc1 | hd2-hd1 | re2-re1 |
| 1 | Veh+Veh | 0.0001 | 0.05 | 0.0001 |
| 2 | Veh+AFDX 384 | 0.05 | 0.05 | 0.05 |
| 3 | Scop+Veh | 0.0001 | NS | NS |
| 4 | Scop+AFDX 384 | 0.05 | NS | 0.05 |

**Table 3.3 (a, b)**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on hole board activity. Treatment groups: vehicle+vehicle (Veh+Veh, n=24), vehicle+AFDX 384 (Veh+AFDX 384, n=8), scopolamine+Veh (Scop+Veh, n=24) and scopolamine+AFDX 384 (Scop+AFDX 384, n=16); a) mean±SEM values, P values for the overall comparison between groups (ANOVA) and t-test P values for inter-group comparisons; b) within group comparisons (Wilcoxon signed-rank test), for all values measured, between the first and the second half of the experiment.

### 3.3.3 T-maze

An overall analysis of alternation percentages revealed a significant difference across all treatment groups (P< 0.05).

No significant difference was found between groups Veh+Veh and Veh+AFDX 384. The rats injected with scopolamine (Scop+Veh) scored a significantly (P< 0.05) lower percentage of alternation than the rats injected only with water for injection (Veh+Veh). The Scop+AFDX 384 group had a significantly higher alternation percentage (P< 0.05) with respect to the Scop+Veh group. The outcome of the experiment is shown in Fig 3.4 and Table 3.4.
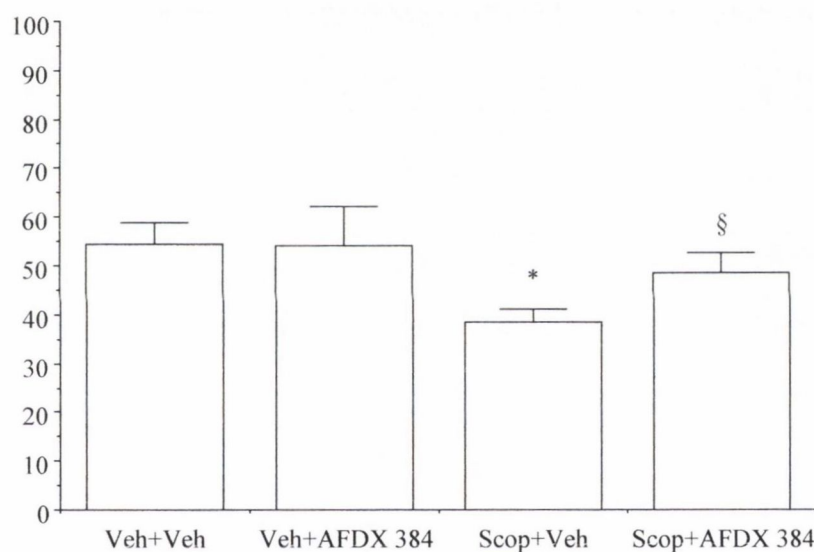


**Fig 3.4**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on spontaneous alternation in the T-maze task. Treatment groups: vehicle+vehicle (Veh+Veh, n=16), vehicle+AFDX 384 (Veh+AFDX 384, n=8), scopolamine+vehicle (Scop+Veh, n=24) and scopolamine+AFDX 384 (Scop+AFDX 384, n=16). Asterisk (*) and symbol "§"show statistically significant difference from groups Veh+Veh and Scop+Veh, respectively. Values are means ±SEM.

| Group | Alternation (%) |
|---|---|
| Veh+Veh | 55±4 |
| Veh+AFDX 384 | 54±8 |
| Scop+Veh | 38±3 |
| Scop+AFDX 384 | 49±4 |
| Overall ANOVA P value (P<) | 0.01 |

**t-test P values (P<)**

| Gr.1 Vs. Gr. 2 | NS |
|---|---|
| Gr.1 Vs. Gr. 3 | 0.05 |
| Gr.3 Vs. Gr. 4 | 0.05 |

**Table 3.4**

Effects of scopolamine (1.5 mg/kg) and AFDX 384 (5 mg/kg) on spontaneous alternation in the T-maze task. Treatment groups: vehicle+vehicle (VehVeh, n=16), scopolamine+vehicle (Scop+Veh, n=24), vehicle+AFDX 384 (Veh+AFDX 384, n=8) and scopolamine+AFDX 384 (Scop+AFDX 384, n=16). Table shows alternation percentages (mean±SEM), the overall ANOVA P value and t-test P values for inter-group comparisons.

## 3.5 Discussion

The results of the present study show that treatment with AFDX 384 had no overall significant effect on normal performance in any of the behavioural tasks used. It is interesting though that, in the watermaze, the rats treated with this drug (Veh+AFDX 384) spent less time swimming in the periphery of the tank. The tendency of animals to explore the periphery of a maze (thigmotaxis) has been reported in rats exposed to behavioural tasks such the watermaze and the open field (e.g., Sutherland, 1985; Whishaw and Kolb, 1984; Clement and Chapouthier, 1998; Ragnauth et al., 2001). It has been suggested that thigmotaxis can be considered a phylogenetically selected strategic behaviour meant to avoid predators in the wild (Grossen and Kelley, 1972; Treit and Fundytus, 1989) and that the more thigmotactic, the more emotional (or anxious or fearful) an animal is (Valle, 1970). The validity of thigmotaxis in measuring emotionality has been demonstrated in mice (Choleris et al., 2001; Simon et al., 1994) and rats (Treit and Fundytus, 1989; Van der Staay et al., 1990). There is also evidence showing that damage to the hippocampus, thalamus, or neocortex increases watermaze periphery swimming (Sutherland, 1985; Whishaw and Kolb, 1984), suggesting that thigmotactic behaviour can be a general response to compromised brain functions. Further, other studies suggest that thigmotaxis can be induced by the disruption of muscarinic receptor-mediated cholinergic transmission in the brain. In these studies, rats trained in the watermaze task and treated with muscarinic antagonists (such atropine sulphate) did not learn the strategies useful to maze acquisition as readily as normal rats do. Instead, they persist in swimming around the perimeter of the tank, close to the wall (Paylor et al., 1990; Whishaw et al., 1985, 1987). Given these considerations, and the experimental evidence showing that AFDX 384 significantly increases acetylcholine levels in the hippocampus and in the cortex (Stillmann et al., 1996; Vannucchi et al., 1997), it could be speculated that the enhancement of

the cholinergic transmission in the brain induced by this drug might have had anxiolytic effects in the animals tested in the watermaze, improving their search strategy. However, further studies should aim at confirming these results in the watermaze task and also at evaluating the effects of AFDX 384 on thigmotactic behaviour in tasks such as the hole board and the open field.

The lack of an effect of this treatment on locomotion and exploratory behaviour in the hole board suggests that AFDX 384 had no significant effect on motor activity or on the exploration in novel environments. Also, the fact that habituation was not affected suggests that AFDX 384 did not impair spatial learning related processes. Habituation of exploratory behaviour involves several behavioural processes. These are, on one hand, responses to novelty, including arousal, emotionality, stress-related factors and on the other hand a diminished response due to familiarity, which requires learning-related processes and recognition or recall (Dai et al., 1995; Gerhardt et al., 1993-94; Platel et al., 1982; Sadile et al., 1979; Tomaz et al., 1990). Further, the data of the experiments carried out on the T-maze task suggest that, on its own, AFDX 384 did not have any significant effect on working memory processes (Olton et al., 1976; Spowart-Manning, 2005). These results add to what has been found by Vannucchi et al. (1997) in other behavioural paradigms. They showed that the same dose of AFDX 384 (5 mg/kg), administered 20-30 min before test, did not affect object recognition memory or retention of a passive avoidance task, two tests that measure two different types of memory, namely episodic (Ennaceur et al., 1988) and short-term reference memory, respectively. Taken together, the present results show that, in normal conditions (in rats that received no other drug treatment), AFDX 384 administration had a limited overall effect on cognition. It has been observed that in the cortex and hippocampus of cognitively impaired aged rats, the presynaptic $M_2$ receptor population is increased relative to other receptor subclasses (Aubert et al.,

1995). The increased density in $M_2$ receptors on presynaptic terminals may reduce acetylcholine release, possibly leading to cognitive impairment (Vannucchi et al., 1997; Aubert et al., 1995). Thus, it could be speculated that, in the brain of young healthy animals, the blockade of $M_2$ receptors has a relatively minor role in the modulation of cognitive functions.

Daily administration of scopolamine disrupted normal watermaze acquisition and probe test performance. Also, acute administration of this drug increased exploration and reduced habituation in the hole board task, and disrupted spontaneous alternation in the T-maze task. The comparison between groups Scop+AFDX 384 and Scop+Veh, showed that the administration of AFDX 384 did not attenuate the scopolamine-induced watermaze performance disruption during both acquisition and probe tests. Even though scopolamine is a relatively non-selective muscarinic antagonist with ten times higher affinity for postsynaptic than for presynaptic receptors (Szerb et al., 1977; Sethy and Francis, 1988), and it is believed that its disruptive effects on cognition are mediated through $M_1$ postsynaptic receptors (Hunter and Roberts, 1988; Messer et al., 1990; Bymaster et al., 1993; Carey et al., 2001; see also Table 1.2a-b for more details on scopolamine's affinity for $M_1$ vs $M_2$ receptors), it is possible that, at this dose, its effects were mediated by both $M_1$ and $M_2$ receptors. Thus, it may be speculated that AFDX 384, when administered in combination with scopolamine, was ineffective at the dose tested because its occupancy of presynaptic $M_2$ receptors may have been relatively limited and therefore unable to induce an increase of acetylcholine concentration sufficient to overcome the putative postsynaptic $M_1$ muscarinic receptor blocking action of scopolamine. Also, given that, in this study, scopolamine had disruptive effects on both watermaze acquisition and probe test performances, it is possible that the cognitive disruption induced by this dose of scopolamine was too marked for AFDX 384 to overcome it. Further research should be carried out to evaluate

the specific effects of the AFDX 384 on both memory formation and memory consolidation, for example using a dose of scopolamine at which only probe test performance is disrupted and/or testing higher doses of the $M_2$ antagonist.

The analysis of the hole board data revealed that exploratory behaviour (number of head dips) in the Scop+AFDX 384 group was lower relative to the Scop+Veh group during the first half of the experiment. During the second half, the Scop+AFDX 384 group still showed poor exploratory behaviour but also higher locomotor activity relative to normal rats. Moreover, the animals in the Scop+AFDX 384 group showed a reduced level of habituation. It is likely that these effects were induced primarily by scopolamine and that AFDX 384 played a minor role, but it is also possible that AFDX 384 contributed to increase the hyperactivity induced by scopolamine, producing an imbalance between the cholinergic and one or more other neurotransmitter systems. Several lines of evidence suggest that the disruption of cholinergic transmission may produce hyperactivity through direct or indirect interactions with dopaminergic systems (e.g., Stoof et al., 1979; Hertting, 1980). However, the mechanism through which AFDX 384, in combination with scopolamine, may have induced hyperactivity remains undetermined and further studies should be undertaken to propose a biochemical mechanism for the effects reported here. It is interesting that the hyperactivity observed in the hole board was not observed in the watermaze task as an increase of swimming speed. This might be due to the different nature of the two tasks, which involve different sets of motor skills for the rats in order to navigate in the two mazes (ambulation in the hole board and swimming in the watermaze).

In the T-maze, treatment with AFDX 384 brought the alternation percentage values to normal, demonstrating its efficacy at attenuating the working memory deficits induced by scopolamine injection. Again, the interaction with other transmitter systems could be considered. It has been

suggested that scopolamine-induced working memory deficits might be related to indirect stimulation of dopamine receptors and by direct modulation of $D_2/D_3$ receptor activity (Bushnell and Levin, 1993). Also, $D_1$ and $D_2$/ligands interact with compounds specific for muscarinic and nicotinic cholinergic receptors (Levin et al., 1990). In particular, working memory has been shown to be sensitive to the activity of postsynaptic $D_1$ receptors, as the administration of $D_1$ agonist SKF 38393 or $D_1$ antagonist SCH 23390, attenuate scopolamine induced working memory deficits (Levin et al., 1991). Given these considerations, a line of study should be addressed to evaluate the role of AFDX 384 in the modulation of dopaminergic systems.

Taken together, the present results show that AFDX 384, at a dose previously reported to increase hippocampal acetylcholine concentration and to improve a scopolamine-induced performance deficit in an object recognition task (Vannucchi et al., 1997) differentially affected performance in the tasks used. Although from this study it is not possible to determine whether the effects of this $M_2$ antagonist were task specific (treatments were given repeatedly in the watermaze and acutely in the T-maze task and hole board) the beneficial effects of AFDX 384 on T-maze performance suggest that this drug can, at least partly, attenuate the disruption of cognitive functions due to the unselective blockade of muscarinic cholinergic receptor-mediated transmission in the brain.

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# CHAPTER 4

# Effects of M$_2$ antagonist, BIBN 99, on scopolamine-induced cognitive impairment

## 4.1 Introduction

BIBN 99 is a lipophilic muscarinic M$_2$ receptor antagonist endowed with central nervous system activity and 30-fold M$_2$ vs. M$_1$ selectivity (Dorjie et al., 1991). As mentioned in the introduction to Chapter 3, the blockade of presynaptic M$_2$ receptor activity antagonists has shown promising results in studies that investigated their effects on cognitively impaired animals (e.g., Quirion, 1995; Rowe, 2003; see also Clader and Wang, 2005 for a review).

The higher selectivity of BIBN 99 for M$_2$ receptors over M$_1$ receptors might be crucial for the potential therapy of cognitively disrupting conditions in which cholinergic transmission is reduced (Rowe, 2003). Given the data that suggest that a proportion of M$_2$ receptors may be located on presynaptic cholinergic terminals and autoinhibitory (e.g., Levey et al., 1991; Quirion et al., 1993) selective interaction with these receptors might reduce the likelihood that its presynaptic action, namely release of acetylcholine, may be counteracted by blocking other receptors located postsynaptically and hence reducing cholinergic transmission.

Indeed, the results of preliminary studies show that, subcutaneous injections of 0.25-0.5 mg/kg BIBN 99 significantly increased acetylcholine release in hippocampal areas in aged rats with learning deficits (Wilson et al., 1992; Doods et al., 1993). Moreover, it was observed that BIBN 99 markedly improved, in a dose time dependent manner, the performance of aged

cognitively impaired animals in the Morris watermaze task (Quirion et al., 1995, see also Table 4.1 for some of the most representative findings showing the ability of BIBN 99 to attenuate or reverse cognitive deficits). Taking these findings into consideration, in order to further estimate the value of BIBN 99 as a potential treatment of cognitive impairment such as occurs in AD, the present study was designed to evaluate whether this drug can attenuate the cognitive disrupting effects of scopolamine on motility, exploration, habituation, reference and working memory. To do so, rats were tested in the hole board, Morris watermaze and in the T-maze task.

| Model | Behavioural Task | Dose and method of administration | Effect | Reference |
|-------|------------------|-----------------------------------|--------|-----------|
| Scopolamine (0.1 mg/kg) given 30 min training | Eight/arm maze | 0.5 mg/kg, s.c., 60 min session after 48 hrs from administration of scopo-lamine | ↑ | Quirion et al., 1995 |
| Age impaired (AI) Rats | watermaze | 0.5 mg/kg, s.c.,60 min and 4hrsbefore testing | ↑ | Quirion et al., 1995 |
| Traumatic brain injury (TBI) | watermaze | 0.5 and 1.0 mg/kg, s.c., daily, 60 min before testing | ↑ | Pike and Hamm, 1995 |
| Age impaired (AI) Rats | watermaze | 0.5 and 1.0 mg/kg, s.c., daily, 45 min before training or 60 min before testing | ↑ | Geofferey et al., 2002 |
| Age impaired (AI) Rats | watermaze | 0.5 mg/kg, s.c., daily, 60 min before training | ↑ | Rowe et al., 2003 |

**Table 4.1**

Most representative findings showing the efficacy of BIBN 99 at improving cognitive deficits in the rat. The arrows indicate improvement of performance in a given task.

## 4.2 Methods

Male Wistar rats, weighing 200-250 g at the beginning of the experiment, were tested in the Morris watermaze, the hole board and theT-maze.

In the initial phase of the study, a comparison was made between two groups of rats, one injected intraperitoneally (i.p.) with water for injection and subcutaneously (s.c.) with saline (Veh+Sal), and another one (Veh+BIBN 99) injected i.p. with vehicle and s.c. with 0.5 mg/kg BIBN-99 (dissolved in saline). Subsequently, the effects of BIBN-99 on scopolamine-induced deficits were tested. Two groups of animals were compared, one group i.p. injected with 1.5 mg/kg scopolamine (same dose as that used in the study described in Chapter 3) and the same volume of saline (Scop+Sal) and the other one with the same dose of scopolamine combined with 0.5 mg/kg BIBN-99 (Scop+BIBN 99). Each rat received one injection/treatment. In each experiment, a maximum of 16 rats (8 rats/group) was tested. Rats were tested in all tasks 30 minutes after injections (see section 2.4 for details on randomization of treatments and Fig. 2.5a for the timelines of injections relative to behavioural tests). The dose of the BIBN 99, method of administration and the medium in which it was dissolved (saline) were chosen according to Rowe et al., (2003) who found that this treatment, administered 1 hr before test, reversed watermaze performance deficit in aged cognitively impaired rats (see also Table 4.1 for further details).

### 4.2.1 Treatment groups

At the beginning of every experiment rats were randomly assigned to a treatment group. In each leg of the experiment only two treatments were used. Statistical comparisons were carried out combining the same treatment groups from different experiments. Thus, since in every experiment only two treatment

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groups (8 animals/group) were tested, the total number of animals/group differed across groups. Treatment groups were:

1) Water for injection+saline (Veh+Sal; watermaze, n=16; hole board, n=16; T-maze, n=16).

2) Water for injection+BIBN 99 (Veh+BIBN 99; watermaze, n=15; hole board, n=8; T-maze, n=8).

3) Scopolamine+saline (Scop+Sal; watermaze, n=24; hole board, n=12; T-maze, n=20).

4) Scopolamine+ BIBN 99 (Scop+ BIBN 99; watermaze, n=24; hole board, n=12; T-maze, n=20).

## 4.3 Results

### *4.3.1 Watermaze*

#### *4.3.1.1 Acquisition*

An overall analysis of the escape latencies scored during the 5-day training revealed that all animals tested learned the task [$F(4, 72)=65$, $P< 0.0001$)], and that there was a significant difference between treatment groups [$F(3, 75)=12$, $P< 0.0001$].

Individual analyses of escape latencies showed that all groups significantly learned the task [Veh+Sal: $F(4, 12)=25$, $P< 0.0001$; Scop+Sal: $F(4, 20)=2137$, $P< 0.0001$; Veh+BIBN 99: $F(4, 11)=19$, $P< 0.0001$; Scop+BIBN 99: $F(4, 20)=7$, $P< 0.05$].

The comparison of the learning profiles of groups Veh+Sal and Veh+BIBN 99 (Fig. 4.1a) revealed no significant difference and a significant day/treatment interaction [$F(4, 26)=3$, $P< 0.05$]. A t-test carried out on the escape latencies for each trial revealed no significant inter-group difference. Also, the acquisition profiles of groups Veh+Sal and Scop+Sal (Fig 4.1a) were significantly different [$F(1, 38)=16$, $P< 0.001$] but had no significant day/treatment interaction. Finally, a comparison between groups Scop+Sal and Scop+BIBN 99 (Fig. 4.1c) revealed no significant difference and a significant day/treatment interaction [$F(4, 43)=3.2$, $P< 0.05$]. A t-test carried out on the escape latencies for each trial revealed no significant inter-group difference. F and P values for inter-group comparisons are shownin Table 4.2.

| Comparisons | df | F | P< |
|---|---|---|---|
| **Overall (between groups)** | 3, 75 | 12 | 0.0001 |
| **Overall (between trials)** | 4, 72 | 65 | 0.0001 |
| **Veh+Sal vs Veh+BIBN 99** | 1, 29 | 2 | NS |
| **Veh+Sal vs Scop+Veh** | 1, 38 | 16 | 0.001 |
| **Scop+Sal vs Scop+BIBN 99** | 1, 46 | 0.05 | NS |

**Table 4.2**

Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on acquisition of the watermaze task. Treatment groups vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=15), scopolamine+saline (Scop+Sal, n=24) and scopolamine+BIBN 99 (Scop+BIBN 99, n=24). Table shows F scores and P values for inter-group comparisons.

**a**
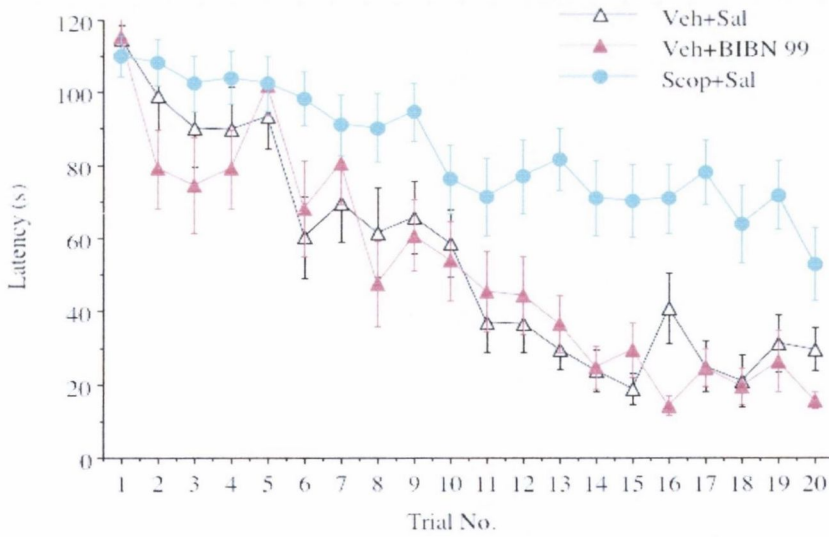
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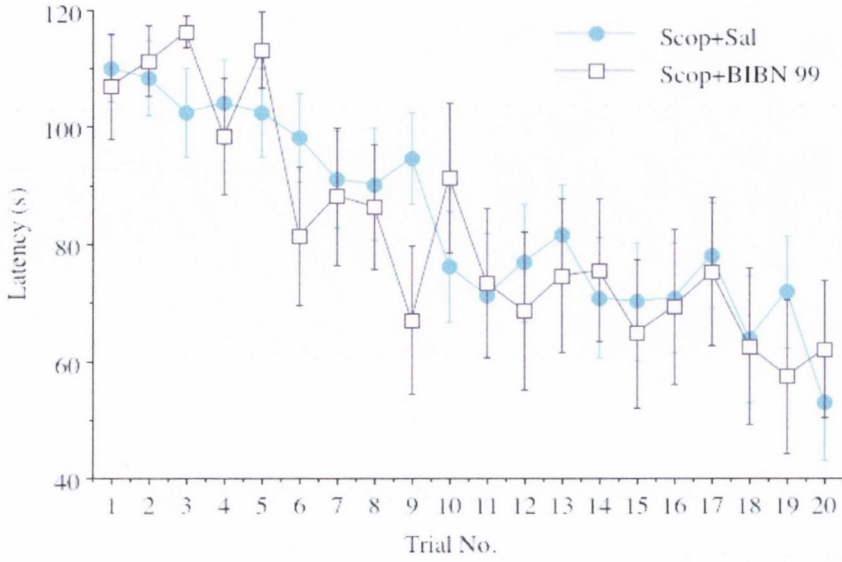


**Fig. 4.1 (a-b)**

Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on acquisition performance in the watermaze. Treatment groups vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=15), scopolamine+saline (Scop+Sal, n=24) and scopolamine+BIBN 99 (Scop+BIBN 99, n=24); a) Veh+Sal, Veh+BIBN 99 and Scop+Sal; b) Scop+Sal and Scop+BIBN 99. Values are means±SEM.

