ACS Chemical Neuroscience

Article

The First Structure-activity Relationship Studies for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Xin Chen, Hyunah Choo, Xi-Ping Huang, Xiaobao Yang, Orrin Stone, Bryan L Roth, and Jian Jin

ACS Chem. Neurosci., **Just Accepted Manuscript** • DOI: 10.1021/cn500325v • Publication Date (Web): 14 Jan 2015

Just Accepted

ACS Chemical Neuroscience is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

The First Structure-activity Relationship Studies for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Xin Chen,[‡,†] Hyunah Choo,[§,¶,†] Xi-Ping Huang,[§] Xiaobao Yang,[‡] Orrin Stone,[§] Bryan L. Roth,*[,§] Jian Jin *[,‡]

[‡]Departments of Structural and Chemical Biology, Oncological Sciences, and Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, New York 10029, United States

[§]Department of Pharmacology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States

[¶]Center for Neuro-Medicine, Korea Institute of Science and Technology, Seongbuk-gu, Seoul 136-791, Republic of Korea

RECEIVED DATE (to be automatically inserted after your manuscript is accepted)

Abstract

Over the past decade, two independent technologies have emerged and been widely adopted by the neuroscience community for remotely controlling neuronal activity: optogenetics which utilize engineered channelrhodopsin and other opsins, and chemogenetics which utilize engineered G protein-coupled receptors (Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)) and other orthologous ligand-receptor pairs. Using directed molecular evolution, two types of DREADDs derived from human muscarinic acetylcholine receptors have been developed: hM3Dq which activates neuronal firing, and hM4Di which inhibits neuronal firing. Importantly, these DREADDs were not activated by the native ligand acetylcholine, but selectively activated by clozapine *N*-oxide (CNO), a pharmacologically inert ligand. CNO has been used extensively in rodent models to activate DREADDs and although CNO is not subject to significant metabolic transformation in mice, a small fraction of CNO is apparently metabolized to clozapine in humans and guinea pigs, lessening the translational potential of DREADDs. To effectively translate the DREADD technology, the next generation of DREADD agonists are needed and a thorough understanding of structure – activity relationships (SAR) of DREADDs is required for developing such ligands. We therefore conducted the first SAR studies of hM3Dq. We explored multiple regions of the scaffold represented by CNO, identified interesting SAR trends, and discovered several compounds that are very potent hM3Dq agonists but do not activate the native human M3 receptor (hM3). We also discovered that the approved drug perlapine is a novel hM3Dq agonist with >10,000-fold selectivity for hM3Dq over hM3.

Introduction

To elucidate how neuronal ensembles interactively encode higher brain processes, new and improved methods for both recording and manipulating neuronal activity will be required.[1, 2] The ability to selectively modulate the activity of defined neuronal populations and to elucidate the behavioral consequences of this selective neuronal modulation affords powerful approaches for studying mammalian brain function in health and disease. Historically, important methods include Wilder Penfield's pioneering studies of focal electrical stimulation of the human cortex.[3] The development of the optogenetics technology pioneered by Diesseroth and colleagues to visualize and activate neuronal activity with exquisite temporal resolution using engineered channelrhodopsin[4,5] and other opsins[6] has provided an expanding toolbox for decoding the neuronal correlates of brain function.[4, 6-10] More recently, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) have been developed as a powerful chemogenetics technology for remotely controlling neuronal activity[11, 12] and have been widely adopted by the neuroscience and greater biological communities.[13-18]

DREADDs, first revealed in 2005,[5] were developed using directed molecular evolution of human muscarinic acetylcholine receptors.[11, 12] After multiple rounds of random mutagenesis, DREADDs derived from the human muscarinic acetylcholine M3 receptors (hM3Dq) to be insensitive to the endogenous ligand acetylcholine but potently and selectively activated by the pharmacologically inert clozapine *N*-oxide (CNO) were discovered. Importantly, CNO lacks appreciable affinity (K_i > 1 μM) for all relevant native CNS (central nervous system) targets[11, 19, 20] The DREADDs have no detectable constitutive activity *in vitro*[11] and, thus, provide an attractive orthologous receptor-effector chemogenetic platform for modifying neuronal activity remotely with minimal invasiveness. In addition to hM3Dq, which activates neuronal firing upon

the CNO stimulation in part by depolarization and elevation of intracellular calcium levels,[12] hM4Di was developed from human muscarinic acetylcholine M4 receptors for inhibiting neuronal firing via activation of G-protein inwardly rectifying potassium (GIRK) channels.[11] Since the introduction of the DREADD technology, a large number of papers have independently validated the utility of excitatory and inhibitory DREADDs.[12, 21-34] In addition, no effect related to the ectopic expression of hM3Dq or hM4Di has been observed.

In addition to being pharmacologically inert, CNO, the "chemical switch" of this chemogenetic approach, is orally bioavailability and CNS penetrant[35, 36, 21, 37] and is not subject to significant metabolic transformation in mice and rats. However, a small fraction of CNO is apparently metabolized to clozapine in humans, non-human primates and guinea pigs.[38-40, 41] Because clozapine modulates the activity of many native CNS receptors,[42] thus interfering with the selective activation of the DREADDs in defined neuronal populations, the 'back-metabolism' issue presents a hurdle for translating the DREADD technology forward. To ultimately develop the next generation of DREADD ligands that can selectively activate defined neuronal populations in primates including human, a thorough understanding of structure – activity relationships (SAR) of DREADDs is needed. To date, no SAR studies have been reported for any DREADDs including hM3Dq and hM4Di.

Here, we report the first SAR studies of hM3Dq. We extensively explored multiple regions of the scaffold represented by CNO, which resulted in the discovery of compounds **13** and **21** that are very potent hM3Dq agonists but do not activate the native human M3 receptor (hM3). We describe the design, synthesis, and pharmacological evaluation of new CNO analogs and discuss the interesting SAR trends revealed from the studies. We also report the discovery

that perlapine, a hypnotic agent first reported in 1966,[43-45] is a novel, potent, and selective agonist of hM3Dq (> 10,000-fold selective for hM3Dq over hM3).

Results and Discussion

Design and Synthesis. To understand the SAR of CNO analogs as hM3Dq agonists, we explored several regions of the CNO scaffold. In particular, we focused on investigating the R^1 and R^2 substituents as well as modifications to the piperazine ring (highlighted in red in Fig. 1). We also studied whether the chloro group (R^3, highlighted in blue in Fig. 1) on the tricyclic core is required and whether a different tricyclic core (e.g., the core of the perlapine scaffold, see below) can be tolerated.

