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α-2 AGONISTS INDUCE AMNESIA THROUGH ACTIVATION OF THE Gi-PROTEIN SIGNALLING PATHWAY

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Abstract—The post-receptorial mechanism of the amnesic action of the α_2-agonists clonidine and guanabenz was investigated in the mouse passive avoidance test. Animals were i.c.v. injected with pertussis toxin (PTX) or with antisense oligonucleotides, complementary to the sequence of the α-subunit mRNA of Gi_1, Gi_2, Gi_3, Go_1 and Go_2 proteins. The administration of PTX (0.25 μg per mouse i.c.v.) reversed the amnesia induced by both α_2-agonists. Similarly, anti-$Gi\alpha_1$ (6.25–12.5 μg per mouse i.c.v.), anti-$Gi\alpha_3$ (3.12–12.5 μg per mouse i.c.v.), anti-$Go\alpha_1$ (12.5–25 μg per mouse i.c.v.) antagonised the detrimental effect induced by clonidine and guanabenz. By contrast, pretreatment with anti-$Gi\alpha_2$ (3.12–25 μg per mouse i.c.v.) and anti-$Go\alpha_2$ (12.5–25 μg per mouse i.c.v.) never modified the impairment of memory processes induced by the α_2-agonists. At the highest effective doses, none of the compounds used impaired motor coordination (rota rod test), nor modified spontaneous motility and inspection activity, (hole board test). These results indicate the involvement of Gi_1, Gi_3, and Go_1, but not Gi_2 and Go_2, protein subtypes in the transduction mechanism responsible for the induction of amnesia by clonidine and guanabenz. **© 2004 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: clonidine, guanabenz, analgesia, Gi proteins, antisense oligonucleotides.

Agonists of α_2-adrenoceptors produce a wide variety of central and peripheral effects. These include an antihypertensive action, alleviation of opiate-withdrawal syndrome, antinociception, cardiovascular control, feeding and sedation (Ruffolo et al., 1993; McDonald et al., 1997). They have many clinical uses: to lower blood pressure (van Zwieten, 1999), promote anesthesia (Khan et al., 1999), ameliorate symptoms in neuropsychiatric disorders such as attention deficit hyperactivity disorders (Scahill et al., 2001). This broad range of effects is consistent with the broad projections of the noradrenergic system along the length of the neuroaxis.

One aspect of the α_2-adrenoceptor function receiving increasing examination is its role in cognitive functions. Despite of the fact that there is a large consensus on the ameliorative effect produced by α_2-adrenoceptor agonists on memory functions, there are also some studies indicating the induction of a detrimental effect induced by the abovementioned compounds on cognitive processes. Clonidine treatment provoked memory disturbances in rats in a step-down (Genkova-Papasova and Lazarova-Bakurova, 1988; Genkova-Papasova et al., 1997) and shuttle-box (Hawkins and Monti, 1979; Kostowski et al., 1980) paradigms. Recent humans studies showed that systemic administration of clonidine disrupted memory accuracy in delayed matching to sample test in Alzheimer's disease patients (Riekkinen et al., 1999) and disrupted spatial working memory in healthy subjects (Jäkälä et al., 1999). The infusion of the α_2-adrenoceptor agonists dexmedetomidine and clonidine impaired memory processes and reduced performance on the digit symbol substitution test in healthy young volunteers (Hall et al., 2000, 2001).

α_2-Adrenoceptors are cell-surface receptors widely expressed in both the CNS and peripheral nervous system (Eason and Ligget, 1993). Three distinct α_2-adrenoceptor subtypes have been characterized: α_{2A}, α_{2B} and α_{2C} (Harrison et al., 1991). The three α_2-adrenoceptor subtypes share many common properties. They are G protein-coupled receptors with seven transmembrane domains. G-proteins are a ubiquitous family of proteins that play a crucial role in transducing extracellular signals to cellular targets, thus transmitting messages from cell surface receptors to cellular effectors including adenylate cyclase, phospholipase C and ion channels (Sprang, 1997). G-proteins are heterotrimeric molecules composed of three different subunits termed α, β and γ. The α subunits can be classified into families, depending on whether they are targets for cholera toxin (Gs), pertussis toxin (PTX; Gi and Go) or neither (Gq and G_{12}; Simon et al., 1991). All three α_2-adrenoceptor subtypes are coupled to the Gi signaling system, inhibiting activity of adenylate cyclase, inhibiting the opening of voltage-gated Ca^{2+} channels and opening K^+ channels (McDonald et al., 1997).

The post-receptorial mechanism involved in the induction of a detrimental effect on cognitive processes by α_2-adrenoceptor agonists has not yet been established. By taking into account that α_2-adrenoceptors are Gi-protein coupled receptors, we investigated the involvement of Gi proteins into the mechanism of action of two α_2-adrenoceptor agonists, clonidine and guanabenz, by means of an antisense strategy. The Gi protein family is composed by several subtypes, named Gi_1, Gi_2, Gi_3, Go_1 and Go_2 (Simon et al., 1991). In the current study we used antisense oligonucleotides (aODN) against the α subunits of the Gi_1, Gi_2, Gi_3, Go_1 and Go_2 proteins in order to determine the role of each subtype in the memory impairment induced by clonidine and guanabenz in a mouse passive avoidance paradigm. In order to exclude that the

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Abbreviations: aODN, antisense oligonucleotide; dODN, degenerated antisense oligonucleotide; ODN, oligonucleotide; PTX, pertussis toxin.

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effects produced by aODN treatments were due to the induction of side effects, some additional behavioral tests (rota rod, hole board) were performed.

