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Antisense Inhibition of δ-Opioid Receptor Gene Function In Vivo by Peptide Nucleic Acids

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ABSTRACT

Peptide nucleic acids (PNA) are synthetic analogs of DNA that hybridize to complementary oligonucleotide sequences with exceptional affinity and target specificity. The stability of PNA in biological fluids together with the unique hybridization characteristics of these structures suggests that PNA may have considerable potential as antisense agents for experimental use in vivo. To test this hypothesis, we attempted to modulate supraspinal δ-opioid receptor function in rats using PNA sequences designed to be complementary to a region of the rat δ-opioid receptor. Repeated i.c.v. administration of PNA over a period of 5 days significantly inhibited the antinociceptive response and locomotor response to selective δ-opioid receptor agonists. PNA attenuated δ-opioid receptor function in a sequence-specific, target-specific, and reversible manner characteristic of the functional inhibition caused by an antisense mechanism. There were no apparent toxicities arising from the PNA treatment based on the behavior of the animals and inspection of the treated tissues. Saturation binding studies on brain homogenates did not reveal any significant difference in receptor B_{max} between treatment groups. However, [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate binding assays demonstrated a significant decrease in agonist efficacy in homogenates prepared from antisense-treated rats. Taken together, these results demonstrate that peptide nucleic acids are effective antisense agents in vivo and suggest that PNA may be a useful alternative to phosphodiester or phosphorothioate oligonucleotides, or variants thereof, for determination of gene function in vivo.

Antisense technology has already proven to be useful both as an experimental tool in functional genomics (Wahlestedt et al., 1993) and as a source of novel therapeutics. However, antisense studies performed with phosphodiester- or phosphorothioate-based oligonucleotides are often limited by the appearance of incomplete knockdown of the gene product and sequence-independent effects in brain and other tissues. These limitations are likely to be characteristic of the oligodeoxynucleotide chemistry and thus may be circumvented by using alternative antisense molecules (Fraser and Wahlestedt, 1997).

Peptide nucleic acids (PNAs) are synthetic analogs of deoxynucleotide bases (Nielsen et al., 1991) capable of hybridizing with complementary DNA or RNA sequences via Watson-Crick base pairing and helix formation (Egholm et al., 1993; Brown et al., 1994). PNA oligomers have demonstrated sufficient uptake to support antisense activity in cultured cells (Taylor et al., 1997; Good and Nielsen, 1998) and primary cultures of rat cortical neurons (Aldrian-Herrada et al., 1998). In addition, it has been reported that naked (Tyler et al., 1998) or modified PNA oligomers are effective antisense agents in vivo (Pooga et al., 1998). PNA oligomers probably inhibit gene function by hybridizing with target mRNA to sterically obstruct translation and the consequent synthesis of target protein (Bonham et al., 1995; Knudsen and Nielsen, 1996).

The achiral, charge-neutral polyamide backbone of the PNA molecule cannot contribute to the electrostatic interaction essential for protein binding. Thus, PNA oligomers can avoid the sequence-independent effects of traditional antisense oligonucleotides, which interact indiscriminately with a variety of endogenous proteins (Stein, 1996). PNA oligomers also do not induce ribonuclease H activity (Bonham et al., 1995) and consequently are not prone to sequence-dependent side effects resulting from ribonuclease H-mediated cleavage of nontarget mRNA (Weidner and Busch, 1994; Lima and Crooke, 1997). In addition, PNA oligomers are not susceptible to degradation by endogenous nucleases or proteases and consequently demonstrate improved stability in biological fluids compared with the traditional antisense oligonucleotides (Demidov et al., 1998). Finally, the charge-neutral backbone of PNA oligomers increases both the affinity and specificity of hybridization to complementary nucleotides (Egholm et al., 1993). Together, these characteristics suggest that PNA oligomers may provide a more complete knockdown of the target gene product with an improved

ABBREVIATIONS: PNA, peptide nucleic acid; %MPE, percentage of maximal possible effect; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin; HLA, horizontal locomotor activity.

toxicity profile over traditional antisense oligonucleotides in vivo.

To investigate the potential of PNA as antisense agents in the living brain, PNA sequences were designed complementary to the rat δ-opioid receptor gene. The δ-opioid receptor was chosen as a target for PNA treatment based on its susceptibility to antisense treatment in vivo using conventional oligonucleotides (Bilsky et al., 1996; Negri et al., 1999). Receptor function was evaluated in antinociceptive and locomotor behavioral assays in keeping with the predicted role of supraspinal δ-opioid receptors in the rat (Longoni et al., 1991; Ossipov et al., 1995). In this report, we demonstrate sequence- and target-specific inhibition of δ-opioid receptor gene function in the rat and suggest that PNA oligomers are a viable alternative to phosphodiester- or phosphorothioate-based oligonucleotides for use in antisense studies in vivo.

