Title: Double Dissociation of the Effects of Haloperidol and the Dopamine D3 Receptor Antagonist ABT-127 on Acquisition vs. Expression of Cocaine Conditioned Activity in Rats.

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Running Title: DOPAMINE D2-LIKE RECEPTOR DOUBLE DISSOCIATION

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Non-standard abbreviations: L-DOPA, L-3,4-dihydroxyphenylalanine; ABT-127, 2,3-di-tert-butyl-6-{4-[3-(4,5-dimethyl-4H-[1,2,4] triazol-3-yisulfanyl)-propyl]-piperazin-1-y1]-pyrimidine hydrochloride; Haloperidol (4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidyl]-1-(4-fluorophenyl)-butan-1-one; NSD-1015, 3-hydroxybenzylydrazine dihydrochloride; DMEM, Dulbecco’s Modified Eagle medium; GBL, gamma-butyrolactone 1,4-lactone, 4-butyrolactone; DHBA, 3,4-dihydroxybenzylamine; HPLC, High Performance Liquid Chromatography; DOPAC, 3,4-dihydroxyphenylacetic acid; DA, Dopamine.

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Abstract

Dopamine receptors play a critical role in reward-related learning but receptor subtypes may be differentially involved. D2-preferring receptor antagonists, e.g., haloperidol, attenuate acquisition of cocaine-conditioned motor activity at doses that fail to block expression. We compared haloperidol to the D3 receptor-preferring antagonist 2,3-di-tert-butyl-6-{4-[3-(4,5-dimethyl-4H-[1,2,4] triazol-3-ylsulfanyl)-propyl]-piperazin-1-yl}-pyrimidine hydrochloride (ABT-127), given at D3-receptor selective doses (i.e., no displacement of $[^3H]$raclopride binding, no effects on gamma-butyrolactone-induced striatal L-DOPA accumulation; no attenuation of apomorphine-induced stereotypy). We hypothesized that haloperidol and ABT-127 will produce a doubly dissociable effect on acquisition vs. expression of cocaine-conditioned activity. Rats received three 1-hr habituation sessions to activity monitors followed by three 1-hr cocaine (10 mg/kg) conditioning sessions. The expression phase (no cocaine injections) took place 48 hrs later. Haloperidol (50 $\mu$/kg) given during the conditioning phase blocked the acquisition of conditioned activity but failed to block the expression of conditioning when given on the test day. In contrast, ABT-127 (1.0 mg/kg), when given during conditioning failed to block the acquisition of conditioned activity but blocked the expression of conditioning when administered on the test day. Results suggest that D2 receptors are more critically involved in acquisition than initial expression and D3 receptors are more critically involved in expression than acquisition of conditioned activity based on cocaine.
Introduction

A number of behavioural test procedures have been used to study the role of dopamine (DA) receptors in reward-related learning. One of the easiest and simplest ways to study this type of learning is conditioned activity (Pickens and Crowder 1967). During this procedure the effects of a pro-DA drug such as cocaine are associated with environmental stimuli. In a subsequent test without drug, the stimulant locomotor response evoked by contextual stimuli alone is termed “conditioned activity”. From a reward-related learning point of view, the environment paired with increased DA neurotransmission produced by cocaine acquired an increased ability to elicit approach and other responses during conditioning (acquisition) that was manifested as conditioned activity during test (expression) (Beninger and Hahn, 1983).

DA receptors are of five subtypes grouped into two families, all of which are G-protein coupled (Jaber et al., 1996). The D1-like family includes D1 and D5 receptors and stimulates the second messenger enzyme adenylyl cyclase, and consequently, cyclic adenosine monophosphate (cAMP). Conversely, the D2-like family includes D2, D3 and D4 receptors and inhibits adenylyl cyclase (Anderson and Pierce, 2005).

Treatment with the D2-preferring receptor antagonists, pimozide (Beninger and Hahn, 1983; Beninger and Herz, 1986), haloperidol, raclopride or sulpiride (Fontana et al., 1993; Dias et al., 2006 but see Cervo and Samanin, 1996), but not metoclopramide (Mazurski and Beninger, 1991) given during the conditioning phase, prevented the acquisition of conditioned activity. However, during the expression test, pimozide (Beninger and Hahn, 1983; Beninger and Herz, 1986) and raclopride (Fontana et al., 1993 but see Cervo and Samanin, 1996) failed to block the expression of conditioned
activity. Results suggest that D2 receptors play a more important role in acquiring conditioning than in its expression during the test phase (see Beninger and Banasikowski, 2008).

D3 receptors may play a more important role in the expression than in the acquisition of conditioned activity. The selective D3 antagonist SB-277011 blocked the expression of conditioned activity based on cocaine in mice (Le Foll et al., 2002) or nicotine in rats (Pak et al., 2006). Similarly, the dopamine D3 receptor-preferring partial agonist BP 897 blocked the expression of amphetamine- or cocaine-induced conditioned activity in rats (Aujla et al., 2002; Aujla and Beninger, 2004) and mice (Le Foll et al., 2002). Partial DA agonists are thought to moderately stimulate the receptor during times when endogenous DA tone is lacking or diminished but to antagonize the receptor from full activation during conditions of increased DA release produced by drug-associated cues (Gyertyan et al., 2007). Thus, BP 897 might have acted like a D3 receptor antagonist in these experiments. In the same studies, Aujla et al. (2002) and Aujla and Beninger (2004) found that BP 897 given during the pairing phase had no significant effect on the acquisition of conditioned activity.

