

Novel C-1 Substituted Cocaine Analogues Unlike Cocaine or Bzotropine

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ABBREVIATIONS: β -CFT, 2 β -carbomethoxy-3 β -(4-fluorophenyl)tropane; β -CPT, 2 β -carbomethoxy-3 β -phenyltropane; CPP, conditioned place preference; DA, dopamine; DAT, dopamine transporter; LeuT, leucine transporter; MDMA, 3,4-methylenedioxy-*N*-methylamphetamine; NET, norepinephrine transporter; NSS, neurotransmitter/sodium symporter; PDSP, Psychoactive Drug Screening Program; SARs, structure-activity relationships; SBFI, sodium-binding benzofuran isophthalate; SERT, serotonin transporter.

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Abstract

Despite a wealth of information on cocaine-like compounds, there is no information on cocaine analogues with substitutions at C-1. Here, we report on (*R*)-(-)-cocaine analogues with various C-1 substituents: methyl (**2**), ethyl (**3**), *n*-propyl (**4**), *n*-pentyl (**5**) and phenyl (**6**). Analogue **2** was equipotent to cocaine as an inhibitor of the dopamine transporter (DAT), whereas **3** and **6** were 3- and 10-fold more potent, respectively. None of the analogues, however, stimulated mouse locomotor activity, in contrast to cocaine. Pharmacokinetic assays showed compound **2** occupied mouse brain as rapidly as cocaine itself; moreover, **2** was behaviorally active in mice in the forced swim test model of depression and the conditioned place preference test. Analogue **2** was a weaker inhibitor of voltage-dependent Na⁺ channels than cocaine, though **6** was more potent than cocaine, highlighting the need to assay future C-1 analogs for this activity. Receptorome screening indicated few significant binding targets other than the monoamine transporters. Benztropine-like 'atypical' DAT inhibitors are known to display reduced cocaine-like locomotor stimulation, presumably by their propensity to interact with an inward-facing transporter conformation. However, **2** and **6**, like cocaine—but unlike benztropine—exhibited preferential interaction with an outward-facing conformation upon docking in our DAT homology model. In summary, C-1 cocaine analogues are not cocaine-like in that they are not stimulatory *in vivo*. However, they are not benztropine-like in binding mechanism and appear to interact with the DAT similarly to cocaine. The present data warrant further consideration of these novel cocaine analogues for antidepressant or cocaine substitution potential.

Introduction

Cocaine contains four asymmetric carbons (Fig. 1), implying sixteen hypothetically possible stereoisomers. However, only eight of these isomers can exist, due to the geometric constraints imparted by the tropane bridgehead amine. The natural plant alkaloid (*R*)-(-)-cocaine (**1**) is the only isomer that possesses psychostimulant activity (Carroll et al., 1992). Different substituents at nearly all of the positions of the cocaine molecule—except C-1 of the tropane skeleton (Fig. 1)—have been introduced, providing a wealth of knowledge on structure-activity relationships (SARs) at monoamine transporters (Carroll et al., 1992). The major difficulty in the asymmetric synthesis of optically pure (*R*)-(-)-cocaine and analogues is the problem of introducing tropane substituents stereoselectively, with the required *cis* relationship between the C-2 and C-3 tropane substituents, and the C-2 carbomethoxy moiety occupying the thermodynamically unfavorable β (*exo*) configuration (Fig. 1). No cocaine analogues possessing substituents at the C-1 bridge position existed until recently, when we targeted such analogues with our novel sulfinimine (*N*-sulfinyl-imine) chemistry (Zhou et al., 2004; Senanayake et al., 2005; Davis, 2006; Morton and Stockman, 2006). Our asymmetric synthesis (Davis et al., 2010; Davis et al., 2012) generated chiral (*R*)-(-)-cocaine analogues with various C-1 substituents, including: methyl (**2**), ethyl (**3**), *n*-propyl (**4**), *n*-pentyl (**5**) and phenyl (**6**) moieties (structures shown in Fig. 1).

Cocaine exhibits non-selective binding to the three monoaminergic neurotransmitter-sodium symporter (NSS) proteins—the dopamine (DA), serotonin (5-HT) and norepinephrine (NE) transporters (DAT, SERT and NET)—where it acts as a non-translocated inhibitor. However, the strong locomotor stimulant and addictive effects of cocaine are mediated almost exclusively by its interaction with the DAT (Chen et al., 2006; Thomsen et al., 2009). Preliminary data obtained for (-)-1-methyl-cocaine (**2**) indicated broad-spectrum inhibition of monoamine uptake with potency similar to cocaine; but, unexpectedly, little or no locomotor stimulatory activity in behavioral tests. This curious finding raised the possibility that C-1 modification produces psychoactive compounds that inhibit monoamine uptake, but lack cocaine-like psychostimulant activity. In this context, it is of

interest that certain 'atypical' DAT inhibitors exhibit limited stimulatory and reinforcing properties compared to cocaine (Rothman et al., 2008; Newman and Katz, 2009). Examples are the tropane compound benztropine (**7**) and various benztropine derivatives that are devoid of benztropine's antimuscarinic and antihistaminergic side effects (Katz et al., 2004; Campbell et al., 2005; Li et al., 2011; Desai et al., 2005). We thus sought to investigate whether C-1 substitution yields novel DAT ligands with a cocaine-like structure but an atypical-like pharmacological profile.

Our studies addressed the following properties of the analogues, with particular focus on two of the compounds: the series prototype (–)-1-methyl-cocaine (**2**) and the most potent DAT inhibitor of the series, (–)-1-phenyl-cocaine (**6**). First, pharmacokinetic experiments were carried out to assess the entry of **2** into the brain. Second, the ability of all five analogues to inhibit radiolabeled substrate uptake at DAT, SERT, and NET was determined. Third, because cocaine has local anesthetic properties, analogues **2** and **6** were tested for their ability to interact with voltage-dependent sodium channels. Fourth, potential locomotor-stimulating properties of all analogues were monitored and compared to those engendered by cocaine and compounds **2** and **6** were tested in a conditioned place preference assay. Fifth, as the non-stimulatory analogue **2** displayed a monoamine transporter binding profile indicative of a triple uptake inhibitor (TUI)—compounds which have shown promising antidepressant action in preclinical studies (Dutta et al., 2008 and refs. therein)—it was tested for potential antidepressant activity in an animal model of depression-like symptoms (Gopishetty et al., 2011). Finally, we performed *in silico* molecular modeling to assess whether **2** and **6** preferentially interact with an open-to-out DAT conformation—like cocaine and the phenyltropane stimulant β -CFT (Reith et al., 2001; Beuming et al., 2008)—or instead interact with a closed-to-out (inward-facing or occluded) state—like benztropine (**7**), various benztropine analogues and other atypical DAT inhibitors such as modafinil (**8**) (Schmitt et al., 2008; Loland et al., 2008; Schmitt and Reith, 2011).

The results of this work indicate that C-1 modification of the cocaine molecule can generate compounds that are taken up into the brain as readily as cocaine, but are not cocaine-like in that

they are not stimulatory *in vivo*, but yet are not benztropine-like in their preferential molecular interaction with an inward-facing DAT conformation. This warrants further work on C-1 substituted phenyltropanes as novel leads in medication development as well as pharmacological probes to assist in mapping inhibitor-binding SARs in NSS proteins.

Methods

Animals, Cells and Reagents. Details on animals (all male mice) are listed below under each test. All radioligand uptake assays were performed with stably transfected human embryonic kidney (HEK) 293 cells. Cultured cells expressing the human DAT were as described by us previously (Chen et al., 2004; Dutta et al., 2008). The human SERT expressing cells were those described by Eshleman and co-workers (Eshleman et al., 1995) and the human NET expressing cells used were those described by Reith and co-workers (Reith et al., 2005). Voltage-gated sodium channel inhibition assays were performed with cultured neocortical neurons obtained from Swiss-Webster mice, as described briefly in the section below and in further detail elsewhere (Cao et al., 2008; Jabba et al., 2010). All of the C-1 cocaine analogues were synthesized by us (Davis lab) (Davis et al., 2010; Davis et al., 2012). Other reagents were from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich Chemical Co. (St. Louis, MO) if not indicated in the sections below. All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.

Brain Uptake of C-1 Cocaine Analogue 2. Pharmacokinetic analysis for brain uptake of compound **2** was performed by Mr. Jeffrey Crabtree, Dr. Sanford Mendonca and Dr. Pat Noker of Southern Research Institute (Birmingham, AL). Six male CD-1 mice (Charles River Laboratories, NC), age 8 weeks, were injected i.p. with 30 mg/kg of **2** and sacrificed at time points of 5 and 30 min (3 animals per time point). Whole brain and plasma levels of **2** were determined by mass spectrometry.

