High-potency ligands for DREADD imaging and activation in rodents and monkeys

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Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are a popular chemogenetic technology for manipulation of neuronal activity in uninstrumented awake animals with potential for human applications as well. The prototypical DREADD agonist clozapine N-oxide (CNO) lacks brain entry and converts to clozapine, making it difficult to apply in basic and translational applications. Here we report the development of two novel DREADD agonists, JHU37152 and JHU37160, and the first dedicated 18F positron emission tomography (PET) DREADD radiotracer, [18F]JHU37107. We show that JHU37152 and JHU37160 exhibit high in vivo DREADD potency. [18F]JHU37107 combined with PET allows for DREADD detection in locally-targeted neurons, and at their long-range projections, enabling noninvasive and longitudinal neuronal projection mapping.
Results

New DREADD ligands with high in vitro affinity and potency

Recently, a new second-generation DREADD ligand, Compound 21 (C21), was put forward as an effective DREADD agonist with excellent brain penetrance that does not convert to clozapine<sup>3</sup>, was put forward as an effective DREADD agonist with high affinity and potency for CNS applications. In biological studies, we would like to identify new ligands with good in vivo DREADD selectivity and function. DREADD ligands developed to date have characteristics that limit their utility for translational central nervous system (CNS) applications<sup>1,3</sup>. The prototypical DREADD agonist, clozapine N-oxide (CNO), has poor brain penetrance and, via metabolic degradation, gives rise to the antipsychotic drug clozapine, which is the main active in vivo CNS DREADD agonist<sup>3</sup>. Therefore, new, potent DREADD agonists and selective, high-affinity DREADD PET radioligands are needed to advance the translational potential of this powerful chemogenetic technology. Here, we use an array of complementary in vitro, ex vivo and in vivo approaches in rodents and in monkeys to report the development of JHU37152 and JHU37160, the first DREADD agonists with high in vivo potency for CNS applications. We also report the development of the first <sup>18</sup>F-labeled high-affinity DREADD PET radioligand. [<sup>18</sup>F]JHU37107, which enables noninvasive and longitudinal DREADD detection and localization in locally targeted neurons and at long-range projection sites. Together, these new tools expand the power of DREADD chemogenetic technology to encompass translational applications for noninvasive manipulation and visualization of neuronal circuits.

JHU37152 and JHU37160 exhibit high in vivo DREADD occupancy. In contrast to CNO and C21, mice injected (IP) with a 0.1 mg kg<sup>−1</sup> dose of either JHU37152 or JHU37160 (Fig. 2a) showed high brain to serum concentration ratios (~eightfold higher in the brain than serum at 30 min), indicating active sequestration in brain tissue (Fig. 2b). Neither JHU37152 nor JHU37160 were P-gp substrates (Supplementary Fig. 5). The CSF concentration of JHU37160 at this same dose in the monkey was below our system’s detection limit. However, JHU37160 was detected in serum where it showed a similar profile as in the mouse (Supplementary Fig. 6). At this same dose, 0.1 mg kg<sup>−1</sup> JHU37152 and JHU37160 occupied approximately 15–20% of striatal DREADD receptors in mice (Fig. 2c, d). In rats, 0.1 mg kg<sup>−1</sup> JHU37160 occupied approximately 80% of cortical hM4Di (Fig. 2e, f). In monkey, 0.1 mg kg<sup>−1</sup> JHU37160 (and to a lesser extent, 0.01 mg kg<sup>−1</sup>) produced <sup>11</sup>C]-clozapine DREADD displacement at hM4Di expressed in the amygdala (Fig. 2g, h, and Supplementary Fig. 6).