The goal of this study was to compare ABT-127, a novel DA D3-preferring receptor antagonist (Drescher et al., 2002; Unger et al., 2005), and haloperidol, a DA D2-preferring receptor antagonist (Vanhauwe et al., 1999), in acquisition and expression of conditioned activity based on cocaine. In the present study, D3-selective doses of ABT-127 were established using tests of apomorphine-induced stereotypy, basal and quinpirole-inhibited striatal L-DOPA accumulation as well as in striatal dopamine D2 receptor occupancy using [3H]raclopride. We hypothesized that systemic treatment with
haloperidol will block acquisition but not expression of conditioned activity and that systemic injections with ABT-127 will block the expression but not the acquisition of conditioned activity based on cocaine.
Methods

Tests of D2 receptor occupancy, L-DOPA accumulation, and apomorphine-induced stereotypy were carried out at Abbott Laboratories using male Sprague Dawley rats. Rats were obtained from Janvier DeGeneste St Isle, France; (body weight of 195 – 245 grams) were housed in a dedicated quiet room maintained on a 12:12 h light-dark schedule (lights on at 06:00 hr), with food and water available ad libitum. Maintenance, compound treatment and sacrificing of animals were done in compliance with the German Animal Protection law (Tierschutzgesetz, Neufassung vom 25.05.1998) and with the EC directive 86/609 (J of the EC no.L358/1 dated 18 December 1986). All experiments were conducted in accordance with Abbott’s Animal Welfare Office and National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines in the facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All animals were treated according to approved protocols, and the experiments were conducted during the light portion of the light/dark cycle.

Conditioned activity studies were carried out at Queen’s University using male Wistar rats. Experimentally naïve albino Wistar rats (N = 161) weighing 200-225 g upon arrival from Charles River Canada (St. Constant QC) were housed in pairs or threes in clear Plexiglas cages (45.0 x 25.0 x 22.0 cm). Average temperature in the colony room was 21°C, humidity 40-70 % with reversed light-dark cycle (lights off from 0700 to 1900 hr). Rats were handled for about 1 min every day for 5 days prior to starting the experiment and were maintained with food (LabDiet 5001, PMI Nutrition International, Brentwood, MO, USA) and water continuously available. Treatment of rats was in
accordance with guidelines of the Animals for Research Act, the Canadian Council on Animal Care, and was approved by the Queen’s University Animal Care Committee.

Drugs

ABT-127 was synthesized at Abbott (Ludwigshafen, Germany) and was dissolved in distilled water. Haloperidol, (4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidy1]-1-(4-fluorophenyl)-butan-1-one (Sigma, St. Louis, MO, USA) was prepared in a 20% water solution of dimethylsulfoxide (DMSO; Sigma, St. Louis, MO, USA). Injections were administered intraperitoneally (i.p.) in a volume of 1 ml/kg. Apomorphine, 6aR-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinoline-10,11-diol (Sigma, St. Louis, MO, USA) was dissolved in 0.5 % ascorbic acid. Quinpirole, 3-hydroxybenzyldrazine dihydrochloride (NSD-1015, 3-hydroxybenzyldrazine dihydrochloride; DHBA, 3,4-dihydroxybenzylamine; from Sigma, Deisenhofen, Germany), and gamma-butyrolactone, 1,4-lactone, 4-butyrolactone (GBL; Aldrich, Steinheim, Germany) were dissolved in distilled water. Cocaine hydrochloride, 3b-Hydroxy-1aH,5aH-tropane-2b-carboxylic acid methyl ester benzoate hydrochloride (Sigma, St. Louis, MO, USA) was dissolved in 0.9 % isotonic saline.

Statistical Analysis:

All statistical tests were carried out using SPSS 16.0 for Windows. Hypothesis tests were completed using $\alpha = 0.05$ and pair-wise comparisons were made with Tukey’s HSD test.

Experiment 1: ABT-127 and haloperidol binding comparison

Cloned rat and human dopamine D3 and D2 receptors were stably expressed in Sf9 cells (rD3) and HEK-293 cells (hD2L, rD2, hD3) that were cultured in Dulbecco's
Modified Eagle Medium (DMEM) NUT MIX F-12 medium supplemented with 10 % fetal calf serum, 1-2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (R15-802). Detached cells were pelleted at 750 x g for 5 min, and cell ghosts (permeabilized cells) were prepared by osmotic shock using lysis buffer containing 50 mM Tris-HCl, 10% glycerol, pH 7.4 for 20 minutes at 4°C, pelleted at 750 x g for 20 minutes at 4°C, and then stored at −80°C until use.

The affinities of the compounds for human and rat dopamine D3 or D2 receptors were measured by competition experiments against 0.1 nM \(^{125}\text{I}\)iodosulpride and 0.01 nM \(^{125}\text{I}\)iodospiperone and 1-5 μg cell protein/tube in a total volume of 0.25 ml (D3) or 1 ml (D2), respectively. Assay buffer contained 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 2 mM MgCl\(_2\), 2 mM CaCl\(_2\), 0.1% BSA; pH 7.4. Non-specific binding was defined with 1 μM spiperone. Radioligand binding reactions were carried out at room temperature for 60 min and were terminated by rapid filtering through Whatman GF/B glass fiber filters followed by 3 ml rinses with ice-cold buffer (50 mM Tris-HCl, pH 7.4). Data derived from liquid scintillation counting were analyzed by iterative non-linear regression analysis (SAS). Fitting was performed according to formulae described by Feldman (1972). The test compounds were analyzed in triplicate at five to eleven concentrations ranging from 0.1 nM to 10 μM using half- or full-log increments in each receptor-binding assay.

**Experiment 2: Rat striatal dopamine D2 receptor occupancy (in vivo displacement of \(^{3}\text{H}\)raclopride)**

Rats were randomly assigned to seven groups (n = 5 per group) and were administered ABT-127 (3, 10 and 30 mg/kg, i.p.), haloperidol (0.5 mg/kg, i.p.) or saline,
followed by high specific activity $[^{3}H]$raclopride ($[^{3}H]3,5$-Dichloro-N-[[2S]-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide; NEN Boston, U.S.A.; 88.2 Curie/mm$^3$, 8 µCi/0.4 ml/animal i.v.) 60 min later. The animals were sacrificed (under slight carbon dioxide anesthesia) 90 min after ABT-127, haloperidol or saline administration. The striata (excluding nucleus accumbens) and the cerebellum of each hemisphere (excluding lobule 9 and 10) were immediately dissected, weighed, dissolved in one ml Soluene-350 (Packard) and transferred into a 60°C water bath for 4 h. Next, 15 ml scintillation fluid (Ultima Gold XR, Packard) was added, and the concentration of accumulated radioactivity (cpm/mg) was determined by liquid scintillation counting (Tricarb 2250 CA, Packard). The ratio of specific (striatal minus cerebellar) to non-specific binding (cerebellar) at the time of sacrifice was taken as a measure of available dopamine D2 receptors. Occupancy (%) in ABT-127- or haloperidol-treated animals was calculated as: Occupancy (%) = 100 x (1 – D2 drug/D2 vehicle).