Inhibition of Monoamine Uptake by DAT, SERT, and NET. The ability of test compounds to inhibit radiolabeled [³H]substrate uptake via human monoamine transporters was monitored as described by us previously (Reith et al., 2005). Briefly, DAT and SERT cells were grown in DMEM medium containing 5% fetal bovine serum, 5% bovine calf serum, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 2 µg/ml puromycin at 37°C and 5% CO₂. NET cells

were grown the same way, except that serum was only of the fetal bovine type (FBS, 10%) and the selective antibiotic was geneticin (G-418; 250 µg/ml). All buffers and solutions were prepared with purified water (Nanopure Diamond, Barnstead, Thermo Scientific, Dubuque, IA). Cell suspensions were prepared and incubated as previously described (Reith et al., 2005) in 122 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 10 mM glucose, 1 mM CaCl₂, 15 mM NaH₂PO₄ plus 7.5 mM Na₂HPO₄, mixed to achieve pH 7.4 at room temperature (21°C) and containing 0.1 mM tropolone for inhibition of COMT. In 96-well plates, cells and test inhibitor compound were first preincubated at room temperature, tritiated substrate was added, incubation was continued, and the assay was terminated by rapid filtration through Whatman GF/C glass fiber filtermats with a 96-pin Brandel harvester (Brandel Inc., Gaithersburg, MD, USA). Following filtermat drying, tritium radioactivity was assessed using a Wallac Microbeta Plus liquid scintillation counter (Perkin Elmer, Boston, MA). The conditions for uptake assays with HEK hDAT, hSERT, and hNET cells, respectively, were as follows: the particular radiolabeled substrates used were [³H]dopamine (ring 2,5,6-[³H]DA, 38.7 Ci/mmol; Perkin-Elmer, Boston, MA), [³H]serotonin (28.0 Ci/mmol, Perkin-Elmer), and [³H]dopamine (ring 2,5,6-[³H]DA, 38.7 Ci/mmol; Perkin-Elmer). For each assay, test inhibitor ligands were preincubated with cells for 6 minutes, followed by addition of radiolabeled substrate and further incubation for 5, 8, or 7 min, respectively. Nonspecific uptake was defined in the presence of 100 µM cocaine (for DAT), 100 µM citalopram (for SERT), or 10 µM desipramine (for NET). It is to be noted here that dopamine has a high affinity for the NET (somewhat higher than norepinephrine itself) (Gu et al., 1994; Buck and Amara, 1994) and is readily translocated by the NET, with a V_{max} value in the range of that of norepinephrine (Gu et al., 1994; Buck and Amara, 1994). Hence, [³H]dopamine is well-suited for use as a radiolabeled substrate in NET uptake assays. All compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted out in 10% (v/v) DMSO. Additions from the latter stocks resulted in final concentrations of DMSO of 1%, which by itself did not interfere with radioligand binding or uptake. The cocaine analogues did not need DMSO for dissolving, but

were assayed as part of a larger series of compounds with varying solubility. At least five concentrations of the test compound were studied, spaced evenly around its IC₅₀ value.

Receptorome Radioligand Binding Screen. Broad-spectrum *in vitro* receptor binding data for analogues **2** and **6** were kindly provided by Dr. Bryan L. Roth (University of North Carolina at Chapel Hill, NC) as part of the National Institute of Mental Health's (NIMH) Psychoactive Drug Screening Program (PDSP). In brief, the PDSP assesses binding of novel compounds at a wide array of cloned human G-protein coupled receptors, membrane transporters and other proteins known to be psychoactive drug targets (e.g. the σ_1 and σ_2 binding sites and the mitochondrial translocator protein TPSO, originally known as the 'peripheral' benzodiazepine receptor). For detailed description of the PDSP experimental protocols, please refer to the PDSP Web site at <http://pdsp.med.unc.edu/> and select the "Binding Assay" link.

Neocortical Neurons for Monitoring Voltage-dependent Sodium Channels. Primary cultures of neocortical neurons were obtained from embryonic day 17 Swiss-Webster mice as described elsewhere (Cao et al., 2008; Jabba et al., 2010). Briefly, pregnant mice were euthanized by CO₂ asphyxiation and embryos were removed under sterile conditions. Neocortices were collected, stripped of meninges, minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase containing isolation buffer, centrifuged, and resuspended in Eagle's minimal essential medium with Earle's salt (MEM) and supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 10% horse serum, 100 IU/mL penicillin, and 0.10 mg/mL streptomycin (pH 7.4). Cells were plated onto poly-L-lysine-coated 96-well (9 mm), clear-bottomed, black-well culture plates (MidSci, St. Louis, USA) at a density of 1.5×10^5 cells/well. Cells were then incubated at 37°C in a 5% CO₂ and 95% humidity atmosphere. Cytosine arabinoside (10 mM) was added to the culture medium on day 2 after plating to prevent proliferation of nonneuronal cells. The culture media was changed every other day, starting from day 5 *in vitro* using a serum free growth medium containing

Neurobasal Medium supplemented with B-27, 100 IU/mL penicillin, 0.10 mg/mL streptomycin, and 0.2 mM L-glutamine. Neocortical cultures were used in experiments between 8-10 days *in vitro*. All animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.

Intracellular Sodium Concentration Measurement. $[Na^+]_i$ measurement and full *in-situ* calibration of sodium-binding benzofuran isophthalate (SBFI) fluorescence ratio were performed as described previously (Jabba et al., 2010). Cells grown in 96-well plates were washed four times with Locke's buffer (8.6 mM HEPES, 5.6 mM KCl, 154 mM NaCl, 5.6 mM glucose, 1.0 mM $MgCl_2$, 2.3 mM $CaCl_2$, 0.1 mM glycine, pH 7.4) using an automated microplate washer (BioTek Instruments, Winooski, VT). After measuring the background fluorescence of each well, cells were incubated for 1 hour at 37°C with dye-loading buffer (100 μ L/well) containing 10 μ M SBFI-AM (Invitrogen) and 0.02% Pluronic F-127 (Invitrogen). Cells were washed five times with Locke's buffer, leaving a final volume of 100 μ L in each well. The plate was then transferred back to the incubator for 15 min to allow the cells to equilibrate after washing and placed in a FlexStation II (Molecular Devices, Sunnyvale, CA) chamber to detect Na^+ -bound SBFI emission at 505 nm (cells were excited at 340 and 380 nm). Fluorescence readings were taken once every 5 sec for 60 sec to establish the baseline, and then 50 μ L of different concentrations of either the cocaine analogues (**2** and **6**), lidocaine or cocaine was added to each well from the compound plate at a rate of 26 μ L/s. After 180 seconds, 50 μ L veratridine was added in each well, yielding a final volume of 200 μ L/well. After correcting for background fluorescence, SBFI fluorescence ratios (340/380) and concentration-response graphs were generated using GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Locomotor Activity in Mice. Locomotor activity of male C57BL/6 mice, 8-9 weeks of age, was measured in activity boxes equipped with infrared beams as described by us previously (Sershen et al., 2002). Briefly, an Opto-Varimex Auto-Track System (version 4.10, 8 Opto-Varimex-3 photocell activity monitors, Columbia Instruments, OH) was used. Mice are housed singly in a

standard mouse cage (7 x 12 x 5 inches) a day before activity monitoring. The cage is placed in the activity monitor and activity measured starting after intraperitoneal (i.p.) drug injection (1-30 mg/kg). Locomotor activity was calculated based on total ambulatory counts (consecutive beams broken during ambulation, single beams broken repeatedly are not counted). Data were expressed as ambulatory beam breaks over a 60-120 min period (in 5 min segments or total counts).

Forced Swim Test. Antidepressant-like activity of analogues **2** and **6** was tested in the forced swim test model as previously described (Porsolt et al., 1977; Castagne et al., 2011). Briefly, adult male C57BL/6 mice ($N = 6-10/\text{group}$) were placed into individual glass cylinders (45 cm high and 21 cm in diameter) containing clean 25°C ($\pm 1^\circ\text{C}$) water, 15 cm deep for 15 minutes. Mice were removed, dried and returned to their home cages. A second 6-min swim test was given 24 hours after the first. Saline (3 ml/kg, i.p.), **2** (30 mg/kg, i.p.), **6** (3 mg/kg i.p.), or desipramine (10 mg/kg, i.p.; a well-known antidepressant, used as a positive control) were administered 23.5 hours, 5 hours, and 30 min before the second swim test (*i.e.*, 30 minutes, 19 hours and 23.5 hours after the first swim). Test sessions were video recorded. Behavior during the 6-minute swim test was scored from the videotapes. The cumulative amount of time that the mouse was immobile in the water during the last 4 minutes of the test was measured, as was the latency for the mouse to show the first bout of immobility behavior. Immobility was defined as the mouse performing no movements except those necessary to keep the head above the water (*e.g.*, no swimming or climbing behaviors). The dose of compound **2** was set at 30 mg/kg i.p. with the consideration that (i) **2** and cocaine have comparable DAT affinity, (ii) 30 mg/kg i.p. is a behaviorally active dose for cocaine, and (iii) this dose of **2** did not produce locomotor stimulation (see Results).

