R-Modafinil (Armodafinil): A Unique Dopamine Uptake Inhibitor and Potential Medication for Psychostimulant Abuse

Supplemental Information

Supplemental Materials and Methods

Compounds

(±)-Modafinil and the R- and S-enantiomers were synthesized in the Department of Medicinal Chemistry, University of Kansas (TP) and in the Medicinal Chemistry Section, National Institute on Drug Abuse (NIDA)-Intramural Research Program (IRP) (JC and OMO) according to the published procedures (1, 2). JHW 007 was synthesized in the Medicinal Chemistry Section, NIDA-IRP (JC) according to the published procedure (3).

(-)-Cocaine hydrochloride (Sigma-Aldrich, St. Louis, MO or NIDA) was solubilized in sterile saline and administered intraperitoneal (i.p.), 10 ml/kg. Modafinil and its enantiomers were dissolved in a vehicle containing DMSO 10%, Tween-80 15%, and sterile water 75% (V/V), and injected i.p. 10 ml/kg. Injections of saline or vehicle (10 ml/kg) served as controls.

Molecular Biology

Synthetic complementary DNAs encoding the human DAT (synDAT) were subcloned into pcDNA3 (Invitrogen, Carlsbad, CA) (4). All mutations were generated by the QuickChange method (adapted from Stratagene, La Jolla, CA) and confirmed by restriction enzyme mapping and DNA sequencing. Positive clones were amplified by

transformation into XL1 blue competent cells (Stratagene) and positive colony picked and grown in lysogeny broth media over night at 37ºC in an orbital incubator (Infors, Basel, Switzerland) @ 200 rpm. Plasmids were harvested using the maxi prep kit provided by Qiagen (Hilden, Germany).

[^{3}H]Dopamine Uptake Experiments

Uptake assays were performed essentially as described (4) using 3,4-[Ring-2,5,6-3H]-dihydroxyphenylethylamine (30-60 Ci/mmol) (Perkin Elmer, Waltham, MA, NET67300). Briefly, transfected COS7 cells were plated in either 24-well dishes (10^5 cells/well) or 12-well dishes (3 x 10^5 cells/well) coated with poly-ornithine (Sigma) to achieve an uptake level of no more than 10% of total added [^{3}H]DA. The uptake assays were carried out 2 days after transfection in uptake buffer (UB) (25 mM HEPES, 130 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM L-ascorbic acid, 5 mM D-glucose, and 1 µM of the catechol-*O*-methyltransferase inhibitor Ro 41-0960 (Sigma), pH 7.4). Prior to the experiment, the cells were washed once in 500 µl of UB and indicated non-labeled compounds were added to the cells in a total volume of 500 µl. The assay was initiated by the addition of 6-10 nM [^{3}H]DA. Nonspecific uptake was determined with 1 µM nomifensine (Sigma-Aldrich). After 5 min of incubation at room temperature (RT), the cells were washed twice with 500 µl of ice cold UB, lysed in 250 µl (24 well) or 300 µl (12 well) 1% sodium dodecyl sulfate and left for 30 min at 37°C. All samples were transferred to 24-well counting plates (Perkin Elmer), 500 µl of Opti-phase Hi Safe 3 scintillation fluid (Perkin Elmer) was added followed by counting of the

plates in a Wallac Tri-Lux β-scintillation counter (Perkin Elmer). All determinations were performed in triplicate.

[^{3}H]WIN 35,428 Binding Experiments

Binding assays were carried out essentially as described by the [^{3}H]DA uptake experiments on whole cells only using [^{3}H]WIN 35,428 (83-87 Ci/mmol) (Perkin Elmer) as the radioligand. Previous to the binding experiment, cells were washed once in ice cold UB and, after the addition of unlabeled ligand and [^{3}H]WIN 35,428, the reactions were incubated at 5˚C until equilibrium occurred (~100 min).

MTSET Labeling Experiments

Two days after transfection, COS7 cells expressing either DAT E2C (a DAT mutant with two endogenous cysteines removed, C90A-C306A, rendering it essentially insensitive to MTSET reactivity, (5)) or DAT E2C I159C, seeded in 12- or 24-well plates, were washed once with 500 μl UB. Subsequently, 400 μl UB and 50 μl of either UB or ligand dissolved in UB were added in the following concentrations: (±)-modafinil: 100 μM, R-(-)-modafinil: 100 μM, S-(+)-modafinil: 100 μM, DA: 100 μM, cocaine: 30 μM and JHW 007: 5 μM. Note that the concentration of inhibitor was chosen as the highest possible concentration that could be washed away to allow subsequent proper [^{3}H]DA analysis. MTSET ([2-(trimethylammonium)ethyl]-methanethiosulfonate) (Toronto Research Chemicals, Toronto, Canada) was added at a final concentration of 0.5 mM and the cells were incubated at RT for 10 min. The stock MTSET solution was freshly prepared in H_2O and immediately diluted 10-fold by application to the transfected cells