**Experiment 3: L-DOPA accumulation in the rat striatum**

Rats were assigned to the different treatment groups and received the following compounds: ABT-127 (3.16, 10, 31.6 and 100 mg/kg, i.p.) 90 min before sacrifice, quinpirole (0.1 mg/kg, i.p.) 45 min before sacrifice, and/or NSD-1015 (100 mg/kg, i.p.) immediately followed by GBL (750 mg/kg, i.p.) 30 min before sacrifice. All compounds were dissolved in distilled water. The rats were sacrificed by cervical dislocation. The brains were removed and different brain regions dissected on ice. Brain regions were rapidly frozen in liquid nitrogen and stored at –80 °C until assayed. The brain tissue was homogenized (by 10 s sonification) in 1 ml 0.1 M perchloric acid containing 50 ng/ml DHBA as internal standard. Homogenates were centrifuged at 10000 x g for 20 min, and
the resulting supernatant was mixed with 500 μl 2 M Tris acetate (pH 8.7) to adjust the pH to 8.4. Samples were added to 10 mg aluminum oxide powder and mixed for 15 min. The aluminum oxide was spun down by centrifugation (30 s at 10000 x g) and then washed twice with 1 ml water. The pellet was mixed with 200 μl 0.2 M perchloric acid for 15 min. The samples were centrifuged at 10000 x g for 30 s, and the resulting supernatants were stored at –80 °C until analyzed by HPLC (High Performance Liquid Chromatography). The eluate (20 μl) was injected onto a C18 reverse phase column (SUPELCOSILTM LC-18, 3 μM, 15 cm x 4 mm, SUPELCO, Bellefonte, USA). The reference mixture used was: 50 ng/ml DHBA, 50 ng/ml L-DOPA, 50 ng/ml DOPAC (3,4-dihydroxyphenylacetic acid) and 100 ng/ml DA. The mobile phase consisted of 90 mM NaH2PO4, 3 mM octyl sulfate, 20 mM Na2EDTA and 50 mM triethyl amine, and it contained 10 % methanol. The HPLC system consisted of an AS3000 autosampler (Thermo Finnigan, San Jose, CA, USA), a P1000 isocratic pump (Thermo Separations, San Jose, CA, USA), a PE Nelson 900 Series Interface (Perkin Elmer, Ueberlingen, Germany) and an Intro-electrochemical detector (Antec, Leyden, The Netherlands). As the analytical cell, a VT-03 cell (Antec, Leyden, The Netherlands) was used with a 3 mm glass carbon working electrode and a salt bridge Ag/AgCl reference electrode. Oxidation currents were measured at a working potential of 650 mV. Data were integrated and processed with TotalChromTM chromatography software (Perkin Elmer, Ueberlingen, Germany). The L-DOPA content was calculated as ng/mg tissue wet weight.

Experiment 4: Apomorphine-induced stereotypy

Rats were placed into individual experimental chambers (wire mesh cages of 20 x 26 cm turned upside down) for a 60-min habituation period. The rats were then pretreated with ABT-127 and after 60 min administered apomorphine HCl (1.21 mg/kg,
s.c.). Control animals received the corresponding vehicle. The measurement of stereotypy was begun 10 min after apomorphine treatment, and the time intervals of 20, 30, 40, 50 and 60 min post-apomorphine injection were assessed.

Stereotypy was scored from 0 to 4 according to the following criteria: 0 = absence of stereotyped behavior, similar to control; 1 = presence of continuous exploration, intermittent sniffing, and movements of head; 2 = intense stereotyped movements of head and/or licking and sniffing, continuous exploration; 3 = intense licking, with intense sniffing, or head weaving without exploration and locomotion; 4 = intense gnawing confined to a small area without exploration and locomotion.

The scoring of apomorphine induced stereotypy was done by a technician who was unaware of the nature of compound that was being tested. During the testing the experimental chambers were arranged in a circle around the experimenter who continually rated animals, going from one to the next; this technique would have further reduced any possibility of experimental bias. We have previously compared this procedure to one in which the rater is blind to all drug treatments and found no significant differences.

Experiment 5: Conditioned activity between group comparisons:

For conditioned activity six automated activity chambers were made of clear Plexiglas, measuring 41.0 x 50.0 x 37.0 cm high and housed in wooden, styrofoam-insulated, outer boxes. A set of seven infrared emitters and detectors was positioned at a height of 5.0 cm above the metal rod floor (three on each 41-cm side, four along the 50-cm front and back walls) spaced 10 cm apart. The photocells formed a matrix of 20 squares of equivalent dimensions (approximately 10 x 10 cm) over the chamber surface.
The rat had to enter and leave a beam for an activity count to be recorded. Each chamber was illuminated with a 2.5 W incandescent bulb and ventilated by a small fan that also provided background noise. Beam breaks were recorded on an experimenter-controlled circuit board connected to a 1 GHz IBM compatible computer. For further details of the apparatus see Aujla et al (2002).

The general experimental protocol consisted of three phases: habituation (3 sessions), conditioning (3 sessions in experiment 5; 4 sessions in experiment 6) and testing (1 session). All sessions occurred during the dark phase (0700-1900 hrs) and consisted of a 1-hr placement into one of the activity chambers. An individual rat was always placed into the same chamber and all groups included at least one rat tested in each of the six chamber.

Animals (N = 125) in all groups received three 1-hr habituation trials, one each day, over three days during which no drug was administered. The conditioning phase began on the next day and consisted of three 1-hr sessions, conditioning days 1-3, one every 48-hrs. The single 1-hr test session took place 48-hrs following conditioning day 3.

