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Increased Levels of Proneurotensin/ Neuromedin N mRNA in Rat Striatum and Nucleus Accumbens Induced by 7-OH-DPAT and Nafadotride

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The D_3 dopamine receptor has been proposed as a potential antipsychotic site. In this study, the effects of the D_3-preferring compounds 7-OH-DPAT and nafadotride on levels of proneurotensin/neuromedin N (proNT/N) were assessed. Adult, male, Sprague-Dawley rats were injected subcutaneously (s.c.) with the agonist 7-OH-DPAT (0.1 mg/kg) or antagonist nafadotride (1 mg/kg) at doses previously shown to produce negligible occupancy of D_2 receptors in vivo. *As a positive control, an additional group of animals was treated with haloperidol (3 mg/kg, s.c.). ProNT/N mRNA levels were determined by* in situ *hybridization. 7-OH-DPAT increased proNT/N mRNA in the nucleus accumbens shell. Nafadotride increased proNT/N mRNA levels in the nucleus accumbens shell and dorsomedial caudate nucleus to levels comparable to those produced by haloperidol. Nafadotride also increased proNT/N mRNA in the anterior and dorsal caudate but to a lesser extent than haloperidol. These data indicate that 7-OH-DPAT and nafadotride increase proNT/N mRNA levels in brain areas affected by antipsychotic drugs and suggest that the D_3 receptor may regulate proNT/N mRNA expression in the nucleus accumbens shell.*

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KEY WORDS: *D_3 dopamine receptor; 7-OH-DPAT; Nafadotride; Neurotensin; Striatum; Nucleus accumbens*

The D_3 dopamine receptor, cloned in 1990 by Sokoloff and colleagues, is similar in sequence and pharmacology to the D_2 receptor (Sokoloff et al. 1990). Although present in roughly 10-fold lower density than D_1 or D_2 receptors, the D_3 receptor is of particular interest because it is expressed primarily in brain regions such as the nucleus accumbens, olfactory tubercle, and islands of Calleja (Bouthenet et al. 1991; Bancroft et al. 1998), terminal fields of the mesolimbic dopamine projection that has been hypothesized to mediate psychotic symptoms (Stevens 1973). Unlike the D_2 receptor, only very low densities of D_3 receptors are detected in either the caudate nucleus or the pituitary, brain areas associated with the untoward neurological and endocrine effects, respectively, associated with most conventional antipsychotics. These observations suggest that the D_3 receptor, alone or in conjunction with other receptors, may be a target for novel antipsychotic drugs with an improved side effect profile (Sokoloff et al. 1990).

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Although it is well established that clinically efficacious antipsychotic drugs are dopamine antagonists (for review see Seeman 1981), it is likely that the therapeutic effects of these drugs are not mediated solely by dopamine receptors. Considerable evidence suggests that the peptide neurotransmitter neurotensin (NT) may also play a role in the pharmacological effects of antipsychotic drugs (for review see Nemeroff et al. 1992). Of note, the behavioral and physiological effects of NT in animals are similar to those produced by antipsychotic drugs. Moreover, NT concentrations in the cerebrospinal fluid (CSF) of some schizophrenic patients is lower than that of controls. Treatment of these patients with antipsychotic drugs is associated with increases in CSF NT concentrations to control levels. Similarly, both acute and chronic treatment with typical antipsychotic drugs, such as haloperidol, increase NT concentrations and density of proneurotensin/neuromedin N (proNT/N) mRNA, the mRNA encoding NT, in specific brain regions, most notably the nucleus accumbens and caudate nucleus, of the rat (Govoni et al. 1980; Merchant et al. 1991). These observations suggest that antipsychotic drugs produce an increase in regional NT concentrations that results, at least in part, from an increase in biosynthesis (Merchant et al. 1992a, 1994b).

