AMITRIPTYLINE AND CLOMIPRAMINE ACTIVATE GI-PROTEIN SIGNALING PATHWAY IN THE INDUCTION OF ANALGESIA

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ORIGINAL ARTICLE

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Amitriptyline and clomipramine activate Gi-protein signaling pathway in the induction of analgesia

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Abstract The post-receptorial mechanisms of the analgesic action of amitriptyline and clomipramine, two tricyclic antidepressants, were investigated in the mouse hot plate test by using an antisense strategy. Mice were injected i.c.v. with antisense oligonucleotides (aODN), complementary to the sequence of the mRNA sequence of the α-subunit of Gi_1, Gi_2 and Gi_3-proteins, 18–24 h prior to the hot plate test. Treatment with aODN against $Gi_1\alpha$, $Gi_2\alpha$ and $Gi_3\alpha$ dose-dependently reduced the analgesia induced by both amitriptyline (15 mg/kg s.c.) and clomipramine (25 mg/kg s.c.). This antagonistic effect disappeared 7 days after the end of the i.c.v. treatment, indicating the absence of irreversible damage or toxicity. Treatment with aODN against $Gi_1\alpha$, $Gi_2\alpha$ and $Gi_3\alpha$, at the active doses, did not modify the animals' pain threshold, indicating the absence of any hyperalgesic effect. Amitriptyline, clomipramine and the aODN employed, at the maximal effective doses, did not produce any alteration of motor coordination of the mice, as revealed by rotarod experiments, and spontaneous motility, as revealed by the Animex apparatus. These results indicate that amitriptyline and clomipramine induce their analgesic effect by activating all three subtypes of the Gi-proteins.

Keywords Amitriptyline · Clomipramine · Analgesia · Gi-proteins · Antisense oligonucleotides · Mice · Hot plate test

Introduction

From the 1960s up to now, antidepressants have been prescribed for chronic pain syndromes with successful results. These include diabetic neuropathy, postherpetic neuralgia, headaches, arthritis, chronic back pain, cancer pain, phantom limb pain (Saunders 1963; Woodforde et al. 1965; McQuay and Moore 1997; Richeimer et al. 1997; Watson 2000). Some authors claim that the analgesic effects of these drugs occur simultaneously with antidepressant effects following a latent period (Lee and Spencer 1977). Others defend the view that tricyclic antidepressant (TCAs) have a direct antinociceptive effect since the analgesia induced by TCAs is also seen in patients suffering from different types of pain syndromes without accompanying depression (Richeimer et al. 1997; Watson 2000). TCAs showed analgesic properties also in laboratory animals (Spiegel et al. 1983; Tura and Tura 1990). The analgesic effect of TCAs in animals seems to be independent of their antidepressant activity since the doses used for analgesia are lower than those considered effective in the treatment of depression (Acton et al. 1992).

It has been suggested that TCAs exert their antinociceptive properties through an inhibition of serotonin and noradrenaline reuptake in the central nervous system (Basbaum and Fields 1978; Dubner and Bennett 1983). Antidepressants have also been reported to be effective as analgesics through activation of the opioid system. Some studies showed that TCAs have direct effects on the opioid receptors (Biegon and Samuel 1980; Spiegel et al. 1983), but some of them gave indication for an indirect activation of the opioid system (Botney and Fields 1983; Sacerdote et al. 1987).

Nearly all inhibitory neurotransmitters able to enhance the pain threshold utilize Gi-proteins as signal transduction system. Gi-proteins represent the most widespread modulatory signaling pathway in neurones (Holz et al. 1986) and their involvement in the modulation of pain perception has been well established. The administration of pertussis toxin (PTX), which selectively inactivates Gi-proteins, produced hyperalgesia and allodynia in laboratory animals (Galeotti et al. 1996; Womer et al. 1997), clearly indicating that a lack of functionality of Gi-proteins enhances the sensitivity to pain. Hypofunctionality

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of Gi-proteins also produced insensitivity to analgesic treatments. Thus, it has been observed that PTX prevents the enhancement of pain threshold induced by a wide variety of drugs with analgesic properties such as opioids, antihistamines, and α_2-agonists (Parenti et al. 1986; Sanchez-Blazquez and Garzon 1991; Galeotti et al. 1996). Recently, amitriptyline and clomipramine analgesia has been reported to involve signal transduction mechanism operated by PTX-sensitive G-proteins (Galeotti et al. 1996, 1997).