Experiment 5a: Establishing conditioned activity:

Two groups were used to establish the conditioned activity paradigm. The unpaired/sal group (n = 9) received saline (sal) before conditioning and test sessions and cocaine (10 mg/kg) was injected in the home cage 1 hr following each conditioning session. The paired/sal group (n = 9) received cocaine (10 mg/kg) prior to each conditioning session and saline prior to the test; this group was injected with saline in the home cage 1 hr following conditioning sessions.
Experiment 5b: Finding the dose of haloperidol in conditioning sessions:

Six groups (n = 9) were used to evaluate the effects of haloperidol on the acquisition of conditioning activity. Groups were injected with haloperidol (0.1, 1.0, 25, 50, 75, 150 µg/kg) 1 hr before being placed into the activity chamber. Immediately before placement in the activity chambers rats received cocaine (10 mg/kg). These groups received a saline injection in their home cage 1 hr following the end of each conditioning session. These groups are identified as hal x + paired/sal where x indicated the dose of haloperidol in µg/kg. On the test day all animals received saline immediately before placement in the activity chambers.

Experiment 5c: Finding the dose of ABT-127 in the test session:

Two groups (n = 9) were used to evaluate the effects of ABT-127 on the expression of conditioned activity. During conditioning, rats received cocaine (10 mg/kg) immediately before placement in the activity chambers. One hr following the end of each conditioning session rats received a saline injection in their home cage. On the test day animals were pretreated with ABT-127 (1.0 or 10 mg/kg) 30 min before being injected with saline and placed in the activity chambers. These groups are identified as paired/ABT x + sal, where x indicates the dose of ABT-127 in mg/kg.

Experiment 5d: Haloperidol in the test session or ABT-127 in conditioning:

Two additional groups enabled a direct comparison between haloperidol and ABT-127 in acquisition versus expression of cocaine-conditioned activity based on the results from experiments 5b and 5c. One group (n = 8) evaluated the role of haloperidol on the test day (expression). The dose of haloperidol (50µg/kg) was chosen on the basis
of the results of experiment 5b (see below). During conditioning, rats received cocaine (10 mg/kg) immediately before placement in the activity chambers. One hr following the end of each conditioning session rats received a saline injection in their home cage. On the test day animals were pretreated with haloperidol (50 µg/kg) before being injected with saline and placed in the activity chambers. This group is identified as paired/hal 50 + sal.

One group (n = 9) examined the role of ABT-127 during conditioning (acquisition). The dose of ABT-127 (1.0 mg/kg) was chosen on the basis of the results of experiment 5c (see below). Rats were injected with ABT-127 (1.0 mg/kg) 30 min before being placed into the activity chamber. Immediately before placement in the activity chambers rats received cocaine (10 mg/kg). Rats received a saline injection in their home cage 1 hr following the end of each conditioning session. The group is identified as ABT 1.0 + paired/sal. On the test day animals received saline immediately before placement in the activity chambers.

Experiment 5e: Haloperidol or ABT-127 in the test session following conditioning with saline:

Two further groups (ns = 9) evaluated the effects of haloperidol (50 µg/kg) or ABT-127 (1.0 mg/kg) during the test session following conditioning sessions with saline. During conditioning, rats received saline immediately before placement in the activity chambers. One hr following the end of each conditioning session rats received cocaine (10 mg/kg) in their home cage. On the test day animals were pretreated with haloperidol 1 hr or ABT-127 30 min before being injected with saline and placed in the activity chambers. These groups are identified as unpaired/hal 50 + sal and unpaired/ABT 1.0 +
Activity data from the last habituation day were compared among the groups using one-way between-subjects analysis of variance (ANOVA) to test for pretreatment differences. Activity counts averaged across the three conditioning sessions and from the single test day were similarly analyzed using separate one-way between-subjects ANOVA. Significant main effects were followed by pair-wise comparisons.

**Experiment 6: Conditioned activity within group comparisons:**

The purpose of this experiment was to investigate the effects of haloperidol and ABT-127 on expression of conditioned activity in the same rats using a counterbalanced repeated measures design. Thus, rats received DA receptor antagonists only before the saline test session but not during the conditioning phase. Two groups of rats (ns = 9) received three 1-hr habituation sessions over three consecutive days. The conditioning phase began on the next day and consisted of 1-hr sessions, one each day. On conditioning days 1-4 (wk 1), 5-8 (wk 2), 9-12 (wk 3), 13-16 (wk 4), and 17-20 (wk 5), the paired/sal group received cocaine (10 mg/kg) and the unpaired/sal group received saline immediately before being placed into the activity chambers. One hr following each conditioning session the unpaired/sal group received cocaine in their home cages and the paired/sal group received saline.

On test days 1, 2 and 4 (wk 1, 2 and 4), all rats received a saline injection prior to placement into the activity chambers. On test day 3 (wk 3) rats from the paired/sal (n = 5) and unpaired/sal (n = 5) groups were pretreated with 50 µg/kg of haloperidol (paired/hal 50 + sal; unpaired/hal 50 + sal ) 1 hr before saline injection and placement in the activity chamber. The remaining 4 animals in each group were pretreated with 1.0 mg/kg of ABT-127 (paired/ABT 1 + sal; unpaired/ABT 1 + sal) 30-min prior to the saline injection
and placed in the activity chambers. Test day 5 (wk 5) was like test day 3 except that animals that were previously pretreated with haloperidol on wk 3 received ABT-127 and those pretreated with ABT-127 on wk 3 received haloperidol. Activity data from the last habituation day were compared among the groups using one-way between-subjects ANOVA to test for pretreatment differences. Test day activity data were analyzed using mixed factorial ANOVA. Significant main effects were followed by pair-wise comparisons.
Results

Experiment 1: ABT-127 and Haloperidol binding comparison

ABT-127 exhibited high affinity in the radioligand binding assays for cloned human dopamine D3 receptors: $K_i = 0.98\, \text{nM}$ (see Table 1). All inhibition curves were best fitted by a one site model which also was evident by the Hill slopes coefficients approaching unity (not shown). ABT-127 had much lower affinity for the human dopamine D2L receptor ($K_i = 145\, \text{nM}$), resulting in a selectivity for dopamine D3 receptors of 148-fold. For the rat dopamine D2 and D3 receptors, respective $K_i$ values for ABT-127 were 52 and 2.3 nM, resulting in a selectivity ratio for the rat dopamine D3 over D2 receptors of approximately 23-fold. In contrast, haloperidol was binding with higher affinity to D2 receptors (both human and rat) with the D2 affinity being approximately 14-fold lower than that for D3 receptors (Table 1).