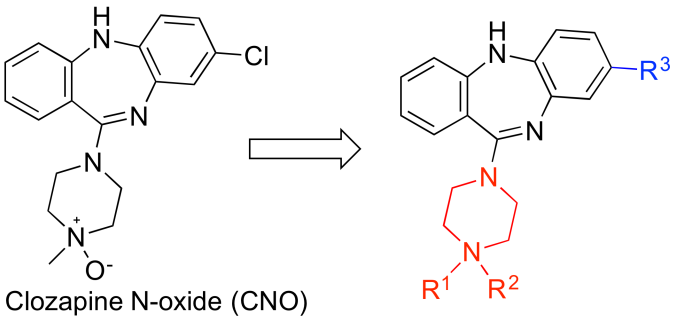
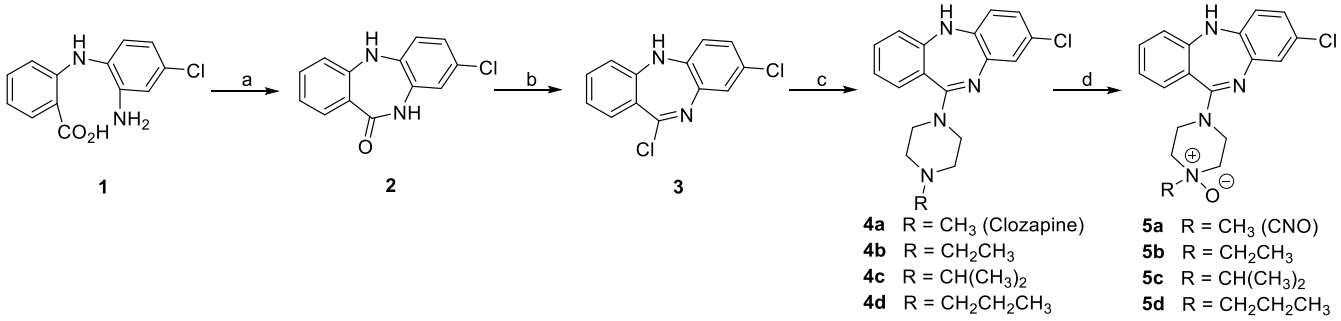


Figure 1. SAR studies of the CNO scaffold.

We first explored the size of the alkyl group (R^1) on the N4' position of the CNO scaffold and synthesized a set of close analogs as outlined in Scheme 1. The commercially available 2-((2-amino-4-chlorophenyl)amino)benzoic acid (**1**) was refluxed in xylene for 48 hours to give the cyclized compound **2**, which was then treated with $POCl_3$ and *N*,*N*-dimethylaniline in toluene at 95°C for 2 hours to afford the chloride **3**. Compound **3** was reacted with various *N*-alkylpiperazines in toluene at 120°C for 2 hours to yield compounds **4a** to **4d**, which were then converted to their corresponding *N*-oxides **5a** to **5d** by treating with *meta*-chloroperoxybenzoic

acid (*m*CPBA) at room temperature in CH_2Cl_2 for 10 minutes. The synthetic route to compounds **4a** – **4d** was described previously.[46, 47]

Scheme 1. Synthesis of N4'-alkyl substituted CNO analogs[a]

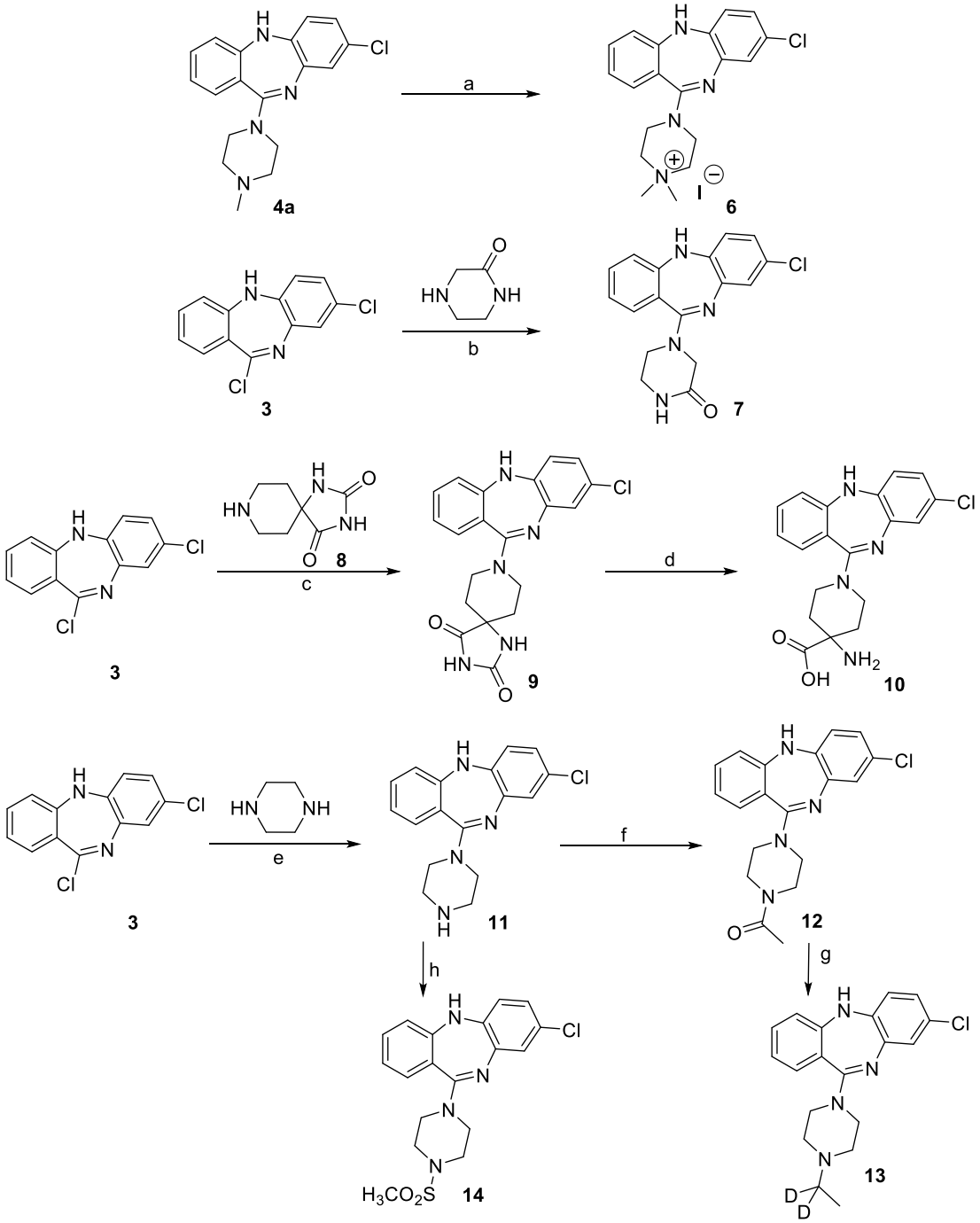


[a]Reagents and conditions: a) xylene, reflux, 48 h, 95% yield; b) $POCl_3$, *N*,*N*-dimethylaniline, toluene, 95°C, 2 h, 67% yield; c) *N*-alkylpiperazines, toluene, 120°C, 2 h, 69 – 80% yield; and d) *m*CPBA, CH_2Cl_2, rt, 10 min, 65 - 75% yield.