Since little is known about the intracellular effectors involved in the mechanism of action of TCAs as analgesics, we decided to further investigate the role of Gi-proteins in the amitriptyline- and clomipramine-induced enhancement of the pain threshold by using an antisense strategy. In particular, we used antisense oligonucleotides (aODN) against the α subunits of the Gi_1, Gi_2 and Gi_3 proteins in order to determine the role of each subtype in the analgesia induced by amitriptyline and clomipramine.

Materials and methods

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 a.m. Animals were used a single time. All experiments were carried out according to the guidelines of the European Community Council for experimental animal care.

I.c.v. injection of oligonucleotides. Intracerebroventricular (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 determined to be 95.

Hot plate test. O'Callaghan and Holtzman (1975) described the method used. Mice were placed inside a stainless steel container, which was set thermostatically at 52.5±0.1°C in a precision waterbath from KW Mechanical Workshop (Siena, Italy). Reaction times (s) were measured with a stopwatch before and 15, 30, 45 and 60 min after amitriptyline and clomipramine administration. The endpoint used was the licking of the fore or hind paws. Those mice scoring less than 12 s and more than 18 s in the pretest were rejected (30%). To prevent tissue injury, an arbitrary cut-off time of 45 s was adopted. The licking latency values reported in the figures were recorded 30 min after amitriptyline and clomipramine administration in correspondence with their maximum analgesic effect.

Rotarod 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 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s, according to Vaught et al. (1985). Performance time was measured before and 15, 30 and 45 min after subcutaneous administration of the investigated compounds.

Spontaneous activity meter (Animex). Locomotor activity in mice was quantified using an Animex activity meter Type S (LKB, Farad, Sweden) set to maximum sensitivity. Mice were placed on the top of the Animex activity meter and each movement produced a signal due to variation in inductance and capacity of the apparatus resonance circuit. These signals were automatically converted to numbers. On the day of the experiment the mice were s.c. treated with saline solution and then the cage, containing five mice, was put on the measurement platform. Activity counts were made every 15 min for 45 min starting immediately after injection of the drug. Because of the arbitrary scale adopted to quantify movements, aODN-treated mice were always compared with dODN-treated ones.

Antisense oligonucleotides. Phosphodiester oligonucleotides (ODNs) protected from terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Tib Molbiol. The sequences of the 33-mer antisense oligonucleotides 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'. All oligonucleotides have been previously characterised (Raffa et al. 1994; Sanchez-Blazquez et al. 1995; Sanchez-Blazquez and Garzon 1998). A 33-mer fully degenerated ODN (dODN) 5'-N*N*N NNN NNN NNN NNN NNN NNN NNN N*N*N-3' (where N is G, or C, or A, or T) was used as a control. ODNs were vehiculated intracellularly by DOTAP to enhance both uptake and stability. AODN or dODN were preincubated at 37°C for 30 min with 13 µM DOTAP, an artificial cationic lipid, and supplied to mice by i.c.v. injection of 5 µl solution 18–24 h prior to the behavioural tests.

Drugs. The following drugs were used: clomipramine (Anafranil, Ciba Geigy); amitriptyline, DOTAP (Sigma). Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 5 µl per mouse by i.c.v. injection and 10 ml/kg by subcoutaneous (s.c.) injection. All drugs were dissolved in saline solution immediately before use.

Statistical analysis. All experimental results are given as the means ± SEM. An analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) 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

The effects produced by the aODN to $Gi_1\alpha$, $Gi_2\alpha$ and $Gi_3\alpha$ on amitriptyline (15 mg/kg s.c.) and clomipramine (25 mg/kg s.c.) analgesia were evaluated by using the mouse hot plate test.

