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Supplementary Material for

Chemogenetics revealed: DREADD occupancy and activation via converted clozapine

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Materials and Methods

Experimental subjects

Mice (C57BL/6J) were ordered from Jackson Laboratory and rats (Sprague-Dawley) were ordered from Charles River. All mice and rats were male and ordered at ~6-weeks of age. Transgenic mice expressing the enzyme cre recombinase under the control of the dopamine D1 receptor (D1-Cre, FK150 line, C57BL/6J congenic, Gensat, RRID: MMRRC_036916-UCD) were crossed with transgenic mice with cre recombinase inducible expression of hM4Di DREADD (R26-hM4Di/mCitrine, Jackson Laboratory, stock no. 026219). All experiments and procedures were approved by the National Institute on Drug Abuse and Johns Hopkins Medicine animal care and use committees.

Cell culture and transfection

Human embryonic kidney (HEK-293, ATCC) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotic/antimycotic and 10% heat-inactivated fetal bovine and kept in an incubator at 37°C and 5% CO_2. Cells were routinely tested for myclopasma contamination. Cells were seeded on 60 cm^2 dishes at 30,000 cells/dish 24 hours before transfection. The indicated amount of cDNA was transfected into HEK-293 cells using polyethylenimine (PEI) in a 1 to 2 DNA:PEI ratio. Cell harvesting for radioligand binding experiments or Ca^{2+} mobilization assays were performed approximately 48 hrs after transfection.

Radioligand binding assays

HEK-293 cells were transfected with 5 µg/dish of AAV packaging plasmids encoding for hM3Dq (Addgene #89149), hM4Di (Addgene #89150) or a control vector and harvested 48 hrs after transfection. Cells were suspended in Tris-HCl 50 mM pH 7.4 supplemented with protease inhibitor cocktail (1:100). The dissected brain tissue was diluted in Tris-HCl 50 mM buffer supplemented with protease inhibitor cocktail (1:1000). HEK-293 cells or brain tissue were disrupted with a Polytron homogenizer. Homogenates were centrifuged at 48,000 g (50 min, 4 °C) and washed 2 times in the same conditions to isolate the membrane fraction. Protein was quantified by the bicinchoninic acid method. For saturation experiments, pellets were resuspended and membrane suspensions (50 µg of protein/ml) were incubated in 50 mM Tris-

HCl (pH 7.4) containing 10 mM $MgCl_2$ and increasing concentrations of [^{3}H]clozapine (83 Ci/mmol) or [^{3}H]CNO (83 Ci/mmol) during 2 hours at RT. Non-specific binding was determined in the presence of 10 µM clozapine or 10 µM CNO, respectively. For competition experiments, membrane suspensions (50 µg of protein/ml) were incubated in 50 mM Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$, 2.5 nM of [^{3}H]clozapine and increasing concentrations of clozapine (0.1 nM to 10 µM) or CNO (1 nM to 100 µM) during 2hr at RT. In all cases, free and membrane-bound radioligand were separated by rapid filtration of 500-µl aliquots in a 96-well plate harvester and washed with 2 ml of ice-cold Tris-HCl buffer. Microscint-20 scintillation liquid (65 µl/well) was added to the filter plates, plates were incubated overnight at RT and radioactivity counts were determined in a MicroBeta2 plate counter with an efficiency of 41%. Saturation curves were fitted to one-site binding hyperbolic curves (for saturation experiments) or one-site competition curves. K_i values were calculated using the Cheng-Prusoff equation.

AAV injections

Animals were anesthetized with isoflurane or a mix of ketamine/xylazine and prepped on a stereotaxic apparatus. Based on corresponding mouse and rat brain atlases (Paxinos and Watson), the following coordinates were used to target the dorsal striatum: Mouse – AP=1.00, ML=±1.50, DV=-3.55; Rat – AP=1.70, ML=±2.50, DV=-5.50, rat cortex: Rat – AP=1.70, ML=±2.50, DV=-3.50. For behavioral tests, the accumbens/basal forebrain was targeted in rats using the following coordinates: Rat – AP=1.70, ML=±0.90, DV=-7.40. All injections were performed using a 5µl Hamilton Neuros 33G syringe at a flow rate of 1.0 µl/min.