EXPERIMENTAL PROCEDURES

Animals

Male Swiss albino mice (23–30 g) from Morini (San Polo d'Enza, Italy) were used. The mice were housed 15 per cage. The cages were placed in the experimental room 24-h before the test for adaptation. The animals were fed a standard laboratory diet and tap water *ad libitum* and kept at 23±1 °C with a 12-h light/dark cycle, light on at 7:00 a.m. All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC) or the National Institutes of Health Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, revised 1996. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

I.c.v. injection of oligonucleotides (ODN)

I.c.v. administration was performed under ether anesthesia, according to the method described by Haley and McCormick (1957). Briefly, during anesthesia, mice were grasped firmly by the loose skin behind the head. A 0.4 mm external diameter, hypodermic needle attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl 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 into the right or left ventricle randomly. To ascertain that the drugs were administered exactly into the cerebral ventricle, some mice (20%) were injected with 5 μl of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the injection technique was evaluated and the percentage of correct injections was 95.

Passive-avoidance test

The test was performed according to the step-through method described by Jarvik and Kopp (1967). The apparatus consisted of a two-compartment acrylic box with a lighted compartment connected to a darkened one by a guillotine door. As soon as the mouse entered the dark compartment, it 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. The maximum entry latency allowed in the training and retention sessions was, respectively, 60 and 180 s. In this test, clonidine (0.125 mg kg^{-1}) and guanabenz (0.3 mg kg^{-1}) were i.p. injected, respectively, 60 min before and immediately after the training session, PTX (0.25 μg per mouse) was i.c.v. injected 11 days prior to the training session whereas aODNs (1.56–25 μg per mouse) were i.c.v. injected 24 and 18 h before training. Between 11 and 25 mice were tested.

Rota-rod test

The apparatus consists of a base platform and a rotating rod of 3 cm diameter with a non-skid 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-rotation speed of 16 r.p.m. 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). Performance time was measured before and 15, 30 and 45 min after s.c. administration of the investigated compounds.

Hole-board test

The hole board test consisted of a 40 cm square plane with 16 flush-mounted cylindrical holes (3 cm diameter) distributed four by four in an equidistant, grid-like manner. Mice were placed on the center of the board one by one and allowed to move about freely for a period of 10 min each. Two electric eyes, crossing the plane from midpoint to midpoint of opposite sides, thus dividing the plane into four equal quadrants, automatically signaled the movement of the animal (counts in 5 min) on the surface of the plane (locomotor activity). Miniature photoelectric cells, in each of the 16 holes, recorded (counts in 5 min) the exploration of the holes (exploratory activity) by the mice. The test was performed 18 h after the last i.c.v. injection of degenerated ODN (dODN; 25 μg per mouse) or aODN (12.5–25 μg per mouse). Twelve mice per group were tested.

aODNs

Phosphodiester ODNs protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Tib Molbiol (Genoa, Italy). The sequences of the 33-mer aODNs used in the present study were the following: anti-$Gi_1\alpha$: 5′-G*C*T GTC CTT CCA CAG TCT CTT TAT GAC GCC G*G*C-3′; anti-$Gi_2\alpha$: 5′-A*T*G GTC AGC CCA GAG CCT CCG GAT GAC GCC C*G*A-3′; anti-$Gi_3\alpha$: 5′-G*C*C ATC TCG CCA TAA ACG TTT AAT CAC GCC T*G*C-3′; anti-$Go\alpha_1$: 5′- A*G*G CAG CTG CAT CTT CAT AGG TG*T *T -3′; anti-$Go\alpha_2$: 5′- G*A*G CCA CAG CTT CTG TGA AGG CA*C *T -3′. All ODNs were previously characterized by *in vitro* (immunoblotting) and *in vivo* (tail flick) experiments (Kleuss et al., 1991; Raffa et al., 1994; Sanchez-Blazquez et al., 1995; Sanchez-Blazquez and Garzon, 1998). The immunoblotting results were also confirmed in our laboratory. A 33-mer fully dODN 5′-N*N*N NNN NNN NNN NNN NNN NNN NNN NNN NNN N*N*N -3′ (where N is G, or C, or A, or T) and a 25-mer fully dODN 5′-N*N*N NNN NNN NNN NNN NNN NNN NN*N *N -3′ (where N is G, or C, or A, or T) were used as a control respectively for anti-Giα and anti-Goα. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP; Sigma) to enhance both uptake and stability, as described previously (Capaccioli et al., 1993). aODN or dODN were preincubated at 37 °C for 30 min with 13 μM DOTAP and supplied to mice by i.c.v. injection of 5 μl solution 18 and 24 h prior to the behavioral and *in vitro* tests.

Western blot analysis

Membrane homogenates (20 μg) made from hippocampus region of control and antisense-treated mice were solubilized in SDS buffer and separated on 10% polyacrylamide gels (1.5 mm). Proteins were transferred onto nitrocellulose (1.5 h at 190 mA), and the membrane was blocked in PBS containing 5% nonfat dry milk for 1 h. Following washings, blots were incubated overnight at 4 °C with specific antibodies (Santa Cruz Biotechnology) against $Gi_{1\alpha}$, Gi_{2a}, $Gi_{3\alpha}$ or Go_α (1:200 dilution). After being washed with PBS containing 0.1% Tween, the nitrocellulose was incubated with goat anti-rabbit horseradish peroxidase-conjugated conjugate secondary antisera (1:10,000) in PBS/0.1% Tween and left for 1 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using ECL detection system (Amersham). Exposition and developing time used was standardized for all the blots. We measure the density of the bands obtained using the Scion Image program.