Materials and Methods

PNA Constructs. PNA sequences inhibit functional gene expression by the steric hindrance of proteins involved in the process of translation. Antisense agents that inhibit protein function in this manner seem to be most effective when directed to areas near the initiation codon, where the secondary and tertiary structure of the mRNA facilitates protein interaction (Bonham et al., 1995). Consequently, the antisense PNA sequence (5′-GTGTCCGAGACGTTG-3′) was designed complementary to a region proximal to the start codon of the δ-opioid receptor mRNA (Evans et al., 1992; Kieffer et al., 1992). A mismatch sequence (5′-GTTGCCGAGACTGTG-3′) in which two base pairs are reversed was designed as a measure of the sequence-specificity of the antisense oligomer. The mismatch sequence maintained the base composition and oligomer polarity of the antisense sequence and thus provided a stringent control. A search of the GenBank database confirmed that the PNA sequences were not homologous to any known nontarget genes in the rat. Unmodified PNA sequences were synthesized and HPLC purified by PerSeptive Biosystems (Framingham, MA). The 15-mer PNA antisense oligomer presented in this report proved to be the most effective of three PNA sequences tested in preliminary assays (data not shown).

Preparation of Animals for Administration of PNA Constructs and Opioid Agonists. Animals were handled in strict adherence to the guidelines established by the Canadian Council for Animal Care. Male Sprague-Dawley rats (250–300 g) were anesthetized with 80 mg of ketamine/xylazine solution per kilogram of body weight (RBI, Natick, MA) and placed in a stereotaxic device. Each animal was then implanted with a 23-gauge cannula extending into the right lateral ventricle (coordinates from bregma: anterior-posterior, 0.8 mm; medial-lateral, 1.5 mm; dorsal-ventral, 3.5 mm) and fixed into place with dental cement. Correct cannula placement was confirmed by histology performed on brains obtained from control rats. Rats were allowed 3 or more days to recover from the surgery before random allocation into treatment groups and subsequent administration of PNA. PNA constructs were diluted in sterile 0.9% saline solution (Astra Canada, Mississauga, Ontario, Canada) and administered via the guide cannula at a dose of 0.45 nmol twice daily for 5 days. Twelve hours after the final PNA treatment, the antinociceptive response to opioid agonists was measured, using either the paw pressure assay or the locomotor activity assays. The opioid agonists [D-Ala^2,*N*-MePhe^4,Gly-ol^5]-enkephalin (DAMGO), deltorphin II (supplied by RBI, Natick, MA), or SNC80 (supplied by Tocris Cookson Inc., Ballwin, MO) were dissolved in 0.9% saline solution and administered to rats via the guide cannula immediately before testing. All PNA and drug treatments were injected via the guide cannula in a volume of 10 μl using a 50-μl Hamilton syringe attached to a catheter (15 cm) constructed from PE20 polyethylene tubing and terminating in a 30-gauge needle. Solution was injected slowly over a period of 60 s and the needle was left within the guide cannula for an additional 30 s after the injection. In all cases, rats were treated concomitantly with 0.9% saline solution as a control for the PNA/drug treatment paradigm.

Paw Pressure Assay. The antinociceptive response to opioid agonists was measured using an analgesy-meter (Ugo Basile, Comerío, Italy). Briefly, an increasing amount of force is applied to the right hind paw of each rat until a threshold force is determined (i.e., the amount of force causing the rat to attempt to withdraw its paw). A maximal cut-off force of 510 g was implemented for this study. Data, presented as the percentage of maximal possible effect (%MPE), were determined using the following calculation:

$$\%\text{MPE} = [(\text{response} - \text{baseline}) / (\text{cut-off} - \text{baseline})] \times 100\%.$$

Locomotor Activity Testing. Activity was measured using the AM1051 Activity Monitor (Benwick Electronics, Cambridge, UK). The plastic cage within the monitor measured approximately 30 × 18 × 18 cm. The monitor was equipped with a 12 × 7 cm infrared beam matrix (i.e., 2.54-cm grid) on both the lower level (set at a height of 3 cm) and the upper level (set at a height of 12 cm). The activity monitor operates by recording the number of times the infrared beams change from broken to unbroken (or vice versa) and incrementing the relevant counters. Horizontal locomotor activity (HLA) and rearing (vertical movement) were recorded for each 10-min interval throughout the duration of the experiment. Rats were habituated in the activity monitor cages for approximately 1 h before drug administration. To minimize disturbing these habituated animals, rats were injected with either deltorphin II (0.3 nmol) or 0.9% saline solution in the activity monitor cage with minimal handling. Data recording was started immediately after the injection. All activity experiments were conducted with parallel treatment groups between 8:00 AM and 3:00 PM.

