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Specific gene blockade shows that peptide nucleic acids readily enter neuronal cells in vivo

Beth M. Tyler[a,*], Daniel J. McCormick[b], Clark V. Hoshall[a], Christopher L. Douglas[a], Karen Jansen[a], Benjamin W. Lacy[a], Bernadette Cusack[a], Elliott Richelson[a]

[a] *Neuropsychopharmacology, Mayo Clinic, Birdsall Medical Research Building, 4500 San Pablo Rd., Jacksonville, FL 32224, USA*
[b] *Biochemistry and Molecular Biology, Mayo Clinic, 200 First St., SW, Rochester, MN 55905, USA*

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Abstract Peptide nucleic acids (PNAs) are DNA analogs that can hybridize to complementary sequences with high affinity and stability. Here, we report the first evidence of intracellular delivery of PNAs in vivo. Two CNS receptors, an opioid (mu) and a neurotensin (NTR-1), were targeted independently by repeated microinjection of PNAs into the periaqueductal gray. Behavioral responses to neurotensin (antinociception and hypothermia) and morphine (antinociception) were lost in a specific manner. Binding studies confirmed a large reduction in receptor sites. The loss of behavioral responses was long lasting but did fully recover. The implications of specifically and readily turning off gene expression in vivo are profound.

Key words: Peptide nucleic acid; Neurotensin; Morphine; Antinociception; Hypothermia

1. Introduction

Peptide nucleic acids (PNAs) are a relatively new class of molecules that have a neutral polyamide backbone with nucleobases that allow complementary hybridization to DNA or RNA. PNAs are stable against nucleases and proteases, bind independently of salt concentration, and, due to their neutral backbone, have a stronger affinity for nucleic acids than do DNA/DNA duplexes [1]. Additionally, PNA/DNA duplexes are less tolerant of mismatches than DNA/DNA duplexes, as a single mismatch in a 15-mer PNA/DNA duplex lowers the T_m on average by 15°C, thus making PNAs much more gene specific than oligomers [2].

In vitro antisense studies targeting a variety of mRNA and DNA sequences have suggested that PNAs are potent inhibitors of protein production. This mechanism, while not completely understood, seems to be dependent on the base content of the PNA: mixed purine/pyrimidine sequences inhibit translation by forming duplexes with mRNA, while homopyrimidines inhibit transcription elongation by forming triplexes with ds DNA [3,4].

With these superior properties of PNAs, initial enthusiasm for their use as antisense or antigene drugs was dampened by the observations that these molecules passed poorly into cells [5,6]. This poor permeability is likely due to their low phospholipid membrane permeability [7]. Although researchers have made derivative forms of PNAs to enhance transport into cells [8,9], there is no information whether these altered PNAs are able to inhibit protein expression.

Despite these reports, we chose to use unmodified PNAs to examine the role of neurotensin receptors in behavioral and physiological responses to neurotensin (NT) in vivo. NT is an endogenous tridecapeptide that is found throughout the mammalian central nervous system. Many studies show that NT is a neurotransmitter or neuromodulator capable of exerting potent effects, including hypothermia and antinociception [10,11]. NT receptors (NTRs) are also distributed heterogeneously in the central nervous system with both high and low affinity (levocabastine-sensitive) sites [12]. NTR-1 (high affinity) has been molecularly cloned from both human and rat cDNA [13–15]. NTR-2 and its species homolog, NTRL, were identified in rat hypothalamic and mouse brain cDNA library, respectively [16,17].

Many groups, including ours, have long hypothesized that the hypothermic and antinociceptive effects of NT are mediated by yet uncharacterized receptor subtypes. This hypothesis is supported by the observation that SR48692, a non-peptide NT antagonist, fails to antagonize the antinociceptive and hypothermic effects of NT [18,19]. SR48692 is much less potent at binding to NTR-2 (IC_{50} = 300 nM) compared to that at NTR-1 (IC_{50} = 5.6 nM) [20]. Additionally, D-Trp^{11}-NT, a potent hypothermic and antinociceptive analog has a relatively high affinity at the NTR-2 (IC_{50} = 25 nM), compared to that at the rat NTR-1 (IC_{50} = 320 nM) [21]. Lastly, we have demonstrated that the levocabastine-sensitive NTR-2, is not likely responsible for the hypothermic and antinociceptive responses of NT [22].