Experiment 2: Rat striatal in vivo dopamine D2 receptor occupancy

Figure 1 displays the D2 receptor occupancy using $[^3H]$raclopride. ANOVA revealed a significant effect of group [$F(3,21) = 24.64, p<0.0001$]. Post-hoc pair-wise comparisons found that significant displacement of $[^3H]$raclopride was seen in rats treated with 10 and 30 but not 3 mg/kg of ABT-127.

Experiment 3: L-DOPA accumulation in the rat striatum

For striatal L-DOPA accumulation was examined (Fig. 2). ANOVA revealed a significant effect of group [$F(7,97) = 20.57, p<0.0001$]. Post-hoc pair-wise comparisons found that only high doses of ABT-127 (30 and 100 but not 3 or 10 mg/kg) were significantly different from the quinpirole group ($p<0.05$). Also, it was found that the
Experiment 4: Apomorphine-induced stereotypy

ABT-127 at doses of 10 and 30 mg/kg but not 3 mg/kg, significantly attenuated apomorphine-induced stereotypy in rats \([F(3,46) = 12.0, p<0.0001;\) see Fig. 3).
with increasing dose of haloperidol during conditioning. A one-way between-groups ANOVA, including the two groups (paired/sal and unpaired/sal) from experiment 5a, revealed a significant group effect \[F(7, 64) = 7.73, p<0.001\]. Post-hoc pair-wise comparison found that groups pretreated with cocaine plus 0.1, 1.0, 25 µg/kg but not 50, 75 or 150 µg/kg haloperidol during conditioning were different from unpaired/sal controls \((p<0.01)\). The paired/sal group differed from groups conditioned with cocaine plus 50, 75 and 150 µg/kg \((p<0.05)\) but not 0.1, 1.0 and 25 µg/kg haloperidol. Results indicate that 50 µg/kg haloperidol during conditioning did not block the unconditioned stimulant effect of cocaine but led to decreased conditioned activity during the test session. This dose was used in the subsequent studies.

In experiment 5c (Finding the dose of ABT-127 in the test session), groups were conditioned with 10 mg/kg of cocaine for three sessions and pretreated with a dose of ABT-127 \(1.0\) or \(10.0\) mg/kg) on the saline test day. ANOVA revealed no significant difference in activity among the paired/ABT 10 + sal, paired/ABT 1 + sal and paired/sal groups across the three conditioning sessions \[F(2,24) = 0.261, \text{n.s.;Fig. 4A}\]. However, on the test day, rats pretreated with either dose of ABT-127 exhibited an attenuation of activity compared to the paired/sal group (Fig. 4B). ANOVA including the two groups from experiment 5a (paired/sal and unpaired/sal) showed a significant group effect \[F(3, 32) = 12.11, p<0.001\]. Post-hoc pair-wise comparisons found that the groups pretreated with 1.0 and 10.0 mg/kg ABT-127 were different from the paired/sal group \((p<0.01)\) but not significantly different from the unpaired/sal group indicating that both doses of ABT-127 blocked conditioned activity during the test session. The lower dose \(1.0\) mg/kg) was used in subsequent studies.
In experiment 5d (Haloperidol in the test session or ABT-127 in conditioning) we aimed to directly compare the effects of haloperidol and ABT-127 on acquisition and expression of conditioned activity. Based on the results obtained in experiment 5b and 5c, we evaluated the 50 µg/kg dose of haloperidol in the test, and the 1.0 mg/kg dose of ABT-127 in conditioning. The results for these groups are shown in Fig. 4B along with the hal 50 + paired/sal group from experiment 5b and the paired/ABT 1 + sal group from experiment 5c (see inset). Analysis of the four groups revealed that haloperidol during conditioning blocked the acquisition of conditioned activity but haloperidol during the test did not block expression; ABT-127 during conditioning did not block acquisition but ABT-127 during the test blocked expression. This description was supported by a significant treatment x phase interaction [F(1,31) = 5.21, p<0.05] using a two-way ANOVA.

The results of experiment 5e (Haloperidol or ABT-127 in the test session following conditioning with saline) showed that unpaired/sal, unpaired/hal 50 + sal and unpaired/ABT 1 + sal groups did not differ significantly from one another on the test day [F(2, 26) = 0.08, n.s.; Fig. 4B]. These findings suggest that animals conditioned with saline and given haloperidol or ABT-127 during test did not exhibit motor impairments.

Experiment 6: Conditioned activity within group comparisons:

The purpose of experiment 6 was to examine the effects of haloperidol and ABT-127 in the same animals during expression of conditioned activity using a counterbalanced repeated measures design. The groups did not exhibit significant differences in activity during the last day of habituation [F(1,16) = 1.26, n.s.] (data not shown). Rats conditioned with cocaine across week 1-5 showed greater activity in
comparison to rats conditioned with saline [Mean (±SEM) = 1320.57 (± 57.36), 628.98 (± 55.10), respectively; F(1,16)=75.60, p<0.001]. On the saline test at the end of wk 1, 2 and 4, the paired/sal group exhibited significantly higher activity than the unpaired/sal group showing the conditioned activity effect (Fig. 5). On test wk 3 and 5, rats were administered haloperidol or ABT-127 in a counterbalanced fashion depending on the week. A mixed factorial ANOVA with independent groups (paired and unpaired) and repeated measures on treatment (saline, haloperidol, ABT-127) revealed a significant interaction [F(2,32) = 7.13, p<0.01] and a significant treatment effect [F(2, 32) = 6.83, p <0.01] (Fig. 5). Simple effects ANOVA found a significant difference between paired/hal 50 + sal and unpaired/hal 50 + sal groups [F(1,8) = 8.61, p<0.05] but no significant difference was observed between paired/ABT 1 + sal and unpaired/ ABT 1 + sal groups. These results show that pretreatment with ABT-127, but not haloperidol, before the test blocked the conditioned activity effect based on cocaine in rats.

