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## Antisense ‘knockdowns’ of M<sub>1</sub> receptors induces transient anterograde amnesia in mice

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### Abstract

The effect on memory processes of inactivation of the M<sub>1</sub> gene by an antisense oligodeoxyribonucleotide (aODN) was investigated in the mouse passive avoidance test. Mice received a single intracerebroventricular (i.c.v.) injection of M<sub>1</sub> aODN (0.3, 1.0 or 2.0 nmol per injection), degenerated ODN (dODN) or vehicle on days 1, 4 and 7. An amnesic effect, comparable to that produced by antimuscarinic drugs, was observed 12, 24, 48 and 72 h after the last i.c.v. aODN injection, whereas dODN and vehicle, used as controls, did not produce any effect. Reduction in the entrance latency to the dark compartment induced by aODN disappeared 7 days after the end of aODN treatment, which indicates the absence of any irreversible damage or toxicity caused by aODN. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that a decrease in M<sub>1</sub> mRNA levels occurred only in the aODN-treated group, being absent in all control groups. Furthermore, a reduction in M<sub>1</sub> receptors was observed in the hippocampus of aODN-treated mice. Neither aODN, dODN nor vehicle produced any behavioral impairment of mice. These results indicate that the integrity and functionality of M<sub>1</sub> receptors are fundamental in the modulation of memory processes. © 1999 Elsevier Science Ltd. All rights reserved.

**Keywords:** Learning; Memory; M<sub>1</sub> receptors; Antisense oligonucleotide; Amnesia; Central cholinergic system

### 1. Introduction

Cholinergic activity has long been associated with memory processes. Morphological and neurochemical studies of Alzheimer's disease, the major type of dementia, revealed marked decreases in the cholinergic innervation of the cortex and hippocampus (Bartus et al., 1982; Mash et al., 1985; Whitehouse, 1986). Drugs involving cholinergic stimulation alleviated cognitive dysfunctions in Alzheimer's disease (Bartus et al., 1982) and in particular M<sub>1</sub>-selective agonists have been proposed as a promising treatment strategy in this pathology (Mash et al., 1985; Whitehouse, 1986; Fisher et al., 1989; Gualtieri et al., 1995). On the other hand, cholinergic blockade produces significant impairments of cognitive functions. A delay-dependent disruption fol-

lowing treatment with scopolamine and atropine, that appeared to resemble that occurring spontaneously in aged subjects and in Alzheimer patients, has been reported (Duetsch, 1971; Bartus and Johnson, 1976). Animals treated with the M<sub>1</sub> selective antagonist pirenzepine (Hammer et al., 1980), had impaired passive avoidance learning (in mice) (Caufield et al., 1983) and impaired spatial learning (Hagan et al., 1987; Hunter and Roberts, 1988), radial arm maze performance (Sala et al., 1991) and active avoidance acquisition (Sen and Bhattacharya, 1991) in rats. Moreover, the M<sub>1</sub> selective antagonists dicyclomine (Nilvebrant and Sparf, 1986) and *S*-(–)-ET-126 (Ghelardini et al., 1996) were able to induce amnesia in a mouse passive avoidance task (Ghelardini et al., 1997b; Matucci et al., 1997).

In order to further investigate the role of the M<sub>1</sub> muscarinic receptor subtype in learning and memory processes, an antisense oligodeoxyribonucleotide (aODN) able to inactivate M<sub>1</sub> gene expression was

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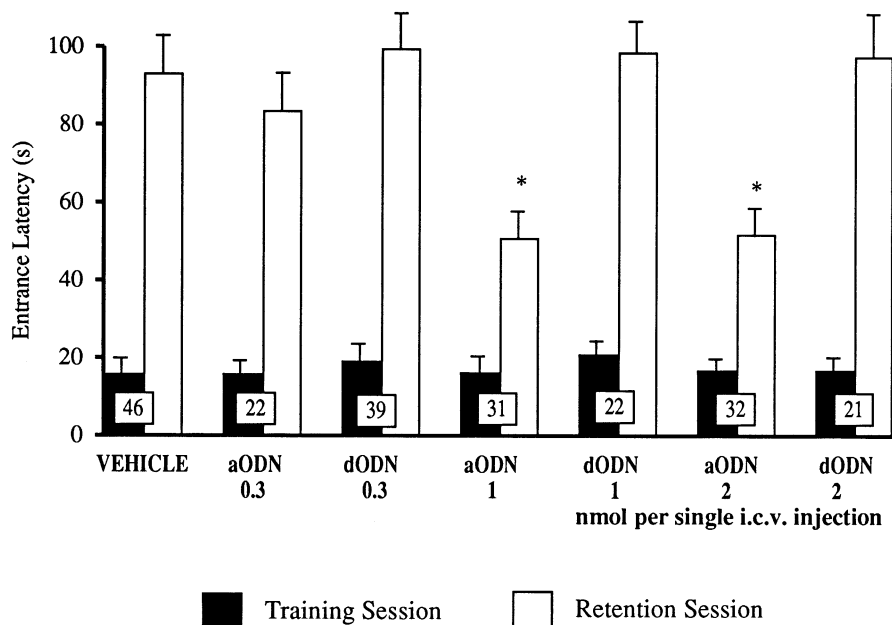


