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Differential involvement of the sigma$_1$ (σ_1) receptor in the anti-amnesic effect of neuroactive steroids, as demonstrated using an *in vivo* antisense strategy in the mouse

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1 The sigma$_1$ (σ_1) receptor cDNA was cloned in several animal species. Molecular tools are now available to identify its endogenous effectors, such as neuroactive steroids, and to establish its precise physiological role. In particular, the σ_1 receptor is involved in memory processes, as observed in pharmacological and pathological rodent models of amnesia.

2 In order to establish the involvement of σ_1 receptors in memory, a 16-mer oligodeoxynucleotide antisense to the σ_1 receptor cDNA (aODN), and its mismatched control (mODN) were prepared and centrally administered into the mouse brain. The anti-amnesic effects induced by the selective σ_1 agonist PRE-084 and the steroid dehydroepiandrosterone (DHEA) sulphate or pregnenolone sulphate were examined in ODN-treated animals.

3 The aODN treatment failed to affect the dissociation constant (K_d) but significantly decreased the number of σ_1 sites (B_{max}) labelled with [^{3}H]-(+)-SKF-10,047 in the hippocampus and cortex. In these structures, the *in vivo* binding levels were also diminished, according to the dose and number of injections, as compared with control animals injected with saline or mODN.

4 Cannulation and injections failed to affect the open-field behaviour of the animals. However, the anti-amnesic effects of PRE-084 and DHEA sulphate against the dizocilpine-induced impairments were blocked after aODN treatment in the short- and long-term memory tests. The anti-amnesic effects of pregnenolone sulphate remained unchanged.

5 These observations bring a molecular basis to the modulatory role of σ_1 receptors in memory, and reveal that the anti-amnesic action of neuroactive steroids may not similarly involve an interaction with σ_1 receptors.

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Abbreviations: aODN, antisense oligodeoxynucleotide; DHEA, dehydroepiandrosterone (3β-hydroxy-5α-androsten-17-one); mODN, mismatch oligodeoxynucleotide; NMDA, N-methyl-D-aspartate; ODN, oligodeoxynucleotide

Introduction

The sigma$_1$ (σ_1) receptor mediates in the nervous system a potent neuromodulatory effect affecting different neurotransmission systems (for reviews, Walker *et al*., 1990; Maurice *et al*., 1999). In particular, selective σ_1 receptor agonists have been reported to facilitate the cholinergic and N-methyl-D-aspartate (NMDA)-type of glutamatergic neurotransmission systems, which are directly involved in learning and memory encoding in forebrain structures such as the hippocampus or cortex (Matsuno *et al*., 1992; Monnet *et al*., 1990). Relevant to these observations, selective σ_1 receptor agonists attenuated the learning deficits induced by NMDA receptor or acetylcholine receptor antagonists (Maurice *et al*., 1994a, b; 1999; Ohno & Watanabe, 1995; Matsuno *et al*., 1994; Urani *et al*., 1998). The σ_1 protein was purified and the cDNA cloned in the guinea-pig (Hanner *et al*., 1996), mouse (Seth *et al*., 1997; Pan *et al*., 1998), rat (Seth *et al*., 1998) and human (Kekuda *et al*., 1996; Jbilo *et al*., 1997). The amino-acid sequences were structurally unrelated to known mammalian proteins, the sequences sharing some homology only with a fungal sterol C_8-C_7 isomerase (Hanner *et al*., 1996).

The availability of the cDNA sequence now allows a molecular strategy to study in a reliable manner the involvement of the σ_1 receptor in different physiological functions, namely the inactivation of receptor functions by the use of antisense oligodeoxynucleotides (aODN) (Wahlestedt *et al*., 1993; Zhang & Creese, 1993; Wagner, 1994; Zhou *et al*., 1994; Standaert *et al*., 1996). This strategy has already been used *in vivo* to selectively block the mRNA translation into functional receptor proteins (for reviews, Wagner, 1994; Weiss *et al*., 1997). King *et al*. (1997) used an *in vivo* antisense strategy directed against the cloned σ_1 receptor to assess its functions in the analgesic effect of opiates. They reported that the aODN probe enhanced the analgesic activity induced by a κ_1- and a κ_3-opioid receptor agonists, thus confirming the role of the σ_1 receptor in an anti-opiate analgesic system (King *et al*., 1997). More recently, this strategy was also used *in vivo* to demonstrate that activation of the σ_1 receptor is

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involved in cocaine-induced convulsions and lethality or conditioned place preference in mice (McCracken *et al*., 1999; Romieu *et al*., 2000). Preliminary experiments demonstrating the implication of σ_1 receptors in learning and memory processes were briefly reported using such an approach (Maurice *et al*., 1997b; Kitaichi *et al*., 1997).

Several neuroactive steroids, including progesterone, pregnenolone, dehydroepiandrosterone (DHEA) and their sulphate esters, have been shown to significantly inhibit the binding of radioligands labelling the σ_1 site *in vitro* or *in vivo* (Su *et al*., 1988; Maurice *et al*., 1996). The physiological evidence and pathological consequences of such interaction between the neuroactive steroids and the σ_1 receptor have recently been reviewed (Maurice *et al*., 1999). Among these steroids, progesterone behaves as a σ_1 antagonist by blocking several effects induced by σ_1 receptor agonists, including potentiation of NMDA-mediated responses *in vitro* (Monnet *et al*., 1995) and *in vivo* (Bergeron *et al*., 1996), modulation of intracellular calcium fluxes (Hayashi *et al*., 2000), and anti-amnesic or antidepressant effects (Maurice & Privat, 1997; Reddy *et al*., 1998; Maurice *et al*., 1998; 1999). Pregnenolone and DHEA sulphates showed σ_1 receptor agonist-like effects, since their effects on these models could be blocked by selective σ_1 antagonists. However, if DHEA sulphate behaved consistently as an efficient σ_1 receptor agonist in the different studies, pregnenolone sulphate led to conflicting results. The steroid showed a clear agonist action on the mobilization of calcium from intracellular stores, behaving like PRE-084 or (+)-pentazocine (Hayashi *et al*., 2000) or on behavioural responses (Maurice & Privat, 1997; Maurice *et al*., 1997a; 1998; Reddy *et al*., 1998). However, it acted as an inverse agonist on the NMDA-evoked response *in vitro* (Monnet *et al*., 1995), while being inefficient *in vivo* (Bergeron *et al*., 1996). These observations questioned the relevance and consequences of the interaction of pregnenolone sulphate with the σ_1 receptor.

The aim of this study was to investigate the role of the cloned σ_1 receptor in mediating the previously reported anti-amnesic effect in the mouse, and to clarify the involvement of this receptor in the behavioural effects induced by the neuroactive steroids DHEA and pregnenolone sulphates. For this purpose, antisense and mismatch ODN were designed to target the mouse clone of the σ_1 protein cDNA and administered centrally. The antisense efficiency was checked using radioligand binding and Western blotting. The anti-amnesic effects of either the selective σ_1 receptor agonist PRE-084 (Su *et al*., 1991; Maurice *et al*., 1994b) or the two steroids were then examined vs the learning impairments induced by the non-competitive NMDA antagonist dizocilpine.

