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In Vivo Occupancy of D_2 Dopamine Receptors by Nafadotride

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Nafadotride has been proposed as a selective antagonist for the D_3 dopamine receptor. This drug has been shown to exhibit selectivity between D_2 and D_3 dopamine receptors in in vitro assay systems; however, the in vivo D_2/D_3 selectivity of the compound has not been determined. In this study, protection against inactivation by EEDQ was used as a measure of in vivo occupancy of D_2 receptors by behaviorally relevant doses of nafadotride (0.1–10 mg/kg, SC and IP) in adult, male Sprague-Dawley rats. Ex vivo [^{3}H]spiperone binding was then determined in striatal membranes. l-Nafadotride (10 mg/kg) protected 71% of D_2 receptors after SC administration; 40% after IP administration. Protection of 13% of D_2 receptors was observed at a dose of 3 mg/kg (SC). These data suggest that blockade of D_2 receptors contributes to the pharmacological effects of nafadotride when administered at doses above 1 mg/kg (SC) or 3 mg/kg (IP).

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KEY WORDS: *Nafadotride; [^{3}H]Spiperone; EEDQ (N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline); D_2 dopamine receptor; Striatum; In vivo occupancy*

Numerous pharmacological studies have been performed to date with the aim of elucidating the function(s) of the D_3 dopamine receptor. A member of the family of D_2-like receptors, the D_3 subtype is of particular interest, because its mRNA is expressed preferentially in brain regions such as the nucleus accumbens, olfactory tubercle, and islands of Calleja (Bouthenet et al. 1991; Sokoloff et al. 1990). These brain regions are terminal fields of the mesolimbic dopamine projection, which is hypothesized to be involved in producing psychotic symptoms in schizophrenia. Unlike mRNA for the D_2 receptor, D_3 mRNA is expressed in low density in the caudate/putamen (Bouthenet et al. 1991; Sokoloff et al. 1990). This suggests that the D_3 receptor may be a target for novel antipsychotic drugs that might be free of the extrapyramidal effects.

Until recently, the utility of pharmacological approaches to studying this novel receptor in vivo has been limited by availability of selective compounds. D_3-selective agonists, such as 7-OH-DPAT, have been identified. Numerous studies using these drugs have suggested possible involvement of the site in a variety of behaviors, including the modulation of locomotor activity and the reinforcing properties of cocaine (Daly and Waddington 1993; Caine and Koob 1993; McElroy et al. 1993). A D_3-selective antagonist, nafadotride, has recently been identified (Sautel et al. 1995). This drug exhibits roughly 10-fold higher affinity for D_3 receptors over D_2 in in vitro binding studies in transfected cell lines and produces effects consistent with antagonist activity at dopamine receptors in a variety of in vitro and in vivo assays. Initial studies of the behavioral effects of nafadotride in the rat also indicate the involvement of the D_3 site in the modulation of locomotor activity and further suggest the utility of this compound in the study of D_3-mediated effects.

To adequately interpret the effects of systemically administered drug in terms of activity at specific recep-

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tors, it is essential to determine the sites with which that compound interacts in vivo as well as in vitro. This study uses protection from inactivation by the alkylating agent *N*-ethoxycarbonyl-2-ethoxy-1,2 dihydroquinoline (EEDQ) as a measure of receptor occupancy. This type of assessment has been employed to determine in vivo occupancy of D_2 receptors by antipsychotic drugs (Meller et al. 1985; Nowak et al. 1989; Saller et al. 1989; Matsubara et al. 1993). We have previously used a modification of this approach to assess the occupancy of D_2 receptors by the D_3-selective dopamine agonist 7-OH-DPAT (Levant et al. 1996). This method will now be used to determine the occupancy of D_2 sites after the administration of behaviorally relevant doses of the D_3-selective dopamine antagonist nafadotride. Although it would be desirable to simultaneously assess D_3 receptor occupancy in animals treated with nafadotride, we have found the D_3 site to be insensitive to inactivation by EEDQ (Levant 1995). Hence, the scope of the present study is limited to the D_2 receptor.

MATERIALS AND METHODS

Experimental Procedure

All experiments were carried out in accordance with the declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals. Adult, male, Sprague-Dawley rats (200–300 g; Harlan Labs, Indianapolis, IN) were housed three to a cage with free access to laboratory diet and water. The animal facility was temperature controlled and maintained on a 12-h light/dark cycle. Animals were obtained at least 5 days before experiments and were accustomed to handling.

