In Vivo Characterization of a Novel Phenylisothiocyanate Tropane Analog at Monoamine Transporters in Rat Brain

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ABSTRACT

Previous studies have shown that the phenylisothiocyanate tropane analog 2-β-propanoyl-3-β-(2-naphthyl)-8-[4-isothiocyanatophenylethyl] ester hydrochloride; GTP

Although cocaine binds to dopamine, serotonin, and noradrenaline transporters (DAT, SERT, and NET, respectively) with approximately equal affinities, it is hypothesized that an increase in extracellular dopamine levels due to blockade of DAT is responsible for its psychostimulant properties (Ritz et al., 1987). The role of DAT in these effects has been studied in DAT knockout mouse models, which exhibit both a higher level of baseline locomotor activity, as well as a blockade of cocaine-induced locomotor activity (Rocha et al., 1998; Carboni et al., 2001). Recent behavioral studies using a mutant DAT knock-in mouse strategy eliminated cocaine effects of locomotor activity and conditioned place preference (Chen et al., 2006). Therefore, DAT is a primary target to study the mechanisms involved in the psychostimulant properties of cocaine, and it serves as a target for development of therapeutics agents to treat cocaine addiction.

Various structure-activity studies have resulted in the synthesis of cocaine analogs with differing affinities and selectivities at monoamine transporters (Boja et al., 1990; Davies et al., 1993). These analogs have made important contributions to understanding the role of DAT in mediating cocaine effects.

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Various structure-activity studies have resulted in the synthesis of cocaine analogs with differing affinities and selectivities at monoamine transporters (Boja et al., 1990; Davies et al., 1993). These analogs have made important contributions to understanding the role of DAT in mediating cocaine effects.

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ABBREVIATIONS: DAT, dopamine transporter; SERT, serotonin transporter; NET, norepinephrine transporter; SF-23, 2-propanoyl-3-(2-naphthyl) tropine; AD-96-129, 4-(2-diphenylmethoxy)ethyl)-1-benzyl piperidine; GA-II-34, N-(n-butyl)-4-(4'-azido-3'-iodophenyl)-4',4'-difluoro-3-(diphenylmethoxy)tropane (RTI-55) on brain sections obtained 24 h after injection showed a highly localized blockade of binding in striatum, with maximal blockade of binding by 1 to 3 nmol HD-205. Similar blockade of SERT binding (using [3H]citalopram) was observed in the same area. No blockade of DAT or SERT binding was observed after intrastriatal injections of the reversible analog 2-β-propanoyl-3-β-(2-naphthyl)-8-benzyl nortropane (HD-206), and HD-205 treatment had no effect on DAP- and μ-opioid-stimulated guanosine 5′-O-[3H-thio]triphosphate binding in sections from the same animals. In a time course study, rats administered with 1 nmol HD-205 showed recovery of 50% DAT binding after 3 to 4 days postinjection, and full recovery after 6 weeks. Rats implanted with bilateral cannulae were tested for cocaine-induced locomotor activity. Two days after intrastriatal injection of 1 nmol of HD-205, systemic (20 mg/kg i.p.) cocaine-induced locomotor activity was not affected; however, locomotor activity induced by intrastriatal administration of cocaine (6 nmol) was eliminated. This strategy of site-specific chemical blockade of transporters could serve as a valuable tool to evaluate the neuroanatomical basis of DAT-mediated cocaine effects.

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actions. For example, some tropane analogs have been used as radioligands at monoamine transporters, both in vitro (Boja et al., 1991a; Letchworth et al., 2000) and in vivo (Spencer et al., 2007). Other studies have focused on acute (Daunais et al., 1998) and chronic (Porrino et al., 1994; Hemby et al., 1995; O'Connor et al., 2004, 2005) effects of tropane analogs as psychostimulants, and several analogs have been reported to attenuate the effects of cocaine in animal models of psychostimulant abuse (Desai et al., 2005; Carroll et al., 2006).

Synthesis of tropanes using novel schemes involving rhodium catalysts (Davies et al., 1994) has resulted in a particularly diverse set of analogs; one of which, the 2-naphthyl analog WF-23, is one of the most potent ligands at DAT and SERT (Bennett et al., 1995).

One class of tropane analogs is designed as irreversible ligands at monoamine transporters. These compounds include azido-based photoaffinity ligands such as [125I]DEEP, 125I-AD-96-129 (piperazine-based derivatives), and [125I]GA-II-34 (a benzoptine derivative); [125I]RTI-82 and [125I]MFZ-2-24 (tropane derivatives) (Vaughan et al., 2001; Parnas et al., 2008); and isothiocyanate and bromoacetamide analogs such as rimcazole and tropane derivatives (Boja et al., 1991a; Letchworth et al., 2000) and isothiocyanate and bromoacetamide analogs such as rimcazole and tropane derivatives (Boja et al., 1991b; Husbands et al., 1997; Lever et al., 2005). These compounds provide an opportunity to precisely map the location of the binding site of cocaine on DAT. For example, recent studies with photoaffinity ligands [125I]RTI-82 and [125I]MFZ-2-24 have identified TM6 and TM1 as binding sites, respectively, on DAT (Vaughan et al., 2007; Parnas et al., 2008), whereas similar studies with [125I]DEEP and [125I]GA-II-34 have implicated TM1 and TM2 for cocaine binding sites on DAT (Vaughan et al., 2005).