JHU37152 and JHU37160 exhibit high in vivo DREADD potency. As predicted from the above findings, JHU37152 and JHU37160 were potent in vivo DREADD agonists, selectively inhibiting locomotor activity in D1-hM3Dq and D1-hM4Di mice at doses ranging from 0.01 to 1 mg kg<sup>−1</sup> without any significant locomotor effects observed at these doses in WT mice (Fig. 3a–c).
Fig. 1 New DREADD ligands displaying high in vitro DREADD affinity and potency. a Compound 13 (C13) and Compound 22 (C22) structures. b Binding competition curves of [3H]CLZ versus increasing concentrations of C13 and C22 in HEK-293 cells expressing DREADDs. C13 and C22 exhibit comparable DREADD affinity to clozapine (CLZ) with C13 showing ~twofold greater affinity than C22. CLZ and C21 competition curves from Supplementary Fig. 1 are overlaid for comparison. c, d C13 selectively blocks [3H]CLZ binding to DREADDs in mouse slices at 10 nM. Representative images of sections collected from 3 different mice are displayed and quantified in (d) as mean ± SEM. Two-way ANOVA followed by Dunnett’s test, *p < 0.05 and **p < 0.01 compared with the respective vehicle. e–h [3H]C13 binds with greater selectivity than [3H]CLZ to DREADDs in mouse and monkey brain tissue expressing AAV-hM3Dq and AAV-hM4Di, respectively. i Intraperitoneal (IP) injection of [3H]C13 readily enters the brain and accumulates in DREADDs expression areas in D1-DREADD mice. Representative images from 3 mice per condition. j–l JHU37107 (J07), JHU37152 (J52), and JHU37160 (J60) are high-affinity DREADD ligands. m Docking and molecular dynamics simulation of J60 in the ligand binding pocket of a hM4Di model. n, o J60 and J52 selectively displace [3H]CLZ at a concentration of 1 and 10 nM from hM3Dq and hM4Di expressed in mouse brain sections (n = 3 mice per condition). p, q J60 and J52 activate hM3Dq and hM4Di expressed in HEK293 cells with high potency (experiments performed 3–5 times). In all cases, data are represented as mean ± SEM. Scale bars are 1 mm. Source data are provided as a Source Data file.
Fig. 2 JHU37152 and JHU37160 exhibit high in vivo DREADD occupancy. a Structures of JHU37152 (J52) and JHU37160 (J60). b Brain and serum concentrations and ratios of J52 and J60 in mice (n = 4 mice per condition) at different time points after a 0.1 mg kg⁻¹ (IP) injection. C21 (1 mg kg⁻¹, IP) data are same as shown in Supplementary Figures for comparison purposes. c, d J52 and J60 (0.1 mg kg⁻¹, IP) displace in vivo [¹¹C]clozapine binding to DREADDs in AAV-DREADD-expressing mice (n = 5 mice). e, f J60 (0.1 mg kg⁻¹, IP) selectively blocks in vivo [¹¹C]clozapine binding to hM4Di in rats (n = 3 rats). g, h J60 (0.1 mg kg⁻¹) blocks in vivo [¹¹C]clozapine binding to hM4Di in the monkey. All data represented are mean ± SEM except in (h) where individual values are displayed. Source data are provided as a Source Data file.
Fig. 3 JHU37152 and JHU37160 exhibit high in vivo DREADD potency. a–c J60 and J52 produce potent inhibition of locomotor activity in transgenic D1-DREADD mice but not in wild-type (WT) mice (n = 7 to 19 mice per condition). Two-way repeated measures ANOVA followed by Dunnett’s multiple comparison tests were performed, *p < 0.05 and **p < 0.01 compared with the respective vehicle. d, e DREADD-assisted metabolic mapping (DREAMM) using [18F]FDG in D1-hM3Dq and D1-hM4Di mice (n = 4 mice per condition) reveals opposing and differential recruitment of whole-brain functional networks. f, g J52 and J60 produce potent activation of locomotor activity in rats (n = 7 rats per condition) expressing hM3Dq in tyrosine hydroxylase (TH)-expressing neurons in the ventral tegmental area. One-way repeated measures ANOVA followed by Dunnett’s multiple comparison tests were performed, *p < 0.05 and **p < 0.01 compared with the respective vehicle. h–j Design of in vivo electrophysiological experiment and IHC showing hM4Di (green) and ChrimsonR (red) expression in the medial division of the medial geniculate nucleus (MGM) and lateral amygdala (LA). k, l J60 (0.1 mg kg$^{-1}$) produces rapid and potent hM4Di-driven inhibition of light-evoked neuronal activation. Data are represented as mean ± SEM, *p < 0.05, ***p < 0.001. Source data are provided as a Source Data file.
Using DREADD-assisted metabolic mapping (DREAMM) with the [18F]fluorodeoxyglucose (FDG) tracer to assess changes in regional brain activity after hM3Dq or hM4Di activation of D1 neurons, 0.1 mg kg\(^{-1}\) (IP) JHU37160 produced metabolic changes in distinct and largely nonoverlapping brain networks in D1-hM3Dq (Fig. 3d) versus D1-hM4Di (Fig. 3e) mice and caused no significant brain metabolic changes in WT mice (Supplementary Fig. 7). The recruitment of distinct, almost mutually exclusive networks was paralleled by metabolic changes with opposite directionality upon differential modulation of D1 neurons with hM3Dq and hM4Di: decreased metabolism in D1-hM3Dq and increased metabolism in D1-hM4Di mice, effects likely mediated via activation and inhibition of striatal GABAergic D1-expressing neurons respectively.

In a competitive binding screen, JHU37152 and JHU37160 exhibited lower affinity than clozapine at 5-HT receptors (Supplementary Fig. 8). Although the overall target profile of both compounds was similar to clozapine, they did not produce any agonistic effect in functional assays performed in HEK-293 cells lacking DREADDs, but expressing endogenous dopamine-binding targets (Supplementary Fig. 4). As such, they are expected to behave as antagonists at these receptors, competing with endogenous neurotransmitters at these same binding sites. In contrast, JHU37152 and JHU37160 evidenced DREADD activation at lower concentrations than clozapine, indicating that the former compounds are more selective DREADD agonists.

In TH-hM3Dq rats, 0.01–0.3 mg kg\(^{-1}\) JHU37152 and JHU37160 led to robust, selective increases in hM3Dq-stimulated locomotion (Fig. 3f, g). To further characterize the performance of JHU37160 as an in vivo DREADD agonist, we performed in vivo electrophysiology experiments in which hM4Di was co-expressed with a new light-drivable channelrhodopsin, ChrimsonR (Supplementary Fig. 9), in the terminals of the medial division of the medial geniculate nucleus (MGM) to striatum/lateral amygdala (LA) pathway (Fig. 3h–j) in mice. Mice were implanted with optrodes in the LA. A dose of 0.1 mg kg\(^{-1}\) (IP) JHU37160 elicited rapid inhibition of ChrimsonR-induced terminal activation; 60% inhibition was observed at ~10 min (36 ± 9% of baseline), and maximal inhibition at 30 min after injection (19 ± 2% of baseline) (Fig. 3k, l). These effects were hM4Di-dependent; the same injection of JHU37160 had no effect on electrical stimulation evoked responses in animals without DREADD expression (Supplementary Fig. 10).