Methods

Animals

Male Swiss OF1 mice (Breeding centre of the Faculty of Pharmacy, Montpellier, France), aged 5–6 weeks and weighing 30 ± 2 g were used. Animals were housed in plastic cages in groups before surgery and then individually. They had free access to laboratory chow and water, except during behavioural experiments, and they were kept in a regulated environment (23 ± 1°C, 40–60% humidity) under a 12 h light-dark cycle (light on at 07:00 h). Experiments were carried out between 09:00 and 17:00 h, in a soundproof and air-regulated experimental room, to which mice were habituated at least 30 min before each experiment. All animal procedures were conducted in strict adherence to the European Community Council Directive of 24 November 1986 (86-609/EEC) and Decree of 20 October 1987 (87-848/EEC).

Design of the oligodeoxynucleotides

Based on the mouse cDNA sequences for the σ_1 receptor, 16-mer phosphorothioate-modified oligodeoxynucleotide (ODN) sequences were designed. They were targeted to the area from −15 to +1 around the initiation codon, 5′-CGCGGCCCAC-GGCATT-3′ (=antisense oligodeoxynucleotide, aODN). The sequence in question was selected because no homology was found with any of the other known cDNA sequences in the GeneBank database (Bainbridge Island, WA, U.S.A.). As a control, a mismatched analogue, including randomly designed defects, 5′-CACGTCCCTCTCCATT-3′, was designed (=mismatch oligodeoxynucleotide, mODN). The ODN were synthesized and purified by high-pressure liquid chromatography by Eurobio (Les Ulis, France). They were dissolved in sterile saline solution and stored at −20°C until use under sodium pentobarbitone anaesthesia, mice were implanted with a polyethylene cannula (0.75-mm inner diameter, 6-mm length), fixed using acrylic cement. The tip of the cannula was placed on the right ventricle, with stereotaxic co-ordinates from the Bregma being, in mm, A −0.5, L −1, V 2.5 (Franklin & Paxinos, 1997). Injections began 24 h after surgery. Under mild ether anaesthesia, the needle of a Hamilton microsyringe was inserted through the cannula and ODN (1 μl) were slowly injected over 1 min, followed by an additional 1 min wait before removing the needle. Animals were used for *in vivo* binding assays or behavioural observations at least 10 h after the last injection.

Drugs

[^{3}H]-(+)-SKF-10,047 (1820 GBq mmol^{-1}, 37 MBq ml^{-1}) was from NEN Life Science Products (Boston, MA, U.S.A.). Dizocilpine ((+)-MK-801 maleate) was from RBI (Natick, MA, U.S.A.). PRE-084 was donated by Dr T.-P. Su (IRP, NIDA/NIH, Baltimore, MD, U.S.A.). Pregnenolone (3ß-hydroxy-5α-pregnen-20-one) sulphate and dehydroepiandrosterone (3ß-hydroxy-5α-androsten-17-one, DHEA) sulphate were from Sigma (Saint-Quentin Fallavier, France). Haloperidol was from Janssen (Boulogne-Billancourt, France). DHEA sulphate was dissolved in dimethyl sulphoxide 5% in saline, and pregnenolone sulphate was dissolved in dimethyl sulphoxide 5% in water. Other compounds were dissolved in saline. [^{3}H]-(+)-SKF-10,047 was injected intravenously (i.v.) in a volume of 100 μl. Other compounds were injected subcutaneously (s.c.) or intraperitoneally (i.p.), in a volume of 100 μl per 20 g body weight.

In vitro *(+)-[^{3}H]-SKF-10,047 binding assays in mouse brain*

Mice were sacrificed by decapitation. The hippocampus and cerebral cortex were dissected out at 4°C, pooled, and

homogenized in 25 volumes of 50 mM Tris/HCl buffer, pH = 7.4, using a Polytron homogenizer for 20 s. Each homogenate was centrifuged for 15 min at 45,000 × *g* at 4°C. The pellet was re-suspended in 5 mM Tris/HCl buffer, pH = 7.4. The homogenate was centrifuged again for 15 min at 45,000 × *g* at 4°C, and the pellet re-suspended in the same buffer at a final concentration of 2 mg protein ml^{-1}. Various concentrations of (+)-[^{3}H]-SKF-10,047, ranging from 1 nM to 1 μM, were incubated in a total volume of 2 ml of 5 mM Tris/HCl buffer for 60 min at 25°C. The bound radioactivity in 500 μl aliquots was separated by filtration through Whatman GF/C filters pre-soaked with 0.05% polyethyleneimine. The total radioactivity was determined by counting 100 μl aliquots of the homogenates. The non-specific binding levels were determined using NE-100 (100 μM). The protein concentration in the homogenates was determined using bovine serum albumin (Sigma) as standard (Bradford, 1976). Binding parameters (K_d, B_{max}) were determined following single-site Scatchard analyses.

In vivo *(+)-[^{3}H]-SKF-10,047 binding assays in mouse brain*

Mice were injected with 150 kBq of [^{3}H]-(+)-SKF-10,047 and sacrificed 30 min later by decapitation. The hippocampi and one cerebral cortex were dissected out, homogenized in a 5 mM Tris-HCl pH = 7.4 buffer at 4°C. Two aliquots of 1 ml were filtered under vacuum through GF/B filters, pre-soaked in 0.05% polyethyleneimine. The total radioactivity was determined by counting aliquots of the homogenates. Non-specific binding was defined using haloperidol, 2 mg kg^{-1}, administered i.p. 10 min before the tracer. Experiments were carried out 10 h or at various times after the last intracerebroventricular (i.c.v.) injection of the ODN.

Western blotting

The hippocampi were dissected out and homogenized with a Dunce glass homogenizer at 4°C in 25 volumes of a 5 mM Tris/HCl pH = 7.4 buffer containing 0.32 M sucrose and protease inhibitors (100 μg ml^{-1} phenylmethylsulphonylfluoride, 50 μg ml^{-1} aprotinin, 100 μl ml^{-1} soya bean trypsin inhibitor). The homogenate was centrifuged at 1000 × *g* at 4°C and the supernatant centrifuged at 45,000 × *g* for 20 min at 4°C. The pellet was re-suspended in 5 mM Tris/Hcl pH = 7.4 buffer and centrifuged again. The final pellet was suspended in 5 mM Tris/Hcl pH = 7.4 buffer containing 1 mM of ethylenediamine tetra-acetic acid. Proteins (30 μg) were diluted in an equal volume of Laemmli buffer, boiled for 5 min and loaded on a 12% polyacrylamide gel. The resolved proteins were transferred to a polyvilinidene difluoride membrane (Hybond-PVDF, Amersham, France). The transfer of proteins was evaluated before immunostaining using Ponceau red staining. The membrane was then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (Tris-HCl 20 mM, NaCl 137 mM, pH = 7.6) containing 0.1% Tween-20 (TBS-T). The membrane was incubated for 90 min at room temperature with a polyclonal anti-σ_1 receptor antibody (1 : 2000 in TBS-T), rinsed for 30 min in TBS-T and then incubated for 60 min at room temperature with a peroxidase-labelled goat anti-rabbit IgG antibody (1 : 4000 in TBS-T). The peroxidase activity was revealed by using an enhanced chemoluminescence detection kit (ECL kit, Amersham), according to the manufacturer's protocol. The anti-σ_1 receptor antibody was prepared, purified and characterized for specificity and selectivity in the laboratory, as recently detailed (Alonso *et al*., 2000).