In a recent study (Murthy et al., 2007), we reported on the synthesis and pharmacological properties of HD-205, a phenylisothiocyanate analog of WF-23 (Fig. 1). Consistent with the actions of an irreversible ligand, HD-205 produced wash-resistant blockade of radioligand binding at DAT, SERT, and NET in rat brain membranes. Moreover, its ability to covalently label DAT was confirmed by synthesis of a 125I derivative of HD-205 that labeled DAT protein after SDS gel electrophoresis of rat striatal membranes (Murthy et al., 2007).

Although irreversible analogs have been extensively studied to understand the binding sites of cocaine on DAT, little is known about the in vivo properties of these analogs. Intracranial injection of such compounds could produce a long-lasting (but not permanent) blockade of monoamine transporters and thus provide an opportunity to examine the role of specific transporters in the behavioral effects of cocaine. Such studies would not be practical for photoaffinity agents, but they could be feasible for alkylating agents such as phenylisothiocyanates and bromoacetamides. In one study, the phenylisothiocyanate tropane analog RTI-76 was administered by intrastriatal injection to produce a prolonged blockade of DAT binding in striatal membranes, with a half-life of 6 days (Fleckenstein et al., 1996b). Similar results, with somewhat shorter time course of action, were obtained with i.c.v. administration on RTI-76, which also produced increases in locomotor activity in rats (Kimmel et al., 2000). However, no detailed anatomical studies have been performed to show that irreversible tropanes could be used to trace neuroanatomical loci of cocaine actions in brain.

In the present studies, we examine in vivo effects of the phenylisothiocyanate analog HD-205 after direct injection into rat striatum. Using autoradiography, these results show the localization of the effects of an irreversible tropane analog on transporter binding in striatum. Moreover, these results also demonstrate that intrastriatal administration of an irreversible drug blocks the locomotor effects of cocaine when injected into the same site, thus providing the potential for using this compound to explore the neuroanatomical localization of the role of DAT in mediating behavioral effects of cocaine.

Materials and Methods

Materials. HD-205 and HD-206 were synthesized as described previously (Murthy et al., 2007). [55S]GTPγS (1150 Ci/mmol), [125I]-RTI-55 (2200 Ci/mmol), [3H]citalopram (81.2 Ci/mmol), Kodak BioMax MS films, Kodak BioMax HE TranScrees, and TR tritium-sensitive phosphorimaging screens were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, IL). Phenicillin G procaine was purchased from Butler Vet (Columbus, OH). Intracranial stainless steel guide cannulae were purchased from Plastics One (Roanoke, VA). Citrafol, hydrobromide, DAMGO, 1,3-di-[3H]propoxylxanthine (DPCPX), fluoxetine, GDP, R-[4]-propylnorapomorphine (NPA), and 8-hydroxy-2-propylaminotetralin were obtained from Sigma-Aldrich (St. Louis, MO). X-ray films were obtained from Phenix Research (Hayward, CA). Other chemicals used were reagent grade chemicals from Sigma-Aldrich and Thermo Fisher Scientific (Waltham, MA).

Animals. Male Fisher F-344 rats (Harlan, Indianapolis, IN), 270 to 350 g, were used in all studies. Animals were pair-housed before surgery and singly housed after surgery in a climate-controlled room with a 12-h light/dark cycle. Food and water were available ad libitum except for the locomotor chamber sessions. All animals were adapted to vivarium conditions for 7 days before surgery. Injections and testing occurred during the dark phase of the cycle (5:00 AM–5:00 PM). All procedures were carried out in accordance with established practices as described in the National Institutes of Health Guide for Care and Use of Laboratory Animals, reviewed and approved by the Animal Care and Use Committee of Wake Forest University.