[18F]JHU37107 enables noninvasive neuronal projection mapping. The high-affinity profiles of JHU37152, JHU37160, and JHU37107 stimulated efforts to develop them into 18F-labeled PET imaging probes. Unfortunately, the discrete positions of the fluorine atoms in JHU37152 and JHU37160 made radiosynthesis efforts challenging and inefficient. The most radiochemically favorable structure was [18F]JHU37107 which we radiolabeled with high yield, molar activity and radiochemical purity (Fig. 4a). In D1-DREADD transgenic mice, [18F]JHU37107 exhibited robust uptake in DREADD-expressing brain regions as compared to areas devoid of DREADD expression (Fig. 4b–d). This signal was displaceable by 0.1 mg kg\(^{-1}\) (IP) JHU37160 (Fig. 4e–d) indicating specific binding of [18F]JHU37107 at DREADD sites. We also tested [18F]JHU37107 in rats with unilateral hM3Dq (Fig. 4e) or hM4Di (Fig. 4f–j) expression in the right motor cortex. [18F]JHU37107 permitted hM4Di visualization in both the AAV injection site as well as at known proximal and distal anatomical projection sites such as striatum, corticalalateral cortex, and motor thalamus. The in vivo localization of the [18F] JHU37107 signal matched the ex vivo expression of DREADDs established by post hoc immunohistochemistry staining (Fig. 4f–j). Finally, we tested [18F]JHU37107 in a monkey expressing hM4Di in the right amygdala (Fig. 4k). [18F] JHU37107 exhibited favorable pharmacokinetic properties (Fig. 4l, m) and metabolite profile (Supplementary Fig. 11) in this species. More importantly, it was able to directly label hM4Di receptors (Fig. 4k–m), allowing robust detection of DREADDs with a dedicated 18F-labeled radioligand for the first time in nonhuman primates.

Discussion

The human muscarinic receptor-based DREADDs are the most popular chemogenetic technology for basic research and are used by a large number of laboratories around the world. Although the majority of DREAD use has been in rodents, DREADDs have also been applied for experimental use in monkeys recently, a critical step before human translation. Results from prior studies, and now from the current study, indicate that the DREADD agonists developed to date, while efficacious in certain applications, do not display sufficient potency or selectivity in others. In rodents (rats and mice), we show here that C21 activated hM3Dq at doses as low as 0.1 mg kg\(^{-1}\), however, it was less potent at activating hM4Di, which required at least 1 mg kg\(^{-1}\). In mice without DREADDs, doses higher than 1 mg kg\(^{-1}\) produced off-target effects. Furthermore, in WT mice, the 1 mg kg\(^{-1}\) dose of C21 required to activate hM4Di produced changes in brain metabolic activity (FDG uptake) even though we and others did not detect any behavioral effects using this dose. In contrast, an equipotent dose of clozapine (0.1 mg kg\(^{-1}\) did not produce any significant changes in brain metabolic activity. In monkeys, the minimal doses required to achieve DREADD occupancy also extensively displaced [11C]clozapine from endogenous targets and produced nonspecific effects. In summary, C21 has a small window of selectivity to activate hM4Di in rodents, which may potentially be compensated by overexpression of the DREADD receptor, and furthermore displays a wide range of off-target effects at the minimal hM4Di-effective doses in monkeys.

In addition to the characterization of C21, here we report the development of a new set of DREADD agonists that exhibit high in vivo potency and CNS DREADD occupancy in both rodents and in old world monkeys. While their selectivity is not ideal (i.e., comparable to clozapine), their high in vivo potency allows for dose adjustments with minimal off-target effects and importantly they exhibit promising characteristics for DREADD use in monkeys. Our data suggest that further steps to improve selectivity require divergence from the dibenzodiazepine (clozapine-based) scaffold and/or require new rationally engineered mutations in the DREADD binding pocket to differentiate it from endogenous wild-type receptors.

The other notable advance in this study is the development of the first, high-affinity 18F-labeled DREADD PET ligand. The use of 18F, with six times longer half-life than 11C, allows for this ligand to be shipped to facilities without cyclotrons or that lack the necessary radiosynthesis infrastructure and capabilities. Moreover, it offers the possibility to scan several animals using one synthesis or to perform longer scans for extensive kinetic modeling and occupancy studies. Finally, this new PET ligand provides strong somatic signaling of receptor expression in both rodents and monkeys, and in rodents, at least, there is signal that represents projections to remote locations from the primary viral injection site, making noninvasive and longitudinal visualization of cell type-specific neuroanatomical projections possible in the living mammalian subject.

Chemogenetic technologies, like DREADDs confer the ability to manipulate neuronal activity across distributed brain circuits without the need for implantable devices, thereby making them...
**Fig. 4** [18F]JHU37107 enables noninvasive detection of DREADD in locally-targeted cells and at their long-range projections. 

**a** Structure of [18F]JHU37107. 

**b–d** [18F]JHU37107 selectively binds to DREADDs in the brain of transgenic D1-DREADD mice (n = 3 mice per condition) and is blocked by 0.1 mg kg\(^{-1}\) of JHU37160. 

**e–j** [18F]JHU37107 selectively binds to AAV-DREADDs expressed in the rat cortex and enables noninvasive and longitudinal mapping of both local (injection site) and long-range projections of motor cortex circuitry (ventrolateral thalamus shown as a main hub). Representative immunohistochemical images showing GFP (green) or HA-tagged DREADDs (red) from representative rats are shown side by side with their corresponding [18F]JHU37107 PET images. The white arrows point at corresponding anatomical regions. 