Fig. 1. Dose-response curve of an aODN to  $M_1$  gene in the mouse passive-avoidance test. Mice received a single i.c.v. injection of aODN, dODN or vehicle on day 1, 4 and 7. Training session was performed 24 h after the last i.c.v. injection. The number of mice is inside the column. \*  $P < 0.01$  in comparison with dODN-treated mice.

used. An aODN is a short segment of synthetic DNA having a sequence complementary to a portion of targeted mRNAs preventing translation and/or mediating mRNA cleavage by the enzyme RNase H. aODNs specifically bind to targeted mRNAs and, therefore, down-regulating the synthesis of the encoded useful pharmacological tools for exploring a variety of neurobiological processes at the molecular level by specifically turning off gene expression, and can be considered as a new potential class of 'informational' drugs (Wahlestedt, 1994; Crooke and Bennett, 1996; Galeotti et al., 1997; Ghelardini et al., 1997a; Meiri et al., 1997). aODN can transiently inactivate a single gene and, therefore, can inactivate receptor functions in a more specific and selective manner than receptor antagonists. The effects on memory processes of anti- $M_1$  aODN were evaluated in mice. The aODN was conjugated to an artificial cationic lipid (DOTAP) used as the vehicle able to improve aODN intracellular stability (Cappacioli et al., 1993) and was administered by intracerebroventricular (i.c.v.) injection. Vehicle and degenerated ODN (dODN, an oligonucleotide accounting for the eventual aODN specific effects) were used as controls.

## 2. Materials and methods

### 2.1. Animals

Male Swiss albino mice (24–26 g) from Morini (San Polo d'Enza, Italy) were used. Fifteen mice were housed

per cage. The cages were placed in the experimental room 24 h before the test for acclimatization. The animals were fed a standard laboratory diet and tap water ad libitum and kept at  $23 \pm 1^\circ\text{C}$  with a 12 h light–dark cycle, light at 07:00 h.

### 2.2. Antisense oligonucleotides

Low cell permeability and the high degradation of natural phosphodiester oligomers are considerable drawbacks in the application of aODNs both in vitro and in vivo. To overcome these drawbacks, phosphorothioate-capped phosphodiester oligonucleotides were used. The above-mentioned compounds are a class of ODN derivatives shown to maintain more stable and effective concentrations in the brain when compared with their unmodified counterpart (Whitesell et al., 1993). Phosphodiester oligonucleotides (ODNs) protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Genosys (Cambridge, England) and purified by high-performance liquid chromatography (HPLC). The 18-mer antisense ODN 5'-CAC TGA GGT GTT CAT TGC-3' (phosphorothioate residues are underlined) complementary to the residues 112–129 of the published mouse  $M_1$  cDNA sequence (Shapiro et al., 1988) and the 18-mer fully degenerated ODN (dODN) 5'-NNN NNN NNN NNN NNN-3' (where N is G, or C, or A, or T and phosphorothioate residues are underlined) were vehiculated intracellularly by an artificial cationic lipid (DOTAP, Boehringer–Mannheim, Ger-

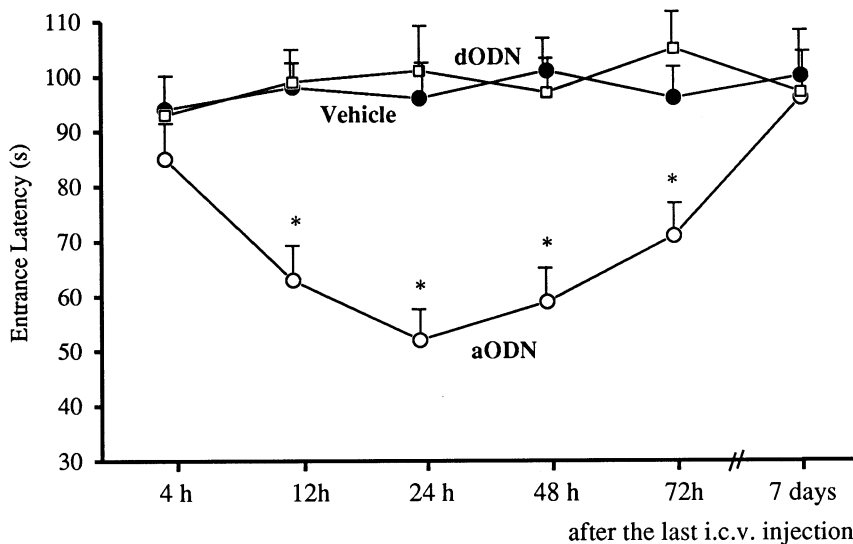


Fig. 2. Time-course of the amnesic effect induced by an aODN to  $M_1$  gene in the mouse passive-avoidance test. Mice received a single i.c.v. injection of aODN (2 nmol per single i.c.v. injection), dODN (2 nmol per single i.c.v. injection) or vehicle on day 1, 4 and 7. Each value represents the entrance latency in the retention session. The number of mice ranged from 18 to 24. \*  $P < 0.01$  in comparison with dODN-treated mice.

many) to enhance both uptake and stability, as described previously (Capaccioli et al., 1993; Quattrone et al., 1994b). aODN or degenerated ODN (100–400  $\mu\text{M}$ ) were preincubated at 37°C for 30 min with 13  $\mu\text{M}$  DOTAP, sterilized through a 0.2  $\mu\text{m}$  filter and supplied to mice by i.c.v. injection of a 5  $\mu\text{l}$  solution as described in the next section.