Tissue Preparation. Immediately after the behavioral testing, rats were decapitated and brains (minus cerebellum) were rapidly removed and stored at −70°C. Previous studies with phosphorothioate oligodeoxynucleotides indicate that these structures have limited distribution proximal to the injection site after i.c.v. administration (Grzanna et al., 1998). Based on these findings, the brain hemisphere ipsilateral to the injection site was used to prepare membrane homogenates in this study. On the day of homogenate preparation, brain hemispheres were thawed and washed in 0.25 mM EDTA/0.5 M phosphate buffer solution, pH 7.4, 4°C. Tissues were individually homogenized in a 20-ml solution of 50 mM Tris buffer, 2.5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.0. P_2 homogenate fractions were prepared after two consecutive low-speed (1200*g*) centrifugation steps and the collection and pooling of the subsequent supernatants. The supernatant was than centrifuged twice at 48,000*g* (20 min for each spin) at 4°C. The P_2 pellet was resuspended in 50 mM Tris buffer, pH 7.4, and incubated at 37°C for 15 min to dissociate any receptor-bound endogenous opioid peptides. Membranes were centrifuged a third time at 48,000*g* as before, and the final pellet was resuspended in 5 ml of 50 mM Tris buffer/0.32 M sucrose solution, pH 7.0. Protein content was determined by modified Lowry assay with SDS. Membrane aliquots were rapidly frozen in dry ice/ethanol and stored at −70°C until the day of the binding assays. [^{3}H]Naltrindole and [^{35}S]guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) binding assays were run in parallel using a common membrane aliquot.

Saturation Binding Assay. Saturation binding curves were performed on rat brain homogenates with the selective δ-opioid receptor radioligand [^{3}H]naltrindole (specific activity, 34.7 Ci/mmol; DuPont-NEN, Wilmington, DE). The incubation buffer was comprised of 50 mM Tris, pH 7.4, with 3 mM $MgCl_2$ and 1 mg/ml BSA, with the peptide CTOP (50 nM; RBI) added to block residual binding of the radioligand to μ-opioid receptors. The binding assay was performed on samples containing 70 to 90 μg of tissue protein in a total assay volume of 300 μl. Nonspecific binding was determined by the addi-

tion of diprenorphine (1 μM; RBI). Samples were incubated for 2 h at room temperature. The assay was terminated by filtration (Brandel M-24 harvester, Gaithersburg, MD) 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 wash buffer (50 mM Tris, pH 7.0, with 3 mM $MgCl_2$). Radioactivity was measured using a liquid scintillation counter (Tri-carb 2100TR; Packard, Meriden, CT).

[^{35}S]GTPγS Binding Assay. This assay was adapted from published procedures (Traynor and Nahorski, 1995). The incubation buffer was composed of 50 mM HEPES, pH 7.4, 20 mM NaOH, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1% BSA, and 120 μM GDP. In addition, 2 μM CTOP was added to the incubation buffer to block any residual SNC80-mediated increases in [^{35}S]GTPγS binding caused by activation of μ-opioid receptors. SNC80 (0.1–10,000 nM), [^{35}S]GTPγS (final concentration of 0.14–0.17 nM) and rat brain membranes (32–34 μg of tissue protein/sample) were combined in a final assay volume of 300 μl. Basal [^{35}S]GTPγS binding was determined in parallel in the absence of SNC80. All samples were incubated for 1 h at room temperature before filtration (Brandel M-24 harvester) through Whatman GF/B filters that were presoaked for 1 h in water. Filters were washed three times with 4 ml of ice-cold wash buffer (50 mM Tris, 5 mM $MgCl_2$, 50 mM NaCl, pH 7.0). [^{35}S]GTPγS binding was measured using a liquid scintillation counter (Tri-carb 2100TR, Packard, Meriden, CT).

Data Analysis. All analyses were performed using Prism (version 2.01) from GraphPad Software (San Diego, CA). The data from the behavioral assays were analyzed by one-way ANOVA and Dunnett's test (where applicable) for each time-point. Comparisons were made between the saline-treated (+drug) group and the antisense and mismatch-treated groups. Receptor binding data were subjected to nonlinear least-squares regression analysis appropriate for saturation binding to a single site. [^{35}S]GTPγS binding data were analyzed by nonlinear regression analysis using a sigmoidal dose-response (variable slope) model. Maximal stimulation of SNC80 induced [^{35}S]GTPγS binding is defined as the peak increase over basal levels observed in brain homogenates prepared from saline-treated animals. The data for percentage of maximal stimulation presented in Table 1 were determined from the upper plateau of the dose-response curve determined from the nonlinear regression analysis. EC_{50} values were determined relative to the maximal effect of SNC80 on [^{35}S]GTPγS binding for individual homogenate samples. Statistical analysis of these data was performed by one-way ANOVA followed by Dunnett's post hoc test (comparison to the saline-treated group) where applicable.