**k–m** [18F]JHU37107 binds to hM4Di expressed in the monkey amygdala and at putative projection sites. All data are represented as mean ± SEM except in (l) and (m) where individual values are displayed. Scale bars are 1 mm. Source data are provided as a Source Data file and the raw PET data are available upon request.
especially useful in awake and even unrestrained animals. The development of [3H]JHU37107 and our new agonists provide means to perform diagnostic imaging of DREADDs in neurotherapeutic contexts,—i.e., “neurotheranostics”16, making it possible to imagine future development of cell type- and circuit-specific neuromodulation for humans. In the same manner, since FDG-PET is a routine human procedure, DREAMM15,16 can also be used to evaluate, longitudinal, noninvasive assessment of whole-brain, functional circuit activity as a function of chemogenetic-based therapies. In sum, if the novel pharmacological tools and approaches we describe here are extended to humans, DREADD-based neurotheranostics16 would comprise a novel precision-medicine approach that could be used for developing chemogenetic-based cell type- and circuit-specific neuromodulation for the precision or personalized treatment of various brain disorders.

Methods

Experimental subjects. Wild-type mice (C57BL/6J) were ordered from Jackson Laboratories and rats (Sprague–Dawley) were ordered from Charles River. Rodents were male and ordered at ~6 weeks of age. Transgenic mice were bred at NIDA breeding facility. Transgenic mice expressing the enzyme cre recombinase under the control of the dopamine D1 receptor promoter (D1-Cre, Fk510 line, C57BL/6 congenic, Genats, RKID: MMRC_056916-UCD) were crossed with transgenic mice with cre recombinase-inducible expression of hM3Dq DREADD (R26-hM4Di/mCitrine, Jackson Laboratory, stock no. 026219) or hM3Dq DREADD (R26-hM3Dq/mCitrine, Jackson Laboratory, stock no. 026220). Three male rhesus monkeys (Macaca mulatta) weighed 8–12 kg. All experiments and procedures complied with all relevant ethical regulations for animal testing and research and followed NIH guidelines and were approved by each institute’s 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; Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 2 mM l-glutamine, anti-biotic/antimycotic (all supplements from Gibco) and 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Inc. Flowery Branch, GA, USA) and kept in an incubator at 37°C and 5% CO2. Cells were routinely tested for mycoplasma contamination (Mycoplasma Detection Kit, Lonza). Cells were seeded on 60 cm2 dishes at 4 × 10^6 cells/dish 24 h before transfection. The indicated amount of the recombinant proteins were suspended in Tris-HCl 50 mM pH 7.4 supplemented with protease inhibitor (1:100). HEK-293 cells or brain tissue were disrupted with a Polytron homogenizer (Kinematica, Basel, Switzerland). Homogenates were centrifuged at 48,000 × g for 20 min and the supernatant was stored at −80°C. The postnuclear fraction. Protein was quantified using BCA Protein Assay Kit (ThermoFisher Scientific). Protein concentrations were determined using the microplate reader (BMG Labtech) to consider the protein concentration in the samples. The protein concentration was considered to be 1 mg/mL.

Radioligand binding assays. HEK-293 cells were transfected with 5 µg/dish of pAAX plasmids encoding for hM3Dq (Addgene #89149) or hM4Di (Addgene #89510) and 7 µg/dish of Gα12/13. Forty-eight hours after transfection, cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS). Approximately, 200,000 cells/well were distributed in 96-well plates, and 5 µM Coelenterazine H (substrate for luciferase) was added to each well. Five minutes after addition of Coelenterazine H, ligands were added to each well. The fluorescence of the acceptor was quantified (excitation at 480 nm and emission at 540 nm for 1-s recordings) in a PheraStar FSX plate reader (BMG Labtech) to confirm the constant expression levels across experiments. In parallel, the BRET signal from the same batch of cells was determined as the ratio of the light emitted by mVenus (510–540 nm) over that emitted by RLuc (485 nm). Results were calculated for the BRET change (BRET ratio in the absence of the drug) 5 min after the addition of the ligands.

Intracellular Ca2+ concentration was monitored using the fluorescent Ca2+ biosensor GCaMP6F. HEK-293 cells were transfected with 7 µg/dish of the cDNA encoding for hM3Dq (Addgene #89149) or hM4Di (Addgene #89510) and 7 µg/dish of Gα12/13. Forty-eight hours after transfection, cells were harvested, washed, and resuspended in Mg2+-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. Increasing concentrations of the indicated compound were added to the cells and fluorescence intensity (excitation at 480 nm, emission at 530 nm) was measured at 18-s intervals during 250 s using a PHERAstar FSX (BMG Labtech). The net change in intracellular Ca2+ concentration was expressed as F/F0 where F is the fluorescence at a given concentration of ligand and F0 is the average of the baseline values (fluorescence values of buffer-treated wells).