### 2.3. GenBank accession numbers

The accession number of the cDNA sequence for the mouse muscarinic receptor subtype reported in this paper ( $M_1$ ) is J04192.

### 2.4. I.c.v. injection of oligonucleotides

Mice were randomly assigned to anti- $M_1$  aODN, dODN, vehicle, saline or naive group. The antisense and dODNs were dissolved in a vehicle constituted by DOTAP. Each group received a single i.c.v. injection on days 1, 4 and 7 whereas naive animals did not receive any treatment. I.c.v. administration was performed under ether anesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick (1957). During anesthesia, mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10  $\mu\text{l}$  syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5  $\mu\text{l}$  ODNs were then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that ODNs were

administered exactly into the cerebral ventricle, some mice were injected with 5  $\mu\text{l}$  of diluted 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated with 95% of injections being correct.

### 2.5. Passive avoidance

The test was performed according to the step-through method described by Jarvik and Kopp (1967). The apparatus consists of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. Mice, as soon as they entered the dark compartment, received a punishing electrical shock (0.5 mA, 1 s). The latency times for entering the dark compartment were measured in the training test and after 24 h in the retention test. For memory disruption animals were i.c.v. injected with the amnesic drugs scopolamine, pirenzepine, dicyclomine and *S*-(-)-ET 126, administered immediately after termination of the training session. The maximum entry latency allowed in the training and retention session was 60 and 180 s, respectively. The memory degree of received punishment was expressed as latencies recorded in the training and retention sessions.

### 2.6. Hole-board test

The hole board test consists of a 40 cm square plane with 16 flush-mounted cylindrical holes (diameter 3 cm) distributed 4 by 4 in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and left to move about freely for a period of 5 min each. Two electric eyes, crossing the plane from mid-

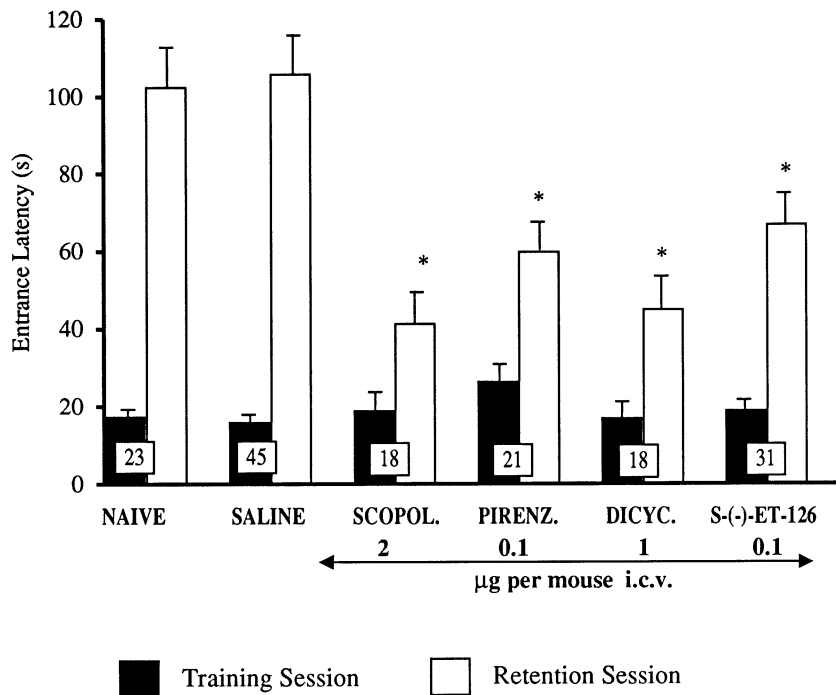


Fig. 3. Amnesic effect of scopolamine, pirenzepine, dicyclomine and *S*(-)-ET-126 in the mouse passive-avoidance test. All drugs were injected immediately after the training session. The number of mice is inside the column. \*  $P < 0.01$  in comparison with naive or saline-treated mice.

Table 1  
Lack of effect of an aODN to  $M_1$  gene in the mouse rota-rod test<sup>a</sup>

Treatment	Dose per single i.c.v. injection	No. of falls in 30 s			
		0 min	15 min	30 min	45 min
Saline	5 µl	3.7 ± 0.3	2.6 ± 0.2	1.2 ± 0.3*	0.9 ± 0.3*
Vehicle	5 µl	3.6 ± 0.4	2.8 ± 0.3	1.5 ± 0.4*	1.0 ± 0.2*
aODN	2 nmol	3.8 ± 0.4	2.5 ± 0.4	1.4 ± 0.3*	1.1 ± 0.2*
dODN	2 nmol	3.6 ± 0.3	2.4 ± 0.4	1.6 ± 0.3*	0.7 ± 0.3*

<sup>a</sup> Mice were injected with aODN, dODN saline or vehicle on day 1, 4 and 7. Rota-rod was performed 24 h after the last i.c.v. injection. Each value represents the mean of 15 mice.

