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Effects of antisense oligonucleotides on brain delta-opioid receptor density and on SNC80-induced locomotor stimulation and colonic transit inhibition in rats

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1 To reduce the density of δ-opioid receptor protein, five antisense phosphorothioate oligodeoxynucleotides (aODN), targeting the three exons of rat δ-opioid receptor mRNA (DOR), were injected twice daily for 4 days or continuously infused for 7 days into brain lateral ventricles (i.c.v.) of Sprague-Dawley rats. Rats acting as controls were infused or injected with a mismatch sequence (mODN) of each aODN. The density of opioid receptors in rat brain membranes was measured by saturation binding experiments using selective ligands for δ, μ and κ opioid receptors.

2 aODNs injected twice a day for 4 days left rat brain δ-opioid receptor density unchanged. The ODN targeting the DOR nucleotide sequence 280–299 ($aODN_{280-299}$, exon 2), decreased brain δ-opioid receptor density significantly more than aODNs targeting exon 1 ($aODN_{239-258}$), exon 2 ($aODN_{361-380}$), or exon 3 ($aODN_{741-760}$) (to 52% vs 79, 72, and 68%). None of the aODNs to the DOR changed the brain density of μ- or k-opioid receptors.

3 When in a novel environment (but not when kept in their home cages), the locomotor activity of $aODN_{280-299}$ treated rats was significantly lower than that of saline or mODN treated rats. The δ-opioid agonist SNC80 (5 mg kg^{-1}, s.c.) significantly and potently stimulated locomotion and delayed colonic propulsion in saline- and mODN-infused rats, but left motor behaviour and colonic transit of δ-knockdown rats unchanged.

4 The baseline nociceptive threshold and the antinociceptive response to morphine were unchanged in δ-knockdown rats.

Keywords: Antisense knockdown; δ-opioid receptors; rat brain; locomotion; colonic propulsion; nociception; morphine analgesia

Abbreviations: AD_{50}, dose of morphine inducing a half-maximal antinociception; ANOVA, analysis of variance; aODN, antisense oligodeoxynucleotide; DOR, δ-opioid receptor; MANOVA, multivariate analysis of variance; mODN, mismatch oligodeoxynucleotide

Introduction

Pharmacological experiments in rats and mice using selective δ-opioid receptor agonists and antagonists implicates δ-opioid receptors in locomotor and stereotyped behaviours (Michael-Titus *et al*., 1989; Longoni *et al*., 1991; Negri *et al*., 1991), in opioid-induced antinociception (Jiang *et al*., 1990; Adams *et al*., 1993; Negri *et al*., 1995) and in central and peripheral control of intestinal motility (Porreca *et al*., 1986; Kaufman *et al*., 1988; Broccardo & Improta, 1992; Broccardo *et al*., 1998). Receptor-mediated functions can now be selectively impaired with the use of antisense oligodeoxynucleotides (aODN) targeting mRNAs for receptor proteins. This strategy has been used in '*in vivo*' studies for selectively blocking the antinociceptive effects of μ-opioid agonists in rats (Rossi *et al*., 1994; Chen *et al*., 1995), the spinal and supraspinal antinociception induced by δ-opioid receptor agonists in mice (Standifer *et al*., 1994; Tseng *et al*., 1994; Lai *et al*., 1994; Bilsky *at al*., 1996) and the antinociceptive activity of k-agonists in mice (Chien *et al*., 1994). No studies have yet described the behavioural and pharmacological responses after antisense knockdown of brain δ-opioid receptors in rats. In this study, to reduce δ-receptor protein levels in the rat brain we used five antisense oligodeoxynucleotides (aODN), targeting different nucleotide sequences of mRNA of the cloned rat δ-opioid receptor (Abood *et al*., 1994). Our primary aim was to investigate further the role of δ-opioid receptors in modulating locomotion, nociception and colonic motility.

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Methods

Animals

Male Sprague-Dawley rats (Charles River, Como, Italy), weighing 220–240 g, at the start of the experiments, were housed in individual $30 \times 30 \times 30$ cm plastic cages in an air-conditioned unit maintained at $22 \pm 2^{\circ}C$ and 50–60% humidity with a 12 h dark/light cycle. During the second week after arrival, animals underwent surgery for i.c.v. cannula implantation. All animal studies were conducted in accordance with the Italian Law for Care and Use of Laboratory Animals and the research project was approved by the Ministry of the University and Scientific and Technological Research (MURST, Italy).