We, therefore, reasoned that if the mechanisms of NT-mediated hypothermia and antinociception were controlled through different NT receptor subtypes, inhibiting production of NTR-1 should have no effect on these NT-mediated responses. However, this would leave us with a negative result, which would be difficult to prove and could be ascribed to lack of transport of the PNA delivery into cells. In addition, no one has shown that PNAs can affect any cellular function. Thus, for a positive control, we also designed and developed PNAs to inhibit production of the mu receptor upon which morphine specifically acts to produce antinociception.

2. Materials and methods

2.1. PNA synthesis

PNAs were synthesized with Fmoc-*N*-(2-aminoethyl) glycyl PNA monomers on an Expidite 8909 Nucleic Acid Synthesizer according

*Corresponding author. Fax: +1 (904) 953-7117.
E-mail: tyler.beth@mayo.edu

Abbreviations: PNA, peptide nucleic acid; NT, neurotensin; NTR, neurotensin receptor; MPE, maximum physiological effect; PAG, periaqueductal gray

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to the chemistry and protocols developed by the manufacturer (Perseptive Biosystems, Inc., Framingham, MA). The exocyclic amines of the bases adenine, guanine, thymine and cytosine of each Fmoc-PNA monomer were protected with the blocking group benzhydryloxycarbonyl (Bhoc). Synthesis of the PNAs (2×2 μmole) was on PAL-Polyethylene Glycol-Polystyrene resin (Perseptive Biosystems) which produces a carboxamide group ($CONH_2$) at the COOH terminus (3′) end. After synthesis, the PNAs were deprotected and removed from the polystyrene-resin by treatment with a mixture of 80% trifluoracetic acid (TFA) containing 20% m-Cresol for 90 min at 22°C. PNAs were then precipitated in diethyl ether, and purified by RP-HPLC on a Vydac C_{18} column at 60°C with a buffer of 0.1% aqueous TFA and a linear gradient of 0.1% TFA containing 80% acetonitrile/20% water. A major peak (at A_{300nm}) was collected, lyophilized, and verified for its correct mass weight by ESI mass spectrometry on a Sciex 165 MS System (Perkin-Elmer, Foster City, CA).

2.2. Animal testing

Male Sprague-Dawley rats (Harlan, Prattville, AL; 180–200 g) were stereotaxically implanted with stainless steel guide cannulae (26 gauge) into the PAG under sodium pentobarbital anesthesia (50 mg/kg, i.p.) as described [23]. The PAG cannulations were performed using the following coordinates −5.6 (posterior) from bregma, lateral 1.0, 5.5 mm down from the dura. The first microinjection did not start until 5–7 days after surgery. Animals were maintained on a normal light/dark cycle and testing occurred during the light cycle.

Using separate animals for each type of PNA, we microinjected the +103 and RMOR in parallel into rats surgically cannulated in the periaqueductal gray (PAG) region of the brain [11]. The PAG was chosen because it is the region of the brain primarily involved in processing nociceptive information and has a high density of NTR-1 and mu receptors [24,25]. Injections of 1.3 nmol in artificial cerebrospinal fluid (ACSF) were given on days −4, −2, 0 and testing with either NT (Mayo Protein Core Facility, Rochester, MN) or morphine (Research Biochemicals Inc., Natick, MA) began on day 1 and continued until the response returned to baseline.