\*  $P < 0.01$  in comparison with the respective pre-test value.

point to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animals on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded the exploration of the holes (head plunging activity) by the mice (exploratory activity). The test was performed 24 h after the last i.c.v. injection of aODN or dODN. Naive animals were used as non pretreated controls.

### 2.7. Rota-rod test

The apparatus consisted of a base platform and a rotating rod of 3 cm diameter with a nonslippery surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus up to five mice were tested

simultaneously on the apparatus, with a rod-rotating speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. (1985). The performance time was measured before and 15, 30 and 45 min after the beginning of test. The test was performed 24 h after the last i.c.v. injection with aODN or dODN. Naive animals were used as untreated controls.

### 2.8. RT-PCR-based analysis of $M_1$ mRNA

Twenty-four hours after the last i.c.v. injection of vehicle, aODN or degenerated ODN, three mice for each group were sacrificed and their brains were rapidly removed and stored ( $-80^{\circ}\text{C}$ ). Mouse brain levels of  $M_1$  mRNA were determined by a quantitative RT-PCR method optimized for other genes (Quattrone et al.,

1994a, 1995a). Frozen mouse brain samples (0.2–0.3 g wet wt.) were homogenized in three vol. of RNazol to extract total RNA according to the manufacturer's instructions. RNA was treated with RQ1 RNase-free DNase, purified by ethanol precipitation, dissolved in water containing an RNase inhibitor (RNasin at 1 U/ml) and reversely transcribed to cDNA with random hexamers. A qualitative PCR reaction was preliminarily carried out for 30 cycles in a standard hot-start PCR procedure (Chou et al., 1992) with the primer pairs 5'-CCA CCT GGC TGC AAT GAA C-3'; residues 102-120 and 5'-GTT GAC TGT CTT GAG CTC TGT G-3'; residues 273-294, according to the M<sub>1</sub> cDNA published sequence (Shapiro et al., 1988). RNase treated before RT or reverse transcriptase-omitted samples were the negative PCR controls in order to exclude any possible contamination by endogenous genomic DNA and/or by amplified DNA carry-over. Primers 5'-GCG GGA AAT CGT GCG TGA CAT-3'; residues 2104-2125 and 5'-GAT GGA GTT GAA GGT AGT TTC GTG-3'; residues 2409-2432, according to the published sequence (Ng et al., 1985), were used in the quantitative protocol for the amplification of  $\beta$ -actin cDNA as an internal standard (Quattrone et al., 1995b). For both primer pairs, the three-step PCR cycles consisted of 1 min denaturation at 92°C, 1 min annealing at 58°C and 1 min extension at 72°C. PCR products were electrophoresed on 2% agarose gel and the M<sub>1</sub> product was first identified by sequencing with the fmol sequencing kit (Promega, Madison, WI). For quantitative analysis, different volumes of M<sub>1</sub> or  $\beta$ -actin cDNA were separately amplified for 30 cycles of PCR and the resulting agarose bands were analyzed by densitometry. M<sub>1</sub> mRNA levels were evaluated by referring three densitometric values in the linear range obtained with the M<sub>1</sub> PCR products to those obtained with the standard  $\beta$ -actin PCR products, and normalizing for the relevant cDNA volumes.

## 2.9. Binding assay-membrane preparation

Male mice used for the [<sup>3</sup>H]telenzepine binding assays were killed by decapitation 24 h after the last i.c.v. injection of aODN, dODN or vehicle. The brain was removed, rinsed with ice-cold 0.9% saline solution and dissected into two regions; cortex and hippocampus. Tissues were homogenized in 40 vol. Hepes buffer containing 20 mM Hepes, 100 mM NaCl, 10 mM MgCl<sub>2</sub> and 0.01 mM phenyl-methyl-sulfonyl-fluoride (PMSF), pH = 7.4 for 15 s using an Ultraturrax homogenizer at half of the maximum speed. Homogenates were centrifugated at 1000 × *g* for 5 min; supernatants were again centrifuged at 48 000 × *g* for 30 min and the resultant sediments were immediately frozen at -80°C until binding experiments were performed.

## 2.10. Ligand binding assay

Frozen pellets were dispersed in Hepes buffer and then spun at 48 000 × *g* for 30 min. The resulting pellets were rehomogenized in the above-mentioned buffer and used for the binding assays; an aliquot was taken for protein determinations. Aliquots of membranes (0.4–0.6 mg ml<sup>-1</sup>) were incubated in a final vol. of 0.5 ml ligands in Hepes buffer at 37°C for 2 h; [<sup>3</sup>H]telenzepine was present at 0.4 nM in tubes containing increasing concentrations (0.3–10 000 nM) of unlabeled telenzepine and at 0.05–0.4 nM in tubes without unlabeled ligand. All measurements were obtained in duplicate. After incubation, bound radioactivity was separated by filtration through Whatman GF/C filters presoaked in 0.1% polyethyleneimine (PEI) using the Brandel M-48R cell harvester (Gaithersburg, MD). Filters were washed twice with 5 ml of 50 mM Tris-HCl buffer, pH = 7.4 containing 10 mM MgCl<sub>2</sub> and counted in 5 ml of FilterCount (Packard) in a liquid scintillation counter TRI-CARB 1900 TR (Packard). Protein content was measured using the Pierce protein assay reagent (Pierce Chemical, Rockford, IL), according to the standard assay procedure recommended by the manufacturer and based on the method of Bradford (1976) using bovine serum albumin as a standard.