A previous study using the antipsychotic drug haloperidol suggests that, in contrast to D_2 blockade, blockade of D_3 receptors may decrease levels of proNT/N mRNA (Diaz et al. 1994). This observation suggests that blockade of D_3 sites may ultimately decrease NT neurotransmission in some brain areas. Since the cloning of the D_3 site, several D_3-preferring compounds have been identified including the agonist 7-OH-DPAT and antagonist nafadotride. In this study, the effects of 7-OH-DPAT and nafadotride on proNT/N mRNA levels in rat striatum and nucleus accumbens were assessed. We will show that 7-OH-DPAT increased the density of proNT/N mRNA in the nucleus accumbens shell while nafadotride increased proNT/N mRNA levels in the nucleus accumbens shell and dorsal striatal regions.

MATERIALS AND METHODS

All experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques.

Drug Treatments

Adult, male Sprague Dawley rats (200–250 g; Harlan Sprague-Dawley, Indianapolis, IN; n = 5–7 per group) were injected subcutaneously (s.c.) with 7-OH-DPAT (0.1 mg/kg) or *l*-nafadotride (1 mg/kg) in saline vehicle. As a positive control and for the purposes of comparison, an additional group of rats was treated with haloperidol (3 mg/kg, s.c.) in 0.3% tartaric acid vehicle. Control animals were treated with 0.3% tartaric acid in saline. All drugs were administered in a volume of 1 ml/kg. Rats were sacrificed by decapitation three hours after drug treatment. Maximal induction of proNT/T mRNA by haloperidol has been previously shown to occur within three hours after treatment (Merchant et al. 1992b). Given the relatively short half-lives of 7-OH-DPAT and nafadotride in previous studies (Levant et al. 1996; Levant and Vansell 1997), the three hour time point was selected for all compounds tested. Following decapitation, brains were rapidly removed, frozen in isopentane, and stored at −70°C.

In Situ Hybridization Histochemistry

Details of proNT/T mRNA detection by *in situ* hybridization histochemistry are published previously (Merchant et al. 1992b). Briefly, coronal sections (20 μm) were cut at the level of the nucleus accumbens and caudate-putamen (Bregma 2.20 mm-Bregma 0.9 mm) using Leica Jung Frigocut 2800N motor-driven cryostat and thaw-mounted, two per slide, onto Superfrost/Plus glass slides (Fisher, Pittsburgh, PA). Slide-mounted sections were post-fixed in paraformaldehyde, acetylated, delipidated, and dehydrated. Sections were then hybridized overnight with a saturating concentration of a ^{35}S-labeled cRNA probe complementary to nucleotides 626–961 of the proNT/N mRNA. Hybridization solution was made up in Tris-EDTA buffer (pH 7.4) containing 0.3 M NaCl and 50% formamide. After an overnight incubation in a humid chamber, sections were treated with RNase A prior to high stringency washes in 0.1X SSC at 55°C. Slides were then dehydrated through a graded alcohol series and air dried.

Slides were apposed to Kodak Bio-Max film (Eastman-Kodak, Rochester, NY) for seven days. Films were developed according to the manufacturer's instructions. Slides were subsequently coated with Kodak NTB2 emulsion, exposed for three weeks, developed, and counterstained with Cresyl violet for microscopic analysis. A ^{35}S-labeled cRNA sense probe produced negligible hybridization signal (Merchant et al. 1992b).

Data Analysis

Hybridization signal was considered to be specific if it was bilaterally symmetrical and consistent from section to section and from brain to brain. Autoradiograms were quantified according to the methods of Chesselet and Weiss-Wunder (1994). Autoradiographic images were digitized and quantified using the Macintosh-based video densitometry program NIH "Image" version 1.61. An optical density (OD) standard curve was