As can be seen in Fig. 5, activity levels tended to increase in both groups over the course of this 5-wk experiment. This change may reflect growth of the animals as we have seen previously that activity counts increase with age (Beninger et al., 1986).
Discussion

The results can be summarized as follows: In experiment 1, ABT-127 bound preferentially to rat and human D3 receptors, whereas haloperidol bound preferentially to D2 receptors. In experiment 4, ABT-127 (3 and 10 mg/kg) failed to antagonize apomorphine-induced stereotypy suggesting that ABT-127 at these doses does not block striatal D2 receptors and can be regarded to be D3-receptor selective. Further evidence for ABT-127 D3 receptor specificity at low doses was found in experiments 2 and 3. Doses of 10 mg/kg or above were necessary for significant inhibition of \([^{3}\text{H}]\text{raclopride}\) binding and quinpirole-induced L-DOPA accumulation in the rat striatum suggesting that higher doses of ABT-127 are needed to affect D2 receptor transmission in the rat brain. A displacement of \([^{3}\text{H}]\text{raclopride}\) binding and attenuation of L-DOPA accumulation were observed with low doses of the dopamine D2-preferring receptor antagonist haloperidol (data not shown).

It is noteworthy that there are no good radioligand tools that would allow us to measure D2 vs D3 receptor occupancy. Both \([^{3}\text{H}]\text{raclopride}\) that is commonly used in preclinical studies (McCormick et al., 2009; Kapur et al., 2000) and \([^{11}\text{C}](+)-\text{PHNO}\) \(([{^{11}\text{C}}](+)-4\text{-propyl-3,4,4a,5,6,10b-hexahydro-2H-naphtho[1,2-b][1,4]oxazin-9-ol})\) that was recently used in humans as a D3-preferring tool (Graff-Guerrero et al., 2009; Boileau et al., 2009) are not selective enough and therefore additional methods/approaches are necessary. One such approach is to evaluate radioligand binding in a brain area that is enriched in one receptor subtype and not the other. This is why in vivo \([^{3}\text{H}]\text{raclopride}\) binding was performed in the striatum where D2 expression is high and D3 expression is
low but not absent (Bouthenet et al., 1991). Using this approach, ABT-127 (10, 30 mg/kg) significantly reduced specific striatal [3H]raclopride binding suggesting that these doses occupied D2 receptors. Additional studies confirmed significant D2 receptor occupancy by high doses of ABT-127: antagonism of quinpirole-induced decrease of L-DOPA accumulation at 30 mg/kg and antagonism of apomorphine-induced stereotypy at 10 and 30 mg/kg. Brain concentrations achieved at the studied doses of ABT-127 would be quite informative. In an unpublished study, rats were dosed (ip) with 30 mg/kg of ABT-127 resulting in ca. 54 nM free brain levels. Considering the in vitro binding data (rat D3 Ki = 2.3 nM, rat D2 Ki = 52 nM), it is quite likely that at 30 mg/kg, ABT-127 reaches D2 receptor occupancy (~50%) that is significant enough to observe behavioral and neurochemical effects. These results suggest that the lower doses (1.0 mg/kg) of ABT-127 that were observed to be effective in conditioned activity studies were selective for D3 receptors.

In experiments 5 and 6, animals receiving cocaine during conditioning and saline in the test were more active in the test than control animals that had never received cocaine in the test environment; thus they acquired conditioned activity. Co-administration of cocaine plus haloperidol (50 µg/kg) during conditioning did not have a significant effect on the unconditioned stimulant properties of cocaine but blocked the acquisition of conditioned activity. Treatment with haloperidol (50 µg/kg) during the test session had no significant effect on the expression of conditioned activity. Co-administration of cocaine plus ABT-127 (1.0 mg/kg) during conditioning had no significant effect on the stimulant properties of cocaine and did not disrupt the acquisition of conditioned activity. Treatment with ABT-127 (1.0 mg/kg) during the test session
following conditioning with cocaine blocked the expression of conditioned activity. Thus, haloperidol blocked acquisition, but not expression, of conditioned activity based on cocaine, and ABT-127 blocked expression, but not acquisition, of conditioned activity based on cocaine, a double dissociation.

It is noteworthy that this double dissociation was observed using compounds with limited rat D2/D3 receptor selectivity. The current approach was based on identifying doses of ABT-127 that did not affect D2 receptor-mediated signaling. Cocaine conditioning experiments were conducted using a dose of ABT-127 that did not produce behavioral or neurochemical effects typical for D2 receptor blockade. Results were similar to what would be expected when using highly selective D3 receptor antagonists (see below).

The observation of enhanced activity in a drug-free state in a test environment previously paired with cocaine is consistent with reports of conditioned activity based on cocaine (Beninger and Herz, 1986) and other psychomotor stimulants (Pickens and Crowder 1967; Mazurski and Beninger, 1991). Control groups that received environment-saline pairings on conditioning days and then received cocaine in their homecage environment one hr later did not exhibit increased activity during the test session, as reported previously for amphetamine (Mazurski and Beninger, 1991). Therefore, the effect of increased activity in rats having received environment-cocaine pairings can be attributed to the association of environmental stimuli with cocaine rather than a previous history of cocaine treatment.

The finding that haloperidol, given during cocaine conditioning, blocked the acquisition of conditioned activity agrees with other reports using D2-preferring receptor
antagonists (Beninger and Hahn, 1983; Beninger and Herz, 1986; Fontana et al., 1993; Dias et al., 2006). Reimer and Martin-Iverson (1994) did not observe a block of acquisition of conditioned activity with haloperidol but they used one dose and their purpose was explicitly to use a sub-threshold dose of haloperidol that could be tested for synergistic effects with the calcium channel blocker nimodipine. Previous reports have found that the expression of conditioned activity based on cocaine or amphetamine is resistant to the effects of dopamine D2-preferring receptor antagonists (Beninger and Hahn, 1983; Beninger and Herz, 1986; Fontana et al., 1993). Inhibition of the unconditioned stimulant effect of cocaine was not necessary to block the acquisition of conditioned activity, as previously reported by Fontana et al. (1993). Thus, at appropriate dose levels, D2-preferring receptor antagonists block the acquisition but not the initial expression of conditioned activity based on amphetamine or cocaine.