Drugs

The following drugs were used: clonidine hydrochloride, guanabenz acetate, PTX (RBI, Milan). All drugs were dissolved in isotonic (NaCl 0.9%) saline solution immediately before use, except

Table 1. Effect of PTX on amnesia induced by clonidine and guanabenz in the mouse passive avoidance test[a]

Pre-treatment (i.c.v.)	Treatment (i.p.)	Entrance latency (s)	
		Training	Retention
Vehicle	Saline	17.6±1.8	121.7±12.6
PTX 0.25 μg per mouse	Saline	16.5±1.7	118.6±13.2
Vehicle	Clonidine 0.125 mg kg^{-1}	15.4±3.0	49.8±8.3*
PTX 0.25 μg per mouse	Clonidine 0.125 mg kg^{-1}	18.5±6.1	101.5±15.6
Vehicle	Guanabenz 0.3 mg kg^{-1}	15.0±2.8	52.3±7.5*
PTX 0.25 μg per mouse	Guanabenz 0.3 mg kg^{-1}	17.8±5.1	128.1±12.5

[a] Pretreatment was performed 11 days before the test, whereas the treatment was administered 60 min before (clonidine) or immediately after (guanabenz) the training session. Data are reported as mean±S.E.M. The number of animals ranged between 20 and 26 per group; at least two trials were performed per group.
* $P<0.01$ versus vehicle+saline group.

for PTX which was dissolved in a water solution containing 0.01 M sodium phosphate buffer, pH=7.0, with 0.05 M sodium chloride. Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 10 ml kg^{-1} by i.p. injection or 5 μl per mouse by i.c.v. injection.

Statistical analysis

All experimental results are given as the means±S.E.M. An analysis of variance, followed by Fisher's protected least significant difference procedure for post hoc comparison, was used to verify the significance of differences between two means. Data were analyzed with the StatView software for the Macintosh (1992).

RESULTS

Effect of PTX on α_2-agonist-mediated amnesia

Clonidine (0.125 mg kg^{-1} i.p.), injected 60 min before the test and guanabenz (0.3 mg kg^{-1} i.p.), injected immediately after the training session, induced amnesia in the mouse passive avoidance test (Table 1). The amnesia induced by the α_2-agonists was of the same intensity of that produced by the well-known amnesic drug scopolamine (1.5 mg kg^{-1} i.p.) used as the reference drug (data not shown). Pretreatment with PTX, injected i.c.v. at the

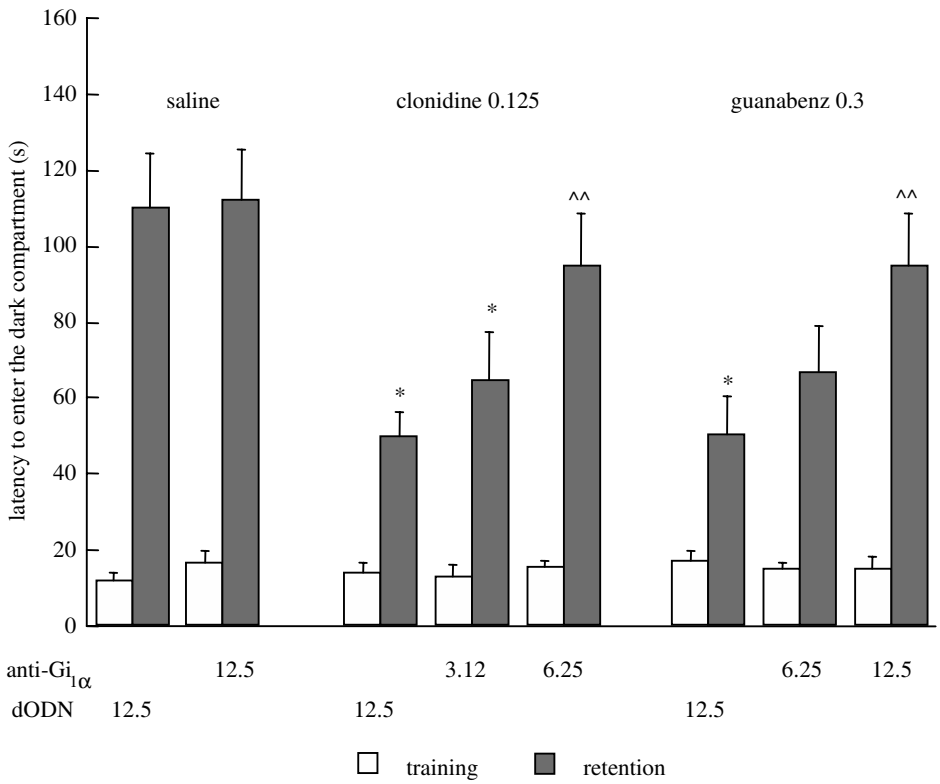

Fig. 1. Prevention by pretreatment with an aODN to the α subunit of Gi_1-protein gene (3.12–12.5 μg per mouse i.c.v.) of clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) amnesia in the mouse passive avoidance test. The test was performed 18 h after the last i.c.v. injection of dODN (12.5 μg per mouse i.c.v.) or aODN. Vertical lines represent S.E. mean; the dose administered is reported in each column. * $P<0.05$ in comparison with dODN+saline group; ^^ $P<0.01$ in comparison with the corresponding amnesic compound. The number of animals ranged between 22 and 27 per group; at least two trials were performed per group.

