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Short Communication

Altering behavioral responses and dopamine transporter protein with antisense peptide nucleic acids

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Abstract

The dopamine transporter (DAT) plays a role in locomotion and is an obligatory target for amphetamines. We designed and synthesized an antisense peptide nucleic acid (PNA) to rat DAT to examine the effect of this antisense molecule on locomotion and on responsiveness to amphetamines. Rats were injected intraperitoneally daily for 9 days with either saline, an antisense DAT PNA, a scrambled DAT PNA, or a mismatch DAT PNA. On days 7 and 9 after initial motility measurements were taken, the animals were challenged with 10 mg/kg of amphetamine and scored for motility. On day 7, there was no significant difference between the baseline levels of activity of any of the groups or their responses to amphetamine. On day 9, the antisense PNA-treated rats showed a statistically significant increase in their resting motility ($P < 0.01$). When these rats were challenged with amphetamine, motility of the saline-, scrambled PNA-, and mismatch PNA-treated animals showed increases of 31-, 36-, and 20-fold, respectively, while the antisense PNA-treated animals showed increases of only 3.4-fold ($P < 0.01$). ELISA results revealed a 32% decrease in striatal DAT in antisense PNA-treated rats compared with the saline, scrambled PNA, and mismatch PNA controls ($P < 0.001$). These results extend our previous findings that brain proteins can be knocked down in a specific manner by antisense molecules administered extracranially. Additionally, these results suggest some novel approaches for the treatment of diseases dependent upon the function of the dopamine transporter.

Keywords: Peptide nucleic acids; Dopamine transporter; Locomotor behavior; Antisense

1. Introduction

The DAT, a member of the Na/Cl-dependent transporters containing 12 transmembrane domains [1], is thought to control both the spatial and temporal activity of released dopamine by rapidly transporting dopamine into presynaptic terminals [2]. The dopamine system has been implicated in the control of locomotion, cognition, and neuroendocrine functions [3]. The DAT is a target of psychoactive drugs as well as drugs of abuse, including cocaine and amphetamines. Other investigators have used knockout mice, in which the DAT gene was inactivated, to establish the critical role of DAT in controlling dopamine levels and locomotion, and its role as an obligatory target for cocaine and amphetamines [2,3]. These knockout mice, in which both copies of the DAT were inactivated, exhibited spontaneous hyperlocomotion and indifference to the behavioral effects of both cocaine and amphetamines. Interestingly, these knockouts still self-administered cocaine [4].

We were interested to see if we could knock down the expression of DAT using antisense methods. Previously, we showed that peptide nucleic acids can be used to knock down expression of receptors in the brain [5,6]. Others have also reported *in vivo* success using PNAs directed at δ-opioid [7] and galanin receptors [8]. In addition, despite problems with poor cellular uptake of PNAs *in vitro*, researchers have successfully targeted a number of other genes with PNAs, such as the bovine papillomavirus E2 protein [9], Ha-*ras* [10], c-*myc* [11,12], and telomerase [13].

For experiments involving antisense strategies, there are several advantages to using PNAs as opposed to traditional oligonucleotides. PNAs are electrically neutral oligomers,

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Abbreviations: DAT, dopamine transporter; and PNA, peptide nucleic acid.

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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 [14,15].

The strategy of selectively studying the roles of specific proteins by the use of PNAs is potentially superior to studying knockout animals, which 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. We were, therefore, eager to apply our antisense PNA strategy to the DAT target in adult rats to see if we could replicate results obtained in knockout mice and expand upon existing knowledge regarding DAT.