## 4.3.1.2 Probe test

Fig.4.2 (a-e) and Table 4.3 show the outcome of probe tests for each of the treatment groups.

An overall comparison across treatment groups revealed a significant difference for all values measured except for the time spent in the opposite quadrant and the target bias as measured by the ratio between the percent time spent in target versus opposite quadrants. All animals, except the animals of group Scop+Sal, showed a significant target quadrant bias (Veh+Sal: $P < 0.01$; Veh+BIBN 99: $P < 0.01$; Scop+BIBN 99: $P < 0.05$).

The animals of the Veh+BIBN 99 group spent significantly less time in the periphery of the tank with respect to the animals of the Veh+Sal group. No other difference was shown by this analysis. Comparison between groups Veh+Sal and Scop+Sal revealed that the animals of the Scop+Sal group had higher escape latency values, spent less time in the target quadrant, travelled longer distances, had faster swim speeds and spent more time in the periphery of the tank. Finally, there was no difference, for any all the values measured, between groups Scop+Veh and Scop+BIBN 99.

a
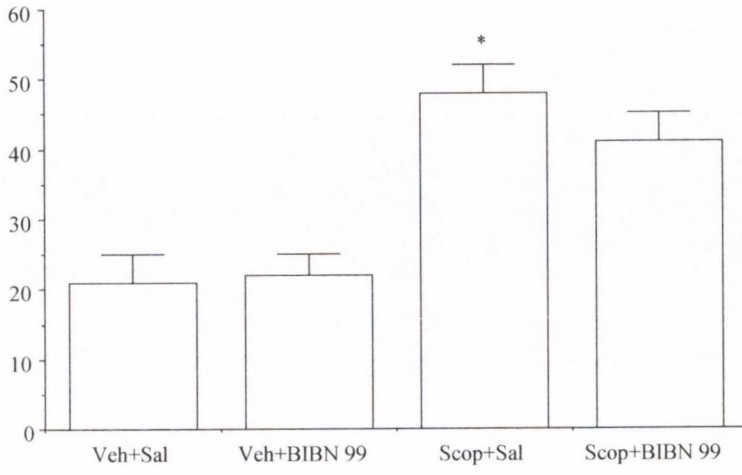


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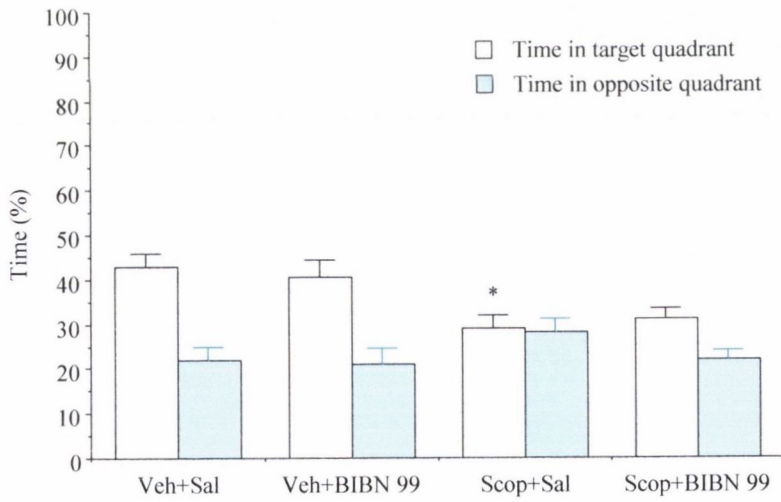
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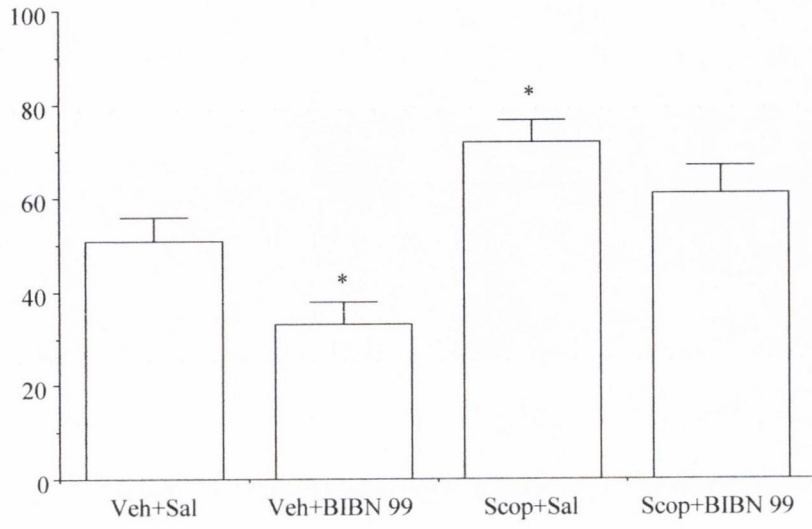


**d**
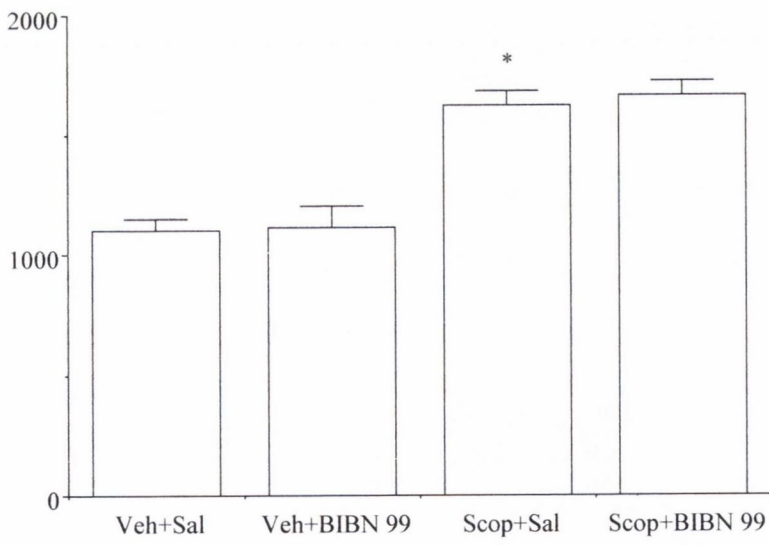
**e**



**Fig. 4.2 (a-e)**

Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on probe test performance in the watermaze. Treatment groups: vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=15), scopolamine+saline (Scop+Sal, n=24) and scopolamine+BIBN 99 (Scop+BIBN 99, n=24); a) latency to the platform location; b) percent time spent in the target quadrant vs. percent time spent in the opposite quadrant; c) percent time spent in the periphery of the tank; d) total distance travelled; e) swim speed. Asterisk (*) indicates statistical difference from the Veh+Sal group. Values are means±SEM

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| | Group | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Veh+Sal | 21±4 | 43±3 | 22±3 | 21±6 | 3±0.5 | 1102±48 | 20±1 | 51±5 |
| 2 | Veh+BIBN 99 | 22±4 | 40.5±4 | 21±3.5 | 19±7 | 3.5±1 | 1114±90 | 21±1 | 33±5 |
| 3 | Scop+Sal | 48±4 | 29±3 | 28±3 | 1±5.5 | 2±1 | 1618±62 | 27±1 | 72±4.5 |
| 4 | Scop+BIBN 99 | 41±4 | 31±2.5 | 22±2 | 9±4 | 2±0.2 | 1664±59 | 28±1 | 61±6 |
| | Overall ANOVA (P <) | 0.0001 | 0.05 | NS | 0.05 | NS | 0.0001 | 0.0001 | 0.0001 |

t-test P values (P<)

| | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (%) |
|---|---|---|---|---|---|---|---|---|
| Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS | NS | 0.05 |
| Gr.1 Vs. Gr. 3 | 0.0001 | 0.05 | NS | 0.05 | NS | 0.0001 | 0.0001 | 0.05 |
| Gr.3 Vs. Gr. 4 | NS | NS | NS | NS | NS | NS | NS | NS |

**Table 4.3**
Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on probe test performance in the watermaze. Treatment groups: vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=15), scopolamine+saline (Scop+Sal, n=24) and scopolamine+BIBN 99 (Scop+BIBN 99, n=24). Table shows mean±SEM values, P values for the overall comparison between groups (ANOVA) and t-test P values for inter-group comparisons.

### 4.3.2 Hole board

An overall analysis revealed no significant difference for all measures except for the value indicating rearing activity during the first half of the experiment (Table 4.4a).

There was no difference between groups Veh+Sal and Veh+BIBN 99 for all values measured during both the first and the second half of the experiment.

Comparison between groups Veh+Sal and Scop+Sal revealed that the Scop+Sal group scored a higher number of lines crossed during the second half of the experiment, and a higher number of head dips during both the first and the second half of the experiment. During the first half of the experiment, the Scop+BIBN 99 group had a significantly lower number of rears relative to group Scop+Sal.

Finally, a within group comparison (Wilcoxon signed-rank test) of the values scored during the first and the second half of the experiment showed that in the group Veh+Sal all values decreased during the second half. In groups Veh+BIBN 99 and Scop+Sal only the number of lines crossed and rears were reduced during the second half of the experiment whereas, in the group Scop+BIBN 99, only the number of lines crossed was significantly reduced (Table 4.4b). These results are illustrated in Fig. 4.3.

**Fig. 4.3**

Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on performance in the hole board task. Treatment groups: vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=8), scopolamine+saline (Scop+Sal, n=12) and scopolamine+BIBN 99 (Scop+BIBN 99, n=12). Asterisk (*) indicates statistical difference from the Veh+Sal group and symbol "§" indicates statistical difference from the Scop+Sal group. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing. Values are means ± SEM.

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| a | Group | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|---|
| | | 1st half | 2st half | 1st half | 2nd half | 1nd half | 2nd half |
| 1 | Veh+Sal | 62±4 | 27±5 | 5±1 | 3±0.5 | 11±1 | 4±2 |
| 2 | Veh+BIBN 99 | 76±4 | 36±8 | 6.5±1.5 | 4±1 | 1.5±3 | 4±1 |
| 3 | Scop+Sal | 74±7 | 51±8 | 8±1 | 6±1 | 11±2 | 6±1.5 |
| 4 | Scop+BIBN 99 | 72±8 | 45±11 | 6±1 | 4±1 | 6±2 | 6±2 |
| | Overall ANOVA P value (P<) | NS | NS | NS | NS | 0.05 | NS |
| | t-test P values (P<) | | | | | | |
| | Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS |
| | Gr.1 Vs. Gr. 3 | NS | 0.05 | 0.05 | 0.05 | NS | NS |
| | Gr.3 Vs. Gr. 4 | NS | NS | NS | NS | 0.05 | NS |

| b | Groups | P Values (P<) | | |
|---|---|---|---|---|
| | | lc2-lc1 | hd2-hd1 | re2-re1 |
| 1 | Veh+Sal | 0.0001 | 0.05 | 0.05 |
| 2 | Veh+BIBN 99 | 0.05 | NS | 0.05 |
| 3 | Scop+Sal | 0.05 | NS | 0.05 |
| 4 | Scop+BIBN 99 | 0.05 | NS | NS |

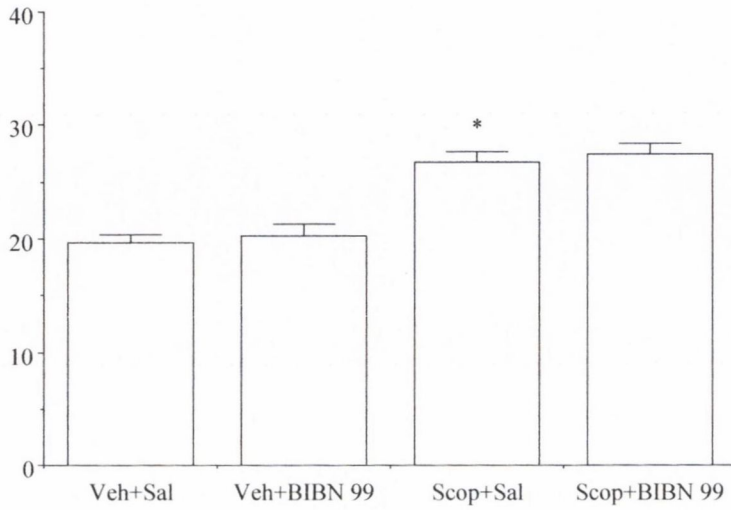**Table 4.4 (a, b)**

Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on performance in the hole board task. Treatment groups: vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=8), scopolamine+saline (Scop+Sal, n=12) and scopolamine+BIBN 99 (Scop+BIBN 99, n=12); a) mean±SEM values, P values for the overall comparison between groups (ANOVA) and t-test P values for inter-group comparisons; b) within group comparisons (Wilcoxon signed-rank test), for all values measured, between the first and the second half of the experiment.

### 4.3.3 T-maze

An overall analysis of the alternation percentages revealed a significant difference across groups ($P< 0.05$).

Comparisons between pairs of groups revealed that there was no significant difference between the Veh+Sal and Veh+BIBN 99 groups. The Scop+Sal group had significantly reduced percentage alternation ($P< 0.05$) relative to the Veh+Sal group. Finally, the Scop+BIBN 99 group had significantly higher percentage alternation relative to the Scop+Sal group ($P< 0.05$). Fig.4.4 and Table 4.5 summarize the outcome of the experiment.
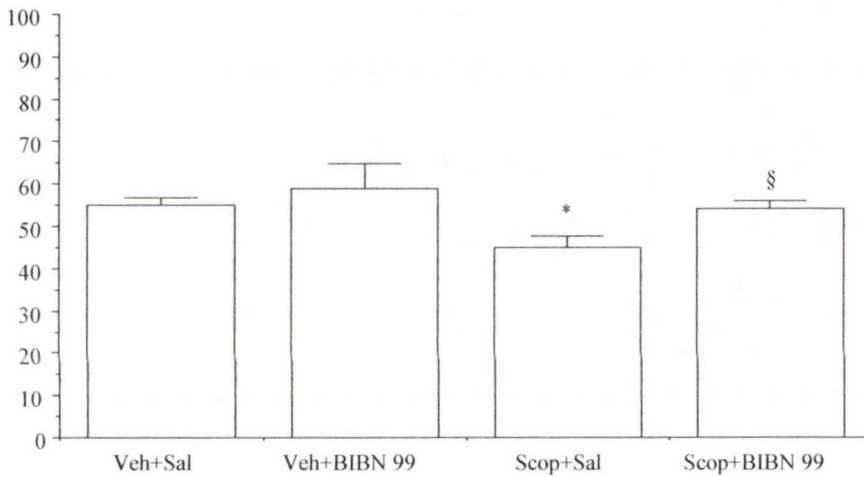


**Fig 4.4**

Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on performance in the T-maze task. Treatment groups: vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=8), scopolamine+saline (Scop+Sal, n=20) and scopolamine+BIBN 99 (Scop+BIBN 99, n=20). Asterisk (*) and symbol "§"show statistically significant difference to group Veh+Sal and Scop+BIBN 99, respectively. Values are means ±SEM.

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| Group | Alternation (%) |
|---|---|
| Veh+Sal | 55±2 |
| Veh+BIBN 99 | 59±6 |
| Scop+Sal | 45±3 |
| Scop+ BIBN 99 | 54±1.4 |
| Overall ANOVA P value (P<) | 0.05 |

**t-test P values (P<)**

| Gr.1 Vs. Gr. 2 | NS |
|---|---|
| Gr.1 Vs. Gr. 3 | 0.05 |
| Gr.3 Vs. Gr. 4 | 0.05 |

**Table 4.5**
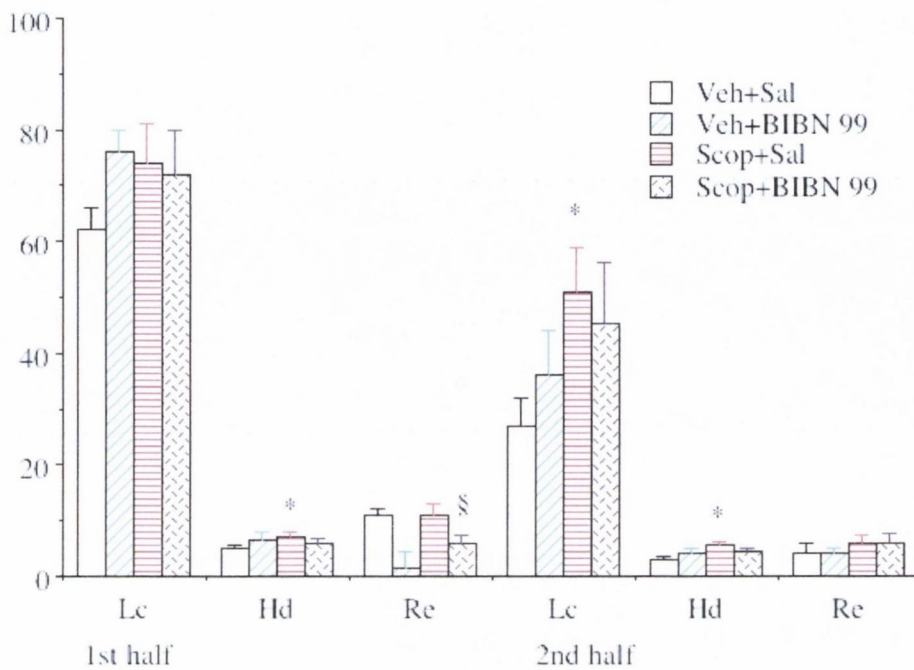
Effects of scopolamine (1.5 mg/kg) and BIBN 99 (0.5 mg/kg) on performance in the T-maze task. Treatment groups: vehicle+saline (Veh+Sal, n=16), vehicle+BIBN 99 (Veh+BIBN 99, n=8), scopolamine+saline (Scop+Sal, n=20) and scopolamine+BIBN 99 (Scop+BIBN 99, n=20). Table shows alternation percentages (mean±SEM), the overall ANOVA P value and t-test P values for inter-group comparisons.

## 4.4 Discussion

The analysis of the watermaze acquisition escape latencies (Fig.4.1a) showed that BIBN 99 had no significant effect on the learning curve when administered in combination with saline. Also, the effect of the BIBN 99, given alone, on probe test performance (Fig.4.2 a-e and Table 4.3) suggests that BIBN 99 improved normal search strategy in this task. This finding agrees with and supports the results reported in Chapter 3, where it was shown that AFDX 384, another $M_2$ antagonist, had similar effects on spatial navigation.

The lack of an effect of BIBN 99 on locomotion and exploratory behaviour observed in the hole board, shows that this drug did not have effects on motor activity and/or the exploration of a novel environment. Furthermore, BIBN 99, on its own, did not affect habituation, suggesting that its administration in normal rats did not reduce spatial learning related processes and recall. The data of the experiments carried out on the T-maze task suggest that BIBN 99, given alone, did not have any significant effect on working memory processes (Olton, 1976; Spowart-Manning and Staay, 2005). Taken together, these results show that in normal rats, BIBN 99 administration, at a dose that is known to increase hippocampal acetylcholine release and to improve memory in the watermaze (Quirion et al., 1995), had a limited overall influence on cognitive functions.

BIBN 99 had no significant effect on the scopolamine-induced disruption of normal watermaze acquisition. During probe tests, it improved search strategy (the animals in the Scop+BIBN 99 group spent significantly more time in the target quadrant relative to the animals of the Scop+Sal group). This may indicate that, in this version of the Morris watermaze, BIBN 99 attenuated the memory consolidation deficits induced by scopolamine but had no significant effect on memory formation. Thus, these results support the evidence that $M_2$ receptor antagonists such as AFDX 116, AFDX 384, BIBN 99, SCH 57790,

known to enhance acetylcholine release, improve spatial memory in the watermaze (Baratti et al., 1993; Vannucchi et al., 1997; Carey et al., 2001; Quirion et al., 1995). On the other hand, it is important to consider the evidence that suggests that $M_2$ receptor activity might influence more acquisition rather retention of spatial information (Seeger et al., 2004), and also that scopolamine has been shown to primarily interfere with acquisition rather than with memory retention in spatial memory tasks (Hagan et al., 1986; Decker et al., 1990; Anagnostaras et al., 1995). Thus, further research is needed to confirm the ability of BIBN 99 to improve consolidation memory deficits in this version of watermaze.

In the hole board, treatment with BIBN 99 had only minimal effects on the scopolamine-induced hyperactivity (Fig. 4.2 and Table 4.4a). Furthermore, this drug did not attenuate the disruptive effects of scopolamine on habituation. The results in the T-maze task show that BIBN 99 normalized spontaneous alternation in animals treated with scopolamine. These results confirm the data of reported in Chapter 3, and suggest that the overall effect of BIBN 99, injected peripherally, can overcome working memory deficits induced by unselective blockade of muscarinc cholinergic transmission in the brain. Also, this data adds to the evidence showing that peripheral administration of BIBN 99 attenuates scopolamine-induced working memory deficits in the 8-arm radial maze (Quirion et al., 1995; Pike et al., 1995).

The possible relevance of these findings to cognitively impairing diseases such as AD is certainly worthy of consideration. In fact, while significant losses in both cortical and hippocampal choline-acetyl transferase activities (Davies and Maloney, 1976; Perry et al., 1977; Coyle et al., 1983; Mullan and Crawford, 1993) and muscarinic $M_2$ receptors (Mash et al, 1985; Araujo, 1988; Aubert et al., 1992) have been observed in AD, often up to 40-45% of the normal levels of these two markers are still present, even in advanced AD cases (Mash et al,

1985; Araujo, 1988; Aubert et al., 1992). It has also been shown that post-mortem cortical and hippocampal AD tissues can synthesize and release acetylcoline (Rylett et al., 1983; Nilsonn et al., 1986-87), and that a proportion of muscarinic receptors is still functional (Pearce and Potter, 1991). Thus, the use of peripherally injected molecules that selectively block $M_2$ autoreceptors, such as BIBN 99, could relieve the remaining feedback inhibition and facilitate acetylcholine release from presynaptic terminals. This strategy of treatment could have greater therapeutic efficacy than other cholinergic drug interventions that may cause activation of negative autoreceptors either by increasing the half-life of acetylcholine in the synaptic cleft (using cholinesterase inhibitors; Summers et al., 1986; Tariot et al., 1988; Davis et al., 1992) or by directly activating these autoreceptors using agonists with low selectivity (Whitehouse, 1988; Gauthier et al., 1991). Also, given that other neurotransmitter systems may also be involved in the modulation of the activity of $M_2$ antagonists (Hajos, 1998; Smiley et al., 1998) further research should be carried out to evaluate their influence on the modulation of $M_2$ receptor-mediated control of reference and working memory.