Antisense oligodeoxynucleotides

All phosphorothioate oligodeoxynucleotides (ODNs) were synthesized by Geneco (M-Medical s.r.l. Florence, Italy). Throughout the text, the ODN sequences are indicated with subscripts referring to targeted nucleotide sequences in the cloned rat δ-opioid receptor (DOR: Genbank, U00475; Abood

et al., 1994). Targeted DOR-RNA regions and aODN sequences were: untranslated region, $aODN_{24-23}$, 5′-CTC-CGCGTCCGTCTCCACCG-3′; exon-1, $aODN_{239-258}$, 5′-G-ACGAGCACGTTGCCCAGCA-3′; exon-2, $aODN_{280-299}$, 5′-GTGGCCGTCTTCAGCTTAGT-3′, and $aODN_{361-380}$, 5′-ATCAGGTACTTGGCGCTCTG-3′; exon-3, $aODN_{741-760}$, 5′-GCAGCATGAGGCCATAGCAC-3′.

For each aODN a mismatch sequence (mODN) was also synthesized by exchanging the positions of nucleotides 4 and 5, 11 and 12, and 15 and 17. These changes kept the base composition of the mODN the same as aODN while abolishing the sequence specificity for the DOR. Database searches ensured the specificity of hybridization of the aODN and the lack of specificity for any known gene for mODN.

Oligonucleotides were dissolved in sterile 0.9% NaCl solution immediately before use at a concentration of 0.3 nmol μl^{-1} for i.c.v. injection and 1 nmol μl^{-1} for i.c.v. infusion.

Intracerebroventricoular (i.c.v.) injection Under ketamine-xylazine anaesthesia (60 mg kg^{-1} −10 mg kg^{-1}, i.p.), each rat was implanted surgically with a plastic guide cannula (Linca, Tel-Aviv, Israel), stereotaxically inserted through a skull hole drilled over the left lateral ventricle of the brain (AP = −1 mm, L = +1.8 mm relative to the bregma, according to data from Paxinos & Watson, 1982). The cannula was screwed into the skull hole until it reached a depth of 1 mm below the external surface of the skull and was secured to bone with dental cement. After surgery, the rats were allowed to recover for 3 days in individual plastic cages. Food and water were available *ad libitum* and the animals were maintained on a natural day/night, light/dark cycle. Two days before i.c.v. treatments, the proper position of the cannula was checked by measuring the drinking response of the rats to i.c.v. administration of 50 ng of angiotensin II. Saline, aODN and mODN solutions were injected into the left lateral ventricle, in a constant volume of 5 μl, using a Hamilton 10-microliter syringe fitted with a 26-gauge needle that was inserted through the guide cannula to a depth of 4.2 mm below the external surface of the skull. The needle was left *in situ* for 30 s to allow the drug to diffuse. Saline, aODN and mODN solutions were i.c.v. injected twice daily for 4 days in awake rats.

I.c.v. infusion An L-shaped cannula (Alzet brain infusion kit, Charles River, Calco, Italy) was stereotaxically inserted through a skull hole drilled over the left lateral ventricle of the brain (AP = −1 mm, L = +1.8 mm relative to the bregma, according to data from Paxinos & Watson, 1982) to a depth of 4.2 mm below the external surface of the skull. The infusion cannula was connected with plastic tubing to an osmotic minipump (Alzet 1007D Charles River, Calco, Italy) that was placed under the skin in the rat lumbar region. Before implantation, the entire infusion apparatus (cannula, tubing and minipump) was prefilled with the ODN solution or saline and immersed for 24 h in sterile saline at 37°C to start the osmotic pumping. Saline, aODN or mODN solutions were infused i.c.v. to rats for 7 days at a rate of 0.5 $\mu l\ h^{-1}$.

Preliminary *in vitro* tests demonstrated that osmotic pumping remained constant for at least 7 days and then declined to zero within 24 h.

Brain receptor binding

For saturation binding experiments, brains of two rats, without cerebellum, were united and homogenized in 50 volumes of Tris-HCl buffer (50 mM, pH 7.4, 4°C) using a PT 3000 Polytron (20 s, speed 16 000 r.p.m.) (Kinematica AG, Littau, Switzerland). The homogenate was centrifuged at 41 000 × *g* for 20 min at 4°C, pellets were re-suspended in 50 volumes of buffer and incubated at 25°C for 30 min to remove endogenous opioids. After centrifugation, pellets were re-suspended in buffer, containing 5% glycerol, to give a final $w\,v^{-1}$ of 20 mg ml^{-1} fresh tissue. Saturation binding experiments were run on each pellet sample (two rats) using 15 concentrations of each of the following ligands: δ-opioid agonist, [^{3}H][D-Ala^2, Glu^4]deltorphin; δ-opioid antagonist, [^{3}H] naltrindole; μ-opioid ligand, [^{3}H]DAMGO; and k-opioid ligand, [^{3}H]U69593. Each assay was carried out with 0.5 ml membrane preparation in a final volume of 2 ml Tris-HCl buffer (50 mM, pH 7.4), at 35°C for 120 min and non-specific binding was determined using 10 μM naloxone. Reagents and membranes were distributed by a robotic sample processor (RSP 5000-series, Tecan AG, Hombrechtikon, Switzerland). Using a Brandel M-24 harvester (Semat Technical Ltd., St. Albans, U.K.), assays were terminated by filtration through Whatman GF/B filter strips previously soaked in 0.5% polyethylenimine for 1 h. Filters were washed three times with 4 ml of ice-cold buffer and radioactivity was counted in a liquid scintillation spectrometer (Betamatic, Kontron, Milano, Italy).