Intracranial Surgery and Drug Treatment. Rats were anesthetized with a combination of atropine methyl nitrate (10.0 mg/kg i.p.) and pentobarbital (50.0 mg/kg i.p.) and placed into a stereotaxic frame (Stoelting, Wood Dale, IL). Cannulae (unilateral cannula for

Fig. 1. Chemical structures of tropane analogs, including cocaine, WF-23, HD-205, and HD-206 (Murthy et al., 2007).
autoradiography studies, and bilateral cannulae in locomotor studies) were implanted into striatum (stereotoxic coordinates: lambda, +7.5 mm, midline, +3.0 mm; depth, −4.5 mm from skull). Cannulae were secured to the skull with stainless steel screws and dental acrylic, and the skin incision was closed with 4.0 chromic gut suture. Animals were administered 75,000 U of penicillin G procaine i.m. and allowed to recover from surgery for at least 7 to 10 days before their involvement in any experiments. Placement of cannulae was verified by initial set of autoradiography experiments with $^{[125I]}$RTI-55 binding (see below).

In all experiments involving intrastratial administration of tropanes, drugs (HD-205 and HD-206) were dissolved in DMSO as vehicle. Cocaine and citoprolam hydrobromide were dissolved in 0.9% saline. For autoradiography experiments, animals received a single unilaterial administration of 1 μl of either HD-205 or HD-206 (at the doses indicated under Results) or vehicle. Rats were euthanatized at various time points after drug administration (1 day to 6 weeks), and brains were removed and frozen for autoradiography as described below. For studies of cocaine-induced locomotor activity, animals received one injection of systemic cocaine administration (20 mg/kg i.p.), and then 2 to 5 days later, they received bilateral intrastratial injections of HD-205 (1 nmol in 1 μl) or vehicle (1 μl). Two days later, rats received another systemic injection of cocaine (20 mg/kg i.p.), and then 5 days later, they received bilateral intrastratial injections of cocaine (2 μg or 6 nl). All of the intrastratial injections were administered via internal cannulae connected to a Hamilton syringe through polyethylene tubing. All injections were administered at a flow rate of 0.2 μl/min and the injector was left in place 5 min after the injection to allow for pressure equilibration.

Radioligand Autoradiography. For autoradiography experiments, rats were euthanatized by rapid decapitation; brains were removed immediately and immersed slowly in isopentane at −40 to −50°C. Coronal sections (20 μm in thickness) were made at the level of striatum using a cryostat at −20°C and thaw-mounted onto gelatin-coated slides. The sections were stored at −80°C until use. In autoradiography experiments, triplicate sections of brain from at least five animals were used for each assay. DAT binding was performed using $^{[125I]}$RTI-55 (Boja et al., 1991a), with fluoxetine to block binding of $^{[125I]}$RTI-55 at SERT (Boja et al., 1992; Tatsumi et al., 1997). Initial results of $^{[125I]}$RTI-55 binding without fluoxetine showed binding outside the striatum in cortical regions; this binding was eliminated by the addition of fluoxetine (data not shown). Sections were preincubated for 10 min in TME buffer and then $^{[3H]}$citalopram (0.4 nM) was added, and the sections were incubated at 25°C for 2 h. Nonspecific binding was assayed at various time points after drug administration (1 day to 6 weeks), and brains were removed and frozen for autoradiography as described below. For studies of cocaine-induced locomotor activity, animals received one injection of systemic cocaine administration (20 mg/kg i.p.), and then 2 to 5 days later, they received bilateral intrastratial injections of HD-205 (1 nmol in 1 μl) or vehicle (1 μl). Two days later, rats received another systemic injection of cocaine (20 mg/kg i.p.), and then 5 days later, they received bilateral intrastratial injections of cocaine (2 μg or 6 nl). All of the intrastratial injections were administered via internal cannulae connected to a Hamilton syringe through polyethylene tubing. All injections were administered at a flow rate of 0.2 μl/min and the injector was left in place 5 min after the injection to allow for pressure equilibration.

Results

Effects of HD-205 on DAT and SERT Binding in Rat Striatum. Our previous studies (Murthy et al., 2007) showed that the phenylisothiocyanate tropane analog HD-205 binds covalently to DAT in rat brain membranes. To determine whether this compound produced irreversible effects on DAT in vivo, we administered HD-205 (as well as the reversible control analog HD-206) directly into rat striatum using unilater all indwelling cannulae, and we examined the resulting blockade of DAT binding by in vitro autoradiography at several time points after injection. Figure 2A shows the results of injection of 1 nmol HD-205 or HD-206 compared with vehicle (DMSO) on $^{[125I]}$RTI-55 binding to DAT, with sections prepared from rats 24 h after injection. Administration of HD-205 produced a highly localized blockade of $^{[125I]}$RTI-55 binding in striatum, whereas injection of HD-206 or vehicle had no discernible effect on $^{[125I]}$RTI-55 binding measured 24 h after injection. The rostral-caudal spread of the effects of HD-205 was examined in multiple adjacent sections on either side of the site of injection (data not shown), which showed decreasing effect of HD-205 until it was virtually absent 600 μm away from the injection site.