Conditioned Place Preference Testing. For conditioned place preference (CPP) the Opto-varimex platform was divided into two sections (8 squares each) by a plastic partition (painted black to within 2 inches of the bottom) containing a 3 x 3 removable door. The two sections had different floor surfaces (smooth and rough textures). One side was covered by black paper, and

had a metal mesh floor. The other side was uncovered and had a smooth metal floor. The 9-inch high sides had a plastic cover, half of which was black colored. The room light was diminished, so that the light side had only a soft illumination. One side corresponded to the least preferred side of the animal during a preconditioning trial, when the mouse had access to both sides through a 3 x 3 inch opening in the center divider. Previous to the trial (pre-conditioning), the mouse was put in the apparatus and allowed to explore freely for 1 hour to decrease novelty during the trial. After conditioning, they were tested for preference again to see whether the time spent on the conditioned (non-preferred) side had increased.

Animals were handled gently several times per day for 2-3 days before they were allowed to explore the test area. Between sessions in the test box, the box was dismantled and thoroughly cleaned with 70% ethanol. The first day of testing the mice had equal access to both sides, and were monitored for time-in-box for 30 minutes to determine preference for light or dark side. On days 2-4, the mice were given an i.p. injection of either cocaine, the designated C-1 analogue test compound or saline and confined to the non-preferred side for 30 minutes during one session. They were given saline on the preferred side during another session. Time of the sessions was alternated, morning or afternoon. On day 5, they were again given access to both sides, and the difference in time spent on the non-preferred side was calculated (post- minus pre-condition time in seconds in non-preferred side – these two times were subjected to statistical analysis, see below). The cocaine dose (10 mg/kg i.p.) was chosen based on the literature (for references see Bardo and Bevins, 2000). Compound **2** with comparable DAT affinity (see Results) was also tested at 10 mg/kg i.p.; the dose of compound **6**, which was ~ 5 times more potent at DAT, was set at 3 mg/kg i.p..

DAT Homology Modeling and Flexible Ligand Docking. Homology modeling of the hDAT protein and flexible ligand docking was performed according to procedures previously described by us (Schmitt et al., 2010; Schmitt and Reith, 2011 and refs. therein). Briefly, the crystal structure of the bacterial leucine transporter LeuT—a prokaryotic NSS protein from *Aquifex*

aeolicus—bound to the ligands leucine and the tricyclic antidepressant desipramine (Zhou et al., 2007; PDB Index 2QJU) was used as the structural template, employing the NSS-family protein amino acid sequence alignment as by Beuming et al. (2008). The sodium ions were initially placed in the DAT model based upon their location in the LeuT crystal, but were allowed to move freely during energy minimization, docking and optimization rounds. The chloride ion that is co-transported during the DAT translocation cycle was initially placed at the position corresponding to E290 in the LeuT structure (see Schmitt and Reith, 2011 for further discussion of NSS protein Cl⁻ ion binding and our molecular model).

Homology modeling was performed using the MODELLER algorithm and the resultant lowest energy structure was imported into the Molecular Operating Environment (MOE) program suite (Version 2009.10; Chemical Computing Group, Montreal, CA). The Protonate3D function in MOE was used in combination with the AMBER99 forcefield and generalized Born (GB/VI) implicit solvation model to prepare the hDAT protein and assign partial charges. Following multiple minimization rounds, model stereochemical quality was analyzed with PROCHECK and Ramachandran plots. Ligand binding sites in the DAT model were identified with the Site Finder tool implemented in MOE—two binding pockets (approximately overlapping with the central ‘S1’ and the putative vestibular ‘S2’ substrate sites of LeuT) were identified. For review of the structural characteristics of LeuT and discussion of the two-site substrate-translocation model, see Nyola et al. (2010). For the present analysis of cocaine, bupropion and C-1 analogue interaction with the DAT, we focused on binding at the central S1 substrate-binding site. This decision was made to facilitate comparison of our docking results with other reported DAT molecular modeling studies, which indicate that both phenyltropane and bupropion-based DAT inhibitors preferentially bind at the S1 site (Beuming et al., 2008; Bisgaard et al., 2011). Dummy atoms were placed at the centroids of alpha spheres defining the S1 site to assist in ligand docking. For docking, ligand structures were imported into MOE, protonated, assigned partial charges and energy minimized ($< 0.001 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$) using the MMFF94x forcefield with

GB/VI implicit solvation. Energetically favorable poses were output to a database and a representative pose was then refined by further minimization rounds; protein backbone atoms were weakly tethered ($1 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ force constant) and the side-chain and ligand atoms completely unconstrained to allow for flexible “ligand adaptive” docking—for the last minimization round, the backbone tethering constant was increased to $10 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ and the convergence gradient was set at $0.01 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$. Final ray-traced models depicted in figures were rendered with PyMOL (Schrödinger LLC, New York, NY, USA). All MOE simulations were performed on a standard quad-core x64 computer running Windows 7.

Data Analysis and Statistics. The half-inhibition concentration (IC_{50}) for a compound to inhibit radiolabeled [^3H]substrate uptake was estimated by logistic fitting of data using the Origin 7.5 software suite (OriginLab, Northampton, MA), and IC_{50} values were converted to K_i values with the Cheng-Prusoff equation as described previously (see Schmitt et al., 2010). IC_{50} values for inhibition of voltage-gated sodium channel activity were obtained via nonlinear regression analysis using GraphPad Prism (GraphPad Software, San Diego, CA). Transporter data and immobility scores on the forced swim test were analyzed by a one-way ANOVA, followed by the Dunnett Multiple Comparisons Test or Bonferroni post-hoc analysis, respectively. Locomotor data were compared between equal drug doses tested within the same experimental set by Student’s t -test. CPP post- and pre-conditioning times were compared by paired Student’s t -test. Data were log-transformed where appropriate for comparing groups with equal variation. The accepted level of significance was $P < 0.05$.

Results

Brain Uptake of C-1 Analogue 2. Upon intraperitoneal administration to mice of 30 mg/kg of (–)-1-methyl-cocaine (**2**), the plasma and whole brain levels at 5 and 30 min (Fig. 2) were comparable to the cerebrocortical levels found by us after i.p. administration of 25 mg/kg (–)-cocaine (Benuck et al., 1987). For brain, the levels of **2** and cocaine were 8.6 ± 0.7 and 6.7 ± 1.2 $\mu\text{g/gm}$, respectively, at 5 min, indicating rapid brain entry for the C-1 analogue. The brain levels were 3.8 ± 0.2 and 2.3 ± 0.3 $\mu\text{g/gm}$ at 30 min; for plasma, the respective values were 3.3 ± 0.2 and 1.1 ± 0.1 $\mu\text{g/ml}$ at 5 min, and 1.7 ± 0.3 and 0.40 ± 0.05 $\mu\text{g/ml}$ at 30 min (mean \pm SEM, compare data in Fig. 2 with results of Benuck et al. (1987)). The dose-independent parameter, half-life of disappearance from the brain was estimated at 27 min for compound **2** (data Fig. 2) and 18 min for cocaine (Benuck et al., 1987); the brain/plasma ratios for the two compounds were 3 and 7. Thus, analogue **2** appears to enter the brain quickly, accumulates in brain at levels above those in plasma (but somewhat less pronounced than cocaine itself), and is eliminated from brain at a slightly slower rate than cocaine.

The predicted octanol-water partition coefficients ($\log P$ values) computed for compounds **2** and **6** are 2.67 and 3.77, respectively, compared with 2.30 for (–)-cocaine and 2.72 for β -CFT (calculated using the XLogP3 algorithm of Cheng et al., 2007). Predicted $\log P$ values for analogues **3**, **4**, and **5** are 3.20, 3.56, and 4.64, showing the expected increase in lipophilicity with increasing alkyl chain length. Because there is a general correlation between $\log P$ and BBB permeability, increasing chain length could be expected to enhance permeability (Rapoport, 1992); but this correlation becomes less predictable for compounds with $\log P$ values exceeding 2, which is the case for our compounds including cocaine. However, the above pharmacokinetic data for compound **2** show it to be rapidly taken up into brain—as are cocaine and other phenyltropane stimulants, such as β -CFT (Cline et al., 1992; Stathis et al., 1995).

Uptake Inhibitory Profile of all Analogues at DAT, SERT, and NET. In comparison with (–)-cocaine itself, the 1-methyl- and 1-ethyl-analogues (compounds **2** and **3**) had similar or somewhat higher potency (lower K_i) with respect to the DAT; these substitutions had little or no effect on

inhibitory potency at the SERT or NET (Table 1). Extending the length of the C-1 alkyl substituent to *n*-propyl and *n*-pentyl (compounds **4** and **5**, respectively) reduced DAT potency. This also appeared to be the case for SERT and NET. However, the C-1 phenyl analogue (**6**), was highly interesting in displaying an order of magnitude increase in affinity for the DAT compared with cocaine, whereas its affinity for SERT and NET was more comparable to that of **3** and **5**, respectively (Table 1). Further work focused on analogues with potent DAT activity. Thus, amongst compounds **2-5**, we chose analogue **2** which inhibited transmitter uptake mediated by DAT with a K_i of 163 ± 23 nM, a value not statistically different from 326 ± 106 nM observed for cocaine. In addition, compound **2** had the interesting feature of nonselectively inhibiting transmitter uptake by all three biogenic amine transporters with K_i values within a 3-fold window (Table 1). Finally, compound **6** was selected for more extensive characterization because of its selectivity for DAT (K_i of 32.3 nM) versus SERT (974 nM) and NET (1,980 nM).