Autoradiography. Flash frozen tissue (both rodents and the monkey) was sectioned (20 µm) on a cryostat (Leica, Germany) and thaw mounted onto ethanol-washed glass slides. Slides were pre-incubated (10 min, RT) in incubation buffer (50 mM Tris-HCl pH 7.4 with 10 mM of MgCl2), then slides were incubated (60 min) in incubation buffer containing [3H]clonazepam (3.5 nM), [3H]C21 (10 nM, 41 Ci/mmol, Novandi, Sweden) or [3H]C13 (3.5 nM, 13 Ci mmol−1 Novandi, Sweden) in the presence or without increasing amounts of the indicated cold ligands (Tocris (clonazepam, C21) or custom synthesis). Slides were air dried and placed in a Hyercassette® (Amersham Biosciences) and covered with a BAS-TR2025 Storage Phosphor Screen (FujiFilm, Japan). The slides were exposed to the screen for 5–7 days and imaged using a phosphor imager (Typhoon FLA 7000; GE Healthcare).

BRET and binding target profile screen. These experiments were performed by an outside vendor (Eurofins, France). Briefly, membrane homogenates from stable cell lines expressing each receptor/enzyme were incubated with the respective radioligand in the absence or presence of clonazepam or C21 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.

P-glycoprotein (P-gp) substrate assay. These experiments were performed by an outside vendor (Eurofins, France). Briefly, P-gp substrate assay was performed at 10 µM. The A to B and B to A permeability was measured in Caco-2 cells outside vendor (Eurofins, France).

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Lentivirus expressing an hM3Δ-Di-GFP fusion protein under an hSyn promoter11 with a titer of >10^9 infectious particles was loaded into a 100 µL glass syringe (Hamilton, Bonaduz, Switzerland). The 31-gauge needle of the syringe was sheathed with a silica capillary (450 µm OD) to create a step 1 mm from the base of the aperture. The syringe was mounted in a Nanomite pump (Harvard Apparatus, Cambridge, MA). The needle was lowered through the incision in the dura mater to each of the pre-calculated target sites and 10 or 20 µl was infused at a rate of 1 µl min⁻¹. Each monkey received a total of between 10 and 12 injections (for a total injection volume of 120–240 µL). Post-infusion, the needle was left in situ for 10 min after each injection to allow pressure from the infusate to dissipate. The needle was then slowly removed. At the completion of the injection series the surgical sites were sutured together in anatomical layers.

Immunochemistry. Rodents were anesthetized with a ketamine and xylazine mixture and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA). The brains were post-fixed in 4% PFA (overnight, 4 °C) and then placed in 30% sucrose for 3–4 days. The brains were frozen and sectioned on a cryostat (40 µm) and collected in PBS with 0.1% Tween-20 (washing buffer). Slices were blocked with bovine serum albumin 3% in washing buffer (blocking buffer, 2 h). Then, incubated the primary antibody (anti-Churchil light) and rinsed with 10 mL sterile saline. The product was eluted with 1000 µL of PBS-10% sucrose for 3–4 days. The brains were frozen and sectioned on a cryostat (35 µm). Mobile Phase A consisted of water (0.1% formic acid, 5 µl of internal standard using a polytron homogenizer and centrifuged at 10 min for 16,200 × g at 4 °C. A 300 µl of supernatant was dried under a stream of nitrogen and resuspended in 150 µl methanol. The resuspended solution was then centrifuged and 100 µl of supernatant was transferred to the autosampler vial for analysis.

Data were acquired using a Nexera XR HPLC (Shimadzu) coupled with a QTRAP 6500 (SCIEX), and was analyzed with Analyst 1.6 (SCIEX). The positive ion mode data were obtained using multiple reaction monitoring (MRM). The instrument settings for curtain gas, collision gas, and source gas were 25, 25, and 30, respectively. The collision activated dissociation was set to medium and the entrance potential was 10 V. C21 was monitored using the MRM ion transition (278.80 → 166.10), and was eluted under positive ion mode for 40 V. C21 was monitored using the MRM ion transitions (278.80 → 166.10) with DP 80 V; 80 V, CXP 10 V and collision energies (CE) = 50 V. JHU37160 and JHU37152 were monitored using the MRM ion transitions (359.10 → 288.10) with DP = 70 V, CXP = 8 V and CE = 28 V. Clozapine was monitored using the MRM ion transitions (327.30 → 270.10) with DP = 100 V; 80 V, CXP = 11 V and CE = 40 V.

Separation of the C21, JHU37160, and clozapine was accomplished using a C18 Security Guard cartridge (4.6 × 4 mm) and an Eclipse XDB-C18 column (4.6 × 250 mm, 5 µm, Agilent) at 35 °C. Mobile phase A consisted of water containing 0.1% formic acid and mobile phase B was methanol containing 0.1% formic acid. The following linear gradient was run for 21.0 min at a flow rate of 0.4 ml min⁻¹: 0–2.0 min 20% B, 2.0 min 80% B, 12 min 90% B, 18.0 min 90% B, 18.1 min 20% B. Twelve-plateau calibration curves were prepared in standard solution by a 0.5 ml serial dilution of standards from 0.92 µg ml⁻¹ for JHU37160; and 0.2 µg/ml for JHU37152 and 0.4 µg ml⁻¹ for clozapine. The injection volume per sample was 10 µl. Samples were kept at 4 °C until extraction. To 25 µl of serum, 5 µl of internal standard was added.