To confirm that the effect of HD-205 administration was restricted to monoamine transporter binding, adjacent sec-
administration of 1 nmol of HD-205. Results (Fig. 4) showed highly localized blockade of [3H]citalopram binding in the area of injection with HD-205, similar to blockade of DAT binding observed in the same sections. These results confirm blockade of both SERT and DAT binding by in vivo treatment of HD-205, as predicted from in vitro effects of HD-205 on DAT and SERT (Murthy et al., 2007). Because HD-205 had very weak irreversible effects on NET binding in vitro (Murthy et al., 2007), NET binding was not performed in the present study.

Time Course Effects of HD-205 in Rat Striatum. Irreversible ligands would be expected to produce a prolonged blockade of transporters, but this blockade should not be permanent. To determine the recovery of DAT binding after intrastratal injection of HD-205, rats were sacrificed at different time points (1 day to 6 weeks) after unilateral injection of 1 nmol of HD-205. The results (Fig. 5) are plotted as area of striatum affected by HD-205 administration. The results show that DAT binding does recover in proportion to the time after HD-205 injection, with approximately 50% recovery of DAT binding 3 to 4 days after injection, approximately 80% recovery after 14 days, and full recovery 42 days (6 weeks) after injection. These results confirm that blockade of DAT binding by HD-205 treatment was not permanent, with an estimated half-life of recovery of 3 to 4 days.

Long-term blockade of DAT by intrastratal injection of HD-205 would, in theory, lead to chronic elevated dopamine levels that could potentially produce long-term changes in dopamine receptor response, including desensitization. To test this possibility, brain sections were assayed for agonist-stimulated [35S]GTPγS binding (top), and 1 nmol of HD-206 (1 μl of DMSO, 1 nmol of HD-205 (1 μl), and 1 nmol of HD-206 (1 μl), with sections obtained 24 h after injection. DAT binding was performed using [125I]RTI-55 as described under Materials and Methods. B, lack of effect of HD-205 administration on receptor-stimulated [35S]GTPγS binding. Shown are the representative autoradiograms of sections from rat brains obtained 24 h after injection of HD-205. Coronal sections were assayed for agonist-stimulated [35S]GTPγS binding; D2 (left) using 10 μM NPA and μ-opioid (right) using 10 μM DAMGO.

Fig. 2. Effects of intrastratal injection of HD-205 on DAT binding (top) and μ-opioid- and D2 dopamine-stimulated [35S]GTPγS binding (bottom), in rat brain sections. A, blockade of DAT binding in striatum 24 h after intrastratal injection of HD-205, showing representative coronal sections from rats treated with unilateral injections of 1 μl of DMSO, 1 nmol of HD-205 (1 μl), and 1 nmol of HD-206 (1 μl), with sections obtained 24 h after injection. DAT binding was performed using [125I]RTI-55 as described under Materials and Methods. B, lack of effect of HD-205 administration on receptor-stimulated [35S]GTPγS binding. Shown are the representative autoradiograms of sections from rat brains obtained 24 h after injection of HD-205. Coronal sections were assayed for agonist-stimulated [35S]GTPγS binding; D2 (left) using 10 μM NPA and μ-opioid (right) using 10 μM DAMGO.

Effects of HD-205 on [125I]RTI-55 binding were quantified using densitometric analysis of autoradiograms of brain sections prepared from rats 24 h after administration of various doses (0.001–3 nmol in 1 μl of DMSO) of HD-205. Since these were all unilateral administrations, each animal served as its own control, and results were expressed as percentage of [125I]RTI-55 binding on the contralateral side. Results (Fig. 3, top) showed that increasing doses of HD-205 increased the area of blockade of [125I]RTI-55 binding in striatum, with 1 to 3 nmol of HD-205 providing maximal area of blockade. In contrast, there was no dose-related effect of HD-205 on optical density of binding in the affected area of striatum (Fig. 3, bottom). Representative sections are shown on the right (Fig. 3). These results are consistent with the effects of an irreversible ligand, which at sufficient doses would be predicted to block virtually all of specific [125I]RTI-55 binding within the immediate area of injection.

Our previous results (Murthy et al., 2007) showed that HD-205 produced irreversible blockade of radioligand binding to both DAT and SERT in brain membranes. To determine whether in vivo administration of HD-205 blocked binding to SERT as well as DAT, SERT binding was assayed with [3H]citalopram in brain sections obtained from rats 24 h after administration of 1 nmol of HD-205. Results (Fig. 4) showed highly localized blockade of [3H]citalopram binding in the area of injection with HD-205, similar to blockade of DAT binding observed in the same sections. These results confirm blockade of both SERT and DAT binding by in vivo treatment of HD-205, as predicted from in vitro effects of HD-205 on DAT and SERT (Murthy et al., 2007). Because HD-205 had very weak irreversible effects on NET binding in vitro (Murthy et al., 2007), NET binding was not performed in the present study.
ments (a period in which all rats were tested to make sure that the locomotor activity was completely back to baseline), 1 μl of either DMSO or HD-205 (1 nmol) was administered bilaterally directly into striatum. Both groups were tested for locomotor activity immediately after the intrastriatal injections to test the direct effects of HD-205 on locomotor activity. Results (DMSO/HD-205 direct column in Table 2) showed that, although DMSO had no effect on activity, the direct