into a final volume of 500 μl UB. After incubation, the cells were washed three times in 500 μl UB (RT) before initiation of [^{3}H]DA uptake, performed as described above, but here only the maximal uptake (without unlabeled DA) and the nonspecific uptake (in the presence of 1 μM nomifensine) were determined, both in triplicate. The reaction was stopped and uptake counted as described above. The effects of the added compound on MTSET reactivity were determined by calculating the effect of preincubation with the compound alone and with MTSET all performed in parallel on the same plate using triplicate determinations.

Modeling of DAT/Ligand Complexes

Similar to that described previously (6), the complexes between DAT and modafinil enantiomers were modeled using a well established induced-fit docking (IFD) protocol (7). Briefly, to expand the conformational space being sampled, we used distinct DAT models that were bound with either substrate or inhibitor and were equilibrated in explicit water and lipid environment as described before (8, 9). Modafinil enantiomers were constructed and prepared for docking using LigPrep (Schrodinger Inc., Portland, OR). Docking with the IFD method (7) was carried out with default options, using the SP scoring function in the first stage and XP scoring function in the second stage.

In-vivo Experiments

Male, experimentally naive Swiss-Webster mice (Taconic, Germantown, NY), weighing 30-40 g, were housed in groups of four in temperature- and humidity-

controlled rooms, and maintained on a 12-h light/dark cycle (lights on from 0700-1900 hours). For microdialysis studies mice had free access to food and water except during sample collections, performed between 0900 and 1800 hours. For drug-discrimination studies mice were individually housed and maintained at ~85% of their unrestricted-feeding weights for the duration of the study. All testing was performed between 1300 and 1600 hours, and mice were fed daily rations at least 1 h after behavioral testing. The animals were maintained in an AAALAC International accredited facility in accordance with NIH Policy Manual 3040-2, Animal Care and Use in the Intramural Program (released 1 November 1999).

Microdialysis

Surgery. Mice were anesthetized with a mixture of ketamine (60.0 mg/kg) and xylazine (12.0 mg/kg) administered i.p.. Mice were then placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA), the skull was exposed and a hole was drilled to expose the dura. Mice were randomly implanted in the right or the left side of the brain with a concentric dialysis probe, under continuous perfusion. The probe was implanted into the nucleus accumbens shell, according to the mouse brain atlas by Paxinos and Franklin (10) (anterior = +1.5, lateral = ±0.6, vertical = -5.2; mm relative to the bregma). After surgery, mice received a subcutaneous injection of saline to replenish body fluids and were allowed to recover overnight in square cages equipped with overhead quartz-lined fluid swivels (Instech Laboratories Inc., Plymouth Meeting, PA) for connections to the dialysis probes. All subsequent studies were conducted in these cages (11).

Dialysis Experiment. Concentric dialysis probes were prepared using AN69 dialyzing membranes (Hospal Dasco, Bologna, Italy) as described previously (11). The exposed dialyzing surface of the membrane, i.e. that not covered by glue, was limited to the lowest 1.0 mm portion of the probes, and the probes were less than 18 mm in total length. Experiments were performed in freely moving mice in the same cages in which they recovered from surgery. Microdialysis test sessions started at 9:00 a.m., approximately 42-47 hours after the surgical procedures. Probes were connected to fluid with swivels (375/D/22QM; Instech Laboratories) and were perfused with Ringer's solution (147.0 mM NaCl, 2.2 mM $CaCl_2$, and 4.0 mM KCl) delivered by a 1.0 ml syringe, operated by a BAS Bee Syringe Pump Controller (BAS West Lafayette, IN), through the dialysis probes at a constant flow rate of 1 µl/min. Collection of dialysate samples (10 µl) started after about 30 minutes, and samples collected every 10 min were immediately analyzed for DA content. Mice received cocaine (10, 20 mg/kg), (±)-, S- or R-modafinil (30, 100, 300 mg/kg), or saline injections only when stable DA values (less than 10% variability) were obtained for at least three consecutive samples (approximately after 1 hour). Sample collection continued every 10 min typically during the first 2 hours after treatment, and every 20 min thereafter (but only 10 of the 20 µl collected were analyzed).