# CHAPTER 5

# Effects of dopaminergic $D_1$ ligands SKF 38393, A 68930 and SCH 23390 on cognition in the rat

## 5.1 Introduction

While a great deal of experimental evidence supports the important role of dopamine (DA) in motor function and motivation, its role in learning and memory functions is less well established. Many stimulant drugs enhance locomotion, via activation of DA systems. Treatments that impair central DA activity interfere with motor responses (Beninger, 1983; Rebec, 1984). These changes in motor function frequently complicate the interpretation of pharmacologic investigations of the role of DA in memory.

It has been shown that drugs that selectively affect subtypes of DA receptors (Kebabian and Calne, 1979) affect acquisition and performance of conditioned behaviours (Beninger et al., 1983). In particular, $D_1$ receptor activity can modulate the effects induced by the muscarinic receptor antagonist scopolamine on cognition, while $D_2$ receptors appear to not have any appreciable role (Levin, 1991). Also, there is evidence that both the stimulation and blockade of $D_1$ receptors can attenuate cognitive deficits. There is evidence that the $D_1$ partial agonist SKF 38393 and the $D_1$ antagonist SCH 23390 attenuate radial maze choice accuracy deficits induced by lesions of basal forebrain projections or by muscarinic receptor blockade (Levin, 1988; McGurck et al., 1989). It is unusual that both an agonist and an antagonist of the same receptor would have a similar effect. Thus, it has been speculated that it is possible that some non-dopaminergic side effects of SCH 23390 might be responsible for its effects on cognition. For example, it is known that SCH

23390 has antagonistic effects on both $5\text{-}HT_2$ receptors and $D_1$ receptors (Bishoff et al., 1986). It has been suggested that SCH 23390 reverses the scopolamine-induced choice accuracy deficit by means of $5\text{-}HT_2$ receptor blockade (McGurck, 1992). Relevant to this hypothesis is the finding that serotonin inhibits acetylcholine release in the hippocampus, an effect which is reversed by $5\text{-}HT_2$ blockers (Muramatsu et al, 1988). Levin and Rose (1991) have suggested that SCH 23390 and SKF 38393 might preferentially act at different subclasses of $D_1$ receptors, whose activity might differentially modulate the interaction between dopaminergic and cholinergic systems, depending on their anatomical localization in the brain. For example, DA input from the ventral tegmental area (VTA) has inhibitory influence over septohippocampal ACh cells (Costa et al., 1983; Robinson et al., 1979), and the inhibition of $D_1$ receptor activity in this area leads to an increase of the firing rate of septohippocampal ACh neurons (Durkin et al., 1986). Other evidence shows that in the basal forebrain, DA fibers from the substantia nigra and VTA provide innervation (Jones et al., 1989; Oades et al., 1987; Vertes, 1988) and appear to exert excitatory influence on the basolocortical ACh projections (Casamenti et al., 1986).

The present study was designed to provide more data that might help to clarify the effects that both the stimulation and the blockade of $D_1$ receptors may have on learning and memory. In particular, given the results of McGurk et al. (1992), who found that, in the radial arm-maze, both SKF 38393 (3 mg/kg) and SCH 23390 (0.05 mg/kg), injected systemically 15-20 before testing, improve the retention deficits induced by lesions of the cholinergic medial pathway in the rat, the present study aimed at investigating the effects of these compounds on acquisition and retention, rather than only retention, using a version of the Morris watermaze task. Further, a hole board and a T-maze were used to investigate the effects of these compounds on locomotor activity and

working memory, respectively. Moreover, in order to provide further evidence on the role of $D_1$ receptor activity on the modulation of cognitive functions the effects of the full $D_1$ agonist A 68930 were investigated in the same behavioural tasks. Finally, SKF 38393 or A 68930 were combined with the muscarinic receptor antagonist scopolamine in order to investigate the influence of $D_1$ receptor stimulation on cholinergic muscarinic cognitive disruption. As SCH 23390, when tested in all tasks, induced catalepsy when combined with just saline, its effects were never investigated in combination with scopolamine. The data relative to SCH 23390 were included in order to provide evidence that the same dose that McGurk et al., (1992) found to improve memory in the radial arm-maze had cognitively disrupting effects in the behavioural tasks used in this study.

## 5.2 Methods

Male Wistar rats, weighing 200-250 g (6-7 weeks old) at the beginning of the experiment, were tested in the Morris watermaze, hole board and T-maze.

The effects of SKF 38393 (6 mg/kg) on behaviour were first evaluated by comparison with rats injected with water for injection (vehicle) and saline (Veh+Sal), and rats injected with water for injection and SKF 38393 (Veh+SKF 38393). Subsequently, a comparison was made between a group of animals injected with 1.5 mg/kg scopolamine (same dose as that used in the study described in Chapter 3) and the same volume of saline (Scop+Sal), and a group injected with scopolamine and SKF 38393 (Scop+SKF 38393).

The experiments with A 68930 (6 mg/kg) and SCH 23390 (0.05 mg/kg) were part of a study carried out separately from the experiments with SKF 38393 in collaboration with an other experimenter who had assessed watermaze control learning with trials lasting 90s instead of the usual 120s, and with probe tests run immediately after the last trial of the 5$^{th}$ day of training (for this reason, the statistical analyses of this part of the study were carried out separately from those of the experiments with SKF 38393). Thus, controls, injected with saline (Sal+Sal), were compared with animals that received injections of saline and A 68930 (Sal+A 68930) or SCH 23390 (Sal+SCH 23390). Subsequently, a group of animals was injected with A 68930 in combination with scopolamine (1.5 mg/kg) (Scop+A 68930) and compared to a group of animals injected with the same dose of scopolamine and saline (Scop+Sal). The effects of A 68930 in combination with scopolamine were not investigated in the T-maze as this drug, on its own (combined with just saline), induced spontaneous alternation deficits. Similarly, SCH 23390 was never administered in combination with scopolamine as, when combined to just saline, it induced catalepsy in all the behavioural tasks used. Scopolamine was dissolved in water for injection and injected intraperitoneally (i.p.), whereas SKF 38393 and SCH 23390 were dissolved in

saline and injected i.p. (McGurk et al., 1992). Previously A 68930 has been dissolved in saline and injected subcutaneously (Isacson et al., 2004). In the present study A 68930 was dissolved in saline but was administered i.p. so that its effects could be compared to those of SKF 38393 and SCH 23390. All drugs were administered 20-30 min before tests in all behavioural tasks (McGurk et al., 1992; Isaacson, 2004) and immediately before or after the injection of saline or scopolamine. The order of injections was randomized across days for all tasks (see also Fig.25a for a description of the timeline of injections). The dose and method of administration of SKF 3893 and A 68930 were chosen on the basis of prelimimary results (not reported here) of experiments carried out on a small sample of rats (n= 6) whose locomotor activity was not affected by the administration of the drug relative to normal rats (n= 6). The dose and method of administration of SCH 23390 were chosen according to McGurk et al., (1992).

## 5.2.1. Treatment groups

### 5.2.1.1 SKF 38393

1) Water for injection+saline (Veh+Sal; watermaze, n=10; hole board, n=10; T-maze, n=10).

2) Water for injection+SKF 38393 (Veh+ SKF 38393; watermaze, n=10; hole board, n=10; T-maze, n=10).

3) Scopolamine+saline (Scop+Sal; watermaze, n=10; hole board, n=10; T-maze, n=10).

4) Scopolamine+SKF 38393 (Scop+ SKF 38393; watermaze, n=10; hole board, n=10; T-maze, n=10).

*5.2.1.2 A 68930 and SCH 23390*

1) saline+saline (Sal+Sal; watermaze, n=10; hole board, n=10; T-maze, n=10).

2) Saline+A 68930 (Sal+A 68930; watermaze, n=10; hole board, n=10; T-maze, n=9*).

3) Saline+SCH 23390 (Sal+SCH 23390; watermaze, n=10; hole board, n=10).

4) Scopolamine+ A 68930 (Scop+A 68930; watermaze, n=10; hole board, n=10).

*The T-maze alternation number of 1 rat of the Sal+A 68930 was excluded from analysis as it did not complete the 14-trial session within 30 min from start.

## 5.3 Results

### 5.3.1 Watermaze

*5.3.1.1 Acquisition*

*5.3.1.1.1 SKF 38393*

An overall comparison of the latencies scored during the 5 days of training revealed that all animals (n=10/group) learned the task [$F_{(4, 33)}=12$, $P< 0.0001$] and that there was a significant difference across treatment groups [$F_{(3, 36)}=29$, $P< 0.0001$]. All treatment groups, except the Scop+SKF 38393 group, showed statistically significant learning [Veh+Sal: $F_{(4, 6)}=23$, $P< 0.05$; Scop+Sal: $F_{(4, 6)}=5$, $P< 0.05$; Veh+SKF 38393: $F_{(4, 6)}=16$, $P< 0.05$]. Comparison between groups Veh+Sal and Veh+SKF 38393 (Fig. 5.1a) showed no significant difference for their learning profiles. Also, a day/group comparison revealed a significant day/treatment interaction [($F_{(4, 15)}=7.2$, $P< 0.05$]. The learning curves of groups Veh+Sal and Scop+Sal (Fig. 5.1a) were significantly different [$F_{(1, 18)}=17$, $P< 0.001$]. Finally, no significant inter-group difference or

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day/treatment interaction was found between groups Scop+SKF 38393 and Scop+Sal (Fig. 5.1b). Table 5.1 summarizes the outcome of overall and inter-group comparisons.

| Comparisons | df | F | P< |
|---|---|---|---|
| Overall (between groups) | 3, 36 | 29 | 0.0001 |
| Overall (between trials) | 4, 33 | 12 | 0.0001 |
| Veh+Sal vs Veh+SKF 38393 | 1, 18 | 0.13 | NS |
| Veh+Sal vs Scop+Veh | 1, 18 | 17 | 0.001 |
| Scop+Sal vs Scop+SKF 38393 | 1, 18 | 1 | NS |

**Table 5.1**

Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on acquisition of the watermaze task. Treatment groups: vehicle+saline (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393 (Scop+SKF 38393, n=10). Table shows F scores and P values for inter-group comparisons.

**a**
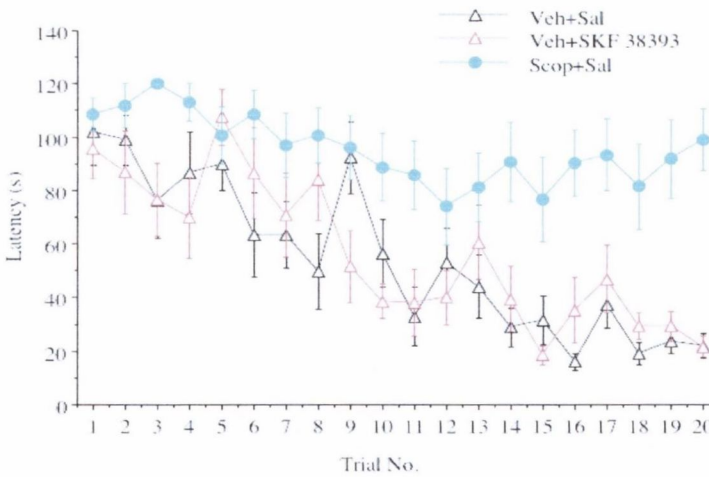
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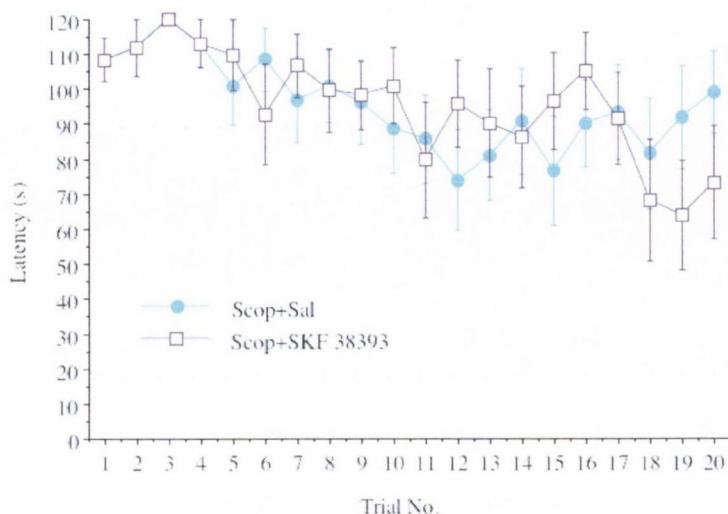


**Fig 5.1 (a-b)**

Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on acquisition in the waremaze. Treatment groups: vehicle+saline (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393 (Scop+SKF 38393, n=10); a) Veh+Sal, Veh+SKF 38393, Scop+Veh; b) Scop+Sal and Scop+SKF 38393. Values are means±SEM.

*5.3.1.1.2 A 68930 and SCH 23390*

An overall comparison of the escape latencies scored during the 5 days of training revealed that all animals (n=10/group) learned the task [$F_{(4, 42)}=40$, $P< 0.0001$] and that there was a significant difference across treatment groups [$F_{(4, 45)}=55.5$, $P< 0.0001$].

All treatment groups, except groups Scop+Sal, Scop+A 68930 and Sal+SCH 23390, showed statistically significant learning [Sal+Sal: $F_{(4, 6)}=34$, $P< 0.001$; Sal+A 68930: $F_{(4, 6)}=21.5$, $P< 0.05$].

Comparison between groups Sal+Sal and Sal+A 68930 showed no significant difference for their acquisition profiles and also no significant day/treatment interaction. Conversely, a significant difference was found between groups Sal+Sal and Sal+SCH 23390 [$F(1, 17)=43.6$, $P< 0.0001$]. A significant day/treatment interaction was also found for this comparison [$F(4, 14)=10$, $P< 0.001$]. Comparison between groups Sal+Sal and Scop+Sal showed a significant difference [$F(1, 18)=126$, $P< 0.0001$] and also a significant day/treament interaction [$F(4, 15)=126$; $P< 0.0001$]. Finally, a significant difference was found between groups Scop+Sal and Scop+A 68930 [$F(1, 18)=4.6$, $P< 0.05$]. No significant day/treatment interaction was found for this comparison. These results are shown in Table 5.2 and Fig. (5.2a-b).

| Comparisons | df | F | P< |
|---|---|---|---|
| **Overall (between groups)** | 4, 45 | 55.5 | 0.0001 |
| **Overall (between trials)** | 4, 42 | 40 | 0.0001 |
| **Sal+Sal  vs Sal+A 68930** | 1, 18 | 1 | NS |
| **Sal+Sal  vs Sal+SCH 23390** | 1, 17 | 43.6 | 0.0001 |
| **Scop+Sal  vs Scop+Sal** | 1, 18 | 126 | 0.0001 |
| **Scop+Sal  vs Scop+ Sal+A 68930** | 1, 18 | 4.6 | 0.05 |

**Table 5.2**

Effects of scopolamine (1.5 mg/kg) and A 68930 (6 mg/kg) and SCH 23390 (0.05 mg/kg) on acquisition of the watermaze task. Treatment groups: saline+saline (Sal+Sal, n=10), saline+A 68930 (Sal+A 68930, n=10), saline+SCH 23390 (Sal+SCH 23390, n=10), and scopolamine+saline (Scop+Sal, n=10); a) Sal+Sal, Sal+A 68930, Sal+SCH 23390, and Scop+Sal; b) Scop+Sal and Scop+A 68930. Table shows F scores and P values for inter-group comparisons.

**a**
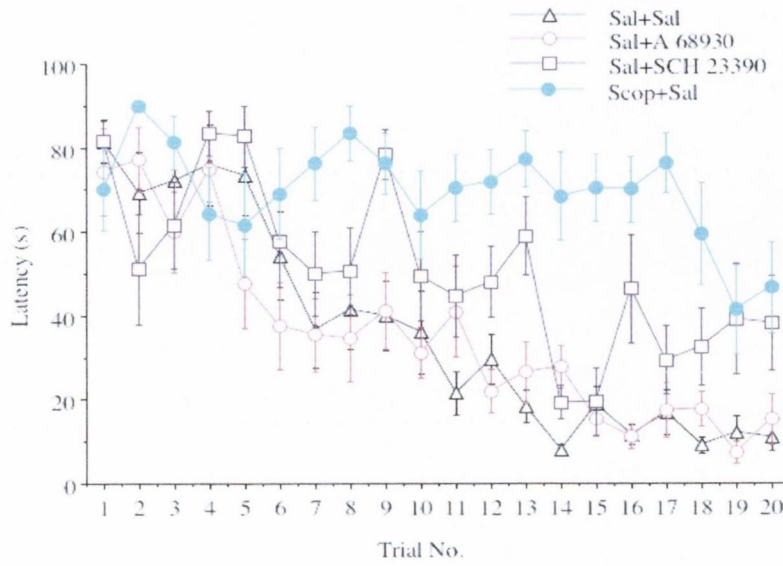


**b**
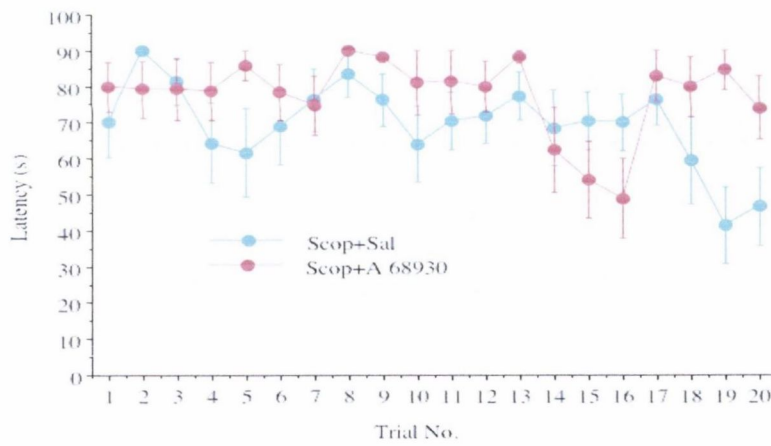


**Fig 5.2 (a-b)**

Effects of scopolamine (1.5 mg/kg), A 68930 (6 mg/kg) and SCH 23390 (0.05 mg/kg) on acquisition in the watermaze. Treatment groups: saline+saline (Sal+Sal, n=10), saline+A 68930 (Sal+A 68930, n=10), saline+SCH 23390 (Sal+SCH 23390, n=10), and scopolamine+saline (Scop+Sal, n=10); a) Sal+Sal, Sal+A 68930, Sal+SCH 23390, and Scop+Sal; b) Scop+Sal and Scop+A 68930. Values are means±SEM.

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## 5.3.1.2 Probe test

### 5.3.1.2.1 SKF 38393

None of the animals in the Scop+SKF 38393 group found the platform location. Thus, a latency of 60 seconds was assigned to all of them. Since this measure had a standard error mean equal to zero, a non-parametric test (Wilcoxon/Kruskall-Wallis test) was used to carry out statistical comparisons that included group Scop+SKF 38393. The outcome of probe tests showed an overall significant difference across groups for all values measured except for the time spent in the opposite quadrant. A within group analysis of quadrant bias (paired t-test) revealed that the animals of the Veh+Sal and Veh+SKF 38393 groups spent significantly more time in the target rather than in the opposite quadrant (Veh+Sal: $P < 0.05$; Veh+SKF 38393: $P < 0.05$) whereas, no quadrant bias was observed in groups Scop+Sal and Scop+SKF 38393.

No statistical difference was found between groups Veh+Sal and Veh+SKF 38393. A comparison between groups Veh+Sal and Scop+Sal revealed that the animals of group Scop+Sal had higher latency values, spent less time in the target quadrant, travelled a longer distance and had a faster swim speed. Finally, group Scop+SKF 38393 scored significantly higher escape lantencies than group Scop+Sal. These results are shown in Fig. 5.3 (a-e) and in Table 5.3.

a



b



132
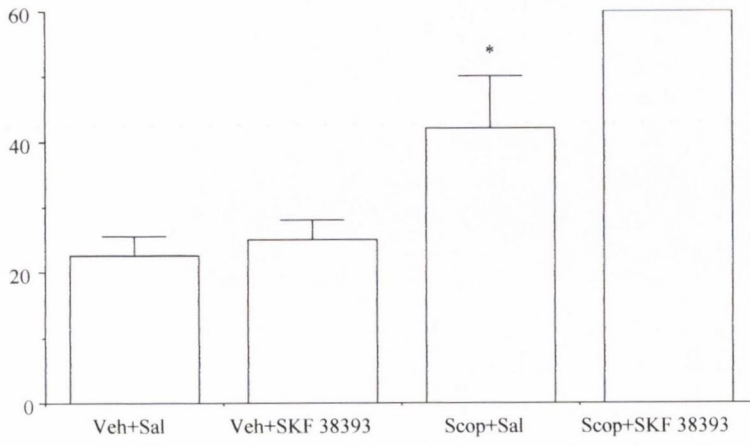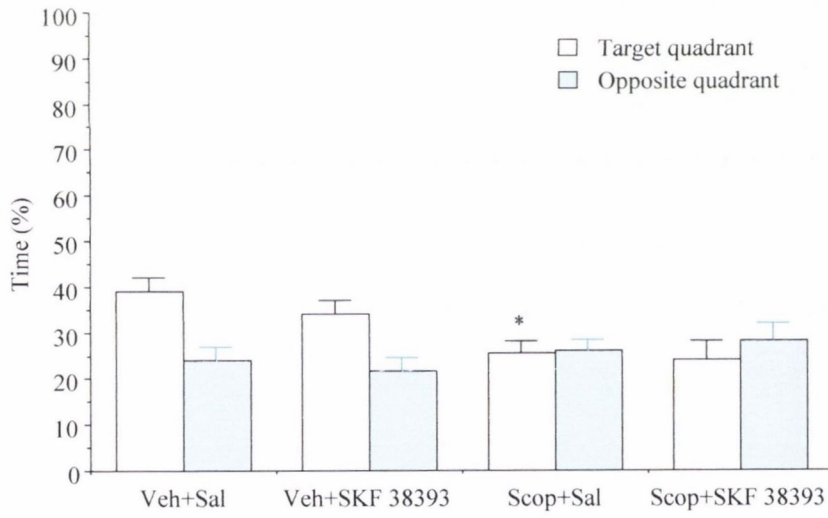
**c**



**d**

**e**



**Fig. 5.3 (a-e)**

Effects of scopolamine (1.5 mg/kg), and SKF 38393 (6 mg/kg) on probe test performance. Treatment groups: vehicle+vehicle (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10), and scopolamine+SKF 38393 (Scop+SKF 38393, n=10); a) latency to the platform location; b) percent time spent in the target quadrant vs. percent time spent in the opposite quadrant; c) percent time spent in the periphery of the tank; d) total distance travelled; e) swim speed. Asterisk (*) and symbol "§" indicate statistical difference from groupsVeh+Sal and Scop+Sal, respectively. Values are means±SEM.