Pretreatment with aODN to $Gi_1\alpha$ (6.25–25 µg per mouse i.c.v.), $Gi_2\alpha$ (6.25–25 µg per mouse i.c.v.) and $Gi_3\alpha$ (6.25–25 µg per mouse i.c.v.) produced a dose-dependent reduction of amitriptyline (15 mg/kg s.c.)-induced analgesia (Fig. 1). The greatest effect was reached at a dose of 25 µg per mouse i.c.v. whereas the dose of 6.25 µg was ineffective (Fig. 1). Similarly, clomipramine (25 mg/kg s.c.)-induced analgesia was dose-dependently

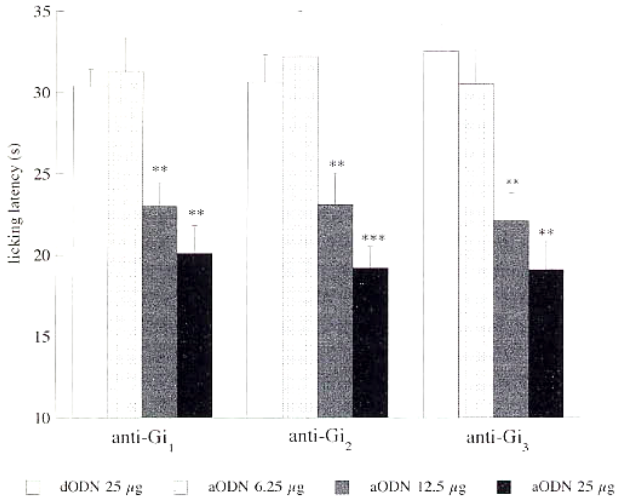

Fig. 1 Prevention by aODN against the α subunit of Gi_1, Gi_2 and Gi_3 proteins of amitriptyline-induced analgesia in the mouse hot plate test. Amitriptyline was administered s.c. at a dose of 15 mg/kg. *Vertical lines* represent SEM; between 19 and 25 mice were tested. $^{**}P<0.01$, $^{***}P<0.001$ in comparison with dODN-treated mice

prevented by pretreatment with aODN to $Gi_1\alpha$ (6.25–25 μg per mouse i.c.v.), $Gi_2\alpha$ (6.25–25 μg per mouse i.c.v.) and $Gi_3\alpha$ (6.25–25 μg per mouse i.c.v.) as illustrated in Fig. 2. The dose of 6.25 μg per mouse i.c.v. was unable to significantly modify the increase of the pain threshold induced by clomipramine administration. The maximum antagonistic effect was obtained at a dose of 25 μg per mouse for all aODN-treated groups (Fig. 2).

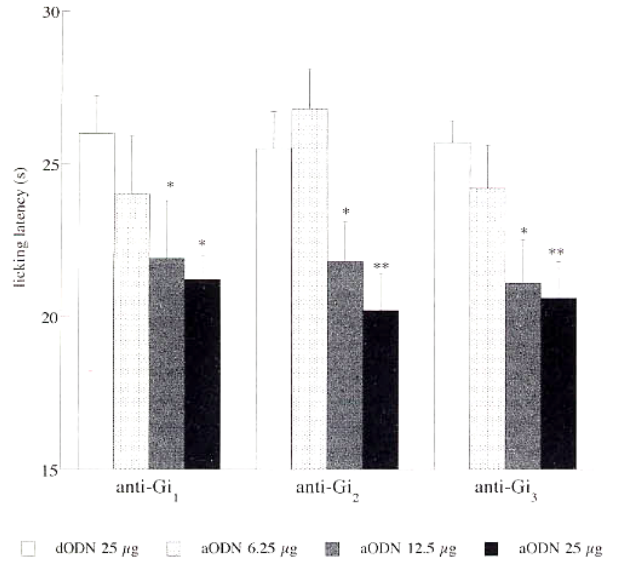

Fig. 2 Prevention by aODN against the α subunit of Gi_1, Gi_2 and Gi_3 proteins of clomipramine-induced analgesia in the mouse hot plate test. Clomipramine was administered s.c. at a dose of 25 mg/kg. *Vertical lines* represent SEM; between 18 and 23 mice were tested. $^{*}P<0.05$, $^{**}P<0.01$ in comparison with dODN-treated mice