We next synthesized the analogs outlined in Scheme 2 to determine whether the positive charge of CNO is required for activating hM3Dq. For example, compound **6**, which contains a quaternary ammonium moiety, has a permanent positive charge while compounds **7**, **9**, **12**, **14** and **15** do not possess a basic amino group, therefore do not contain a positive charged group. As illustrated in Scheme 2, compound **4a** (clozapine) was converted to the quaternary ammonium iodide **6** by stirring overnight with CH_3I in acetone at room temperature. Compound **3** was treated with piperazin-2-one at 99°C overnight in the 1:1 mixture of 1,4-dioxane and ethanol to give compound **7**. Similarly, compound **9** was produced by treating compound **3** with commercially available 1,3,8-triazaspiro[4.5]decane-2,4-dione (**8**) in the 2:1 mixture of 1,4-dioxane and *N*,*N*-dimethylformamide (DMF) at 130°C for 24 hours. Hydrolysis of compound **9** using 0.5 N aqueous NaOH solution in 1,2-dimethoxyethane under microwave irradiation afforded compound **10**. Likewise, compound **11** was prepared from compound **3** and piperazine in toluene at 120°C for 2 hours. Acetylation of compound **11** by AcCl in CH_2Cl_2 at 0°C in the presence of triethylamine (TEA) yielded compound **12**, which was subsequently reduced to the

deuterated compound **13** using $LiAlD_4$ under reflux conditions, followed by quenching with CD_3OD at 0°C. In addition, compound **11** was reacted with MsCl in CH_2Cl_2 at 0°C in the presence of diisopropylethylamine (DIPEA) to give the methylsulfonamide **14**.

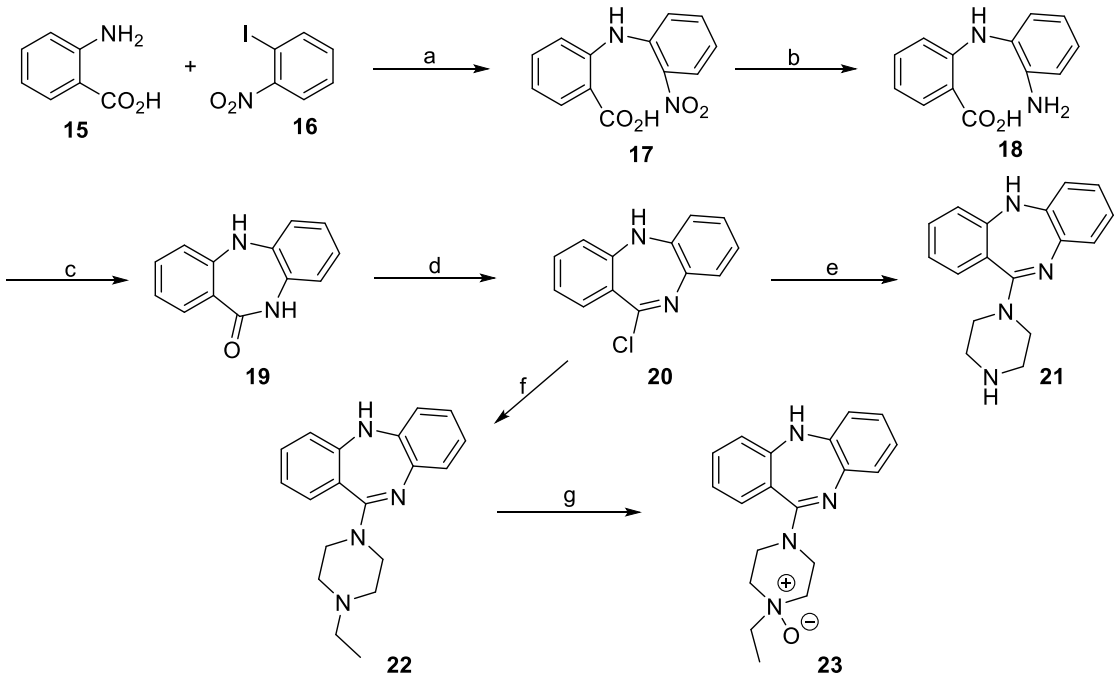
Scheme 2. Synthesis of compounds 6, 7, and 9 – 14[a]



[a]Reagents and conditions: a) CH_3I, acetone, rt, overnight, 55% yield; b) 2-oxypiperazine, 1,4-dioxane/ethanol 1:1, 99°C, overnight, 65% yield; c) 1,3,8-triazaspiro[4.5]decane-2,4-dione (**8**), 1,4-dioxane/DMF (2:1), 130°C, 24 h, 66% yield; d) 1,2-dimethoxyethane, 0.5 N NaOH, microwave, 150°C, 10 min, 16% yield; e) piperazine, toluene, 120°C, 2 h, 69% yield; f) AcCl, TEA, CH_2Cl_2, 0°C, 1 h, 86% yield; g) (1) $LiAlD_4$, THF, N_2, reflux, 2h, (2) CD_3OD, 0°C, (3) NH_4OH, 0°C, 84% yield; h) MsCl, DIPEA, CH_2Cl_2, 0°C, 1 h, 93% yield.

To determine whether the 8-Cl group on the tricyclic core is required to activate hM3Dq, we prepared compounds **21** – **23** according to the synthetic route outlined in Scheme 3.[48] The commercially available 2-aminobenzoic acid (**15**) and 2-nitrophenyl iodide (**16**) were subjected to Ullmann coupling conditions[49] to afford the aniline **17**. Reduction of the nitro moiety of compound **17** yielded compound **18**, which was refluxed in xylene to generate the benzodiazepine **19**. Treatment of compound **19** with $POCl_3$ provided the chloride **20**, which was then displaced with piperazine to afford compound **21**. Similarly, compound **22** was prepared by the displacement reaction of the chloride **20** with 1-ethylpiperazine in toluene under reflux conditions. In addition, the oxidation of compound **22** by *m*CPBA in CH_2Cl_2 afforded the *N*-oxide **23**.