The product was eluted with 400 µL of ethanol into a sterile, pyrogen-free bottle (Waters XBridge C18 4.6 mm × 100 mm) and eluted with 35:65 (v:v) acetonitrile:water (0.1% ammonium hydroxide) at a flow rate of 1.0 ml min⁻¹. The radioactive peak corresponding to [1³C]clozapine (t₁/₂ = 9.2 min), was detected with a radioactivity detector (Waters 2487, Milford, MA) and diluted with 4.0 mL of saline. A 10 µl aliquot of the final product was injected onto an analytical high-performance liquid chromatography (HPLC) column (Waters XBridge C18 4.6 mm × 100 mm) and eluted with 35/65 (v:v) acetonitrile:water (0.1% ammonium hydroxide) at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 1.0 ml min⁻¹. The radioactive peak corresponding to [1¹C]clozapine was separated by an analytical C18 column (Waters XBridge C18 4.6 mm × 100 mm) and eluted with 35/65 (v:v) acetonitrile:water (0.1% ammonium hydroxide), and no acetic acid was added to the reservoir. The molar activity for both [¹³C]clozapine ranged from 351 to 483 GBq µmol⁻¹ (9482–13,041 mCi µmol⁻¹) at end of synthesis. The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 1.0 ml min⁻¹. The radioactive peak corresponding to [¹³C]clozapine was separated by an analytical C18 column (Waters XBridge C18 4.6 mm × 100 mm) and eluted with 35/65 (v:v) acetonitrile:water (0.1% ammonium hydroxide), and no acetic acid was added to the reservoir. The molar activity for both [¹³C]clozapine ranged from 351 to 483 GBq µmol⁻¹ (9482–13,041 mCi µmol⁻¹) at end of synthesis. The reaction was kept at room temperature for 2 min. The solution was then diluted with 200 µl of 4006 (v:v) acetonitrile:water (0.1% ammonium hydroxide) and injected onto the liquid chromatography (HPLC) column (Waters XBridge C18, 4.6 mm × 100 mm) and eluted with 35/65 (v:v) acetonitrile:water (0.1% ammonium hydroxide) at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA). The product was eluted with 1.25 ml of 100% acetonitrile containing 0.1% acetic acid at a flow rate of 2 ml min⁻¹. The radioactivity detector was cryogenically cooled to below −20 °C before use (RapidRadionics, BioDynamics, USA).
saline was added through the same filter. The final product \(^{18}\text{F}\)[HU37107] was then analyzed by analytical HPLC (Luna C18, 10 micron, 46 mm × 250 mm, mobile phase: methanol:water:acetonitrile:fluoroacetic acid (80:20:0.1:0.01; by volume), flow rate of 0.5 mL min\(^{-1}\); λ = 6.4 min) using a UV detector at 254 nm to determine the radiochemical purity (>95%) and specific radioactivity (152–188 GBq μmol\(^{-1}\)) at the time synthesis ended.

\(^{[1]}\text{H}\)clozapine and \(^{[6]}\text{C}\)clozapine imaging using PET. Mice and rats were anesthetized with isoflurane and placed in a prone position on the scanner bed of an ARGUS small animal PET/CT scanner (Sedecal, Spain) or a NanoScan PET/CT scanner (Mediso, USA) injected intravenously (~100–200 μL) with \(^{[1]}\text{H}\)clozapine (~700 μCi) or \(^{[6]}\text{C}\)clozapine (~350 μCi) and dynamic scanning commenced. When indicated, animals were pretreated with vehicle or the indicated drug 10 min before the injection of the PET radiotracer. Total acquisition time was 60 min.

All macroscuqe samples were acquired on the Focus 220 PET scanner (Siemens Medical Solutions, Knoxville, TN). The Focus 220 is a dedicated preclinical scanner with a transaxial FOV of 19 cm and an axial FOV of 7.5 cm. Image resolution is <2 mm within the central 5 cm FOV.

After initial evaluation the monkey was sedated with ketamine (10 mg kg\(^{-1}\)) followed by keta-proten (as an analgesic) and glycopyrrolate (for salivation reduction), all weight dependent IM injections. The monkey would then be placed in the supine position, intubated with a tracheal tube. Anesthesia was maintained by 1–3.5% isoflurane and oxygen, the monkey’s head was positioned and immobilized for optimal positioning of the brain and moved into the scanner. A 10 min transmission scan was performed for attenuation correction followed by coregistration. One iv was inserted, if possible, in the right arm and one in the right leg for injection of tracer and blocking agent. The monkey was always monitored while anesthetized. HR, BP, O₂ saturation, RR, three lead ECG, rectal temperature was documented every 15 min.

Arterial blood sampling was acquired throughout the study using an indwelling femoral port. The first 2 min samples were collected every 15 s then at 3, 5, 10, 30, 60, 90, and 120 min post tracer injection. All scans were acquired for 120 min using list mode acquisition.

Scan data were histogrammed into 33 frames (6 × 30 s, 3 min, 2 μmin, and 22 × 5 s) was performed by Filtered Back Projection with scatter correction. After completion of the last study of the day, isoflurane was cut off. The monkey was gradually awakened, moved to the housing facility and fully recovered.

In all cases, the PET data were reconstructed and corrected for dead-time and radioactive decay. All qualitative and quantitative assessments of PET images were performed using a dedicated software tool (ROIS TOOL, Biomedica, Switzerland). Binding potential \(BP_{ND}\) (a relative measure of specific binding) was calculated using a reference tissue model using the cerebellum as a reference tissue in rodents. In macaques, the kinetic data were fitted to a two-tissue compartment model and the concentration of parent in plasma was used as an input function, then the volume of distribution ratios compared to cerebellum were calculated to establish \(BP_{ND}\). In all cases, the dynamic PET images were coregistered to MRI templates and time-activity curves were generated by predefined volumes of interest (macaques) or manually drawn in rodents and the described analyses were performed. Receptor occupancy was calculated using the formula: occupancy = (\(BP_{ND}\) – measured \(BP_{ND}\)) / \(BP_{ND}\), where \(BP_{ND}\) is the baseline condition and \(BP_{ND,0}\) the binding potential when the animals were pretreated with the drug. In an independent manner, \(BP_{ND}\) parametric maps were generated by pixel-based kinetic modeling using a multilinear reference tissue model using the cerebellum as a reference region and the start time \((t = 0)\) was set to 16 min.

