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Peptide nucleic acids targeted to the neurotensin receptor and administered i.p. cross the blood–brain barrier and specifically reduce gene expression

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ABSTRACT **Intraperitoneal injection of an unmodified antisense peptide nucleic acid (PNA) complementary to mRNA of the rat neurotensin (NT) receptor (NTR1) was demonstrated by a gel shift assay to be present in brain, thus indicating that the PNA had in fact crossed the blood–brain barrier. An i.p. injection of this antisense PNA specifically inhibited the hypothermic and antinociceptive activities of NT microinjected into brain. These results were associated with a reduction in binding sites for NT both in brain and the small intestine. Additionally, the sense-NTR1 PNA, targeted to DNA, microinjected directly into the brain specifically reduced mRNA levels by 50% and caused a loss of response to NT. To demonstrate the specificity of changes in behavioral, binding, and mRNA studies, animals treated with NTR1 PNA were tested for behavioral responses to morphine and their mu receptor levels were determined. Both were found to be unaffected in these NTR1 PNA-treated animals. The effects of both the antisense and sense PNAs were completely reversible. This work provides evidence that any antisense strategy targeted to brain proteins can work through i.p. delivery by crossing the normal blood–brain barrier. Equally important was that an antigene strategy, the sense PNA, was shown *in vivo* to be a potentially effective therapeutic treatment.**

Peptide nucleic acids (PNAs), a new type of DNA analog (Fig. 1), hold great promise as antisense or antigene drugs, because they are electrically neutral oligomers that are stable against nucleases and proteases, bind independently of salt concentration to their complementary nucleic acids, and have higher affinity for nucleic acids than do DNA/DNA duplexes (1, 2). Additionally, PNA/DNA duplexes are much more gene specific, because they are less tolerant of mismatches than are DNA/DNA duplexes (3). Initial enthusiasm for their use as antisense or antigene drugs was dampened by the fact that these molecules pass poorly into cells (4, 5). Our laboratory reported that unmodified (carrier-free) PNAs, on their direct injection into rat brain, enter neuronal cells and inhibit protein synthesis in a gene-specific manner (6).

To determine both the mechanism of action of PNAs and whether PNAs could pass the blood–brain barrier (BBB), brain neurotensin (NT) receptors (NTR1) again were targeted. After a single i.p. injection of antisense PNA to NTR1 (targeted to mRNA) behavioral and physiological responses to NT (antinociception and hypothermia) were specifically and almost completely lost. These results were accompanied by specific reductions in receptor sites as determined by radioligand binding assays. However, there were no changes in mRNA levels. A sensitive assay developed to detect the amount of PNAs in tissue (gel shift assay) confirmed the presence of PNA in brain after i.p. injection. Therefore, these results provided evidence that any antisense strategy targeted to brain proteins can work by i.p. delivery and by crossing the normal (i.e., not compromised by malignancy) BBB. Also, of great interest was the fact that a sense-NTR1 PNA, targeted to the DNA sequence (in this case injected directly into brain) caused the same blockade of the responses to NT and significantly reduced NTR1 mRNA levels. Thus, this sense PNA acted as a true antigene agent *in vivo*.

METHODS

PNA Synthesis. PNA oligomers were made on an Expedite 8909 synthesizer as described (6) or manually synthesized on a 50 μmol scale by using phosphatidylserine-polyethylene glycol-PAL (PerSeptive Biosystems, Framingham, MA, polyethylene glycol-polystyrene) resin (1 equivalent) and fluorenylmethoxycarbonyl-protected nucleobase monomers (6 equivalent; PerSeptive Biosystems) in the presence of diisopropylethylamine and 2,6-lutidine. PNAs were removed from the resin support, and blocking groups were removed by 2-hr cleavage at room temperature in 80% trifluoroacetic acid containing 20% (vol/vol) m-cresol. The PNAs were precipitated into cold diethyl ether and were purified by RP-HPLC on a Vydac Q8 column (25 mm × 250 mm) at 55°C.

Animal Testing. Male Sprague–Dawley rats (Harlan, Prattville, AL) were injected with PNAs [AS-NTR1, mismatch AS-NTR1, or AS-MOR1 (morphine receptor)] at 10 mg/kg i.p or were microinjected with sense-NTR1 PNA directly into the periaqueductal gray (PAG). Twenty-four hours postinjection, animals received either 18 nmol of NT microinjected into the PAG and 30 min later were examined for body temperature (rectal) and antinociception (hot plate at 52°C), or they received morphine 5 mg/kg i.p. and 30 min later were examined for antinociception (tail flick) as described (6). Antinociception scores were calculated as percent of maximum possible effect (%MPE) with the following equation: %MPE = [(postdrug latency time − predrug latency time)/(cutoff time − predrug latency time)] ×100; where the cutoff time (i.e., the time when the animal was removed from the device) was 30 s for hot plate and 12 s for tail flick.