2. Materials and methods

2.1. PNA

All PNAs were synthesized on a 200 μmol scale on MBHA resin (Advanced Chemtech) using 3.6 equivalents of HATU [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] and 4 equivalents each of diisopropylethylamine, 2,6-lutidine (as a carboxyl-activating agent), and the protected PNA monomers. Monomers either were synthesized using a modification of a published procedure [16,17] or were purchased directly from PE Biosystems. The monomers employed in the synthesis were Boc-2-aminoethylglycine derivatives of 9*N*-carboxymethyl-*N*6-benzyloxycarbonyladenine, 9*N*-carboxymethyl-*N*2-benzloxycarbonylguanine, 1*N*-carboxymethylthymine, and 1*N*-carboxymethyl-4*N*-benzyloxycarbonylcytosine. The monomers were deprotected with 5% *m*-cresol solution in trifluoroacetic acid. The cleavage of the benzyloxycarbonyl groups and the removal of the PNA were carried out by treatment of the resin-bound PNA with trifluoroacetic acid: trifluoromethanesulfonic acid:*m*-cresol:thioanisole (6:2:1:1, by vol.). HPLC was used to purify each synthesis (Vydac preparative column, C-18, 10 μm particle size, 22 $\times$ 250 mm; and the elution conditions were: 10% B, 90% A to 100% B in 360 min, 8 mL/min: A = 0.1% trifluoroacetic acid, B = 80% acetonitrile, 0.1% trifluoroacetic acid; UV detection at 260 nm). Additionally, an aliquot of each synthesis was verified by ESI mass spectrometry on a Sciex 165 MS System (Perkin-Elmer) and MALDI-TOF (Perseptive BioSystem Voyager DE-STR) in order to confirm the molecular weight. PNA sequences were: antisense PNA DAT (5′-TCT GCT CCT TGA-3′); mismatch PNA (5′-**G**CT **T**CT **T**CT **C**GA-3′) where **bold** indicates the mismatch; and scrambled PNA (5′-AGT CTT CGT CTC-3′). The antisense PNA was targeted to the mRNA of the DAT starting at base 101 within the coding sequence.

2.2. Motility studies

Sprague–Dawley male rats (250 g, Harlin) were injected with one of the three PNAs (antisense, mismatch, or scrambled) to the DAT (10 mg/kg, i.p.) or with saline (vehicle). Following injection, the animals were placed in a plexiglass Opto-Varimax minor motility chamber (Columbus Instruments) for 30 min for habituation. After the habituation, the motility of the rats was recorded for 3 hr. On days 7 and 9, the animals were challenged with 10 mg/kg of amphetamine administered i.p. All groups of animals were monitored in the motility chambers for 3 hr. The results were analyzed by ANOVA using Dunn's method of multiple comparisons, with $P < 0.05$ considered significant.

2.3. Protein studies for DAT

To determine the effect of PNA treatment on DAT protein levels, we analyzed striatal brain tissue from control and PNA-treated rats using an ELISA. Briefly, striatal tissue was homogenized in 10 vol. of PBS, centrifuged, and washed three times at 10,000 *g* for 3 min at 4°. The pellets were washed and resuspended in coating buffer. Aliquots (100 μL) of each sample were used to coat wells of ELISA plates. Plates were incubated overnight at 4° and washed. A rabbit anti-rat DAT antibody (Chemicon) was added, and the plates were incubated for 1 hr at room temperature. The plates were washed again, and a goat anti-rabbit horseradish peroxidase secondary antibody (Jackson Immunoresearch) was added and incubated for 1 hr at room temperature. Plates were washed and developed with the TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories). OD was recorded 30 min later at 650 nm. Protein concentrations were determined by bicinchoninic acid (Pierce), and OD was normalized to protein content.