Results

Antinociceptive Response to Opioid Agonists in the Paw Pressure Assay. Concentration-response curves were established for the opiate receptor agonists DAMGO, deltorphin II, and SNC80 in the paw pressure assay of acute mechano-nociception (Fig. 1). All three opioid agonists had a similar response profile; antinociception was maximal 15 min postinjection and the duration of response lasted less than 1 h for each dose. Each opiate agonist was able to reduce the nociception index by up to 80% within the dose ranges tested. Agonist concentrations giving 80% of maximal response (EC_{80}) were determined for each compound (i.e., 60, 400, and 0.2 nmol for deltorphin II, SNC80, and DAMGO, respectively). These agonist concentrations were used in subsequent studies investigating the capacity of PNA oligomers to inhibit agonist-induced antinociception.

Inhibition of δ-Opioid Receptor-Mediated Antinociception by PNA. The antinociceptive response to EC_{80} concentrations of the selective δ-opioid receptor agonists deltorphin II and SNC80 are shown in Fig. 2, A and B, respectively. As expected, the antinociceptive response to both compounds peaked at 15 min after injection and was barely detectable at 1 h after injection. Treatment with the PNA antisense sequence significantly reduced the antinociceptive response to deltorphin II and SNC80 over the course of the test session ($P < .001$ and $P < .01$, respectively). By comparison, treatment with the PNA mismatch sequence did not significantly alter the antinociceptive response to either δ agonist at any time interval ($P > .05$). In addition, neither PNA antisense nor PNA mismatch treatment were effective in inhibiting the antinociceptive response to an EC_{80} concentration of the μ agonist DAMGO (Fig. 2C). Finally, treatment with PNA antisense or PNA mismatch did not alter the baseline nociceptive responses of animals in the paw pressure assay measured before the administration of the opiate agonists (Fig. 2, A–C).

The restoration of the antinociceptive response to deltorphin II was measured after the termination of PNA treatment (Fig. 3). A recovery period of 5 days was chosen to accommodate the delay contingent on the rate of δ-opioid receptor turnover (Jiang et al., 1991). Full recovery of deltorphin II-mediated antinociception was observed in rats previously treated with PNA antisense.

Inhibition of δ-Opioid Receptor Mediated Locomotor Activity by PNA. PNA antisense treatment did not alter baseline exploratory activity in rats compared with saline-treated control animals (data not shown). However, PNA antisense treatment significantly attenuated deltorphin II-mediated increases in HLA and rearing activity compared with saline and mismatch-treated control animals at the 10- and 20-min intervals of the test session (Fig. 4, A and B). The mismatch-treated group did not vary significantly from the saline-treated group at any test interval in these locomotor assays ($P > .05$).

General Observations Pertaining to PNA Toxicity. At no time during the course of the antisense (or mismatch) treatment did the animals display any behavior indicating a toxic response to the PNA. Comparison of body weights before and after PNA treatment revealed no significant differences compared with saline-treated control rats ($P > .05$,

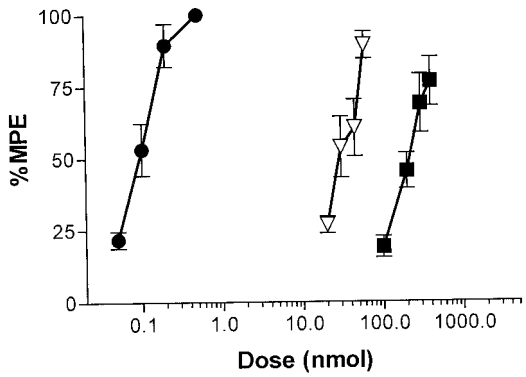

Fig. 1. Antinociceptive effects of DAMGO (●), deltorphin II (▽), and SNC80 (■) in the paw pressure assay. The data represent the peak antinociceptive effects for each agonist measured at 15 min after injection (i.c.v.). %MPE is a measure of the antinociceptive effect of each opioid agonist (compared with saline-treated control rats) as a %MPE that can be measured using this paradigm. Data is presented as mean ± S.E. (n = 8–12 rats).

data not shown). Also, visual inspection of brain tissues did not show any gross signs of tissue necrosis in response to PNA treatment.

δ-Opioid Receptor Density in Brain Homogenates. Binding of the δ-opioid selective radioligand [^{3}H]naltrindole was saturable and best fit to a one-site model in brain membrane homogenates prepared from all treatment groups (data not shown). Analysis of [^{3}H]naltrindole saturation binding revealed an 11 to 13% decrease in whole brain δ-opioid receptor density after antisense treatment compared with that of mismatch- and saline-treated control groups, as shown in Table 1. This difference in receptor B_{max} was not significant ($P > .05$). In addition, there was no significant difference between the associated K_d values determined for each treatment group ($P > .05$).