Broad-spectrum Receptorome Binding Screen of Analogues 2 and 6. In order to assess whether the lack of locomotor stimulatory effect of the C-1 analogues is mediated by activity at targets other than monoamine transporters, compounds **2** and **6** were submitted to the NIMH Psychoactive Drug Screening Program (PDSP) (data not shown). Other than the expected activity at the DAT, SERT and NET, the only notable binding 'hits' ($K_i < 10$ μ M) revealed by the receptorome screen were the two σ receptor subtypes. Compound **2** lacked appreciable affinity for the σ_1 receptor, but bound to the σ_2 receptor with low micromolar affinity ($K_i = 1.13$ μ M). Compound **6**, on the other hand, showed non-selective sigmaergic activity, binding to the σ_1 receptor with an affinity of 524 nM and to the σ_2 receptor with an affinity of 198 nM. The results of the PDSP screen indicate that, except for sigma receptors, activity at sites other than the DAT is unlikely to underlie the unique properties of the C-1 analogues **2** and **6**. It should be noted here that a recent study by Hiranita et al. (2011) shows that compounds with dual DAT affinity and sigma antagonist activity are capable of attenuating cocaine self-administration. In fact, compound **6** (more than **2**) has a relatively significant affinity for sigma receptors (0.2-0.5 μ M), similar to the

sigma affinity of rimzacole (0.2-0.9 μM), and both compound **6** and rimcazole display appreciable DAT affinity (for rimzacole activity see Hiranita et al., 2011).

Cocaine Analogue Inhibition of Veratridine-stimulated Na^+ Influx in Neocortical Neurons.

The ability of cocaine, lidocaine and cocaine analogues to block voltage-gated sodium channels was assessed in neocortical neurons loaded with SBFI. We specifically determined the ability of these compounds to antagonize veratridine-stimulated Na^+ influx in these neurons. As depicted in Fig. 3, both cocaine analogues (**2** and **6**), lidocaine and cocaine produced concentration-dependent antagonism of the increase in neuronal $[\text{Na}^+]_i$ produced by veratridine. The concentration-response curves for the cocaine analogues **2** and **6**, lidocaine and cocaine, were best fit by a three-parameter logistic equation yielding IC_{50} values of $16.01 \pm 1.90 \mu\text{M}$, $0.29 \pm 0.07 \mu\text{M}$, $39.6 \pm 2.4 \mu\text{M}$ and $6.99 \pm 2.43 \mu\text{M}$ respectively. These results indicate that 1-phenyl-cocaine (**6**) is approximately 55-fold more potent than the 1-methyl analogue (**2**). Furthermore, in comparison with the IC_{50} value of $6.99 \mu\text{M}$ for (-)-cocaine itself, analogue **2** is 2.3-fold less and analogue **6** is 24-fold more active as an inhibitor of voltage-dependent Na^+ channels than the parent compound (Fig. 3). Previously, cocaine has been demonstrated to have an IC_{50} of $6 \mu\text{M}$ and lidocaine an IC_{50} of $73.6 \pm 10 \mu\text{M}$ as inhibitors of veratridine-induced Na^+ flux (Matthews and Collins, 1983, Deffois et al., 1996). This potency of cocaine and lidocaine is therefore in good agreement with the data presented here.

Effect of all C-1 Substituted Cocaine Analogues on Locomotor Activity. The first compounds tested, the 1-methyl- and 1-propyl-cocaine analogues (**2** and **4**), had little if any effect on locomotor activity in adult mice, at doses up to 30 mg/kg i.p. (Fig. 4; compare with saline in left panel and with cocaine). Subsequently tested compounds **3**, **5**, and **6** (the 1-ethyl, 1-pentyl and 1-phenyl derivatives, respectively) were also non-stimulatory, showing no significant effects on locomotion at doses of 10 or 30 mg/kg i.p. (Fig. 5). In contrast, (-)-cocaine dose-dependently increased locomotion, with intraperitoneal administration of 10 mg/kg producing a slight elevation above saline, and 30 mg/kg producing pronounced increases (Figs. 4, 5). Overall, each of the novel C-1

analogues, even at a dose of 30 mg/kg, did not stimulate locomotion to levels above those seen with 10 mg/kg of cocaine. This finding is remarkable for compounds **3** and **6**, which exhibit greater potency than cocaine in blocking the DAT (Table 1). This is especially true for **6**, which is 10-fold more potent than cocaine at blocking DAT-mediated substrate uptake in HEK-hDAT cells (the K_i values for inhibition of [3 H]DA uptake for compound **6** and cocaine were 32.3 nM and 326 nM, respectively). Thus, compared with cocaine, in the context of DAT activity, doses of 10 and 30 mg/kg of analogue **6** are relatively high, and we therefore additionally tested lower doses of 1 and 3 mg/kg (Fig. 5B). Administration of 1 and 3 mg/kg of compound **6** produced locomotor counts comparable to the counts observed with 10 mg/kg. This indicates it is unlikely we somehow missed an inverted U-shaped dose-response curve for locomotion with higher doses becoming inhibitory or inducing motor stereotypy that might mask increases in locomotor behavior. In this context, doses of compounds **4** and **5** higher than 30 mg/kg would be expected to be needed for behavioral activity involving DA based on their lower DAT affinity compared with cocaine (~ 3- to 4-fold); such higher doses, however, were not tested in order to avoid complications from potentially deleterious off-target effects.

Effect of Analogue 2 and 6 in Forced Swim Test Sensitive to Depression-like Symptoms.

Saline, analogue **2** (30 mg/kg, i.p.), analogue **6** (3 mg/kg, i.p.) or the classical tricyclic antidepressant desipramine (10 mg/kg, i.p.) were administered 23.5 hours, 5 hours and 1 hour prior to the 6-min test swim (Fig. 6). Compound **2** was as effective as the antidepressant desipramine in reducing immobility time (panel A; $F_{2,22} = 21.26$; $P = 0.0001$). In addition, the latency to the first episode of immobility was significantly increased over saline, similar to desipramine (panel B; $F_{2,22} = 20.52$; $P < 0.0001$). In a separate experiment, a significant immobility reduction and latency increase was also observed for compound **6** (Fig. 6 panels C and D, with, respectively, $F_{2,20} = 5.336$; $P = 0.013$ and $F_{2,20} = 6.191$; $P < 0.0085$). Together, these results suggest that (–)-1-methyl-cocaine (**2**) and (–)-1-phenyl-cocaine (**6**) have antidepressant-like action.

Effect of Analogues 2 and 6 in Conditioned Place Preference Test. In the conditioned place preference (CPP) test, analogue **2** (10 mg/kg), as cocaine (**1**) (10 mg/kg), increased the postconditioning time (over preconditioning time) in the non-preferred side (first two bars in each block in Fig. 7A). That is, analogue **2** produced CPP, without reducing the CPP engendered by cocaine itself (Fig. 7A, third bar); saline was ineffective in increasing the postconditioning time in the non-preferred side as expected. In a separate experiment in a different batch of mice, analogue **6** (3 mg/kg) did not produce CPP, with postconditioning time in the non-preferred side being equal to the preconditioning time (first bar in each block in Fig. 7B). Because only one dose of **6** was tested here, caution is needed in concluding the compound completely lacks CPP properties. Intriguingly, pretreatment with compound **6** (3 mg/kg) prior to cocaine (10 mg/kg) (last bar in Fig. 7B) caused the difference between post- and pre-conditioning time to only approach statistical significance ($P = 0.053$; paired Student's *t*-test); in contrast, the difference observed with cocaine alone in a separate experiment (first bar in right-hand cluster in Fig. 7A) indicated a statistically significant CPP at $P = 0.013$. The results taken together suggest that although some C-1 analogues may possess rewarding qualities, other C-1 substitutions can give compounds that (i) may not produce CPP, (ii) may interfere with CPP engendered by cocaine, and (iii) are not locomotor stimulants. To find a compound that engenders CPP but does not stimulate locomotion (both DA-associated behaviors) is not in itself surprising. Notably, dissociation among genetically different mouse strains for cocaine to produce CPP and locomotor stimulation has been reported (Eisener-Dorman et al., 2011) with not entirely overlapping brain circuits for reward and locomotion. Likewise, a dissociation between the locomotor effect and the discriminative stimulus effect of cocaine-like compounds has been reported for the C2-phenyl ester-substituted cocaine analogue RTI-15 (Cook et al., 1998).