Open-field behaviour

General motility of the mice was examined in a circular wooden arena (75-cm diameter). On its floor, two concentric circles were drawn, with a diameter of 15 and 45 cm, respectively. Open-field session consisted in placing a mouse in the centre circle and monitoring its movements for 10 min using a video camera. The following parameters were then measured: (1) the time taken to move out from the centre circle; (2) locomotion activity, in terms of the number of partitions crossed; (3) time of immobilization; (4) locomotion activity in the five central partitions; (5) frequency of rearing; (6) frequency of grooming.

Spontaneous alternation performances

Recording the spontaneous alternation behaviour in a Y-maze assessed spatial working memory performance. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries, including possible returns into the same arm, was recorded using an Apple IIe computer. An alternation was defined as entries into all three arms on consecutive occasions. The number of maximum alternations was therefore the total number of arm entries minus two and the percentage of alternation was calculated as (actual alternations/maximum alternations) × 100. The σ_1 receptor ligand or steroid was administered 30 min and dizocilpine 20 min before the session.

Passive avoidance test

The apparatus consisted of a transparent acrylic cage (30 × 30 × 40 cm high) with a grid-floor, inserted in a soundproof outer box (35 × 35 × 90 cm high). The cage was lit by a 15 W lamp during the experimental period. A wooden platform (4 × 4 × 4 cm) was fixed at the centre of the grid-floor. Electric shocks (1 Hz, 500 ms, 45 V DC) were delivered to the grid-floor using an isolated pulse stimulator (Model 2100, AM Systems, Everett, WA, U.S.A.). The test consisted of two training sessions, at 90-min time interval, and a retention session, carried out 24 h after the first training. During training sessions, each mouse was placed on the platform. When it stepped down and placed its four paws on the grid-floor, shocks were delivered for 15 s. Step-down latency and the numbers of vocalizations and flinching reactions were measured. Shock sensitivity was evaluated by adding these two numbers. None of the treatments used in the present study significantly affected the shock sensitivity. Animals, which did not step down within 60 s during the second session, were considered as remembering the task and taken off, without receiving further electric shocks. This procedure, used in routine, permits the intra-group variability to be minimized without affecting the relevance of the behavioural measure. The retention test was performed in a similar manner as training, except that the shocks were not

applied to the grid-floor. Each mouse was again placed on the platform, and the latency recorded, with an upper cut-off time of 300 s. Two parametric measures of retention were analysed: the latency and the number of animals reaching the avoidance criterion, defined as correct if the latency measured during the retention session was greater than 3 fold the latency showed by the animal during the second training session and, at least, greater than 60 s. Basically, the median latency could be considered as a qualitative index of memory capacities, whereas the percentage of animals to criterion could be considered as a quantitative index (Maurice *et al*., 1998). The σ_1 receptor ligand or steroid was administered 30 min and dizocilpine 20 min before the first training, and once, injections were not repeated before the second training or the retention test.

Statistical analyses

Statistical differences between the parameters determined after Scatchard analyses were evaluated using the Dunn's multiple comparisons test after a two-way analysis of variance. *In vivo* binding data were expressed as bound-to-free radioactivity ratios and analysed using the Newman-Keuls' multiple comparisons test after a two-way analysis of variance (F values). Open-field results were analysed using a two-way analysis of variance. Alternation percentages were analysed using Dunnett's multiple comparisons test after a two-way analysis of variance. Step-down latencies measured during the passive avoidance test retention did not show a normal distribution, since cut-off times were set. They were thus represented as median and interquartile range and analysed using the Kruskal-Wallis non-parametric analysis of variance (KW values), group comparisons being made with Dunn's non-parametric multiple comparisons test. Percentage of animals to criterion were analysed using the χ^2 test.

Results

Effects of the ODN treatments on σ_1 receptor binding and expression

The efficiency of the ODN treatments was initially examined in terms of *in vitro* binding parameters (K_d, B_{max}) using (+)-[^{3}H]-SKF-10,047 for labelling the σ_1 sites (Table 1). The ODN (10 μg in 1 μl) were administered every 12 h for 3 days and the binding parameters were determined from membrane preparations in the hippocampus and cortex. The Scatchard analyses showed that affinity constants failed to be affected by the treatments, being in the 8–12 nM range (Table 1). The number of sites, however, was significantly diminished in aODN-treated animals in the ipsilateral hippocampus (−60.3%) and in the cortex (−32.6%). These results are consistent with a selective decrease in the σ_1 receptor expression in the structures easily reached by the diffusion of the aODN repeatedly administered through the i.c.v. route.

The efficiency of the ODN treatments was then examined using the (+)-[^{3}H]-SKF-10,047 binding *in vivo* (Figure 1). The radioligand allows a highly specific and selective labelling of the σ_1 sites in the mouse brain *in vivo* (Ferris *et al*., 1988; Weissman *et al*., 1990; Bouchard *et al*., 1993; Maurice *et al*., 1996). Indeed, control non-cannulated animals exhibited bound-to-free radioactivity ratios about 69% in the hippocampus (white column, Figure 1a) and 58% in the cortex (white column, Figure 1b). These levels corresponded to 90 and 93% specific binding, respectively, as determined using a pre-treatment with haloperidol (black columns, Figure 1a,b).

A fixed dose of ODN (10 μg) was administered every 12 h for 1, 3 or 5 days, which corresponded to 2, 6 or 10 injections. In the mouse hippocampus (Figure 1a) and cortex (Figure 1b), the saline or mODN treatments failed to affect the binding levels as compared to control non-cannulated animals. The aODN treatment led to decreases in binding levels, which became significant after 6–10 injections as compared with the saline-treated groups, in each brain structure (Figure 1a,b).

The dose-response curve (4–20 μg) was examined using the 3 days (six injections) procedure. Within each structure, significant decreases in binding levels were observed at the highest doses (Figure 1c,d). Inhibition, however, reached a plateau value, corresponding to 43% of the binding level measured in saline-treated animals. From these experiments, the protocol corresponding to the injection of 10 μg of ODN during 3 days was established for the behavioural studies.