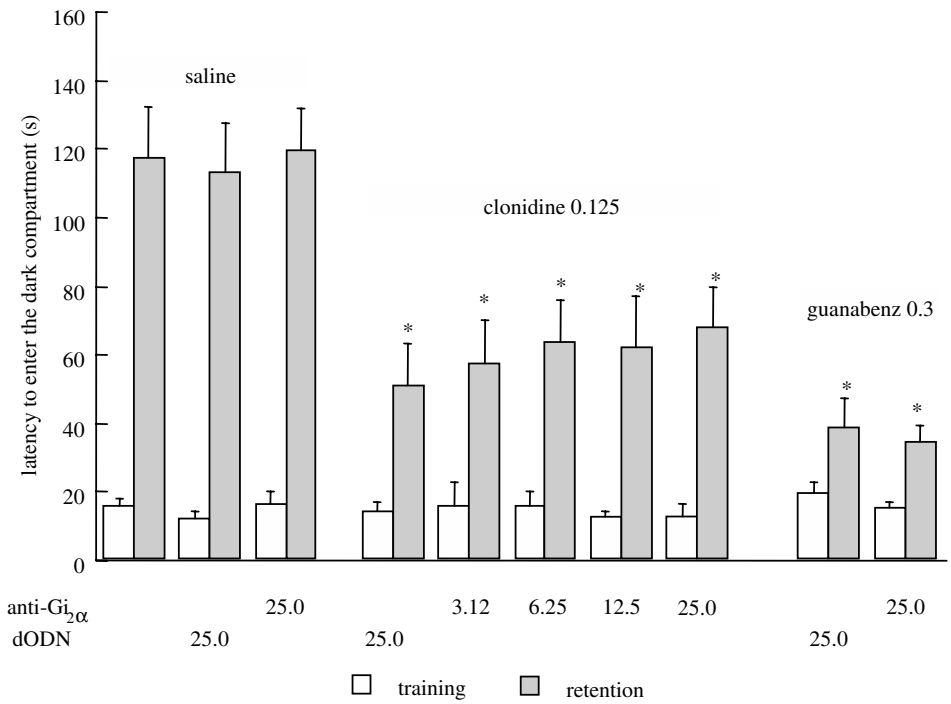

Fig. 2. Lack of effect of pretreatment with an aODN to the α subunit of Gi_2-protein gene (3.12–25 μg per mouse i.c.v.) on clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) amnesia in the mouse passive avoidance test. The test was performed 18 h after the last i.c.v. injection of dODN (25 μg per mouse i.c.v.) or aODN. Vertical lines represent S.E. mean; the dose administered is reported in each column. * $P<0.05$ in comparison with dODN+saline group. The number of animals ranged between 20 and 25 per group; at least two trials were performed per group.

dose of 0.25 μg per mouse 11 days before the test, completely prevented the clonidine- and guanabenz-induced amnesia. PTX, when injected alone, was devoid of any effect on memory processes in comparison with vehicle+saline-treated mice. No difference between the entrance latencies of each group in the training session of the passive avoidance test was observed (Table 1).

Effect of aODN against Giα subunits on α_2-agonist-mediated amnesia

The amnesia induced by clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) was prevented, in the mouse passive avoidance test, by pretreatment with the aODN against the α subunit of the Gi_1 proteins (Fig. 1). Anti-$Gi\alpha_1$ (3.12–12.5 μg per mouse i.c.v.) produced a dose-dependent antagonism of the clonidine-induced amnesia. The dose of 3.12 μg per mouse i.c.v. was completely ineffective, while the doses of 6.25 μg per mouse i.c.v. enhanced the entrance latency in the retention session up to a value comparable to that produced by control animals. Similarly, the amnesia induced by guanabenz was prevented by anti-$Gi\alpha_1$ (12.5 μg per mouse i.c.v.) pretreatment (Fig. 1). Anti-$Gi\alpha_1$ (12.5 μg per mouse i.c.v.) did not produce any effect in the mouse passive avoidance test in comparison with saline- and dODN-treated mice when given alone (Fig. 1).

The administration of an aODN against the α subunit of the Gi_2 proteins (3.12–25 μg per mouse i.c.v.), in contrast to anti-$Gi\alpha_1$, was unable to prevent clonidine- and guanabenz-induced amnesia (Fig. 2). At the highest dose employed, anti-$Gi\alpha_2$ did not modify the entrance latency in mice in comparison with saline-saline and dODN-saline-treated mice, used as control groups (Fig. 2).

The administration of an aODN against the α subunit of the Gi_3 proteins (3.12–12.5 μg per mouse i.c.v.) antagonised the memory disruption produced by both α_2-agonists without showing any memory facilitating activity when given alone (Fig. 3). Anti-$Gi\alpha_3$ was inactive in preventing clonidine- and guanabenz-induced amnesia at lower doses (1.56 and 3.12 μg per mouse i.c.v., respectively) as illustrated in Fig. 3.

No difference between the entrance latencies of each group in the training session of the passive avoidance test was observed (Figs. 1–3).

Effect of aODN against Goα subunits on α_2-agonist-mediated amnesia

Pretreatment with an aODN against the α subunit of the Go_1 proteins prevented, in a dose-dependent manner, the amnesia induced by clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) in the mouse passive avoidance test. The dose of 6.25 μg per mouse was ineffective whereas at 12.5 and 25 μg per mouse the anti-$Go\alpha_1$ enhanced the entrance latency in the retention session up to a value comparable to that produced by dODN- and saline-treated mice (Fig. 4). By contrast, the administration of an anti-$Go\alpha_2$ never modified the detrimental effect on memory processes produced by the investigated α_2-agonist (Fig. 5).