All samples were assayed in duplicates. Saturation curves were fitted and binding parameters (K_d and B_{max}) were determined using a computer program (PRISM, GraphPad, San Diego, CA, U.S.A.).

Protein concentrations were determined by BCA Protein Assay reagent kit (Pierce, Rockford, IL, U.S.A.), using bovine serum albumin as the standard.

Locomotor activity

Spontaneous locomotor activity of each animal was studied in two environments, the home cage (30 × 30 × 30 cm) as the familiar environment and a square open-field compartment (100 × 100 × 60 cm) as the novel environment. SNC80-stimulated locomotor activity was studied in the open field. The movements of rats were recorded by a videocamera and analysed by a computer connected to an image analyser to calculate motor activity as the distance travelled (Noldus Information Technology, Wageningen, The Netherlands), and rearing as scores. The spontaneous locomotor activity of each rat in the familiar environment (its home cage) was recorded daily from 19:00 h to 20:00 h from the beginning of ODN treatments to the first day after treatments ended. The spontaneous locomotor response of each rat to the open-field environment was tested only once, two days after i.c.v. treatments ended. The spontaneous motor activity in the open field was recorded for 1 h. After recording, the rat was removed, subcutaneously (s.c.) injected with SNC80 (5 mg kg^{-1}) or saline, immediately returned to the open field cage, and the motor activity recorded again, for 1 h. Open-field sessions were conducted between 08:30 h and 13:00 h. During each test, data were collected every 2 min and the distance travelled was calculated for each 2-min period by the computer. The rearing score was calculated by an observer, by counting the number of events in 2-min observation periods. For each observation period data are expressed as mean ± s.e.mean of events. In SNC80 experiments the distance travelled was expressed as m h^{-1} and rearing as events h^{-1}.

Colonic bead expulsion time

The expulsion time of a glass bead inserted into the distal colon was measured (Raffa & Jacoby, 1990) in a different group of rats. Immediately after assessment of spontaneous locomotor

activity in the open-field environment, rats were s.c. injected with saline or SNC80 (5 mg kg^{-1}), and a single 5-mm glass bead was inserted 3 cm into the distal colon of each rat. The time required for expulsion of the glass bead was taken as a measure of colonic propulsion and a significant increase in this time over control values was considered to indicate inhibition of colonic propulsion.

Test of antinociception

Nociceptive responses were evoked in rats by exposing the tail to radiant heat (D'Amour & Smith, 1941). Rats were tested after completing the spontaneous locomotor test in the open-field environment. To assess its baseline tail-flick time, before receiving morphine, each rat was exposed to the nociceptive stimulus three times at 30-min intervals. After morphine injection, animals not flicking their tails within 15 s (cut-off time) were removed from the nociceptive stimulus and assigned a maximal antinociceptive response. The antinociceptive response was tested every 15 min for 2 h and was calculated according to the following formula: per cent antinociception = 100 × (test latency − baseline latency)/(cut-off time − baseline latency). Five doses of morphine HCl (0.8, 1.5, 3.0, 6.0 and 10 mg kg^{-1}) were used, each dose being injected s.c. to a different group of four rats. Morphine AD_{50} was expressed as mg kg^{-1}.

Experimental design

I.c.v. injected rats Rats were injected twice daily for 4 days with 5 μl saline or aODNs (1.5 nmol/5 μl) or mODNs (1.5 nmol/5 μl). Spontaneous locomotor activity in home cages was recorded daily from day 1 – 5 in all animals. On day 6 the rats were put in the open field and spontaneous locomotor activity was recorded for 1 h. Immediately afterwards the animals were killed by inhaling CO_2 and the brain was removed for use in the receptor binding assay.

I.c.v. infused rats Each aODN or mODN or saline was infused i.c.v. to rats for 7 days at a constant rate of 0.5 nmol h^{-1} with Alzet 1007D osmotic minipumps (ALZA, Charles River, Calco, Italy), at a nominal pumping rate of 0.5 ± 0.1 μl h^{-1}, and reservoir volume of 90 ± 10 μl. Spontaneous locomotor activity in home cages was recorded daily from day 1 – 8. On day 9 the animals were put in the open field and spontaneous locomotor activity was recorded for 1 h. Immediately afterwards the animals were killed by inhaling CO_2 and the brain was removed for use in the receptor binding assay.