Groups of four to five rats were pretreated with either nafadotride, haloperidol, or the respective vehicle before treatment with EEDQ. *l*-Nafadotride (0.1–10 mg/kg, SC or IP; Bioprojet, Paris) was administered in 0.9% saline 20 min before treatment with EEDQ. The pretreatment interval was determined on the basis of the detection of overt behavioral effects in preliminary studies. Haloperidol (1 mg/kg, IP; RBI, Natick, MA) was administered in 0.3% tartaric acid 1 h before EEDQ. The dose of EEDQ (Aldrich, Milwaukee, WI) was 10 mg/kg (SC) administered in 50% ethanol. All drugs were administered in a volume of 1 ml/kg. Three hours after treatment with EEDQ, rats were killed by decapitation. Brains were rapidly removed, frozen on dry ice, and stored at −70°C until assayed.

[^{3}H]Spiperone Binding Assays

[^{3}H]Spiperone binding was performed in membranes prepared from caudate/putamen as previously described (Seeman et al. 1985). Striatal tissue was dissected from each brain and homogenized in 20 vol (w/v) of buffer (50 mmol/L Tris-HCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, and 2 mmol/L $CaCl_2$; pH 7.4 at 23°C) using a PRO homogenizer (setting 4 for 10 s). The crude homogenate was centrifuged twice (15 min at 48,000 × *g*), resuspending the pellet each time in 20 vol of buffer. The final pellet was resuspended in buffer to yield a final tissue concentration of 1.5 mg original wet weight/ml. Binding assays were performed in duplicate in polystyrene tubes in a final volume of 0.5 ml. The final concentration of [^{3}H]spiperone (24 Ci/mmol; Amersham, Arlington Heights, IL) was ~100 pmol/L. Nonspecific binding was defined in the presence of 1 μmol/L (+)-butaclamol (RBI, Natick, MA). Binding was initiated by the addition of membrane homogenate. Tubes were incubated for 2 h at 23°C. The reaction was terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester. Filters were washed 3 times with 3 ml ice-cold buffer (50 mmol/L Tris, pH 7.4 at 23°C), and placed in scintillation vials. After the addition of Beckman Ready Protein+ cocktail, vials were shaken, allowed to equilibrate for 2 h, and counted on a Beckman 6500 scintillation counter. Protein concentrations were determined using the BCA method (Pierce, Rockford, IL). Specific [^{3}H]spiperone binding is expressed as fmol/mg protein.

Data Analysis

Receptor protection was calculated from data from the three experimental conditions using the following equation: percent protection = [(drug + EEDQ) − (vehicle + EEDQ)]/[(vehicle + vehicle) − (vehicle + EEDQ)]. Data were analyzed for statistically significant effects by ANOVA followed by Dunnett's test or the Student-Newman-Keuls multiple comparison's test as appropriate. Significance was assumed at $p < .05$.

RESULTS

The ability of the nafadotride to protect D_2 receptors from inactivation by EEDQ was assessed. In preliminary studies *l*-nafadotride (10 mg/kg, SC) produced an inhibition of locomotor activity within 20 min of administration, which persisted for a period of approximately 3 h (data not shown). Because of this relatively short duration of action, a post-EEDQ treatment interval of 3 h was selected for these experiments.

For initial studies, four treatment groups were included in these experiments: vehicle + vehicle (control), drug + vehicle, drug + EEDQ, and vehicle + EEDQ (Figure 1). *l*-Nafadotride (10 mg/kg, IP) produced significant protection of [^{3}H]spiperone binding sites from inactivation by EEDQ. The amount of [^{3}H]spiperone binding detected in animals treated with this dose of *l*-nafadotride followed by vehicle was not significantly