Animals being tested with NT (18 nmol) microinjected into the PAG, had baseline hot plate and body temperatures determined immediately prior to the experiment, while those tested for morphine (5 mg/kg i.p.) response had baseline tail flick scores recorded. Thirty minutes after microinjection of NT, the rat was placed on the hot plate and latency was measured as described [11]. Immediately after the 30 min hot plate trial, body temperature was measured using a thermistor probe inserted 3 cm into the rectum. Animals receiving morphine were tested using the tail flick assay as described [26] except the distal 3 cm of a restrained rat's tail was placed in mineral oil at 60°C and elapsed time was measured until the animal flicked its tail. Hot plate and tail flick assays were scored as the percent of maximum possible effect (% MPE) and calculated using the following equation: % MPE = [(post-drug latency−pre-drug latency)/(cut-off−pre-drug latency)]×100; where the cut-off was 30 s for hot plate and 12 s for tail flick. Cannulated animals were decapitated and the brains removed for verification of the cannula placement by comparison to stereotaxic plates [27]. Only data from animals with a correct cannula placement were included in the study.

2.3. Binding experiments

For NT competition binding assay, homogenates were prepared from freshly obtained PAG and hypothalamus of adult rats as previously described [28]. Competition binding assays were carried out as previously described [29] with the following modifications: binding buffer also contained 0.5% glycerol, 0.01 mM phenylmethylsulfonylfluoride, 7 μM pepstatin, 0.8 μM aprotonin, 12 μM leupeptin, 0.1 mM iodoacetamide and 0.25 mM EDTA (all from Fisher Scientific, Pittsburgh, PA), but did not contain *N*-benzyloxycarbonyl prolyl prolinal. Brain homogenate (1 mg wet wt. of protein) was incubated with [^{125}I]NT (0.2 nM) (NEN, Boston, MA) and increasing concentrations of unlabeled NT in total volume of 100 μl. For the morphine competition binding assay, homogenates and competition binding assays were prepared from freshly obtained PAG of adult rats as previously described [30] with the following modifications: assays were performed in 1 ml 96 well plates, 2.5 mg wet wt. protein per well and 2 nM [^{3}H]morphine (NEN, Boston, MA).

3. Results

We chose to target the mu and the NTR-1 at the genomic level as well as at the mRNA level by designing PNAs that could bind to either mRNA or the parallel DNA sequence. The PNA to the mu receptor (RMOR) was pyrimidine rich to aid in ds DNA strand invasion, while the PNA to the NTR-1 (+103) had mixed types of bases. Previous antisense work on the mu receptor elucidated an effective exon target, so we designed our PNA to bind to this region [26]. The NTR-1 gene has been molecularly cloned and we targeted our PNA to an area starting +103 bp from the start codon (within the coding region), an area predicted to have high secondary structure. The scrambled control (SCR) had the same number and type of bases as the RMOR, only randomly ordered (Table 1).

Rats given +103 and tested with NT had antinociceptive responses at approximately 10% MPE ($P < 0.001$ vs. NT alone; Fig. 1A) and body temperature change of approximately −0.4°C ($P < 0.001$ vs. NT alone; Fig. 1B). RMOR treated rats tested with morphine scored approximately 30% MPE ($P < 0.001$ vs. morphine alone; Fig. 1A). The +103 animals recovered more quickly than the animals treated with RMOR, with a significant return of MPE by day 5 ($P > 0.06$ vs. baseline; Fig. 2) and a significant BT return by day 6 ($P > 0.1$ vs. baseline; Fig. 2), both responses were recovered fully by day 11 ($P > 0.9$ vs. baseline; Fig. 2). The RMOR treated animals took until day 14 to fully recover MPE ($P > 0.9$ vs. baseline; Fig. 2). Thus, for both PNA treated groups, the corresponding response to the drug was significantly decreased but all responses did recover to the baseline level of untreated animals.

In order to determine that these losses were specific to the proteins targeted, animals treated with +103 were tested with morphine, and animals treated with RMOR were tested with NT. There was no significant difference between the antinociceptive or body temperature responses of these groups and animals that received no PNAs ($P > 0.5$; Fig. 1A, B). Additionally, to ensure that there was no nonspecific PNA interaction in the cells, SCR was given to another set of animals in the exact manner described. These animals showed no significant change in their antinociceptive response to morphine compared to controls ($P > 0.3$). Vehicle control, ACSF, animals also had no change in their body temperature and antinociceptive response to NT or morphine compared to untreated control animals ($P > 0.9$).