The content in σ_1 protein was then examined in the hippocampus and cortex using a Western blot analysis. As shown in Figure 2 for a representative analysis in the hippocampus, the ODN treatments failed to affect the intensity of the immunolabelling.

Table 1 Effects of the ODN treatments on the equilibrium saturation binding of (+)-[^{3}H]-SKF-10,047 to mouse brain membranes preparations

i.c.v treatment	K_i (nM)	B_{max} (fmol mg^{-1} protein)	*Variation vs Saline* (%)
Hippocampus			
Saline	7.5±0.6	131±15	
mODN	9.1±1.2	154±16	
aODN	7.2±0.2	52±1	−60.3%**,##
Cortex			
Saline	12.3±1.0	298±20	
mODN	13.1±2.7	338±72	
aODN	9.6±1.2	201±7	−32.6%*

Non-specific binding level was defined using 100 μM of NE-100. Results are the mean±s.e.mean of three experiments performed in triplicate. *$P<0.05$, **$P<0.01$ *vs* Saline-treated group; ##$P<0.01$ *vs* the mODN-treated group (Dunn's test).

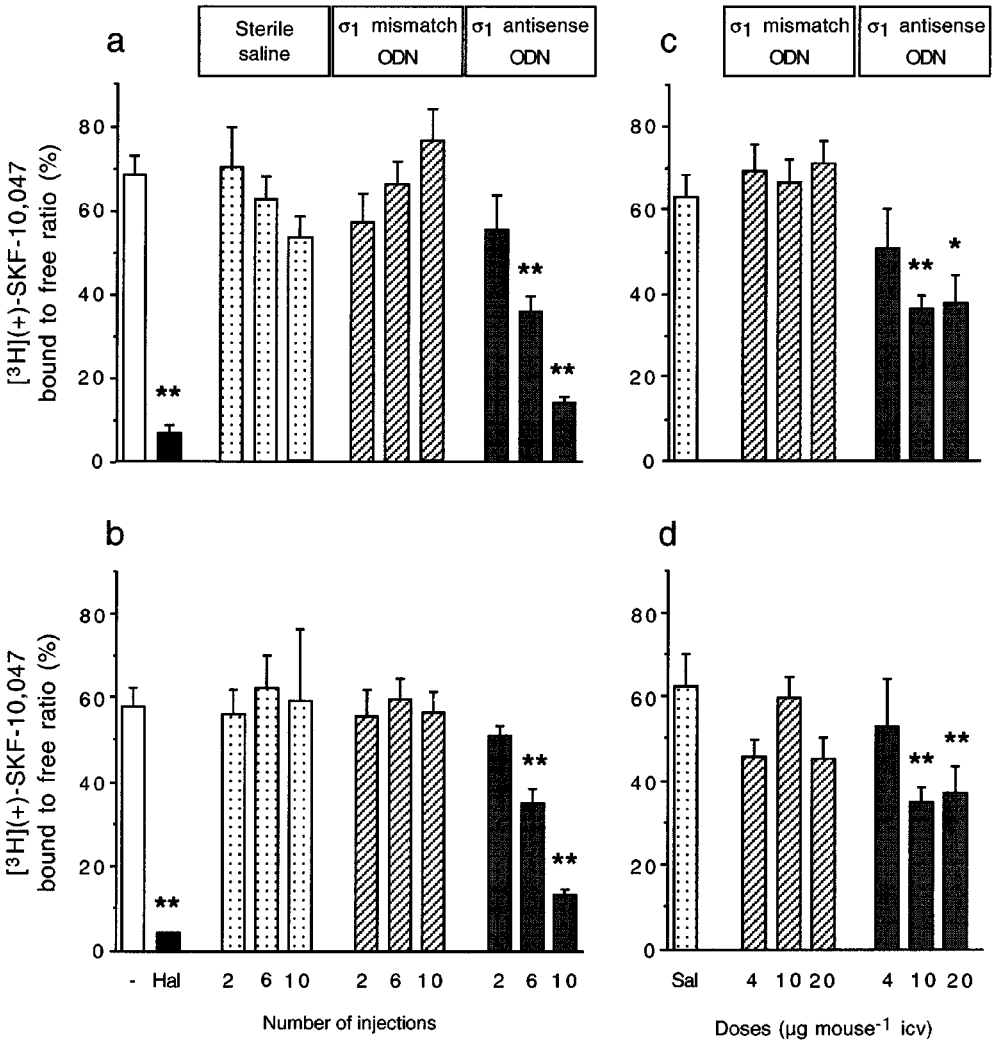

Figure 1 Effect of the ODN treatments on the *in vivo* binding levels of [^{3}H]-(+)-SKF-10,047 to σ_1 sites in the mouse hippocampus (a,c) and cortex (b,d). Effect of repeated injections (a,b): the ODN, or saline (Sal), was administered at 10 μg μl^{-1} (2.25 nmol) twice daily for 1, 3 or 5 days. Dose-response effect (c,d): the ODN or saline was administered at 4 μg (1 nmol), 10 μg (2.25 nmol) or 20 μg μl^{-1} (4.5 nmol) twice daily for 3 days. In (a) and (b), the binding levels are compared with non-treated animals and the level of non-specific binding was determined in animals treated i.p. with haloperidol (Hal, 2 mg kg^{-1}), 10 min before the tracer. The number of animals per group was $n=5-6$. In (a), $F_{10,54}=11.11$, $P<0.001$; in (b), $F_{10,52}=4.75$, $P<0.001$; in (c), $F_{6,35}=18.16$, $P<0.001$; in (d), $F_{6,34}=15.65$, $P<0.001$. *$P<0.05$, **$P<0.01$ *vs* the respective saline-treated group; Newman-Keuls' test.

Effects of the ODN treatments on the anti-amnesic effect of the selective σ_1 receptor agonist PRE-084

Preliminary to any behavioural investigation, the general ability of cannulated animals was checked in an open-field paradigm. As presented in Table 2a, none of the parameters examined was affected neither by the cannulation nor the treatment used. These observations, relevant to locomotion, exploration or anxiety behaviours, indicated that the animals did not show deficits in their general behaviour which, in turn, would have impeded the validity of the memory tests.

The effects of the ODN treatments on the anti-amnesic ability of a selective σ_1 receptor agonist, PRE-084, were then examined. Deficits in short-term and long-term memory were induced using the NMDA receptor non-competitive antagonist dizocilpine.

In the short-term memory test, the spontaneous alternation behaviour in the Y-maze, the dizocilpine treatment led to significant diminution in the spontaneous alternation behaviour in animals treated centrally with saline (black columns, Figure 3a). The PRE-084 did not affect the alternation behaviour by itself, but allowed a significant attenuation of the dizocilpine-induced deficits in saline- and mODN-treated animals (Figure 3a). A similar profile was observed in mODN-treated animals. In aODN-treated animals, dizocilpine induced deficits of similar extent, but PRE-084 failed to attenuate the dizocilpine-induced deficit.