3. Results and discussion

Figure 1 shows the locomotor results of a subchronic experiment using four groups of rats. Each group was injected daily for 9 days with either saline, antisense DAT PNA, mismatch DAT PNA, or scrambled DAT PNA (10 mg/kg, i.p.). Measurements were taken on days 7 and 9 after initial motility; rats were challenged with 10 mg/kg of amphetamine (i.p.) and scored for motility. The antisense DAT PNA-treated animals showed a trend of increased baseline motility on day 7, but it was not statistically significant. There was no significant difference in the responses of any of the rats to amphetamine on that day. On day 8, animals were not tested for amphetamine, but were allowed to rest; PNA or vehicle injections were still given. On day 9, the antisense PNA-treated rats showed a statistically significant increase in their resting motility levels, compared with that for the saline-, mismatch-, and scrambled PNA-treated animals ($P < 0.01$). In response to a challenge

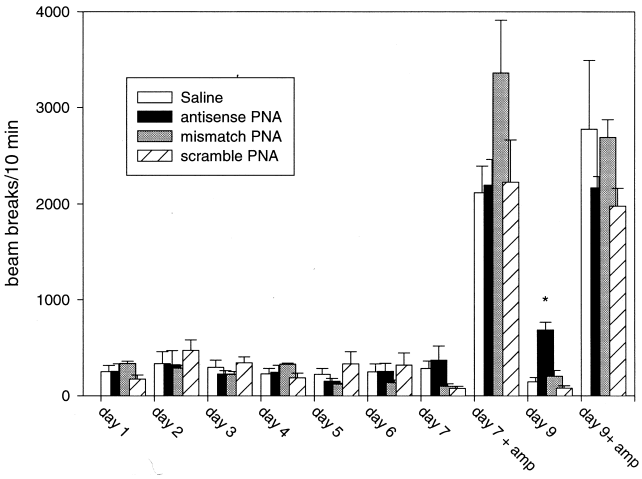

Fig. 1. Motility of rats treated with antisense PNA targeted to dopamine transporter (10 mg/kg, i.p.), mismatch PNA (10 mg/kg, i.p.), scrambled PNA (10mg/kg, i.p.), or saline. Rats (N = 6 for saline and antisense groups; N = 4 for mismatch and scrambled groups) were injected daily for 9 days, and motility was scored for 3 hr post-habituation in an Opto-Varimax minor motility chamber (means ± SEM). Key: (*) $P < 0.01$ vs saline, mismatch, and scrambled PNA.

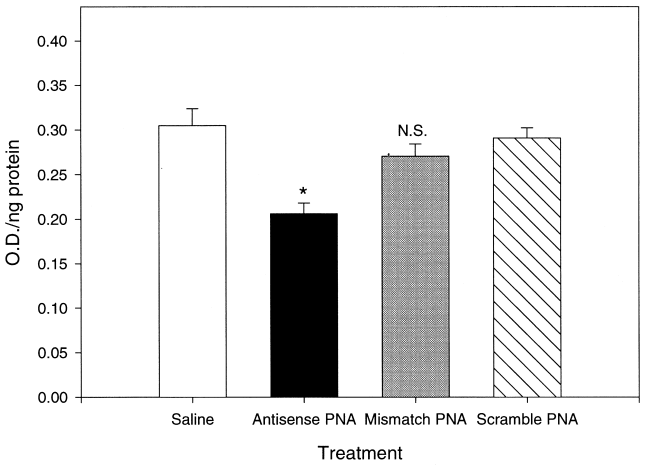

Fig. 3. DAT levels post treatment in the striata of rats treated with antisense DAT PNA, mismatch PNA, scrambled PNA, or saline. Rats were injected for 9 days with antisense PNA, mismatch PNA, scrambled PNA, or saline, and the striata were microdissected from the brain (N = 6 for saline and antisense groups; N = 4 for mismatch and scrambled groups). DAT protein levels were determined by ELISA and were normalized to protein content (means ± SEM). Key: (*) $P < 0.001$ vs saline, mismatch, and scrambled. N.S. = not significant vs saline.

with 10 mg/kg of amphetamine, the saline-, mismatch-, and scrambled PNA-treated rats showed a robust increase in motility of 31-, 20-, and 36-fold above their respective baselines, while the antisense PNA-treated animals showed an increase of only 3.4-fold ($P < 0.01$ vs saline, mismatch, and scrambled) (Fig. 2). No significant difference was found between the saline and mismatch or scrambled groups.