Scheme 3. Synthesis of compounds 22 – 24[a]



[a]Reagents and conditions: a) K_2CO_3, Cu, 3-methylbutan-1-ol, reflux, 4 h; b) NaS_2O_4, NH_4OH/H_2O 3:2, 80°C, 30 min, 75% yield in two steps; c) xylene, reflux, Dean-Stark conditions, 48 h, 96% yield; d) $POCl_3$, *N*,*N*-dimethylaniline, toluene, reflux, 3 h, 52% yield; e) piperazine, toluene, reflux, overnight, 63% yield; f) 1-ethylpiperazine, toluene, reflux, 2 h, 72% yield; and g) *m*CPBA, CH_2Cl_2, rt, 10 min, 78% yield.

Biological Evaluation. The newly synthesized compounds were evaluated in the hM3Dq and hM3 Ca^{2+} mobilization fluorometric imaging plate reader ($FLIPR^{TETRA}$) assays according to the protocols reported previously.[11, 50, 51] Agonist activities of these compounds in the hM3Dq and hM3 functional assays are summarized in Table 1.

Table 1. Agonist activities of new compounds in hM3Dq and hM3 FLIPR assays.*

Compound	hM3Dq		hM3	
	EC_{50} (nM)	**E_{max}** (relative to CNO)	**EC_{50} (nM)**	**E_{max}** (relative to acetylcholine)
4a	1.1	95	360	88
4b	7.0	91	>30,000	NA
4c	13	45	>30,000	NA
4d	71	50	>30,000	NA
5a	6.0	100	>30,000	NA
5b	19	50	>30,000	NA
5c	190	45	>30,000	NA
5d	740	79	>30,000	NA
6	0.069	100	9.5	92
7	>30,000	NA	>30,000	NA
9	>30,000	NA	>30,000	NA
10	>30,000	NA	>30,000	NA
11	2.1	95	490	86
12	>30,000	NA	>30,000	NA
13	9.6	86	>30,000	NA
14	>30,000	NA	>30,000	NA
21	1.7	100	>30,000	NA
22	1.3	81	>30,000	NA
23	220	59	>30,000	NA

*EC_{50} values are the average of at least 2 duplicate experiments with standard deviations (SD) values that are 3-fold less than the average. NA: not applicable.

For the size of the *N*-alkyl group in compounds **4a** – **4d** and **5a** – **5d**, we observed a clear trend showing that the longer and/or bulkier the *N*-alkyl group, the weaker the compounds' potency for hM3Dq. The replacement of the methyl group in compounds **4a** and **5a** with the *n*-propyl group in compounds **4d** and **5d** resulted in a potency decrease of approximately 70- and 100-fold, respectively. In addition to the loss in potency, the compounds with a longer or bulkier *N*-alkyl group (e.g., compounds **4b** – **4d** and **5b** – **5d**) in general displayed lower agonist efficacy for hM3Dq and became partial agonists of hM3Dq rather than full agonists as seen for compounds **4a** and **5a**. Interestingly, compounds **4c** and **5c**, which contain an *i*-propyl group, were more potent than compounds **4d** and **5d**, which contain a *n*-propyl group, suggesting that

the length of the *N*-alkyl group plays a more significant role than the bulkiness of the *N*-alkyl group in reducing agonist potency. We were also pleased to find that compounds **4b** – **4d** and **5b** – **5d** did not display any agonist activity (EC_{50} > 30,000 nM) for the native human M3 receptor (hM3), in contrast to compound **4a** (clozapine), which was a hM3 agonist with sub-μM potency. In addition, compounds **4a** – **4d** were in general more potent than their corresponding *N*-oxides **5a** – **5d** at activating hM3Dq, suggesting that the negative charge on the *N*-oxides is not only not required for activating hM3Dq, but also reduces agonist potency.

The quaternary ammonium salt **6** was an extremely potent full agonist of hM3Dq with an EC_{50} value of 69 pM and about 15-fold more potent than compound **4a** (clozapine). However, compound **6** was also a potent full agonist of hM3 (EC_{50} = 9.5 nM, E_{max} = 92) even though it achieves >100-fold higher potency for hM3Dq over hM3. On the other hand, compounds **7**, **12**, and **14**, which do not contain a basic amino group or a group with permanent positive charge, did not display any agonist activity for hM3Dq. As expected, these compounds did not activate hM3 either. Taken together, these results suggest that either a basic amino group as in compounds **4a** and **4b** or a group with permanent positive charge as in compounds **5a** and **6** is required to retain hM3Dq agonist activity. In addition, compound **9** that contains a hydantoin moiety and compound **10** that contains an amino acid moiety in this region did not activate hM3Dq and hM3. On the other hand, compound **11**, which is the des-methyl clozapine, showed similar potency and efficacy for hM3Dq and hM3 as clozapine, suggesting that the *N*-methyl group is not required for activating hM3Dq. Interestingly, compound **13**, which is a deuterated analog of compound **4b**, exhibited similar potency and efficacy (EC_{50} = 9.6 nM, E_{max} = 86%) for hM3Dq as compounds **4b** and **5a** (CNO) (Fig. 2). Importantly, compound **13** did not display any agonist activity for hM3. Because compound **13** contains an α,α-di-deutero ethyl group, it is likely that

the *N*-de-alkylation, the major metabolic pathway that converts clozapine to des-methyl clozapine,[39, 40] will be significantly reduced on the basis of the well-documented primary kinetic isotope effect[52] in similar systems.[53, 54]