Long-term memory capacities were measured in ODN-treated animals by assessing the passive avoidance ability. PRE-084 and/or dizocilpine were administered once before the training session and retention was examined after 24 h, in terms of latencies (Figure 3b) and percentages of animals to criterion (Figure 3c). In saline- and mODN-treated animals, PRE-084 did not affect the latencies by itself, but it attenuated the dizocilpine-induced deficits (Figure 3b). In aODN-treated animals, the dizocilpine-induced decrease in

latency was unchanged but remained unaffected by the PRE-084 pre-treatment. The percentages of animals of criterion showed that the dizocilpine-induced deficits were similar in all three experimental groups (Figure 3c). The PRE-084 administration alone did not affect the percentages but allowed a blockage of the dizocilpine-induced deficits in the saline- and mODN-treated animals. PRE-084 failed to attenuate the dizocilpine-induced deficits in the aODN-treated animals (Figure 3c). It must be noted that neither the cannulation, nor the ODN administration affected the ability of animals to perform the test. This was checked during the first training session by measuring the step-down latency or shock sensitivity (Table 2b).

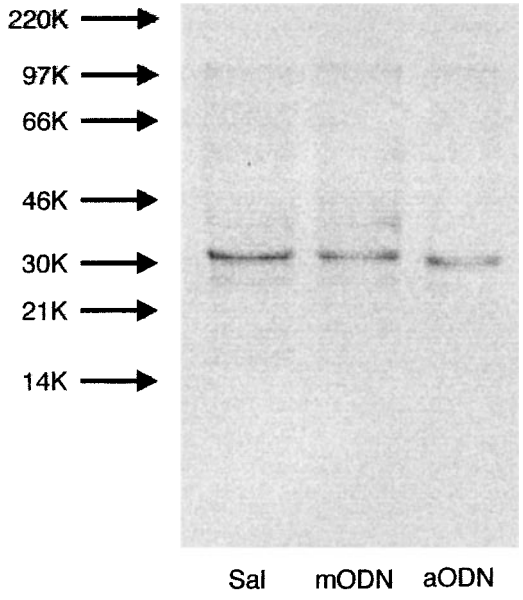

Figure 2 Western blotting of Swiss mouse hippocampus homogenates following the ODN treatments. The σ_1-receptor immunoreactivity is detected by the polyclonal antibody as a major protein species migrating with an apparent molecular weight of 30 KDa. The different treatments failed to affect the intensity of the immunolabelling.

Effects of the ODN treatments on the anti-amnesic effect of neuroactive steroids

The anti-amnesic effects of DHEA sulphate and pregnenolone sulphate against the dizocilpine-induced deficits were examined using the same paradigm in saline-, mODN-, and aODN-treated animals.

In the spontaneous alternation behaviour test, DHEA sulphate produced a significant attenuation of the dizocilpine-induced deficits in saline- and mODN-treated animals (Figure 4b). In aODN-treated animals, the steroid failed to affect the dizocilpine-induced decrease in alternation (Figure 4b). In the passive avoidance test, DHEA sulphate attenuated the dizocilpine-induced deficits observed during the retention session, for the saline- and mODN-treated animals, both in terms of latencies (Figure 3b) and percentages of animals to criterion (Figure 4c). However, in aODN-treated animals, the steroid failed to affect the dizocilpine-induced impairments (Figure 4b,c).

Different results were obtained with pregnenolone sulphate. In the spontaneous alternation behaviour test, pregnenolone sulphate allowed a significant attenuation of the dizocilpine-induced deficits in saline-, mODN-, and aODN-treated animals (Figure 5a). In the passive avoidance test, the steroid administration attenuated the dizocilpine-induced deficits observed during the retention session, for the saline-, mODN-, and aODN-treated animals, both in terms of latencies (Figure 5b) and percentages of animals to criterion (Figure 5c). Inactivation of the σ_1 receptor by *in vivo* aODN treatment failed to affect the anti-amnesic properties of pregnenolone sulphate.

Discussion

In the present study, we report that a phosphorothioate aODN targeting the nucleotide sequence $-15 \sim +1$ of the cloned mouse σ_1 receptor injected into the mouse lateral ventricle decreased the binding parameters to σ_1 sites in the ipsilateral hippocampus and cerebral cortex, and blocked the anti-amnesic effects of a selective σ_1 agonist in both short- and long-term memory tests.

Table 2 Behavioural parameters in ODN-treated mice in the open-field test and during the first passive avoidance training session

Parameter	*Control mice*	*Cannulated mice*			*ANOVA*
		Saline	*Mismatch ODN*	*Antisense ODN*	
(a) Open-Field behaviours:					
Latency to depart (s)	2±1	3±1	6±2	6±2	$F_{(3.63)}=2.124$, NS
Locomotion (m)	34.50±2.53	35.71±2.51	33.09±3.67	35.24±4.08	$F_{(3.63)}=0.104$, NS
Immobility (s)	49±6	54±8	99±23	78±16	$F_{(3.63)}=2.646$, NS
Speed (m/min)	3.75±0.26	3.93±0.27	3.87±0.31	3.94±0.39	$F_{(3.63)}=0.088$, NS
Rearings	24±3	26±6	18±5	19±4	$F_{(3.63)}=0.713$, NS
Groomings	8±2	7±1	7±1	9±2	$F_{(3.63)}=0.248$, NS
Locomotion in centre (%)	31.8±1.3	26.4±3.1	26.7±4.0	29.1±3.8	$F_{(3.63)}=0.664$, NS
n	20	15	15	17	
(b) Passive avoidance training:					
Step-down latency (s)		21[6–30]	19[5–30]	10[8–15]	KW=0.969, NS
Shock sensitivity		11±1	9±1	11±1	$F_{(2.35)}=1.765$, NS
n		12	12	12	