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| a<br>Group | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (s) |
|---|---|---|---|---|---|---|---|---|
| 1 Veh+Sal | 22.5±3 | 39±3 | 24±3 | 15±5 | 2±0.5 | 905±108 | 19±1.5 | 57±5 |
| 2 Veh+SKF 38393 | 25±3 | 34±2 | 21.5±3 | 13±4.5 | 2.6±1 | 1205±106 | 19.5±2 | 48±5 |
| 3 Scop+Sal | 42±8 | 25.5±3.5 | 26±2.5 | -1±5 | 2±0.3 | 1613±135 | 25±3 | 70±8 |
| 4 Scop+SKF 38393 | 60 | 24±3 | 28±4 | -4±6.5 | 1±0.3 | 1629±144 | 27±2.5 | 77±6 |
| Overall ANOVA (P <) | 0.001* | 0.05 | NS | 0.05 | NS | 0.001 | 0.05 | 0.05 |

t-test P values (P<)

| | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (s) |
|---|---|---|---|---|---|---|---|---|
| Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS | NS | NS |
| Gr.1 Vs. Gr. 3 | 0.05 | 0.05 | NS | 0.05 | NS | 0.001 | 0.05 | NS |
| Gr.3 Vs. Gr. 4 | 0.05* | NS | NS | NS | NS | NS | NS | NS |

**Table 5.3**
Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on probe test performance. Treatment groups: vehicle+vehicle (Veh+Sal, (n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393, n=10). Table shows mean±SEM values, P values for the overall comparison between groups (ANOVA) and P values for inter-group comparisons (t-test). Asterisks (*) indicate comparisons with the Scop+SKF 38393 group (this group's values had no SEM) using a Wilcoxon/Kruskall-Wallis test.

*5.3.1.2.2 A 68930 and SCH 23390*

An overall comparison revealed a significant difference across treatment groups for all values measured. An analysis of the target quadrant bias revealed that all groups except the Sal+Sal group (P< 0.05) had no preference for the target quadrant. The animals of group Sal+A 68930 travelled a significantly longer distance and had a significantly higher swim speed relative to group Sal+Sal. Also, relative to group Sal+Sal, the animals of group Sal+SCH 23390 spent less time in the target quadrant, more time in the opposite quadrant, swam a significantly shorter distance and had a lower swim speed. The animals of the Sal+SCH 23390 group had normal latency values during probe tests although, after reaching the target location, they swam in a different direction (away from the target quadrant) and spent the rest of the trial floating in a confined area of the tank. The animals of the Scop+Sal group reached the platform location with higher latency values and spent more time in the periphery of the tank than the animals of the Sal+Sal group. No significant difference was found between the animals of groups Scop+A 68930 and Scop+Sal. These results are shown in Fig. 5.4 (a-e) and in Table 5.4.

**a**



**b**



137
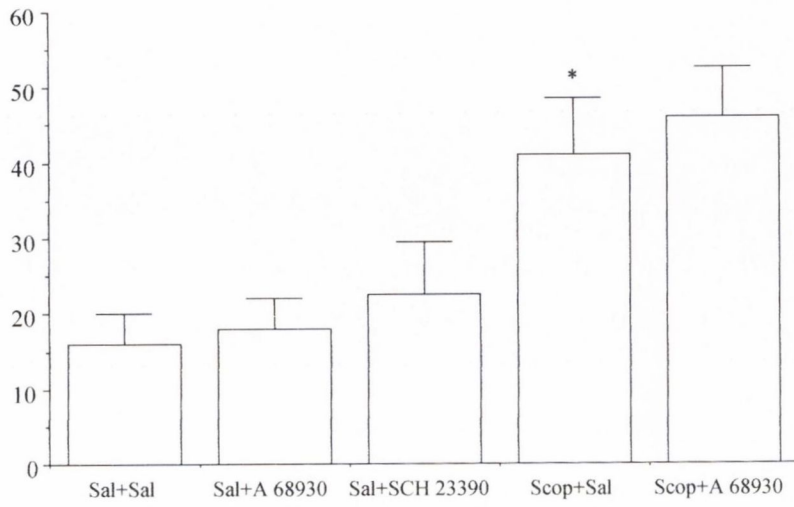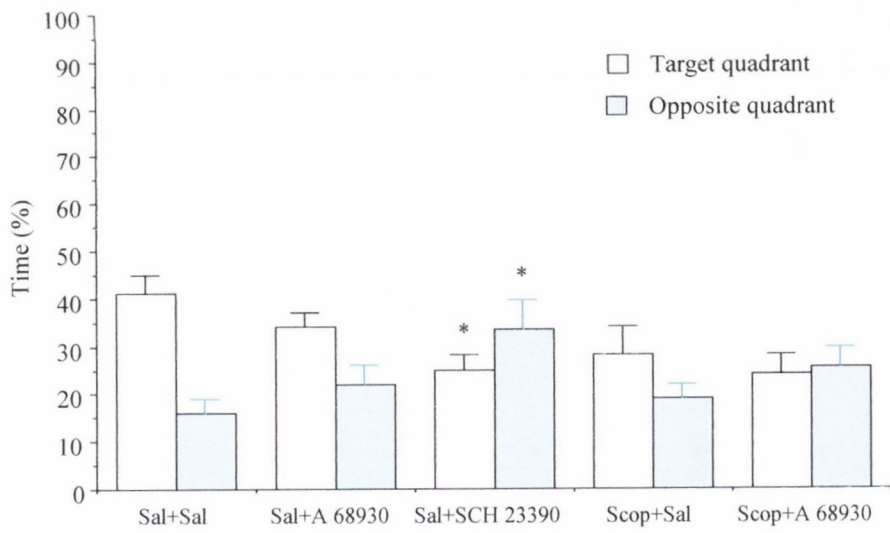
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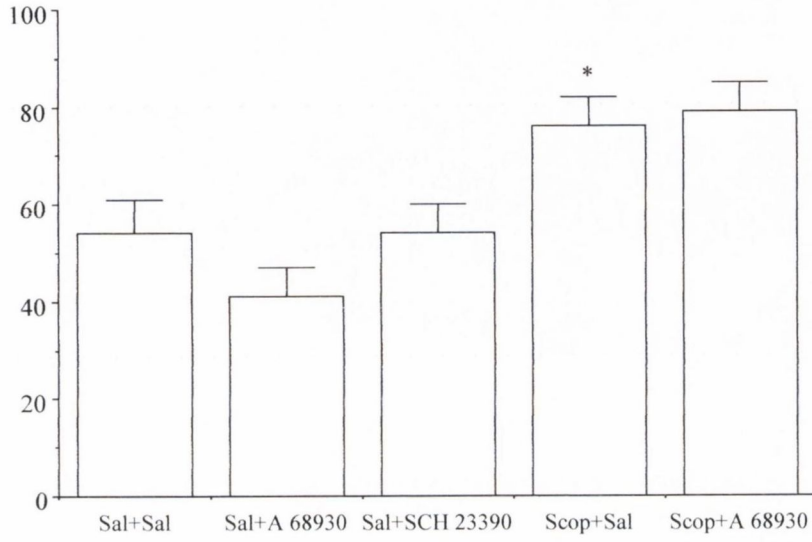


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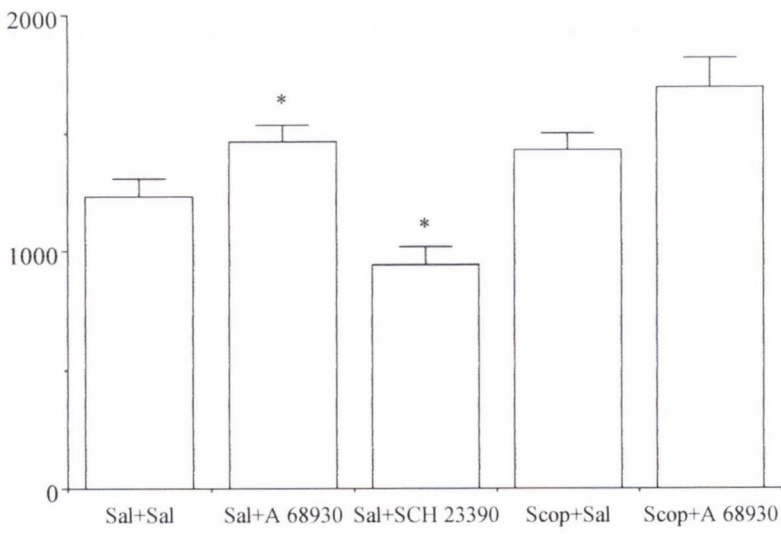
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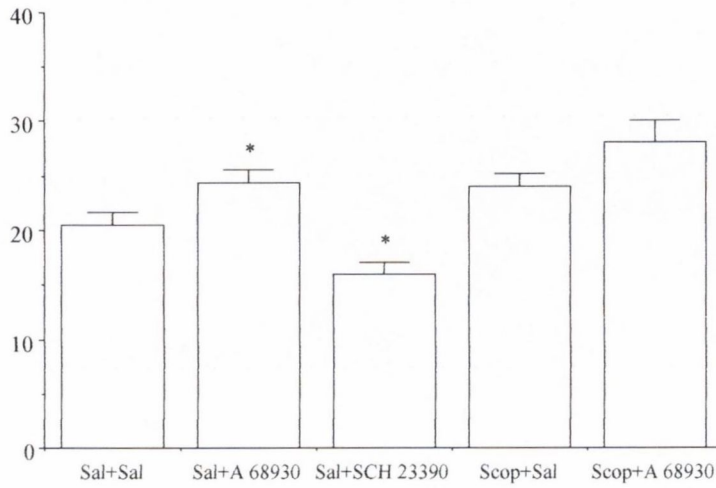


**Fig. 5.4 (a-e)**

Effects of scopolamine (1.5 mg/kg), A 68930 (6 mg/kg) and SCH 23390 (0.05 mg/kg) on probe test performance. Treatment groups: saline+saline (Sal+Sal, n=10), saline+A 68930 (Sal+A 68930, n=10), saline+SCH 23390 (Sal+SCH 23390, n=10), scopolamine+saline (Scop+Sal, n=10), and scopolamine+A 68930 (Scop+A 68930, n=10); a) latency to the platform location; b) percent time spent in the target quadrant vs. percent time spent in the opposite quadrant; c) percent time spent in the periphery of the tank; d) total distance travelled; e) swim speed. Asterisk (*) indicates statistical difference from the Sal+Sal group. Values are means±SEM.

**a**

| | Group | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (s) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Veh+Sal | 22.5±3 | 39±3 | 24±3 | 15±5 | 2±0.5 | 905±108 | 19±1.5 | 57±5 |
| 2 | Veh+SKF 38393 | 25±3 | 34±2 | 21.5±3 | 13±4.5 | 2.6±1 | 1205±106 | 19.5±2 | 48±5 |
| 3 | Scop+Sal | 42±8 | 25.5±3.5 | 26±2.5 | -1±5 | 2±0.3 | 1613±135 | 25±3 | 70±8 |
| 4 | Scop+SKF 38393 | 60 | 24±3 | 28±4 | -4±6.5 | 1±0.3 | 1629±144 | 27±2.5 | 77±6 |
| | Overall ANOVA (P <) | 0.001* | 0.05 | NS | 0.05 | NS | 0.001 | 0.05 | 0.05 |

t-test P values (P<)

| | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) | Time in periphery (s) |
|---|---|---|---|---|---|---|---|---|
| Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS | NS | NS |
| Gr.1 Vs. Gr. 3 | 0.05 | 0.05 | NS | 0.05 | NS | 0.001 | 0.05 | NS |
| Gr.3 Vs. Gr. 4 | 0.05* | NS | NS | NS | NS | NS | NS | NS |

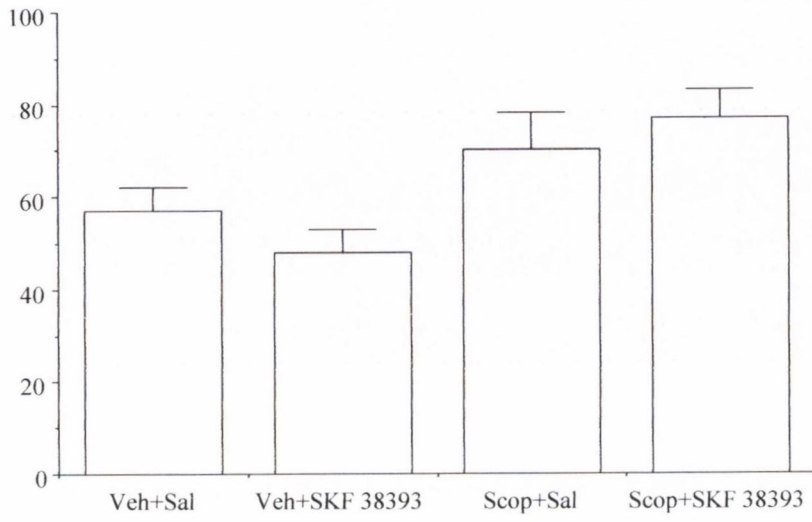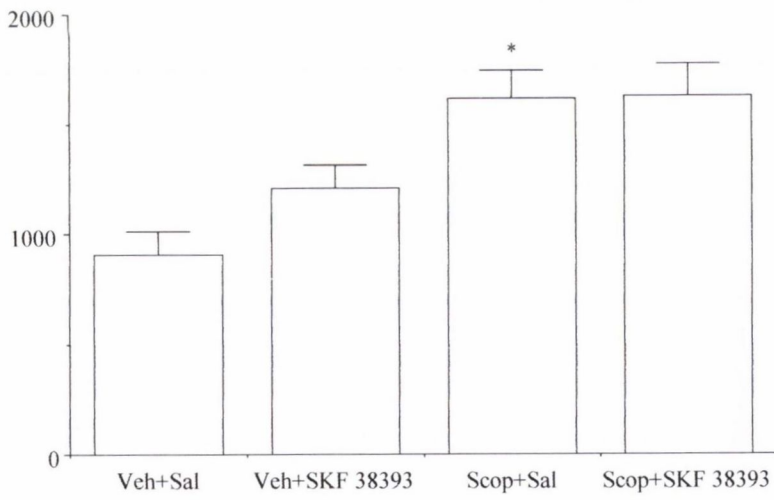**Table 5.3**
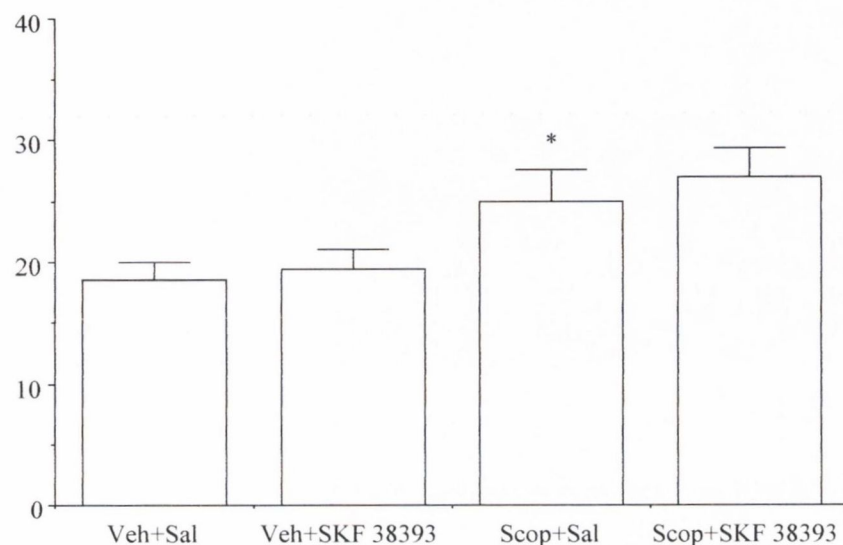Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on probe test performance. Treatment groups: vehicle+vehicle (Veh+Sal, (n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393, n=10). Table shows mean±SEM values, P values for the overall comparison between groups (ANOVA) and P values for inter-group comparisons (t-test). Asterisks (*) indicate comparisons with the Scop+SKF 38393 group (this group's values had no SEM) using a Wilcoxon/Kruskall-Wallis test.

### 5.3.2 Hole board

*5.3.2.1 SKF 38393*

No overall difference was observed across treatment groups (n=10/group) during the first half of the experiment (Fig. 5.5 and Table 5.5), whereas during the second half, there was a significant difference only for the number of head dips and rears.

No difference was found between groups Veh+Sal and Veh+SKF 38393. Comparison between groups Veh+Sal and Scop+Sal revealed a smaller number of lines crossed in the Scop+Sal group during the first half of the experiment, whereas no inter-group difference was found during the second half. Finally, no difference was found between groups Scop+Sal and Scop+SKF 38393. Groups Veh+Sal and Veh+SKF 38393 showed a statistically significant reduction of all the values measured in the second half of the experiment, whereas in groups Scop+Sal and Scop+SKF 38393, the number of lines crossed was the only value significantly reduced. The above results are shown in Fig. 5.5 and Table 5.5 (a, b).

**a**
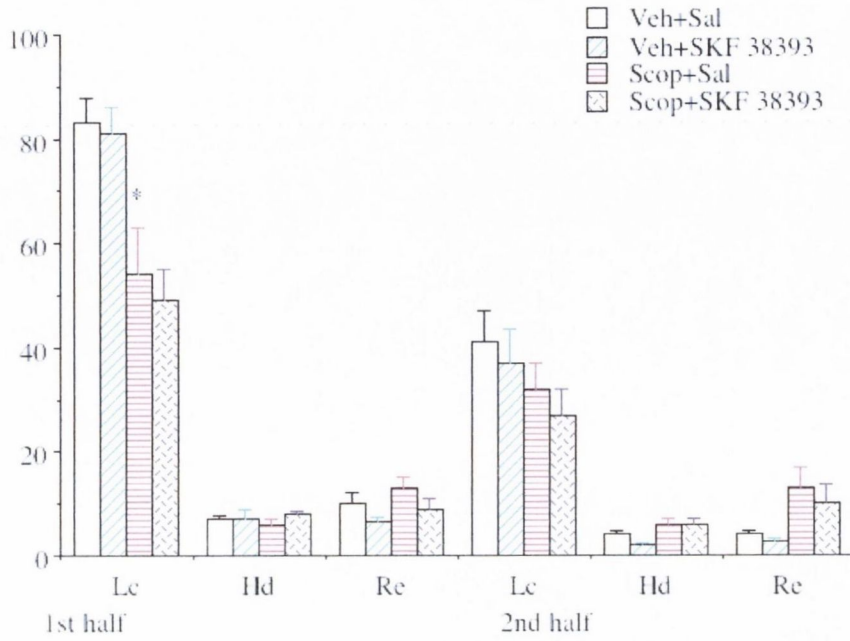


**Fig. 5.5**

Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on performance in the hole board task. Treatment groups: vehicle+saline (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10), and scopolamine+SKF 38393 (Scop+SKF 38393, n=10). Asterisk (*) indicates statistically significant difference from the Veh+Sal group. Vaues are means±SEMS.

**a**

| | Group | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|---|
| | | 1st half | 2st half | 1st half | 2nd half | 1nd half | 2nd half |
| 1 | Veh+Sal | 83±5 | 41±6 | 7±1 | 4±1 | 10±2 | 4±1 |
| 2 | Veh+SKF 38393 | 81±5 | 37±6.5 | 7±1 | 2±0.5 | 7±1 | 3±1 |
| 3 | Scop+Sal | 54±9 | 32±5 | 6±1 | 6±1 | 13±2 | 13±4 |
| 4 | Scop+SKF 38393 | 49±6 | 27±5 | 8±1 | 6±1 | 9±2 | 10±3.5 |
| | Overall ANOVA P value (P<) | NS | NS | NS | 0.05 | NS | 0.05 |

t t-test P values (P<)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Gr.1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS |
| | Gr.1 Vs. Gr. 3 | 0.05 | NS | NS | NS | NS | NS |
| | Gr.3 Vs. Gr. 4 | NS | NS | NS | NS | NS | NS |

**b**

| | Groups | P values (P<) | | |
|---|---|---|---|---|
| | | lc2-lc1 | hd1-hd2 | re2-re1 |
| 1 | Veh+Sal | 0.05 | 0.05 | 0.05 |
| 2 | Veh+SKF 38393 | 0.05 | 0.05 | 0.05 |
| 3 | Scop+Veh | 0.05 | NS | NS |
| 4 | Scop+SKF 38393 | 0.05 | NS | NS |

**Table 5.5 (a-b)**

Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on performance in the hole board task. Treatment groups: vehicle+saline (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393, n=10); a) mean±SEM values, P values for the overall comparison between groups (ANOVA) and t-test P values for inter-group comparisons; b) within group comparisons (Wilcoxon signed-rank test) between the levels of activity scored during the first and the second half of the experiment. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing.

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*5.3.2.2 A 68930 and SCH 23390*

There was an overall difference across treatment groups (n=10/group) in the first half of the experiment, for the number of lines crossed and rears but not for the number of head dips. During the second half, there was a significant difference only for the number of lines crossed.

Relative to the Sal+Sal group, the animals of group Sal+A 68930 had a significantly lower number of lines crossed, head dips and rears during the first half of the experiment but had no difference during the second half. Comparison between groups Sal+Sal and Sal+SCH 23390 revealed that the animals of group Sal+SCH 23390 had a significantly smaller number of lines crossed and rears during the first half of the experiment and that there was no inter-group difference during the second half. The animals of the Scop+Sal group had a higher number of lines crossed during the second half of the experiment relative to group Sal+Sal. Finally, relative to group Scop+A 68930, the Scop+Sal group scored a higher number of lines crossed and rears during the first half of the experiment. No significant difference was observed during the second half of the experiment.

Group Sal+Sal showed significant reduction for all the values measured during the second half of the experiment. Groups Scop+Sal and Scop+A 68930 showed reduction for none of the values measured during the second half of the experiment. In the group Sal+SCH 23390, the numbers of lines crossed and head dips were reduced during the second half of the experiment relative to the first half whereas, in group Sal+A 68930, only the number of lines crossed was significantly reduced. These results are shown in Fig. 5.6 and Table 5.6 (a-b).

