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DIPHENHYDRAMINE-INDUCED AMNESIA IS MEDIATED BY GI-PROTEIN ACTIVATION

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Abstract—The effect of the i.c.v. administration of antisense oligodeoxynucleotides directed against the α subunit of different Gi-proteins (anti-Gi α_1 , anti-Gi α_2 , anti-Gi α_3 , anti-Go α_1 , anti-Go α_2) on the amnesia induced by the H₁-antihistamine diphenhydramine (20 mg kg⁻¹ s.c.) was evaluated in the mouse passive avoidance test. Pretreatment with anti-Gi α_1 (12.5–25 μ g per mouse i.c.v.) and anti-Gi α_2 (25 μ g per mouse i.c.v.), administered 24 and 18 h before test, prevented antihistamine-induced amnesia. By contrast, pretreatment with an anti-Gi α_3 (25 µg per mouse i.c.v.), anti-Go α_1 (25 µg per mouse i.c.v.) and anti-Go α_2 (25 µg per mouse i.c.v.) did not modify the detrimental effect induced by diphenhydramine. At the highest effective doses, none of the compounds used impaired motor coordination, as revealed by the rota rod test, nor modified spontaneous motility and inspection activity, as revealed by the hole board test.

These results suggest the important role played by the Gi_1 - and Gi_2 -protein pathway in the transduction mechanism involved in the impairment of memory processes produced by the H₁-antihistamine diphenhydramine. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: Gi-proteins, memory, amnesia, H₁-antihistamines, diphenhydramine, passive avoidance.

The H₁-receptor antagonists are amongst the most widely used medications in the world. These compounds produce the inhibition of the effects of histamine mediated by H_1 receptors such as smooth-muscle contraction in the respiratory and gastrointestinal tracts, pruritus, sneezing by sensory-nerve stimulation, vasodilation (Simons and Simons, 1994). Therefore, their most common use is in the treatment of allergic disorders (Rimmer and Church, 1990). In addition to these well-known peripheral effects, H₁-receptor antagonists produce various central inhibitory actions (Simons and Simons, 1994). The first generation of H₁ antagonists can both stimulate and depress the CNS. Stimulation occasionally is encountered in patients given therapeutic doses, but it is a striking feature of poisoning, which can result in convulsions, particularly in infants (Faingold and Berry, 1972). Central depression, on the other hand, usually accompanies therapeutic doses of the H₁ antagonists, which appears to be related to occupancy

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

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of cerebral H_1 receptors (Simons and Simons, 1994). Diminished alertness, slowed reaction times or somnolence are common manifestations (Simons and Simons, 1994). Several data also indicate the induction of an impairment of cognitive functions by antihistamines. The post-training i.c.v. administration of histamine has been reported to cause memory facilitation in rats which is antagonized by the simultaneous treatment with both promethazine and cimetidine (de Almeida and Izquierdo, 1986, 1988). Moreover, the administration of diphenhydramine further reduces learning ability in patients with seasonal allergic rhinitis in which learning performances are already impaired by allergy symptoms (Vuurman et al., 1996).

Even if the amnesic properties of antihistamines are documented, the post-receptorial mechanism involved in the induction of this detrimental effect on cognitive processes has not yet been established. It has been reported that first-generation H₁-receptor antagonists are receptor-independent G-protein activators in HL-60 cells, basophils and mast cells (Burde et al., 1996). Recently, it has been reported that one of the central effects induced by H₁-receptor antagonists, the antinociception, underlies the activation of a signal transduction mechanism operated by Gi-proteins. The i.c.v. administration of pertussis toxin (PTX), compound which selectively inactivates Gi proteins, prevented the increase of the pain threshold induced by diphenhydramine, pyrilamine and promethazine in the mouse hotplate test (Galeotti et al., 1996, 1999). Furthermore, the i.c.v. administration of antisense oligodeoxynucleotides directed against the α subunit of different Gi-proteins (anti-Gi α_1 , anti-Gi α_2 , anti-Gi α_3) prevented the antinociception induced by antihistamines (Galeotti et al., 2002). Since Gi-proteins represent the most widespread modulatory signalling pathway in neurones (Holz et al., 1986), we thought it worthwhile to investigate the involvement of Gi proteins into the mechanism of action of the antihistamine diphenhydramine, by means of an antisense strategy. The Gi protein family is composed by several subtypes, named Gi1, Gi2, Gi3, Go1 and Go2 (Simon et al., 1991). In the current study we used antisense oligonucleotides (aODN) against the α subunits of the Gi₁, Gi₂, Gi₃, Go₁ and Go₂ proteins in order to determine the role of each subtype in the memory impairment induced by diphenhydramine in a mouse passive avoidance paradigm. In order to exclude that the effects produced by aODN treatments were due to the induction of side effects, some additional behavioural tests (rota rod, hole board) were performed.