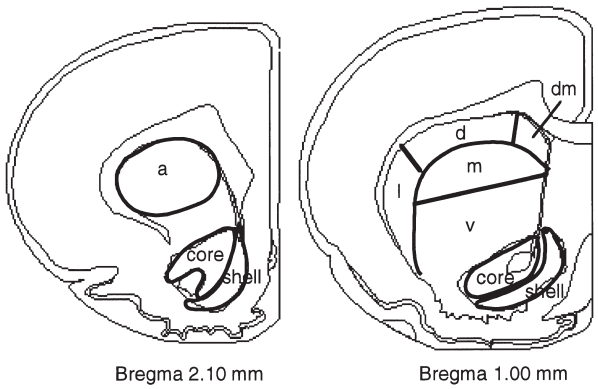

Figure 1. Schematic illustration of brain areas sampled for quantitative analysis of proneurotensin/neuromedin N mRNA. Autoradiograms were quantified by volume analysis in subregions of the caudate-putamen and nucleus accumbens. Hybridization signal optical densities were measured in duplicate sections at four levels through the forebrain with two anterior levels at Bregma 2.20–2.00 mm and two posterior levels at Bregma 1.50–0.9 mm. The nucleus accumbens was divided into shell and core. The caudate-putamen was divided into six subregions: anterior (a), dorsomedial (dm), dorsal (d), lateral (l), medial (m), and ventral (v).

generated using a Kodak Step Tablet. A best-fit curve of optical density was generated using a Rodbard plot. Brain regions were identified in cresyl violet-stained sections according to the atlas of Paxinos and Watson (1986). Autoradiograms were sampled bilaterally. Measurements represent average pixel optical density by volume analysis. Pixel size was calibrated to 5.9 μm^2. Optical densities were measured in duplicate sections at four levels through the forebrain with two anterior levels between Bregma 2.20 and 2.00 mm and two posterior levels between Bregma 1.50 mm and 0.9 mm. Optical density values from each animal were averaged and background hybridization signal subtracted. Background hybridization signal was sampled in frontal cortex where proNT/N mRNA is not expressed. To best describe to effects of the compounds tested on proNT/N mRNA expression in the caudate-putamen, the brain region was subdivided into six subregions for sampling purposes as shown in Figure 1. Because OD values for the two anterior levels and posterior levels were not significantly different, as analyzed by ANOVA (see below), data from the anterior and posterior levels, repsectively, were pooled for subsequent analysis.

Statistics

Quantitative data from autoradiograms are expressed as the mean ± S.E.M. Data were analyzed for statistically significant effects with planned comparisons for each brain region by ANOVA followed by the Student-Newman-Keuls multiple comparisons test. Drug-induced alterations in proNT/N mRNA levels were assumed to be significant at $p < .05$.

Drugs

7-OH-DPAT (7-hydroxy-diphenylaminotetralin) and haloperidol were obtained from RBI, Inc. (Natick, MA). *l*-Nafadotride was generously supplied by Dr. Pierre Sokoloff (INSERM, Paris).

RESULTS

ProNT/N mRNA levels were determined in the striatum and nucleus accumbens of rats treated with the agonist 7-OH-DPAT or the antagonist nafadotride. The effects of 7-OH-DPAT and nafadotride were compared with those of haloperidol.

As previously described (Levant et al. 1992; Merchant et al. 1992a), in vehicle-treated, control animals, proNT/N mRNA hybridization signal was observed primarily in the nucleus accumbens shell (Figure 2). A somewhat lower level of proNT/N mRNA was observed in the nucleus accumbens core. ProNT/N mRNA signal was low to very low in the caudate-putamen of control animals.

In animals treated with the agonist 7-OH-DPAT (0.1 mg/kg), a significant increase in proNT/N mRNA to 150% of control was observed in the nucleus accumbens shell (Figure 3). ProNT/N mRNA levels were not altered in the nucleus accumbens core or caudate-putamen following treatment with the agonist.