[^{3}H[Clozapine and [^{3}H]CNO autoradiography

Flash frozen tissue was sectioned (10 µm) on a cryostat and thaw mounted onto ethanol-soaked glass slides. Pre-incubation consisted of washing slides (10 min) in buffer consisting of 170 mM Tris-HCl, 120 mM NaCl, 1 mM $CaCl_2$, pH 7.4. For binding, slides were incubated (60 min) in wash buffer containing 1.0-3.5 nM [^{3}H]clozapine or [^{3}H]CNO (total binding) or 1.0-3.5 nM [^{3}H]clozapine or [^{3}H]CNO plus 100 µM non-radioactive clozapine or CNO (non-specific binding). Following incubation, slides were washed with washing buffer for 5 min (2x) ending with a 30 second dip in deionized H_2O. Slides were air dried and placed in a Hypercassette™

and covered with a BAS-TR2025 phosphor screen. The slides were exposed to the phosphor screen for 3-5 days and imaged using a phosphor imager.

Immunohistochemistry

Flash frozen tissue was sectioned (10 µm) on a cryostat (Leica) and thaw mounted on ethanol-soaked glass slides. Sections were fixed with paraformaldehyde (4%), permeabilized with PBS with TritonX-100 (0.1%, washing buffer), blocked with bovine serum albumin 5% in washing buffer (2 hours, RT), and then incubated overnight at 4°C with the primary antibodies mix: rabbit anti-mCherry (1:500, ab167453, Abcam Inc.) and chicken anti-GFP (1:2000, ab13970, Abcam Inc.). Sections were then washed again and incubated with Alexa 647 goat anti-rabbit (1:200, A21245, Invitrogen) and Alexa 488 goat anti-chicken (1:400, A11039, Invitrogen) and washed again. Then, sections were covered in mounting medium, and images were acquired either using a confocal microscope with a laser scanning module or a stereo microscope.

Binding and enzyme profile screen

These experiments were performed by an outside vendor. Briefly, membrane homogenates from stable cell lines expressing each receptor/enzyme were incubated with the respective radioligand in the absence or presence of CNO or reference control compounds in a buffer. In each experiment, the respective reference compound was tested concurrently with the test compound to assess the assay reliability. Nonspecific binding was determined in the presence of a specific agonist or antagonist at the target. Following incubation, the samples were filtered rapidly under vacuum through glass fiber filters presoaked in a buffer and rinsed several times with an ice-cold buffer using a 48-sample or 96-sample cell harvester. The filters were counted for radioactivity in a scintillation counter using a scintillation cocktail.

Synthesis of [^{11}C]Clozapine and [^{11}C]CNO

Both [^{11}C]clozapine and [^{11}C]CNO were synthesized using the methods developed by Bender et al.(*13*) with minor modifications. Briefly, 1 mg of N-desmethylclozapine was dissolved in 200 µL of acetonitrile. [^{11}C]methyl triflate was bubbled into the solution until the radioactivity reached a plateau. The reaction was kept at room temperature for 2 min. The solution was then diluted with 200 µL of 40:60 (v:v) acetonitrile:water (0.1% ammonium

hydroxide) and injected onto semi-preparative HPLC. The column (Waters XBridge C_{18} 10 mm x 150 mm) was eluted with 40:60 (v:v) acetonitrile:water (0.1% ammonium hydroxide) at a flow rate of 10 mL/min. The radioactive peak corresponding to [^{11}C]clozapine (t_R = 6.1 min) was collected in a reservoir containing 50 mL of water and 250 mg of L-ascorbic acid. The diluted product was loaded onto a solid phase extraction cartridge and rinsed with 3.0 mL of water. The product was eluted with 400 µL of ethanol into a sterile, pyrogen-free bottle and diluted with 4.0 mL of saline. A 10 µL aliquot of the final product was injected onto an analytical HPLC column (Waters XBridge C_{18} 4.6 mm x 100 mm) and eluted with 35:65 (v:v) acetonitrile:water (0.1% ammonium hydroxide) at a flow rate of 2 mL/min. The radioactive peak corresponding to [^{11}C]clozapine (t_R = 9.2 min) coeluted with a standard sample. The synthesis of [^{11}C]CNO was the same as above with the exception that after methylation, 50 µL of a 12 mg/mL solution of m-chloroperbenzoic acid was added and allowed to react for 1 min at room temperature. The semi-preparative HPLC eluant used was 25:75 (v:v) acetonitrile:water (0.1% ammonium hydroxide), and no ascorbic acid was added to the reservoir. The specific activity for both [^{11}C]clozapine and [^{11}C]CNO ranged from 351 to 483 GBq/µmol (9,482-13,041 mCi/µmol) at end of synthesis.