Analytical Procedure. Dialysate samples (10 µl) were injected without purification into a high-performance liquid chromatography apparatus equipped with a MD 150 x 3.2 mm column, particle size 3.0 µm (ESA, Chelmsford, MA) and a coulometric detector (5200a Coulochem II, or Coulochem III, ESA) to quantify DA. Potentials for the oxidation and reduction electrodes of the analytical cell (5014B; ESA)

were set at +125 mV and -125 mV, respectively. The mobile phase, containing 100 mM NaH_2PO_4, 0.1 mM Na_2EDTA, 0.5 mM n-octyl sulfate, and 18% (v/v) methanol (pH adjusted to 5.5 with Na_2HPO_4), was pumped by an ESA 582 solvent delivery module at 0.50 ml/min. Assay sensitivity for DA was 2 fmoles per sample.

Histology. At the end of the experiment, mice were euthanized by pentobarbital overdose, brains were removed and left to fix in 4% formaldehyde in saline solution (11). Brains were sliced, using a vibratome (Vibratome Plus, The Vibratome Company, St. Louis, MO), in serial coronal slices oriented according to the atlas by Paxinos and Franklin (10) in order to identify the location of the probes. Only data from animals for which probe tracks were within the nucleus accumbens shell boundaries were used for results described in the manuscript.

Drug Discrimination

Cocaine Discrimination. Details are essentially identical to those described previously (12, 13). Experiments were conducted with subjects placed in 29.2 x 24.2 x 21 cm operant-conditioning chambers (modified ENV-001; MED Associates, St. Albans, VT) containing two response keys (levers requiring a downward force of 0.4 N) with pairs of green and yellow light-emitting diodes above each. A dispenser delivered 45 mg food pellets (BioServ, Frenchtown, NJ) to a tray located between the response keys, and a light was mounted near the ceiling to provide overall illumination. The chamber was enclosed in a ventilated enclosure that provided sound attenuation, with white noise to further mask extraneous noise.

Mice were initially trained with food reinforcement to press both levers, and eventually trained to press one after cocaine (10 mg/kg, i.p.) and the other after saline (i.p.) injection. All responses produced audible clicks of a relay mounted behind the front wall of the chamber. The ratio of responses to food pellets (fixed ratio or FR) was gradually increased until, under the final conditions, the completion of 10 consecutive responses on the cocaine- or saline-appropriate lever produced food. Incorrect responses reset the FR response requirement. The right- versus left-assignments of cocaine and saline keys were counterbalanced among subjects. Subjects were injected and placed in chambers, with sessions started after a 5-min time-out period during which lights were off and responses had no consequences, other than the audible click. After the time-out the house light was turned on until the completion of the 10-response requirement and the presentation of food. Sessions ended after 20 food presentations or 15 min, whichever occurred first, and were conducted 5 days per week, with cocaine or saline sessions scheduled in a double alternation sequence (e.g. …CSSCSS…). Testing with different doses of cocaine or modafinil enantiomers was initiated after subjects met the criteria of at least 85% cocaine- or saline-appropriate responding on four consecutive sessions (two sessions of each) over the entire session, and the first FR of the session. Test sessions were conducted with the pre-session administration of different doses of cocaine (5 min), or the modafinil enantiomers (60 min), and were identical to training sessions with the exception that 10-consecutive responses on either lever were reinforced. Pretreatment times were selected based on a previous study of the discriminative stimulus effects of racemic modafinil (14).

Data Analysis

Uptake Data and Binding. Data were analyzed by nonlinear regression analysis using Prism 5.0 from GraphPad Software, San Diego, CA. The Inhibition potency (or K_M value) for dopamine and K_D values were calculated from the pIC_{50} values determined by non-linear regression analysis of [^{3}H]DA uptake and [^{3}H]WIN 35,428 binding data as described previously. The standard error (S.E.) intervals were calculated from pK $\pm$ S.E. The K_i values were calculated from IC_{50} values determined by non-linear regression analysis of [^{3}H]DA uptake or [^{3}H]WIN 35,428 binding data using the equation $K_i = IC_{50}/(1+(L+K))$ where L = concentration of radioligand and K is K_M for [^{3}H]DA uptake or K_D for [^{3}H]WIN 35,428 binding.

Microdialysis. Data are shown as percent increase above basal DA values. Basal DA values were calculated as the mean of three consecutive samples (differing no more than 10%) immediately preceding the first drug or vehicle injection. All results are presented as group means (± standard error of the mean). Statistical analysis was carried out using a two-way analysis of variance (drug dose and time as factors) for repeated measures over time, with results from treatments showing overall changes subjected to post-hoc Tukey`s test. Changes were considered to be significant when $p < 0.05$.

Supplemental References

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