## 2.11. Data analysis

Sequence comparisons of both aODN and RT-PCR primers with the database were carried out by the FASTA program. All experimental results are given as the mean ± SEM. An analysis of variance ANOVA, followed by Fisher's Protected Least Significant Difference procedure for post-hoc comparison, were used to verify significance between two means of behavioral results. Data were analyzed with the StatView software for the Macintosh. The statistical significance of RT-PCR was obtained with the Student's *t*-test. The binding data were evaluated quantitatively using the Ligand (Munson and Rodbard, 1980) computer program; this type of analysis provides optimal estimates of binding parameters (affinity constants, binding capacities, non specific binding). *P* values < 0.05 were considered significant.

## 2.12. Drugs

The following drugs were used: scopolamine hydrobromide (Sigma, Italy); dicyclomine hydrochloride (Le Petit, Italy); *S*-(-)-ET 126 (*S*-(-)- $\alpha$ -(hydroxymethyl)benzene-acetic acid 1-methyl-4-piperidiny ester) prepared in the Department of Pharmaceutical Sciences of Florence; pirenzepine dihydrochloride (RBI, Italy); [<sup>3</sup>H]telenzepine (DuPont NEN, Italy); *D*-amphetamine (De Angeli, Italy). Other chemicals were of

the highest quality commercially available. Drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use. Drug concentrations were prepared so that the necessary dose could be administered in a volume of 5  $\mu$ l per mouse by intracerebroventricular (i.c.v.) injection and 10 ml  $\text{kg}^{-1}$  by subcutaneous (s.c.) injection.

### 3. Results

#### 3.1. Design of oligonucleotides

Considering that the translation start sites of mRNAs are particularly prone to aODN action (Goodchild, 1989; Stein and Cheng, 1993), we compared the sequence encompassing this site among the murine known muscarinic receptors gene family, to design a specific antimouse  $M_1$  aODN. We noted that a region of 18 bases comprising the translation start site region of the mouse  $M_1$  mRNA (Shapiro et al., 1988) is endowed with a very low sequence homology even with the nearest other members of the muscarinic receptor family. We therefore chose this sequence to define an aODN targeting the  $M_1$  murine mRNA. A homology search in the Genbank database confirmed the absolute specificity of this aODN. Considering the described sequence-independent, non-antisense effects of ODNs (Storey et al., 1991; Gao et al., 1992; Blagosklonny and Neckers, 1994; Schick et al., 1995), we designed a fully degenerated phosphorothioate capped phosphodiester ODN as the most suitable control for these potentially confusing effects. The fully degenerated 18-mer is a collection of about  $3 \times 10^{14}$  different molecular species. At concentrations achieved in the nanomolar to micromolar range in *in vitro* antisense experiments, every species, i.e. every ODN of defined sequence, was present at the site of action at a concentration less than  $10^{-18}$  M, which is totally insufficient to achieve any antisense, or generally sequence-dependent, cellular effect. Therefore, if ODN i.c.v. administration per se had achieved any biological response, this would have been present in ODN-treated controls.

#### 3.2. Amnesic effect of aODN to $M_1$ muscarinic receptor

Mice were pretreated with a single i.c.v. injection of aODN, dODN or vehicle on days 1, 4 and 7. The effect of aODN pretreatment on memory processes was then evaluated in the mouse passive avoidance test.

At the dose of 0.3 nmol per i.c.v. injection, aODN did not significantly affect the entrance latency into the dark compartment (Fig. 1), whereas at the dose of 1.0 and 2.0 nmol per i.c.v. injection, aODN produced amnesia (Fig. 1). This effect was detected 24 h after the

last i.c.v. injection. No difference in the amnesic effect produced by the two effective doses of aODN was revealed (Fig. 1). Therefore, higher doses of aODN were not investigated.

Experiments were performed 24 h after the end of ODN treatment since a time-course study showed that the maximum amnesic effect was reached 24 h after the last i.c.v. injection at the dose of 2.0 nmol per i.c.v. injection and then it decreased (Fig. 2). However, amnesia induced by aODN to  $M_1$  receptors appeared at 12 h and was also statistically significant at 48 and 72 h (Fig. 2). In contrast, on day 7, the impairment of cognitive processes produced by aODN disappeared (Fig. 2).