EXPERIMENTAL PROCEDURES

Animals

Male Swiss albino mice (23–30 g) from the Morini (San Polo d'Enza, Italy) breeding farm 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 ± 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 European Communities Council Directive of 24 November 1986 (86/609/EEC) for experimental animal care. All efforts were made to minimise the number of animals used and their suffering.

I.c.v. injection technique

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 μ l syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μ l 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 crebral ventricle, some mice (20%) were injected with 5 μ l of diluted 1:10 India ink and their brains examined macroscopically after sectioning. The accuracy of the 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, diphenhydramine (20 mg kg⁻¹) was i.p. injected immediately after the training session, whereas aODNs (6.5–25 μ g per mouse) were i.c.v. injected 24 and 18 h before training. Between 11 and 25 mice were tested.

Hole board test

The hole board test was performed according to Galeotti et al. (2002). The 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 centre 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 mid-point to mid-point of opposite sides, thus dividing the plane into four equal quadrants, automatically signalled 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 degenerate ODN (dODN; 25 μ g per mouse) or aODN (25 μ g per mouse). Twelve mice per group were tested.

Rota rod test

The apparatus consisted of a base platform and a rotating rod with a diameter of 3 cm and a non-slippery 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 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). Those mice scoring less than three and more than six falls in the pretest were rejected (20%). The performance time was measured before (pretest) and 15, 30 and 45 min after the beginning of the test. Animals were i.c.v. pretreated 24 and 18 h prior to the test with dODN (25 μ g per mouse) or aODN (12.5–25 μ g per mouse). Twelve mice per group were tested.

aODN

Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased by Tib Molbiol (Genoa, Italy). The sequences of the 33-mer aODNs used in the present study were the following: anti-Gi₁ α : 5'-G*C*T GTC CTT CCA CAG TCT CTT TAT GAC GCC G*G*C-3'; anti-Gi₂α: 5'-A*T*G GTC AGC CCA GAG CCT CCG GAT GAC GCC C*G*A-3'; anti-Gi₃a: 5'-G*C*C ATC TCG CCA TAA ACG TTT AAT CAC GCC T*G*C-3'; anti-Goa1: 5'- A*G*G CAG CTG CAT CTT CAT AGG TG*T *T -3'; anti-Go α_2 : 5'-G*A*G CCA CAG CTT CTG TGA AGG CA*C *T -3'. All ODNs were previously characterised 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). We also confirmed the aODN effect on Gi/oa protein levels by performing immunoblotting experiments. We observed a statistically significant reduction of the expression of Gia1 (36.4 \pm 10.6), Gia2 (38.9 ± 13.6) , Gia3 (41.1 ± 6.9) , Goa1 (39.8 ± 11.1) , Goa2 (37.1±12.5) levels after aODN treatment (25 µg per mouse i.c.v.) in comparison with mice treated with dODN (25 µg per mouse i.c.v.). The dODN treatment did not modify the $G\alpha$ protein levels in comparison with saline-treated mice. At the dose of 6.5 µg per mouse i.c.v., we did not observe any significant modification of the protein levels (data not shown). 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 NN*N *N-3' (where N is G, or C, or A, or T) were used as a control respectively for anti-Gi $\!\alpha$ and anti-Goa. ODNs were vehiculated intracellularly by an artificial cationic lipid (DOTAP; Sigma, Milan, Italy) 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 μ M DOTAP and supplied to mice by i.c.v. injection of 5 µl solution 18 and 24 h prior to the behavioural tests.