Treatment with the antagonist nafadotride (1 mg/kg) induced significant increases in proNT/N mRNA levels in nucleus accumbens and caudate-putamen. In the nucleus accumbens shell, proNT/N mRNA signal was increased to 150% of control (Figure 3). In the caudate-putamen, proNT/N mRNA signal was increased roughly 2- and 4-fold, respectively, in the dorsomedial and dorsal portions of the nucleus relative to control (Figure 4). ProNT/N mRNA levels were not altered in the nucleus accumbens core, anterior, lateral, medial, or ventral caudate-putamen following treatment with the antagonist.

As previously described (Merchant et al. 1991, 1992b), the antipsychotic drug haloperidol produced increases in proNT/N mRNA levels in nucleus and caudate-putamen. ProNT/N mRNA was increased 1.8- and 2.7-fold in the nucleus accumbens shell and core, respectively, relative to controls (Figure 3). ProNT/N mRNA levels in the anterior, dorsal, and lateral caudate-putamen were increased to roughly 10-fold that observed in controls (Figure 4). Increases in proNT/N mRNA signal of 2.5- and 4-fold, respectively, were observed in the dorsomedial and medial portions of the caudate nucleus. The increases in the levels of proNT/N mRNA in the anterior, dorsal, and dorsolateral portions