**b**
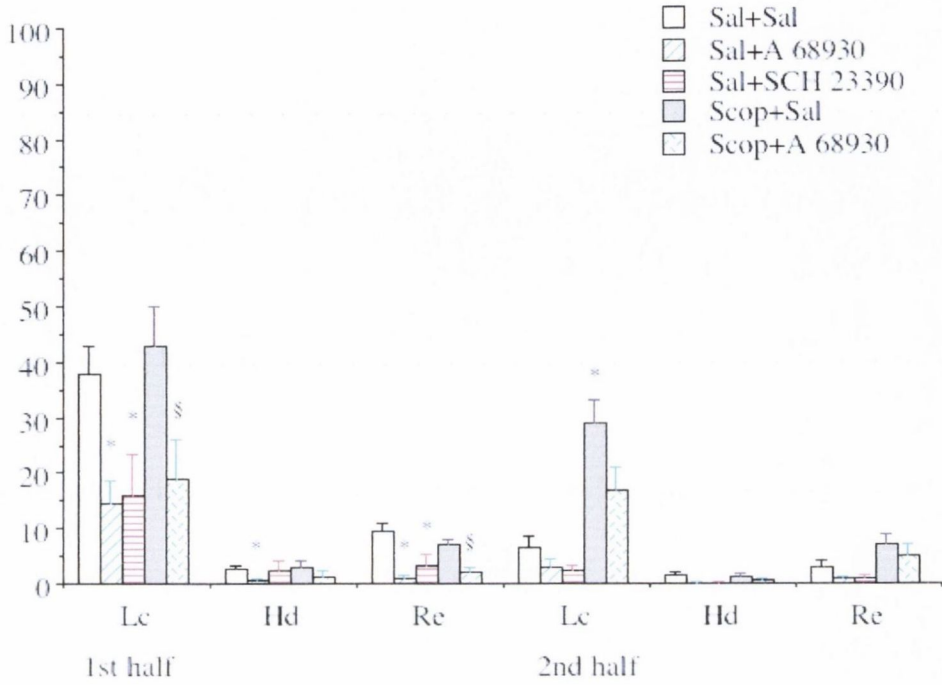


**Fig. 5.6**

Effects of scopolamine (1.5 mg/kg), A 68930 (6 mg/kg) and SCH 23390 (0.05 mg/kg) on performance in the hole board task. Treatment groups: saline+saline (Sal+Sal, n=10), saline+A 68930 (Sal+A 68930, n=10), saline+SCH 23390 (Sal+SCH 23390, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+A 68930 (Scop+A 68930, n=10). Asterisks (*) indicate statistical difference from the Sal+Sal group; Symbol "§" indicates statistical difference from the Scop+Sal group. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing. Values are means ±SEM.

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**a**

| Group | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|
| | 1st half | 2st half | 1st half | 2nd half | 1nd half | 2nd half |
| 1 Sal+Sal | 38±5 | 6.5±2 | 2.5±0.6 | 1.5±0.6 | 9.5±1.4 | 3±1 |
| 2 Sal+A 68930 | 14.6±4 | 3±1.5 | 0.6±0.4 | 0.1±0.1 | 1±0.4 | 1±0.3 |
| 3 Sal+SCH 23390 | 16±7.4 | 2.3±1 | 2.5±1.6 | 0.1±0.1 | 3±2 | 1±0.5 |
| 4 Scop+Sal | 43±7 | 29±4 | 3±1 | 1.3±0.5 | 7±1 | 7±2 |
| 5 Scop+A 68930 | 19±7 | 17±4 | 1.3±1 | 0.5±0.5 | 2±1 | 5±2 |
| Overall ANOVA P value (P<) | 0.05 | 0.0001 | NS | NS | 0.001 | NS |

t-test P values (P<)

| | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|
| Gr.1 Vs. Gr. 2 | 0.05 | NS | 0.05 | NS | 0.001 | NS |
| Gr.1 Vs. Gr. 3 | 0.05 | NS | NS | NS | 0.001 | NS |
| Gr.1 Vs. Gr. 4 | NS | 0.0001 | NS | NS | NS | NS |
| Gr.4 Vs. Gr. 5 | 0.05 | NS | NS | NS | 0.05 | NS |

**b**

| Groups | P Values (P<) | | |
|---|---|---|---|
| | lc2-lc1 | hd2-hd1 | re2-re1 |
| 1 Sal+Sal | 0.0001 | 0.05 | 0.05 |
| 2 Sal+A 68930 | 0.05 | NS | NS |
| 3 Sal+SCH 23390 | 0.05 | 0.05 | NS |
| 4 Scop+Sal | NS | NS | NS |
| 5 Scop+A 68930 | NS | NS | NS |

**Table 5.6 (a-b)**

Effects of scopolamine (1.5 mg/kg), A 68930 (6 mg/kg) and SCH 23390 (0.05 mg/kg) on performance in the hole board task. Treatment groups: saline+saline (Sal+Sal, n=10), saline+A 68930 (Sal+A 68930, n=10), saline+SCH 23390 (Sal+SCH 23390, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+A 68930 (Scop+A 68930, n=10); a) mean±SEM values, P values for the overall comparison between groups (ANOVA) and t-test P values for inter-group comparisons; b) within group comparisons (Wilcoxon signed-rank test) between the levels of activity scored during the first and the second half of the experiment. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing.

### 5.3.3 T-maze

*5.3.3.1 SKF 38393*

Overall comparison (ANOVA) across treatment groups (n=10/group) revealed a significant difference for their alternation percentages (P< 0.0001)

There was no significant difference of alternation percentage between the Veh+Sal and the Veh+SKF 38393 group. Comparison between rats injected with scopolamine (1.5 mg/kg) and saline (Veh+Sal) and rats injected with scopolamine and SKF 38393 (Scop+SKF 38393) showed significantly higher (P< 0.05) alternation percentages in the latter group.

Fig. 5.7 and Table 5.7 summarize the outcome of the experiment.

**a**
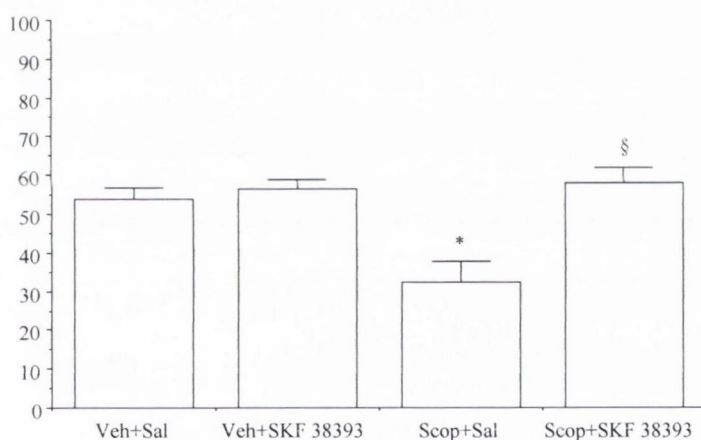


**Fig 5.7**

Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on spontaneous alternation in the T-maze task. Treatment groups: vehicle+saline (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393, n=10). Asterix (*) and symbol "§" indicate statistically significant difference from groups Veh+Sal and Scop+Sal, respectively. Values are means±SEM.

| | Group | Alternation (%) |
|---|---|---|
| 1 | **Veh+Sal** | 54±2 |
| 2 | **Veh+SKF 38393** | 56±3 |
| 3 | **Scop+Sal** | 32±5.5 |
| 4 | **Scop+SKF 38393** | 58±4 |
| | **Overall ANOVA P value (P<)** | 0.0001 |

**t-test P values (P<)**

| | |
|---|---|
| **Gr.1 Vs. Gr. 2** | NS |
| **Gr.1 Vs. Gr. 3** | 0.05 |
| **Gr.3 Vs. Gr. 4** | 0.05 |

**Table 5.7**

Effects of scopolamine (1.5 mg/kg) and SKF 38393 (6 mg/kg) on performance in the T-maze task. Treatment groups: vehicle+saline (Veh+Sal, n=10), vehicle+SKF 38393 (Veh+SKF 38393, n=10), scopolamine+saline (Scop+Sal, n=10) and scopolamine+SKF 38393 (Scop+SKF 38393, n=10). Table shows means±SEM, P values.for overall analysis (ANOVA) and P values for inter-group comparisons (t-test).

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## 5.3.3.2 A 68930 and SCH 23390

There was a significant overall difference (P< 0.05) across treatment groups, for the alternation percentages scored.

Treatment with A 68930 only (Sal+A 68930, n=9) reduced normal (Sal+Sal, n=10) alternation (P< 0.001). In particular, one rat of group Sal+A 68930 did not complete the 14-trial session within 30 minutes, and for this reason its alternation number was excluded from statistical analysis. Similarly, all animals of group Sal+SCH 23390 (n=10) were excluded from analysis. The dose of SCH 23390 used in the present experiments seemed to reduce locomotion and exploration in this paradigm (see results in the hole board task for comparison), as most of the rats stopped moving and exploring 2-3 trials after the forced trial. Given the outcome of the experiments when both A 68930 and SCH 23390 were given alone, their effects on T-maze performance were not tested in combination with scopolamine. Fig. 5.8, and Table 5.8 summarize the outcome of the experiment.
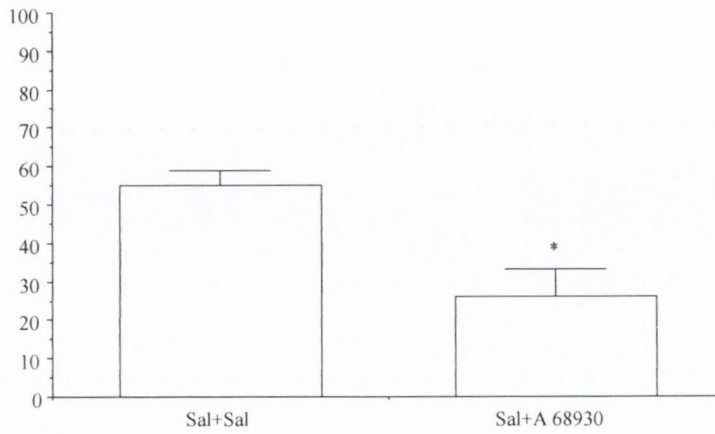
**Fig. 5.8**

Effects of A 68930 (6 mg/kg) on spontaneous altenation in the T-maze task test for the treatment groups: saline+saline (Sal+Sal, n=10) and saline+A 68930 (Sal+A 68930, n=9). Asterix (*) indicates statistically significant difference to group Sal+Sal. Values are means±SEM.

| | Group | Alternation (%) |
|---|---|---|
| 1 | Sal+Sal | 55±7 |
| 2 | Sal+A 68930 | 26±7 |
| | t-test P value (P<) | 005 |

**Table 5.8**

Effects of scopolamine (1.5 mg/kg) and A 68390 (6 mg/kg) on performance in the T-maze task test for the treatment groups: saline+saline (Sal+Sal, n=10) and saline+A 68930 (Sal+A 68930, n=9). Table shows means±SEM, P values for the overall (ANOVA) comparison and for the comparison between groups Sal+Sal and Sal+A 68930 (t-test).

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## 5.4 Discussion

The results of the present study suggest that SKF 38393 (6 mg/kg) alone did not have an overall effect on cognitive functions and/or general motor activity. Conversely, the effects of A 68930 (6 mg/kg) or SCH 23390 (0.05 mg/kg), given alone, suggest that both cognition and motor activity were affected by these two dopamine receptor ligands. In particular, A 68930 had disruptive effects on watermaze probe test performance, but the deficit induced by this treatment was less marked than that caused by SCH 23390, which also disrupted acquisition and decreased exploration during probe tests. The reduction of hole board activity induced by A 68930 and SCH 23390 may be interpreted as the result of an influence on motor activity and motivation to explore novel environments. Also, an increase in the level of anxiety caused by the exposure to a novel environment or to a new task cannot be ruled out in the groups that received A 68930. Increased anxiety may have induced a rise in the swim speed in the watermaze probe tests. The absence of the escape platform in the probe tests may have worked as an anxiogenic stimulus.

Given that SKF 38393 has been reported to exert just 30-50% of the efficacy of DA (O'Boyle et al., 1989) the comparison of the effects induced by SKF 38393 and A 68930 on rat behaviour in this study suggests that an optimal level of stimulation of $D_1$ receptors might be crucial to control cognitive functions and that, an over stimulation of these receptors can trigger events that induce memory disruption and suppression of motor activity. The marked effects that SCH 23390 had in the watermaze and in the hole board suggest that the disruption of spatial navigation and the reduction of motor and exploratory activity might be related to $D_1$ receptor blockade in the brain. Motor slowing and catalepsy are a prominent feature of many dopaminergic agents that frequently complicates the interpretation of the behavioural effects of these compounds (e.g., Bushnell and Levin, 1993; Isacson et al., 2004). However,

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previous studies have shown that SCH 23390 can have both beneficial and disruptive effects on memory. For example, it has been found that SCH 23390 blocked the dihydrexidine-induced acetylcholine increase in the rat striatum and frontal cortex (Steele et al., 1997), but that it could also diminish or reverse the adverse effects on working memory induced by medial cholinergic pathway lesions (McGurk et al., 1992). From the analysis of the present results it is difficult to evaluate the effects SCH 23390 on cognition because of its marked effects on motor functions. On the basis of these considerations it would be important to evaluate the effects of A 68930 and SCH 23390 in the same behavioural tasks at lower/higher doses.

Although SKF 38393 did not reverse the memory deficits caused by scopolamine (1.5 mg/kg) in the watermaze, it normalized the alternation percentage in the T-maze task. In the hole board, SKF 38393 treatment had no effect on scopolamine-induced suppression of locomotion. However, given that usually systemic administration of scopolamine induces locomotor hyperactivity (Abood and Biel, 1962) and that in this study its administration had very little or no effect on general activity or on habituation, it is difficult to determine whether these results were due to the administration of SKF 38393. The results in the T-maze task are consistent with the evidence that shows that SKF 38393 can facilitate in vivo acetylcholine release in the hippocampus (Imperato et al., 2003) and that working memory deficits caused by cholinergic hypofunction (induced by either lesion of basal forebrain projections or muscarinic receptor blockade) can be attenuated by the stimulation of $D_1$ receptors following the administration of SKF 38393 (Levin and Rose, 1991; Steele, 1997). The inability of SKF 38393 to influence spatial memory in the watermaze could be due to its low intrinsic activity (O'Boyle et al., 1989). The $D_1$ receptor is positively coupled to adenylate cyclase activity. In the hippocampus, striatum and prefrontal cortex, the stimulation of this receptors

leads to the formation of cAMP that subsequently activates a cAMP-dependent protein kinase A (PKA) by binding to its regulatory subunits. There is a general agreement that the cellular mechanisms triggered by the intraction cAMP/PKA in these brain areas influence synaptic plasticity and memory mechanisms (see Jay, 2003 for a review). Thus, it could be speculated that the low intrinsic activity of a partial agonist such as SKF 38393 might explain its lack of an effect in the watermaze. Perhaps the level of adenylate cyclase stimulation needs to be higher in order to attenuate memory scopolamine-induced deficits in this version of watermaze, although the experiments with A 68930 (a full agonist) showed that the administration of this drug had no beneficial effect on the adverse effects of scopolamine in the same task. Given the behaviour observed in the hole board when A 68930 was given alone (it reduced general activity) it might be suggested that strong stimulation of $D_1$ receptors (at least at the dose used here) exacerbated the disruptive effects on watermaze spatial memory induced by scopolamine. Similar considerations might be made for the behaviour of rats tested in the hole board after injection of A 68930 and scopolamine (animals injected with A 68930 and scopolamine failed to habituate and there was a dramatic reduction of locomotion relative to animals that received scopolamine+saline).

Altogether, this study indicates that the dopaminergic system can influence working memory processes through activation of $D_1$ receptors. Also, the present experiments suggest that the blockade and/or stimulation of $D_1$ receptors induced by SCH 23390 and A 68930, respectively can have disruptive effects on cognition and on motor functions. Further studies should be carried out to investigate the effect of these drugs in order to evaluate their role in the control of cognitive functions.

# CHAPTER 6

# Interactive effects of 5-HT$_{1A}$ agonist, ipsapirone with a sub-amnesic dose of scopolamine on rat spatial navigation and exploratory activity.

## 6.1 Introduction

It is well known that serotonergic transmission in the brain plays an important role in mnemonic processes (e.g., Meneses and Hong, 1997; also see Meneses, 2003 for a recent review on the role of serotonin in learning and memory). Experimental evidence has shown that serotonergic agents can influence learning and memory through a variety of receptor subclasses, producing variable effects depending on the receptor subtype, site of administration (systemic or central), type of drug used, timing of drug administration (pre-training, post-training, pre-test) and behavioural task used (Meneses, 2003; also, Table 6.1 shows a summary of the effects on learning and memory induced by a variety of agents binding specific serotonergic receptor subclasses).

The serotonergic system can control cognitive processes, through an interaction with cholinergic mechanisms (e.g., Cassel and Jeltsch, 1995; Steckler and Sahgal, 1995) that are believed to play a crucial role in spatial learning and memory. This research was started by Vanderwolf (1987), who found that acquisition in the Morris watermaze was synergistically impaired by the combination of a scopolamine-induced cholinergic blockade and the inhibition of serotonin (5-HT) synthesis by p-chlorophenylalanine (pCPA). Nilsson et al. (1988) subsequently demonstrated that serotonergic lesions

induced by intracerebral administration of the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) exacerbated the spatial memory deficits in the Morris watermaze task caused by radio frequency lesions of the septum. One of the drawbacks of this work was that the disruption of the cholinergic system alone was enough to impair spatial memory. This made it difficult to evaluate the serotonergic contribution to the deficits observed when both the cholinergic and the serotonergic systems were disrupted. This issue was addressed by Richter-Levin and Segal (1989). They showed that the combined administration of pCPA and a low dose of the muscarinic cholinergic reeptor blocker atropine disrupted normal performance in the Morris watermaze task. Neither of these treatments was found to affect performance on their own. More recently, other studies have shown that a combination of 5,7-DHT with the cholinergic immunotoxin, 192 IgG-saporin, injected directly in the hippocampus, induced working-memory deficits in theT-maze, watermaze and radial maze, whereas neither toxin produced any of these deficits when injected separately (Lehmann, et al., 2000-2002).

Fig. 6.1 summarizes the anatomical interactions between the serotonergic and cholinergic systems. There is evidence showing serotonergic projections to the medial septum (MS) and the vertical limb of the diagonal band of Broca (vDBB). In particular, the MS and the vDBB receive projections mainly from the medial raphe (MR), whereas the dorsal raphe (DR) appears to be the primary source of the serotonergic projections to the horizontal limb of the diagonal band of Broca (hDBB) and to the nucleus basalis magnocellularis (NBM) (Zaborszky et al., 1991; Molliver et al., 1987). Similarly, there are projections from the MR and the DR to the pedunculopontine tegmental nucleus (PPTg) and the latero-dorsal tegmental nucleus (LTDg) (Steininger et al., 1992; Vertes et al., 1991, 1994). PPTg and LTDg in turn innervate the raphe nuclei (Woolf et al., 1989).

There is also evidence for interactive processes in areas in which the two neurotransmitter systems converge. For example, there is a serotonergic input to the olfactory bulb (Parent et al., 1981, Steinbush et al., 1981), which is the main projection area of cholinergic neurons located in the hDBB (this projection is not shown in Fig. 6.1). The MR sends serotonergic inputs to the hippocampus, which is the primary target of MS and vDBB cholinergic projections. The hippocampus also receives inputs from the cortical areas and the amygdala, which in turn are innervated by the NBM and the DR. Moreover, there are serotonergic projections from the DR to thalamic, hypothalamic nuclei, and to the substantia nigra (SN), which also receive cholinergic innervation from the brainstem cholinergic nuclei and/or the NBM (Steckler and Sahgal, 1995). Given these anatomical findings, it has been suggested that three different serotonergic-cholinergic interactive systems might be considered, namely the "(MR)-MS/vDBB-hippocampal system", the "DR/MR-PPTg/LTDg-thalamic/nigral system" and the "DR-NBM-cortical/amigdaloid system". Activation or inhibition of these three different systems could differentially influence cognition (Steckler and Sahgal, 1995).

With regard to the receptors that might be involved in the cholinergic and serotonergic modulation of learning and memory, a fair amount of attention has focused on muscarinic cholinergic and $5-HT_{1A}$ serotonergic receptor subtypes, which are highly concentrated in limbic areas such as the neocortex and the hippocampus, but also in the raphe nuclei (Hoyer, et al., 1986; Pazos et al., 1988). One often-used approach to studying these receptor subtypes is to determine the effects that specific pharmacological agents have on cognition.

In this regard, the effects of the muscarinic antagonist scopolamine and the $5-HT_{1A}$ full agonist 8-OH-DPAT have been extensively studied. For example, Riekkinen et al. (1995) found that 8-OH-DPAT, potentiated the disruptive effects of systemically given scopolamine on spatial memory.

Conversely, administration of 8-OH-DPAT in the dorsal raphe has been shown to alleviate learning and memory impairments caused by intrahippocampal injection of scopolamine (Carli et al., 1998-2000). Similar results were found in other studies in which scopolamine and 8-OH-DPAT were given systemically (Cole et al., 1994; Meneses and Hong, 1999). In particular, the study of Cole et al. (1994) showed that 8-OH-DPAT improved scopolamine-induced memory deficts at low doses but had opposite effects when given at higher doses. In the same study, the partial $5\text{-HT}_{1A}$ agonist ipsapirone improved the memory impairments induced by scopolamine. This evidence suggests that a fine regulation of $5\text{-HT}_{1A}$ receptor activity is crucial to overcome the cognitive impairments induced by the disruption of cholinergic muscarinic transmission in the brain.

Evidence for the importance of serotonergic modulation of cholinergic activity also derives from other studies, showing that 8-OH-DPAT and the partial $5\text{-HT}_{1A}$ agonists buspirone, ipsapirone and MDL 73005 increase cortical and/or hippocampal ACh release in freely moving animals (Bianchi et al., 1989; Siniscalchi et al., 1991, 1994; Wilkinson et al., 1996). In an "operant delayed matching to position" task, Cole et al. (1994) have shown that a low dose of 8-OH-DPAT (0.1 mg/kg) as well as the partial agonist ipsapirone (which enhanced performance when given alone) antagonized the effect of scopolamine. They suggested that this effect is due to a primary action of $5\text{-HT}_{1A}$ autoreceptors. Conversely, Barrett and Rowan (1992) observed that the co-administration of sub-amnesic doses the muscarinic antagonist, atropine (10 and 20 mg/kg) and the $5\text{-HT}_{1A}$ agonist ipsapirone (5mg/kg) impaired rat spatial navigation in the Morris watermaze.