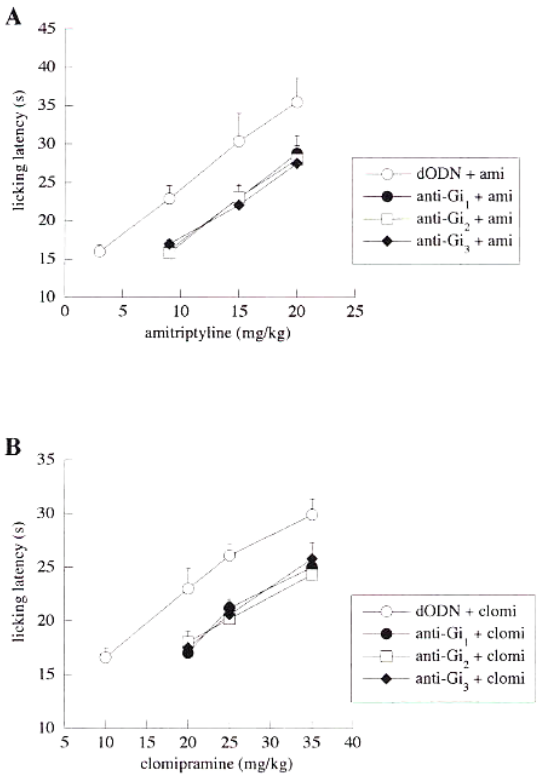

Fig. 3 Effect of i.c.v. pretreatment with aODN against the α subunit of Gi_1, Gi_2 and Gi_3 proteins on the antinociceptive effect produced by increasing concentrations of **A** amitriptyline (3–20 mg/kg s.c.) and **B** clomipramine (10–35 mg/kg s.c.) in the mouse hot plate test. *Vertical lines* give SEM; each point represents the mean for at least nine mice

Pretreatment with the degenerate oligonucleotide (dODN) never modified amitriptyline- and clomipramine-induced antinociception in comparison with untreated mice (naive) or vehicle- and saline-injected animals (data not shown).

Both amitriptyline (3–20 mg/kg s.c.) and clomipramine (10–35 mg/kg s.c.) produced dose-dependent antinociception (Fig. 3). Pretreatment with aODN to $Gi_1\alpha$, $Gi_2\alpha$ and $Gi_3\alpha$ (25 μg per mouse i.c.v.) prevented the antinociception induced by increasing concentrations of amitriptyline (Fig. 3A) and clomipramine (Fig. 3B), displacing to the right the amitriptyline (Fig. 3A) and clomipramine (Fig. 3B) dose-response line.

The prevention of amitriptyline (Fig. 4A) and clomipramine (Fig. 4B) analgesia produced by anti-$Gi_1\alpha$, anti-$Gi_2\alpha$ and anti-$Gi_3\alpha$ at the highest active dose (25 μg per mouse i.c.v.), disappeared 7 days after the end of the aODN pretreatment.

The aODN pretreatment, at the doses able to prevent TCAs-induced analgesia, did not reduce the pain threshold in mice in comparison with saline-, vehicle- and dODN-treated animals, showing no hyperalgesic effect (Table 1).

Both amitriptyline and clomipramine produce dose-dependent antinociception. In order to restrict observations

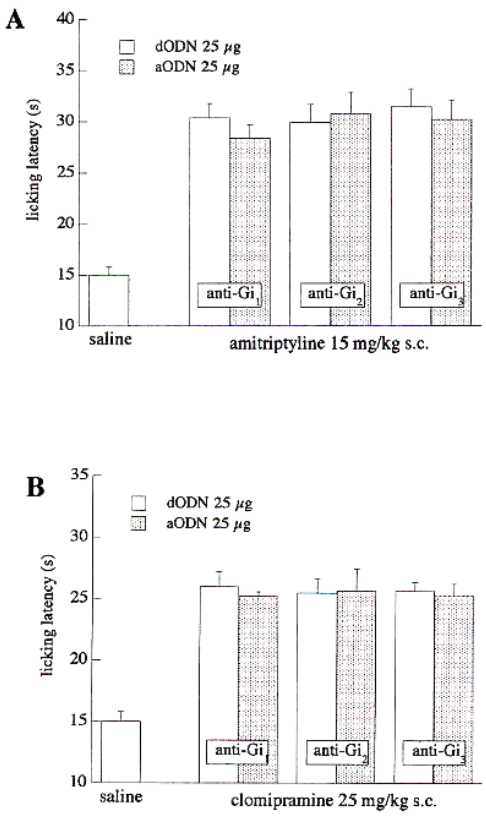

Fig. 4 Lack of effect by pretreatment with an aODN to the α subunit of Gi_1– (25 μg per mouse i.c.v.), Gi_2– (25 μg per mouse i.c.v.) and Gi_3– (25 μg per mouse i.c.v.) protein gene on **A** amitriptyline and **B** clomipramine analgesia 7 days after the i.c.v. injection of dODN (25 μg per mouse i.c.v.) or aODN. *Vertical lines* represent SEM; 15 mice per group were tested