Fig. 3. Dose-response effects of HD-205 in vivo. Rats were treated with unilateral intrastriatal injections of various doses (0.001–3 nmol) of HD-205 in 1 μl of DMSO, and sections were obtained 24 h later. DAT binding was determined with [125I]RTI-55 as described under Materials and Methods, and autoradiograms were calculated densitometrically using two different parameters: top, by area (number of pixels) affected; bottom, optical density (plotted as per cent OD of contralateral side in each autoradiogram). Shown on right are typical autoradiograms from rats administered 0.003, 1.0, and 3.0 nmol of HD-205. In all cases, data were obtained from sections at the point of injection, determined by locating the section of maximal area affected in a series of at least 20 serial sections around the point of injection. Data are expressed as mean values ± S.E.M. from triplicate sections from the same rat, using five to six rats.

Fig. 4. Blockade of SERT binding in rat striatum after HD-205 injection. Rats were injected unilaterally with 1 nmol of either HD-205 or HD-206, coronal sections at the level of striatum were obtained 24 h later, and SERT binding was determined with [3H]citalopram as described under Materials and Methods. Shown are representative autoradiograms from rats treated with HD-205 (left) and HD-206 (right).

Fig. 5. Time course of recovery of DAT binding in rat striatum after intrastriatal injection of HD-205 (1 nmol in 1 μl of DMSO). Rats were treated with unilateral injection of HD-205 and coronal sections at the level of striatum were obtained at various time points (1–42 days) after injection. DAT binding was determined with [125I]RTI-55 as described under Materials and Methods, and the area affected by DAT blockade was calculated densitometrically from autoradiograms (see Fig. 3). Data represent mean values ± S.E.M. of area calculations (in pixels) of triplicate autoradiograms from each animal (n = 5–9 rats in each group).
TABLE 1
Long-term (14-day) effect of HD-205 on receptor-stimulated [35S]GTP*S binding

Rats were injected intrastriatally with either 1 nmol of HD-205 in 1 μl of DMSO alone, and then they were euthanized after 14 days and brain sections prepared for autoradiography. Sections were assayed for agonist-stimulated [35S]GTP*S binding, using 10 μM NPA for D2 and 10 μM DAMGO for μ opioid as described under Materials and Methods. Data represent nanocuries per gram of [35S] from densitometric analysis of autoradiograms (with percentage of contralateral side in parentheses) and are mean values ± S.E.M. of triplicate sections of brains of same rat from five HD-205-treated and five DMSO-treated control animals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Receptor</th>
<th>Contrailateral</th>
<th>Ipsilateral</th>
<th>nCi/g</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>D2</td>
<td>37.5 ± 6</td>
<td>41.8 ± 5 (110)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD-205</td>
<td>D2</td>
<td>49.4 ± 8</td>
<td>30.8 ± 8 (80)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>μ</td>
<td>135 ± 15</td>
<td>133 ± 15 (95)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HD-205</td>
<td>μ</td>
<td>146 ± 9</td>
<td>135 ± 11 (93)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05, significantly different from contralateral side (ANOVA).

The effect of HD-205 was not to stimulate activity but, surprisingly, to produce a significant inhibitory effect. Subsequently, activity returned to normal in less than 1 h after injection, and it remained at baseline for 2 days following intrastriatal injections (data not shown). Two days following injections, both groups were administered i.p. cocaine at 20 mg/kg (cocaine i.p. post column in Table 2). This cocaine post-treatment produced the same activity in both groups, which was not different from activity observed in the cocaine pretreatment groups. Both groups were allowed to recover for 2 days (during which time their activities were at baseline; data not shown), and then 6 nmol of cocaine was injected intrastriatally through the same cannulae in which HD-205 or DMSO had been injected 5 days earlier, and rats were tested for locomotor activity immediately. Results (cocaine intrastriatal column in Table 2) showed that intrastriatal cocaine produced significant increase in activity (190% baseline) in the vehicle group; however, in the HD-205-treated rats, intrastriatal cocaine not only produced no increase in activity but actually produced a small but significant decrease in activity compared with baseline. Therefore, the highly localized irreversible blockade of DAT by HD-205 did not affect cocaine-induced activity when cocaine was administered systemically, but it did produce a total block of activity when cocaine was administered in the same location in striatum as HD-205.