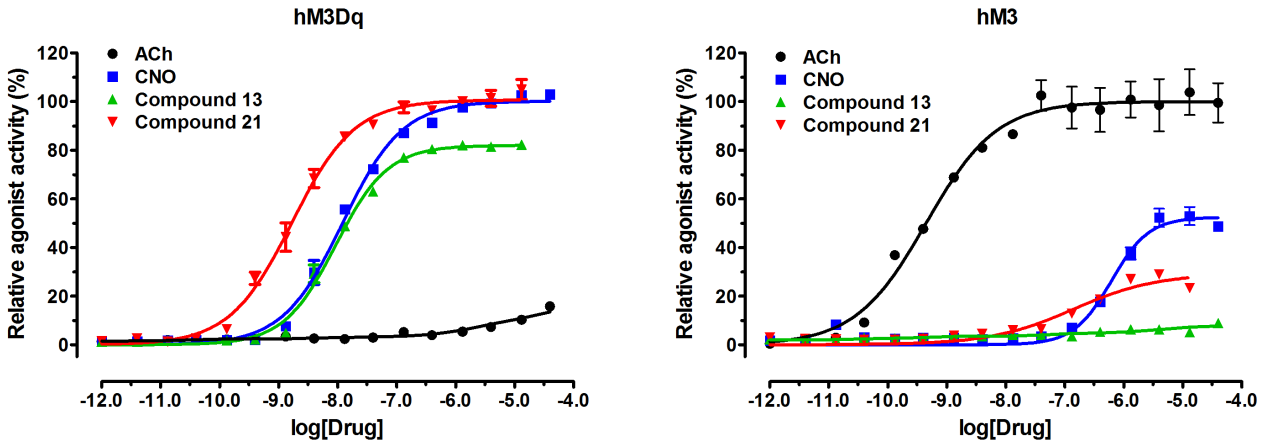


Figure 2. Compounds **13** and **21** are potent hM3Dq agonists and do not activate hM3 being similar to compound **5a** (CNO). The endogenous ligand acetylcholine (ACh), on the other hand, is a potent hM3 agonist and does not activate hM3Dq.

We were also pleased to find that the 8-chloro group was not required to maintain high agonist potency and efficacy for hM3Dq. In particular, compound **21** was a potent full agonist (EC_{50} = 1.7 nM, E_{max} = 100%) of hM3Dq (Fig. 2). In contrast to compound **11**, a full hM3 agonist with sub-μM potency, compound **21** did not display any agonist activity for hM3 (EC_{50} > 30,000 nM). In addition, compound **22** was found to be a potent hM3Dq agonist (EC_{50} = 1.3 nM, E_{max} = 81%), which was more potent than the corresponding chloro analog, compound **4b** (EC_{50} = 7.0 nM, E_{max} = 91%). On the other hand, the *N*-oxide **23** (EC_{50} = 220 nM, E_{max} = 59%) was about 10-fold less potent for hM3Dq than compound **5b**, the corresponding chloro analog (EC_{50} = 19 nM, E_{max} = 50%). Similar to compound **21**, both compounds **22** and **23** did not exhibit any agonist activity for hM3.

We next selected a subset of the above hM3Dq agonists that are inactive against hM3 and assessed their binding affinities to other aminergic GPCRs. Because compound **4a** (clozapine) showed high binding affinities to $5HT_{2A}$ and $5HT_{2C}$ serotonin, α_{1A} adrenergic, and H_1 histamine receptors with K_i values of 5.4, 9.4, 1.6, and 1.1 nM, respectively (Table 2), we tested compounds **4b**, **4c**, **5b**, **5c**, **13** and **21** in $5HT_{2A}$, $5HT_{2C}$, α_{1A}, and H_1 radioligand binding assays. The assay results are summarized in Table 2.

Table 2. Binding affinities of selected hM3Dq agonists to other GPCRs.*

Compound	K_i (nM)			
	$5HT_{2A}$	**$5HT_{2C}$**	**α_{1A}**	**H_1**
4a	5.4	9.4	1.6	1.1
4b	29	24	46	1.9
4c	16	17	37	4.6
5b	1900	5100	>10,000	160
5c	5200	6700	320	6200
13	71	280	67	5.0
21	66	170	280	6.0

*K_i values are the average of at least 2 duplicate experiments with standard deviation (SD) values that are 3-fold less than the average.

Compounds **4b** and **4c** had reduced binding affinities to $5HT_{2A}$, $5HT_{2C}$, and α_{1A} (K_i = 16 – 46 nM) compared with compound **4a** (clozapine), but retained high binding affinities to H_1 (K_i < 5.0 nM). On the other hand, the *N*-oxide **5b** displayed weak binding affinities for $5HT_{2A}$, $5HT_{2C}$, and α_{1A} (K_i > 1,000 nM) and was about 8-fold selective for hM3Dq over H_1 while the *N*-oxide **5c** displayed poor binding affinities to $5HT_{2A}$, $5HT_{2C}$, and H_1 (K_i > 5,000 nM) but was only about 2-fold selective for hM3Dq over α_{1A}. Interestingly, compound **13**, a deuterated analog of compound **4b**, exhibited reduced binding affinities to all four receptors compared with compound **4b**. Compound **13** was selective for hM3Dq over $5HT_{2A}$ (7-fold), $5HT_{2C}$ (29-fold), and α_{1A} (7-fold), but was not selective over H_1. We were pleased to find that compound **21** displayed much improved selectivity compared with compound **4a** (clozapine). In addition to

being inactive at hM3, compound **21**, a potent full agonist of hM3Dq (EC_{50} = 1.7 nM), was 40-fold selective over $5HT_{2A}$, 100-fold selective over $5HT_{2C}$, and 165-fold selective over α_{1A}. Although it was only 3.5-fold selective for hM3Dq over H_1, the overall selectivity profile of compound **21** is significantly better than compound **4a** (clozapine).

Lastly, to identify an alternative compound that might activate hM3Dq, we conducted a screen of the commercially available Library Of Pharmaceutically Active Compounds (LOPAC; N = 1,280 compounds) and Prestwick Chemical Library (N = 1,280 compounds) using the hM3Dq FLIPR assay. From this screen, we discovered perlapine as a novel, potent agonist of hM3Dq (Fig. 3). Importantly, perlapine was > 10,000-fold selective for hM3Dq over hM3. Interestingly, perlapine contains a different tricyclic core in comparison with CNO. The high hM3Dq potency of perlapine suggests that the benzodiazepine tricyclic core of the CNO (compound **5a**) scaffold is not required for maintaining high hM3Dq agonist activity.

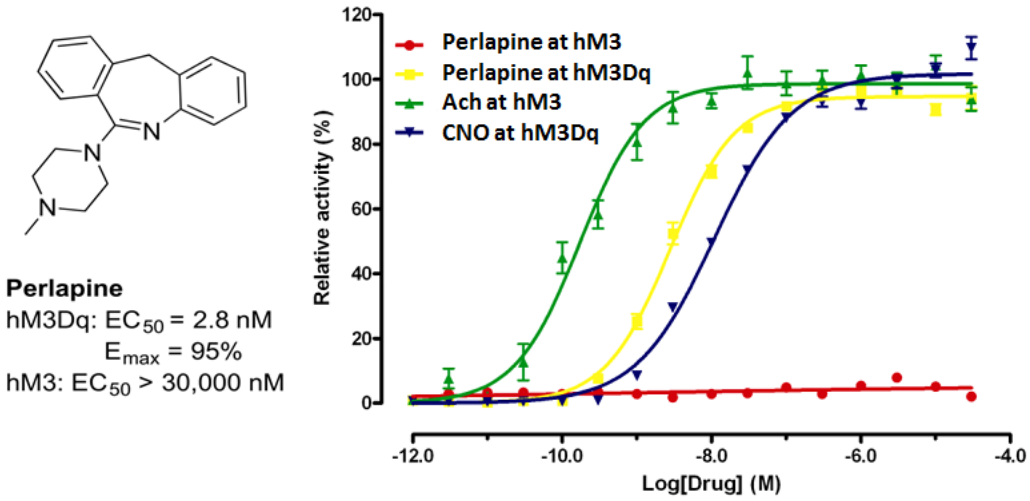


Figure 3. Perlapine is a potent full agonist of hM3Dq and does not activate hM3. CNO (compound **5a**) was used as a positive control in the hM3Dq FLIPR assay and acetylcholine (ACh) was used as a positive control in the hM3 FLIPR assay.