The finding that ABT-127 blocked expression but not acquisition of conditioned activity is in agreement with the observation that the selective D3 receptor antagonist SB-277011 blocked the expression of conditioned activity based on cocaine in mice (Le Foll et al., 2002) or nicotine in rats (Pak et al., 2006). The dopamine D3 receptor-preferring partial agonist BP 897 is thought to moderately stimulate the receptor during times when endogenous DA tone is lacking or diminished but to antagonize the receptor from full activation during conditions of increased dopamine release produced by drug-associated cues (Gyertyan et al. 2007). Consistent with action as a receptor antagonist, BP 897 blocked expression of conditioned activity based on amphetamine or cocaine in rats or mice (Aujla et al., 2002; Le Foll et al., 2002). In the same study, Aujla et al. (2002) found that BP 897 given during the pairing phase had no significant effect on the acquisition of
conditioned activity. Thus, D3 receptor antagonists block expression but not acquisition of conditioned activity at doses that have no significant effect on unconditioned activity.

It may be D3 receptors in the nucleus accumbens (NAc) and basolateral amygdala (BLA) that play a role in the expression of conditioned activity. Thus, Aujla and Beninger (2004) found that bilateral injections of BP 897 into NAc or BLA during expression of conditioned activity based on intra-NAc amphetamine blocked the effect. Similar injections of BP 897 into either structure during conditioning had no significant effect.

This is the first study to systematically compare the effects of a D2 and D3 receptor-preferring antagonist in acquisition and expression of conditioned activity, showing a double dissociation. Our results with conditioned activity are consistent with related studies using stimulant self-administration, reinstatement or conditioned place preference (CPP) techniques. We recently reviewed these studies (Beninger and Banasikowski, 2008) and will provide an overview of the results here. In self-administration studies, when the maintenance of responding relied heavily on conditioned stimuli (e.g., on lean second-order schedules), D3 receptor antagonists (Di Ciano et al., 2003, 2008; Claytor et al., 2006; but see Martelle et al., 2007) or partial agonists decreased responding (Pilla et al., 1999; Martelle et al, 2007). Similarly, in self-administration studies using progressive ratios, D3 agents decreased break points for cocaine (Xi et al., 2005) and nicotine (Ross et al., 2007). D3 receptor antagonists or partial agonists generally reduced the response-reinstating effects of cues associated with self-administered cocaine (Cervo et al., 2003; Gilbert et al., 2005) or ethanol (Vengeliene et al., 2006). Some studies have reported that D2 receptor antagonists also reduce the
response-reinstating effects of cocaine-associated cues (Cervo et al., 2003; Gal & Gyertyan, 2006). These authors suggest the possibility that D2 and D3 receptors may act at different sites in the brain but further studies are needed to delineate the possible role for D2 and D3 receptors in reinstatement of responding for cocaine produced by cocaine-associated cues.

In CPP studies, D3 receptor antagonists or partial agonists usually blocked expression based on cocaine (Vorel et al., 2002; Duarte et al., 2003; Cervo et al., 2005), nicotine (Pak et al., 2006), d-amphetamine (Aujla and Beninger, 2005), morphine (Frances et al., 2004; Vazquez et al., 2007; but see Duarte et al., 2003) or heroin (Ashby et al., 2003) but not food (Duarte et al., 2003). The effects of D3 receptor antagonists or partial agonists on the acquisition of CPP based on cocaine (Gyertyan and Gál, 2003, 2007; Cervo et al., 2005; but see Vorel et al., 2002; Duarte et al., 2003), d-amphetamine (Aujla and Beninger, 2005) or opiates (Duarte et al., 2003; but see Ashby et al., 2003) often were non-significant.

Changes in D3 receptors have been seen in behaviorally conditioned animals. Rats with a history of cocaine self-administration, after undergoing 30-31 days of withdrawal, received a reinstatement test and were killed for assay of D3 receptor binding 24 hr later. Increases were found in the ventral caudate and in NAc core (Neiswander et al., 2004). Le Foll et al. (2002) injected mice with cocaine or saline and exposed them to a test environment; saline-treated mice (the unpaired group) received cocaine later in the home cage. On the test day, mice were placed into the test environment following a saline injection for a 30-min session, during which conditioned activity was seen in the paired group, and then killed. Results revealed a significant increase in D3 (but not D1 or
D2) receptor mRNA and receptors in NAc but not striatum of paired but not unpaired mice. Results showed that changes in D3 receptors were specific to mice that had received drug-environment pairings that produced conditioned activity and implicate D3 receptors in the expression of conditioned activity.

In summary, ABT-127 is a D3 receptor-preferring antagonist. The D2 receptor-preferring antagonist haloperidol blocks the acquisition of conditioned activity based on cocaine at a dose that does not significantly affect expression; the D3 receptor-preferring antagonist ABT-127 blocks the expression of conditioned activity at a dose that does not significantly block acquisition, a double dissociation. Results agree with a number of findings from conditioned activity, drug self-administration and CPP studies that point to a more important role for D3 receptors in the expression of conditioned behaviors than in their acquisition. Some studies suggest that behavioral conditioning leads to changes in D3 receptors in the NAc and possibly the ventral striatum. The mechanism underlying the contribution of D3 receptors to the expression of conditioned behaviors remains to be elucidated.
References


Cervo L, Burbassi S, Colovic M and Caccia S (2005) Selective antagonist at D3 receptors, but not non-selective partial agonists, influences the expression of


Reimer AR and Martin-Iverson MT (1994) Nimodipine and haloperidol attenuate behavioural sensitization to cocaine but only nimodipine blocks the establishment of conditioned locomotion induced by cocaine. *Psychopharmacol* **113**:404-410.