Given that HD-205 irreversibly blocks both DAT and SERT in striatum (Fig. 4), it was important to confirm whether effects of HD-205 on cocaine-induced locomotor activity were mediated specifically by DAT. One method to restrict irreversible actions of HD-205 to DAT in vivo and to remove its irreversible actions at SERT, is to pretreat rats with a SERT-specific blocker before injection with HD-205, thus protecting SERT from reaction with HD-205. To accomplish this, rats were injected with citalopram (7.5 mg/kg i.p.), a highly potent SERT blocker (Tatsumi et al., 1997; David et al., 2003). This set of experiments was conducted similar to the previous locomotor studies (Table 2), except that both groups of rats (i.e., DMSO- and HD-205-injected) were pretreated with citalopram (7.5 mg/kg i.p.) 20 min before the intrastriatal injections of HD-205 and DMSO. Table 3 shows the results of locomotor activity in these citalopram-treated rats. These results are essentially the same as those obtained in normal (i.e., not administered citalopram) rats (Table 2). Once again, i.p. cocaine pretreatment demonstrated that all animals responded to systemic cocaine administration with the same increase in activity, whereas bilateral intrastriatal injection of HD-205, 2 days later, had a small inhibitory effect. Furthermore, as observed previously (Table 2), the effects of i.p. cocaine (20 mg/kg) were not affected 2 days after HD-205 injection, whereas the increase in activity observed by intrastriatal injection of cocaine (6 nmol, or 2 μg) in DMSO-treated rats (331% baseline) was completely blocked (104% baseline) in rats injected with HD-205. Therefore, pretreatment of rats with citalopram had no effect on the ability of HD-205 to block intrastriatal effects of cocaine.

TABLE 2
Effects of intrastriatal administration of HD-205 on cocaine-induced locomotor activity

Rats were surgically implanted with bilateral cannulae as described under Materials and Methods, and then after 7 to 10 days recovery, they were pretested for locomotor activity with 20 mg/kg i.p. cocaine (cocaine i.p. PRE column). After 2 days of baseline sessions, rats were injected either with 1 μl of DMSO (n = 6) or 1 nmol of HD-205 in 1 μl of DMSO (n = 6), and they were tested directly for activity (DMSO/HD-205 direct column). After two further days of baseline sessions, rats were injected i.p. with 20 mg/kg cocaine (cocaine i.p. post column in Table 2). Finally, after another 2 days of baseline sessions, rats were injected intrastriatally with 6 nmol of cocaine through the same cannulae (cocaine intrastriatal column). Data are expressed as percentage of baseline activity, and they represent mean values ± S.E.M.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cocaine: i.p. PRE</th>
<th>DMSO or HD-205: Direct</th>
<th>Cocaine: i.p. POST</th>
<th>Cocaine: Intrastriatal 6 nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>980 ± 129g</td>
<td>100 ± 18</td>
<td>896 ± 133i</td>
<td>190 ± 28f</td>
</tr>
<tr>
<td>HD-205</td>
<td>601 ± 115k</td>
<td>51 ± 9i</td>
<td>811 ± 78*</td>
<td>85 ± 8*</td>
</tr>
</tbody>
</table>

* P < 0.05, significantly different from DMSO controls.
† P < 0.05, significantly different from baseline (ANOVA).

TABLE 3
Effects of HD-205 on cocaine-induced locomotor activity after pretreatment with citalopram

Rats were surgically implanted with bilateral cannulae as described under Materials and Methods, and then after 7 to 10 days recovery, they were pretested for locomotor activity with 20 mg/kg i.p. cocaine (cocaine i.p. PRE column). After 2 days of baseline sessions, rats were injected i.p. with citalopram (7.5 mg/kg), and then 20 min later they were injected intrastriatally with either 1 μl of DMSO (n = 5) or 1 nmol of HD-205 in 1 μl of DMSO (n = 6). Locomotor activity was tested directly after HD-205 treatment (direct column). After i.p. injection of 20 mg/kg cocaine (cocaine post column), and after intrastriatal injection of 6 nmol of cocaine (cocaine intrastriatal column), as described in Table 2. Data are expressed as percentage of baseline activity, and they represent mean values ± S.E.M.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cocaine: i.p. PRE + Citalopram, then DMSO or HD-205: Direct</th>
<th>Cocaine: i.p. POST</th>
<th>Cocaine: Intrastriatal 6 nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>820 ± 125i</td>
<td>126 ± 35</td>
<td>983 ± 202i</td>
</tr>
<tr>
<td>HD-205</td>
<td>1062 ± 200i</td>
<td>72 ± 19i</td>
<td>1433 ± 305i</td>
</tr>
</tbody>
</table>