Pretreatment with the dODN did not modify the entrance latency into the dark compartment in com-

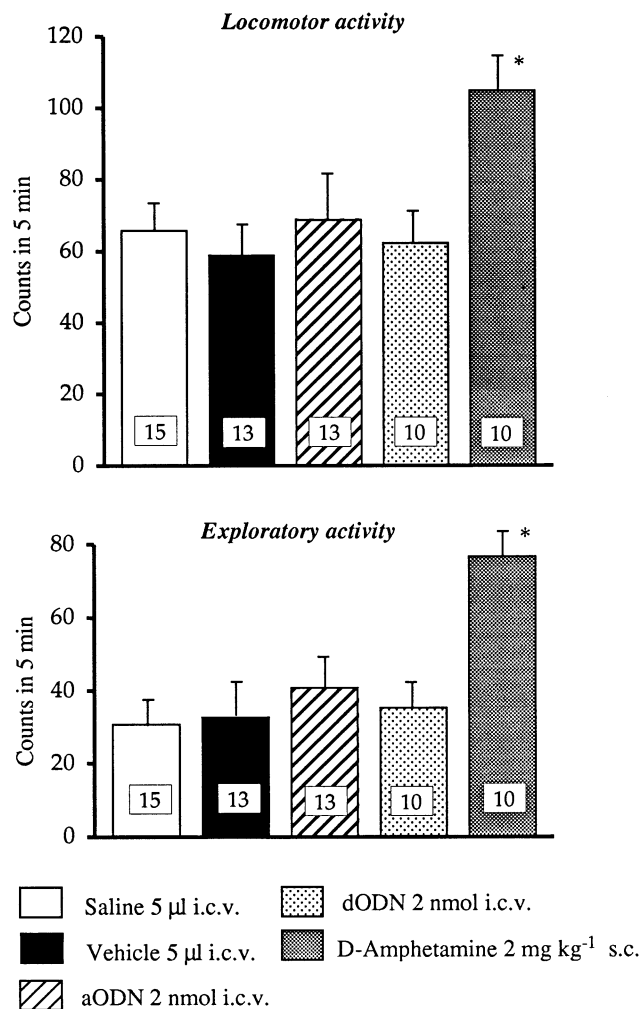


Fig. 4. Lack of effect of an aODN to  $M_1$  gene in comparison with D-amphetamine in the hole board test. Mice received a single i.c.v. injection of aODN, dODN, saline or vehicle on day 1, 4 and 7. The responses for saline, vehicle, aODN and dODN were recorded 24 h after the last i.c.v. injection. D-Amphetamine response was recorded 15–25 min after administration. The number of mice is inside the column. \*  $P < 0.01$  in comparison with saline-treated mice.

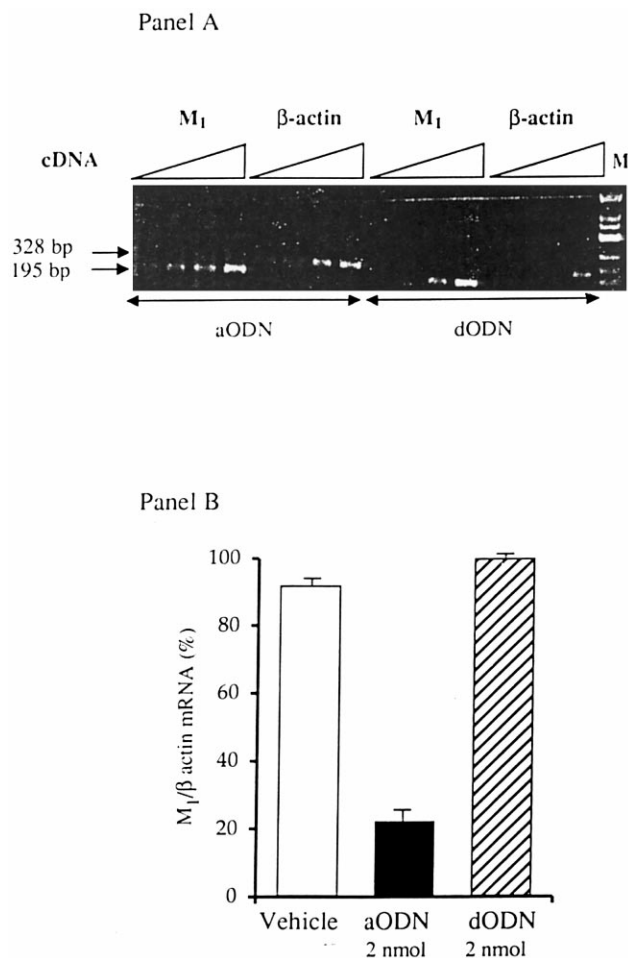


Fig. 5. Quantitative RT-PCR analysis of  $M_1$  mRNA. Total RNA was extracted from the brains of three mice for each group behaviorally processed 24 h after the last ODN injection.  $M_1$  and  $\beta$ -actin mRNA were submitted to RT-PCR as reported in the 'experimental procedures'. aODN and dODN were administered i.c.v. on day 1, 4 and 7. (A) RT-PCR analysis of increasing cDNA aliquots of retrotranscribed RNA. Primers specific for murine  $M_1$  mRNA and  $\beta$ -actin mRNA were used. (B) Quantification of  $M_1$  mRNA steady-state levels performed by densitometric analysis of PCR products taken in the range of linearity. Data are the mean of three determinations. Values are expressed as a percentage (%).

parison with mice injected with vehicle i.c.v. as shown in Fig. 1.