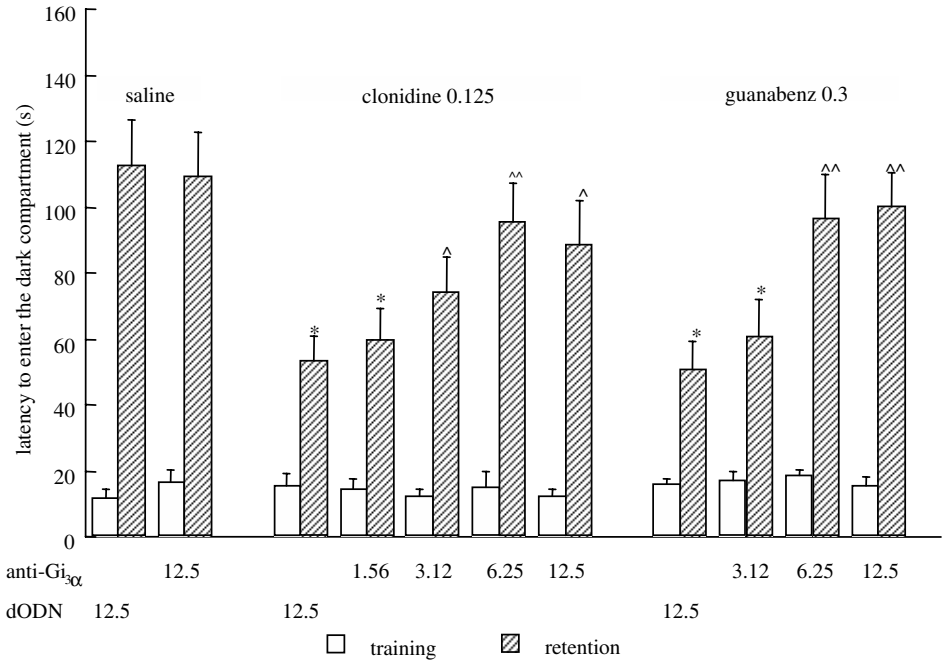

Fig. 3. Prevention by pretreatment with an aODN to the α subunit of Gi_3-protein gene (1.56–12.5 μg per mouse i.c.v.) of clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) amnesia in the mouse passive avoidance test. The test was performed 18 h after the last i.c.v. injection of dODN (12.5 μg per mouse i.c.v.) or aODN. Vertical lines represent S.E. mean; the dose administered is reported in each column. * $P<0.05$ in comparison with dODN+saline group; ^ $P<0.05$, ^^ $P<0.01$ in comparison with the corresponding amnesic compound. The number of animals ranged between 22 and 26 per group; at least two trials were performed per group.

Effect of aODN against Giα subunits on mouse rota rod and hole board tests

The administration of the aODN against the α subunits of the Gi proteins used in the present investigation elicited their effect on cognitive processes without changing either gross behavior or motor coordination as revealed by the rota rod test (Table 2). None of the aODNs, administered at the highest active doses, increased the number of falls from the rotating rod in comparison with dODN-treated mice (Table 2). The number of falls in the rota rod test progressively decreased since mice learned how to balance on the rotating rod.

The spontaneous motility and exploratory activity of mice was not modified by administration of the abovementioned aODNs as revealed by the hole-board test in comparison with saline-, vehicle- and dODN-treated mice. In the same experimental conditions D-amphetamine (1 mg kg^{-1} i.p.), used as the reference drug, increased both parameters evaluated (Fig. 6).

Effect of aODN against Giα subunits on G protein α subunits

Mice were treated with the aODNs (25 μg per mouse i.c.v.) 18 and 24 h prior to the experiments. Eighteen hours after the last i.c.v. injection, mice were killed and the hippocampus was dissected and examined for the levels of the individual G protein α subunit. The dODN did not significantly change the immunoreactivity when compared with that of naive anaimals. A statistically significant reduction ($P<0.05$) of the expression of $Gi_{1\alpha}$ (36.4±10.6), $Gi_{2\alpha}$ (38.9±13.6), $Gi_{3\alpha}$ (45.1±6.9), $Go_{1\alpha}$ (39.8±10.5) and $Go_{2\alpha}$ (37.9±11.1) after aODN treatment in comparison with mice treated with the corresponding dODN was observed (Fig. 7).

DISCUSSION

The current study examined the involvement of the Gi-protein system in the detrimental effect on memory processes induced by activation of α_2-adrenoceptors in the mouse passive avoidance test. To this purpose two chemically diverse α_2-adrenoceptor agonists, the imidazoline derivative clonidine and the guanidine derivative guanabenz, were investigated. The results showed that clonidine- and guanabenz-induced amnesia requires the selective activation of several Gi protein subtypes.

In the present experimental conditions, post-training i.p. administration of clonidine and guanabenz produced an impairment of memory functions that was completely prevented by pretreatment with PTX. PTX is a bacterial toxin produced by *Bordetella pertussis* that ADP-ribosylates and inactivates the α subunit of Gi proteins (Katada and Ui, 1982), indicating the important role played by the Gi proteins in the signal transduction mechanism activated by α_2-adrenoceptor agonists.

The Gi protein subfamily is composed of different members, such as Gi_1, Gi_2, Gi_3, Go_1 and Go_2 (Simon et al., 1991). Since PTX inactivates all members of the Gi protein family, the role of each subtype was investigated by pretreating animals with aODNs against the α subunits of these Gi protein subtypes. The inhibition of the expression