The present study was designed with the purpose of expanding the findings of Barrett and Rowan (1992) using the muscarinic antagonist scopolamine rather than atropine. Thus, the effects of a combination of sub-

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amnesic doses of scopolamine (0.2 mg/kg) and ipsapirone (5mg/kg) on cognition were evaluated using the Morris watermaze and the hole board.

| Receptor | Effective dose (mg/kg) | Effect | Antagonized | No effect | 5-HT depletion (PCA) effect |
|---|---|---|---|---|---|
| 5HT$_{1A}$ | 8-OH-DPAT (0.062) | ↑ | WAY100635<br><br>S-UH-301 | GR127935<br>MDL100907<br>SB-200646<br>Ro 04-6790 | PCA annulled agonist effect |
| 5HT$_{1B}$ | GR46611X (10.0) | ↓ | 5-HT-moduline<br>SB-224289<br>GR127935 | | Eliminated |
| 5HT$_{1B/1D}$ | TFMPP (5.0–10.0)<br><br>mCPP (5.0–10.0) | ↓<br><br>↓ | SB-224289<br>Ketanserin | | Eliminated. No effect on agonist effect |
| 5-HT$_{1F}$ | LY344864<br>(5.0–10.0) | ↓ | | | |
| 5HT$_{2A}$ | DOI (0.01–0.1) | ↑ | MDL100907 | SB-200646<br>LY215840<br>(low dose) | Eliminated |
| 5HT$_{2B/2C}$ | 1-NP (0.1–5.0)<br>Mesulergine<br>(0.2–0.4) | ↓<br><br>↓ | SB-200646<br>SB-200646 | | |
| 5HT$_3$ | mCPBG (1.0) | ↓ | Ondansetron<br>Tropisetron | | Eliminated |
| 5HT$_4$ | BIMU8 (5.0)<br>BIMU8 (5.0) | ↓<br><br>↓ | GR125487D<br>SDZ205-557 | | Unaffected |
| 5HT$_6$ | Ro 04-6790 (5.0) | ↑ | Ritanserin | WAY100635<br>GR127935<br>Ketanserin<br>Ondansetron<br>GR125487D | Unaffected |
| 5HT$_7$ | 8-OH-DPAT<br>(0.0.62) | ↑ | LY215840<br>Ritanserin<br>(high dose)<br>DR4004<br>SB-269970 | | Eliminated |

**Table 6.1**

Effects of 5-HT receptor agonists and antagonists on memory consolidation in a learning task. ↑indicates facilitation; ↓ indicates impairment; PCA: p-chloroamphetamine, a 5-HT synthesis inhibitor (modified from Meneses, 2003).

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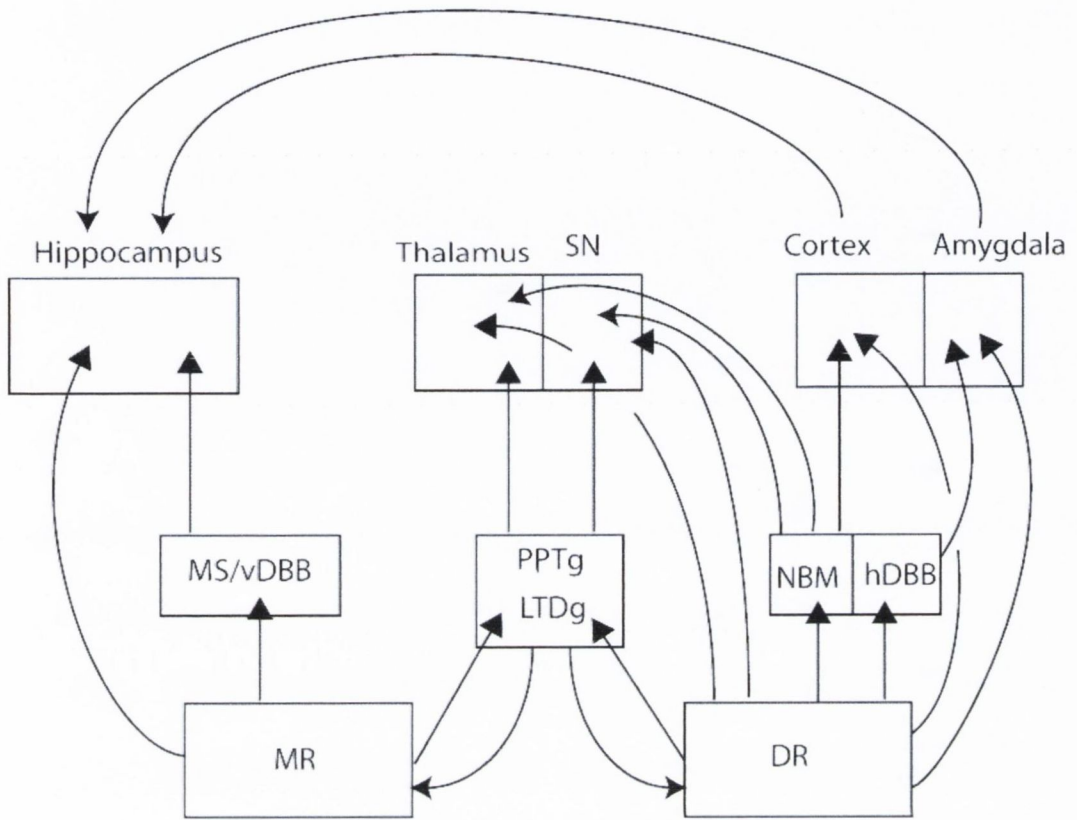
**Fig. 6.1**

Possible sites where cholinergic and serotonergic systems may interact in the mediation of cognitive processes. Three systems may be of special interest, the "MR-MS/vDBB-hippocampal system" the "DR/MR-PPTg/LTDg/thalamic/nigral system" and the "DR-NBM-cortical/amygdaloid system" (modified after Steckler and Sahgal, 1995).

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## 6.2 Methods

Male Wistar rats, weighing 200-250 g (6-7 weeks of age) at the beginning of the experiment, were randomly assigned to four different treatment groups (n=13/group). Solutions of scopolamine hydrobromide (0.2 mg/kg) or ipsapirone (5 mg/kg) and water for injection were prepared daily, before each experimental session. The dose of scopolamine (0.2 mg/kg) was chosen on the basis of preliminary experiments showing that its administration in normal rats induced no significant disruption of watermaze performance or hole board activity.

All treatments were administered intraperitoneally (i.p.). 30 minutes before test both in the watermaze or the hole board (see Fig 2.5a for the timeline of injections). Injections were given in immediate succession and randomized across days for a given rat in all tasks. The dose and method of administration of ipsapirone were chosen according to Barrett and Rowan (1992).

### 6.2.1 Treatment groups

1) Water for injection (Veh+Veh; watermaze, n=13; hole board, n=13).
2) Water for injection+ipsapirone (Veh+Ipsa; watermaze, n=13; hole board, n=13).
3) Scopolamine+water for injection (Scop+Veh; watermaze, n=13; hole board=13).
4) Scopolamine+ipsapirone (Scop+Ipsa; watermaze, n=13; hole board, n=13).

## 6.3 Results

### 6.3.1 Watermaze

#### 6.3.1.1 Acquisition

An overall analysis of the latencies scored during the five days of training, revealed that all animals significantly learned the task [$F_{(4, 45)}=85$, $P< 0.0001$].

No significant difference across groups was observed. A within group analysis showed that each of the treatment groups significantly learned the task [Veh+Veh: $F_{(4, 9)}=45$, $P< 0.0001$; Scop+Veh: $F_{(4, 9)}=16.5$, $P< 0.001$; Veh+Ipsa: $F_{(4, 9)}=17$, $P< 0.001$; Scop+Ipsa: $F_{(4, 9)}=10$, $P< 0.05$]. There was a significant difference between the learning profiles of groups Veh+Veh and Veh+Ipsa [$F_{(1, 24)}=5$, $P< 0.05$] but no significant day/group interaction was found for this comparison. No inter-group difference or day/group interaction was found for the comparisons Veh+Veh/Scop+Veh, Veh+Ipsa/Scop+Ipsa and Scop+Veh/Scop+Ipsa. Table 6.2 summarizes the outcome of overall and inter-group comparisons and Fig 6.2 shows the acquisition profiles of the groups tested.

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| Comparisons | df | F | P< |
|---|---|---|---|
| **Overall (between groups)** | 3, 48 | 3 | NS |
| **Overall (between trials)** | 4, 45 | 85 | 0.0001 |
| **Veh+Veh vs Veh+Ipsa** | 1, 24 | 5 | 0.05 |
| **Veh+Veh vs Scop+Veh** | 1, 24 | 2 | NS |
| **Scop+Veh vs Scop+Ipsa** | 1, 23 | 1.4 | NS |
| **Veh+Ipsa vs Scop+Ipsa** | 1, 24 | 0.1 | NS |

**Table 6.2**

Effects of scopolamine (0.2 mg/kg) and ipsapirone (5 mg/kg) on acquisition of the watermaze task. Treatment groups (n=13/group): vehicle+vehicle (Veh+Veh), vehicle+ipsapirone (Veh+Ipsa), scopolamine+vehicle (Scop+Veh), scopolamine+ipsapirone (Scop+Ipsa). Table shows F scores and P values for inter-group comparisons.
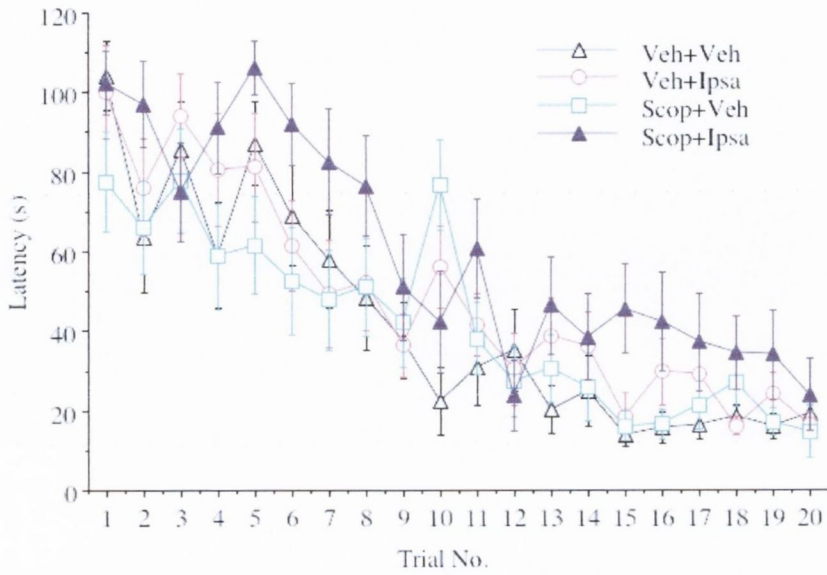
**Fig. 6.2**

Effects of scopolamine (0.2 mg/kg), and ipsapirone (5 mg/kg) on acquisition in the watermaze task. Treatment groups (n=13/group): vehicle+vehicle (Veh+Veh), scopolamine+vehicle (Scop+Veh), vehicle+ipsapirone (Veh+Ipsa), scopolamine+ipsapirone (Scop+Ipsa). Values are means±SEM.

## 6.3.1.2 Probe test

No overall difference was found across treatment groups for any of the values measured. A within group analysis showed that groups Veh+Veh and Scop+Veh but not groups Veh+Ipsa and Scop+Ipsa spent significantly more time in the target quadrant than in the opposite quadrant (Veh+Veh: $P < 0.05$; Scop+Veh: $P < 0.05$). No difference, for any of the values measured, was found between groups Veh+Veh and Veh+Ipsa. Also, no difference was found between groups Veh+Ipsa and Scop+Ipsa. Comparison between groups Scop+Veh and Scop+Ipsa showed that the animals of the Scop+Ipsa group spent significantly more time in the opposite quadrant and travelled a significantly longer distance. These results are shown in Fig. 6.3 (a-d) and Table 6.3.

**a**
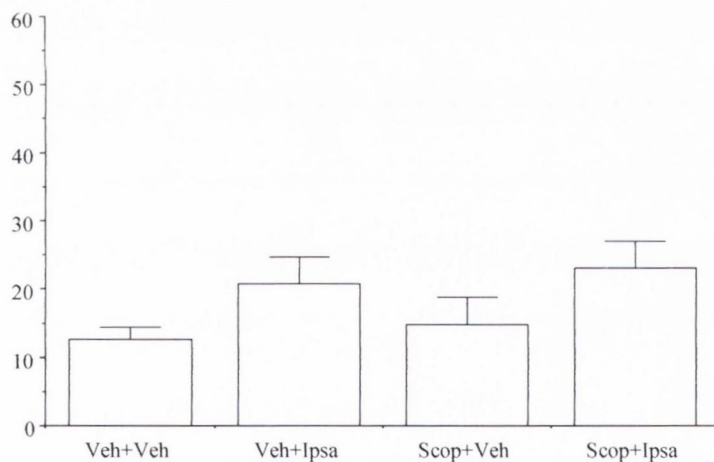
**b**
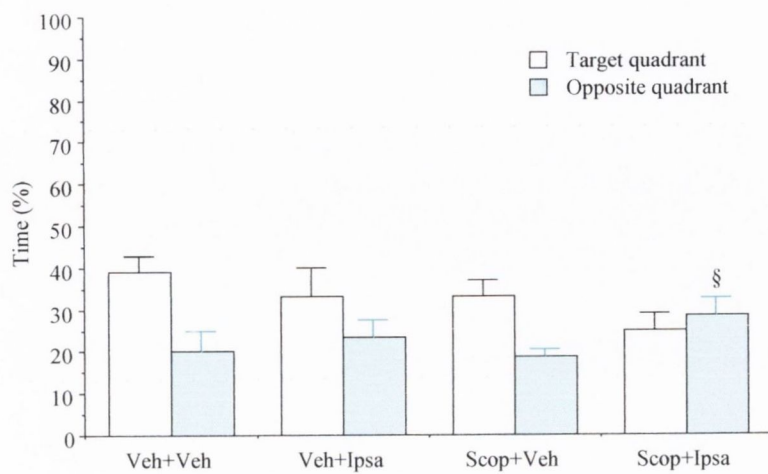


**c**
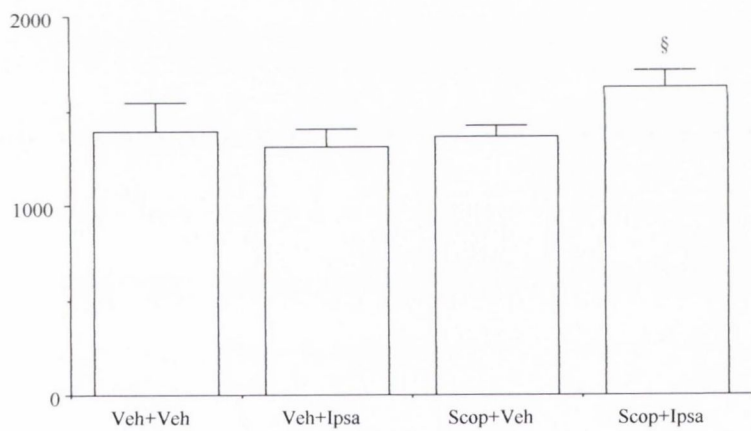
**d**
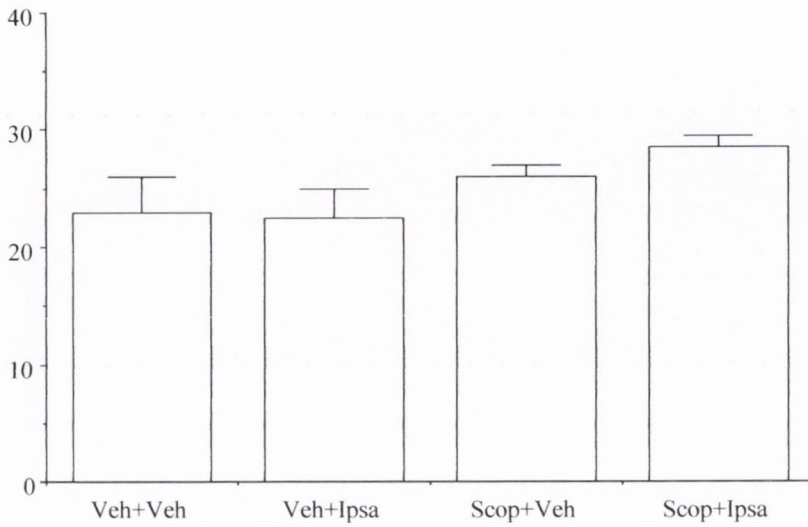


**Fig. 6.3 (a-d)**

Effects of scopolamine (0.2 mg/kg) and ipsapirone (5 mg/kg) on watermaze probe test performance. Treatment groups (n=13/group): vehicle+vehicle (Veh+Veh), vehicle+ipsapirone (Veh+Ipsa), scopolamine+vehicle (Scop+Veh), scopolamine+ipsapirone (Scop+Ipsa); a) latency to the platform location; b) percent time spent in the target quadrant vs. percent time spent in the opposite quadrant; c) percent time spent in the periphery of the tank; d) total distance travelled; e) swim speed. Symbol "§" indicates statistical difference to the Scop+Veh group. Values are means±SEM.

| | Group | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) |
|---|---|---|---|---|---|---|---|---|
| 1 | Veh+Veh | 12.5±2 | 39±4 | 20±5 | 19±8 | 5±2 | 1392±152 | 23±3 |
| 2 | Veh+Ipsa | 21±4 | 33±7 | 23.5±4 | 10±10 | 3.5±1.5 | 1307±97 | 22.5±2.5 |
| 3 | Scop+Veh | 15±4 | 33±4 | 18.5±2 | 15±5 | 2±0.4 | 1363±57 | 26±1 |
| 4 | Scop+Ipsa | 23±4 | 25±4 | 28.5±4 | -3±7 | 1.5±0.4 | 1693±90 | 28.5±1 |
| | Overall ANOVA (P <) | NS | NS | NS | NS | NS | NS | NS |
| | | | | | | | | |
| | t-test P values (P<) | | | | | | | |
| | Gr. 1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS | NS |
| | Gr. 1 Vs. Gr. 3 | NS | NS | NS | NS | NS | NS | NS |
| | Gr. 2 Vs. Gr. 3 | NS | NS | NS | NS | NS | NS | NS |
| | Gr. 2Vs. Gr. 4 | NS | NS | NS | NS | NS | NS | NS |
| | Gr. 3Vs. Gr. 4 | NS | NS | 0.05 | NS | NS | 0.05 | NS |

**Table 6.3**
Effects of scopolamine (0.2 mg/kg) and ipsapirone (5 mg/kg) on watermaze probe test performance. Treatment groups (n=13/group): vehicle+vehicle (Veh+Veh), vehicle+ipsapirone (Veh+Ipsa), scopolamine+vehicle (Scop+Veh), scopolamine+ipsapirone (Scop+Ipsa). Table shows mean±SEM values, P values for the overall comparison between groups (ANOVA), and inter-group comparisons (t-test).

### 6.3.2 Hole board

An overall comparison revealed no significant difference across groups. Comparisons Veh+Veh/Veh+Ipsa and VehVeh/Scop+Veh revealed no difference during both the first and the second half of the experiment. Relative to group Veh+Ipsa, the Scop+Veh group scored a higher number of rears in the second half of the experiment. Comparison between groups Scop+Veh and Scop+Ipsa revealed that the animals of the Scop+Ipsa group scored a significantly smaller number of lines crossed and rearing during the first half of the experiment. No difference was found during the second half. Finally, no difference was found between the groups Veh+Ipsa and Scop+Ipsa groups.

A within group analysis showed that, in groups Veh+Veh and Scop+Veh, all values were significantly reduced during the second half of the experiment relative to the first half. In the Veh+Ipsa group, the number of lines crossed but not the number of head dips and rears, were significantly reduced during the second half of the experiment relative to the first half. In the Scop+Ipsa group all values, excepting the number of rears, were significantly reduced during the second half of the experiment. Fig. 6. 4 and Table 6.4 (a, b) show these results.
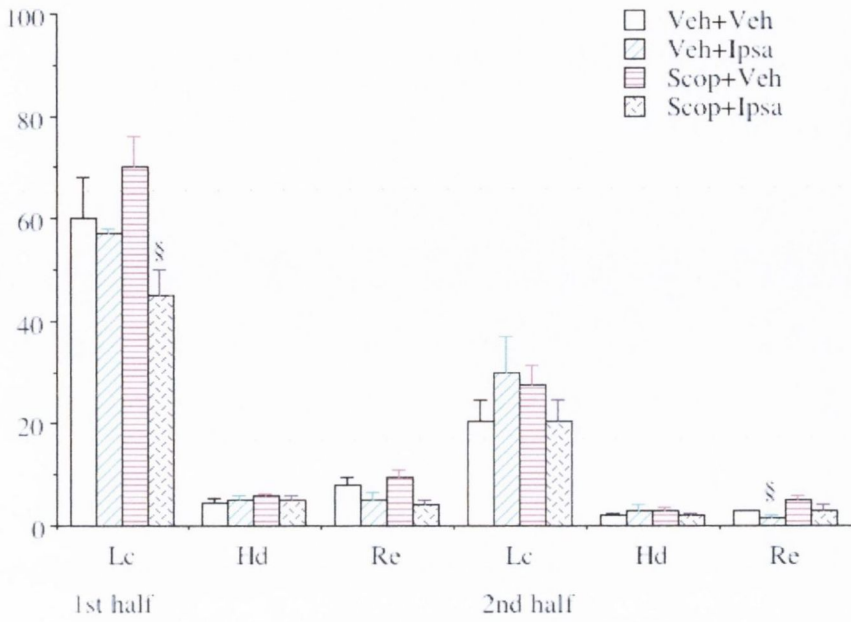
**Fig.6.4**

Effects of scopolamine (0.2 mg/kg) and ipsapirone (5 mg/kg) on hole board activity. Treatment groups (n=13/group): vehicle+vehicle (Veh+Veh), vehicle+ipsapirone (Veh+Ipsa), scopolamine+vehicle (Scop+Veh), scopolamine+ipsapirone (Scop+Ipsa). Symbol "§" indicates statistical difference to the Scop+Veh group. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing. Values are means±SEM.

a

| | Group | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|---|
| | | 1st half | 2st half | 1st half | 2nd half | 1nd half | 2nd half |
| 1 | Veh+Veh | 60±8 | 20.5±4 | 4.4±1 | 2±0.5 | 8±1.5 | 3±0.1 |
| 2 | Veh+Ipsa | 57±1 | 30±7 | 5±1 | 3±1 | 5±1.5 | 1.5±0.5 |
| 3 | Scop+Veh | 70±6 | 27.5±4 | 6±0.1 | 3±0.5 | 9.5±1.5 | 5±1 |
| 4 | Scop+Ipsa | 45±5 | 20.5±4 | 5±1 | 2±0.5 | 4±1 | 3±1 |
| | Overall ANOVA P value (P<) | NS | NS | NS | NS | NS | NS |
| | t-test P values (P<) | | | | | | |
| | Gr. 1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS |
| | Gr. 1 Vs. Gr. 3 | NS | NS | NS | NS | NS | NS |
| | Gr. 2 Vs. Gr. 3 | NS | NS | NS | NS | NS | 0.05 |
| | Gr. 2 Vs. Gr. 4 | NS | NS | NS | NS | NS | NS |
| | Gr. 3 Vs. Gr. 4 | 0.05 | NS | NS | NS | 0.05 | NS |

b

| | Groups | P Values (P<) | | |
|---|---|---|---|---|
| | | lc2-lc1 | hd2-hd1 | re2-re1 |
| 1 | Veh+Veh | 0.0001 | 0.05 | 0.05 |
| 2 | Veh+Ipsa | 0.05 | NS | NS |
| 3 | Scop+Veh | 0.0001 | 0.05 | 0.05 |
| 4 | Scop+Ipsa | 0.05 | 0.05 | NS |

**Table 6.4 (a, b)**

Effects of scopolamine (0.2 mg/kg) and ipsapirone (5 mg/kg) on hole board activity. Treatment groups (n=13/group): vehicle+vehicle (Veh+Veh), scopolamine+vehicle (Scop+Veh), vehicle+ipsapirone (Veh+Ipsa), scopolamine+ipsapirone (Scop+Ipsa); a) mean±SEM values, P values for the overall comparison between groups (ANOVA) and for inter-group comparison (t-test); b) within group comparisons (Wilcoxon signed-rank test) between the levels of activity scored during the first and the second half of the experiment. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing.