Brains were harvested and striata microdissected from rats treated with saline, antisense PNA, mismatch PNA, or scrambled PNA. Figure 3 shows the results of an ELISA examining DAT levels in striata of these animals. A significant, 32% decrease was found in antisense PNA-treated rats ($P < 0.001$ vs saline-, mismatch-, and scrambled PNA-treated rats). Scrambled PNA- and mismatch PNA-treated animals showed no significant change compared with saline-treated animals.

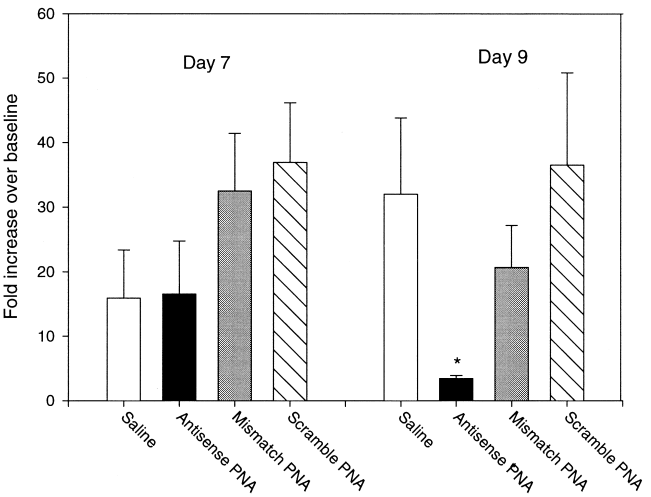

Fig. 2. Motility: fold increase over baseline with amphetamine challenge. Rats (N = 6 for saline and antisense groups; N = 4 for mismatch and scrambled groups) were injected with amphetamine (10 mg/kg, i.p.) on day 7 and day 9. Motility was scored in an Opto-Varimax minor motility chamber, and each group was compared to its resting baseline for fold increase in locomotion (means ± SEM). Key: (*) $P < 0.01$ vs saline, mismatch, and scrambled PNA.

These results suggest that we accomplished our goal to knock down DAT in the brain by treating mature rats extracranially with an antisense PNA targeted to this transporter. Thus, the antisense PNA targeted to DAT produced an effect that phenotypically mimicked knockout DAT animals (mice) [2], since the rats showed an increased basal locomotor activity and a significantly decreased response to amphetamine. Additionally, the rats showed a significant decrease in DAT protein levels in the striatum, an area of the brain rich in dopaminergic nerve endings. This study provides support for results with knockout animals, as these PNA-treated rats exhibited phenotypically similar behaviors, but had the added advantage of normal development up until the time of the antisense treatment.

The antisense PNA acted in a sequence-specific manner, since the mismatch PNA and the scrambled PNA had no statistically significant effect on DAT protein levels, on basal locomotor activity, or locomotor responses to an amphetamine challenge. The length of the effect, the reversibility of the reduction in DAT protein, and behavioral changes will be areas of further experimentation.

A significant increase in basal locomotor activity did not occur until day 9 of treatment at which point the marked reduction in response to amphetamine also occurred. This delay is in accordance with the reported long half-life of the dopamine transporter (at least 5 days) [18], although a recent report using a more direct way of measuring the

turnover rate estimates the turnover to be around 2 days [19]. In any case, the long half-life of the transporter may suggest a lack of an acute regulation of dopamine transmission *in vivo*, which would be consistent with other data that demonstrate a need for chronic stimulant treatment to cause a change in DAT mRNA levels [20].

As with our previous work targeting other proteins [5,6], the extracranially administered (i.p.) antisense PNA to DAT had to cross the blood–brain barrier to cause its biological effects. The ability to selectively turn off a protein, particularly in the brain, is a powerful tool, which may lead to the development of novel therapeutic agents against known and novel targets.

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