Drugs

The following drugs were used: diphenhydramine hydrochloride, D-amphetamine hydrochloride (De Angeli). Drugs were dissolved in isotonic (NaCl 0.9%) saline solution. Drug and ODNs concentrations were prepared in such a way that the necessary dose could be administered in a volume of 5 μ l by i.c.v. injection or 10 ml kg⁻¹ by s.c. administration.

Statistical analysis

All experimental results are given as the mean \pm S.E.M. Analysis of variance, followed by Fisher's protected least significant difference procedure for post hoc comparison, was used to verify significance between two means. Data were analysed with the StatView software for the Macintosh (1992). *P* values of <0.05 were considered significant.



Fig. 1. Prevention by aODN against the α subunit of Gi₁ protein (6.25–25 μ g per mouse i.c.v.) of diphenhydramine (20 mg kg⁻¹ s.c.) amnesia in the mouse passive avoidance test. Vertical lines represent S.E.M. * *P*<0.05, ** *P*<0.01 in comparison with dODN+diphenhydramine-treated mice.

RESULTS

Effect of aODN against $Gi\alpha$ subunits on diphenhydramine amnesia

Diphenhydramine (20 mg kg⁻¹ s.c.) induced amnesia in the mouse passive avoidance test that was prevented by pretreatment with an aODN against the α subunit of the Gi₁ proteins. Anti-Gi α_1 (6.25–12.5 µg per mouse i.c.v.) produced a dose-dependent antagonism of the detrimental effect produced by the H₁-antihistamine. The dose of 6.25 µg per mouse i.c.v. was completely ineffective; 12.5 µg per mouse i.c.v. partially prevented diphenhydramine amnesia whilst the doses of 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 (Fig. 1).

The administration of an aODN against the α subunit of the Gi₂ proteins (6.25–25 µg per mouse i.c.v.), dosedependently prevented diphenhydramine-induced amnesia. At the concentration of 25 µg per mouse i.c.v., a complete prevention was obtained, at the dose of 12.5 µg partially prevented diphenhydramine amnesia, even if the statistical significance was not reached, whereas, at 6.25 µg per mouse i.c.v., the aODN pretreatment was ineffective (Fig. 2).

The administration of an aODN against the α subunit of the Gi₃ proteins (12.5–25 µg per mouse i.c.v.), in contrast to anti-Gi α_1 and anti-Gi α_2 , was unable to prevent diphenhydramine-induced amnesia (Fig. 3). At the highest dose employed, anti-Gi α_2 did not modify the entrance latency in mice in comparison with the control groups (Fig. 3).

A time-course of recovery from antihistamine amnesia after aODN treatment was performed. The prevention of diphenhydramine-induced impairment of memory processes produced by anti-Gi α_1 and anti-Gi α_2 at the highest active dose (12.5–25 μ g per mouse i.c.v.), disappeared 7 days after the end of the aODN pretreatment (data not shown).

Effect of aODN against $Go\alpha$ subunits on diphenhydramine amnesia

The administration of an aODN against the α subunit of the Go₁ and Go₂ proteins (25 µg per mouse i.c.v.) was unable to prevent diphenhydramine-induced amnesia (Fig. 4). At the highest dose employed, anti-Gi α_2 did not modify the entrance latency in mice in comparison with the control groups (Fig. 4).

Effect of diphenhydramine on mouse behaviour

Diphenhydramine, at the dose used in the present work, elicited its detrimental effect on memory functions without changing gross behaviour of mice. The H_1 -antihistamine investigated did not alter the mice motor coordination, as revealed by the rota rod test, the spontaneous motility and inspection activity, as revealed by the hole board test (data not shown).

Effect of aODNs on mouse behaviour

The motor coordination of mice pretreated with aODN to $Gi\alpha_1$ (25 µg per mouse i.c.v.), $Gi\alpha_2$ (25 µg per mouse i.c.v.), $Gi\alpha_3$ (25 µg per mouse i.c.v.), $Go\alpha_1$ (25 µg per mouse i.c.v.), and $Gi\alpha_2$ (25 µg per mouse i.c.v.), was



Fig. 2. Prevention by aODN against the α subunit of Gi₂ protein (6.25–25 μ g per mouse i.c.v.) of diphenhydramine (20 mg kg⁻¹ s.c.) amnesia in the mouse passive avoidance test. Vertical lines represent S.E.M. ** *P*<0.01 in comparison with dODN+diphenhydramine-treated mice.

evaluated by using the rota rod test. The motor coordination of aODN-treated groups was not impaired when compared with that of dODN-treated mice (Fig. 5).