Small animal positron emission tomography (PET)

Rats were anesthetized with isoflurane and placed in a prone position on an ARGUS small animal PET/CT scanner bed and injected intravenously (~500 µL) with [^{11}C]clozapine or [^{11}C]CNO (~700 µCi) and dynamic scanning commenced. Total acquisition time was 60 min. The PET data were reconstructed using the two-dimensional, ordered-subsets-expectation maximization algorithm (2D-OSEM) and corrected for dead time and radioactive decay. Qualitative and quantitative assessments of PET images were performed using the PMOD software environment. Briefly, regions of interest (ROIs) were delineated on an MRI rat brain template provided with the software. Then all PET images were coregistered and spatially normalized to the combined MRI–ROI template using the Fusion module in PMOD. This led to coregistered PET–MRI images with a voxel size of 0.2 mm isotropic. ROI values (counts/sec) were extracted from target (GFP, hM4Di injections sites) and cerebellum and converted to binding ratios.

Ex-vivo biodistribution of [^{3}H]Clozapine and [^{3}H]CNO uptake

Mice were injected (IP) with [^{3}H]clozapine or [^{3}H]CNO (1 µCi/g), euthanized 30 min later and brain, blood, and tissues were collected for radiometric analyses. The brains were flash frozen in 2-methylbutane and stored at -80ºC until use. The blood was centrifuged (13,000 rpm, 10 min at RT) and serum was collected. The tissues were solubilized and bleached with hydrogen peroxide. Serum and tissue samples were dissolved in scintillation cocktail and radioactivity counts were determined in a scintillation counter.

P-gp substrate assay

These experiments were performed by an outside vendor. CNO and Clozapine were tested in P-gp substrate assessment assays at 10 µM. The A to B and B to A permeability was measured in Caco-2 cells in the presence and absence of verapamil, a P-gp inhibitor. Efflux ratios (*E*) were calculated based on the apparent B-A and A-B permeability with and without verapamil. In each experiment, the respective reference compound was tested concurrently with the test compound to assess the assay reliability. Fluorescein was used as the cell monolayer integrity marker. Fluorescein permeability assessment (in the A-B direction at pH 7.4 on both sides) was performed after the permeability assay for the test compound. The cell monolayer that had a fluorescein permeability of less than 1.5 x 10^{-6} cm/s for Caco-2 was considered intact, and the permeability result of the test compound from intact cell monolayer was reported.

[^{11}C]CNO and [^{11}C]Clozapine metabolite extraction

Rats were injected with hM4Di and GFP AAVs into the right and left motor cortex area, respectively. After 3-4 weeks rats were anesthetized, injected IV with [^{11}C]CNO (~148-222 MBq) and euthanized ~35-40 min later. Blood was harvested via intracardiac puncture, separated into plasma, and processed for detection of [^{11}C]CNO and converted [^{11}C]clozapine via a previously reported ultra-sensitive column-switching radio-HPLC method(*19*). Brains were also harvested, the cerebellum was discarded (due to its low [^{11}C]clozapine uptake), and the remaining forebrain tissue from each rat was separated into left (GFP-expressing) and right (hM4Di-expressing) blocks and processed for [^{11}C]CNO and [^{11}C]clozapine detection via the same method. [^{11}C]CNO and its metabolites were extracted from plasma and brain samples using a previously published procedure(*20*). Left and right hemispheres (excluding cerebellum) from animals injected with GFP (left) and hM4Di (right) AAV constructs (see above) were

homogenized in 2 mL of 0.5M citric acid, followed by centrifugation for 5 min at 9500xg and 4ºC. Supernatants were filtered using PTFE 1 µM syringe filters and analyzed on a HPLC system as described below. Approximately 4 mL of blood samples were centrifugated for 5 min at 2200xg and 4ºC to obtain plasma, which was filtered using PTFE 0.45 µM syringe filters prior to HPLC analysis.