Another group of rats were infused for 7 days with $aODN_{280-299}$, or the corresponding mismatch mODN, or saline. On day 9, after a 1-h recording session of spontaneous locomotor activity in the open field, groups of five rats for each of the three treatment groups underwent the following experimental procedure: (a) s.c. injection of SNC80 (5 mg kg^{-1}), or saline (2 ml kg^{-1}) and assessment of the locomotor activity in the open field; (b) s.c. injection of SNC80 (5 mg kg^{-1}) or saline (2 ml kg^{-1}) and assessment of colonic propulsion; (c) s.c. injection of morphine (0.8, 1.5, 3, 6, 10 mg kg^{-1}) or saline (2 ml kg^{-1}) and assessment of analgesia with the tail-flick test.

Statistical analyses

Multivariate analysis of variance (MANOVA) was used to analyse the behavioural data to determine the treatment group effects, within-session effects and the interaction between these variables. Subsequently, if more selective comparisons were needed, a one-way analysis of variance (ANOVA) was used. *Post-hoc* Duncan's multiple range tests were for specific comparisons between groups. A one-way ANOVA and Tukey's multiple comparison test were used to compare K_d and B_{max} of brain opioid receptors in rats with different aODN and mODN treatments.

Drugs and chemicals

[^{3}H][D-Ala^2, Glu^4]deltorphin, [^{3}H]U69593, [^{3}H]DAMGO and [^{3}H]naltrindole were purchased from NEN Products (Du Pont de Nemours Italiana, Milan, Italy) and SNC80 ((+)-4-[(aR)-a-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was obtained from Tocris Cookson (Bristol, U.K.). Morphine HCl was purchased from SALARS (Como, Italy). SNC80 was dissolved in water by adding a few drops of 0.1 N HCl followed by drops of NaOH 0.1 N sufficient to raise the pH to 6.0.

Results

Changes in rat brain δ-opioid receptor density after aODN treatments

I.c.v. injection of antisense oligonucleotides twice daily for 4 days left the δ-opioid receptor density of rat brain unchanged (Table 2).

Conversely, 7-day infusion of aODN sequences directed against coding regions significantly reduced the [D-Ala^2, Asp^4]deltorphin-and naltrindole-binding sites with respect to the saline-infused and mODN-infused rats. Saturation binding curves showed that the most potent in reducing the [D-Ala^2, Asp^4]deltorphin B_{max} was $ODN_{280-299}$ (52% versus saline, $P<0.001$), followed by $aODN_{741-760}$, (68%, $P<0.001$), $aODN_{361-380}$ (72%, $P<0.01$) and $aODN_{239-258}$ (79%, $P<0.05$). The decrease in δ-opioid binding sites induced by the infusion of $aODN_{280-299}$ was significantly higher than that induced by $aODN_{741-760}$, and, of course, by all the others (Table 1).

$aODN_{24-43}$ did not significantly decrease the the [D-Ala^2, Asp^4]deltorphin B_{max} (88%, $P>0.05$). Saturation binding experiments using the selective δ-antagonist naltrindole, as a ligand, yielded similar results. I.c.v. infusion of mODN left brain δ-opioid receptor density unchanged. No aODN treatment affected the K_D values of the δ-opioid receptor ligands. None of the treatments changed the B_{max} and K_D of the μ-selective ligand DAMGO or the k-selective ligand U-69,593 (Table 1).

Rat baseline locomotor activity and behavioural responses to SNC80 after ODN treatments

I.c.v. injections of all the tested aODNs, daily, for 4 days, left baseline locomotor activity of rats in the familiar and in the open field environment unchanged (data not shown).

Seven-day infusion of all the tested aODNs left baseline locomotor activity of rats in the familiar environment unaffected. However rats that received 7-day infusion of $aODN_{280-299}$ or $aODN_{741-760}$ in their brain ventricles showed significant inhibition of locomotor activity in the open-field environment (Figure 1). The multiple comparison of distance travelled across subsequent sampling periods showed that over the initial 0- to 2-min, $aODN_{280-299}$- and $aODN_{741-760}$-infused

Table 1 Rat brain δ-opoid receptor density and affinity after 7-day infusion of antisense (aODN) or mismatch (mODN) oligodeoxynucleotides targeting δ-opoid receptor