Docking of Cocaine, Analogue 2, and Analogue 6 into hDAT Homology Model. It was originally assumed that all ligands acting as non-translocated DAT inhibitors possessed reinforcing effects identical to those of cocaine and differed solely in affinity for the transporter (hence, in the

dose required to elicit cocaine-like behavioral reactions). However, various tropane compounds with a benztropine-type structure can exhibit remarkably high affinity for the DAT; but, rather than being powerful central stimulants like cocaine, these atypical DAT inhibitors show mild (if any) locomotor stimulant effects, as well as attenuate the effects of concomitantly-administered cocaine (Agoston et al., 1997; Li et al., 2011). There is ample evidence that different classes of DAT ligands preferentially bind to (or induce upon binding) distinct transporter conformational states (Reith et al., 2001; Chen et al., 2004; Loland et al., 2008). The lack of cocaine-like locomotor stimulation seen with atypical inhibitors (like benztropine) is thought to result from their propensity to stabilize the DAT in a closed-to-out (inward-facing or occluded) conformational state, as opposed to cocaine-like ligands, which stabilize an open-to-out (outward-facing) transporter conformation (Schmitt et al., 2008; Loland et al., 2008; Schmitt and Reith, 2011). We considered the possibility that the C-1 cocaine analogues fall in the benztropine-like category with respect to their conformational effects on the DAT. Thus, we sought to investigate the interaction of certain C-1 analogue compounds with the DAT *in silico*, using our homology model of the human DAT and flexible ligand-adaptive docking procedures (Schmitt et al., 2010; Schmitt and Reith, 2011). We performed docking simulations with two of the novel C-1 analogues—the prototypical series member 1-methyl-cocaine (**2**) and the most potent DAT inhibitor of the series, 1-phenyl-cocaine (**6**)—as well as (–)-cocaine itself and benztropine to serve as a basis for comparison. The hDAT model was based upon the structure of LeuT, with ligands docked at the primary (S1) substrate-binding site. For each ligand, an energetically favorable docking pose was selected for further geometric optimization of the transporter/ligand complex (see Methods).

Final energy-minimized poses of the model hDAT/ligand complexes are shown in Fig. 8. Following flexible docking, cocaine was oriented such that the protonated tropane amine faced the S1 site residues of TM1 and exhibited strong molecular interactions with residues D79 (via a hydrogen bond) and F76 (via a cation- π interaction with the aromatic side-chain of F76). The 3 β -benzoyloxy and 2 β -carbomethoxy moieties were positioned towards TMs 3 and 8, enveloped by

the side-chains of residues V152, G153, Y156 and N157, as well as S422 and A423 (Fig. 8A). The docking orientation of cocaine and the binding-pocket residues found were consistent with those reported by Beuming et al. (2008) in their docking model of cocaine at the DAT S1 site. The docking model of **2** suggested that the analogue binds in a similar manner as cocaine, with the extra methyl group oriented towards D79 and surrounding residues of TM1 (F76, A77 and V78), but still readily accommodated within the binding pocket (Fig. 8B). In contrast, the additional steric heft of the C-1 phenyl substituent of **6** was not as readily accepted and the compound adopted an entirely different binding orientation than cocaine and **2** (Fig. 8C). The extra C-1 phenyl group was positioned downward (toward the cytosolic vestibule), adjacent to the aromatic side-chains of residues F76 and F326. In addition, the 3 β -benzoyloxy moiety was oriented upward, slightly above the extracellular vestibule gating network residues R85, F320 and D476, enabling formation of a cation- π interaction between the benzoyloxy aromatic ring and R85 (Fig. 8C). In the benztropine docking model, the diphenylmethoxy ring system faced V152, G153 and Y156 of TM3 and the tropane *N*-methyl amine group was surrounded by F76 (which formed a cation- π interaction with the amine nitrogen), V78 and D79 of TM1 (Fig. 8D). Similar to the docking results for benztropine reported by Bisgaard et al. (2011), we found that one of the diphenylmethoxy system rings was positioned relatively high up in the S1 binding site, close to residues A479 and A480 of TM10 (Fig. 8D).

Despite their benztropine-like lack of locomotor stimulatory activity *in vivo* (Figs. 4 and 5), our *in silico* docking results do not support the theory that the C-1 analogues interact with the DAT in an 'atypical' manner like benztropine. In their study combining DAT modeling and site-directed mutagenesis, Beuming et al. (2008) showed that the presence or absence of a hydrogen bond between D79 and Y156 in a given DAT/ligand complex offers an indication of the DAT conformation bound by the ligand. Tyrosine residue Y156 (which is highly conserved across NSS protein family members) interacts with substrates upon binding at the S1 site and also participates in the vestibular gating network—consisting of D79, R85, Y156, F320 and D476—that partitions the S1 and S2 sites

(Shan et al., 2011). When DAT substrates (such as dopamine, dextroamphetamine and MDMA) bind at the S1 site, a hydrogen bond formed between the side chain oxygen atoms of D79 and the hydroxyl moiety of Y156 helps to close the vestibular gate, protecting the substrate and ions from permeation of extracellular water into the S1 site (Beuming et al., 2008). Hence, an interatomic distance of less than 3.5 Å (indicative of an intact hydrogen bond) between the oxygen atoms of D79 and Y156 during ligand binding in the S1 site suggests the ligand interacts with a closed-to-out transporter state, whereas D79-Y156 distances greater than the 3.5 Å maximum for hydrogen bonding signify interaction with an open-to-out (outward-facing) state. In our case, cocaine binding resulted in a terminal D79-Y156 distance of 6.03 Å—consonant with many other literature findings of cocaine's selective interaction with an outward-facing DAT conformation—and benztropine binding resulted in a D79-Y156 distance of 3.29 Å, indicating preferential interaction with an inward-facing conformation (Figs. 8A and 8D). Unexpectedly, docking of the C-1 analogues **2** and **6** yielded respective terminal D79-Y156 distances of 4.40 Å and 4.89 Å (Figs. 8B and 8C), suggesting that these compounds, like cocaine, exhibit preferential interaction with an outward-facing DAT conformation.

Binding of C-1 Analogues to Different DAT Conformations. Binding affinity for conformationally biased DAT mutants can be indicative of preferential interaction with either an outward or inward facing conformation of the transporter (Loland et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011). Thus, a compound that prefers an inward-facing form of the DAT displays a loss of binding affinity for a mutant biased towards an outward-facing conformation, whereas an outward preferring compound gains affinity at such a mutant. The latter is what we observed for compounds **2**, **3** and **6** when comparing their affinities for displacement of [³H]CFT binding at W84L—a DAT mutant with an outward-facing bias—and WT transporters (Table S1, Supplemental Data). This is consonant with the docking data presented above for compounds **2** and **6** (see Supplemental Results and Discussion).

Discussion

C-1 Modification of the Cocaine Molecule and Brain Uptake. The lack of appreciable locomotor effect of the C-1 compounds could be explained if they have difficulty crossing the blood-brain barrier (BBB). This, however, would be unexpected for cocaine-like structures, with rapid brain entry observed for phenyltropane cocaine analogues (Cline et al., 1992; Stathis et al., 1995). Another hypothesis for the lack of cocaine-like locomotor stimulant effects of **2** is that the compound is either metabolized to inactive products more rapidly than cocaine, or is actively expelled from the brain by the ATP-coupled multidrug efflux transporter P-glycoprotein, which is expressed in the endothelial cells forming the blood-brain barrier. However, the fact that **2** was detected in the brain at 30 minutes post-administration—in concentrations similar to those achieved following administration of cocaine—makes this explanation implausible. Furthermore, central effects observed for **2** in the forced-swim and CPP tests strongly indicate brain penetration of this analogue.

Properties of C-1 Analogues are Unlike Either Cocaine or Benztropine. All C-1 cocaine analogues synthesized thus far (Fig. 1) were devoid of the locomotor stimulant activity associated with cocaine (Figs. 4 and 5). At the same time, analogues **3** and **6** were more potent inhibitors of the DAT than cocaine and analogue **2** was at least as potent. This combination of non-stimulatory character with potent DAT interaction equally applies to benztropine analogues (Li et al., 2011), some of which attenuate behavioral effects of cocaine (Desai et al., 2005). However, we do not think that the C-1 cocaine analogues investigated here are in the same pharmacological category as benztropine (and derivatives), as our *in silico* modeling data indicate that, unlike benztropine, the C-1 analogues do not appear to interact with an inward-facing DAT conformation (Fig. 8). In contrast, the C-1 analogues appear to bind, like cocaine, to an open-to-out state: compare the terminal distance between oxygen atoms of DAT residues D79-Y156 for cocaine, **2** and **6** (Fig. 8A-C) with that of benztropine (Fig. 8D). Only in the benztropine docking model is the interatomic distance lower than the 3.5 Å maximum required for formation of a hydrogen bond. Hence, of the four ligands tested in our docking models, only benztropine allows the DAT extracellular gating network

to operate, giving rise to a closed-to-out conformation. Results from interaction experiments involving W84L, a DAT mutant conformationally biased towards the outward-facing transporter state, point to the same conclusion for compounds **2**, **3**, and **6** (see Supplemental Data). In this context it is of interest that modafinil (**8**), which possesses a benzhydrylsulfinyl moiety that is similar to the diphenylmethoxy group of benztropine (albeit with a sulfinyl moiety in lieu of oxygen), has shown promise as a therapeutic for cocaine addiction and interacts with a closed-to-out, inward-facing DAT conformation (Schmitt and Reith, 2011 and refs. therein). The novel C-1 cocaine analogues described here are interesting probes for mapping inhibitor-binding properties in monoamine transporters.