The spontaneous motility and exploratory activity of mice was not modified by administration of the above-

mentioned aODNs as revealed by the hole-board test in comparison with dODN-treated mice (Fig. 6). In the same experimental conditions D-amphetamine (1 mg kg⁻¹ i.p.), used as the reference drug, increased both parameters evaluated (Fig. 6).



Fig. 3. Lack of effect by aODN against the α subunit of Gi₃ protein (12.5–25 μ g per mouse i.c.v.) of diphenhydramine (20 mg kg⁻¹ s.c.) amnesia in the mouse passive avoidance test. Vertical lines represent S.E.M. * P<0.05, ** P<0.01 in comparison with dODN+saline-treated mice.



Fig. 4. Lack of effect by pretreatment with an aODN to the α subunit of Go₁- (25 µg per mouse i.c.v.) and Go₂- (25 µg per mouse i.c.v.) protein gene on diphenhydramine (20 mg kg⁻¹ s.c.), amnesia. Vertical lines represent S.E.M.; * *P*<0.05, ** *P*<0.01 in comparison with dODN+saline-treated mice.

DISCUSSION

The important role played by the Gi-protein family in the mechanism of amnesic action of diphenhydramine is indicated by present results. The inhibition of the expression of Gi α_1 and Gi α_2 produced a dose-dependent prevention of diphenhydramine-induced amnesia whereas the administration of aODNs against Gi α_3 , Go α_1 and Go α_2 never exerted any modification of diphenhydramine activity in the mouse passive avoidance test. These results indicate a differential involvement of the Gi protein subtypes in the



Fig. 5. Effect of pretreatment with an aODN to the α subunit of Gi₁-(25 µg per mouse i.c.v.), Gi₂- (25 µg per mouse i.c.v.), Gi₃- (25 µg per mouse i.c.v.), Go₁- (25 µg per mouse i.c.v.) and Go₂- (25 µg per mouse i.c.v.) protein gene on motor coordination in the mouse rota rod test. Vertical lines represent S.E.M.

mechanism of action of the investigated H₁-antihistamine. In particular, the integrity and functionality of Gi₁ and Gi₂ proteins appears essential to produce memory impairment by diphenhydramine. By contrast, the Gi α_3 , Go α_1 and Go α_2 subtypes, in these experimental conditions, appears not to be involved.

The inhibition of antihistamine-induced memory disruption disappeared 7 days after pretreatment with the aODNs. This return of sensitivity implies both the total reversal of aODN-induced specific inhibition of Gia expression and a lack of damage or toxicity associated with aODN treatment. Pretreatment with the anti-Gia ODNs at the highest effective doses did not modify the entrance latency, showing the absence of any effect on memory functions by these pretreatments. Therefore, the prevention of diphenhydramine amnesia cannot be attributable to a direct beneficial effect on memory processes induced by the aODNs. Furthermore, the dODN did not modify diphenhydramine-induced amnesia in comparison with naive or saline i.c.v. injected mice (data not shown). This observation ruled out the possibility that the antagonism exerted by aODNs may have resulted from a sequence-independent action on cerebral structures.

Diphenhydramine is a first-generation H_1 -receptor antagonist (Simons and Simons, 1994). H_1 receptors are G-protein coupled receptors that activate phospholipase C via a PTX-insensitive mechanism (Arrang, 1994). By contrast, the antagonism exerted by anti-Gia indicates that the intracellular mechanism of analgesic action of the investigated H_1 -antihistamine involves the activation of PTX-sensitive G-proteins. It has been reported that first-generation



Fig. 6. Lack of effect of pretreatment with an aODN to the α subunit of Gi₁- (25 µg per mouse i.c.v.), Gi₂- (25 µg per mouse i.c.v.), Gi₃- (25 µg per mouse i.c.v.), Go₁- (25 µg per mouse i.c.v.) and Go₂- (25 µg per mouse i.c.v.) protein gene on inspection activity (A) and spontaneous motility (B) in the mouse hole board test. Vertical lines represent S.E.M.; Amphet: D-amphetamine 1 mg kg⁻¹ i.p. * P<0.05 in comparison with control group.