Treatment	*δ-opoid agonist* *[^{3}H][D-Ala^2, Glu^4]deltorphin*		*δ-opoid agonist* *[^{3}H]naltrindole*		*μ-opoid agonist* *[^{3}H]DAMGO*		*κ-opoid agonist* *[^{3}H]U69593*	
Dose (0.5 nmol h^{-1})	B_{max} (fmol mg^{-1} protein)	K_D (nM)	B_{max} (fmol mg^{-1} protein)	K_D (nM)	B_{max} (fmol mg^{-1} protein)	K_D (nM)	B_{max} (fmol mg^{-1} protein)	K_D (nM)
Saline	118.7±14.5	2.71±0.27	88.9±6.8	0.18±0.02	161.7±11.9	0.92±0.09	28.7±3.1	3.33±0.18
aODN $_{24-43}$	104.8±13.8	2.32±0.29	84.7±6.1	0.11±0.03	154.3±10.4	1.26±0.11	32.3±2.9	2.91±0.20
mODN $_{24-43}$	111.4±14.7	1.95±0.28	92.5±7.3	0.09±0.02	168.3±9.7	1.31±0.14	26.9±3.2	2.83±0.21
aODN $_{239-258}$	95.7±9.9*	1.87±0.21	78.3±5.7*	0.10±0.03	149.7±11.2	1.94±0.23	33.7±3.3	3.31±0.24
mODN $_{239-258}$	122.4±16.3	2.14±0.22	89.7±6.1	0.13±0.03	153.3±9.4	1.12±0.14	27.8±2.4	3.22±0.31
aODN $_{280-299}$	56.6±7.7**°	1.76±0.21	54.3±4.8**°	0.10±0.01	171.1±12.3	1.84±0.21	31.5±1.9	2.98±0.27
mODN $_{280-299}$	108.3±14.5	1.91±0.19	91.8±7.9	0.19±0.03	164.8±12.7	1.22±0.12	28.7±2.5	2.74±0.23
aODN $_{361-380}$	80.9±9.7**	1.79±0.11	69.6±5.3**	0.15±0.02	161.5±13.3	1.45±0.20	34.5±3.2	3.31±0.30
mODN $_{361-380}$	114.5±16.7	1.81±0.21	94.7±8.1	0.18±0.03	175.3±14.5	1.31±0.13	30.9±3.1	3.12±0.28
aODN $_{741-760}$	74.2±8.3**	1.75±0.12	64.8±8.3**	0.14±0.02	159.7±15,9	1.27±0.10	27.8±2.1	3.35±0.31
mODN $_{741-760}$	109.7±14.8	1.94±0.19	87.7±6.8	0.17±0.03	162.7±12,2	1.22±0.14	28.7±2.2	3.07±0.34

Mean±s.e.mean of five experiments; *$P<0.05$ **$P<0.001$, vs related mODN and saline (Tukey's multiple comparison test).

Table 2 Rat brain δ-opoid receptor density and affinity after 4-day treatment with twice daily i.c.v. injection of antisense (aODN) or mismatch (mODN) oligodeoxynucleotides

Treatment	*[^{3}H][D-Ala^2, Glu^4]deltorphin*		*[^{3}H]naltrindole*	
Dose (1.5 nmol, 5μl)	B_{max} (fmol mg^{-1}, protein)	K_D (nM)	B_{max} (fmol mg^{-1}, protein)	K_D (nM)
Saline	118.7±11.5	2.7±0.51	98.9±6.8	0.18±0.06
aODN $_{24-43}$	107.7±10.3	2.8±0.62	92.4±4.8	0.21±0.09
mODN $_{24-43}$	120.1±12.5	3.0±0.52	101.1±11.5	0.41±0.05
aODN $_{239-258}$	108.9±13.2	2.7±0.54	98.7±4.1	0.24±0.07
mODN $_{239-258}$	130.1±16.2	3.9±0.58	102.9±13.1	0.35±0.05
aODN $_{280-299}$	109.3±10.9	2.3±0.31	97.2±3.7	0.14±0.06
mODN $_{280-299}$	116.7±12.4	3.1±0.5	104.2±10.0	0.29±0.04
aODN $_{361-380}$	112.7±13.9	2.9±0.72	91.6±6.7	0.21±0.08
mODN $_{361-380}$	97.0±12.5	3.8±0.80	86.4±11.1	0.18±0.08
aODN $_{741-760}$	103.6±15.5	3.1±0.62	98.7±6.4	0.19±0.06
mODN $_{741-760}$	99.6±10.5	2.1±0.62	90.7±5.6	0.20±0.07

Each value represents mean±s.e.mean of five experiments.

rats moved significantly less than saline- or mODN-infused rats (2.4±0.5 m min^{-1} and 4.1±0.6 m min^{-1} vs 5.8±0.8 m min^{-1}, vs 5.3±0.7 m min^{-1}; $P<0.01$ and; $P<0.05$). Over the course of the open-field session, locomotor activity declined more slowly in δ-knockdown rats than in controls, so that, during the third and fourth sampling periods, antisense-infused and mODN-infused rats showed similar locomotor activity (Figure 1).

When injected s.c. at the dose of 5 mg kg^{-1}, the selective δ-opioid agonist SNC80 (Bilsky *et al*., 1995) stimulated horizontal locomotor activity and rearing in rats infused i.c.v. with saline (48.7±5.6 m h^{-1}, $P<0.001$ and 14±3 events h^{-1}, $P<0.001$) or with mODN$_{280-299}$ (42.8±5.3 m h^{-1}, $P<0.001$; 18±4 events h^{-1}, $P<0.001$), but left locomotion and rearing in aODN$_{280-299}$-infused rats practically unchanged (6.6±1.3 mh^{-1}, and 5±2 events h^{-1}) (Figure 2).