* P < 0.05, significantly different from DMSO controls.
† P < 0.05, significantly different from baseline (ANOVA).
To confirm that this pretreatment with citalopram was effective in blocking irreversible effects of HD-205 at SERT, both DAT and SERT autoradiography was performed in brain sections from these rats immediately after behavioral testing was completed, 5 days after HD-205 or DMSO injection (Fig. 6). Typical autoradiograms of SERT binding (Fig. 6A) show no effect on binding in DMSO-injected rats and a significant localized area of blockade of SERT binding in rats injected with HD-205 alone. In contrast, rats treated with citalopram before injection of HD-205 showed little effect on SERT binding in striatum (Fig. 6A). DAT binding confirmed the specificity of citalopram pretreatment (Fig. 6B): the localized blockade of DAT binding after injection of HD-205 alone was not affected by pretreatment with citalopram before HD-205. Densitometric analysis (Table 4) of autoradiograms of DAT and SERT binding showed that citalopram pretreatment protected approximately 90% of SERT binding sites from alkylation by HD-205, whereas blockade of DAT sites was slightly (but not significantly) reduced by citalopram pretreatment. These results confirm that pretreatment with a specific SERT blocker prevents irreversible effects of HD-205 at SERT.

Discussion

Several irreversible tropanes have been synthesized as covalent ligands for monoamine transporters (Lever et al., 2005), using both photoaffinity and alkylling strategies. Although these compounds have been used for mapping covalent binding sites on DAT (Vaughan et al., 2005, 2007; Parnas et al., 2008), a few studies have shown in vivo effects of irreversible tropanes (Fleckenstein et al., 1996b; Vicentic et al., 1999; Kimmel et al., 2000, 2001). The phenylthiocyanate 2-naphthyl tropane analog HD-205 (Murthy et al., 2007) is an ideal compound for this purpose for several reasons. First, HD-205 is one of the most potent irreversible inhibitors of monoamine transporters, with irreversible IC50 values of 4.1 and 14 nM at DAT and SERT, respectively. Second, since HD-205 uses phenylthiocyanate as an alkylling reagent, it can be administered directly into brain for irreversible blockade of DAT and SERT, unlike photoaffinity analogs where such administration is not practical. This is the first study to report the in vivo effects of an irreversible isothiocyanate at DAT using autoradiography, and to correlate the effects of this blockade on cocaine-induced locomotor activity.

Intrastriatal injection of HD-205 resulted in a highly localized blockade of both DAT and SERT binding in rat striatum, consistent with previous in vitro studies with HD-205 in striatal membranes (Murthy et al., 2007). This highly localized effect is similar to the blockade of μ receptor binding by intrastriatal injection of the μ antagonist 5′-naltrindole-isothiocyanate (Martin et al., 2006), and it may be due to the localized effect of the highly reactive isothiocyanate reacting with other proteins (Murthy et al., 2007). This highly localized effect of HD-205 represents a distinct advantage to provide a neuroanatomical localization of the role of monoamine transporters in mediating cocaine effects in the brain.

The effects of intrastriatal injection of HD-205 were not the result of nonspecific tissue damage, since neither intrastriatal injections of DMSO nor the reversible analog HD-206 had any effects on DAT binding 24 h after injection (Fig. 2A). The specificity of HD-205 was confirmed by showing that HD-205 administration had no effect on D2- and μ-opioid receptor-stimulated [35S]GTPγS binding, using adjacent sections from the same animals 24 h after treatment with HD-205 (Fig. 2B). Interestingly, a decrease in D2-activated G proteins was observed in the injected side 14 days, but not 7 days, after a single injection of HD-205 (Table 1). This seemed to be homologous desensitization (i.e., specific for monoamine receptors), since no effect was observed on μ-stimulated [35S]GTPγS binding in adjacent sections from the same rats. This is consistent with previous reports of desensitization of D2-activated G proteins in striatum after chronic i.p. administration of the potent tropane WF-23 (O’Connor et al., 2004, 2005); in fact, the time course in observing significant desensitization in the D2 system is consistent with these previous results as well.

Increasing doses of HD-205 administration produced increasing areas of blockade of DAT binding. Thus, varying the dose of HD-205 can determine the area affected, and in the future this strategy could be used to vary the portion of brain to be studied in cocaine behavioral studies. It is possible that even larger areas of brain could be affected by intracerebroventricular rather than intrastriatal injections, as shown with RTI-76 (Kimmel et al., 2000); however, the possibility of
nonspecific effects and toxicity may also increase with such injections.

Time course studies revealed that the blockade of DAT binding by intrastral injection of HD-205 was not permanent, with 50% recovery of DAT binding 3 to 4 days after HD-205 treatment, and total recovery on binding in 6 weeks (Fig. 5). These results are consistent with previous studies with the irreversible analog RTI-76 (Fleckenstein et al., 1996b), which showed a half-life of DAT recovery of 6 days after intrastral injection of RTI-76, and a value of 2 to 3 days after i.c.v. administration. These results suggest that optimal studies using HD-205 to examine the role of DAT on cocaine behavioral actions should be accomplished within a week of injection for optimal results. These rates of recovery may reflect the turnover rate of DAT protein in brain; however, this conclusion is complicated by the fact that chronic inhibition of DAT in vivo by cocaine or other tropanes may produce up-regulation of DAT (Tella et al., 1996).