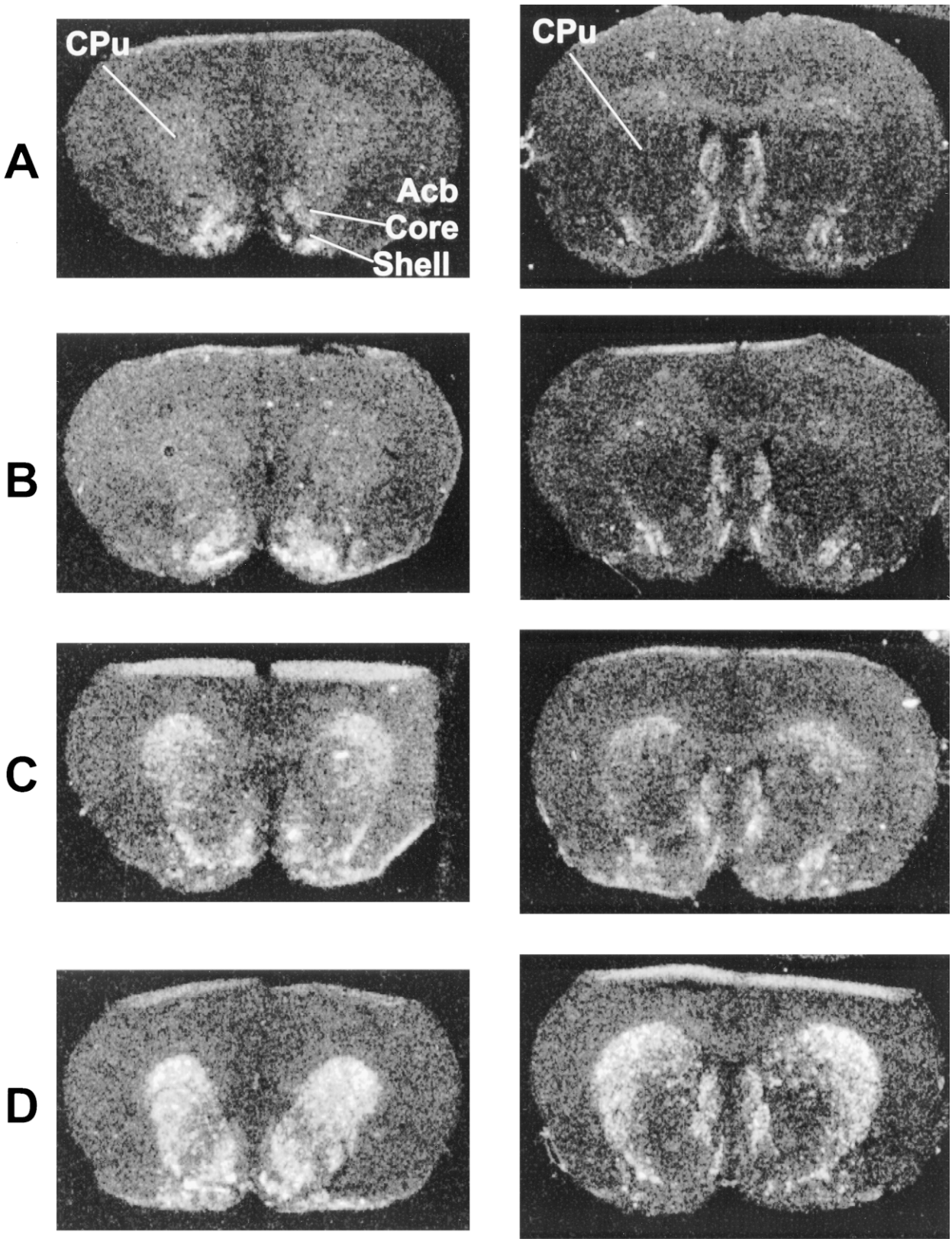

Figure 2. Effects of 7-OH-DPAT, nafadotride, and haloperidol on the density of proneurotensin/neuromedin N mRNA in the caudate-putamen and nucleus accumbens. Rats were treated with (A) vehicle, (B) 7-OH-DPAT (0.1 mg/kg), (C) nafadotride (1 mg/kg), or (D) haloperidol (3 mg/kg). The effects of drug treatment on proNT/N mRNA levels were determined 3 hrs after treatment. *In situ* hybridization for proNT/N mRNA was performed as described in "Material and Methods." Anterior sections shown are at approximately Bregma 2.10 mm, posterior sections at approximately Bregma 1.00 mm.

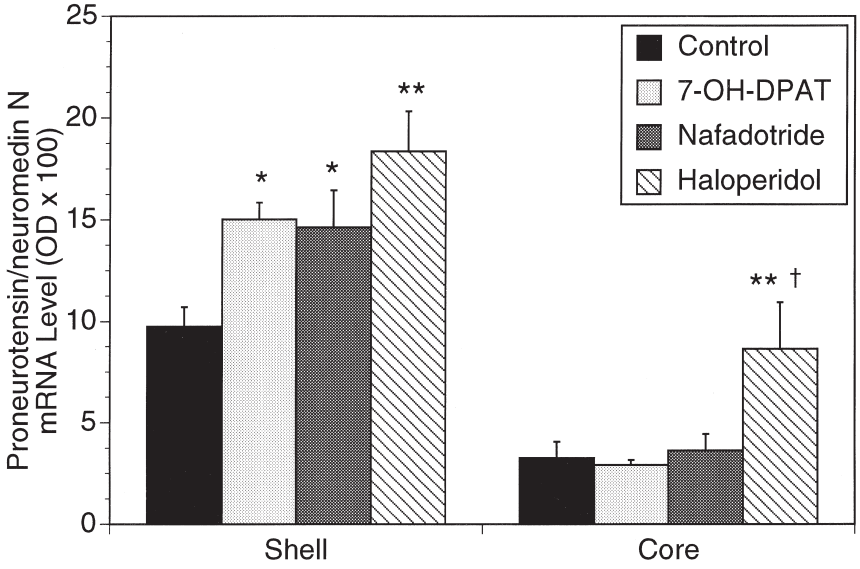

Figure 3. Quantification of the effects of 7-OH-DPAT, nafadotride, and haloperidol on the density of proneurotensin/neuromedin N mRNA in the nucleus accumbens. The intensity of proNT/N mRNA hybridization signal was quantified by volume analysis in subregions of the nucleus accumbens as indicated in Figure 1. Autoradiograms from duplicate brain sections were sampled bilaterally at four levels through the forebrain (Bregma 2.20–0.9 mm). Data represent the mean ± S.E.M.; $n = 5$ to 7 animals per group. *$p < .05$, **$p < .01$ vs. vehicle; †$p < .01$ vs. nafadotride by ANOVA and Student-Newman-Keuls multiple comparisons test.

of the caudate were significantly greater in magnitude than those observed in animals treated with nafadotride. The level of proNT/N mRNA in the ventral portion of the caudate nucleus was not altered by treatment with haloperidol.

