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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Communicated by Susan E. Leeman, Boston University School of Medicine, Boston, MA, April 14, 1999 (received for review January 7, 1999)

ABSTRACT Intrapерitoneal 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 hypothalamic 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 antigen strategy, the sense PNA, was shown in vivo to be a potentially effective therapeutic treatment.

METHODS

PNAs were synthesized as described (6) or manually synthesized on a 50 μmol scale by using phosphoramidite resin–polyethylene glycol–PAI (PerSeptive Biosystems, Framingham, MA, polyethylene glycol–polystyrene) resin (1 equivalent) and fluorenylmethoxycarbonyl-protected nucleoside monomers (6 equivalent; PerSeptive Biosystems) in the presence of diisopropyl ether 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 Q column (25 mm × 250 mm) at 55°C. Male Sprague–Dawley rats (Harlan, PratTVille, AL) were injected with PNAs [AS-NTR1, mismatch AS-NTR1, or AS-MOR1 (morphine receptor)] at 10 μmol/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. 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 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 antigen agent in vivo.

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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the following modifications: the assay buffer contained the peptidase inhibitors 1,10 phenanthroline (1 mM) and aprotonin (5 mg/ml) and the radioligand was $^{[125]}$I[N]. 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[N (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 described (7, 8) with the following modifications: the assay 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[N (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 the cDNA Cycle Kit (Invitrogen). 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 CHCl3/MeOH (2:1) and spun briefly at 1,000 × 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 vivo 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'-CATTGTCTCAAA-C3') and the antisense PNA directed to the mRNA of the NTR1 (AS-NTR1; 5'-CATTGTCTCAAA-C3') was used as a control for the specificity of the PNA sequence. Also, sense PNA directed to DNA (sense-NTR1; 5'-TTTGGAGCAAGAC-3') was used as a control for the specificity of the PNA sequence. Also, sense PNA (AS-NTR1; 5'-CAGCTCTCTCTTCT-3') targeted to the mRNA of the MOR subtype 1 (MOR1) was also 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 NT, rats were tested with NT (18 nmol), which was micro-
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 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
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 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$_{50}$ 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 $[^{3}H]MOR1$ 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 antigenic agent in vivo.

The antisense PNA treatment did not affect mRNA levels, but could 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 triplets 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

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Status of response to:</th>
<th>Binding, % Δ v. control</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>NT</td>
<td>Morphine</td>
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<tr>
<td>PAG</td>
<td>Control</td>
<td>Unchanged</td>
<td>Unchanged</td>
</tr>
<tr>
<td></td>
<td>AS-NTR1</td>
<td>Sig. reduced</td>
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<tr>
<td>Rest of brain</td>
<td>Control</td>
<td>–</td>
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<tr>
<td></td>
<td>AS-NTR1</td>
<td>Sig. reduced</td>
<td>Unchanged</td>
</tr>
<tr>
<td>Small intestine</td>
<td>Control</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>AS-NTR1</td>
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*, 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). 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 because it had complementary sequences to the gene-specific cDNA primer 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.

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 antigen PNA, directed to DNA of the NTR1, 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 (t1/2) 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 (γ = 31,030 x + 375,700; R² = 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. 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 Nielsen (20), but in agreement with the in vivo studies by Pooga et al. (25). In addition, the antigen (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 antigen 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). 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 99mTc and 125I, 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, the report of a sense-NTR1 PNA (targeting DNA) acting as an antigenic 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 antigenic 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 neupeptide. 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).

We thank Dr. Mike McKinney for helpful discussions involving detection methods and Dr. John Hardy for reviewing this manuscript. This work was supported by the Mayo Foundation and grants from the U.S. Public Health Service (National Institute of Mental Health and National Institute of Neurological Disorders and Stroke).