Binding Experiments. For the NT and morphine binding assays, homogenates were prepared from freshly obtained PAG and the rest of brain of adult rats as described (7) with

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Abbreviations: BBB, blood–brain barrier; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOR, morphine receptor; NT, neurotensin; NTR, NT receptor; PAG, periaqueductal gray; PNA, peptide nucleic acid; %MPE, percent maximum possible effect.

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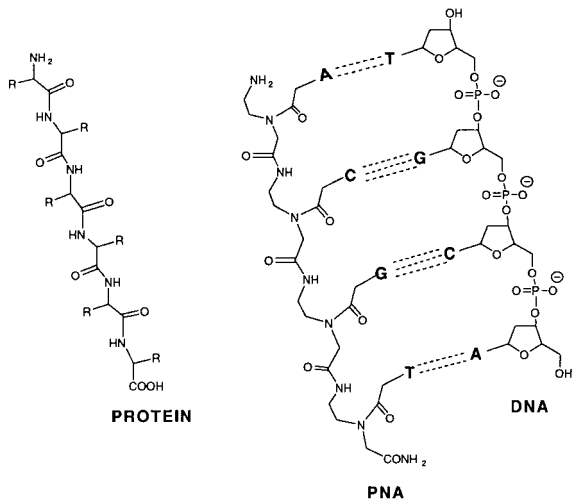

FIG. 1. Structural representations of protein, PNA, and DNA adapted from ref. 1.

the following modifications: the assay buffer contained the peptidase inhibitors 1,10 phenantholine (1 μM) and aprotonin (5 mg/ml) and the radioligand was [^{125}I]NT. For PAG and the rest of the brain binding assays, tissues were incubated with 1 nM [^{3}H]morphine or 0.3 nM [^{125}I]NT (NEN) at room temperature for 60 or 30 min, respectively. Total and nonspecific binding was measured as described (6) except that binding sites were normalized to protein concentrations by BCA protein determination (Pierce). Purified plasma membrane homogenates also were prepared from freshly obtained jejunum and ileum, and radioligand binding assays were performed as described (7, 8) with the following modifications: binding buffer contained the peptidase inhibitors stated above and incubation was carried out at room temperature (1 hr for morphine and 30 min for NT). For these assays, intestinal homogenates (500 μg protein) were incubated with 2 nM [^{3}H]morphine or 80 pM [^{125}I]NT (NEN) in a final volume of 1 ml. Nonspecific binding in brain and intestinal assays was determined by using 1 μM unlabeled morphine (Research Biochemicals) or NT. Binding data were analyzed as specific bound dpm/μg protein and compared with control (no PNA) animals for percent change.

Quantitative PCR: mRNA Level Detection. Total RNA was isolated from approximately 20 mg of tissue by using the S.N.A.P. Total RNA Isolation Kit (Invitrogen). Quantitative reverse transcription–PCR was performed on treated and control animals by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard and PCR fragments of the human NTR and a GAPDH deletion mutant as exogenous standards. First-strand cDNA was synthesized from approximately 500 ng of total RNA by using the cDNA Cycle Kit (Invitrogen). PCR was performed on a GeneAmp PCR System 9600 (Perkin—Elmer) by using end-labeled primer and *Taq* DNA Polymerase (GIBCO/BRL). Products were separated on a 5% acrylamide gel containing 7 M urea and detected by using a Storm860 PhosphorImager (Molecular Dynamics). Data were analyzed by using IMAGEQUANT software (Molecular Dynamics).

Reverse Transcriptase-PNA Blockade Assay. Total RNA was isolated from approximately 20 mg of tissue by using the RNeasy Kit (Qiagen, Chatsworth, CA). Templates for exogenous cRNA standards for NTR1 and GAPDH were constructed by using the PCR MIMIC Construction Kit (CLONTECH) and synthesized by using the Megashort-Script Kit (Ambion, Austin, TX). Total RNA (1 μg) was incubated with 600 ng of AS-NTR1 PNA, mismatch AS-NTR1 PNA, or no PNA at 40°C for 40 min. Exogenous cRNA standards were added, and cDNA was synthesized by using the cDNA Cycle Kit and gene-specific primers for NTR1 and GAPDH (Invitrogen). Quantitative PCR was performed on these samples and products were separated on a 2% agarose gel and stained with Vista Green (Amersham Pharmacia). Products were detected and analyzed as described below.