## 6.4 Discussion

The first part of the study shows that treatment with scopolamine (0.2 mg/kg) alone (Scop+Veh) had no effect on either watermaze acquisition or probe tests. In the hole board, this treatment had no effect on locomotor activity, exploration or habituation. Treatment with ipsapirone (5 mg/kg) alone (Veh+Ipsa) had no effect on watermaze acquisition but, in probe tests, significantly reduced the target quadrant bias. Also, in the hole board, this treatment had no effect on locomotion and exploration but it partially reduced habituation (only the number of head dips was not significantly reduced in the second half of the experiment). The evaluation of the effects of the combination scopolamine+ipsapirone (Scop+Ipsa) revealed that this treatment had no significant effect on acquisition, but impaired performance in probe tests. This is shown by the comparison with the Scop+Veh group. The animals of the Scop+Ipsa group spent significantly more time in the quadrant opposite to the target quadrant and swam a longer distance. Also, a within group comparison of the time spent in the target quadrant with the time spent in the opposite quadrant revealed that there was no target quadrant preference in the Scop+Ipsa group. These results suggest that the combination scopolamine/ipsapirone had disruptive effects on the process of consolidation of the information acquired during acquisition, leading to spatial navigation deficits in the probe test. Although ipsapirone had some disruptive effects on its own, the rats that received this treatment showed significant learning during acquisition and no difference to normal (Veh+Veh) rats for all of the values measured during probe tests. Besides, in the hole board spontaneous locomotor activity was not markedly affected by the administration of ipsapirone alone. Altogether, the present results may relate to the research carried out by of Barrett and Rowan (1992), who observed that atropine (10 and 20 mg/kg) or ipsapirone (5 mg/kg) had no effect if administered alone but, when combined, impaired rat spatial

orientation in the Morris watermaze. Also, these results agree with the evidence which showed that a combination of pre-training sub-amesic doses of scopolamine and the 5-HT$_{1A}$ full agonist 8-OH-DPAT produced impairments on acquisition in the Morris watermaze and on retention in the passive avoidance (Riekkinen et al., 1994-1995b).

In the hole board, treatment with Scop+Ipsa significantly reduced locomotion and rearing relative to group Scop+Veh. Also, habituation was partially reduced by this treatment. There is evidence that habituation of exploratory behaviour involves several behavioural processes. These are, on one hand, responses to novelty, including arousal, emotionality, stress-related factors and on the other hand a diminished response due to familiarity, which requires learning–related processes and recognition or recall (Dai et al., 1995; Gerhardt et al., 1993-94; Platel et al., 1982; Sadile et al., 1979; Tomaz et al., 1990). Also, exploratory activity and habituation are closely related to the hippocampus and its cholinergic input (Carlton et al., 1968; Gray and McNaughton, 1983). Taken together, these considerations might explain both the performance impairment in the Morris watermaze and the reduction (even though not complete) of hole board habituation observed in the Scop+Ipsa group. They may also be considered to strengthen the hypothesis of "interplay" between the cholinergic and the serotonergic system in the modulation of memory processes (Decker and McGaugh, 1991; see also Table 6.1).

However, studies of 5-HT1$_A$ agonists have led to controversial results, as regards their effects on performance in paradigms such as the Morris watermaze, passive avoidance, and hole board. For instance, systemic treatment with the specific 5-HT$_{1A}$ agonist 8-OH-DPAT, impairs performance in the Morris watermaze (Carli and Samanin, 1992; Carli et al., 1995a; Kant et al., 1996, 1998) and radial maze (Winter and Petti, 1987; Helsley et al., 1998), whereas systemic treatment with the 5-HT$_{1A}$ agonist MDL 73005 improves the

deficits caused by scopolamine in versions of the Morris watermaze that test both reference and working memory (Bertrand et al., 2001). Indeed, spatial memory impairments induced by systemic treatment with 8-OH-DPAT persist in 5-HT-depleted rats (Carli and Samanin, 1992) and are antagonized by intra-hippocampal delivery of the 5-HT$_{1A}$ antagonist WAY 100135 (Carli et al., 1995b), suggesting an involvement of postsynaptic 5-HT$_{1A}$ receptors. Also, when infused into the hippocampus (Carli et al., 1992) or the septal region (Bertrand et al., 2000), 8-OH-DPAT impairs watermaze performance.

Conversely, when infused into the dorsal raphe, 8-OH-DPAT reverses the spatial learning impairment caused by intrahippocampal injection of scopolamine (Carli et al., 1998). In the dorsal raphe nuclei, 5-HT$_{1A}$ receptors appear to operate mainly as presynaptic autoreceptors. Their activation inhibits both raphe serotonergic cell firing (Sprouse, 1991; Millan et al, 1993) and 5-HT release in projection areas (Hjorth and Magnunson, 1998; Hutson et al., 1989). These include structures that are not serotonergic, such as the hippocampus and the septal regions (Pazos and Palacios, 1985; Chalmers and Watson, 1991), where 5-HT$_{1A}$ receptors operate as postsynaptic heteroreceptors (e.g., Buhot, 1997; Barnes and Sharp, 1999). These studies suggest that the effects of 5-HT$_{1A}$ agonists on cognition may strongly depend on the activation of presynaptic or blockade of postsynaptic 5-HT$_{1A}$ receptors. Thus, on the basis of these considerations, it may speculated that, in the present study, the effects of ipsapirone were due to its activity on postsynaptic rather presynaptic receptors located in structures such as the hippocampus and the septum.

This study provides evidence that spatial memory formation and recollection are influenced by cholinergic and serotonergic activity within the CNS. Also, the present experimental data should be taken into account when administering 5-HT$_{1A}$ agonists as a treatment for depression or anxiety (Robinson, 1991; Cutler et al., 1993; Cowen, 2000) in cognitively impaired

aged patients or in patients with neurodegenerative diseases, given that, in both conditions, central cholinergic function is reduced (Fibiger et al., 1991; Collerton, 1986).

# CHAPTER 7

# Effects of CO exposure on rat spatial navigation and exploratory activity, and its interaction with a low dose of scopolamine

## 7.1 Introduction

It is well documented that exposure to carbon monoxide (CO) can produce dysfunctions in the central nervous system. For example, Nabeshima et al., (1991) have found that acetylcholine concentration was significantly reduced in the frontal cortex and the striatum of mice, 7 days after exposure to CO.

In animals exposed to CO, necrosis of the cerebral cortex, hippocampus, substantia nigra and globus pallidus has been reported following anatomical investigation (Lapresle and Fardeau, 1967), computer tomography (Sawada et al., 1980-83) and magnetic resonance scanning (Horowitz et al., 1987). Other histological studies have shown that a moderate neuronal loss in the CA1 region of the hippocampal formation occurred 7 days after exposure to CO (Nabeshima et al., 1991). Ultrastructural features of both necrosis and apoptosis were observed by electron microscopy. In the same studies, microdialysis in the cortex and the hippocampus has shown that glutamate release and hydroxyl radical generation were significantly increased immediately after hypoxia, caused by exposure to CO.

Also, it has been found that CO binds to soluble guanylate cyclase (Ingi et al., 1996) competing with nitric oxide (NO). In normal conditions, the modulation of soluble guanylate cyclase by NO plays important roles in the

regulation of synaptic transmission, long-term potentiation and memory processes (Boulton et al., 1995; Hawkins, 1996). It has been shown that CO activates guanylate cyclase but interferes with nitric oxide binding which is necessary to fully activate the enzyme (Brune et al., 1990; Stone and Marletta. 1994). Hernandez et al. (2003) found that, in the rat, acute carbon monoxide exposure reduced activation of soluble guanylate cyclase by nitric oxide. They also observed that acute exposure effects were stronger at 7 days than at 24 hrs after exposure and suggested that this effect may contribute to delayed memory loss and cognitive impairment in humans.

Piantadosi et al., (1997) showed that the learning and memory deficits observed in the radial maze, following exposure to CO, were associated with heterogeneous cell loss in the cortex, globus pallidus, and cerebellum. Also, the neuronal damage appeared 3 days after exposure and gradually increased over the following 3 weeks.

It has been shown that, in mice, systemic administration of (+) MK-801 (a noncompetitive antagonist of NMDA receptors), 1hr before CO-exposure, attenuates "delayed amnesia" (amnesia occurring 5 days after exposure) in the passive avoidance task (Nabeshima et al., 1990). These results suggest that delayed amnesia and neuronal death induced by CO-exposure may be due to an activation of NMDA receptors. As Choi et al. (1987) observed, the strong $Ca^{2+}$ influx, resulting from the hyperactivation of the NMDA receptor complex (induced by an increased release of glutamate), can critically be associated with delayed excitotoxic neuronal death.

There is also evidence that intoxication caused by inhalation of CO involves the disruption of the serotonergic system. For example, Muraoka et al. (1998) observed that serotonin (5-HT) concentration significantly decreased in the frontal cortex, from 1hr to 7 days after exposure to CO. Moreover, Masayuki et al. (1996) found that, in mice previously exposed to CO, the

administration of the κ-opioid receptor agonist U-50488H improves performance in the step-down type avoidance and in spontaneous alternation tasks. These results were confirmed by experiments showing that the ameliorating effects of U-50488H on CO-induced delayed amnesia were inhibited by nor-binaltorphine, a selective antagonist for the same class of receptors. In the same studies, a low dose of scopolamine also blocked the effect of U-50488H. These results suggest that muscarinic receptors may play a crucial role in the control of opioid receptor activity following exposure to CO.

Other evidence suggests that the disruption of cholinergic transmission in the brain might mediate the development of the delayed amnesia induced by exposure to CO. In particular, Hiramatsu et al. (1992) showed that, in mice, scopolamine blocked the anti-amnesic effects that nefiracetam, a cyclic derivative of GABA, had on passive avoidance disruption of performance induced by exposure to CO. Moreover, it has been shown that the administration of nicotine improves CO-induced amnesia, as measured in a step-down type passive avoidance task (Hiramatsu et al, 1994).

The present study was designed to further investigate the role of cholinergic transmission, and in particular of muscarinic receptor activity, in the development of amnesia following exposure to CO. After exposure to pure CO (2400±240ppm) rats were tested in the Morris watermaze and in the hole board, after 3 and 10 days, respectively, in order to evaluate the effects of such a treatment on spatial learning and memory and on spontaneous exploratory activity. Subsequently, the exposure protocol was combined with the administration of a sub-amnesic dose of the muscarinic receptor antagonist scopolamine, in order to study the possible synergistic interactions between the inhibition of activation of muscarinic receptors and the neurotoxicity associated with exposure to CO.

| Effects of Exposure to CO | Animal | Reference |
|---|---|---|
| Delayed amnesia, as measured in the passive avoidance task, is attenuated by systemic administration of (+) MK-801 (a noncompetitive antagonist of NMDA receptors), 1hr before exposure. | Mouse | Nabeshima et al., 1990 |
| Increased release of glutamate in the cortex and hippocampus, immediately after exposure. Neuronal loss in the hippocampus (CA1) and reduction of acetylcholine concentration in the striatum and the cortex after 7 days from exposure. Disruption of passive avoidance performance. | Mouse | Nabeshima et al., 1991 |
| Scopolamine blocks the anti-amnesic effects that nefiracetam, a cyclic derivative of GABA, has on passive avoidance disruption of performance induced by exposure to CO. | Mouse | Hiramatsu et al., 1992 |
| The administration of nicotine improves CO-induced amnesia as measured in a step-down type avoidance task | Mouse | Hiramatsu et al., 1994 |
| $\kappa$-opioid receptor subclass activity is disrupted. Administration of the $\kappa$-opioid receptor agonist U-50488H improves performance deficits induced by exposure to CO in the step-down type avoidance and in spontaneous alternation tasks. | Mouse | Masayuki et al. 1996 |
| Learning and memory deficits in the radial maze, associated with heterogeneous cell loss in the globus pallidus, and cerebellum. Also, the neuronal damage appears 3 days after exposure and gradually increases over the following 3 weeks. | Rat | Piantadosi et al., 1997 |
| 5-HT concentration is significantly reduced in the frontal cortex, from 1hr to 7 days after exposure | Rat | Muraoka et al., 1998 |
| Acquisition in a Y-maze task and recall in a passive avoidance test are impaired 5 and 7 days after exposure, respectively. | Mouse | Hiramatsu and Inoue, 2000 |
| Acute exposure reduces activation of soluble guanylate cyclase by nitric oxide. Also, effects appear to be stronger at 7 days than at 24 hrs after exposure. | Rat | Hernandez et al, 2003 |

**Table 7.1**
Most representative findings, published from 1990 to present time, providing evidence on the disruptive effects of CO exposure on learning and memory in the rat and in the mouse.

## 7.2 Methods

### 7.2.1 Exposure to CO

Exposure of male Wistar rats (200-250 g) to CO was carried out using a hermetically sealed chamber with a pipe feeding into it. Rats were exposed to pure CO (2400±240ppm) for 1 hr and, after exposure, were kept in a heated environment to maintain body temperature at 37-38 $^{\circ}$C (see section 2.3 for further description of the exposure chamber and the method of exposure to the gas; also see section 2.4 and Fig.2.5b for the timeline of treatments relative to testing). Control animals ("Air" group) were exposed to air in the same chamber, for the same amount of time. The dose and time of exposure were chosen according to Piantadosi et al., 1997 (see section 7.1 for details on the main findings reported in this publication). All groups exposed to CO initially consisted of 16 animals but exposure to the gas was lethal in some cases (see section 7.2.3 for the details about the number of animals that were found dead immediately after exposure).

### 7.2.2 Drug treatments

The animals that received drug treatments after exposure to CO (groups CO+Veh and CO+Scop) were injected i.p., daily 30 minutes prior to the test (starting 3 days after exposure to CO) with water for injection (vehicle) or 0.2 mg/kg scopolamine The groups Veh+Veh and Scop+Veh used here for comparison were the same groups of the study shown in Chapter 6. As shown in Chapter 6, this dose was found to have no effects on performance in both the watermaze and the hole board.

## 7.2.3 Treatment groups:

1) Rats exposed to only air for 1 hr (Air, watermaze, n=16; hole board, n=16)

2) Rats exposed to CO for 1 hr (CO, watermaze, n=15; hole board, n=15)

3) Water for injection (Veh+Veh; watermaze, n=13; hole board, n=13)

4) Scopolamine+ water for injection (Scop+Veh; watermaze, n=13; hole board, n=13).

5) Rats exposed to CO for 1hr and injected i.p. with water for injection (CO+Veh, watermaze, n=12; hole board, n=12)

6) Rats exposed to CO for 1hr and injected i.p. with 0.2 mg/kg scopolamine (CO+Scop, watermaze, n=13; hole board, n=13)

The variation of the number of animals/group was due to the toxicity of CO, which caused the death of 1 animal in the CO group, 4 in the CO+Veh group and 3 in the CO+Scop group.

## 7.3 Results

### *Watermaze 7.3.1*

#### *7.3.1.1 Acquisition*

Overall analysis showed that all animals learned the task [$F_{(4, 73)}=183$, $P< 0.0001$] and that there was no difference between groups.

Individual group analysis of escape latency values revealed that all groups significantly learned the task [Air: $F_{(4, 12)}=27.4$, $P< 0.0001$; CO: $F_{(4, 11)}=19$, $P< 0.0001$; Veh+Veh: $F_{(4, 9)}=45$, $P< 0.0001$; Scop+Veh: $F_{(4, 9)}=16.5$, $P< 0.001$; CO+Veh: $F_{(4, 8)}=75$, $P< 0.0001$; CO+Scop: $F_{(4, 9)}=29.6$, $P< 0.0001$]. No difference was found for the comparisons Air/CO, Veh+Veh/Scop+Veh, CO+Veh/CO+Scop. Comparison Veh+Veh/CO+Veh revealed no inter-group difference but a significant day/treatment interaction ($F_{(4, 20)}=4$, $P< 0.05$). Table 7.2 summarizes the outcome of overall and inter-group comparisons and Fig.7.1 shows the acquisition profiles of the treatment groups tested.

| Comparisons | df | F | P< |
|---|---|---|---|
| **Overall (between groups)** | 5, 76 | 2 | NS |
| **Overall (between trials)** | 4, 73 | 183 | 0.0001 |
| **CO vs Air** | 1, 29 | 0.6 | NS |
| **Veh+Veh vs Scop+Veh** | 1, 24 | 2 | NS |
| **CO+Veh vs CO+Scop** | 1, 23 | 0.14 | NS |

**Table 7.2**

Effects of scopolamine (0.2 mg/kg) and CO (2400±240ppm/1hr) on acquisition of the watermaze task. Treatment groups: Air (n=16), CO, Veh+Veh (n=13), Scop+Veh (n=13), CO+Veh (n=12), CO+Scop (n=13). Table shows F scores and P values for inter-group comparisons.

a
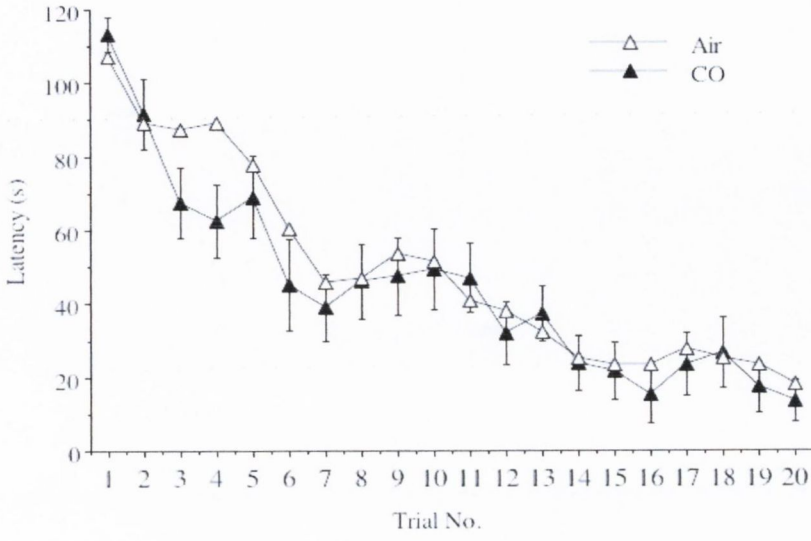


b
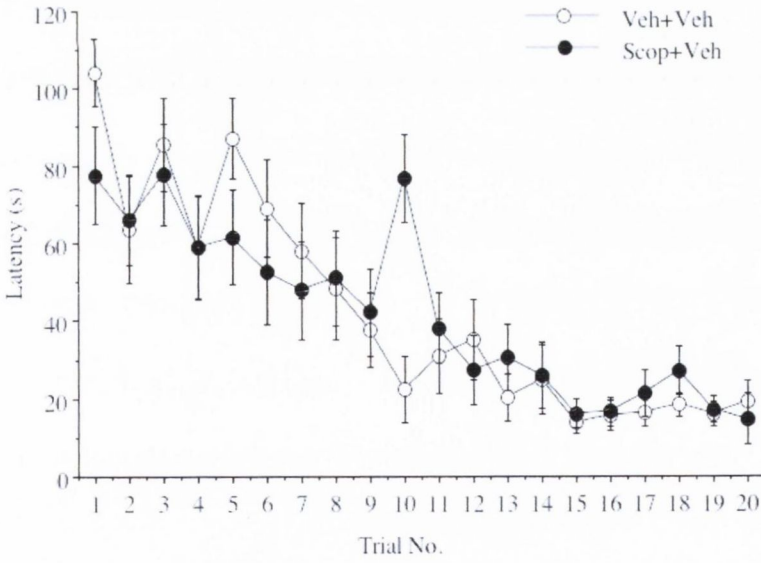
c
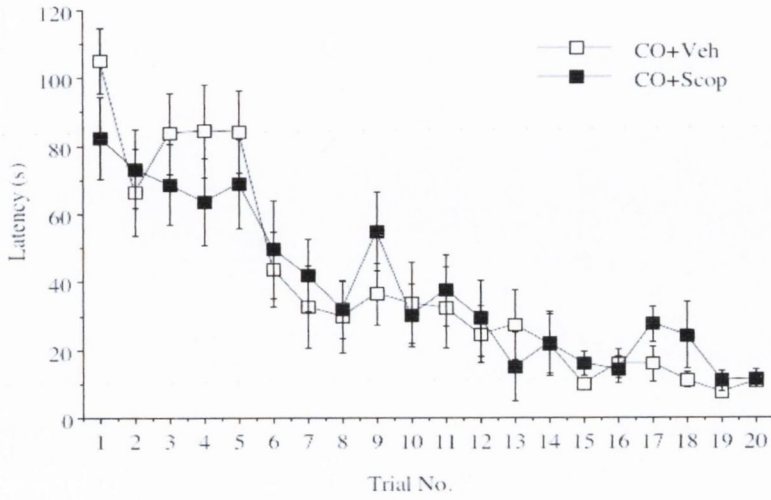


**Fig. 7.2 (a-c)**

Effects of scopolamine (0.2 mg/kg), and exposure to CO (2400±240ppm/1hr) on acquisition in the watermaze task. Treatment groups: Air (n=16), CO, Veh+Veh (n=13), Scop+Veh (n=13), CO+Veh (n=12), CO+Scop (n=13); a) Air and CO; b) Veh+Veh and Scop+Veh; c) CO+Veh and CO+Scop. Values are means±SEM.