Colonic propulsion after ODN treatment

I.c.v. infusion of aODN$_{280-299}$ left the colonic bead expulsion time unchanged (2.1±0.8 min in saline-infused rats; 5.1±1.7 min in aODN infused rats). Subcutaneous injection of SNC80 in saline- or mODN$_{280-299}$-infused rats significantly delayed colonic expulsion of glass bead (51.2±9.1 min and 49.7±6.8 min; $P<0.001$).

Conversely, in aODN$_{280-299}$-infused rats SNC80 failed to inhibit colonic bead expulsion (9.8±1.1 min, $P>0.001$ vs s.c. saline, $P<0.05$ vs mODN$_{280-299}$ group) (Figure 3).

Morphine-induced antinociception in aODN-treated rats

aODN$_{280-299}$-, saline- and mODN-infused rats all had similar baseline tail-flick times (3.2±0.6 s vs 2.9±0.4 s vs 3.3±0.7 s). Dose-response curves of morphine-induced antinociception recorded in saline-infused rats, mODN$_{280-299}$-infused rats and in aODN$_{280-299}$-infused rats yielded similar AD50 (2.18[1.75–2.71] mg kg^{-1}; 2.51[1.98–3.19] mg kg^{-1}; and 3.29[1.15–9.41] mg kg^{-1}). In δ-knockdown rats morphine lost less than 30% of its analgesic potency (potency ratio: aODN/mODN, 0.78; aODN/saline, 0.68) (Figure 4).

Discussion

In this study we found that a phosphorothioate aODN targeting the nucleotide sequence 280–299 in the exon 2 of the cloned rat δ-opioid receptor (rDOR) infused for 7 days into the rat brain lateral ventricles potently inhibited the binding to brain membranes of δ-opioid receptor ligands. Other aODN sequences tested were less active or inactive in reducing the B_{max} of [D-Ala^2, Asp^4]deltorphin or naltrindole. This difference suggests either that the nucleotide sequences 24–43, 239–258, 361–380 and 741–760 of rDOR are poor targets for the antisense strategy or that the related antisense sequences hybridize poorly with the mRNA.

In our experiments, i.c.v. injection of the tested phosphorothioate ODNs (2.5 nmol) twice daily for 4 days in rats failed

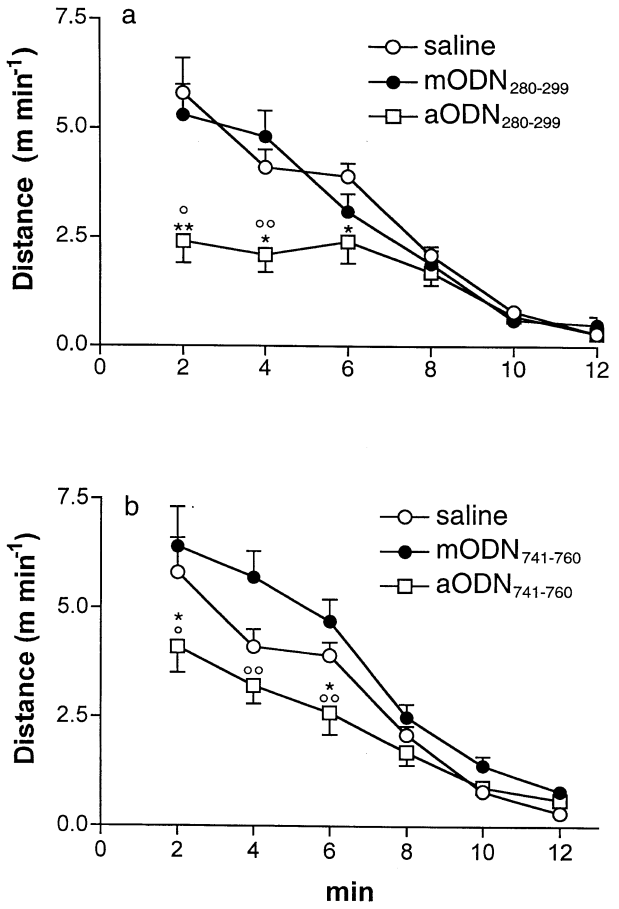

Figure 1 Spontaneous locomotor response to novel environment of aODNs-, mODNs- and saline-infused rats. The distance traveled was calculated for each 2 min period. Rats that received 7-day i.c.v. infusion of $aODN_{280-299}$ or $aODN_{741-760}$ showed significant inhibition of locomotor activity during the first three observation periods. Data are expressed as mean ± s.e.mean ($n=10$). Duncan's multiple range test: $^{*}P<0.05$, $^{**}P<0.01$ vs saline; $^{\circ}P<0.05$, $^{\circ\circ}P<0.01$ vs mODN.