Gel Shift Assay. Flash-frozen brain tissue (minus the PAG and hypothalamus) was homogenized in 1 mM Tris, pH 4.0/0.1 mM EDTA at a concentration of 1.5 ml/g wet weight of tissue. Samples were boiled for 5 min and spun to pellet insoluble material. Supernatants were extracted with 10 vol of $CHCl_3$/MeOH (2:1) and spun briefly at 1,000 $\times$ *g* to separate phases. The supernatants were lyophilized and resuspended in a volume of 40 μl. An oligonucleotide (GIBCO/BRL) complementary to the PNA was end-labeled by using T4 Polynucleotide Kinase (GIBCO/BRL) and [γ-^{32}P]ATP (NEN) according to manufacturer's instructions. Unincorporated nucleotides were removed by using a Chroma-Spin 10 column (CLONTECH) and gel-purified on a 20% acrylamide gel. The portion of the gel containing the probe was excised, and probe was eluted into 100 μl of 10 mM Tris, pH 8.0/1 mM EDTA. Approximately 400 pg of probe was added to each sample. The samples were incubated at room temperature for 20 min and run on a 20% polyacrylamide gel. Gels were incubated in gel drying solution (Bio-Rad) for 30 min and dried on a gel dryer. Signal was detected by using a Storm860 PhosphorImager (Molecular Dynamics), and data were analyzed by using IMAGEQUANT Software (Molecular Dynamics).

Statistical Analysis. Statistical analysis was done by using the rank sum test with $P < 0.05$ being considered significant.

RESULTS

The NT System as a Model for the Effectiveness of PNA Treatment. NT is an endogenous tridecapeptide, which is found throughout the mammalian central nervous system (9). Many studies demonstrate that NT is a neurotransmitter capable of exerting potent effects, including hypothermia and antinociception (10, 11). NT mediates its effects through its well-characterized receptors (NTRs). To date, two NTRs have been molecularly cloned, and both are distributed heterogeneously in the central nervous system (12–16). Previous results suggest that NTR1 mediates the hypothermic and antinociceptive responses to NT (6). NTR2 does not generate a functional response to NT *in vitro* and appears unlikely to be involved in the hypothermic and antinociceptive effects of NT *in vivo* (17).

Effects of PNA Treatment on Responses to NT and Its Binding Sites. Four different PNAs (AS-NTR1, mismatch AS-NTR1, AS-MOR1, and sense-NTR1) were used in this study. The antisense PNA directed to the mRNA of the NTR1 (AS-NTR1; 5′-CATTGCTCAAAC-3′; Table 1) was based on the cDNA sequence of the molecularly cloned NTR1 of the rat (12), targeting an area starting +103 bp from the start codon (within the coding region). This AS-NTR1 PNA was effective when microinjected directly into the brain (6). For the present study, a PNA containing a mismatch at every third base pair (mismatch indicated in bold: mismatch AS-NTR1, 5′-TATGGCACAGAC-3′) was used as a control for the specificity of the PNA sequence. Also, sense PNA directed to DNA (sense-NTR1: 5′-GTTTGAG-CAATG-3′) of the NTR1 was tested to determine the antigene potential of PNAs *in vivo*. As an additional control for nonspecific or toxic PNA effects, the antisense PNA (AS-MOR1; 5′-CAGCCTCTTCCTCT-3′) targeted to the mRNA of the MOR subtype 1 (MOR1) also was used. This AS-MOR1 PNA specifically knocks down MOR1 levels and responses, when it is directly microinjected into brain (6).

Three of the PNAs (all except sense-NTR1) were injected i.p. (10 mg/kg) independently, into separate groups of rats. The sense-NTR1 PNA (5 μg) was microinjected directly into the PAG, the major area in the brain involved with perception of pain (nociception). Twenty-four hours after injection of PNA, rats were tested with NT (18 nmol), which was micro-

Table 1. Gene targets, antisense, and sense sequences

Rat gene production	Gene product abbreviation	PNA sequence	Location of complementary	PNA abbreviation
NTR subtype 1	NTR1	5′-CAT TGC TCA AAC-3′	Coding region of mRNA	AS-NTR1
NTR subtype 1	NTR1	5′-TAT GGC ACA GAC-3′	Coding region of mRNA	Mismatch AS-NTR1
NTR subtype 1	NTR1	5′-GTT TGA GCA ATG-3′	Coding region of DNA	Sense-NTR1
MOR subtype 1	MOR1	5′-CAG CCT CTT CCT CT-	5′ noncoding region of mRNA	AS-MOR1

injected into the PAG because NT is rapidly degraded on systemic administration and does not cross the BBB. Thirty minutes after receiving NT, animals were scored for antinociception (hot plate) and hypothermia (rectal probe). Animals receiving AS-NTR1 PNA scored an average of 25% MPE ($P <$ 0.001 vs. untreated animals) compared with no pretreatment or vehicle-treated (no PNA) animals, which scored 77% and 71% MPE, respectively (Fig. 2). Thus, the animals that received AS-NTR1 PNA and then NT more readily perceived the pain and therefore spent less time on the hot plate because the effects of NT were blocked by this PNA treatment. Animals receiving the mismatch AS-NTR1 PNA scored a mean of 60% MPE, which was not statistically significant compared with untreated animals ($P = 0.38$). The animals receiving sense-NTR1 PNA had an antinociceptive score of 14% MPE ($P =$ 0.002 vs. untreated animals), again indicating that these animals readily responded to the heat stimuli. The animals that received AS-MOR1 PNA (serving as a control for toxic or nonspecific PNA effects) scored 82% MPE in response to NT, a value that was nearly identical to that for animals receiving no pretreatment or vehicle.