NS: not significant

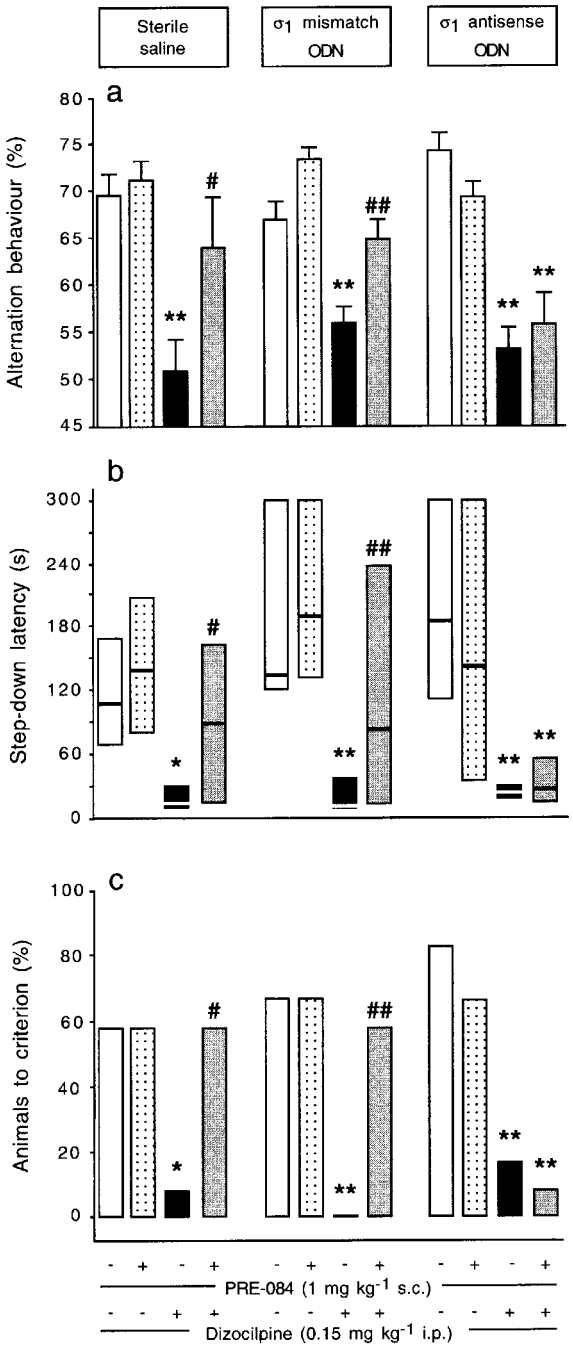

Figure 3 Effect of the ODN treatments on the anti-amnesic effect of the σ_1 receptor agonist PRE-084 against the dizocilpine-induced learning impairments: spontaneous alternation behaviour in the Y-maze (a); latencies (b) and percentage of animals to criterion (c) during retention in the passive avoidance test. PRE-084 (1 mg kg^{-1} s.c.) was administered 10 min before dizocilpine (0.15 mg kg^{-1} i.p.) which was given 20 min before the Y-maze session or the first passive avoidance training session. The number of animals per group was $n=12$. In (a), $F_{3,47}=6.8$, $P<0.001$ for saline-treated animals; $F_{3,47}=16.2$, $P<0.001$ for mODN-treated animals; $F_{3,47}=18.7$, $P<0.001$ for aODN-treated animals. In (b), results are presented as median and interquartile range; KW = 12.3, $P<0.01$ for saline-treated animals; KW = 24.9, $P<0.001$ for mODN-treated animals; KW = 20.6, $P<0.001$ for aODN-treated animals. $^*P<0.05$, $^{**}P<0.01$ *vs* the respective (saline + saline)-treated group; $\#P<0.05$ *vs* the respective (saline + dizocilpine)-treated group; Dunnett's test in (a), Dunn's test in (b), χ^2 test in (c).

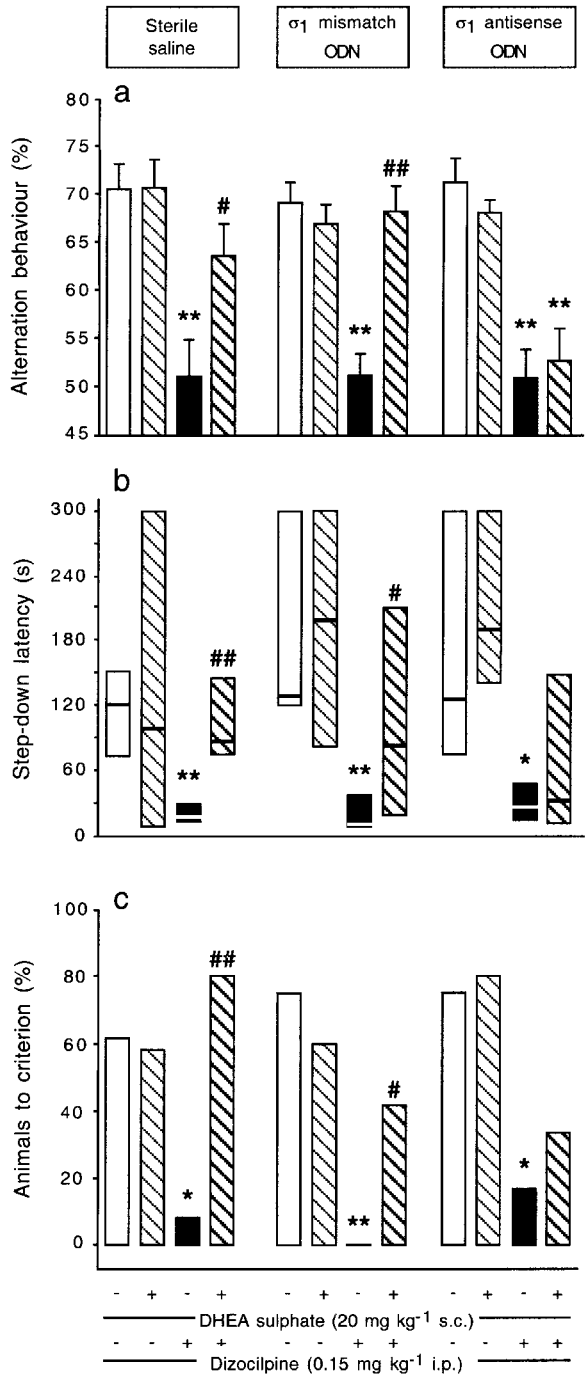

Figure 4 Effect of the ODN treatments on the anti-amnesic effect of the neuroactive steroid DHEA sulphate against the dizocilpine-induced deficits : spontaneous alternation behaviour in the Y-maze (a); latencies (b) and percentages of animals to criterion (c) during retention in the passive avoidance test. DHEA sulphate (20 mg kg^{-1} s.c.) was administered 10 min before dizocilpine (0.15 mg kg^{-1} i.p.) which was given 20 min before the Y-maze session or the first passive avoidance training session. The number of animals per group was $n=10-13$. In (a), $F_{3,47}=8.6$, $P<0.001$ for saline-treated animals; $F_{3,47}=14.7$, $P<0.001$ for mODN-treated animals; $F_{3,47}=16.2$, $P<0.001$ for aODN-treated animals. In (b), KW = 15.8, $P<0.01$ for saline-treated animals; KW = 22.8, $P<0.001$ for mODN-treated animals; KW = 14.7, $P<0.01$ for aODN-treated animals. $^*P<0.05$, $^{**}P<0.01$ *vs* the respective (saline + saline)-treated group; $\#P<0.05$, $++P<0.01$ *vs* the respective (saline + dizocilpine)-treated group; Dunnett's test in (a), Dunn's test in (b), χ^2 test in (c).