The intensity of the amnesia produced by aODN treatment was comparable to that exhibited by the non selective muscarinic antagonist scopolamine (2  $\mu$ g per mouse i.c.v.) and the  $M_1$  selective antagonists pirenzepine (0.1  $\mu$ g per mouse i.c.v.), dicyclomine (1  $\mu$ g per mouse i.c.v.) and *S*-(-)-ET-126 (0.1  $\mu$ g per mouse i.c.v.) (Fig. 3). The antimuscarinic drugs were administered immediately after the training session.

The i.c.v. injection of vehicle or saline did not modify animals' performance in the mouse passive avoidance test in comparison with naive mice (Fig. 3).

### 3.3. Effect of aODN on rota-rod and hole-board tests

Mice pretreated with aODN (2.0 nmol per injection) or dODN (2.0 nmol per injection) were evaluated for motor coordination, spontaneous motility and inspection activity by use of the rota-rod (Table 1) and hole board (Fig. 4) tests. Both tests were carried out 24 h after the last i.c.v. injection. The motor coordination of animals pretreated with aODN and dODN, which was evaluated using the rota-rod test, was not significantly impaired in comparison with saline- and vehicle-treated mice (Table 1). Each group progressively reduced its number of falls since mice learned how to balance on the rotating rod.

The spontaneous motility and inspection activity of mice was unmodified by pretreatment with aODN (2.0 nmol per injection) or dODN (2.0 nmol per injection) as revealed by the hole board test (Fig. 4) in comparison with saline- and vehicle-treated mice. In the same experimental conditions D-amphetamine (1 mg  $\text{kg}^{-1}$  s.c.) increased both parameters evaluated.

### 3.4. Effect of aODN on specific inhibition of $M_1$ gene expression

The lowering of  $M_1$  mRNA following aODN administration as an index of  $M_1$  gene expression inactivation was quantified by RT-PCR. Before quantification, RT products were tested for possible genomic DNA contamination. For this purpose, amplification products—a segment of 192 bp for mouse  $M_1$  cDNA and of bp 232 for  $\beta$ -actin cDNA—were visualized by agarose gel electrophoresis. Qualitative gel analysis showed bands of the expected length and an absence of any contamination in the negative controls (data not shown). Quantitative results of  $M_1$  mRNA brain levels following aODN mouse treatment confirmed that phenotypic effects of anti- $M_1$  aODN on cognitive processes were actually due to the specific inhibition of  $M_1$  gene expression (Fig. 5). Fig. 5, panels A and B, shows that the  $M_1$  mRNA/ $\beta$ -actin mRNA ratio was sharply lowered in anti- $M_1$  aODN-treated mice as compared to dODN-treated mice.

### 3.5. Determination of $M_1$ receptor protein brain levels

Saturation analysis demonstrated that [ $^3\text{H}$ ]telenzepine binds with high affinity and saturable manner to a homogeneous class of sites in membranes from brain, hippocampus and cortex of dODN- and aODN-treated mice. A decrease in [ $^3\text{H}$ ]telenzepine binding was observed in the cortex even if statistical significance was reached only in the hippocampus (Table 2). [ $^3\text{H}$ ]telenzepine binding in the whole brain was not appreciably affected by the treatment (data not shown); in any case  $K_D$  values remained unaffected (Table 2).



Table 2  
 $[^3\text{H}]$ telenzepine binding to mouse cortex and hippocampus membranes by anti-M1 ODN (aODN) and degenerate ODN (dODN)<sup>a</sup>

Treatment	Cortex		Hippocampus	
	$B_{\text{max}}$ (fmol/mg protein)	$K_{\text{D}}$ (nM)	$B_{\text{max}}$ (fmol/mg protein)	$K_{\text{D}}$ (nM)
dODN ( $n = 3$ )	1136.91 $\pm$ 52.85	2.23 $\pm$ 0.29	956.27 $\pm$ 30.32	1.40 $\pm$ 0.10
aODN ( $n = 3$ )	1066.21 $\pm$ 22.58	1.95 $\pm$ 0.12	822.79 $\pm$ 34.82*	1.27 $\pm$ 0.10

<sup>a</sup> Binding experiments were performed 24 h after the last i.c.v. injection. Values indicate mean  $\pm$  SEM.

\*  $P < 0.05$  versus dODN-treated mice.

#### 4. Discussion

The present study provides evidence of the fundamental role played by the M<sub>1</sub> muscarinic receptor subtype in cognitive processes. Pretreatment by i.c.v. administration of an antisense to the M<sub>1</sub> gene coding for the mouse M<sub>1</sub> receptor impairs passive avoidance learning in mice. At active doses, aODN to M<sub>1</sub> receptor does not modify animal gross behavior nor impair motor coordination, spontaneous motility and inspection activity.