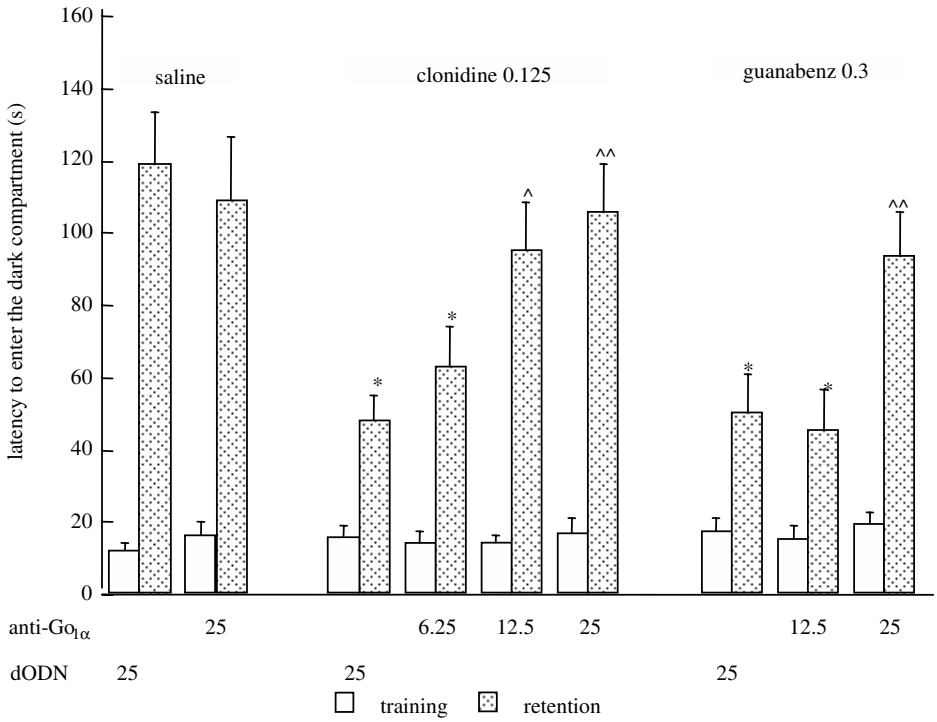

Fig. 4. Prevention by pretreatment with an aODN to the α subunit of Go_1-protein gene (6.25–25 μg per mouse i.c.v.) of clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) amnesia in the mouse passive avoidance test. The test was performed 18 h after the last i.c.v. injection of dODN (25 μg per mouse i.c.v.) or aODN. Vertical lines represent S.E. mean; the dose administered is reported in each column. * $P<0.05$ in comparison with dODN+saline group; ^ $P<0.05$, ^^ $P<0.01$ in comparison with the corresponding amnesic compound. The number of animals ranged between 22 and 28 per group; at least two trials were performed per group.

of $Gi\alpha_1$, $Gi\alpha_3$ and $Go\alpha_1$ produced a dose-dependent prevention of α_2 agonist-induced amnesia whereas the administration of an aODN against $Gi\alpha_2$, as well as the aODN against $Go\alpha_2$, was unable to modify the clonidine and guanabenz detrimental activity. These results indicate a differential involvement of the Gi protein subtypes in the mechanism of action of the investigated α_2-adrenoceptor agonists. In particular, the integrity and functionality of Gi_1, Gi_3 and Go_1 proteins appears essential to produce memory impairment after activation of α_2-adrenoceptors. However, to reverse the α_2 agonist-induced memory deficit, a concentration of anti-$Gi\alpha_1$ and anti-$Go\alpha_1$ respectively two- and four-fold higher than that of anti-$Gi\alpha_3$ was necessary. A preferential interaction of clonidine and guanabenz with the Gi_3 protein subtypes in the induction of amnesia can be supposed. By contrast, the Gi_2 and Go_2 subtypes appear not to be involved, in these experimental conditions.

Among α_2-adrenoceptor subtypes, clonidine and guanabenz have a preferential interaction with the α_{2A} (Newman-Tancredi et al., 1998). α_{2A}-Adrenoceptors can activate all Gi protein subtypes even if a preferential activation of the $Gi\alpha_3$ subtypes over PTX-sensitive G-proteins has been observed. In experiments performed in reconstituted phospholipid vesicles, the α_{2A}-adrenoceptors showed the following preference: Gi_3 greater than Gi_1 greater than or equal to Gi_2 greater than Go (Kurose et al., 1991). Furthermore, prolonged treatment with α_2-adrenoceptor agonists produced a down-regulation of all Gi-protein subtypes, but $Gi\alpha_3$ had the greatest reduction (Gasic and Green, 1995). The abovementioned data can, at least in part, explain the involvement of $Gi\alpha_3$, $Gi\alpha_1$, $Go\alpha_1$ in the mechanism of amnesic action of the investigated α_2-agonists and support the hypothesis of a prominent role played by the Gi_3 subtype.

Although α_{2A}-adrenoceptors show some preference for Gi_3, they also activate the Gi_2 subtype and, to a lesser extent, the Go subtype (McClue and Milligan, 1991; Kurose et al., 1991). The involvement of $Gi\alpha_2$ and $Go\alpha_2$ has been observed for other pharmacological and physiological activities of α_2-agonists, such as analgesia (Garzon et al., 1999; Sanchez-Blazquez et al., 1999; Karim and Roerig, 2000), stimulation of voltage-dependent Ca^{2+} channels (Macrez-Lepretre et al., 1995), proliferation of human intestinal epithelial cells (Schaak et al., 2000). In the present study we observed the lack of effect of an aODN against $Gi\alpha_2$ or $Go\alpha_2$, which implies that these subunits are not a major component of transduction mechanisms leading to amnesia. It is possible that multiple α_2 agonists-induced effects are mediated by different Gi-protein subunits and that clonidine and guanabenz could have different intrinsic activity for each effect.