Radioactive high performance liquid chromatography

Samples of rat plasma and brain tissue were analyzed using a modified column-switching HPLC method(*19*), Briefly, 2 HPLC systems consisting of a 1260 infinity quaternary pump, a 1260 infinity UV detector, a 1260 infinity column compartment module and a radiation detector were controlled by software. Samples loaded onto a 4 mL Rheodyne injector loop were initially directed to the capture column (packed with 33µm polymeric reversed phase sorbent) and detectors with 1% acetonitrile and 99% water mobile phase at 2 ml/min to detect polar species. After 2 min of elution, analytical mobile phase composed of 50% acetonitrile and 50% aqueous solution of 0.2% triethylamine, pH=7.1 adjusted with phosphoric acid was applied to a flash capture column and direct trapped non-polar compounds to an analytical column (Waters, reverse phase XBridge BEH C18 5 µM 4.6x250 mm) and detectors at 2 mL/min. The HPLC system was standardized using non-radioactive clozapine, [^{11}C]clozapine, CNO, [^{11}C]CNO prior to analysis of brain and blood plasma samples.

Ca^{2+} mobilization assays

Intracellular Ca^{2+} was monitored using the fluorescent Ca^{2+} biosensor GCaMP6 described by Chen et al.(*21*) HEK-293 cells transfected with 5 µg/dish of the cDNA encoding for hM3Dq or hM4Di and 5µg/dish of GCaMP6 were harvested, washed, resuspended in Mg^{2+}-free Locke's buffer pH 7.4 (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 2.3 mM CaCl2, and 5 mM HEPES) containing 5.6 mM of glucose and approximately 200,000 cells/well were distributed in black 96-well plates. Fluorescence intensity (excitation at 488 nM, emission at 515 nM, 5 reads per data point) was measured at 15 s-intervals using a Cytation5 multi-mode reader during 75 s to determine the baseline values, then the indicated concentrations of CNO or clozapine were added to the wells and fluorescence was read during 6 min in the same conditions. The change in

intracellular Ca^{2+} concentration was expressed as $\Delta F/F_0$ where ΔF is F minus F_0, F is the fluorescence at a given time point and F_0 is the average of the baseline values.

[^{35}S]GTPγS autoradiography

We injected a rat with AAV-hM4Di-mCherry into the nucleus accumbens/basal forebrain area and the brain was harvested 2 weeks later. Flash frozen tissue was sectioned (10 µm) on a cryostat and thaw mounted on ethanol cleaned glass slides. DREADD expression was confirmed via DREADD-mCherry fluorescence (Fig. 3D) and [^{3}H]clozapine autoradiography (Fig. 3E). Adjacent sections were encircled with a hydrophobic membrane using a PAP pen and then exposed to an agonist-stimulated [^{35}S]GTPγS autoradiography assay, performed as previously described(*22*), where sections were treated with either vehicle, CNO, or clozapine. Pre-incubation buffer was pipetted onto each slide and allowed to incubate for 20 min (500 mM Tris-HCl, 100 mM EDTA, 500 mM $MgCl_2$ and 4 M NaCl). The pre-incubation buffer was removed via aspiration and each slide was loaded with GDP in the presence of DPCPX and allowed to incubate of 60 min (Pre-incubation buffer, 48 mM GDP, 24 mM DPCPX). GDP buffer was removed via aspiration and [^{35}S]GTPγS cocktail (GDP buffer, 20 mM DTT, 48 mM GDP, 24 mM DPCPX, 300 nM [^{35}S]GTPγS) with agonists of interest (CNO 10 nM and 1 µM, clozapine 10 nM and 1 µM), without agonists (basal condition), or with a saturated concentration of non-radioactive GTP (for non-specific binding) was pipetted onto each slide and allowed to incubate for 90 min. The [^{35}S]GTPγS cocktail was removed via aspiration and slides were washed in ice cold washing buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4) for 5 min (2x) followed by a 30 second dip in ice cold deionized water. Hydrophobic membrane was removed with a cotton swab and xylene and slides were placed into a Hypercassette™ covered by a BAS-SR2040 phosphor screen. The slides were exposed to the phosphor screen for 3-5 days and imaged using a phosphor imager.

Locomotor activity assessment

Rats (260-310g) injected with AAV-hM4Di-mCherry or AAV-GFP or sham (see above) in the accumbens/basal forebrain were tested for locomotor activity 3 weeks after virus injection. In a first session rats expressing hM4Di or GFP were habituated for 30 min to the locomotor activity chambers and on the second session rats were injected (IP) with vehicle (NaCl 200 mM)

and placed in the chambers for 30 min. On consecutive sessions, the rats received vehicle, CNO (10 mg/kg body weight), or clozapine (0.1 or 1 mg/kg body weight) in a counterbalanced design and locomotor activity was quantified as distance traveled.