As an added measure of specificity for the NT system, AS-NTR1-treated animals that lacked antinociception when tested with NT were tested with morphine and 30 min later were scored for antinociception by the tail flick method (Fig. 2). These AS-NTR1-treated animals scored 95% MPE, which was essentially identical to that found for animals not injected with PNA and tested with morphine that scored 99% MPE.

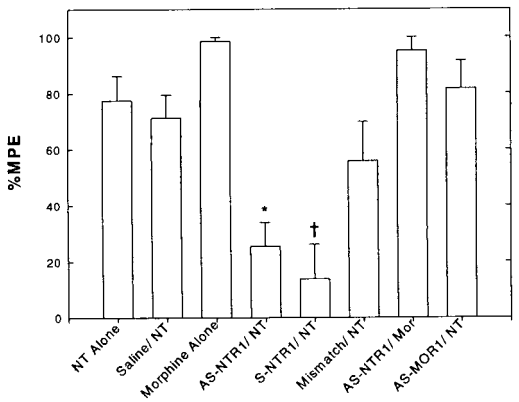

FIG. 2. Antinociceptive response to NT or to morphine in rats treated i.p. with saline, antisense PNA to the NTR1 (AS-NTR1), mismatch AS-NTR1 PNA, or antisense PNA to the MOR (AS-MOR1) or sense-NTR1 PNA microinjected into the PAG. PNA-treated animals received AS-NTR1, mismatch AS-NTR1, or AS-MOR1 10 mg/kg i.p., or sense-NTR1 PNA 5 μg microinjected into the PAG. Testing was 24 hr postinjection. For the these responses, animals either were placed on a hot plate after NT was microinjected into their PAG (18 nmol) or their tails were immersed in heated oil (tail flick assay) after they were treated with morphine (5 mg/kg i.p.). Data are reported as %MPE ± SEM as determined 30 min after drug (NT or morphine) delivery. The description before the slash at the bottom of each bar indicates pretreatment, while the drug listed after the slash indicates the drug used for testing. *, $P <$ 0.001 vs. NT alone; †, $P = 0.002$ vs. NT alone.

Immediately after the 30-min NT antinociception test, animals were examined for body temperature changes in response to NT (Fig. 3). Animals treated with AS-NTR1 or sense-NTR1 had a body temperature change of −0.5°C, which was highly significant compared with untreated animals whose body temperature change was −1.8°C ($P < 0.001$ and $P = 0.008$ vs. untreated animals, respectively). The vehicle control animals had a body temperature change of −1.7°C, which was nearly identical to the no pretreatment group. The mismatch AS-NTR1 control animals had a mean body temperature change of −1.3°C, which was not significantly different when compared with that for untreated animals ($P = 0.83$).

Thus, these i.p.-administered antisense PNA behavioral results were consistent with our previous studies involving direct injection into brain of PNAs (6). Both results indicated that the PNA acted in a gene-specific manner and that the mere injection of a PNA did not, by itself, alter whole animal responses to a test drug. Importantly, the effects of the sense-NTR1 PNA (targeted to DNA) suggested that this PNA exerted its effect at the level of gene regulation for the NTR1.

The time course for recovery of the inhibitory effect of a single PNA injection (10 mg/kg) was determined with another group of animals, which were tested every 24 hr with NT, until the responses to the peptide returned to the level seen in untreated animals (Fig. 4). The responses to NT in animals that had received AS-NTR1 returned to baseline levels within 48 hr after PNA injection and remained normal at 72 hr. Later time points were not included in the analysis, as some animals (including controls) started to respond erratically to NT after receiving more than three doses of the peptide, which is likely because of receptor desensitization. The reversibility of the inhibitory effects of PNA treatment further suggests that the

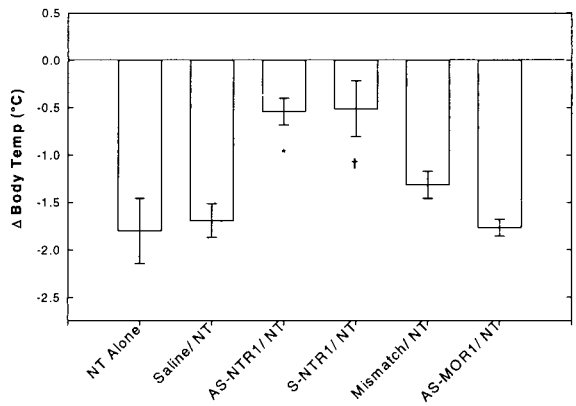

FIG. 3. Hypothermic response to NT in rats treated i.p. with saline, antisense PNA to the NTR (AS-NTR1), mismatch AS-NTR1 PNA, or antisense PNA to the MOR (AS-MOR1) or sense-NTR1 PNA microinjected into the PAG. Animals were treated as described in Fig. 2. Body temperature was measured by use of a thermistor probe inserted 3 cm into the rat's rectum before and 30 min after NT (18 nmol) microinjection into the PAG. The change in body temperature ± SEM is reported. *, $P <$ 0.001 vs. NT alone; †, $P = 0.008$ vs. NT alone.