All Gi-protein subtypes were shown to inhibit the adenylyl cyclase activity with a consequent reduction of intracellular cAMP levels (Wong et al., 1992). The role of the cAMP cascade in memory processes has been recently

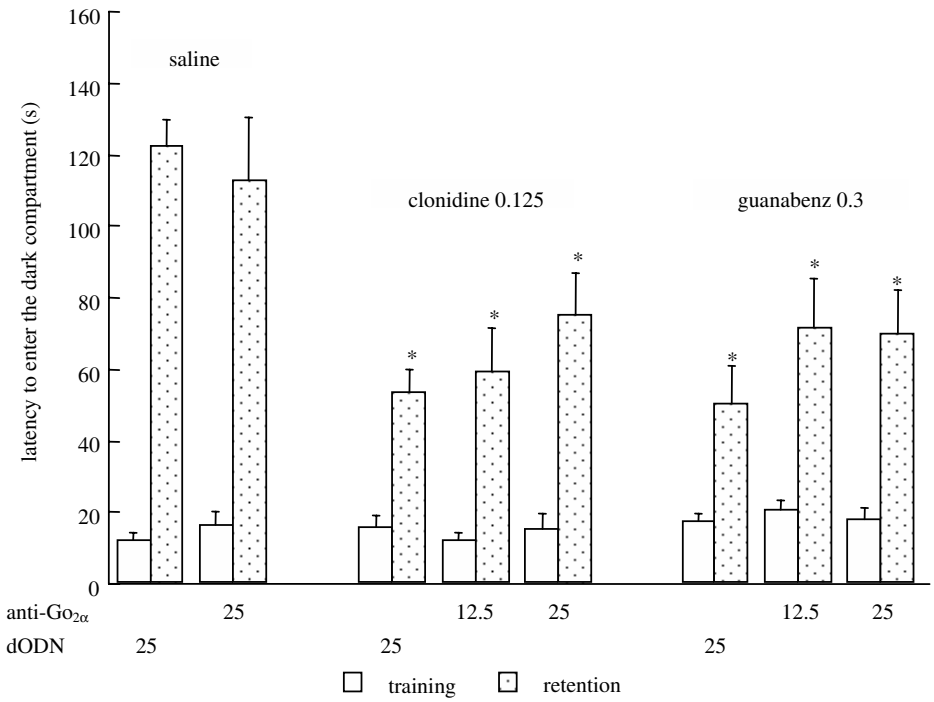

Fig. 5. Lack of effect of pretreatment with an aODN to the α subunit of Go_2-protein gene (12.5–25 μg per mouse i.c.v.) on clonidine (0.125 mg kg^{-1} i.p.) and guanabenz (0.3 mg kg^{-1} i.p.) amnesia in the mouse passive avoidance test. The test was performed 18 h after the last i.c.v. injection of dODN (25 μg per mouse i.c.v.) or aODN. Vertical lines represent S.E. mean; the dose administered is reported in each column. * $P<0.05$ in comparison with dODN+saline group. The number of animals ranged between 20 and 26 per group; at least two trials were performed per group.

evidenced. Genetic and pharmacological studies in mice and rats demonstrated that the cAMP responsive element binding protein is required for a variety of complex forms of memory, including spatial and social learning (Silva et al., 1998). The activation of the cAMP-dependent protein kinase induces long-term memory (Muller, 2000) and the inhibition of cAMP phosphodiesterase reverses memory deficits in the radial arm maze task (Zhang et al., 2000). Several studies have also shown that the regulation of adenylyl cyclase activity is disrupted in Alzheimer's disease patients. The alteration of adenylyl cyclase activity in postmortem brain is related to an impairment of the stimulatory G-proteins (Gs) whereas the Gi-protein-mediated inhibition of the enzyme is unaltered (Schnecko et al., 1994; Fowler et al., 1995). Furthermore, lower Gsα levels and unmodified Giα levels were observed in fibroblasts from familial Alzheimer's disease patients (Shanahan et al., 1997). Recently, it has been reported that amyloid β-peptides cause toxicity through activation of Gi proteins (Soomets et al., 1999; Rymer and Good, 2001). We can hypothesize that an intact Gi-protein functionality is essential for the induction of amnesia. Our results on α_2-agonists amnesia confirm the hypothesis of a Gi-protein involvement in the induction of memory impairment. By consider-

Table 2. Effect of clonidine, guanabenz, PTX and aODN to Gi α subunits in the mouse rota-rod test[a]

Treatment	Number of falls in 30 s			
	Pre-test	After the beginning of the test		
		15 min	30 min	45 min
Saline	5.3±0.4	3.2±0.4	3.1±0.5	2.3±1.0
Clonidine 0.125 mg kg^{-1}	5.6±0.5	4.6±0.5	4.1±0.4	3.8±0.3
Guanabenz 0.30 mg kg^{-1}	5.2±0.8	4.8±1.0	4.4±0.9	2.8±0.9
PTX 0.25 μg $mouse^{-1}$	5.4±0.6	3.9±0.8	2.9±1.0	2.6±1.0
dODN 25 μg $mouse^{-1}$	4.9±0.2	2.9±0.3	2.1±0.4	1.8±0.2
Anti-$Gi_{1\alpha}$ 12.5 μg $mouse^{-1}$	5.0±0.6	2.8±0.9	1.5±0.8	1.0±0.4
Anti-$Gi_{2\alpha}$ 25 μg $mouse^{-1}$	5.2±0.4	3.2±0.6	2.3±0.5	1.3±1.0
Anti-$Gi_{3\alpha}$ 12.5 μg $mouse^{-1}$	4.6±0.8	2.9±1.0	1.6±0.9	1.3±0.9
Anti-$Go_{1\alpha}$ 25 μg $mouse^{-1}$	4.4±0.5	2.1±0.4	1.8±0.5	1.0±0.5
Anti-$Go_{2\alpha}$ 25 μg $mouse^{-1}$	4.8±0.4	2.7±0.5	2.2±0.7	1.9±0.8

[a] Data are expressed as mean±S.E.M. The number of animals was 12 per group; two trials were performed per group.