The maximum amnesic effect obtained following injection of an aODN to the M<sub>1</sub> receptor has similar intensity to that produced by the unselective muscarinic antagonist scopolamine as well as the selective M<sub>1</sub> antagonists pirenzepine, dicyclomine and *S*-(-)-ET 126. These data confirm the important role played by M<sub>1</sub> receptors in the modulation of mnemonic functions (Section 1). Furthermore, the employment of an aODN to the M<sub>1</sub> receptor allows clear elucidation of the role of the M<sub>1</sub> receptor in memory processes without any potential interference due to interaction with the other muscarinic receptor subtypes. The above-mentioned M<sub>1</sub> receptor antagonists, even if they are the most selective M<sub>1</sub>/M<sub>2</sub>-M<sub>3</sub> compounds available, are endowed with selectivity for M<sub>1</sub> receptors comprising between 3 (for dicyclomine; Giachetti et al., 1986) and 176 (for *S*-(-)-ET 126; Ghelardini et al., 1996). This selectivity ratio does not exclude the fact that, at doses active on cognitive processes, these compounds can also interact with other muscarinic receptor subtypes such as M<sub>2</sub>, that are involved in modulation of learning and memory (Packard et al., 1990; Sen and Bhattacharya, 1991; Gualtieri et al., 1995).

The aODN treatment induces a transient anterograde amnesic effect since the impairment of passive avoidance performance disappears 7 days after the last i.c.v. injection of the aODN. This return to normal memory function implies both the total reversal of aODN-induced specific inhibition of M<sub>1</sub> gene expression and a lack of damage or toxicity associated with aODN treatment. In comparison with naive and saline i.c.v. treatment, dODN and vehicle treatments do not modify the latency to enter the dark compartment, ruling out the possibility that the antagonism exerted by aODN could

be caused by sequence-independent effects on cerebral structures. This claim is supported by results obtained from the quantitative RT-PCR analysis of ODN effects on M<sub>1</sub> gene expression. Degenerated ODN does not modify M<sub>1</sub> mRNA brain levels, whereas the anti-M<sub>1</sub> aODN specifically lowers M<sub>1</sub> mRNA brain levels.

A reduction in M<sub>1</sub> mRNA levels, but not in M<sub>1</sub> receptors, is observed by processing the whole brain. Considering that M<sub>1</sub> receptors are expressed at the highest levels in particular brain areas, such as the cortex and hippocampus (Cortes and Palacios, 1986; Cortes et al., 1986), we investigated the effects produced by aODN treatment in M<sub>1</sub> receptor levels in these two areas. These regions appear to be the most appropriate also because they are greatly involved in the modulation of memory processes (Zola-Morgan and Squire, 1993). By performing binding experiments in the hippocampus and cortex of anti-M<sub>1</sub> treated mice, we documented a reduction in the binding sites of  $[^3\text{H}]$ -telenzepine to M<sub>1</sub> receptors in both cerebral structures. These results are in agreement with previous findings in which a reduction of  $[^3\text{H}]$ -QNB binding in rat cortex and hippocampus is observed after treatment with an aODN to the m1 muscarinic receptor mRNA (Zang et al., 1994). Fewer M<sub>1</sub> receptors in the hippocampus of anti-M<sub>1</sub> aODN-treated mice confirms the importance of a complete integrity and functionality of M<sub>1</sub> receptors in the modulation of memory functions. In fact, a lack of expression of M<sub>1</sub> receptors, as well as the intrahippocampal injection of scopolamine (Brito et al., 1983), atropine (Singh et al., 1974) and pirenzepine (Messer et al., 1985), produce amnesia. The amnesic effect in amnesic and Alzheimer's patients is reported to be ascribed to a disturbance of declarative long-term memory or explicit memory, and not to non-declarative memory, in which classical conditioning tasks like passive avoidance are classified (Zola-Morgan and Squire, 1993; Squire and Zola, 1996). These data could mean that the reduction of M<sub>1</sub> receptor levels seen in the cortex and hippocampus of aODN-treated mice might be observed because the M<sub>1</sub> receptors are expressed at the highest levels in these two structures. The detection of an amnesic effect in anti-M<sub>1</sub> treated mice in passive avoidance experiments might indicate that the M<sub>1</sub> muscarinic receptor subtype could

not play a key role in memory processes through the hippocampus, but it may play a modulating role through other structures involved in learning and memory.

In our experimental conditions, the anti-M<sub>1</sub> aODN does not cause any detectable modification in mouse gross behavior. Moreover, mice treated with the highest dose used of aODN have unimpaired motor coordination in comparison with dODN- and vehicle-treated groups or naive mice. Repetition of the test session every 15 min for four times induces a progressively slight decrease in the number of falls in control animals as well as in aODN- and dODN-treated mice. By contrast, the lack of variation, or an increase in the number of falls after treatment indicates impaired mouse motor coordination that could lead to a misinterpretation of the results obtained in the passive avoidance test. Since during the passive avoidance test mice have to move from the light to the dark compartment of a light-dark box, the treatment must not modify either spontaneous motility or inspection activity of animals. The lack of effect by aODN and dODN treatment in the hole board test, which measures both the above-mentioned parameters, excludes that the observed amnesia underlies a behavioral side effect produced by the i.c.v. injection of oligos.

Seen as a whole, our data indicate that the M<sub>1</sub> muscarinic receptor subtype plays a key role in the modulation of memory functions in mice, and also suggests the actual possibility of modulating receptor activity in laboratory animals by the aODN strategy.

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