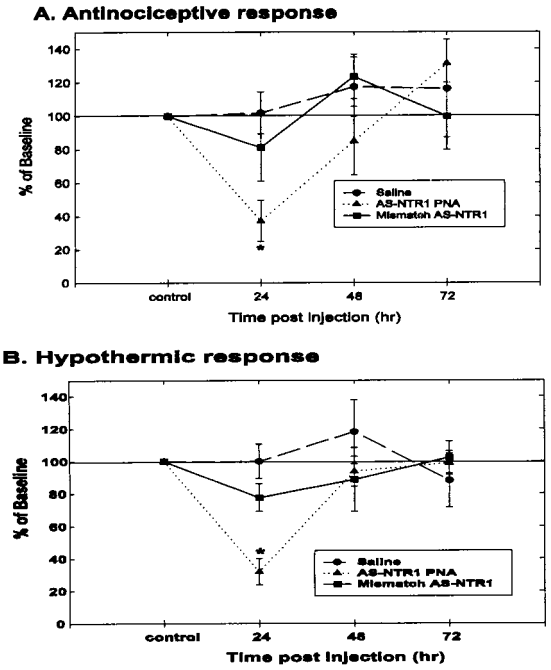

FIG. 4. Time course of antinociceptive and hypothermic responses to NT in rats treated i.p. with saline, antisense PNA to the NTR (AS-NTR1), or mismatch AS-NTR1 PNA. Animals received saline or PNA treatment as described in the legend to Fig. 2. Testing was every 24 hr for 3 days. Untreated control animals tested with NT alone were used to calculate the mean baseline response, which was set to 100. Data are reported as a percentage of the baseline response ± SEM as determined 30 min after drug delivery. Animals given saline (●, $n = 4$), AS-NTR1 PNA (▲, $n = 4$), or mismatch AS-NTR1 PNA (■, $n = 4$) were microinjected with NT (18 nmol) into the PAG and were tested for length of time on the hot plate (%MPE score) (*A*) and for body temperature lowering (*B*).

PNA was acting by gene-specific mechanisms, rather than by a nonspecific or toxic effect on cells.

In the last of the behavioral and physiological studies, dose-response curves for AS-NTR1 PNA were obtained (Fig. 5). The

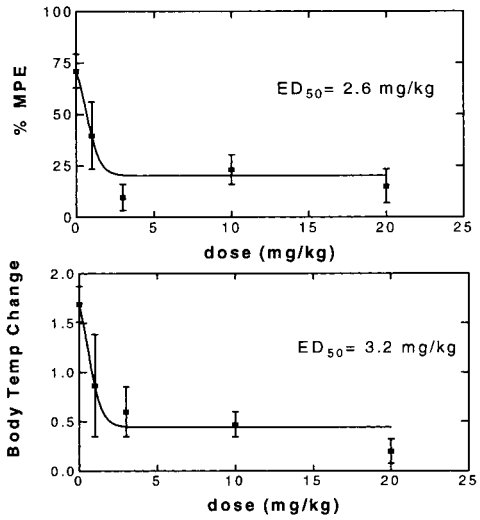

FIG. 5. Effect of dosage of antisense PNA to the NTR (AS-NTR1) given i.p. on responses to NT (antinociception and hypothermia). Rats received varying doses of anti-NTR1 PNA injected i.p. ($n \geq 4$ at each dose) and 24 hr later were microinjected with NT (18 nmol) into the PAG and scored for antinociception on the hot plate (%MPE) and for change in body temperature. ED_{50}s were calculated by determining the effective dose (mg/kg) that gave 50% of the maximal response.

AS-NTR1 PNA produced steep dose-response curves, which are characteristic of antisense effects (18), and appeared to have identical potency at reducing both responses to NT with ED_{50}s of 3.2 and 2.6 mg/kg for hypothermia and antinociception, respectively. The dose-response data shown here are representative results using one batch of PNA. Some variation in potency from different PNA preparations was observed. For this reason, all other experiments were performed at 10 mg/kg, a dose that provided consistent results regardless of PNA batch.