The specific loss of responses to morphine and NT were likely due to the reduced synthesis of the respective receptors caused by their antisense PNAs. To test this hypothesis, we treated animals with either +103 or RMOR injected into the PAG and tested for binding and receptor number at day 1 (lost response) and compared these data to normal controls (Table 2). The K_Ds for NT in the PAG and hypothalamus and morphine in the PAG were unchanged between untreated

Table 1
PNA sequences and names

Target gene	PNA name	PNA sequence
NTR-1	+103	5′-CATTGCTCAAAC-3′
mu	RMOR	5′-CAGCCTCTTCCTCT-3′
none	SCR (scrambled)	5′-TCCTCCTCCACTTG-3′

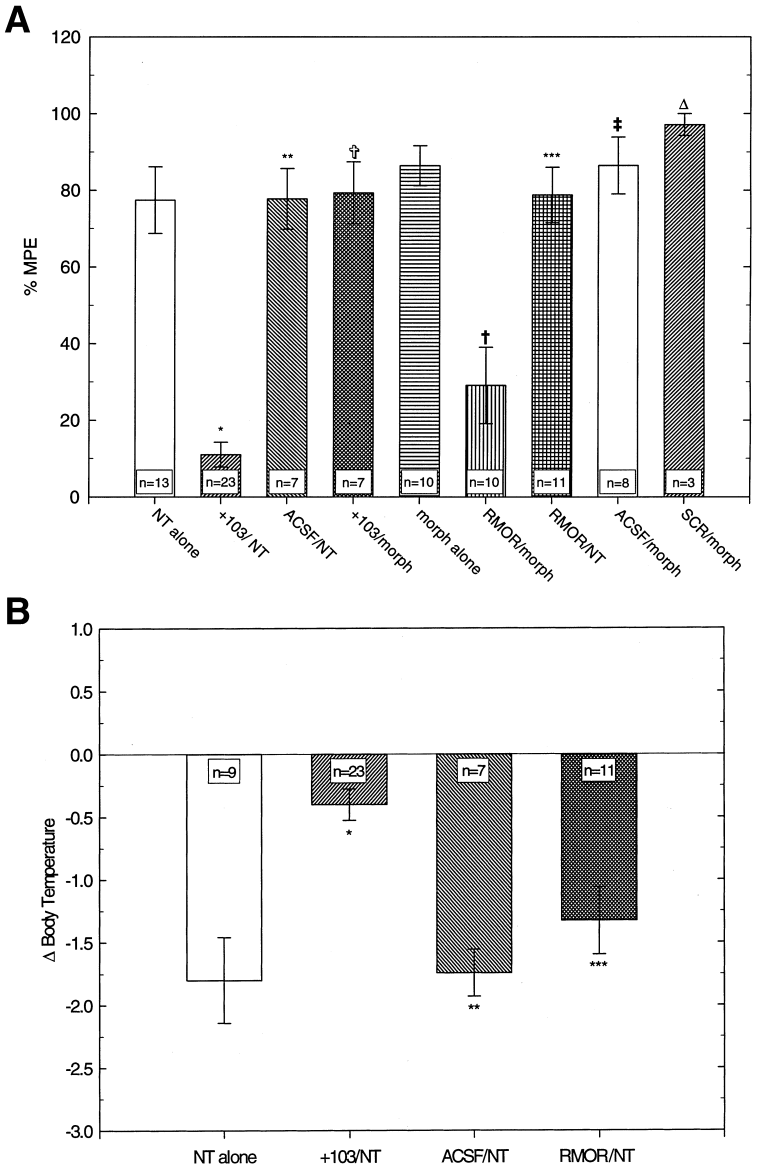

Fig. 1. A: % MPE for untreated, ACSF, +103, or RMOR PNA treated animals. PNA treated animals received 1.3 nmol of either +103 or RMOR microinjected into the PAG on days −4, −2, and 0, and testing was on day 1. Animals were tested for their hot plate antinociceptive response to either NT microinjected into the PAG (18 nmol) or their tail flick antinociceptive response to morphine (5 mg/kg i.p.). Data are reported as % MPE ± S.E.M. as calculated 30 min after drug delivery. The treatment before the '/' indicates pretreatment into the PAG on days −4, −2, 0 while the drug listed after the '/' indicates the drug used for testing. $^{*}P < 0.001$ vs. NT alone, $^{**}P > 0.98$ vs. NT alone, open dagger $P > 0.5$ vs. morphine alone, $\dagger P < 0.001$ vs. morphine alone, $^{***}P > 0.9$ vs. NT alone, $\ddagger P > 0.99$ vs. morphine alone, $\triangle P > 0.3$ vs. morphine alone. B: Body temperature change for untreated, ACSF, +103, or RMOR PNA treated animals. Body temperature was measured using a thermistor probe inserted 3 cm into the rectum prior to and 30 min after NT (18 nmol) microinjection into the PAG. The change in body temperature ± S.E.M. is reported. The treatment before the '/' indicates pretreatment into the PAG on days −4, −2, 0 while the drug listed after the '/' indicates the drug used for testing. $^{*}P < 0.001$ vs. NT alone, $^{**}P > 0.9$ vs. NT alone, $^{***}P > 0.3$ vs. NT alone.