Transgenic male and female mice (25-30g, offspring of D1-Cre (heterozygous) × R26-hM4Di/mCitrine (heterozygous) crossing) either heterozygous for both loci ($Drd1a^{hM4Di}$) or wild-type for D1-Cre and heterozygous for hM4Di/mCitrine (controls) were also tested for locomotor activity. Mice were habituated for 60 min to the locomotor activity chambers. On consecutive sessions, 10 min before being placed in the chambers, mice were injected (IP) with vehicle, CNO (1 mg/kg) or clozapine (0.01, 0.1 or 1 mg/kg) in a counterbalanced design and locomotor activity was measured during 60 min. Mice were then were returned to their home cages, and 60 min later (at 120 min) were placed back in their respective locomotor activity chambers, where activity was measured for another 60 min.

Statistics

Sample sizes were chosen based on our results from previous experiments. Depending on experiment, we used paired/two-sample t-tests or single factor and multifactor ANOVAs with *Sidak or Tukey post-hoc tests*, taking repeated measures into account where appropriate. All statistical tests were evaluated at the $P \leq 0.05$ level.

Figure S1.

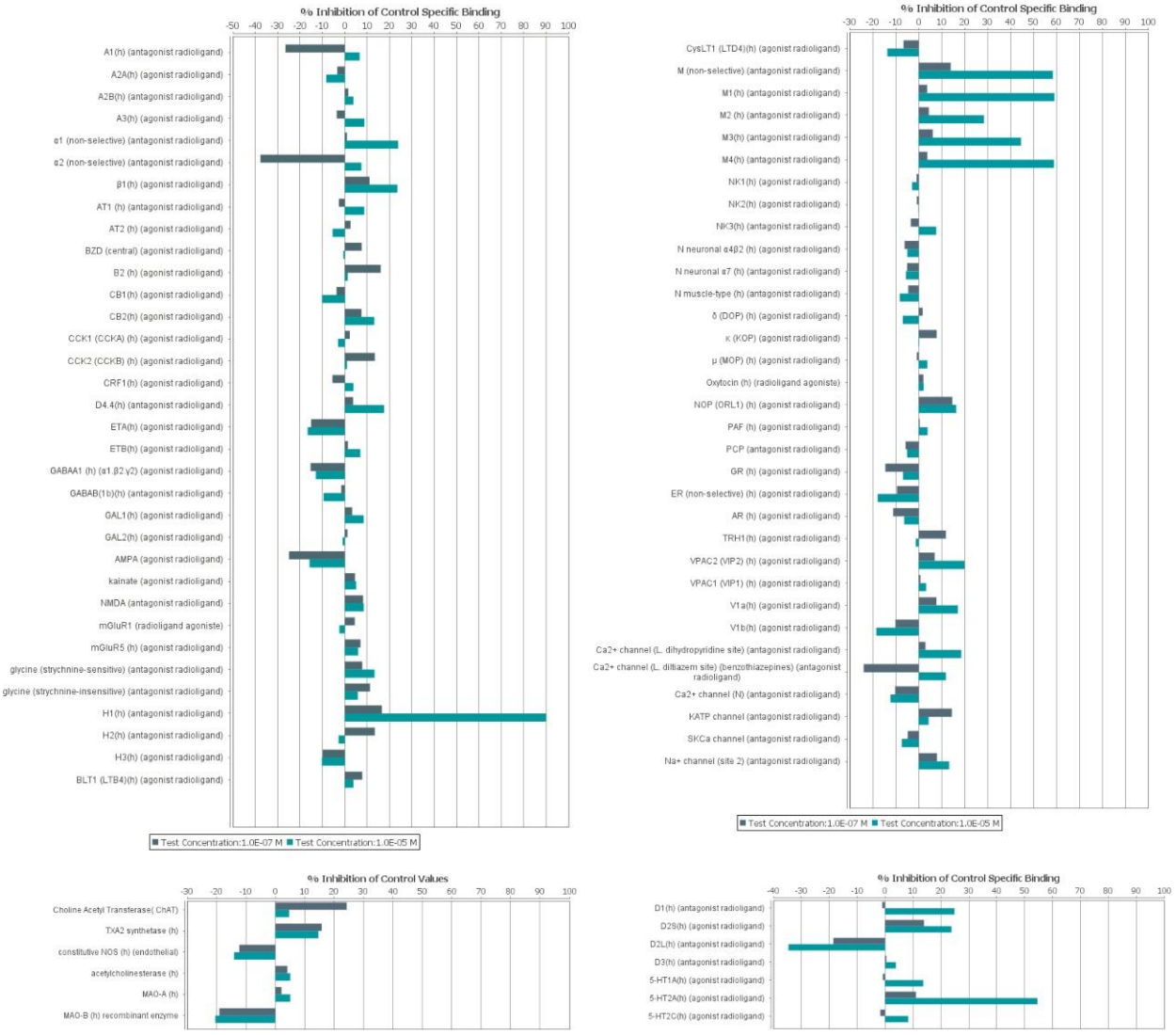

Fig. S1. Binding screen profile of CNO for various receptors and enzymes tested at 100 nM and 10 µM. CNO does not to lead to significant inhibition of specific binding at any examined target at 100 nM (dark color). However, at 10 µM concentration (light color), CNO leads to at least 50% inhibition of specific binding towards histamine H1 receptor, 5-HT2_a receptor, and muscarinic M1 and M4 receptors, including 40% inhibition for M3.

Figure S2.