It was hypothesized that the antisense effects of the PNA treatment blocked the pharmacological effects of NT by reducing the expression of NTR1 protein through inhibition of protein translation. Thus, to determine the effect of PNA treatment on expression of this receptor, animals that received AS-NTR1 PNA by i.p. delivery were sacrificed 24 hr after injection for use in binding assays to measure levels of receptor binding sites. The PAG, the rest of brain, and portions of the small intestine (jejunum and ileum) were harvested from these animals. The rest of brain provided tissue to measure further efficacy of the PNA treatment, and the small intestine was selected because it is a peripheral site containing both NT and morphine binding sites. These tissues then were prepared and used in well-established binding assays with [^{125}I]NT and [^{3}H]morphine (Table 2). The AS-NTR1-treated animals had a 35%, 40%, and 65% reduction in the number of NTR binding sites in the PAG, rest of brain, and small intestine, respectively, compared with untreated animals ($P < 0.05$ in all cases). In these animals, there was no significant difference in the number of morphine binding sites in the rest of brain or the small intestine compared with controls. These brain binding data agree well with those published previously for PNAs injected directly into brain (6). In addition, these results again indicated the specificity of the PNA effects. Finally, these data showed that PNA delivered i.p. crossed not only the BBB, but also the plasma membrane of cells in brain and in the small intestine to reduce specifically protein production.

The Effect of PNAs on mRNA Levels. To explore the possible mechanisms of action of the PNA and to test again the specificity of its effects, the levels of mRNA for the NTR1, the MOR1, and GAPDH in control and PNA-treated animals were measured. Because some mRNAs have diurnal fluctuations, the injection time of these time-course studies was controlled so that the time of day for harvest would be identical in all of the groups. AS-NTR1-treated animals (targeted to mRNA) had no significant change in the ratios of mRNA for NTR1/GAPDH or for MOR1/GAPDH over time (Fig. 6). On the other hand, animals treated with the sense-NTR1 (targeted to DNA; Fig. 6), demonstrated a 50% decrease in the ratio of mRNA for NTR1/GAPDH at 8 hr ($P < 0.05$ vs. control and vs. AS-NTR1 PNA-treated animals at 8 hr), indicating that the PNA treatment was inhibiting transcription of this gene. All mRNA levels were normalized to the housekeeping gene GAPDH to control for efficiency of mRNA extraction and cDNA synthesis. Importantly, there was no effect on the ratios of mRNA for MOR1/GAPDH at any of the times, thus demonstrating that neither the antisense nor sense NTR1 PNA were nonspecifically affecting other genes. The data for sense-NTR1 PNA (targeted to DNA) demonstrated that PNAs were an effective antigene agent *in vivo*.

The antisense PNA treatment did not affect mRNA levels, but did reduce NTR1 protein expression by affecting translation. The mechanism of protein inhibition by PNAs *in vitro* seems to depend on the base content of the PNA, with different targets being sensitive to different base compositions. Both duplexes formed between mixed purine/pyrimidine sequence PNAs and mRNA and triplexes formed by homopyrimidine PNAs and mRNA inhibit translation by steric blocking. Thus, these results are consistent with data from *in vitro* studies (19, 20) showing no activation of RNase H by PNA/RNA complexes, but blockade of protein translation by these complexes.

Table 2. Effect of i.p. antisense PNA treatment targeting the mRNA of NTR on the responses to and the binding sites for NT and morphine in brain and small intestine

Tissue	Treatment	Status of response to:		Binding, % Δ v. control	
		NT	Morphine	NT	Morphine
PAG	Control	Unchanged	Unchanged	–	–
	AS-NTR1	Sig. reduced	Unchanged	−35 ± 9*	–
Rest of brain	Control	Unchanged	Unchanged	–	–
	AS-NTR1	Sig. reduced	Unchanged	−40 ± 1*	2 ± 2
Small intestine	Control	–	–	–	–
	AS-NTR1	–	–	−65 ± 5*	−7 ± 8

*, $P < 0.05$.

To show more directly the specificity of binding of the PNA to mRNA, AS-NTR1 PNA, mismatch AS-NTR1 PNA, or no PNA was incubated with total RNA isolated from the brain of an untreated rat (the sense-NTR1 PNA was not included because it had complementary sequences to the gene-specific cDNA primer) to test for the ability of these PNAs to inhibit reverse transcription. Samples incubated with the mismatch AS-NTR1 PNA or with no PNA had no effect on reverse transcription, whereas there was a 65% decrease in product formed with the AS-NTR1 PNA (data not shown). Appropriate controls (cRNA standards for the target gene and GAPDH) were included to monitor the efficiency of cDNA synthesis and PCR product formation. These results showing that a duplex-forming PNA can inhibit reverse transcriptase are in agreement with those from others (21). Importantly, these behavioral, binding, and mRNA results address all of the criteria established to prove true antisense mechanisms, that is, a direct measurement of the targeted protein, a measurement of a nontargeted protein, and a mismatch control (22).

Direct Evidence that PNAs Cross the BBB. The behavioral, physiological, and binding data very strongly suggested that the AS-NTR1 PNA (directed to mRNA) was not only acting in a gene-specific manner, but also was crossing the BBB in its carrier-free form. However, previous research has shown that transport of PNAs across the BBB is negligible (23, 24). Therefore, it was important to show directly that the PNA was in the brain after i.p. injection.