SNC80-Stimulated [^{35}S]GTPγS Binding in Brain Homogenates. SNC80 (0.1–10,000 nM) induced [^{35}S]GTPγS binding in brain homogenates prepared from all treatment groups. Dose response relationships were best fit to a sigmoidal curve, as shown in Fig. 5. Basal [^{35}S]GTPγS binding did not differ significantly between treatment groups ($P > .05$; data presented in caption for Table 1). SNC80 (10 μM) induced a maximal stimulation of 40.4 ± 2.4% above basal levels in brain homogenates prepared from saline-treated rats; maximal stimulated binding values for each treatment group were determined as a percentage of this value as shown in Table 1. EC_{50} values were determined relative to the maximal effects observed for each treatment group. The EC_{50} value for SNC80 stimulated [^{35}S]GTPγS binding was 20% higher in brain homogenates prepared from antisense-treated rats compared with the control group. However, one-way ANOVA comparison of the treatment groups just failed to indicate a significant difference ($P = .084$). In contrast, maximal SNC80-stimulated [^{35}S]GTPγS binding was significantly lower in homogenates prepared from the antisense-treated group compared with those prepared from the control group (~25% lower, $P < .05$). There was no significant difference in maximal SNC80-stimulated [^{35}S]GTPγS binding between the control group and the mismatch group ($P > .05$).

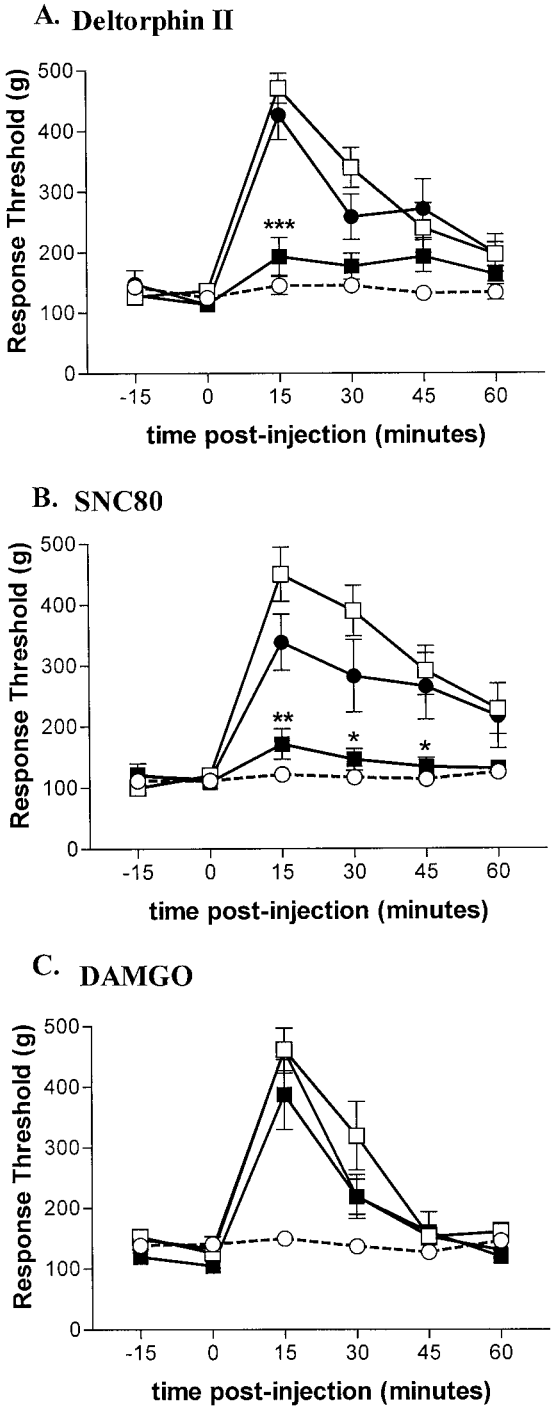

Fig. 2. PNA antisense treatment inhibited the antinociceptive response to deltorphin II (60 nmol) (A) and SNC80 (400 nmol) (B) but not DAMGO (0.2 nmol) (C). Values represent saline-treated control animals (○), saline-treated (vehicle) + agonist (□), antisense-treated + agonist (■), and mismatch treated + agonist (●). *, **, and *** represent significant differences between the antisense group and the saline (+ agonist) and mismatch groups where $P < .05$, 0.01, and 0.001, respectively. Each curve represents the mean ± S.E. response of 7 to 11 rats.