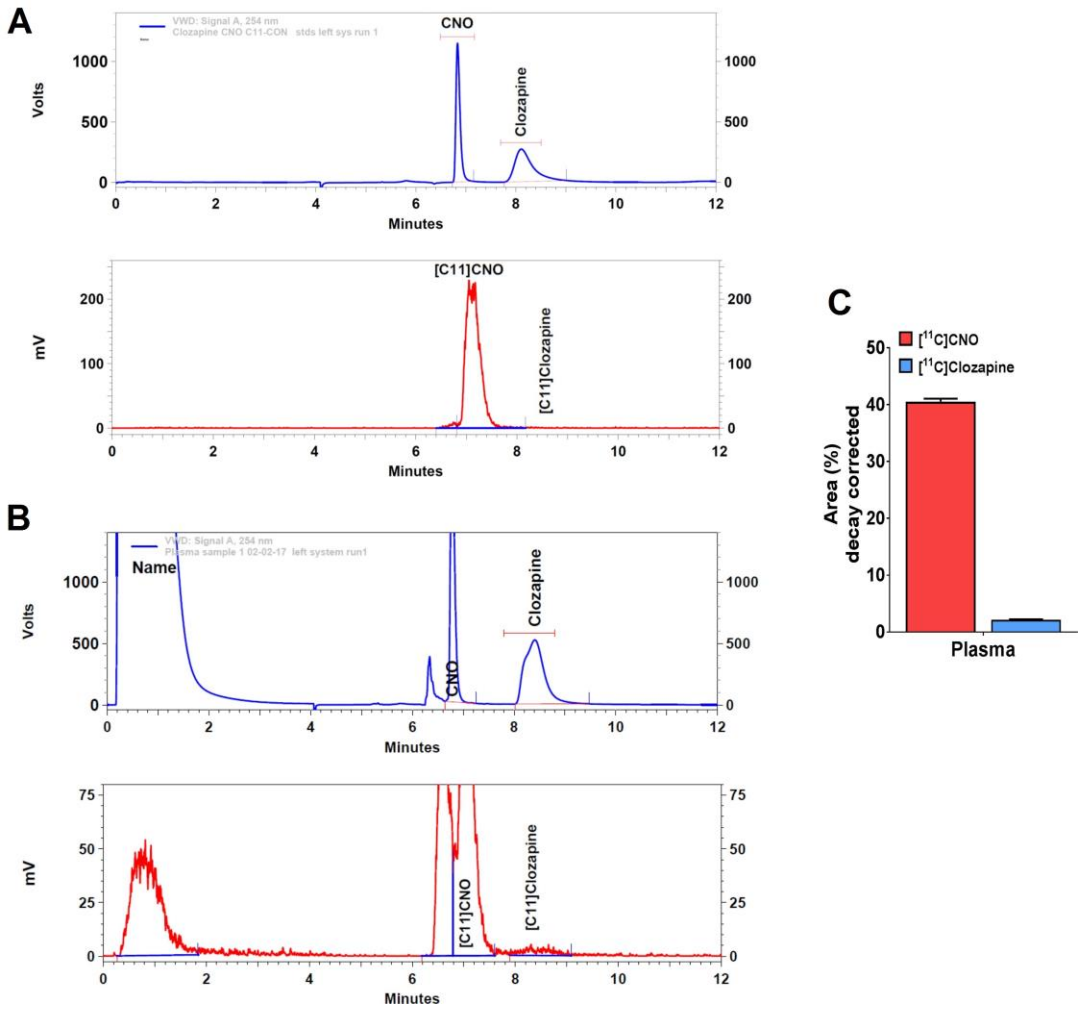

Fig. S2. Representative radio-HPLC chromatograms from radioligand and plasma analysis of metabolites after systemic [^{11}C]CNO administration. (A) UV absorbance (254 nm) chromatogram (blue) showing peaks corresponding to CNO and clozapine standards used for sample spiking of the [^{11}C]CNO radioligand. Radiometric (red) chromatogram showing a single peak corresponding to synthesized [^{11}C]CNO and confirming its purity prior to IV injection. (**B**) UV (blue) chromatogram showing peaks corresponding to CNO and clozapine standards used for plasma sample spiking. Radiometric (red) chromatogram showing an initial peak (polar metabolites), a second peak (unidentified radiometabolite), a third peak corresponding to [^{11}C]CNO and finally a fourth peak corresponding to [^{11}C]clozapine. (**C**) Mean ± S.E.M decay corrected percentage of area under peaks corresponding to [^{11}C]CNO and [^{11}C]clozapine, expressed as a percent from the sum of all four peaks derived from four independent experiments (n=4 rats) indicating conversion in plasma of [^{11}C]CNO to [^{11}C]clozapine.

Figure S3.

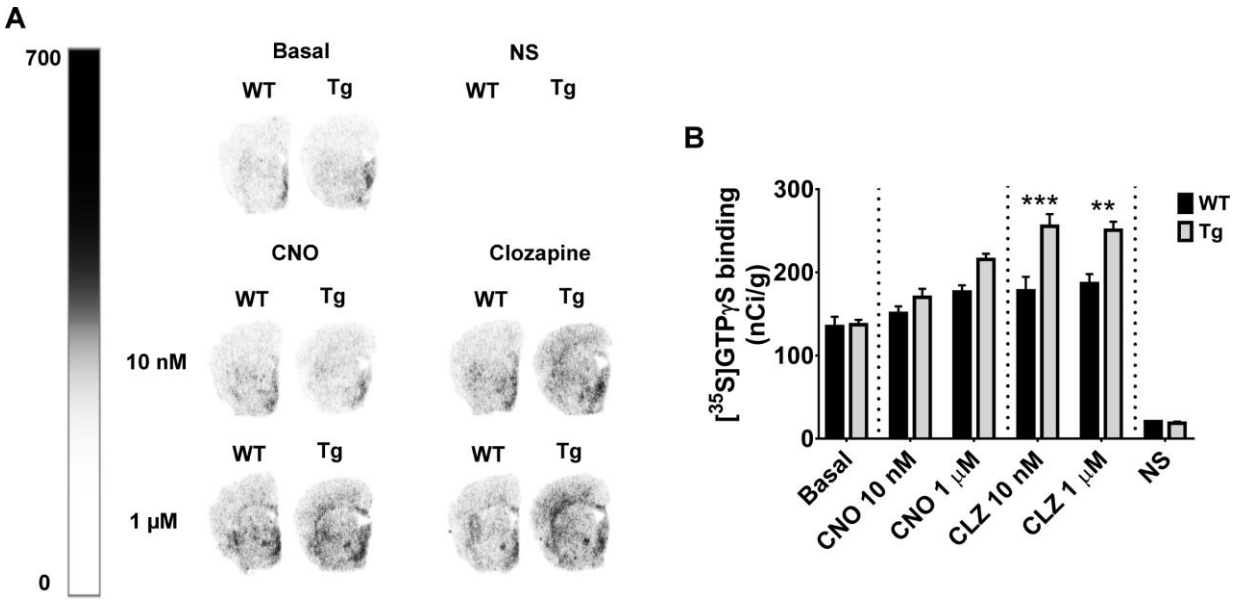

Fig. S3. Clozapine is more potent than CNO in activating hM4Di. (A) Representative autoradiograms and **(B)** densitometric quantitative assessment of [^{35}S]GTPγS binding in cortex from transgenic hM4Di (hM4Di-Tg) and wild-type (WT) mice (n=2) in response to vehicle (Basal), non-specific (NS) condition using non-radioactive GTP, and agonist-stimulated conditions (CNO, clozapine (CLZ) at 10 nM and 1 µM) indicating that CLZ is significantly more potent than CNO in inducing hM4Di activation. Statistical significance was calculated using a two-way ANOVA with treatment and genotype as factors followed by a Sidak's post-hoc multiple comparison test $^{**}P<0.01$, $^{***}P<0.001$ comparing Tg to WT mice).