Because only very low levels of PNA in brain were expected, it was necessary to develop a very sensitive gel shift assay to measure the PNA concentrations in brain. This assay is based on the principle that an oligonucleotide bound to a PNA of complementary sequence would migrate differently on a gel than the unbound oligonucleotide (Fig. 7). This method was capable of detecting as little as 50 pg of PNA per 160 mg wet weight of brain tissue (500 pg/whole brain). Animals were injected i.p. with AS-NTR1 PNA (10 mg/kg) and perfused 8 hr postinjection with sterile saline. Even though others have reported that the elimination half-life ($t_{1/2}$) of PNAs in blood is only 29 min (23), perfusion was performed to remove the possibility that any PNA detected was in the blood in capillaries of brain tissue and not actually in the cellular tissue of the brain itself (although no significant difference in PNA levels was found between perfused versus nonperfused animals). Extract from the brain of a treated animal caused a shift in the mobility of a radioactively labeled cDNA oligomer to the same position found when AS-NTR1 PNA was directly added to the probe in the presence of control brain extract (Fig. 7, lane 3). By using a standard curve of AS-NTR1 PNA in control brain extract ($y = 31{,}030x + 375{,}700$; $R^2 = 0.97$) and correcting for recovery of PNA added to brain extract (60%), the PNA level in brain was calculated as 2.4 ± 0.3 ng/whole brain 8 hr after i.p. delivery. Therefore, the gel shift assay provided direct evidence that the PNA had, in fact, crossed the BBB. Importantly, because this gel shift assay was capable of distinguishing the loss of even a single base (data not shown), the results strongly suggested that the recovered PNA from brains of treated animals was in its original, undegraded form.

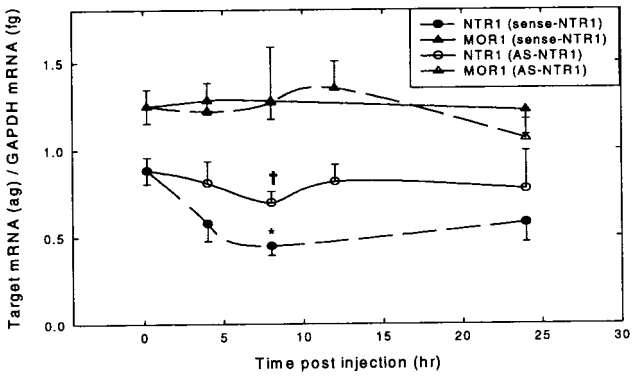

FIG. 6. Effect of antisense PNA to the NTR1 (AS-NTR1) and sense-NTR1 PNA targeted to the NTR1 microinjected directly into brain on mRNA levels of NTR1 and MOR1 over time. Rats were microinjected with either AS-NTR1 PNA (5 μg; open symbols) or sense-NTR1 PNA (5 μg; filled symbols) into the PAG for various times but the PAG harvest time of day was identical for all animals (to avoid possible diurnal variations in mRNA levels). The 0 time represents animals receiving no PNA. Levels of NTR1 (circles) and MOR1 (triangles) mRNA were determined by gene specific reverse transcriptase–PCR and are presented as a ratio normalized to the levels of mRNA for GAPDH. *, $P < 0.05$ vs. control; †, $P < 0.05$ anti-NTR1 (NTR1) 8-hr time point vs. sense-NTR1 (NTR1) 8-hr time point.

DISCUSSION

There are three major findings presented in this work. First, an antisense PNA targeted to the mRNA of the NTR1 and administered i.p. reduced translation of this gene *in vivo* by crossing the BBB. Second, an antigene PNA, directed to DNA of the NTR1,

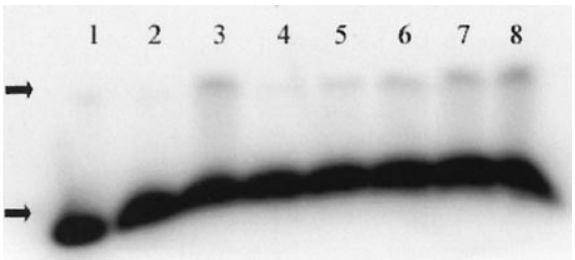

FIG. 7. Detection of antisense PNA to the NTR1 (AS-NTR1) in brains of rats after i.p. injection of PNA. Animals received either saline or 10 mg/kg AS-NTR1 PNA (P1) i.p. Eight hours later these animals were perfused with sterile saline, and brains were harvested and flash-frozen. Brain extract was prepared as described in *Methods*. Standard curves were generated by using control brain extracts to which were added various concentrations of AS-NTR1 PNA. Lane 1, probe alone in Tris-EDTA buffer; lane 2, control brain + probe; lane 3, 10 mg/kg AS-NTR1 PNA treated animal at 8-hr post ip injection + probe. Lanes 4–8 contained control brain extract with probe and 0, 50, 100, 200, and 300 pg of PNA standard, respectively. The top arrow indicates the position of PNA/oligonucleotide probe hybrid, while the bottom arrow indicates the position of excess free oligonucleotide probe.

reduced gene expression *in vivo* by specifically inhibiting transcription of that gene. Third, very small quantities of PNA were required to cause a biological effect that lasted for at least 24 hr.