DAT blockers, including cocaine and other tropanes, increase synaptic levels of dopamine and increase locomotor activity (Porrino et al., 1994; Fleckenstein et al., 1996a; Daunais et al., 1998). Indeed, i.c.v. administration of the irreversible analog RTI-76 increased locomotor activity at relatively high doses (Kimmel et al., 2001), but no studies have yet reported the effects of irreversible DAT blockade on in vivo cocaine actions. In the present study, we report that intrastral injection of HD-205 blocked cocaine-induced locomotor activity when cocaine was injected intrastralatly at the same sites, but not after i.p. administration of cocaine. This lack of effect on systemic actions of cocaine can be explained by the highly localized blockade of DAT binding by the irreversible drug effects. With injections of 1 nmol drug in 1-μl volumes, the effect of HD-205 was localized to a small portion of the total volume of striatum, leaving the remaining portions of the basal ganglia unaffected and allowing systemic cocaine to act on the remaining DAT. HD-205 did block activity produced by intrastral injection of a relatively small dose of cocaine (6 nmol), but the effects of HD-205 were reduced when the dose of intrastral cocaine was raised to 24 nmol (data not shown). This is explained by the fact that larger doses of cocaine diffuse further in striatum and can bind to unaffected DAT to increase locomotor activity. Therefore, these results demonstrate that blockade of cocaine effects by HD-205 is not only dependent on the dose of HD-205 used but also on the dose of cocaine.

The effect of HD-205 to block cocaine-induced locomotor activity may have important implications in understanding of cocaine actions at DAT. Interestingly, intrastral HD-205 produced no increase in locomotor activity by itself. This was unexpected, since previous studies showed that a variety of tropanes with different structures inhibit dopamine uptake in synaptosomes, with potencies similar to their binding potencies at DAT (Bennett et al., 1995). Based on these observations, one might predict that HD-205 would act as a psychostimulant by itself, and that it would produce locomotor activity additive to that of cocaine. The lack of effect of HD-205 on activity was not caused by a difference in the doses of cocaine and HD-205 (2 and 13 nmol, respectively); since HD-205 is approximately 40 times more potent than cocaine in binding to DAT (Murthy et al., 2007), there should have been more than sufficient HD-205 present to increase activity. This lack of activity is probably also not associated with any unique effects of the irreversible HD-205 analog on DAT binding: preliminary studies (data not shown) revealed that bilateral intrastral injection of the reversible analog HD-206 (2 nmol) in the same vehicle (DMSO) also had no effect on locomotor activity by itself. At this point, there is no explanation for this lack of effect of HD-205 and HD-206 on locomotor activity, but it could be associated with the fact that these compounds, administered in DMSO, may diffuse through a smaller area in striatum compared with the same volume of cocaine in saline, and thus recruit fewer DAT molecules to activate behavior. These compounds may also inhibit dopamine uptake somewhat less than other tropanes, and future studies will compare the effects of intrastral cocaine and HD-205 on extrastratypic levels of dopamine by microdialysis.

Not only did HD-205 produce no increase in locomotor activity but it also produced a small decrease in activity by itself (Tables 2 and 3). Do these data suggest that HD-205 is actually a sedative rather than a psychostimulant? Although further studies would have to be performed to explore this question, it is more likely that this decrease in activity is a nonspecific action of the drug. First, this decrease in activity is short-lived, with activity returning to baseline levels within a couple of hours after drug administration (data not shown), despite the fact that both DAT and SERT are blocked for several days after HD-205 administration. In addition, previous studies with other alkylation analogs also showed nonselcetive effects of sedation (Keck and Lakoski, 1997). Thus, intracranial administration of an isothiocyanate in DMSO, reacting with many synaptic proteins in addition to DAT and SERT, could produce a temporary loss in activity.

One disadvantage of HD-205 as a pharmacological tool is the fact that it binds with high affinity to both DAT and SERT. To specifically examine the role of DAT in cocaine actions, it is critical to pharmacologically block the irreversible effects of HD-205 at SERT without affecting its reaction with DAT. Our studies using citalopram, administered 20 min before injection of HD-205, showed that alkylation of SERT by HD-205 could be prevented without affecting alkylation of DAT binding. In theory, the reverse experiment could be performed, protecting DAT from alkylation to explore the role of SERT blockade. Therefore, by carefully choosing the site of injection, as well as the dose of HD-205 and cocaine, together with the use of specific blocking agents, these results demonstrate that HD-205 could be used to evaluate the role of discrete populations of DAT and SERT in the psychostimulant and reinforcing actions of cocaine.

References