## 7.3.1.2 Probe test

No significant overall difference was found for any of the values measured except for the swim speed (Table 7.2). All groups, except the CO+Scop group, showed a significant target quadrant bias (Air: P< 0.05; CO: P< 0.05; Veh+Veh: P< 0.05; Scop+Veh: P< 0.05; CO+Veh: P< 0.05). No significant difference was found between groups Air and CO or between the Veh+Veh and the Scop+Veh groups. The animals of group CO+Veh had a significantly higher swim speed relative to the animals of group Veh+Veh. Finally, the animals of the CO+Scop group spent significantly less time in the target quadrant than the animals of the CO+Veh group. Fig.7.3 shows the comparisons for the time spent in the target quadrant vs. the time spent in the opposite quadrant and the mean swim speed during trial (figures showing other values measured have been omitted, as no significant difference was found for the comparisons made). A summary of the outcome of probe test is shown in Table 7.2.

a

**b**
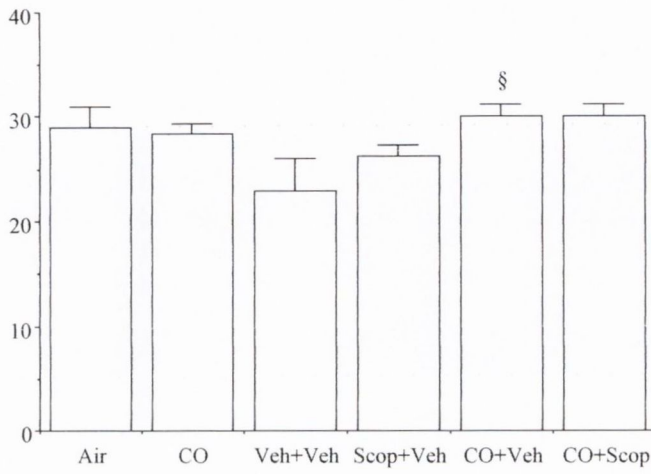


**Fig 7.3**

Effects of scopolamine (0.2 mg/kg), and exposure to CO (2400±240ppm/1hr) on probe test performance in the watermaze task. Treatment groups: Air (n=16), CO, Veh+Veh (n=13), Scop+Veh (n=13), CO+Veh (n=12), CO+Scop (n=13). Symbol "#" indicates statistical difference to group CO+Veh and the symbol "§" indicates statistical diffrence to group Veh+Veh. Values are means±SEM.

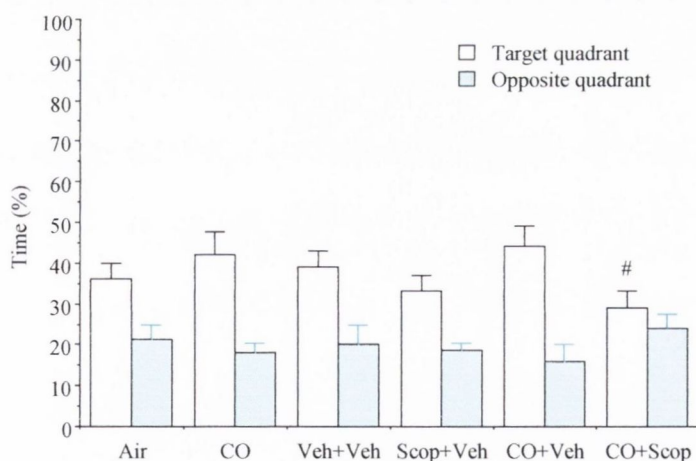| | Group | Latency to platform (s) | Time in tg. q. (%) | Time in opp. q. (%) | Tg.-opp. (%) | Tg./opp. | Total distance travelled (cm) | Mean swim speed (cm/s) |
|---|---|---|---|---|---|---|---|---|
| 1 | Air | 17±4 | 36±4 | 21±3.5 | 15±7 | 10±7 | 1552±54 | 29±2 |
| 2 | CO | 16.5±5 | 42±5 | 18±2.5 | 25±8 | 5±2 | 1502±61 | 28.5±1 |
| 3 | Veh+Veh | 12.5±2 | 39±4 | 20±5 | 29.5±8 | 6±1.5 | 1392±152 | 23±3 |
| 4 | Scop+Veh | 15±4 | 33±4 | 18.5±2 | 15±5 | 2±0.4 | 1363±57 | 26±1 |
| 5 | CO+Veh | 7.5±1 | 44±5 | 16±4 | 28±8 | 6±2 | 1507±53 | 30±1 |
| 6 | CO+Scop | 14±4 | 29±4 | 24±3.5 | 23±6 | 3±1.4 | 1630±54 | 30±1 |
| | Overall ANOVA (P <) | NS | NS | NS | NS | NS | NS | 0.05 |
| | t-test P values (P<) | | | | | | | |
| | Gr. 1 Vs. Gr. 2 | NS | NS | NS | NS | NS | NS | NS |
| | Gr. 3 Vs. Gr. 4 | NS | NS | NS | NS | NS | NS | NS |
| | Gr. 3 Vs. Gr. 5 | NS | NS | NS | NS | NS | NS | 0.05 |
| | Gr. 5 Vs. Gr. 6 | NS | 0.05 | NS | NS | NS | NS | NS |

Table 7.2

Effects of scopolamine (0.2 mg/kg), and exposure to CO (2400±240ppm/1hr) on probe test performance in the watermaze task.Treatment groups: Air (n=16), CO, Veh+Veh (n=13), Scop+Veh (n=13), CO+Veh (n=12), CO+Scop (n=13). Table shows mean±SEM values, P values for the overall comparison between groups (ANOVA), and P values for inter-group comparisons (t-test).

### 7.3.2 Hole board

An overall comparison (ANOVA) across groups revealed no significant difference for all values, except for the number of lines crossed both during the first and the second half of the experiment (Table. 7.3a).

Relative to the animals exposed to just air, the animals exposed to CO scored a higher number of lines crossed and head dips during the first half of the experiment. During the second half, the level of exploratory activity, as measured by the number of lines crossed and rears, was still higher in the CO group. No difference was found between the Veh+Veh and the Scop+Veh groups during the first or the second half of the experiment. Comparison between groups CO+Veh and Veh+Veh showed higher numbers of lines crossed and head dips for the CO+Veh group during the first half of the experiment. During the second half, only the number of lines crossed was significantly higher in the CO+Veh group. Comparison between groups CO+Veh and CO+Scop revealed no significant difference during both the first and the second half of the experiment. A within group comparison (Wilcoxon signed rank test) showed a reduced level of activity during the second half of experiment for all treatment groups (in the CO group only the number of head dips was not significantly reduced) (Table 7.3b).

**a**
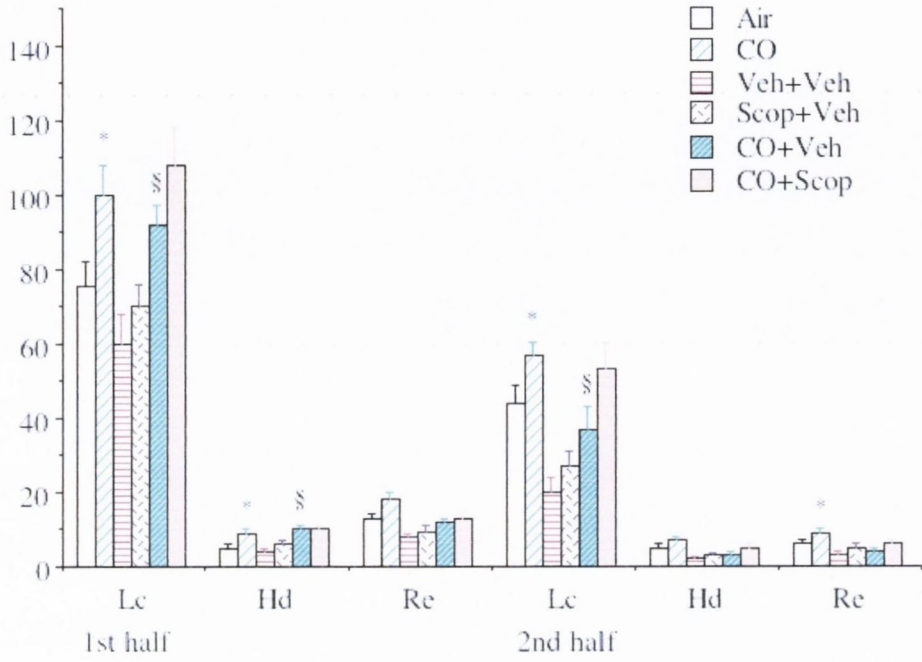


**Fig 7.4**

Effects of scopolamine (0.2 mg/kg), and exposure to CO (2400±240ppm/1hr) on hole board activity. Treatment groups: Air (n=16), CO, Veh+Veh (n=13), Scop+Veh (n=13), CO+Veh (n=12), and CO+Scop (n=13). Asterisk (*) and symbol "§" indicate statistical difference to group Air and Veh+Veh, respectively. Values are means ±SEM. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing.

a

| | Group | Lines crossed | | Head dips | | Rearing | |
|---|---|---|---|---|---|---|---|
| | | 1st half | 2nd half | 1st half | 2nd half | 1st half | 2nd half |
| 1 | Air | 75±7 | 44±5 | 5±1 | 5±1 | 13±1 | 6±1 |
| 2 | CO | 100±8 | 57±3.5 | 9±1 | 7±1 | 18±2 | 9±1 |
| 3 | Veh+Veh | 60±8 | 20.5±4 | 4.4±1 | 1.8±0.5 | 8±1.5 | 3±0.1 |
| 4 | Scop+Veh | 70±6 | 27.5±4 | 6±0.1 | 3±0.6 | 9.5±1.5 | 5±1 |
| 5 | CO+Veh | 92±5 | 37±6 | 10±1 | 3±1 | 12±1 | 4±1 |
| 6 | CO+Scop | 108±10 | 53±7 | 10±1 | 5±1 | 13±1.5 | 6±1 |
| | Overall ANOVA P value (P<) | 0.05 | 0.05 | NS | NS | NS | NS |
| | t-test P values (P<) | | | | | | |
| | Gr. 1 Vs. Gr. 2 | 0.05 | NS | 0.05 | NS | NS | 0.05 |
| | Gr. 3 Vs. Gr. 4 | NS | NS | NS | NS | NS | NS |
| | Gr. 3 Vs. Gr. 5 | 0.05 | 0.05 | 0.05 | NS | NS | NS |
| | Gr. 5 Vs. Gr. 6 | NS | NS | NS | NS | NS | NS |

b

| | Groups | P Values (P<) | | |
|---|---|---|---|---|
| | | lc2-lc1 | hd2-hd1 | re2-re1 |
| 1 | Air | 0.0001 | 0.0001 | 0.0001 |
| 2 | CO | 0.0001 | NS | 0.05 |
| 3 | Veh+Veh | 0.0001 | 0.0001 | 0.0001 |
| 4 | Scop+Veh | 0.0001 | 0.0001 | 0.0001 |
| 5 | CO+Veh | 0.0001 | 0.05 | 0.05 |
| 6 | CO+Scop | 0.0001 | 0.05 | 0.05 |

Table 7.3

Effects of scopolamine (0.2 mg/kg), and exposure to CO (2400±240ppm/1hr) on hole board activity. Treatment groups: Air (n=16), CO, Veh+Veh (n=13), Scop+Veh (n=13), CO+Veh (n=12), CO+Scop (n=13); a) mean±SEM values, P values for the overall comparison between groups (ANOVA) and for inter-group comparisons (t-test); b) within group comparisons (Wilcoxon signed-rank test) between the levels of activity scored during the first and the second half of the experiment. Abbr.: Lc: lines crossed; Hd: head dips; Re: rearing.

## 7.4 Discussion

The first part of this study shows that exposure to CO alone did not have an overall effect on watermaze performance. Previous studies have shown that performance in tasks such as the watermaze or rewarded alternation on the elevated T-maze relies on the function of the dorsal hippocampus (Bannerman et al., 1999; Hock et al., 1998), suggesting, therefore, that it is the dorsal component of the hippocampus that supports spatial learning. Thus, it might be speculated that, in the present study, exposure to CO did not cause significant cell damage in this area. It is also important to consider those data showing that exposure to CO can have delayed effects. Nabeshima et al. (1991) found that neuronal degeneration progresses gradually, having effects on behaviour only 7 to 10 days after exposure to CO. A cytological analysis showed that cell death in each CA1 subfield of the hippocampus could only be observed at least 3 days after exposure and that it was asymmetrical in the two hemispheres. This phenomenon indicates that "delayed" neuronal death in the hippocampal CA1 subfield develops irregularly. In the same studies, cell death in the parietal cortex was not observed until 7 days after exposure and no neurodegeneration in the hippocampal CA2 and CA3 subfields or in the dentate gyrus was observed at any stage (Nabeshima et al., 1991; Koroleva et al., 1999).

Thus, it can be speculated that in the present study, CO inhalation was not sufficient to induce significant cell degeneration in the brain, and to cause a learning impairment in the Morris watermaze when training started 3 days after exposure. In the hole board, the effects of CO exposure were more marked. In fact, during both the first and the second half of the experiment, the animals of the CO group showed a higher level of locomotion and/or exploration relative to the animals of the Air group (hyperactivity was also observed in group CO+Veh relative to group Veh+Veh). For the most part, previous studies have shown that hippocampal damage increases open field locomotor activity (Gray et al., 1982,

1983). It is generally believed that the hyperactivity following the disruption of hippocampal function is due to the damage of efferent glutamatergic projections to medium spiny neurons in the nucleus accumbens. These accumbens cells also receive dopaminergic projections from the ventral tegmental area (Sesack et al., 1990; Totterdell et al., 1989), a pathway that is known to play a pivotal role in the regulation of motor activity. Also, it has been suggested that dorsal hippocampal cell loss could underlie much, if not all, of the hyperactivity observed with complete hippocampal lesions in the open field (Bannerman et al., 1999). It could be suggested that the effects on hole board activity reported in the present study might be attributed to the longer time period elapsed after exposure to CO, which might have induced a more marked disruption of hippocampal activity, although this needs to be confirmed by experiments in the same task carried out at shorter delays from exposure to the gas.

In the second part of the study, the same protocol of exposure to CO was combined with a sub-effective dose of scopolamine administered 3 days after exposure, daily, 30 minutes before training. As mentioned in the introduction, this approach was designed in order to study the possible synergistic interaction between the partial loss of muscarinic receptor activation, and the cell death associated with exposure to CO. The analysis of data revealed that the animals exposed to CO and injected with scopolamine (CO+Scop) performed more poorly in the watermaze than those animals that were injected with just vehicle after exposure to CO (CO+Veh). In fact, even though the animals of the CO+Scop group did not show any difference to the animals of group CO+Veh during acquisition, they spent less time in the target quadrant during the probe test. Also, in the CO+Scop group, a within-group comparison of the time spent in the target quadrant with the time spent in the opposite quadrant showed no preference for the target quadrant. In the hole board there was no difference between groups CO+Scop and CO+Veh. It is of interest that, group CO+Veh

showed no overall performance impairment during both acquisition and probe tests (only the swim speed recorded during probe tests was increased with respect to the Veh+Veh). This evidence suggests, that in the CO+Scop group, the blockade of muscarinic receptors, induced by scopolamine, may have played a role to excerbate the effect of exposure to CO. Given that reduced cholinergic neuronal function may be one of the mechanisms underlying memory dysfunction following exposure to CO (eg., Hiramatsu et al., 1994), these results suggest that the development of the cognitive deficit observed might have been related to the decrease of cholinergic transmission due to disruption of muscarinic receptor activity. This data agrees with other findings that stress the importance of the role of muscarinic receptors in the developement of cognitive deficits following CO inhalation. For instance, it has been shown that a low dose of scopolamine blocks the ameliorating effects of nicotinic agonists in animals exposed to CO and tested in spontaneous alternation tasks 5-7 days after exposure (Masayuki et al., 1996). Furthermore, intoxication caused by inhalation of CO involves also the disruption of the serotonergic system. This is suggested by the studies of Santucci et al. (1995), who found that the dose of scopolamine needed to impair spatial orientation in the Morris watermaze depends on the status of the serotonergic system.

On the basis of these results, further studies should be carried out in order to investigate why combined exposure to CO and a sub-effective dose of scopolamine had more marked effects than exposure to CO combined with vehicle only during the probe test and not during acquisition. If these results are confirmed, it might be suggested the muscarinic cholinergic transmission could be crucial for retrieval but not for acquistion of spatial memory in animals exposed to CO. Moreover, it would be of interest to investigate the effects of the same treatments starting the experiments a longer time after CO exposure (e.g., starting after 10 days), in order to better describe the cognitive effects of this

treatment protocol in the tasks used here, and also to investigate the role played by muscarinic receptor antagonism in memory disruption at stages in which cell death might be more advanced.

# CHAPTER 8

## Overall discussion and conclusions

This research examined the ability of agents that modulate cholinergic, serotonergic and dopaminergic neurotransmission to affect cognition in a watermaze, a hole board and a T-maze. The non-subtype selective muscarinic acetylcholine receptor antagonist, scopolamine, was used to disrupt cognition in order to evaluate neurotransmitter regulation of a compromised cognitive status. The interaction of cholinergic antagonism with low-level exposure to carbon monoxide gas was also assessed.

The experiments in Chapter 2 and Chapter 3 show that, in the behavioural paradigms used, $M_2$ antagonists AFDX 384 and BIBN 99, given alone, had an overall limited effect on the cognitive functions of young adult healthy animals. However, even though these two compounds had no effect on watermaze acquisition, there was some evidence that they improved the search strategy during probe tests run 3 hours after the last acquisition trial. This suggests that the blockade of $M_2$ receptors may stimulate those mechanisms that underlie an efficient use of spatial information, crucial to localize the area where a target is located. Importantly, neither AFDX 384 or BIBN 99 affected behavioural measures in the hole board or in the T-maze thus implying that at the doses used, these agents had no effect on general exploratory locomotor activity and habituation in a novel environment.

Furthermore, neither AFDX 384 or BIBN 99 had a significant overall effect on scopolamine-induced watermaze acquisition deficits. However, probe tests showed that BIBN 99 had an ameliorative effect on the consolidation of the memory for a search strategy that the animals could use to navigate towards

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the escape platform (administration of BIBN 99 improved the target quadrant bias). Interestingly, in scopolamine treated animals, administration of AFDX 384 and BIBN 99 normalized T-maze spontaneous alternation deficits but did not have an overall significant effect on hole board activity. Overall, these results suggest that under conditions in which cholinergic muscarinic postsynaptic transmission is reduced, the blockade of $M_2$ receptors in the brain can influence the mechanisms that underlie reference memory, working memory, exploratory activity and habituation.

Of the three dopamine $D_1$ receptor ligands studied on their own, the partial agonist SKF 38393 was the only one that had no effect on locomotor activity in the hole board, even though it partially reduced habituation. SKF 38393 alone had no effect on performance of the watermaze or T-maze and did not reverse the memory deficits caused by scopolamine in the watermaze. However, it normalized the scopolamine-induced impairment of alternation behaviour in the T-maze task. It is suggested that these results could reflect both a $D_1$ receptor ability to influence those mechanisms involved in the control of working memory and/or an ineffectiveness of SKF 38393 in the modulation of reference memory because of its partial agonist characteristics.

Treatment with the full $D_1$ receptor agonist A 68930 alone did not affect acquistion of the watermaze task but impaired probe test performance. In particular, during the probe test, A 68930 induced an increase of swim speed, which was interpreted as an increase of anxiety. In the hole board and the T-maze, A 68930 reduced general activity and spontaneous alternation, respectively. Moreover, in the watermaze and the hole board, A 68930 had no beneficial effects on the adverse effects of scopolamine. Of note, in the hole board test, combination of A 68930 with scopolamine appeared to cause greater disruption of locomotor activity relative to rats treated with scopolamine and just saline. From these findings it is concluded that strong activation of $D_1$

receptors can impair general locomotor and exploratory activity in novel environments, failing to selectively affect specific cognitive mechanisms. The $D_1$ receptor antagonist SCH 23390, on its own, induced catalepsy making it difficult to evaluate its actions on the behavioural tasks used.

The results reported in Chapters 6 and 7 showed that the combination of a sub-effective dose of scopolamine with either ipsapirone or CO caused performance disruption in the watermaze. Although ipsapirone, at the dose used (5 mg/kg), had disruptive effects on its own (it reduced the target quadrant bias during probe tests), its combination with scopolamine induced further cognitive disruption, as the animals that received this treatment showed further spatial orientation deficits in the watermaze. In the hole board, scopolamine or ipsapirone had little or no effect (in the rats injected with ipsapirone habituation was partially reduced) but the combination scopolamine-ipsapirone appeared to reduce general activity and to disrupt habituation.

In the case of exposure to CO, the levels used had no overall effect on watermaze performance, although there was evidence of an increase in swim speed relative to a group that received no exposure. In the hole board, the effects of CO exposure were more marked as the animals that received this treatment were more active than the animals exposed to only air. These results suggest that this level of CO was not sufficient to induce marked cell degeneration in the brain at the time tested. However, the combination of CO exposure with a subeffective dose of scopolamine induced watermaze probe test performance disruption relative to the animals that received one of the two treatments alone. The combined treatment did not affect hole board activity. Thus blockade of muscarinic receptors appeared to unmask a subliminal toxic effect of exposure to CO. On the basis of these results, it is suggested that further studies should investigate the mechanisms that underlie the muscarinic

modulation of CO exposure-induced cognitive disruption at stages in which the neuroxicity of the gas may have induced further cell degeneration in the brain.

These results are summarized in Table 8.1.

| Treatment | Dose | Watermaze | Hole board | T-maze |
|---|---|---|---|---|
| **AFDX 384** | 5 mg/kg, i.p. | No effect on task acquisition in normal rats. Improvement of probe test search strategy in normal rats. No effect on scopolamine-induced disruption of both acquisition and probe test. | No overall effect in normal rats. No effect on scopola-mine-induced hyperactivity. | No effect on normal rats. Reversal of scopolamine-induced deficit. |
| **BIBN 99** | 0.5 mg/kg, s.c. | No effect on task acquisition in normal rats. Improvement of probe test search strategy in normal and scopolamine treated rats. | No overall effect in normal rats. No effect on scopolamine-induced hyperactivity. | No effect on normal rats. Reversal of scopolamine-induced deficit. |
| **SKF 38393** | 6 mg/kg, i.p. | No effect on both task acquisition and probe test performance in normal rats. No effect on scopolamine-induced disruption of both acquisition and probe test. | Partial reduction of habituation. No other effect in normal rats. No effect on locomotor and exploratory activity of scopolamine treated rats. | No effect on normal rats. Reversal of scopolamine-induced deficit. |

| Treatment | Dose | Watermaze | Hole board | T-maze |
|---|---|---|---|---|
| A 68930 | 6 mg/kg, i.p. | No effect on task acquisition. Impairrment of probe test performance and increase of swim speed in normal rats. No effect on scopolamine-induced disruption of both acquisition and probe tests. | Reduction of general activity in normal rats. No effect in scopolamine-treated rats. | Reduction of spontaneous alternation in normal rats. |
| SCH 23390 | 0.05 mg/kg, i.p | Disruption of acquisition and probe test performance in normal rats (decreased level of exploration and catalepsy). | Catalepsy in normal rats. | Catalepsy in normal rats |
| Scopolamine/ Ipsapirone | 0.2 mg/kg, i.p./ 5 mg/kg, i.p. | No effect on normal acquisition. Disruption of probe test performance. | Reduction of lo-comotor and exploratory activity and of habituation | Not tested. |
| Scopolamine/CO | 0.2 mg/kg, i.p./ 2400±240ppm/1hr | No effect on normal acquisition. Disruption of probe test performance. | No effect | Not tested. |

**Table 8.1** Summary of the main effects of the drugs studied.

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