to doses of amitriptyline and clomipramine that exhibit analgesic activity and are devoid of other behavioural effects, we tested amitriptyline and clomipramine on the rotarod test to evaluate their effect on mouse motor coordination. In this test, each group progressively reduced its number of falls every following session because mice learned how to balance on the rotating rod. The doses of amitriptyline and clomipramine of respectively 20 mg/kg and 35 mg/kg s.c. did not modify the number of falls from the rotating rod in comparison with saline-treated mice, indicating that the presence of drug did not affect either motor expression or learning. Higher doses of amitriptyline (30 mg/kg s.c.) and clomipramine (45 mg/kg s.c.) produced an increase in the number of falls, indicating the presence of motor incoordination (Fig. 5A).

The motor coordination of mice pretreated with aODN to $Gi_1\alpha$ (25 μg per mouse i.c.v.), $Gi_2\alpha$ (625 μg per mouse i.c.v.) and $Gi_3\alpha$ (25 μg per mouse i.c.v.) was evaluated by using the rotarod test. The motor coordination of aODN-treated groups was not impaired when compared with that of dODN-treated mice (Fig. 5B).

The spontaneous motility of mice was not modified by pretreatment with aODN to $Gi_1\alpha$ (25 μg per mouse i.c.v.), $Gi_2\alpha$ (625 μg per mouse i.c.v.) and $Gi_3\alpha$ (25 μg per mouse i.c.v.), in comparison with dODN-treated mice, as revealed by the Animex apparatus (Fig. 6).

Discussion

Present results demonstrate the fundamental role played by the Gi-proteins in the mechanism of analgesic action of amitriptyline and clomipramine. In particular, the involvement of Gi_1, Gi_2 and Gi_3 subtypes has been observed. The administration of aODN against the α subunit of the above-mentioned Gi-proteins produced a dose-dependent prevention of the analgesia induced by the two investigated TCAs in the mouse hot plate test.

Pretreatment with the anti-Giα aODNs at the effective doses did not modify the pain threshold, showing the absence of any hyperalgesic effect. Therefore, the prevention of amitriptyline and clomipramine antinociception cannot be attributed to a direct effect on the pain threshold induced by the aODN. Furthermore, the dODN did not modify amitriptyline- and clomipramine-induced antinociception 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

Table 1 Effect of aODN against $Gi_1\alpha$, $Gi_2\alpha$ and $Gi_3\alpha$ in the mouse hot plate test. Mice received saline as s.c. treatment. Data are reported as means ± SEM; between 12 and 16 mice were tested

Treatment (i.c.v.)	Licking latency (s)				
	Before s.c. treatment	After s.c. treatment			
		15 min	30 min	45 min	60 min
–	15.4±0.5	14.0±1.0	14.8±0.7	15.1±0.9	15.0±1.0
Saline	15.1±0.9	14.8±0.9	14.9±0.9	15.4±0.7	15.0±0.7
DOTAP	15.9±0.8	15.2±0.7	15.0±0.9	14.8±0.9	14.4±0.8
dODN 25 μg	14.9±0.7	14.5±0.8	14.4±0.6	15.0±0.8	14.6±0.9
Anti-$Gi_1\alpha$ 12.5 μg	16.1±1.0	15.1±1.2	14.9±0.7	16.1±1.1	16.1±1.2
Anti-$Gi_1\alpha$ 25 μg	15.1±0.7	14.7±1.4	15.2±1.4	15.6±1.3	16.2±1.6
Anti-$Gi_2\alpha$ 12.5 μg	15.4±1.1	15.2±0.9	14.7±1.4	14.6±1.3	15.0±1.6
Anti-$Gi_2\alpha$ 25 μg	14.6±1.1	14.9±1.4	15.5±1.2	14.8±0.9	14.6±1.1
Anti-$Gi_3\alpha$ 12.5 μg	15.5±0.7	16.7±1.2	15.7±1.1	16.7±0.9	16.7±1.3
Anti-$Gi_3\alpha$ 25 μg	14.3±1.3	15.5±1.2	14.6±1.0	15.2±1.5	15.7±1.1