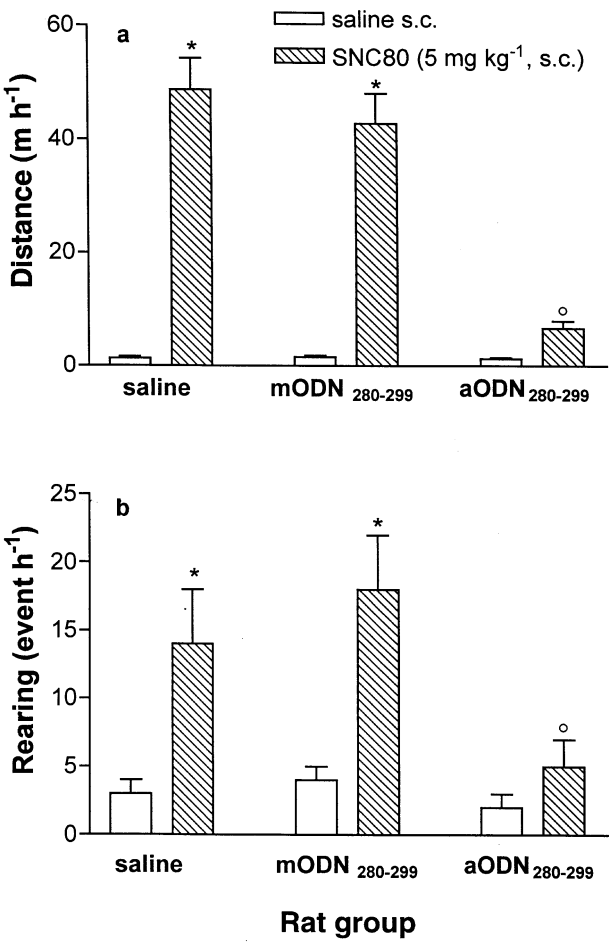

Figure 2 Motor responses to the δ-opioid receptor agonist SNC80 (5 mg kg^{-1}) or saline (2 ml kg^{-1}), of rats infused per 7 days with $aODN_{280-299}$ or $mODN_{280-299}$ or saline. Distance travelled and rearing events were calculated for 1 h period. Data are expressed as mean ± s.e.mean ($n=5$). Tukey's multiple comparison test: $^{*}P<0.001$ vs s.c. saline; $^{\circ}P<0.001$ vs SNC80 injected mODN- or saline-group.

to reduce cerebral δ-opioid receptor density. This finding contrasts with a report that daily i.c.v. or intrathecal injections of phosphodiester aODN (0.8–1.7 nmol) for only 3-days in mice, significantly inhibited δ-opioid receptor synthesis, both in the brain and spinal cord (Rossi *et al.*, 1997). Supposing that phosphorothioate ODNs had lower hybridization efficacy than phosphodiester aODNs, we tried i.c.v. doses higher than 3 nmol, but these doses of phosphorothioate derivatives produced seizures and postural defect in rats (data not shown). Sanchez-Blazquez *et al.* (1997) succeeded in reducing the δ-opioid receptor-related protein in the rat striatum with a 21-day infusion of a phosphorothioate aODN targeting the nucleotide sequence 70–85 of DOR into the rat lateral brain ventricle. In a previous work we showed that 24-day continuous i.c.v. infusion of $aODN_{280-299}$ and $aODN_{741-760}$ down regulated the DOR protein levels on the Western blot of solubilized striatum proteins (Negri *et al.*, 1999). Currently available data therefore indicate that significant knock down of brain δ-opioid receptors requires longer ODN administration in rats than in mice. One reason could be the larger tissue mass and greater target distance in rat brain.

Preliminary data obtained in the present experiments on rat locomotor activity as well as in previous studies on antinociception in mice (Wang *et al.*, 1996), showed that the response to δ-opioid agonists remained attenuated for 2 days and gradually recovered in 10 days after cessation of the

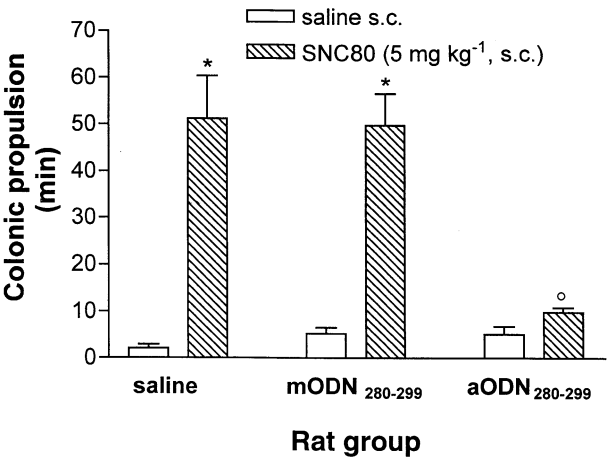

Figure 3 Colonic propulsion after s.c. injection of the δ-opioid receptor agonist SNC80 (5 mg kg^{-1}) or saline, in rats infused per 7 days with $aODN_{280-299}$ or $mODN_{280-299}$ or saline. Colonic propulsion was calculated as the time required to expel a glass bead inserted 2–3 cm into the rat distal colon. Data are expressed as mean ± s.e.mean ($n=5$). Tukey's multiple comparison test: $^{*}P<0.001$ vs s.c. saline; $^{\circ}P<0.001$ vs SNC80 injected mODN- or saline-group.