control, vehicle (ACSF), scrambled (SCR) and treated animals who lost their behavioral response (either +103 or RMOR). However, in +103 treated animals who had lost their antinociceptive and hypothermic responses to NT, the number of NT receptor binding sites decreased 39% and 46% in the PAG and hypothalamus, respectively. Importantly, this change in the number of NT receptor binding sites in the hypothalamus indicates the ability of PNAs to diffuse or migrate readily through CNS tissue. Significantly, there was no change in the number of NT receptors in RMOR or SCR treated animals, indicating the specificity of the PNA for the targeted protein. Furthermore, RMOR treated animals showed a large decrease, 53%, in the number of morphine binding sites as compared to control animals. Again, there was no significant difference between the number of morphine binding sites for the SCR treated, ACSF and control animals indicating the effects of the specific PNAs were, indeed, specific.

4. Discussion

Our results were truly novel and surprising for two major reasons: (1) naked PNAs cross cell membranes in vivo and specifically inhibit protein expression and (2) our original hy-

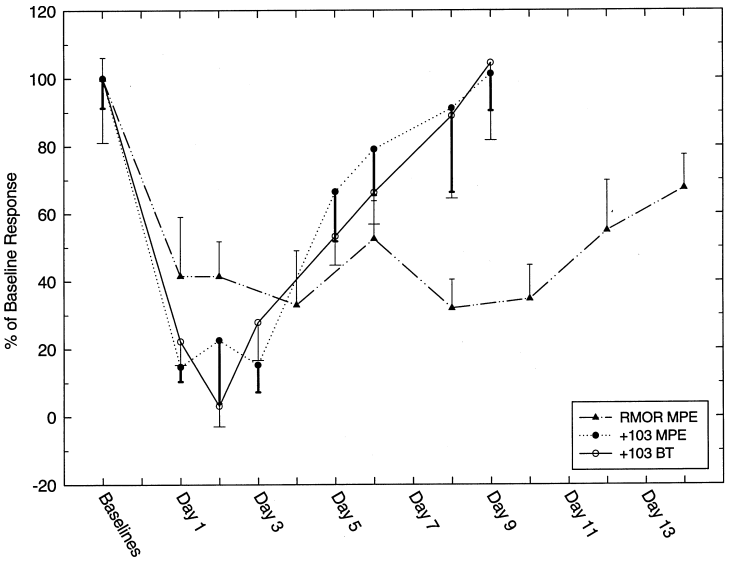

Fig. 2. Time course of hypothermia and antinociceptive responses in +103 or RMOR PNA treated rats. PNA treated animals received 1.3 nmol of either +103 or RMOR microinjected into the PAG on days −4, −2, and 0 and testing began on day 1 and continued until there was no difference compared to untreated animals. Untreated control animals tested with either NT or morphine were used to calculate the baseline responses which were set to 100. Data are reported as a percentage of the baseline response ± S.E.M. as calculated 30 min after drug delivery. +103 treated animals were tested with NT microinjected into the PAG (18 nmol) and were monitored for body temperature (○, $n = 23$) and hot plate MPE scores (●, $n = 23$). RMOR treated animals were tested with morphine (5 mg/kg i.p.) for antinociception using the tail flick assay (▲, $n = 10$).