Novel Pharmacological Tools in Medication Development for Psychiatric Disorders. Some of the C-1 cocaine analogues described here display a novel pharmacology: cocaine-like brain uptake and potent DAT interaction combined with a benztropine-like lack of locomotor stimulation (although molecular interaction with the DAT appears to differ from that of benztropine). The combination of monoamine transporter interaction with lack of stimulatory effect warrants further exploration of this type of compounds for central effects other than stimulation. Analogues **2** and **6** provide proof of principle for C-1 cocaine analogues to be able to exert central effects, but not locomotor stimulatory activity. Thus, **2** and **6** displayed antidepressant-like activity in the forced-swim test as effectively as the classical antidepressant desipramine (Fig. 6). It will therefore be important to further explore SARs for strengthening the antidepressant effect while maintaining the lack of locomotor stimulation. One obvious possibility is to remove the benzoyl ester linkage from the source compounds, generating more metabolically stable phenyltropanes, as in 2 β -carbomethoxy-3 β -phenyltropane (troparil), the prototypical compound of the WIN series of phenyltropane stimulants (Clarke et al., 1973). In addition, it can be expected that the phenyltropane versions will be more potent as monoamine transporter inhibitors than their respective cocaine (benzoyloxytropane) counterparts (Reith et al., 1986; Carroll et al., 1992).

It is also likely that removal of the benzoyl ester moiety will attenuate cocaine-like inhibitory activity at voltage-gated Na⁺ channels, reducing the possible risk of cardiotoxicity (Matthews and Collins, 1983). In this respect, the present data emphasize the importance of monitoring all C-1 cocaine analogues for activity at voltage-gated Na⁺ channels. Analogue **2** was less potent (16.01 μM) than cocaine itself (6.99 μM) in inhibiting Na⁺ flux, reducing the risk of cardiotoxicity. In contrast, **6** was more potent than cocaine (by an order of magnitude, 0.29 μM).

Based on the realization that dopamine dysfunction plays a large role in anhedonia and depression (Nestler and Carlezon, 2006), there has been revival of the idea that DAT blockade can contribute to antidepressant activity, which has given rise to the development of triple uptake inhibitor (TUI) compounds as potential antidepressants (Dutta et al., 2008 and refs. therein). Current antidepressants are either selective for SERT (SSRIs) or target both SERT and NET (SNRIs). These medications all suffer from delayed onset of antidepressant effect, risking patients to abandon treatment within the required 2-week period. In addition, many patients are not responsive to currently available antidepressants and the majority of patients do not experience complete remission of depressive symptoms when treated with SSRIs alone (Stewart et al., 2009). Therefore the search needs to be continued for novel treatments and for lead TUI compounds such as the present C-1 analogues not available until now.

Another obvious area where cocaine-like compounds could be useful is the addiction field. There is no accepted pharmacotherapy currently available for psychostimulant users. Off-label prescription of dopaminergic agents—particularly DAT substrates like dextroamphetamine or atypical DAT blockers like modafinil—have beneficial effects in attenuating drug craving (Shearer, 2008; Herin et al., 2010), but a novel, widely applicable, treatment is in high demand. In this context, it is of interest that analogue **6** does not produce CPP, attenuates CPP engendered by cocaine, and is not a locomotor stimulant (Figs. 5 and 7B). Manipulation of dopaminergic and serotonergic neurotransmission can affect self-administration of psychostimulant drugs (Spealman, 1993; Rothman and Baumann, 2006; Howell et al., 2007); for example, SERT activity has been

shown to attenuate the locomotor stimulator effects of cocaine and amphetamine (Spealman, 1993; Howell et al., 2007). Hence, non-stimulatory C-1 analogues combining DAT and SERT inhibition could be useful. The non-stimulatory analogue **2** displayed this combination. Unlike analogue **6** however, compound **2** did not reduce cocaine CPP, and itself produced CPP (Fig. 7A). It should be noted that CPP tests can be susceptible to effects other than reward reduction or enhancement (Bardo and Bevins, 2000), and more addiction-related tests are needed. In this context, it should also be recalled that compound **6** displays sigma activity (see Results, broad-spectrum receptorome screening), and that sigma antagonists have been reported to block CPP development (see Katz et al., 2011).

In the context of anti-addiction drug development, one issue to consider for C-1 cocaine analogues is the distinction between substitution and antagonist therapies. The goal of antagonist therapy is pharmacologically enforced abstinence via use of a selective, high-affinity antagonist of the addictive drug's binding target (e.g. naltrexone, a potent μ -receptor antagonist used in opioid addiction). In the case of treatment of cocaine addiction, a long-held view has been that we may be able to antagonize cocaine action at the DAT with a compound that does not interfere with DA recognition (a "DA-sparing" compound) (Carroll et al., 1992). Although this is still a theoretical possibility—and may have some limited practical use in treating cocaine overdose—recent attention has shifted to being able to substitute the psychostimulant drug of addiction with a milder, longer-acting 'agonist-like' agent that itself has lower abuse liability (Shearer, 2008; Herin et al., 2010). The potential value of our novel cocaine analogues, we feel, lies in the latter category. The prototypical compound **2** exhibits an antidepressant effect (as indicated by the forced-swim test data) and is not a locomotor stimulant; if it retained some cocaine-like, mildly rewarding effect, it would be of interest to test it preclinically as a cocaine replacement therapeutic. However, its activity in the CPP test necessitates future behavioral work to ensure that this compound and other non-stimulatory C-1 analogues do not have the potential of addictive liability.

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Authorship Contributions

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Additional results are available on line as Supplemental Data.

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Legends to Figures

Fig. 1. Chemical structures of (*R*)-(-)-cocaine, novel C-1 substituted cocaine analogues, as well as the atypical DAT inhibitors bupropion and modafinil. Tropane ring carbon atoms of cocaine and its analogues are numbered and the absolute configuration of the stereoactive carbons in (-)-cocaine is indicated (formally, (-)-cocaine has a 1*R*,2*R*,3*S*,5*S* absolute configuration).

Fig. 2. Pharmacokinetic data for compound (-)-**2** administered to CD-1 male mice with 3 animals per time point. Data shown are mean \pm SEM (vertical bar).

Fig. 3. Nonlinear regression analysis of the response of the Na⁺-sensitive fluorescent indicator SBFI (ratiometric measurement of fluorescence intensity at 505 nm with sequential excitation at 340/380 nm) to veratridine, in the absence and presence of the C-1 cocaine analogues (-)-**2** and (-)-**6** (panels A and B), lidocaine (panel C) or cocaine (panel D). Data shown denote the mean \pm SEM of two representative experiments performed with 2-4 replicates. IC₅₀ values represent the mean \pm SEM of 4-6 experiments, each performed with 2-4 replicates.

Fig. 4. The effect of acute cocaine, saline or the C-1 analogues (-)-**2** (left panels) and (-)-**4** (right panels) on ambulatory counts (measure of consecutive infra-red beams interrupted by C57BL/6 male mice during ambulatory activity) over a 120-minute period after injection (in 5-min intervals). Doses administered (either 10 mg/kg or 30 mg/kg i.p.) are indicated; data are mean \pm SEM (vertical bar). **P* < 0.01, *n* = 5-6, compared with C-1 compound at same dose (Student's *t*-test).

Fig. 5. The effect of acute cocaine, analogues (-)-**3**, (-)-**5** (panel A) or analogue (-)-**6** (panel B) on ambulatory counts. Details are as in Fig. 4; however, a wider dose range (1-30 mg/kg) was tested for compound (-)-**6**, to account for its increased potency as a DAT inhibitor compared to

cocaine. $*P < 0.05$, $n = 6-12$, compared with C-1 compound at the same dose (Student's t -test). Statistics were applied to C-1 groups compared with cocaine groups (at same dose) examined within one and the same experimental set; cocaine values shown are the average of all experiments.

Fig. 6. The effect of analogue (–)-**2** and (–)-**6** in the forced swim test model of antidepressant-like activity in C57BL/6 male mice. Similar to the clinically useful tricyclic antidepressant desipramine (DMI, 10 mg/kg, i.p.), (–)-**2** (30 mg/kg, i.p.) decreased immobility time (panel A) and increased latency to first episode of immobility (B) compared with saline-injected controls. The same was true for (–)-**6** (3 mg/kg, i.p.) and immobility time (C) and latency to first episode of immobility (D). Data are mean \pm SEM (vertical bar). $*P < 0.05$, $***P < 0.001$, $n = 7-10$ (One-way ANOVA and Bonferroni *post-hoc* tests).