Before this work, which has focused on the pharmacodynamic effects of PNAs in whole animals, other researchers studying PNAs in animals had focused solely on the pharmacokinetic aspects of PNAs in the body. Based on these pharmacokinetic studies and other *in vitro* studies, concern has been raised about the usefulness of unmodified PNAs as antisense or antigene molecules, because of their poor penetration into cells and their negligible entry into brain. Thus, researchers have been seeking ways to modify PNAs to enhance their transport into cells and across the BBB.

Despite this background, the promise of PNAs for use as antisense agents remained strong. Thus, the present study was undertaken to address the pharmacodynamic effects of unmodified PNAs in brain after i.p. injection into whole animals. Despite the very low brain levels, the antisense PNA produced readily measurable effects at the behavioral, physiological, and biochemical levels. Although the AS-NTR1 PNA was targeted over 100 bases downstream from the initiation codon, it was effective in blocking translation. This result is in contrast to the *in vitro* findings of Knudsen and Nielson (20), but in agreement with the *in vivo* studies by Pooga *et al.* (25). In addition, the antigene (sense-NTR1) PNA, targeted to the nontemplate DNA strand within the coding region, also was shown to have effects after direct injection into brain, including marked reduction in NTR1 mRNA. Preliminary studies indicate that this antigene PNA is also effective after i.p. delivery.

The transport of PNAs across the BBB is a very important finding, because previous research has suggested that unmodified oligonucleotides and PNAs do not cross this barrier (23, 24, 26), unless a malignancy is present that sufficiently disrupts it (27). Although the levels of unmodified PNA obtained in brain are low, these levels clearly caused biological effects. The highly sensitive gel shift assay used in this study allowed for detection as low as 500 pg in a whole brain. Others may not have had sufficiently sensitive assays to detect PNAs in brain. In addition, this study used small PNAs that were not modified at their N terminal end while others, in their aim to monitor the kinetics of PNA delivery, have attached N-terminal linkers and radioactively labeled-tags such as ^{99m}Tc and ^{125}I, which may inhibit the ability of those PNA molecules to cross the BBB.

Although antisense strategies (targeting mRNA) using oligonucleotides have been used effectively *in vivo* in a variety of settings to inhibit specifically gene expression, this report of a sense-NTR1 PNA (targeting DNA) acting as an antigene agent provides evidence of this action *in vivo.* Literature on *in vitro* studies had reported that homopyrimidine PNAs were capable of DNA double-strand invasion, but the composition of the sense-NTR1 PNA was mixed. Thus, whether the DNA and PNA are forming a duplex by Hoogsteen base-pairing or triplex formation is uncertain at this time. However, it is clear that in either case the disruption of transcription is significant and specific to the targeted gene. Next, it should be determined whether sense-NTR1 PNAs are more potent than antisense PNAs, as theoretically, a much smaller amount of PNA would be needed to be effective as an antigene agent.

The decrease in peripheral sites (small intestine) for NTR1 was greater than that in brain. These results suggest that although PNAs are clearly capable of crossing the BBB and entering neuronal cells, these molecules may more readily enter peripheral sites. However, what is very certain is that, by either route, a significant and specific reduction in NT binding sites occurred in direct relation to the nearly complete loss of the respective behavioral and physiological responses to this neuropeptide. The fact that a 35–40% reduction in NT binding sites led to a nearly complete loss in behavioral and physiological responsiveness to NT suggests that the NT/NTR cascade is a threshold phenomenon.

This strategy of selectively studying the roles of specific proteins by PNAs is potentially superior to that of current methods, such as knockout animals. These models are presently restricted to mice and produce animals lacking the protein of interest for the entire time of development. Thus, compensatory mechanisms may occur that produce animals that may not accurately reflect normal development. The use of PNAs may represent a tool for basic science research to more accurately define the roles of certain genes. Finally, this method of using unmodified PNAs injected i.p. represents a powerful strategy for potentially targeting any gene product at any site inside or outside the brain. The ability of unmodified PNAs in general to cross the BBB and have specific effects in brain seems likely, because ongoing studies with AS-MOR1 PNA injected i.p. into rats also showed loss of responsiveness to morphine, but not to NT. Thus, the use of PNAs in a clinical setting may lead to drugs that can treat a multitude of diseases, including those in brain previously thought to be untreatable (e.g., Huntington's disease and Alzheimer's disease).

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