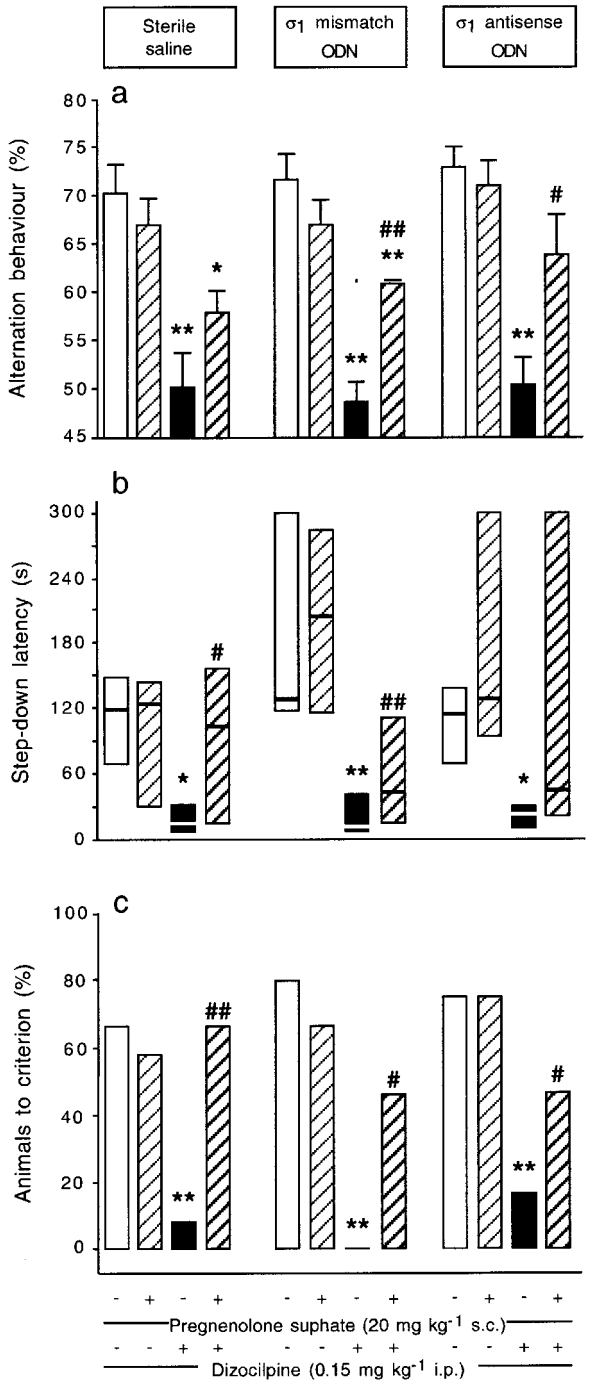

Figure 5 Effect of the ODN treatments on the anti-amnesic effect of the neuroactive steroid pregnenolone sulphate against the dizocilpine-induced deficits: spontaneous alternation behaviour (a); latencies (b) and percentages of animals to criterion (c) during retention in the passive avoidance test. Pregnenolone sulphate (20 mg kg^{-1} s.c.) was administered 10 min before dizocilpine (0.15 mg kg^{-1} i.p.) which was given 20 min before the Y-maze session or the first passive avoidance training session. The number of animals per group was $n=12$. In (a), $F_{3,47}=9.5$, $P<0.001$ for saline-treated animals; $F_{3,47}=22.5$, $P<0.001$ for mODN-treated animals; $F_{3,48}=11.2$, $P<0.001$ for aODN-treated animals. In (b), KW = 10.7, $P<0.05$ for saline-treated animals; KW = 18.7, $P<0.01$ for mODN-treated animals; KW = 8.3, $P<0.05$ for aODN-treated animals. $^*P<0.05$, $^{**}P<0.01$ *vs* the respective (saline + saline)-treated group; $\#P<0.05$, $\#\#P<0.01$ *vs* the respective (saline + dizocilpine)-treated group; Dunnett's test in (a), Dunn's test in (b), χ^2 test in (c).

The ODN treatment, 10 μg twice daily during 3 days, did not lead to a significant decrease in protein content in the ipsilateral hippocampus or cortex, as examined by Western blot analysis. However, it led, in these structures, to significant decreases in the total number of binding sites and *in vivo* binding levels. The Western blot analysis is the most direct assessment of protein levels. However, it presented the least sensitivity to small changes in protein abundance with variability between samples of 10–20% (Standaert *et al*., 1996). *In vitro* and *in vivo* binding experiments concerned directly the number of σ_1 binding sites and bioavailability of the σ_1 receptor. Both approaches indicated that the aODN treatment significantly affected these parameters, in correlation with the alteration of the behavioural response examined afterwards.

It has been proposed that the inactivation of synthesis by aODN treatment may preferentially affect small functional pools of proteins, with a rapid turnover, at synaptic sites for receptors located on the synaptic membrane, without affecting extrasynaptic pools, which have a slower turnover (Wagner, 1994; Zhou *et al*., 1994). The σ_1 receptor is known to be present at the plasmic membrane, particularly associated with the post-synaptic thickening, the nuclear membrane, the membrane of mitochondria and of some cisternae of the endoplasmic reticulum (Phan *et al*., 1999; Alonso *et al*., 2000). The exact functional role of the σ_1 receptor at each of these locations is far from being fully understood, but recent evidence has shown that the protein acts as a sensor/modulator of calcium mobilizations (Hayashi *et al*., 2000). Indeed, it has been demonstrated using NG-108 cell cultures, that selective σ_1 receptor agonists potentiate the inositol 1,4,5-trisphosphate receptor-mediated calcium mobilization from the endoplasmic reticulum stores. The authors also reported that the addition of agonist to the culture provoked a translocation of the receptor from the endoplasmic reticulum to the plasma membrane (Hayashi *et al*., 2000). Thus, the σ_1 receptor activation led to a particular trafficking that remains to be fully characterized. In turn, the effect of aODN treatment may differentially affect the different pools and complex trafficking of the receptor, and, in turn, explain that decreases in binding parameters can be observed without any significant change in protein content using Western blot analysis. Further experiments are, however, necessary to fully elucidate this point. Moreover, it must be noted that a σ_1 aODN treatment in NG-108 cell cultures *in vitro* led to a significant attenuation of the protein immunoreactivity by Western blotting (Hayashi *et al*., 2000). Some *in vitro vs in vivo* discrepancies are thus observed that remain to be clarified.