Fig. 7. CPP test on C57BL/6 male mice treated with saline, cocaine, (–)-**2**, or (–)-**2** preceding cocaine by 20 min (panel A) or treated with (–)-**6**, or (–)-**6** preceding cocaine by 20 min (panel B) (all drugs at 10 mg/kg i.p. except for (–)-**6** which was at 3 mg/kg i.p.). Data are mean \pm SEM (vertical bar). $*P < 0.05$, $n = 8$, compared with preconditioning time in non-preferred side (paired Student's t -test).

Fig. 8. Final energy-minimized poses of DAT/ligand complex following flexible docking of cocaine, the novel C-1-substituted cocaine analogues (–)-**2** and (–)-**6** and benztropine at the DAT primary (S1) substrate-binding site. Selected binding pocket residues are labeled and rendered as sticks; bound ligand molecules (also shown as sticks) are highlighted using gray-colored carbon atoms. Co-transported sodium and chloride ions are labeled and rendered as purple and green spheres, respectively. The distance between the carboxylate oxygen atom of residue D79 and the ring hydroxyl moiety of residue Y156 (accentuated by a green-colored dashed line connecting the two

oxygens) is indicated in the lower right of each panel in green text. Binding of (–)-cocaine (panel A) and the C-1 analogues (–)-**2** (panel B) and (–)-**6** (panel C) disrupts the hydrogen bond between the side-chains of D79 and Y156 (interatomic distance $> 3.5 \text{ \AA}$), indicating that these compounds promote an open-to-out DAT conformational state. In contrast, in the binding model of benztropine (panel D), the D79-Y156 interatomic distance is 3.29 \AA , suggesting that benztropine interacts with a more inward-facing (closed-to-out) DAT conformation.

Tables

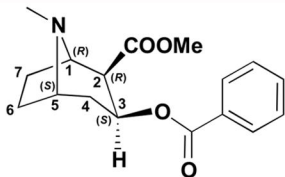
Table 1. K_i values for inhibiting neurotransmitter uptake in HEK-293 cells heterologously expressing human monoamine transporters. Uptake of [3 H]dopamine by DAT or NET expressing cells, and [3 H]serotonin by SERT expressing cells was monitored as described in the Methods section.

| Compound (C-1 Moiety) | K_i at DAT ¹ (nM) | K_i at SERT ¹ (nM) | K_i at NET ¹ (nM) |
|--|--------------------------------|---------------------------------|--------------------------------|
| (-)-1 Cocaine (H) | 326 ± 106 | 513 ± 143 | 358 ± 69 |
| (-)-2 (Me) | 163 ± 23 | 435 ± 77 | 488 ± 101 |
| (-)-3 (Et) | 95.1 ± 17.0* | 1,106 ± 112 | 598 ± 179 |
| (-)-4 (<i>n</i> -Pr) | 871 ± 205* | 2,949 ± 462** | 796 ± 195 |
| (-)-5 (<i>n</i> -C ₅ H ₁₁) | 1,272 ± 199** | 1,866 ± 400* | 1,596 ± 21** |
| (-)-6 (Ph) | 32.3 ± 5.7** | 974 ± 308 | 1,980 ± 99** |

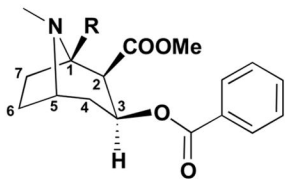
¹Average ± SEM for 3-8 independent experiments.

* $P < 0.05$, ** $P < 0.01$ compared with (-)-1 (cocaine) (One-way ANOVA followed by Dunnett's Multiple Comparisons Test).

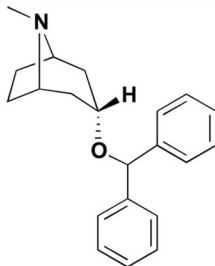
Fig. 1



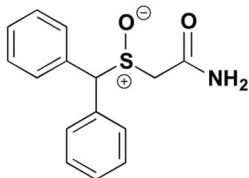
2β-carbomethoxy-3β-benzoyloxytropane
(*R*)-(-)-cocaine (1)



- R = Me:** (-)-1-methyl-cocaine (2)
R = Et: (-)-1-ethyl-cocaine (3)
R = *n*-Pr: (-)-1-propyl-cocaine (4)
R = *n*-C₅H₁₁: (-)-1-pentyl-cocaine (5)
R = Ph: (-)-1-phenyl-cocaine (6)



3α-benzhydryloxytropane
benztropine (7)



2-(benzhydrylsulfinyl)acetamide
(*RS*)-modafinil (8)