Discussion

This study demonstrates that an unmodified PNA oligomer is an effective antisense agent in vivo. In addition, this study confirms that the cloned δ-opioid receptor mediates both the antinociceptive and the locomotor effects of δ agonists admin-

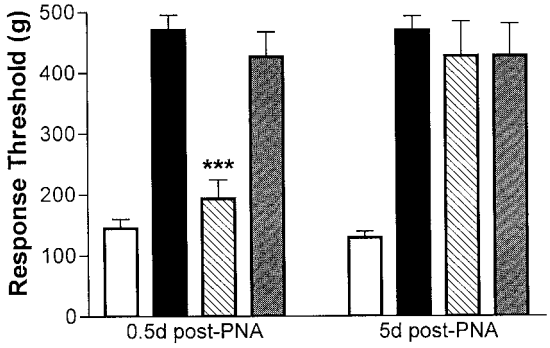

Fig. 3. Recovery of δ-opioid receptor function after PNA treatment. Twice-daily i.c.v. injections of PNA antisense over a period of 5 days inhibited the antinociceptive response to deltorphin II (60 nmol) at 0.5 days but not 5 days after PNA treatment. Testing at 0.5 and 5 days was performed on the same groups of rats. *** represents a significant difference between the antisense group and the saline (+ agonist) group where $P < .001$. Each column represents the mean ± S.E. antinociceptive response to Deltorphin II observed at 15 min postinjection (n = 5–7 rats per group). Open columns, control; filled columns, vehicle + deltorphin II; hatched columns, antisense-treated + deltorphin II; gray columns, mismatch-treated + deltorphin II.

istered directly into the brain of conscious rats. Finally, the findings presented in this report indicate that the [^{35}S]GTPγS binding assay is more sensitive than saturation binding experiments for evaluating the effects of antisense treatment on tissue samples in vitro.

Before antisense testing, the effects of δ- (deltorphin II, SNC80) and μ- (DAMGO) opioid receptor agonists were assessed in the paw pressure assay of antinociception. DAMGO was approximately 1000-fold more potent than the δ agonists consistent with the predominant expression of μ-opioid receptors in supraspinal pain pathways (Mansour et al., 1995).

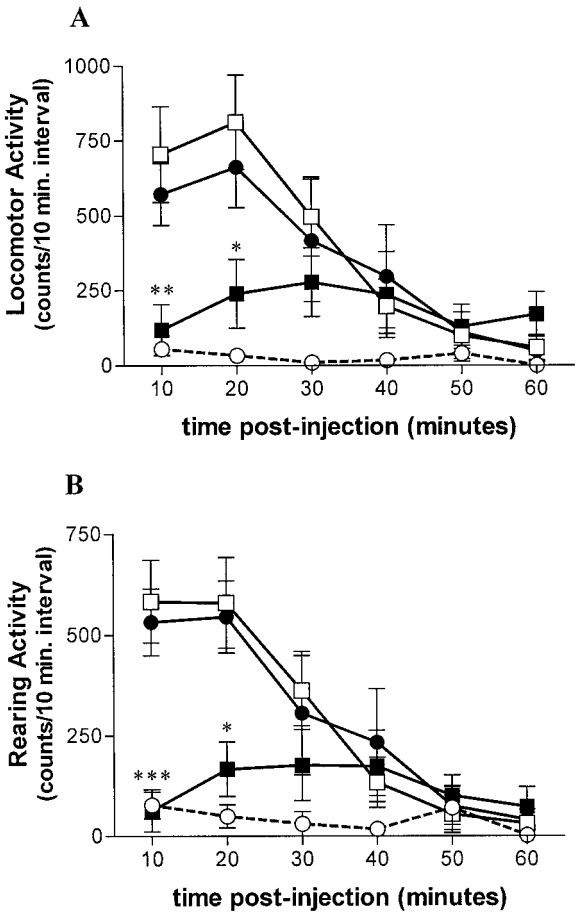

Fig. 4. PNA inhibition of δ-opioid receptor-mediated locomotor activity. PNA antisense treatment inhibits the increased HLA (A) and the increased rearing activity (B) in response to the δ agonist deltorphin II (0.3 nmol, i.c.v.). Values represent saline-treated controls (○), saline-treated (vehicle) + deltorphin II (□), antisense-treated + deltorphin II (■) and mismatch treated + deltorphin II (●). *, **, and *** represent significant differences between the antisense group and the saline (+ agonist) and mismatch groups where $P < .05$, 0.01, and 0.001, respectively. Each curve represents the mean ± S.E. response of 7 to 10 rats.

Pretreatment with the PNA antisense sequence significantly inhibited the antinociceptive response to deltorphin II and SNC80. The sequence-specific nature of inhibition by the antisense but not the mismatch sequence implies that the PNA oligomer is effective via an antisense mechanism. To verify that the effect of the PNA antisense sequence was also target-specific (i.e., selective for δ-opioid receptors), a separate group of rats were treated with PNA and then challenged with the μ-opioid receptor agonist DAMGO. The μ-opioid receptor was chosen as a control target based on its similarity to the δ-opioid receptor in mediating antinociceptive responses and its supraspinal distribution. The δ antisense (and mismatch) PNA sequences were not complementary to any region of the μ-opioid receptor mRNA (Chen et al., 1993). The lack of effect of either PNA sequence on DAMGO-mediated antinociception suggests that the inhibition of response to deltorphin II and SNC80 by PNA treatment in the paw pressure assay is caused by an inhibition of δ-opioid receptor function as opposed to a more general change in the functioning of supraspinal nociceptive pathways.