DISCUSSION

Modulation of NT and proNT/N mRNA in the striatum and nucleus accumbens by antidopaminergic drugs is well established (for review see Nemeroff et al. 1992); however, the roles of the novel dopamine receptors in modulation of regional NT concentrations has yet to be determined. A previous study using high doses of haloperidol suggested that blockade of the D_3 receptor might decrease levels of proNT/N mRNA in the nucleus accumbens shell (Diaz et al. 1994). Accordingly, the aim of this study was to examine the effects of D_3-preferring compounds on proNT/N mRNA levels in specific brain regions as determined by *in situ* hybridization.

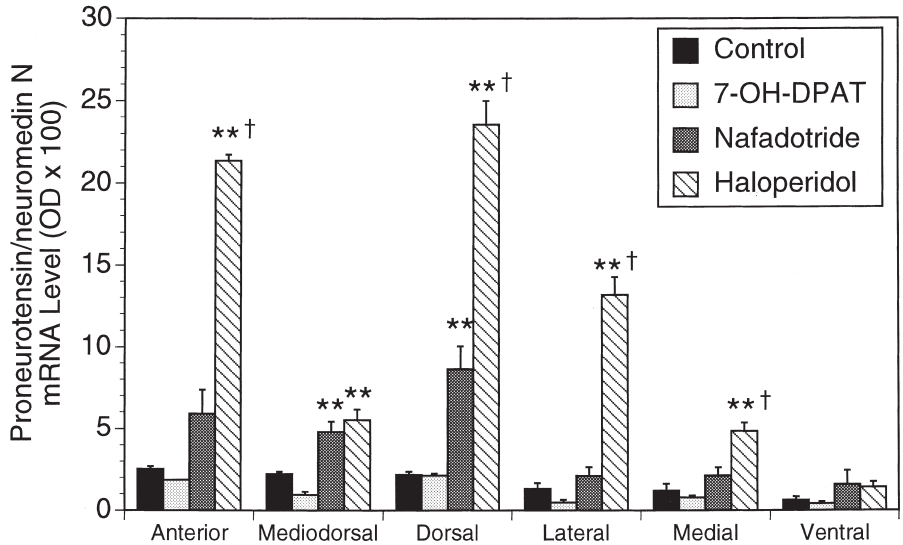

Figure 4. Quantification of the effects of 7-OH-DPAT, nafadotride, and haloperidol on the density of proneurotensin/neuromedin N mRNA in the caudate-putamen. The intensity of proNT/N mRNA hybridization signal was quantified by volume analysis in subregions of the caudate-putamen as indicated in Figure 1. Autoradiograms from duplicate brain sections were sampled bilaterally at four levels through the forebrain (Bregma 2.20–0.9 mm). Data represent the mean ± S.E.M.; $n = 5$ to 7 animals per group. *$p < .05$, **$p < .01$ vs. vehicle; †$p < .01$ vs. nafadotride by ANOVA and Student-Newman-Keuls multiple comparisons test.

One of the primary limiting factors in the study of the functional role of the D_3 receptor has been a lack of selective pharmacological tools. Although several D_3-preferring agonists and antagonists, including 7-OH-DPAT and nafadotride, have been identified, the D_2/D_3 selectivity of these compounds *in vitro* has been controversial (for review see Levant 1997). To aid in elucidating the functional role of the D_3 receptor, we performed *in vivo* occupancy studies to determine doses of 7-OH-DPAT and nafadotride that lack interaction with striatal D_2-like receptors *in vivo* following systemic administration (Levant et al. 1996; Levant and Vansell 1997). These studies indicate that 7-OH-DPAT produced significant occupancy of striatal D_2 receptors only when administered at doses $\geqslant$1 mg/kg (s.c.) in rats; nafadotride, at doses $\geqslant$10 mg/kg (s.c.). Because *in vivo* occupancy studies may underestimate the interaction of a drug with a receptor, the doses selected for use in this study were behaviorally active doses 10-fold lower than the lowest dose required to produce significant occupancy of D_2 receptors. Thus, these doses may be considered to be putatively D_3-selective.