Conclusion

In summary, we conducted the first SAR studies for hM3Dq, a chemogenetic platform for activating neuronal firing, by the design, synthesis, and pharmacological evaluation of new CNO analogs. We explored multiple regions of the CNO scaffold and observed the following interesting SAR trends: (1) a longer or bulkier *N*-alkyl group as in compounds **4c**, **4d**, **5c**, and **5d** reduces both potency and efficacy for hM3Dq; (2) a basic amino group as in compounds **4a** and **4b** or a permanent positive charge group as in compounds **5a** and **6** is required to retain hM3Dq agonist activity; (3) the negative change on the *N*-oxides such as **5a** – **5d** reduces hM3Dq agonist potency; (4) the 8-chloro group is not required to maintain high agonist potency and efficacy for hM3Dq; and (5) modifications to the benzodiazepine tricyclic core of CNO is tolerated. From these SAR studies, we discovered several compounds such as **13** and **21**, which are very potent full agonists of hM3Dq but do not activate the native human M3 receptor (hM3). In addition, the selectivity of compound **21** against a number of aminergic GPCRs is significantly improved compared with clozapine. Furthermore, we discovered perlapine as a novel, potent hM3Dq agonist, which is > 10,000-fold selective for hM3Dq over hM3. These SAR studies lay the foundation for developing the next generation of DREADD ligands that can selectively activate defined neuronal populations in primates.

Methods

Chemistry. *General Methods.* HPLC spectra of all compounds were acquired from an Agilent 6110 Series system with UV detector set to at 220 nm. Samples were injected (5 μL) onto an Agilent Eclipse Plus 4.6 x 50 mm, 1.8 μM, C18 column at room temperature. A linear gradient from 10% to 100% B (MeOH + 0.1% Acetic Acid) in 5.0 min was followed by pumping 100% B

for another 2 minutes with A being H_2O + 0.1% acetic acid. The flow rate was 1.0 mL/min. Mass spectra (MS) data were acquired in positive ion mode using an Agilent 6110 single quadrupole mass spectrometer with an electrospray ionization (ESI) source. HRMS analysis was conducted on an Agilent Technologies G1969A high-resolution API-TOF mass spectrometer attached to an Agilent Technologies 1200 HPLC system. Samples were ionized by electrospray ionization (ESI) in positive mode. Nuclear Magnetic Resonance (NMR) spectra were recorded at Varian Mercury spectrometer with 400 MHz for proton (^{1}H NMR) and 100 MHz for carbon (^{13}C NMR); chemical shifts are reported in ppm (δ). Preparative HPLC was performed on Agilent Prep 1200 series with UV detector set to at 220 nm. Samples were injected onto a Phenomenex Luna 75 x 30 mm, 5 μM, C18 column at room temperature. The flow rate was 30 mL/min. Different linear gradient for different compounds were used with A being H_2O + 0.5% TFA and B being MeOH.

Compounds **2**,[46, 47] **3**,[46, 47] **4a**,[46, 47] **4b**,[46] and **5a**[55, 56] were prepared according to the procedures described previously.

8-Chloro-11-(4-isopropylpiperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine (4c)

A solution of 8,11-dichloro-5*H*-dibenzo[*b*,*e*][1,4]diazepine (**3**, 0.397 g, 1.44 mmol) and 1-isopropylpiperazine (1 g, 7.799 mmol) in 1,4-dioxane (20 mL) was stirred overnight at 120°C. After cooling down, the reaction mixture was concentrated and the residue was dissolved with 50 mL of EtOAc. The resulting solution was washed with 30 mL of aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4, and the filtrate was concentrated and the residue was purified by flash column chromatography with 5-10% MeOH in CH_2Cl_2 to give the desired product **4c** (0.410 g) in 80% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.16 (m, 2H), 7.04 (d, *J* = 1.5 Hz, 1H), 6.99 (t, *J* = 7.5 Hz, 1H), 6.88 – 6.72 (m, 2H), 6.58 (d, *J* = 8.3 Hz, 1H), 4.86 (s, 1H), 3.46

(br, s, 4H), 2.70 (quin, *J* = 6.5 Hz, 1H), 2.58 (br s, 4H), 1.06 (d, *J* = 6.5 Hz, 6H); ^{13}C NMR (101 MHz, , $CDCl_3$) δ 162.84, 152.90, 142.13, 140.59, 132.00, 130.55, 129.24, 126.94, 123.69, 123.18, 123.13, 120.25, 120.15, 54.80 (2C), 48.95 (2C), 47.77, 18.80 (2C); HPLC purity 100%, RT 4.099 min; MS (ESI) 355.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{24}ClN_4^+$ $[M+H]^+$: 355.1689, Found: 355.1693.

8-Chloro-11-(4-propylpiperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine (4d)

Compound **4d** (0.380 g, 70% yield) was prepared via the same procedure as preparing compound **4c** from 8,11-dichloro-5*H*-dibenzo[*b*,*e*][1,4]diazepine (**3**, 0.400 g, 1.52 mmol), 1-propylpiperazine HCl salt (0.500 g, 3.04 mmol), and DIPEA (2 mL, 11.48 mmol) in 1,4-dioxane (20 mL). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.19 (m, 2H), 7.04 (d, *J* = 2.3 Hz, 1H), 6.99 (t, *J* = 7.5 Hz, 1H), 6.79 (dd, *J* = 8.2, 2.0 Hz, 2H), 6.58 (d, *J* = 8.3 Hz, 1H), 4.86 (s, 1H), 3.45 (br s, 4H), 2.50 (br s, 4H), 2.33 (t, *J* = 7.6 Hz, 2H), 1.52 (sixtet, *J* = 7.6 Hz, 2H), 0.90 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.93, 152.90, 142.08, 140.59, 132.03, 130.52, 129.25, 126.96, 123.69, 123.21, 123.20, 120.26, 120.16, 60.93 (2C), 53.40 (2C), 47.47, 20.21, 12.18; HPLC purity 100%, RT 4.113 min; MS (ESI) 355.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{24}ClN_4^+$ $[M+H]^+$: 355.1689, Found: 355.1687.