aODN treatment, while ODNs infused into the brain lateral ventricles were almost completely cleared within 24 h after the

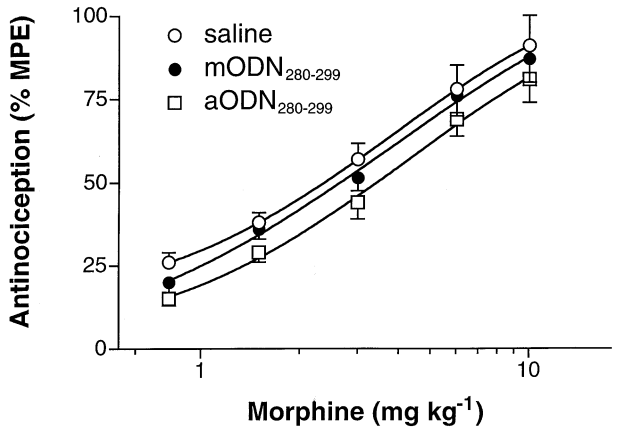

Figure 4 Dose-response relationship of the antinociception produced by s.c. morphine (0.8, 1.5, 3.0, 6.0 and 10 mg kg^{-1}) in rats infused per 7 days with $aODN_{280-299}$ or $mODN_{280-299}$ or saline. Data are expressed as mean ± s.e.mean ($n=5$).

end of infusion (Grzanna *et al*., 1998). To avoid possible interference from residual cerebral ODN concentrations we therefore explicitly measured brain δ-opioid receptor levels and did pharmacological tests 48 h after ODN infusion ended.

In present experiments, we showed the selectivity of the antisense strategy in inhibiting the biosynthesis of δ-opioid receptors by parallel use of both antisense and mismatch ODN sequences and by measuring the density of δ- μ- and k-opioid receptors in the same sample of brain homogenate. Mismatch ODNs never affected brain δ-opioid receptors and no antisense infusion modified brain μ- and k-opioid receptor density.

In $aODN_{280-299}$ δ-knockdown rats we observed decreased locomotor responses to a novel environment but unchanged locomotion in the home cages. In addition, we found no significant difference between colonic propulsion in $aOND_{280-299}$-infused and saline-infused rats. These results indicate that although the endogenous δ-opioid system participates in the expression of the behavioural response to a novel environment, it neither modulates locomotion in a familiar environment nor controls colonic propulsion. In these δ-knockdown rats, the stimulation of locomotor activity and the inhibition of colonic propulsion induced by the δ-opioid agonist SNC80 were strongly reduced, while the number of δ-receptors in the whole brain was reduced by no more than 50%.

Diffusion of the probes from the ventricular system into the brain is limited, leading to reductions in opioid receptors in discrete areas and not in all the brain. In a previous work, we demonstrated that antisense oligonucleotides infused into the lateral ventricle reached the striatum (Negri *et al*., 1999). The striatum is rich in δ-opioid receptors. It is also important in co-ordinating locomotion and is the site where δ-opioid agonists act to produce locomotor effects (Longoni *et al*., 1991; Spina *et al*., 1998). The strongly reduced locomotor response to δ-agonists in aODN-infused rats is therefore consistent with the intense down-regulation of the receptor protein in the striatum. The number of brain receptors measured in whole brain could therefore be only partly reduced whereas the pharmacological effect of the δ-opioid receptor agonist SCN80 is, at the dose used, totally abolished. The loss of SNC80 agonist activity in δ-knockdown rats provides further evidence that when injected subcutaneously this drug selectively acts on the brain to affect locomotor behaviour and colonic propulsion. Finally, our experiments in δ-knockdown rats show that the nociceptive response to radiant heat is preserved and that morphine-induced antinociception did not differ from that evoked by the alkaloid in saline- or mODN-treated rats. Although pharmacological evidence exists for co-operative potentiation of morphine-induced antinociception by exogenous δ-opioid agonists (Adams *et al*., 1993; Negri *et al*., 1995), our results indicate that this co-operation does not occur with the endogenous δ-opioid system. Confirming previous findings in μ-knockout mice (Matthes *et al*., 1996), they also suggest that morphine-induced analgesia is modulated only by μ opioid receptors.

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