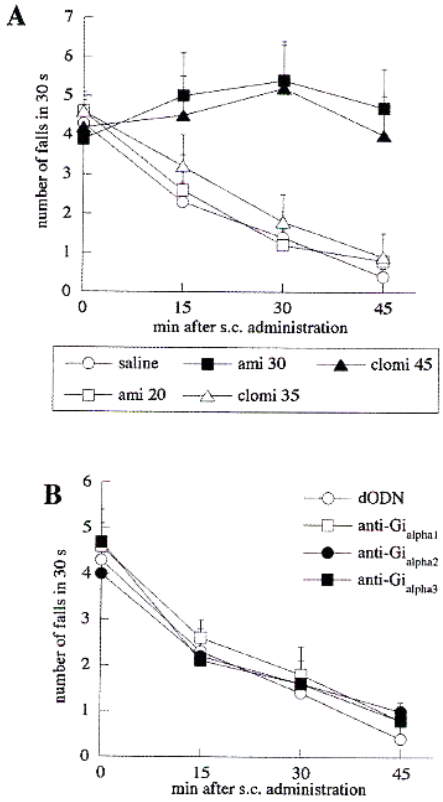

Fig. 5 **A** Effect of amitriptyline and clomipramine on motor coordination in the mouse rotarod test. Amitriptyline (15–30 mg/kg s.c.)-induced and clomipramine (25–45 mg/kg s.c.)-induced were administered immediately before the test. *Vertical lines* represent SEM; 12 mice per group were tested. **B** Effect of pretreatment with an aODN to the α subunit of Gi_1– (25 μg per mouse i.c.v.), Gi_2– (25 μg per mouse i.c.v.) and Gi_3– (25 μg per mouse i.c.v.) protein gene on motor coordination in the mouse rotarod test. The test was performed 18–24 h after the i.c.v. injection of dODN (25 μg per mouse i.c.v.) or aODN. *Vertical lines* represent SEM; 12 mice per group were tested

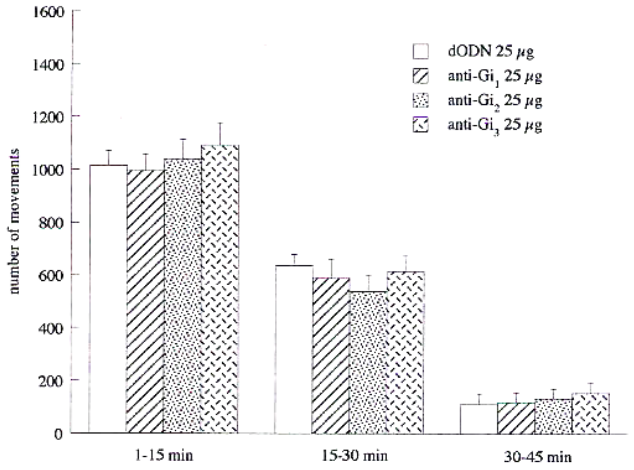

Fig. 6 Lack of effect by pretreatment with an aODN to the α subunit of Gi_1– (25 μg per mouse i.c.v.), Gi_2– (25 μg per mouse i.c.v.) and Gi_3– (25 μg per mouse i.c.v.) protein gene on spontaneous motility in the Animex apparatus. The test was performed 18–24 h after the i.c.v. injection of dODN (25 μg per mouse i.c.v.) or aODN. *Vertical lines* represent SEM; ten mice per group were tested

resulted from a sequence-independent action on cerebral structures.

Amitriptyline and clomipramine are inhibitors of the reuptake of serotonin and noradrenaline, two neurotransmitters able to increase the pain threshold. Selective inhibitors of serotonin reuptake as well as selective inhibitors of noradrenaline reuptake showed analgesic activity (Atkinson et al. 1999). The increase of endogenous serotonin in the synaptic cleft after TCAs administration has been supposed to be responsible for the increase of the pain threshold either directly (Galeotti et al. 1995), or through activation of the opioid system (Botney and Fields 1983; Sacerdote et al. 1987). Furthermore, the involvement of the noradrenergic system has also been observed in TCAs analgesia. It has been recently reported that the increase of the pain threshold induced by amitriptyline and imipramine is mediated by the activation of the α_{2A} adrenoceptors (Ghelardini et al. 2000). We can, therefore, hypothesise that amitriptyline and clomipramine induce analgesia through activation of multiple neurotransmission systems, such as serotoninergic, opioid and noradrenergic ones.