4-(8-Chloro-5*H*-dibenzo[*b*,e][1,4]diazepin-11-yl)-1-ethylpiperazine *N*-oxide (5b)

A solution of compound **4b** (0.100 g, 0.293 mmol) in CH_2Cl_2 (5 mL) was treated with mCPBA (0.064 g, 0.371 mmol) at room temperature. After 10 min, the reaction was completed. The resulting mixture was concentrated and purified by flash column chromatography with 5-15% C (2% NH_4OH in MeOH) in CH_2Cl_2 to give the desired *N*-oxide compound **5b** (0.073 g,) in 70% yield: ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.39 – 7.34 (m, 1H), 7.32 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 7.01 (d, *J* = 8.0 Hz, 1H), 6.97 (d, *J* = 2.3 Hz, 1H), 6.87 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.81 (d, *J* = 8.4 Hz, 1H), 3.88 (br s, 2H), 3.74 (t, *J* = 12.1 Hz, 2H), 3.55 – 3.43 (m, 2H),

3.367 (q, *J* = 7.2 Hz, 2H), 3.19-3.09 (m, 2H), 1.38 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.06, 153.18, 141.54, 140.62, 132.57, 130.29, 129.35, 126.98, 123.90, 123.56, 122.96, 120.45, 120.43, 66.73 (2C), 63.23 (2C), 42.23, 7.69; HPLC purity 100%, RT 4.394 min; MS (ESI) 357.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{22}ClN_4O^+$ $[M+H]^+$: 357.1482, Found: 357.1481.

4-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)-1-isopropylpiperazine *N*-oxide (5c)

Compound **5c** (0.068 g, 65% yield) was prepared similarly as **5b** from compound **4c** (0.100 g, 0.282 mmol) and mCPBA (0.063 g, 0.365 mmol) in CH_2Cl_2 (5 mL). ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.39 – 7.30 (m, 2H), 7.05 (t, *J* = 7.5 Hz, 1H), 7.01 (d, *J* = 7.9 Hz, 1H), 6.97 (d, *J* = 2.3 Hz, 1H), 6.87 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.81 (d, *J* = 8.4 Hz, 1H), 3.90 (br s, 2H), 3.73 (t, *J* = 11.9 Hz, 1H), 3.57 – 3.40 (m, 3H), 3.19-3.12 (m, *2*H), 1.38 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.02, 153.15, 141.64, 140.61, 132.51, 130.31, 129.38, 126.97, 123.81, 123.55, 123.01, 120.43, 120.39, 70.99 (2C), 60.41 (2C), 42.27, 16.54 (2C); HPLC purity 100%, RT 4.305 min; MS (ESI) 371.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{24}ClN_4O^+$ $[M+H]^+$: 371.1639, Found: 371.1642.

4-(8-Chloro-5*H*-dibenzo[*b*,*e*][1,4]diazepin-11-yl)-1-propylpiperazine *N*-oxide (5d)

Compound **5d** (0.075 g, 72% yield) was prepared using the same procedure as preparing **5b** from compound **4d** (0.100 g, 0.282 mmol) and mCPBA (0.063 g, 0.365 mmol) in CH_2Cl_2 (5 mL) ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.39 – 7.34 (m, 1H), 7.32 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 7.00 (d, *J* = 8.0 Hz, 1H), 6.97 (d, *J* = 2.3 Hz, 1H), 6.87 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.81 (d, *J* = 8.4 Hz, 1H), 3.86 (br s, 2H), 3.74 (t, *J* = 11.9 Hz, 3H), 3.57 – 3.44 (m, 2H), 3.29 – 3.21 (m, 2H), 3.20-3.10 (m, 2H), 2.00 – 1.84 (m, 2H), 1.00 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, MeOH-d_4) δ 164.31, 155.62, 143.36, 142.96, 133.85, 131.29, 129.72, 127.45, 124.99,

124.29, 124.04, 121.68, 121.53, 73.69 (2C), 64.26 (2C), 43.23, 16.42, 11.37; HPLC purity 100%, RT 4.439 min; MS (ESI) 371.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{24}ClN_4O^+$ $[M+H]^+$: 371.1639, Found: 371.1640.

4-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)-1,1-dimethylpiperazin-1-ium iodide (6)

A solution of **4a** (0.100 g, 0.306 mmol) in acetone (5 mL) was treated with methyl iodide (21 μL, 0.337 mmol) at room temperature. After overnight, the resulting mixture was concentrated, dissolved in 1 mL of MeOH, and diluted with 10 mL of distilled water. The aqueous solution was washed with EtOAc (2 X 10 mL) and concentrated. The residue was purified by preparative HPLC to give the compound **6** (0.079 g) in 55% yield: ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.57 (t, J = 7.7 Hz, 1H), 7.49 (d, J = 7.9 Hz, 1H), 7.26 (d, J = 2.3 Hz, 1H), 7.27-7.13 (m, 3H), 7.00 (d, J = 8.5 Hz, 1H), 4.03 (br s, 4H), 3.72 (br s, 4H), 3.33 (s, 6H); ^{13}C NMR (101 MHz, MeOH-d_4) δ 166.47, 156.55, 145.82, 136.58, 135.26, 132.80, 130.24, 128.46, 126.86, 125.08, 122.75, 122.70, 119.64, 61.86 (2C), 52.46 (2C), 44.44 (2C); HPLC purity 100%, RT 3.893 min; MS (ESI) 341.2 $[M+H]^+$.

4-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)piperazin-2-one (7)

A solution of compound **3** (0.200 g, 0.760 mmol) and 2-oxypiperazine (0.152 g, 1.52 mmol) in a 1:1 mixture of 1,4-dioxane and ethanol (15 mL) was stirred overnight at 99^{o}C. After concentration, the residue was diluted with EtOAc (50 mL) and the solution was washed with 20 mL of aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4 and the filtrate was concentrated. The residue was purified by flash column chromatography with 30-50% EtOAc in hexanes to afford the desired product (0.162 g) in 65% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (t, J = 7.6 Hz, 1H), 7.23 (d, J = 7.8 Hz, 1H), 7.05 (s, 1H), 7.02 (t, J = 7.6 Hz, 1H), 6.84 (d, J = 8.3 Hz, 1H), 6.81 (d, J = 7.9 Hz, 1H), 6.60 (d, J = 8.3 Hz, 1H), 6.03 (s, 1H), 4.90 (s, 1H), 4.10 (s, 2H), 3.67 (br s, 2H), 3.50 (br s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.89, 161.84, 153.15,

141.39, 140.49, 132.63, 130.04, 129.37, 127.19, 124.02, 123.52, 122.99, 120.48, 120.39, 51.47, 44.12, 41.15; HPLC purity 100%, RT 4.681 min; MS (ESI) 327.1 $[M+H]^+$; HRMS (ESI) calcd for $C_{17}H_{16}ClN_4O^+$ $[M+H]^+$: 327.1007, Found: 327.1006.