H₁-receptor antagonists are receptor-independent G-protein activators in HL-60 cells, basophils and mast cells and that such a mechanism of action might be responsible for the stimulatory effects of these compounds (Burde et al., 1996). We can, therefore, suppose that a direct activation of the Gi-protein-mediated transduction system might also be responsible for the detrimental effect on memory functions induced by antihistamines. The involvement of the Gi-protein system has also been observed for other central effects of antihistamines. Literature data evidenced that diphenhydramine, pyrilamine and promethazine antinociception is prevented by the i.c.v. administration of PTX, which inactivates Gi proteins (Galeotti et al., 1996, 1999) and of aODNs against the α subunit of Gi proteins (Galeotti et al., 2002). These results further support the hypothesis of an involvement of the Gi protein-mediated system in the intracellular events responsible for the induction of amnesia by diphenhydramine. However, first-generation H₁-receptor antagonists induce histamine release from basophils and mast cells (Mota and Da Silva, 1960; Mota, 1966; Lichtenstein and Gillespie, 1975) and increase the histamine levels in the cerebrospinal fluid (Suojaranta-Ylinen et al., 1991). Since it is well known that histamine can modulate the release of several neurotransmitters (Hill, 1990), an involvement of neurotransmitters different from histamine, activating Gi proteins as a signal transduction mechanism, downstream from the initial receptor interaction, cannot be excluded.

In the present study we observed the lack of effect of an aODN against $Gi\alpha_3$ that implies that this subunit is not a major component of transduction mechanisms leading to amnesia. By contrast, other pharmacological activities of diphenhydramine, as analgesia, are mediated by activation of the three Gi protein subtypes (Galeotti et al., 2002). It is plausible to suppose that different-induced effects are mediated by different Gi-protein subunits and that diphenhydramine could have different intrinsic activity for each effect.

Taking into account present observations together with several literature data, it appears that the Gi-protein sys-

tem represents an essential intracellular step in the induction of amnesia. Gi proteins 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 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 Gs α levels and unmodified Gia 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 hypothesise that an intact Gi-protein functionality is essential for the induction of amnesia. Our results on diphenhydramine amnesia confirm the hypothesis of a Gi-protein involvement in the induction of memory impairment. By considering that high cAMP levels are required for mnemonic integrity, we can also suppose that diphenhydramine induces amnesia through a reduction of intracellular cAMP levels via the activation of Gi₁ and Gi₂ proteins.

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. It has been, therefore, necessary to evaluate the possible induction of side effects by the anti-Gi α treatment. The aODNs, at the highest doses used, did not modify animals' gross behaviour. Moreover, additional behavioural tests were performed to illustrate any side effects produced by the treatments that cannot be revealed by the researcher through the observation of the animal's spontaneous behaviour. The aODN treatment did not impair motor coordination, as revealed by the progressive reduction of the number of falls in the rota rod test. Repetition of the test session every 15 min progressively decreases the number of falls since animals learn how to balance on the rotating rod. The lack of variation, or an increase in the number of falls after treatment indicates an impairment of mice motor coordination that could lead to a misinterpretation of the results obtained in the behavioural test. Moreover, aODNs did not modify spontaneous motility and exploratory activity as indicated by the hole board test. We can, thus, suppose that the effects observed in the passive avoidance test were not imputable to compromised behavioural paradigms.

Similarly, the administration of histamine $\rm H_1$ receptor antagonists produces various inhibitory effects including

sedation (Simons and Simons, 1994) whose appearance could lead to a modification of the entrance latency values observed in the passive avoidance test. At the dose employed, diphenhydramine exerted its amnesic activity without showing any alteration of the mice motor coordination, spontaneous motility and exploratory activity.

In conclusion, our results evidence that knockdown of $Gi\alpha_1$ and $Gi\alpha_2$, but not by $Gi\alpha_3$, $Go\alpha_1$ and $Go\alpha_2$, reversed the diphenhydramine-induced amnesia, indicating the involvement of this transduction system in the induction of a detrimental effect on memory functions by H₁-antihistamines.

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