The agonist 7-OH-DPAT, used at a dose that inhibits locomotor activity (Daly and Waddington 1993; Ahlenius and Salmi 1994; Khroyan et al. 1995), produced a significant increase in proNT/N mRNA level selectively in the nucleus accumbens shell. This observation concurs with those of Tremblay et al. (1997) which indicated that D_3 receptor antisense decreased proNT/N mRNA levels in the accumbal shell thus providing additional evidence for a role for the D_3 receptor in the modulation of NT in this brain area. As drug-induced increases in proNT/N mRNA has been shown to precede increases in regional NT concentration (Merchant et al. 1991; Levant et al. 1992), it is likely that this increase in proNT/N mRNA would result in increased NT content in the accumbal shell and ultimately an increase in NT neurotransmission. Interestingly, centrally administered NT also decreases locomotor activity in rats (Rinkel et al. 1983; Elliott et al. 1986). Thus, alterations in NT neurotransmission may play an intermediary role in 7-OH-DPAT-mediated effects on locomotion. Moreover, the effects of 7-OH-DPAT are in clear contrast to the effects of quinpirole, a D_2/D_3 receptor agonist that increases locomotor activity (Dall et al. 1988; Eilam and Szechtman 1989) and decreases NT concentrations in both the nucleus accumbens and striatum (Merchant et al. 1989) further supporting selective actions of 7-OH-DPAT at the D_3 site.

Treatment with the antagonist nafadotride significantly increased proNT/N mRNA levels in the nucleus accumbens shell and the dorsal and mediodorsal striatum. Unlike D_2 receptor antagonists which inhibit locomotor activity in rats, nafadotride, at the dose used in this study, increased locomotor activity (Sautel et al. 1995) suggesting selective activity at D_3 sites which are localized in the mediodorsal striatum as well as in the nucleus accumbens (Bancroft et al. 1998). Likewise, alterations in the activity of ascending pathways from the substantia nigra, which also expresses D_3 sites (Levant 1998), might also contribute to the effects of nafadotride on striatal proNT/N mRNA levels. While it may seem contradictory that an agonist and an antagonist might both increase proNT/N mRNA levels in the accumbal shell, the indirect agonist amphetamine also produces substantial increases in NT concentration and proNT/mRNA levels in the nucleus accumbens and caudate nucleus but in a different population of cells than those affected by haloperidol (Merchant et al. 1987, 1994b). Accordingly, nafadotride and 7-OH-DPAT might increase proNT/mRNA levels in different cell types.

Alternatively, the regional similarity between the effects of nafadotride and haloperidol on proNT/N mRNA levels raises the question of whether this dose of nafadotride is entirely selective for the D_3 receptor. As such, the effects of nafadotride, which exhibits 10-fold D_3/D_2-selectivity *in vitro* (Sautel et al. 1995), on proNT/N mRNA could be due to blockade of D_2 receptors. Of note, nafadotride produced stimulation of locomotor behavior in both D_3 receptor knockout and wild-type mice suggesting possible activity at D_2, or other, receptors (Koeltzow et al. 1998). Should nafadotride prove to be acting at D_2 and D_3 receptors, the smaller magnitude of the increases in proNT/N mRNA levels produced by nafadotride compared to haloperidol could be due to the combined effects of stimulation of proNT/N mRNA expression mediated by D_2 blockade and inhibition of expression mediated by D_3 blockade (Diaz et al. 1994; Tremblay et al. 1997). On the other hand, the differences in the effects of nafadotride and haloperidol may simply be dose-related. The dose of haloperidol used in this study should produce nearly 100% occupancy of D_2-like receptors *in vivo* (Matsubara et al. 1993), and thus produce near-maximal to maximal effects on regional expression to proNT/N mRNA. Because of the limitations imposed in this study by selecting a putatively "D_3-selective" dose of nafadotride, it is likely that the dose selected is blocking a smaller percentage of D_3 and/or D_2 sites. Thus, the haloperidol-mediated increases in proNT/N mRNA in the nucleus accumbens core, and anterior, medial, and lateral caudate, which were not observed following nafadotride treatment, may be the result of greater D_2 receptor blockade. It must be noted, however, although the interaction of haloperidol with the D_2 receptor *in vivo* has been clearly demonstrated (Matsubara et al. 1993), the percentage of D_3 sites occupied *in vivo* by either haloperidol or nafadotride is not known. As such, the contribution of D_3 blockade to the observed effects of either compound on proNT/N mRNA levels cannot currently be discerned.