An advantage of antisense techniques as a method of determining gene function is that inhibition of target gene expression is transient in nature, thus minimizing the development of any compensatory changes as a consequence of the manipulation (Fraser and Wahlestedt, 1997). To confirm that the behavioral effects of PNA antisense treatment in the paw pressure assay were caused by a reversible inhibition of δ-opioid receptor function, the antinociceptive effects of deltorphin II were remeasured in rats after the termination of PNA treatment. The allowed recovery period is consistent with the expected rate of δ-opioid receptor turnover (Jiang et al., 1991). The complete recovery of deltorphin II efficacy in rats formerly treated with PNA antisense supports the proposed δ receptor-specific action of the PNA antisense sequence. In addition, this finding suggests that the inhibited response to δ agonists after PNA treatment was caused by neither a general neurotoxicity nor a long-term change in nonopioid receptor systems.

Distinct populations of δ-opioid receptors in the thalamic and striatal regions of the brain, respectively mediate the antinociceptive and locomotor responses to δ agonists (Mansour et al., 1995). PNA antisense treatment significantly inhibited deltorphin II-mediated increases in locomotor activity in a sequence-specific manner. This finding provides additional evidence that PNA sequences are effective anti-

TABLE 1

Effect of PNA antisense treatment on δ-opioid receptor density

Saturation and [^{35}S]GTPγS binding were performed on homogenates of brain hemispheres from saline-, antisense-, and mismatch-treated rats. The data from each rat brain homogenate was analyzed separately. Basal [^{35}S]GTPγS binding was 3240 ± 120, 3230 ± 150, and 3220 ± 120 cpm for the saline-, antisense-, and mismatch-treated groups respectively; there was no significant difference between treatment groups ($P > .05$). Maximal stimulated binding is defined as the peak increase over basal levels for SNC80-induced [^{35}S]GTPγS binding in brain homogenates prepared from saline-treated animals. Saturation binding and [^{35}S]GTPγS binding were assayed in parallel on the same brain homogenates.

	[^{3}H]Naltrindole Saturation Binding		[^{35}S]GTPγS Binding	
	K_d	B_{max}	SNC80 EC_{50}	% Maximal Stimulated Binding
	nM	*mol/mg protein*	*nM*	
Saline-treated (n = 5)	0.059 ± 0.007	44.3 ± 2.8	71.5 ± 6.2	100.0 ± 5.8
Antisense-treated (n = 5)	0.055 ± 0.010	38.6 ± 2.2	85.5 ± 5.4	74.8 ± 5.0[a]
Mismatch-treated (n = 5)	0.069 ± 0.010	43.4 ± 0.9	65.8 ± 5.8	97.0 ± 8.1

[a] Represents a significant difference in comparison to the saline-treated group ($P < .05$). Data are presented as mean ± S.E.

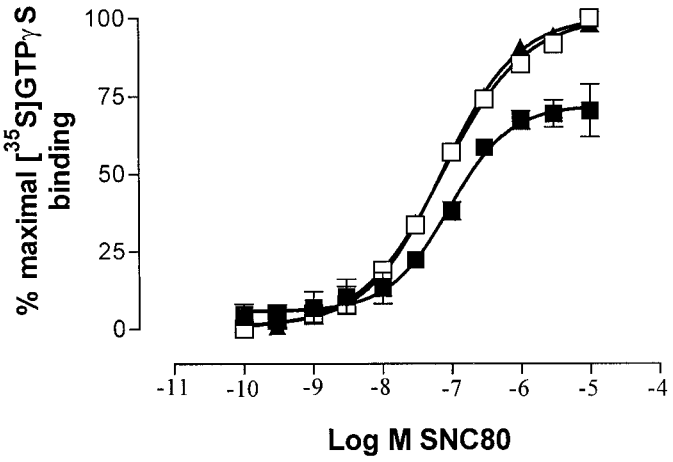

Fig. 5. Representative dose-response curve for [^{35}S]GTPγS (0.14–0.17 nM) binding to rat brain membranes in response to SNC80 (0.1–10,000 nM). Homogenates were prepared separately for each saline- (○), antisense- (■), and mismatch- (▲) treated rat. The data are from a single assay (i.e., one rat per group). Homogenates from each treatment group were assayed in parallel and each binding experiment was performed once with quadruplet samples.

sense agents in vivo. In addition, it confirms that a common δ-opioid receptor subtype mediates the locomotor and antinociceptive effects of deltorphin II. Finally, this observation implies that PNA oligomers are able to penetrate more than one region of the brain after i.c.v. injection.