Some serotonin receptor subtypes (Lucas and Hen 1995), opioid receptors (Law et al. 2000) and α_2 adrenoceptors (Birnbaumer 1990) are Gi-protein-coupled receptors within the central nervous system. Furthermore, Gi-proteins are involved into the mechanism of analgesic action of opioids (Parenti et al. 1986; Chung et al. 1994) and α_2 agonists (Sanchez-Blazquez and Garzon 1991) since pretreatment with PTX, a bacterial toxin produced by *Bordetella pertussis* that ADP-ribosylates and inactivates the α subunit of Gi-proteins, antagonises their analgesic effect. The analgesia induced by activation of these neurotransmission systems is, however, mediated by different Gi subtypes. It has been widely demonstrated that the analgesia induced by μ-opioid agonists is mediated by the activation of the Gi_2 subtype. The administration of selective antibodies against $Gi_2\alpha$ subunit (Sanchez-Blazquez and Garzon 1993; Garzon et al. 1994), as well as the inhibition of its expression by the use of specific antisense oligonucleotides (Raffa et al. 1994; Sanchez-Blazquez et al. 1995; Standifer et al. 1996), prevented the spinal and supraspinal analgesia induced by agonists of μ-opioid receptors. Furthermore, the administration of Myr^+-$Gi\alpha_2$ restores the analgesic efficacy of opioids in G-protein knock-down mice (Garzon et al. 1999). By contrast, the analgesia induced by *k*-opioid agonists is mediated by Gi_1 and Gi_3 subtypes (Standifer et al. 1996), whereas the increase of the pain threshold induced by δ-opioids involves the activation of the Gi_3 subtypes (Sanchez-Blazquez and Garzon 1993). The α_2-adrenoceptor antinociception also involves a selective activation of the Gi_3 subtypes. The use of aODN against $Gi_3\alpha$ demonstrated the essential role played the Gi_3 subtype in the increase of the pain threshold induced by α_2-adrenoceptor agonists (Raffa et al. 1996).

The main clinical use of tricyclic antidepressants is in neuropathic pain and, in particular, their utility is on hyperalgesia and allodynia, the most relevant pain modalities associated with neuropathic pain (Watson 2000). Present data indicate the involvement of Gi-proteins in the mechanism of analgesic action of amitriptyline and clomipramine in an acute model of pain. We cannot, therefore, exclude that the effects of tricyclic antidepressants on hyperalgesia and allodynia may involve other mechanisms not directly related with Gi-proteins.

Amitriptyline and clomipramine exerted their antinociceptive activity without showing any alteration of the mice motor coordination as revealed by the rotarod test. However, the administration of doses of amitriptyline and clomipramine higher than those used in the present study produced impairment of motor coordination whose appearance could lead to a modification of the licking latency values detected in the hot plate test. In order to avoid misinterpretation of data, it has been necessary to limit the investigation to doses of amitriptyline and clomipramine at which these compounds showed antinociceptive properties without any behavioural side effect.

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-Giα. The aODNs, at the highest doses used, did not modify animals' gross behaviour. Moreover, these compounds did not impair motor coordination as revealed by the rotarod test nor modify spontaneous motility as indicated by the Animex apparatus. We can, thus, suppose that the effects observed in the hot plate test were not due to compromised behavioural function. Moreover, the inhibition of TCAs analgesia by anti-Giα disappeared 7 days after administration, indicating an absence of irreversible damage or toxicity on cerebral structures caused by the aODNs.

In conclusion, present results evidence the important role of Gi_1, Gi_2 and Gi_3 proteins in the mechanism of analgesic action of amitriptyline and clomipramine. Besides antidepressants, many analgesic drugs employed clinically acts through the activation of the Gi-proteins regardless the receptor involved in their mechanism of action. The capability to selectively activate the Gi-protein system may represent a novel pharmacological approach for pain relief.

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