While the effects of nafadotride and haloperidol on proNT/N mRNA expression were quite similar, it must be noted that although D_2-like receptor antagonists ap-

pear to produce a common pattern alterations in proNT/N mRNA levels or NT content (Nemeroff et al. 1992), the role of D_2 blockade in these effects has not been definitively demonstrated. For example, BMY 14802, a *sigma* site ligand purported to lack dopaminergic activity, produced increases in NT concentration and proNT/N mRNA that were similar to those produced by haloperidol in both regional distribution and magnitude (Levant and Nemeroff 1990; Levant et al. 1992). Likewise, CI-943, a putative antipsychotic with negligible affinity for a variety of sites including dopamine receptors, also increased NT content in nucleus accumbens and caudate-putamen (Levant et al. 1991a). Thus, if the dose of nafadotride used in this study proves to lack activity at D_2 receptors as suggested by behavioral and *in vivo* occupancy studies (Sautel et al. 1995; Levant and Vansell 1997), the effects of nafadotride, and perhaps haloperidol and other antipsychotics, could be mediated by some other, as yet undetermined, mechanism.

Previous studies suggest that antipsychotic drugs produce specific patterns of alterations in regional NT concentrations or levels of proNT/N mRNA. In rats, both acute and chronic treatment with typical antipsychotic drugs, such as haloperidol and chlorpromazine, increase NT concentrations and proNT/N mRNA levels in the nucleus accumbens and striatum (Govoni et al. 1980; Merchant et al. 1991; Levant et al 1991b). These observations suggest that increased concentrations of NT, secondary to increased levels of proNT/N mRNA, is associated with antipsychotic efficacy (Merchant et al. 1992a; Merchant 1994). Interestingly, clozapine, a highly efficacious antipsychotic with negligible incidence of extrapyramidal effects (Gerlach 1991), increases NT concentrations and proNT/N mRNA levels in the nucleus accumbens but not in the caudate nucleus (Kilts et al. 1988; Merchant and Dorsa 1993; Merchant et al. 1992b, 1994a). Accordingly, it has been hypothesized that drug-induced increases in the concentrations of NT or proNT/N mRNA, particularly in the nucleus accumbens, may be associated with antipsychotic activity (Kilts et al. 1988). The results of this study indicate that 7-OH-DPAT and nafadotride alter proNT/N mRNA levels in brain areas associated with antipsychotic activity. The functional relevance of drug-induced alterations in NT expression, however, requires further elucidation. Future study must determine the functional consequences of the increases levels proNT/N mRNA induced by 7-OH-DPAT and nafadotride.

In conclusion, the results of this study demonstrate that the agonist 7-OH-DPAT and antagonist nafadotride increase levels of proNT/N mRNA in specific subregions of rat nucleus accumbens and striatum. Future study must determine the specific relative contributions of D_2, D_3, and perhaps other, receptors in these observations as well as the functional consequences of this modulation of proNT/N mRNA levels.

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