To determine the arterial input function for the radiotracer injected in rhesus monkeys radioligand concentrations in the arterial plasma were corrected by the mobile phase 23:77 (v:v) acetonitrile:water (0.1% tri-fluoroacetic acid), then analyzed by analytical HPLC (Luna C18, 10 micron, 4.6 mm × 250 mm; mobile phase: MeOH:H₂O:Et₃N (80:20:0.1; by volume) at an isocratic flow rate of 4.0 mL min\(^{-1}\). A 10 min as infrared beam crossings and traveled distance was converted to cm. Animals were repeatedly tested on consecutive sessions (after an initial habituation session with no drug treatment) in a counterbalanced design.

Locomotor activity assessment in mice. Transgenic male and female mice (see above) (20–30 g) expressing hM3Dq or hM4Di and mCitrine reporters (or controls) were tested for locomotor activity. Mice were injected (IP) with the indicated drug (0.1 mg kg\(^{-1}\), C21, HU37160 or vehicle (H2O, saline). Ten minutes after injection, animals were placed in an open field arena (Opto-varimex ATM3, Columbus Instruments) and their locomotor activity was tracked during 60 min as infrared beam crossings and traveled distance was converted to cm. All animals were tested on consecutive sessions (after an initial habituation session with no drug treatment) in a counterbalanced design.

Locomotor activity assessment in rats. Tyrosine hydroxylase (TH):Cre rats (n = 9; founder, K. Deisseroth lab) or WT littermates (n = 9) were bilaterally injected with AAV2 hSyn-DIO-hM3Dq-mCherry (1ml/hemisphere, Addgene), and allowed to recover for 2 + weeks. Following handling, they were habituated to a Med Associates locomotor testing box (43 × 43 × 30.5 cm) for 2 h on 2 days, then repeatedly tested after separate day doses of DREADD agonists and vehicle. Cohort 1 was administered C21 (1 or 5 mg kg\(^{-1}\)) or clozapine (0.001–0.5 mg kg\(^{-1}\)), in vehicle, in counterbalanced order on separate days, with each test separated by at least 48 h. The 2 h locomotor testing session commenced 30 min after each IP injection on each day. Vehicle was administered on 2 separate days, and average locomotion was used for comparison with DREADD agonists. Cohort 2 underwent the same procedures, but with a 4 h testing session, and the following IP injections: vehicle (x2), HU37152 (0.01–0.3 mg kg\(^{-1}\)), HU37160 (0.01–0.3 mg kg\(^{-1}\)). All drugs were dissolved in 5% DMSO in saline, and injected 1 ml kg\(^{-1}\). Following all tests, rats were transcardially perfused, brains sectioned coronally at 40 μm, and VTA-specific expression confirmed using endogenous mCherry reporter expression, which co-localized nearly exclusively with TH immunoreactivity.

ChrimsonR generation and characterization. pcDNA3.1 (+):1Hygro vector (Life Technologies, Carlsbad, CA, USA) was used for expression of Chrimson, oC-Chrimson and oC-Chrimson-t in HEK293A cells (Life Technologies, Carlsbad, CA, USA). Channelrhodopsin inserts (with and without modification) were cloned into the BamHI and Xhol site and the fluorescent protein (tdTomato or citrine) cloned in-frame and a 3’ stop codon between the Xhol and XbaI site. For neuron expression, the channelrhodopsin-FP inserts were placed in an AAV2 vector between BamHI and HindIII sites (Addgene #50954). Transfection in HEK293 cells were achieved with Fugene (Roche, Basel, Switzerland) and electroporation (Lonza, Gaithersburg, MD, USA) was used for expression in neuronal cultures. Cell culture. HEK293 cell experiments were performed 2–3 days post transfection whereas neuronal experiments were performed >10 days post transfection. Cell culture conditions were as described previously.

For visualizing the channelrhodopsin-FP expression, images were taken on a Zeiss Axiocore 200 M microscope (Zeiss, Jena, Germany) with Slidebook software (D. Denver, CO, USA) using a 10× Plan-Neofluar objective (Microscope Photo, Tucson, AZ, USA). Images were taken with a 450× oil objective with NA of 1.2. Citrine images were acquired with 495/10 excitation filter and 535/25 nm filters and tdTomato images were acquired with 580/20 nm excitation and 653/95 nm emission filters (Semrock, Rochester, NY, USA). For analyzing membrane/cytosolic fluorescence, a line profile was drawn across the cell in ImageJ or Fiji. The fluorescence intensity of the two membrane regions and cytosolic portion (including nucleus) were measured from profile. After background subtraction, the mean membrane and cytosolic fluorescence were calculated and the ratio was calculated.

A thin-layer chromatography was performed with an extravascular solution containing 118 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM 4-[(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mM glucose (pH 7.4)
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 Dunnett’s or Tukey post hoc tests, taking repeated measures into account where appropriate. All statistical tests were evaluated at the P ≤ 0.05 level.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The source data underlying the figures are provided as a Source Data file or can be obtained from the authors upon request.