Footnotes

a) Funded, in part, by a grant from the Natural Sciences and Engineering Research Council of Canada, [no. 7861-10] to RJB.

b) This study was presented, in part, at the 37th Annual Society for Neuroscience Meeting. Parts of this study were included in the MSc. thesis of TJB.
Legends for figures

**Figure 1:** Mean (±SEM) displacement of [3H]raclopride by ABT-127. Rats were administered ABT-127 (3, 10 and 30 mg/kg, i.p.) or vehicle, followed 60 min later by [3H]raclopride (i.v.). To measure specific and non-specific binding striatum and cerebellum were dissected 30 min after [3H]raclopride infusion. N=5 per group. * ** significantly different (p<0.001) from the control group.

**Figure 2:** Mean (±SEM) striatal L-DOPA content (ng/mg tissue wet wt) following combined treatment with quinpirole (QUIN) and ABT-127. ABT-127 (3, 10, 30 and 100 mg/kg, i.p.) was given 90 min before sacrifice, quinpirole (0.1 mg/kg, i.p.) 45 min before sacrifice, and NSD-1015 (100 mg/kg, i.p.) immediately followed by gamma-butyrolactone (GBL; 750 mg/kg, i.p.) 30 min before sacrifice. N=11-19 per group. *** significantly different (p<0.001) from the quinpirole control group. ††† significantly different (p<0.001) from the NSD, or NSD/GBL groups.

**Figure 3:** Mean (±SEM) apomorphine-induced stereotypy score in rats co-treated with ABT-127. ABT-127 (3, 10, and 30 mg/kg, i.p.) or its vehicle (“0”) was given 60 min before apomorphine (1.21 mg/kg, s.c.). Behavioral observations started 10 min after the apomorphine injection. N=10-12. *** significant (p < 0.001) and ** significant (p < 0.01) from the control group.

**Figure 4:** Mean (±SEM) activity counts per session averaged across three 1-hr pairing sessions (A: Conditioning) and for the test session (B: Test) from experiment 5 (Between group comparisons) for the group that received cocaine (10 mg/kg) unpaired with the test environment during conditioning and saline (sal) immediately prior to the test session (unpaired/sal) and the group that received cocaine immediately prior to
conditioning sessions and saline immediately prior to the test (paired/sal) from experiment 5a (Establishing conditioned activity), all groups that received haloperidol (hal) doses (µg/kg) 1 hr before plus cocaine immediately before conditioning sessions and were tested with sal (hal x + paired/sal) from experiment 5b (Finding the dose of haloperidol in conditioning sessions), the two groups that received conditioning with cocaine followed by a dose (mg/kg 30 min before the session) of ABT-127 (ABT) and sal (paired/ABT x + sal) in the test from experiment 5c (Finding the dose of ABT-127 in the test session), the group that was conditioning with cocaine and tested with 50 µg/kg hal (paired/hal 50 + sal) and the group that was conditioned with ABT (1.0 mg/kg 30 before the session) plus cocaine and tested following sal (ABT 1 + paired/sal) from experiment 5d (Haloperidol in the test session and ABT-127 in conditioning), and the groups that were given cocaine unpaired with the environment during conditioning and tested with hal (unpaired/hal 50 + sal) or ABT (unpaired/ABT 1 + sal) from experiment 5e (Haloperidol or ABT-127 in the test session following conditioning with saline). Inset in panel B shows that hal blocked acquisition but not expression and ABT blocked expression but not acquisition [Analysis of variance (ANOVA) revealed a significant drug by phase interaction]. *** significant (p < 0.001) and ** significant (p < 0.01) difference from unpaired/sal by ANOVA; † significant (p < 0.05) difference from paired/sal by ANOVA.

**Figure 5:** Mean (±SEM) activity counts per session during the 1-hr saline (sal) test from experiment 2 (Within group comparisons) for the group that received sal paired with the test environment but cocaine in their home cage (unpaired) and the group that received cocaine (10 mg/kg immediately before) paired with the test environment (paired) when
tested with sal on test days 1, 2 and 4 averaged (unpaired/sal and paired/sal), when tested with ABT-127 (1.0 mg/kg 30 min before) plus sal (unpaired/ABT 1 + sal and paired/ABT 1 + sal) and when tested with haloperidol (50 µg/kg 1 hr before) plus sal (unpaired/hal 50 + sal and paired/hal 50 + sal). ** significant (p < 0.01) difference from unpaired/sal by analysis of variance (ANOVA); † significant (p < 0.05) difference from unpaired/hal 50 + sal by ANOVA.
Table 1. Affinities of ABT-127 and haloperidol for cloned human hD2L and hD3 receptors.

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>ABT-127</th>
<th>Haloperidol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human D3</td>
<td>0.98 (0.89-1.08)</td>
<td>7.6 (7.3-7.8)</td>
</tr>
<tr>
<td>Human D2L</td>
<td>145 (130-162)</td>
<td>0.56 (0.52-0.59)</td>
</tr>
<tr>
<td>D2/D3</td>
<td>~148</td>
<td>~0.07</td>
</tr>
<tr>
<td>Rat D3</td>
<td>2.3 (2.1-2.5)</td>
<td>10.4 (9.1-11.9)</td>
</tr>
<tr>
<td>Rat D2</td>
<td>52 (46-58)</td>
<td>0.73 (0.68-0.8)</td>
</tr>
<tr>
<td>D2/D3</td>
<td>~23</td>
<td>~0.07</td>
</tr>
</tbody>
</table>

In vitro affinity (Ki, nM) and D2L:D3 selectivity ratio for ABT-127 and haloperidol determined by inhibition of radioligand binding to cloned human and rat dopamine D2L and D3 receptors. Dopamine D3 receptor binding and dopamine D2L receptor binding were performed with [125I] iodosulpride and [125I] iodospiperone respectively (for detail see material and methods). All values are mean Ki values (with 95% CL) of at least three determinations performed in triplicate.
Figure 1.
Figure 3.
A. Conditioning

B. Test
Figure 5.