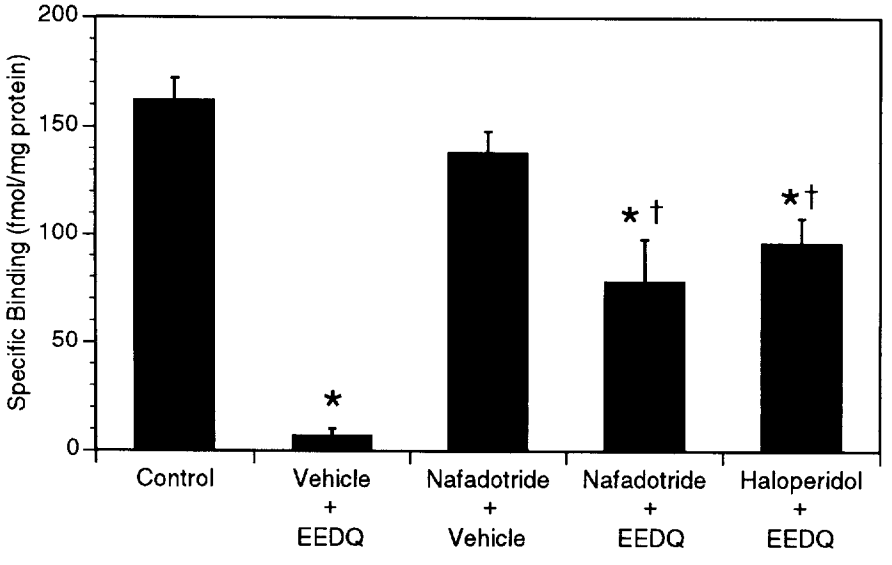

Figure 1. Effects of *l*-nafadotride and EEDQ on ex vivo [^{3}H]spiperone binding and protection of receptors by haloperidol. Rats were pretreated with *l*-nafadotride (10 mg/kg, IP); haloperidol (1 mg/kg, IP) or vehicle before injection with EEDQ (10 mg/kg, SC) or vehicle. Rats were killed 3 h after injection with EEDQ. Striatal membranes were incubated with ~100 pmol/L [^{3}H]spiperone at 23°C for 2 h. Nonspecific binding was defined by 1 μM (+)-butaclamol. Data represent the mean ± SEM (n = 4–5 animals/group). *$p < .01$ vs. control (vehicle + vehicle), †$p < .01$ vs. vehicle + EEDQ by ANOVA and the Student-Newman-Keuls multiple comparisons test.

different from control. This indicates that brain membranes were washed sufficiently to remove any potentially interfering drug before the binding assay. As such, a drug + vehicle treatment group was not included in subsequent experiments. As an additional control, a group of animals was pretreated with haloperidol (1 mg/kg, IP) 1 h before EEDQ. In agreement with previous studies using 4-h and 24-h post-EEDQ intervals (Saller et al. 1989; Levant et al. 1996), haloperidol administration resulted in 57% occupancy of [^{3}H]spiperone labeled sites.

Pretreatment with nafadotride resulted in dose-related protection of [^{3}H]spiperone binding sites (Figure 2). *l*-Nafadotride (10 mg/kg) protected 71% of D_2 receptors after subcutaneous administration. Intraperitoneal administration of the drug (10 mg/kg) protected 40% of [^{3}H]spiperone-labeled sites. Subcutaneous administration of *l*-nafadotride (3 mg/kg) protected 13% of D_2 receptors but failed to reach statistical significance. Negligible protection was observed after administration of *l*-nafadotride at doses below 3 mg/kg (SC) or 10 mg/kg (IP).

DISCUSSION

Initial studies with nafadotride indicate that this putatively D_3-selective antagonist produces differential behavioral effects when administered at low or high doses. For example, in rats, low doses of the drug appear to stimulate locomotor activity, whereas higher doses inhibit locomotion (Sautel et al. 1995). Based on these observations, and those of the effects of D_3-selective agonists, it has been proposed that blockade of D_3 receptors may underlie the stimulatory effects of low doses of nafadotride, whereas the inhibition in locomotor activity, observed at higher doses, results from the nonselective actions of the drug at D_2 receptors. This hypothesis is concordant with the 10-fold D_3/D_2 selectivity reported for the drug in vitro (Sautel et al. 1995). The D_3/D_2 selectivity of the drug in vivo, however, had not been determined. To adequately interpret the effects of systemically administered nafadotride in terms