Pretreatment with PNA antisense oligomers did not alter baseline response thresholds in the paw pressure assay. This observation is consistent with previous reports that the antagonism (Jiang et al., 1991) or inhibition of expression (Bilsky et al., 1996; Kest et al., 1996) of δ-opioid receptors does not alter the baseline response of animals in acute pain models. Similarly, PNA antisense treatment did not alter baseline exploratory locomotor activity in the present study. The finding that repeated i.c.v. injections of PNA did not alter baseline antinociceptive or locomotor responses suggests that there is no toxicity in response to the PNA treatment affecting either the motor response required for paw withdrawal, the cognition and processing of nociceptive signals, or the supraspinal processes that control basic exploratory activity. In addition, there were no obvious changes in the general behavior or the body weight of the animals indicative of any untoward effects of the PNA. Also, there was no indication of tissue damage at the injection site, which compares favorably to the side-effect profile after treatment with phosphorothioate oligonucleotides, where gross tissue necrosis proximal to the injection site is a common outcome (LeCorre et al., 1997).

Saturation binding studies suggest that there may have been a small diminution (i.e., ~13%) in receptor B_{max} in brain homogenates prepared from antisense-treated rats compared with saline-treated control rats. However, this difference in receptor B_{max} is not significant. This finding is consistent with a number of other reports of antisense studies directed against G protein-coupled receptors in vivo in which substantial changes in antisense-mediated behavior were not accompanied by comparable decreases in receptor density. In studies where receptor B_{max} values were reported, examples of antisense modulation of supraspinal opioid or dopamine receptors coincided with either no change (Shah et al., 1997) or a modest change (i.e., <20%) in receptor binding sites (Niesbrand et al., 1995; Qin et al., 1995; Bilsky et al., 1996). Although such small changes in receptor population might seem insufficient to account for the changes in behavior, receptor binding on whole tissue homogenates may dilute highly restricted decreases in protein expression (Grzanna et al., 1998). However, this explanation seems to be insufficient to account for the present findings, in which the effects on both pain and locomotor activity imply that PNA oligomers effectively penetrate multiple brain regions. An alternate hypothesis is that only a small pool of newly synthesized G protein-coupled receptors are functional and that antisense treatment inhibits the replenishment of this receptor pool (Qin et al., 1995; Hua et al., 1998). This hypothesis was tested using the [^{35}S]GTPγS binding assay, which measures the efficacy of ligands at G protein-coupled receptors (Traynor and Nahorski, 1995). Comparison of the EC_{50} values describing SNC80-induced stimulation of [^{35}S]GTPγS suggest a reduced agonist potency in brain homogenates prepared from antisense-treated animals. Moreover, the efficacy of SNC80 was significantly reduced in homogenates prepared from the antisense treatment group. These changes in the SNC80 dose-response relationship are consistent with pharmacological models describing dose-response profiles generated in the presence of a noncompetitive antagonist. The [^{35}S]GTPγS binding data provides an in vitro correlate for the behavioral differences observed in the antisense treatment groups in vivo and seems to be a more sensitive assay than saturation binding for measuring the efficacy of antisense treatment. Taken together, the saturation binding and [^{35}S]GTPγS binding data support the notion that antisense treatment preferentially inhibits the replenishment of a functional receptor pool.

The hybridization properties of PNA have made these synthetic oligomers very useful tools for a diverse number of scientific applications, including hybridization techniques (Perry-O'Keefe et al., 1996), high-throughput DNA or RNA screening (Webb and Hurskainen, 1996; Weiler et al., 1997), and site-directed mutagenesis (Faruqi et al., 1998). In addition, the superior hybridization affinity of PNA increases their versatility as antisense agents compared with phosphodiester or phosphorothioate oligonucleotides. Specifically, the high hybridization affinity of PNA-mRNA hybrids permits the use of short oligomer sequences to achieve antisense effects. Thus, a 15-base sequence was chosen for use in this study, although it has been shown that phosphorothioate oligonucleotides of comparable length are ineffective antisense agents (Monia et al., 1992). Also, the concentration of PNA required to achieve antisense effects in vivo in this study is about 10-fold less than the concentrations of oligonucleotide sequences used in previous reports of antisense knockdown of the δ-opioid receptor in rats (i.c.v.; Negri et al., 1999; G.L.F., P.B.S.C., and C.W., submitted for publication). This is consistent with the improved in vitro antisense potency of PNA sequences compared with their phosphorothioate analogs (Norton et al., 1996). The reduced dose of PNA required is probably a product of the high hybridization affinity and improved stability of these synthetic oligomers (Demidov et al., 1998). The ability to reduce oligomer length and dose when using PNA sequences in vivo may be of benefit in improving the efficiency of cellular uptake and in reducing

the prevalence of nonspecific effects (Woolf et al., 1992; Flanagan et al., 1996).

In conclusion, the sequence- and target-specific inhibition of G protein-coupled receptor function in the living brain described previously (Tyler et al., 1998) and in this report demonstrates that unmodified PNA oligomers are effective antisense agents in vivo. We anticipate continued advances in PNA chemistry to further improve the potency and toxicity profile of PNA oligomers over conventional oligonucleotides for application in the domain of functional genomics.

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