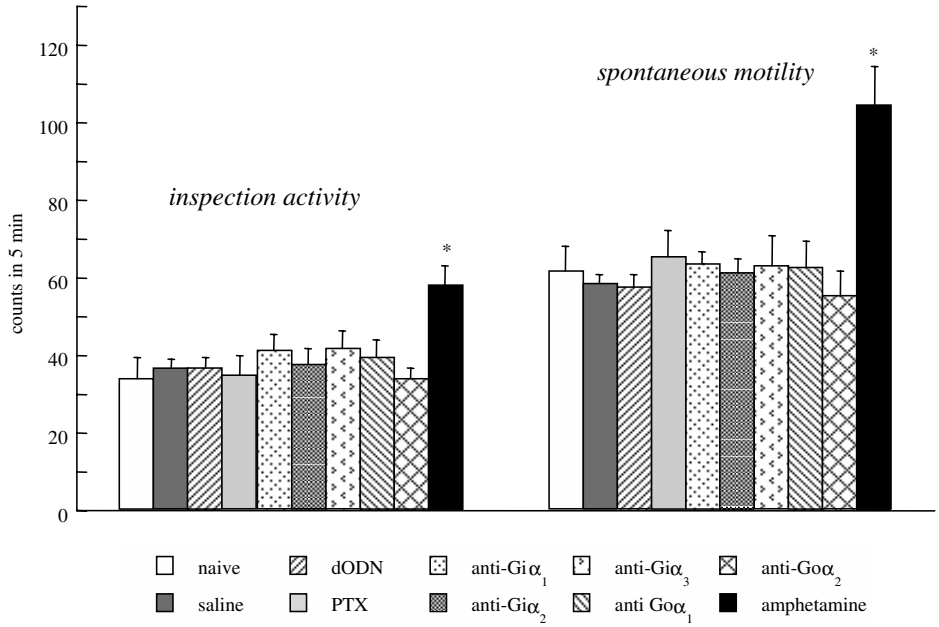

Fig. 6. Lack of effect of pretreatment with PTX (0.25 μg per mouse i.c.v.) and an aODN to the α subunit of Gi_1- (12.5 μg per mouse i.c.v.), Gi_2- (25 μg per mouse i.c.v.), Gi_3- (12.5 μg per mouse i.c.v.), Go_1- (25 μg per mouse i.c.v.), Go_2- (25 μg per mouse i.c.v.) protein gene on spontaneous motility and inspection activity in the mouse hole board test. The test was performed 18 h after the last i.c.v. injections of dODN (25 μg per mouse) or aODN. PTX was administered 11 days before the test. Vertical lines represent S.E. mean. D-Amphetamine was administered at the dose of 1 mg kg^{-1} s.c. * $P<0.05$ in comparison with naive group. The number of animals used was 12 per group; two trials were performed per group.

ing that high cAMP levels are required for mnemonic integrity, we can also suppose that clonidine and guanabenz induce amnesia through a reduction of intracellular cAMP levels via the activation of Gi_3, Gi_1 and Go_1 proteins.

Pretreatment with PTX did not produce any ameliorative effect on memory processes in the mouse passive avoidance test, in agreement with previous results obtained in the rat (Chou and Lee, 1995). Similarly, aODN against the α subunits of Gi proteins never modified the entrance latency in comparison with control animals. We can, therefore exclude that the prevention of a_2 agonist-induced amnesia is due to a procognitive effect exerted by these treatments. Furthermore, pretreatment with dODN, used as the reference ODN, never modified the amnesia

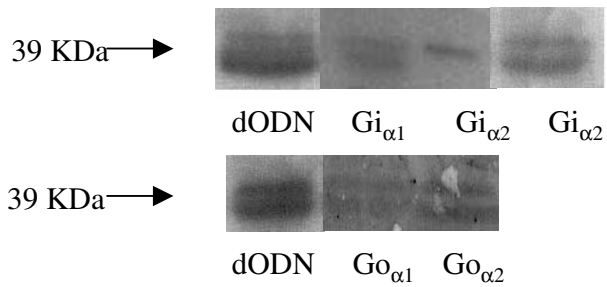

Fig. 7. Effect of aODN treatment on G protein α subunit levels in membranes form mouse hippocampus. Mice received an i.c.v. injection of aODN or dODN (25 μg per mouse) 18 and 24 h prior to the test. Membrane proteins (20 μg) were solubilized in SDS, separated on SDS-polyacrylamide gel (10%), transferred to nitrocellulose, and probed with the indicated antibodies. For each comparison, the assay was repeated at least four times using samples from different animals.

induced by clonidine and guanabenz in comparison with saline-treated animals, excluding the possibility of a sequence-independent effect induced by the aODNs.

As the Gi-proteins are widely distributed in the neural areas, the function of a variety of cellular receptors is expected to be altered by the impairing effect of the antisense treatment. Thus, the administration of PTX and aODN against the α subunits of Gi proteins could induce side effects that make difficult the interpretation of the results obtained. Furthermore, α_2-adrenoceptors induce numerous pharmacological effects, such as sedation and hypotension (Ruffolo et al., 1993), whose appearance could lead to an alteration of the results obtained. It has been, therefore, necessary to choose a range of doses at which these compounds were not endowed with behavioral side effect. PTX, aODNs and α_2 agonists did not impair motor coordination as revealed by the rota rod test nor modify spontaneous motility and inspection activity as indicated by the hole board test. We can, thus, suppose that the effects produced by these treatments were not due to compromised behavioral paradigms.

In conclusion, our results evidence the important role played by $Gi\alpha_3$, $Gi\alpha_1$ and $Go\alpha_1$, but not by $Gi\alpha_2$ and $Go\alpha_2$, in clonidine- and guanabenz-induced amnesia. The elucidation of the post-receptorial mechanisms involved in memory processes can not only help unravel the complex systems mediating cognitive functions, but also the manipulation of Gi-protein activity may lead to novel treatments for pathologies characterized by memory dysfunction.

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