Animals submitted to the *in vivo* σ_1 aODN treatment were then examined for their learning ability and for the efficiency of the anti-amnesic effect of a selective σ_1 receptor agonist, PRE-084 (Su *et al*., 1991). Vehicle- and mODN-treated animals showed marked deficits after dizocilpine administration that could be significantly attenuated when PRE-084 was administered prior to the drug. These anti-amnesic effects were observed using the spontaneous alternation and step-down type of passive avoidance tests, as previously described (Maurice *et al*., 1994b). The aODN-treated animals showed no impairment of their learning ability, when they received saline only. In addition, the dizocilpine administration provoked deficits in both tests that appeared to be similar

in extent as compared with vehicle- or mODN-treated animals. These observations indicate that inactivation of the σ_1 receptor did not affect the memory processes, nor the deficits induced by NMDA receptor blockage. Several previous observations tended to suggest the same result. In particular, (i) selective σ_1 receptor agonists or antagonists alone failed to affect the memory capacities; (ii) selective σ_1 antagonists, such as haloperidol, BMY-14802, or NE-100 failed to affect the learning deficits observed after several pharmacological manipulations, including administration of dizocilpine (Matsuno *et al*., 1994; Maurice *et al*., 1994a, b; 1999; Maurice & Privat, 1997). These observations show that the σ_1 receptor is not necessarily involved in normal learning and memory processes, but rather exerts its neuromodulatory enhancing action when neurotransmission systems appear deficient. The exact mechanism for the involvement of the σ_1 receptor in memory formation remains to be elucidated, but it may imply a phasic regulation of several neurotransmission systems, highly efficient when learning ability is pathologically affected rather than a tonic regulation.

The aODN treatment, however, resulted in a complete blockage of the anti-amnesic effect of PRE-084, in both tests. This result demonstrated that the cloned σ_1 receptor appears to be the one involved in the anti-amnesic effects of selective σ_1 agonists. This experiment brings a molecular basis to a well-documented effect of these drugs, and new molecular approaches will be possible to evaluate the resulting therapeutic potentials.

A first illustration was given by examining the involvement of the σ_1 receptor in the anti-amnesic effects induced by the two neuroactive steroids, DHEA and pregnenolone sulphates. Both steroids are known to induce, after systemic administration, marked pro-mnesic or anti-amnesic effects. Pregnenolone, DHEA and their sulphate esters, enhanced memory retention in an active avoidance learning task in mice after central administration (Flood *et al*., 1992). Pregnenolone sulphate enhanced learning when administered after the first session of training in a spontaneous alternation task in rats (Mayo *et al*., 1993) or in an appetitive reinforced Go-No Go visual discrimination task in mice (Meziane *et al*., 1996). DHEA or DHEA sulphate improved the age-related deficits in footshock active avoidance training in mice (Flood & Roberts, 1988). The free steroid, administered centrally, prevented the amnesia induced by administration of the vehicle, dimethylsulphoxide 5%, alone. Its sulphate ester, administered systemically or centrally immediately after training or given in the drinking water during 2 weeks, facilitated memory retention in a step-down passive avoidance test in mice but did not improve acquisition (Flood *et al*., 1988).

DHEA sulphate was reported to attenuate the learning impairments induced by dizocilpine in mice through an interaction with the σ_1 receptor since its effect could be blocked by a preadministration with the σ_1 receptor antagonist BMY-14,802 (Maurice *et al*., 1997a). The present observation showing that, on both the spontaneous alternation and passive avoidance responses, the anti-amnesic effect of DHEA sulphate was blocked in σ_1 aODN-treated animals, demonstrated that the σ_1 receptor is a necessary target for the anti-amnesic effect of the steroid. On the other hand, pregnenolone had a preserved anti-amnesic effect as potent in σ_1 aODN-treated animals as in vehicle- or σ_1 mODN-treated ones. Both steroids are considered as excitatory steroids. First, they act as negative allosteric modulators of the $GABA_A$ receptor-mediated responses (Majewska & Schwartz, 1987; Majewska *et al*., 1988; 1990). Second, they potentiate several responses mediated through the NMDA receptor. However, when pregnenolone sulphate acted through a specific, extracellularly directed modulatory site, located on the receptor complex, but distinct from either the spermine, glycine, phencyclidine, arachidonic acid, Mg^{2+} and redox sites (Park-Chung *et al*., 1997), DHEA sulphate failed to affect the receptor through a direct interaction (Park-Chung *et al*., 1994; Kimonides *et al*., 1998). However, through its interaction with the σ_1 receptor, DHEA sulphate has been reported to indirectly potentiate several NMDA-mediated physiologic responses *in vitro* and *in vivo*, and at the behavioural level (Monnet *et al*., 1995; Bergeron *et al*., 1996; Maurice & Privat, 1997; Maurice *et al*., 1998; Urani *et al*., 1998). The present *in vivo* antisense strategy confirmed the different pharmacological profiles for these two excitatory neuroactive steroids.

Parallel to this observation, it must be noted that both steroids differently affected the extent of excitotoxic insults resulting from hyperactivation of the NMDA receptors. Pregnenolone sulphate was reported to facilitate the NMDA receptor-mediated excitotoxic cell death in several *in vitro* models of neurodegeneration. First, it potentiated the increase in intracellular free calcium concentration induced by either acute or chronic NMDA exposure in primary cultures of rat hippocampal neurones and thereby exacerbated the resulting dizocilpine-sensitive neuronal death (Weaver *et al*., 1998). Second, it potentiated the neurodegeneration observed in isolated and intact rat retina preparations exposed to NMDA (Guarneri *et al*., 1998). Third, it facilitated the appearance of neurodegeneration and resulting learning deficits in mice exposed to an hypoxic insult *in vivo*, by repetitive exposure to carbon monoxide gas (Maurice *et al*., 2000). These observations suggested that the selective and efficient potentiation of the NMDA receptor activation induced by pregnenolone sulphate led to major consequences in the case of hyperactivation of this receptor. Indeed, the steroid potentiated the excitotoxic neurodegeneration and worsened the resulting behavioural deficits.

DHEA sulphate, on the contrary, prevented or reduced the neurotoxic effects in primary hippocampal cultures exposed to NMDA (Kimonides *et al*., 1998). Subcutaneously implanted DHEA pellets protected the CA_1 hippocampal neurones against the toxicity induced *in vivo* by unilateral infusions of NMDA (Kimonides *et al*., 1998). DHEA sulphate, but not DHEA, presented good neuroprotective efficiency against the glutamate-induced neuronal death in primary cultures of rat hippocampal neurones (Mao & Barger, 1998). Finally, the steroid completely blocked the appearance of neurodegeneration and resulting learning deficits in mice exposed to carbon monoxide (Maurice *et al*., 2000). This neuroprotective effect seemed unrelated to the interaction of the steroid with the σ_1 receptor, since it was not blocked by the selective σ_1 antagonist NE-100. However, these different results confirmed and brought physiopathological consequences for the differential pharmacological profiles presented by both steroids.

Conclusion

In this study, it was demonstrated that, using an *in vivo* antisense strategy, the σ_1 protein cloned in the mouse by Seth *et al*. (1997) and Pan *et al*. (1998) identified the receptor mediating the anti-amnesic effect of selective σ_1 agonists. Such antisense strategy revealed the differential involvement of the σ_1 receptor in the anti-amnesic effects mediated by excitatory neuroactive steroid hormones. Indeed, the anti-amnesic effect induced by DHEA sulphate necessarily involved an interaction with the σ_1 receptor, whereas pregnenolone sulphate could exert its effect by a more direct interaction with the NMDA and/or $GABA_A$ receptor.

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