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References

1. Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S. & Roth, B. L. Evolving the lock to fit the key: a family of G protein-coupled receptors potently activated by an inert ligand. Proc. Natl Acad. Sci. USA 104, 5163–5168 (2007).
2. Gomez, J. L. et al. Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. Science 357, 503–507 (2017).
3. Chen, X. et al. The first structure-activity relationship studies for designer receptors exclusively activated by designer drugs. ACS Chem. Neurosci. 6, 476–484 (2015).
4. Thompson, K. J. et al. DREADD agonist 21 is an effective agonist for muscarinic-based DREADDs in vitro and in vivo. ACS Pharmacol. Transl. Sci. 14, 61–72 (2018).
5. Fendryka, M. et al. Pharmacokinetic and pharmacodynamic actions of clozapine-N-oxide, clozapine, and compound 21 in DREADD-based chemogenetics in mice. ACS Chem. Neurosci. 9, 4522 (2018).
6. Nagai, Y. et al. PET imaging-guided chemogenetic silencing reveals a critical role of primate rostromedial caudate in reward evaluation. Nat. Commun. 7, 13605 (2016).
7. Ji, B. et al. Multimodal imaging for DREADD-expressing neurons in living brain and their application to implantation of iPSC-derived neural progenitors. J. Neurosci. 36, 11544–11558 (2016).
8. Michaelides, M. & Hard, Y. L. DREAMM: a biobehavioral imaging methodology for dynamic in vivo whole-brain mapping of cell-type specific functional networks. Neurropsychopharmacology 40, 239–240 (2014).
9. Michaelides, M. et al. Whole-brain circuit dissection in free-moving animals reveals cell-specific mesocorticolimbic networks. J. Clin. Invest. 123, 5342–5350 (2013).
10. Atwood, B. K., Lopez, J., Wagner-Miller, J., Mackie, K. & Straiker, A. Expression of G protein-coupled receptors and related proteins in HEK293, AT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 12, 1410 (2011).
11. Eldridge, M. A. et al. Chemogenetic disconnection of monkey orbitofrontal and rhinal cortex reversibly disrupts reward value. Nat. Neurosci. 19, 37–39 (2015).
12. Upright, N. A. et al. Behavioral effect of chemogenetic inhibition is directly related to receptor transcription levels in Rhesus Monkeys. J. Neurosci. 38, 7909–7975 (2018).
13. Grayson, D. S. et al. The Rhesus Monkey connectome predicts disrupted functional networks resulting from pharmacogenetic inactivation of the amygdala. Neuron 91, 453–466 (2016).
14. Raper, J. et al. Metabolism and distribution of clozapine-N-oxide: implications for nonhuman primate chemogenetics. ACS Chem. Neurosci. 8, 1570–1576 (2017).
15. Sternson, S. M. & Roth, B. L. Chemogenetic tools to interrogate brain functions. Annu. Rev. Neurosci. 37, 387–407 (2014).
16. Kevdavida, B. D. et al. Neurotheranostics as personalized medicines. Adv. Drug Deliv. Rev. pii: S0169-409X(18)30261-8 (2018).
17. Bender, D., Holschbach, M. & Stocklin, G. Synthesis of n.c.a. carbon-11 labelled clozapine and its major metabolite clozapine-N-oxide and comparison of their biodistribution in mice. Nucl. Med. Biol. 21, 921–925 (1994).
18. Ichiye, M. et al. Noninvasive quantification of dopamine D2 receptors with iodine-123-BFP SPECT. J. Nucl. Med. 37, 513–520 (1996).
19. Gandelman, M. S., Baldwin, R. M., Zoghbi, S. S., Zee-Ponce, Y. & Innis, R. B. Evaluation of ultrafiltration for the free-fraction determination of single
photon emission computed tomography (SPECT) radiotracers: beta-CIT, IBF, and iomazenil. J. Pharm. Sci. **83**, 1014–1019 (1994).

20. Lin, J. Y., Knutsen, P. M., Muller, A., Kleinfeld, D. & Tsien, R. Y. ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nat. Neurosci.* **16**, 1499–1508 (2013).

21. Sherman, W., Day, T., Jacobson, M. P., Friesner, R. A. & Farid, R. Novel procedure for modeling ligand/receptor induced fit effects. *J. Med. Chem.* **49**, 534–553 (2006).

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Author contributions

All coauthors reviewed the paper and provided comments. J.B., M.A.E., F.H., J.L.G., M.S.S., A.M.A, S.I., M.B., C.R., M.F., S.S.S., S.T., S.S.Z., R.L.G., A.M., L.M.G.F., N.A. and J.Y.L. performed the experiments, chemical synthesis, and/or analyzed data. J.B., M.A.E., F.H., J.L.G., M.S.S., A.M.A, A.M., J.Y.L., Y.W.P., R.B.L., R.M., P.M., L.S., D.R.S., S.V.M., S.N., A.G.H., B.J.R. and M.M. designed and/or supervised experiments and syntheses. M.G.P. and A.B. provided access to resources and support. J.B. and M.M. wrote the paper with input from all authors. M.M. conceived the study.

Additional information

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Competing interests: M.M. is a cofounder and owns stock in Metis Laboratories. J.B., J.L.G., F.H., M.S.S., A.G.H., M.G.P., and M.M. are listed as inventors on an application (62/627,527) filed with the U.S. Patent Office regarding the novel DREADD compounds described herein. Remaining authors declare no competing interest.

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