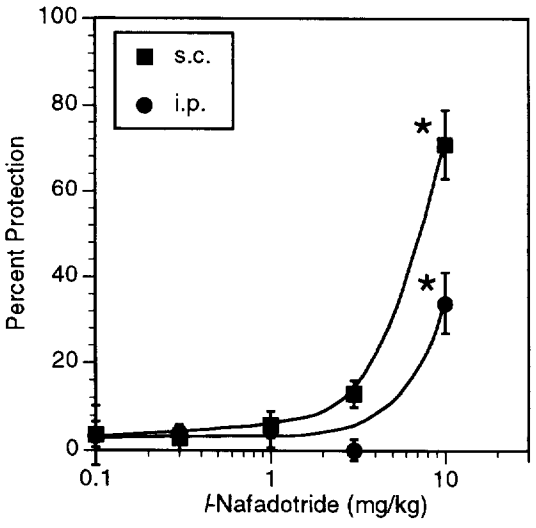

Figure 2. Protection of [^{3}H]spiperone binding sites by *l*-nafadotride. Rats were pretreated with various doses of *l*-nafadotride (0.1–10 mg/kg, SC or IP) or vehicle 20 min before injection with EEDQ (10 mg/kg, SC). Rats were killed 3 h after injection with EEDQ. Striatal membranes were incubated with ~100 pmol/L [^{3}H]spiperone at 23°C for 2 h. Nonspecific binding was defined by 1 μmol/L (+)-butaclamol. Data are expressed as percent protection calculated for each independent experiment as described in Materials and Methods. Data shown represent the mean ± SEM (n = 4–5 animals/group). *$p < .01$ vs. vehicle + EEDQ by ANOVA and Dunnett's test.

of receptor interactions, the selectivity of the compound must be determined in vivo as well as in vitro. The present data provide direct evidence of interactions of nafadotride with D_2 receptors in vivo.

This study used [^{3}H]spiperone binding in striatal membranes to assess the density of D_2 dopamine receptors. Although there is some dispute in the literature regarding the D_2/D_3 selectivity of spiperone, our lab and others have reported significantly higher affinity for this drug at D_2 receptors than D_3 (Sokoloff et al. 1990; Levant and De Souza 1993; Levant et al. 1995). Because there are relatively few D_3 or D_4 receptors in the striatum (for review, see Levant 1996), the contribution of these sites to the [^{3}H]spiperone binding observed in this study is negligible. Thus, the data represent a reasonable assessment of the occupancy of non-D_3, D_2-like receptors and a rational estimate of D_2 receptor occupancy.

The D_3 antagonist *l*-nafadotride produced significant protection of D_2 receptors from inactivation by EEDQ at a dose of 10 mg/kg. Somewhat greater protection of D_2 receptors was observed after subcutaneous administration of the drug than intraperitoneal injection. This difference is most likely due to more extensive first-pass metabolism of the drug after intraperitoneal administration. It has been previously demonstrated that less than 20% receptor occupancy is required for the blockade of dopamine agonist-induced behaviors by D_2 receptor antagonists (Schotte et al. 1993). As such, when administered at a dose of 10 mg/kg, blockade of D_2 receptors is likely to contribute the effects of nafadotride such as catalepsy and decreased locomotor activity (Sautel et al. 1995). Protection of 13% of D_2 sites was observed after treatment with a dose of 3 mg/kg (SC). Whereas this dose did not produce a statistically significant effect, it is likely that effects produced by nafadotride at this dose may also result from the antagonism of the D_2 receptor.

In contrast, negligible D_2 receptor protection was observed after the administration of *l*-nafadotride at the behaviorally active doses of 3 mg/kg (IP) or 1 mg/kg (SC) and below. Although the present data do not directly demonstrate interactions of nafadotride with the D_3 receptor, the findings suggest that the pharmacological effects of these doses of the drug, such as increased locomotor activity (Sautel et al. 1995), may well result from the blockade of these sites. Blockade of the D_3 site may also contribute to catalepsy in a small percentage of animals as well as enhancing the reinforcing properties of food (Sautel et al. 1995; Chaperon and Thiebot 1996). However, there are several limitations on the interpretation of these observations. First, although nafadotride exhibited very low potency at a variety of non-dopaminergic receptors in vitro (Sautel et al. 1995), these findings cannot eliminate the possible activity of the drug at receptors other than D_2 in vivo. Second, the interpretation of the literature in the context of the present data is limited to those studies utilizing subcutaneous or intraperitoneal administration in the rat. Because of pharmacokinetic factors such as absorption and metabolism, these data may not apply to comparable doses administered by other routes or in other species. Third, the present study was limited to the study of striatal membranes. As such, the data may not necessarily reflect D_2 receptor occupancy in other brain regions. Finally, the present data cannot differentiate between protection of D_2 receptors by nafadotride or by an active metabolite(s).

In summary, the present data demonstrate that systemically administered nafadotride produces dose-dependent protection of D_2 dopamine receptors from inactivation by EEDQ. Significant protection of these sites is observed at a dose 10 mg/kg when administered by subcutaneous or intraperitoneal routes. These findings support the interpretation of the biphasic behavioral effects produced by nafadotride (Sautel et al. 1995) and indicate the utility of this drug for further study of the functional role of the D_3 receptor.

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