Figure S4.

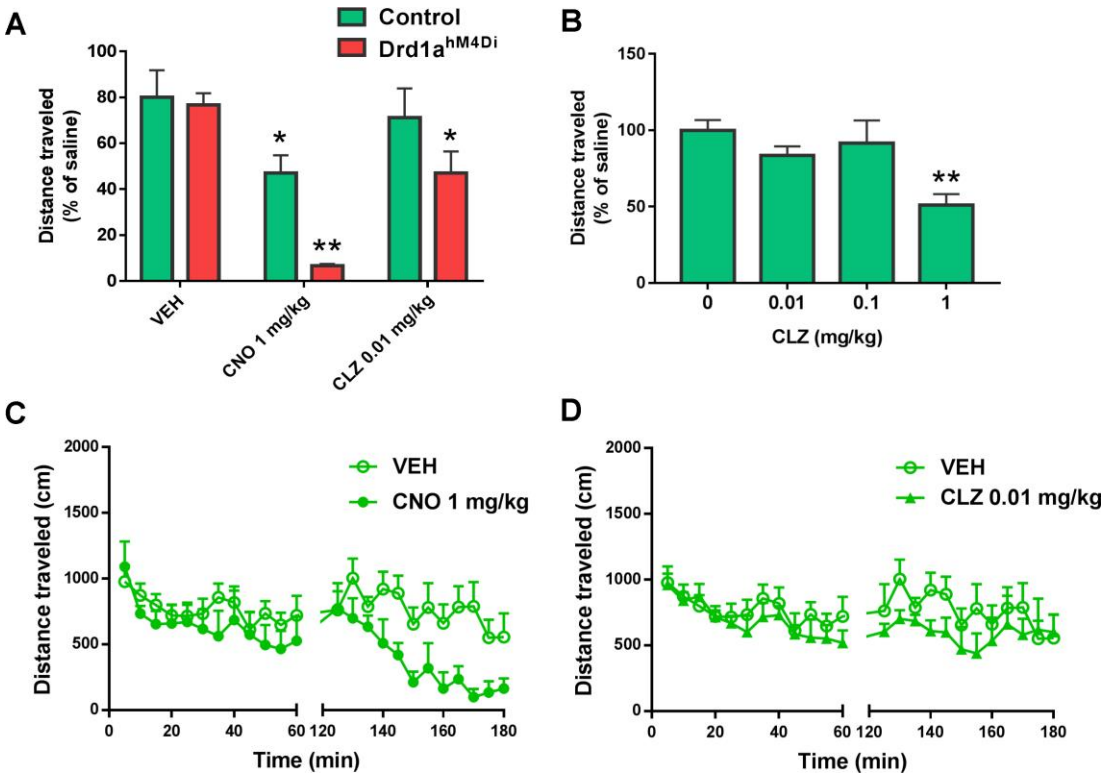

Fig. S4. CNO shows temporally-discrete non-specific effects. After a session of habituation to the chambers, Drd1a^{hM4Di} (red, n = 7) or control (green, n = 7) mice were treated with vehicle, CNO (1 mg/kg) or clozapine (CLZ 0.01, 0.1 or 1 mg/kg) and their ambulatory activity was recorded for 60 min. Animals were returned to their home cages and 1 hr later activity was measured for another 60 min. In (**A**) and (**B**), each bar represents mean ± S.E.M. of the distance traveled during the second hour of recording normalized as the percentage of distance traveled by vehicle treated animals (the data for the first hour is showed in the main text). Statistical significance was calculated using a repeated measures two-way ANOVA with group (Control vs. hM4Di) and treatment as factors followed by a Tukey's post-hoc multiple comparison test (*P<0.05, **P<0.01 compared with the respective vehicle). In (**C**) and (**D**), time course data of control animals where every point (mean ± S.E.M.) represents the distance traveled (cm) in 5-min bins for vehicle (open circles), CNO (1 mg/kg, solid circles) or clozapine (0.01 mg/kg, solid triangles). Statistical significance was calculated using two independent repeated measures two-way ANOVAs with time and treatment as factors. A significant difference was found for CNO compared with vehicle ($p < 0.001$) but not for clozapine compared with vehicle ($p = 0.1736$).

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