pothesis that a PNA directed to the NTR-1 would have no effect on NT-mediated behavioral responses was not correct. Recently [31], it was reported that PNAs could cross cell membranes of immortalized and transformed cell lines, but the functional effect of the PNAs was not examined. Others [32] have reported that Technetium-99m-labeled PNAs are able to hybridize in vivo to beads conjugated with the complementary strand, indicating the stability of PNAs in vivo. Another group [9] has examined the transport of ^{125}I-biotinylated PNA with and without a vector, following intravenous administration and reported that the vector-conjugated PNA was able to cross the blood-brain barrier, further indicating their stability within the CNS. However, none of these groups examined the ability of naked PNAs to cross cell membranes in vivo or their effects on protein production. In some cases the PNAs examined were significantly modified and the delivery was systemic. Thus, the key to PNAs crossing cell membranes in vivo may lie in the fact that when left unmodified, these small neutral molecules are able to cross the cell membrane functionally intact. The addition of other molecules to the PNA in order to add a tag, such as a radiolabel, may allow for visualization of the PNA, but may also hinder the transport of the PNA into the cell.

While our initial hypothesis regarding NT-mediated responses may not be correct, we think it is still possible that another NT receptor subtype is responsible for its hypothermic and antinociceptive effects. In fact, all the evidence to date still strongly suggests that there are other NT receptor subtypes responsible for these NT-mediated effects. The antisense PNA directed to the NTR-1 was targeted to a coding

Table 2
Comparison of binding results with NT and morphine in neuronal homogenates from rats treated in vivo with PNAs

Pretreatment	Status of response to		NT	NT B_{max}	NT B_{max}	Morphine	RMOR B_{max}	RMOR B_{max}
	NT	Morphine	K_D (nM)	fmol/mg wet wt.	% change vs. control	K_D (nM)	fmol/mg wet wt.	% change vs. control
PAG								
none control	normal	normal	10 ± 1	13.5 ± 0.9	–	2.2 ± 0.2	1.7 ± 0.1	–
ACSF	normal	normal	12 ± 1	14 ± 2	7.4	2.2 ± 0.5	1.6 ± 0.1	−5.9
SCR	normal	normal	12 ± 1	13 ± 1	−0.7	2.1 ± 0.1	1.5 ± 0.1	−12
+103	lost	normal	10 ± 1	8.2 ± 0.8*	−39*	N.D.	N.D.	N.D.
RMOR	normal	lost	12 ± 1	13.4 ± 0.2	−0.7	1.6 ± 0.2	0.8 ± 0.1*	−53*
Hypothalamus								
none control	normal	normal	12 ± 1	16.4 ± 0.9	–			
ACSF	normal	normal	13 ± 1	18 ± 2	7.9			
SCR	normal	normal	14 ± 1	17 ± 1	1.2			
+103	lost	normal	11 ± 1	8.9 ± 0.8*	−46*			
RMOR	normal	lost	13 ± 1	15.7 ± 0.2	−4.3			

*$P < 0.05$ vs. none control.
N.D., not determined.

region of this gene and is only a 12-mer. This short coding region may be conserved between the NT receptor subtypes and thus may be inhibiting the synthesis of more than one subtype of NTR.

The long lasting effects of the PNAs, the complete return of the responses, and a comparison of kinetics between the mu receptor and NTR-1 are intriguing. Obviously, further experiments must be done in order to gain a fuller understanding of how PNAs are blocking protein expression and what happens to the PNAs within the animal and within the cell. The two PNAs examined here were directed against cell surface receptors and the applicability to other types of proteins has yet to be examined. However, it is clear, despite evidence in the literature to the contrary, that PNAs do present a new and potentially powerful method of specifically affecting protein regulation in vivo.

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