Fig. 2

**(-)-1-methyl-cocaine (Analogue 2)
Single-dose Pharmacokinetics**

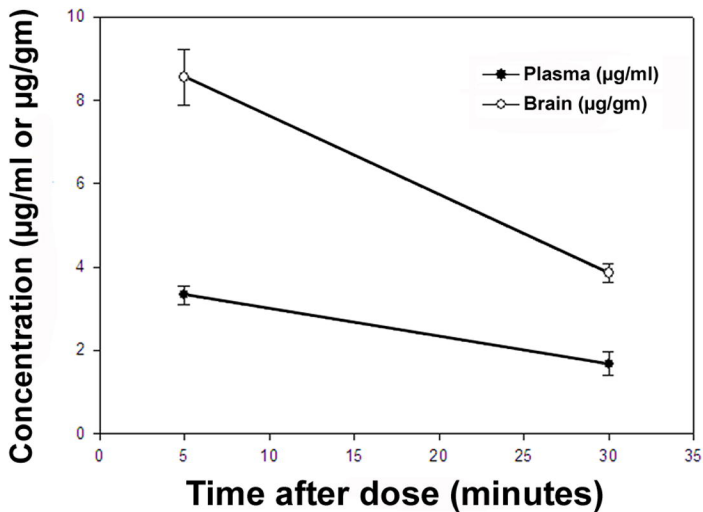


Fig. 3

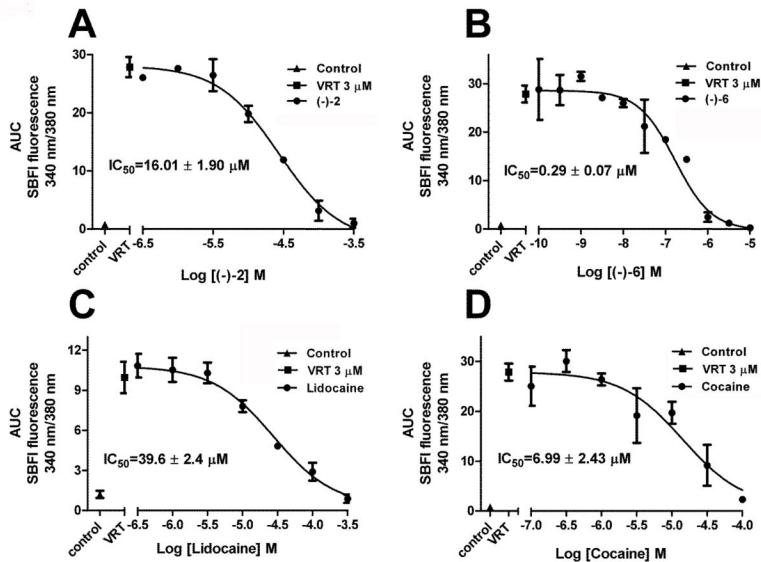


Fig. 4

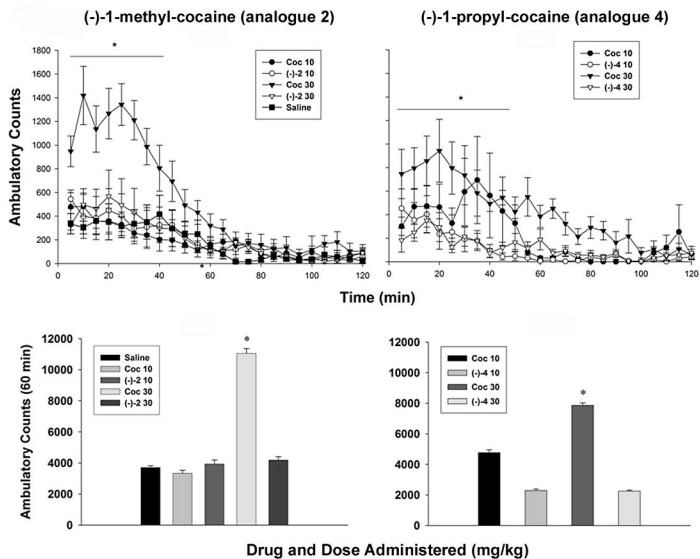


Fig. 5

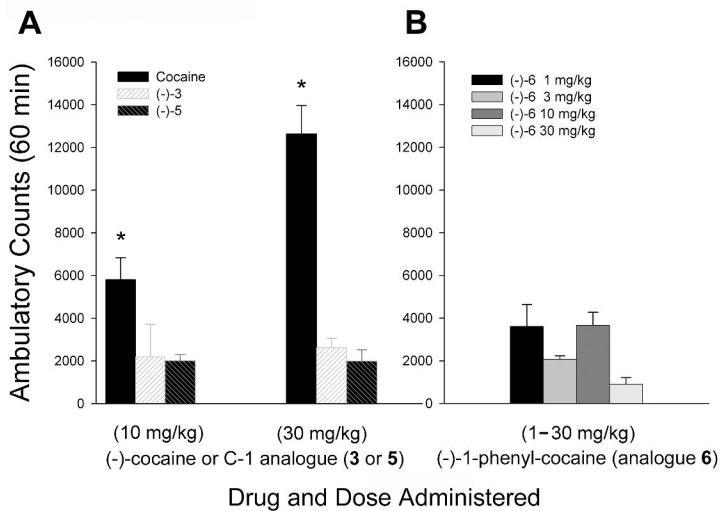


Fig. 6

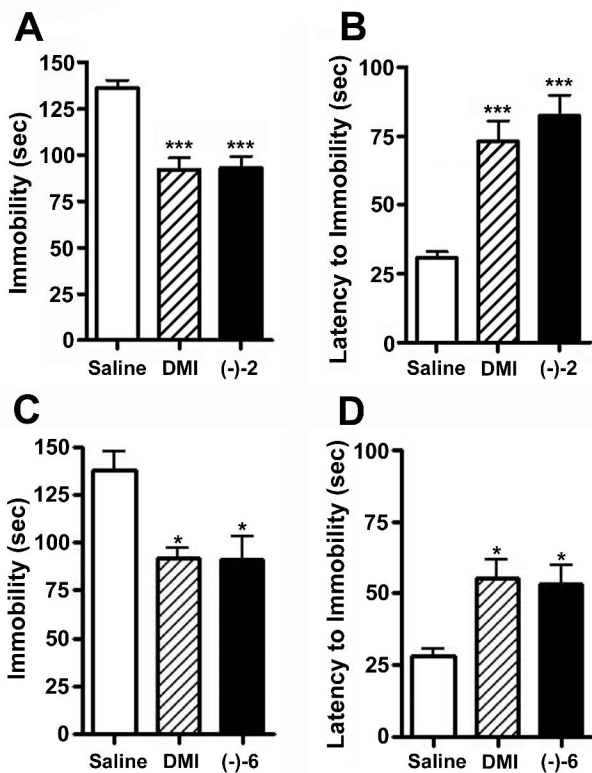


Fig.7

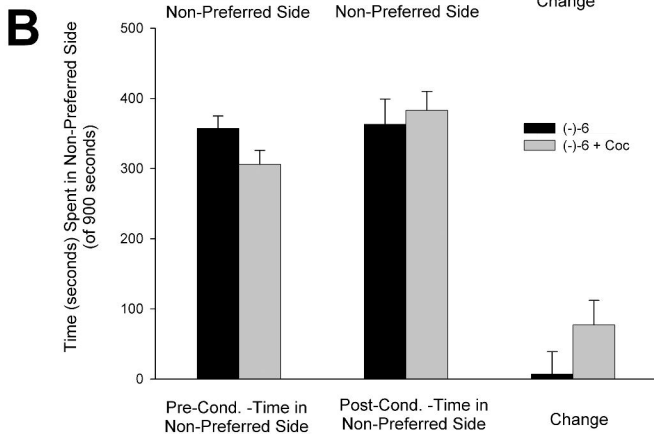
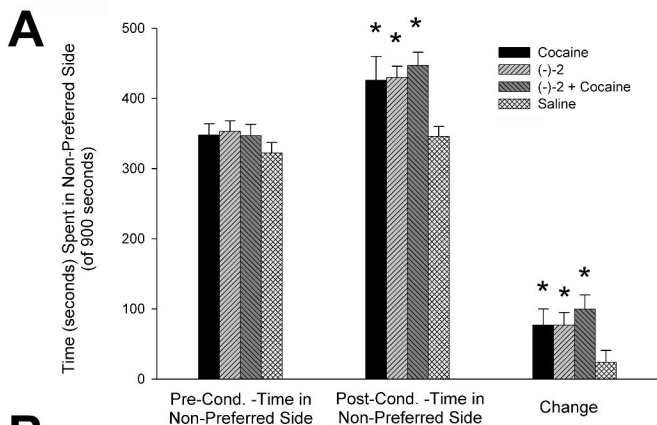
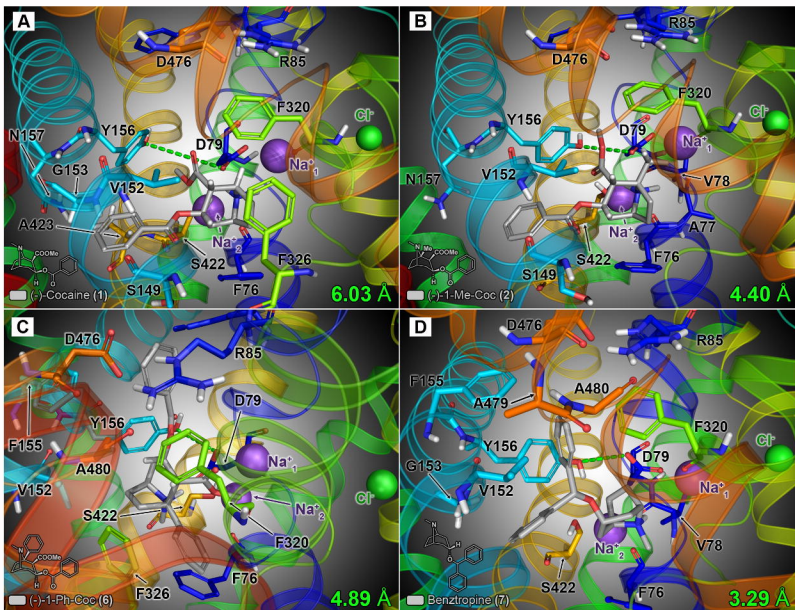


Fig. 8



Supplemental Data

Table S1: Potencies of C-1 cocaine analogues and reference DAT inhibitors, assessed by competition for intact-cell [³H]CFT binding to WT or mutant hDAT.

| Compound (C-1 Moiety) | Whole-Cell hDAT Binding K_i (nM) | | K_i [WT] / K_i [Mutant] Ratio |
|---|------------------------------------|------------|-----------------------------------|
| | Wild-type | W84L | |
| (-)- 2 (Me) | 167 ± 12 | 48.1 ± 4.7 | 3.48 |
| (-)- 3 (Et) | 216 ± 27 | 48.0 ± 6.2 | 4.51 |
| (-)- 6 (Ph) | 38.8 ± 6.1 | 16.5 ± 1.3 | 2.35 |
| (-)- 1 (cocaine, H) ^a | 164 ± 1.2 | 46.7 ± 4.5 | 3.50 |
| 7 (benztropine) ^a | 75.3 ± 7.4 | 190 ± 6.8 | 0.40 |
| (±)- 8 (modafinil) ^b | 2143 ± 215 | 3816 ± 266 | 0.56 |

Binding assays were performed using intact HEK cells stably transfected with either WT or mutant hDAT; values are means ± SEM for 2-6 experiments, each performed in triplicate. ^{a,b}Values previously reported in Schmitt et al. (2008)^a and Schmitt and Reith (2011)^b were obtained with identical methods and are included here for reference.

Supplemental Results and Discussion

The novel 1-alkyl substituted cocaine analogues **2**, **3**, and **6** displayed a higher K_i value at WT DAT than at the conformationally biased W84L mutant. The increase in affinity of the C-1 analogues for the W84L transporter was similar in magnitude to that of cocaine (indeed, the gain in binding affinity seen with cocaine was virtually identical to that of the 1-methyl analogue, compound **2**). This suggests that the C-1 analogues tested here preferentially interact with the outward-facing conformation of the DAT, as does cocaine itself (Loland et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011). In contrast, benztropine and modafinil have been shown to preferentially interact with a more inward-facing “closed-to-out” conformation (Loland et al., 2008; Schmitt et al., 2008; Schmitt and Reith, 2011). For comparison, Table S1 lists their K_i values for interaction with WT and W84L DATs under the same assay conditions employed here. Benztropine and modafinil showed WT over W84L K_i ratios of 0.40 and 0.56, indicating a loss of affinity for the outward-facing W84L mutant versus WT DAT. These mutant affinity shift data are consonant with the docking results in the main paper, which suggest a closed vestibular gate with benztropine bound to the DAT at the S1 site (Fig. 8D). Such a closed vestibular gate also occurs with modafinil binding (Schmitt and Reith, 2011). In contrast, binding of cocaine forces this gate open by disrupting the hydrogen bond between the side chains of DAT residues D79 and Y156 (see Fig. 8A)—resulting in an open-to-out transporter conformation (Beuming et al., 2008).