8-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)-1,3,8-triazaspiro[4.5]decane-2,4-dione (9)

A mixture of compound **3** (0.250 g, 0.951 mmol) and commercially available compound **8** (0.200 g, 1.18 mmol) in 20 mL of a mixture of 1,4-dioxane and DMF (2:1) was heated to 130°C for 24 h. The reaction mixture was cooled down to room temperature and concentrated. The residue was purified by flash column chromatography with 0-10% MeOH in CH_2Cl_2 to give the desired product **9** (0.250 g) in 66% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H), 7.37 – 7.17 (m, 2H), 7.10 – 7.03 (m, 2H), 7.01 (t, *J* = 7.5 Hz, 1H), 6.85 – 6.76 (m, 2H), 6.62 (d, *J* = 8.3 Hz, 1H), 5.06 (s, 1H), 3.90 (br s, 2H), 3.20 (br s, 2H), 2.18 – 2.05 (m, 2H), 1.74 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.84, 163.26, 156.74, 152.90, 141.62, 140.70, 132.47, 130.29, 129.42, 126.97, 123.82, 123.59, 123.43, 120.48, 62.15 (2C), 43.28, 33.19 (2C); HPLC purity 100%, RT 4.289 min; MS (ESI) 396.1 $[M+H]^+$; HRMS (ESI) calcd for $C_{20}H_{19}ClN_5O_2^+$ $[M+H]^+$: 396.1222, Found: 396.1221.

4-Amino-1-(8-chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)piperidine-4-carboxylic acid (10)

A solution of compound **9** (0.100 g, 0.253 mmol) in 1,2-dimethoxyethane (5 mL) was treated with 5 mL of 0.5 N NaOH at room temperature. The resulting mixture was heated under microwave for 10 min (max power 100 W, max temperature 150°C, max pressure 17.0 bar). After cooling down to room temperature, the reaction mixture was quenched with 10% citric acid and filtered. The filtrated was purified with preparative HPLC to afford the desired product (0.015 g) in 16% yield: ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.63 (t, *J* = 7.7 Hz, 1H), 7.54 (d, *J* = 8.0 Hz, 1H), 7.34 (d, *J* = 19.4 Hz, 1H), 7.25 (t, *J* = 7.9 Hz, 3H), 7.08 (d, *J* = 8.6 Hz, 1H), 4.36-3.59 (m, 4H), 2.70-2.40 (m, 2H), 2.40-2.01 (m, 2H); HPLC purity 100%, RT 3.585 min; MS

(ESI) 371.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{20}ClN_4O_2^+$ $[M+H]^+$: 371.1269, Found: 371.1264.

Compound **11** was prepared according to the previously published procedures.[46]

1-(4-(8-Chloro-5H-dibenzo[b,e][1,4]diazepin-11-yl)piperazin-1-yl)ethanone (12)

To the solution of compound **10** (0.400 g, 1.28 mmol) and TEA (0.27 mL, 2.0 mmol) in CH_2Cl_2 was added AcCl (0.10 mL, 1.4 mmol) at 0°C. The resulting mixture was then stirred at 0°C for 1 h. After removing the solvents, the residue was purified by flash column chromatography with 0-5% MeOH in CH_2Cl_2 to give the desired product **12** (0.390 g, 1.10 mmol) in 86% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (td, *J* = 7.7, 1.5 Hz, 1H), 7.26 – 7.22 (m, 1H), 7.04 (d, *J* = 2.4 Hz, 1H), 7.02 (td, *J* = 7.6, 1.0 Hz, 1H), 6.86 – 6.77 (m, *J* = 2.4 Hz, 2H), 6.60 (d, *J* = 8.3 Hz, 1H), 4.89 (s, 1H), 3.67 (br s, 2H), 3.59 – 3.46 (m, 4H), 3.34 (br s, 2H), 2.11 (s, *J* = 11.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.41, 162.90, 153.15, 141.57, 140.65, 132.40, 130.20, 129.25, 126.97, 123.75, 123.39, 123.32, 120.42, 120.34, 47.80, 47.26, 46.13, 41.50, 21.62; HPLC purity 100%, RT 4.919 min; MS (ESI) 355.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{20}ClN_4O^+$ $[M+H]^+$: 355.1320, Found: 355.1315.

8-Chloro-11-[4-(1,1-dideutrioethyl)piperazin-1-yl]-5H-dibenzo[b,e][1,4]diazepine (13)

To the solution of compound **12** (0.100 g, 0.282 mmol) in 15 mL of anhydrous THF was added $LiAlD_4$ (0.024 g, 0.572 mmol) at room temperature under N_2 atmosphere. The reaction mixture was heated under reflux conditions for 2 h. The reaction was quenched with 0.1 mL of CD_3OD at 0°C. The resulting mixture was treated with 0.5 mL of NH_4OH at 0°C and filtered through celite and the filtrate was concentrated. The residue was purified by flash column chromatography with 0-10% MeOH in CH_2Cl_2 to give the desired product **13** (0.081 g) in 84% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.23 (m, 3H), 7.06 (d, *J* = 2.4 Hz, 1H), 7.01 (td, *J* = 7.6, 1.0 Hz, 1H), 6.81 (dd, *J* = 8.3, 2.4 Hz, 2H), 6.60 (d, *J* = 8.3 Hz, 1H), 4.88 (s, 1H), 3.49 (br, s, 4H), 2.54 (br, s, 4H),

1.10 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.90, 152.87, 142.03, 140.59, 132.02, 130.46, 129.17, 126.91, 123.60, 123.17, 120.24, 120.16, 52.87 (2C), 51.78 (q, J = 20.0 Hz), 47.44, 11.86 (2C); HPLC purity 100%, RT 4.072 min; MS (ESI) 343.2 $[M+1]^+$; HRMS (ESI) calcd for $C19H20D_2ClN_4^+$ $[M+H]^+$: 343.1653, Found: 343.1653.

8-Chloro-11-(4-(methylsulfonyl)piperazin-1-yl)-5H-dibenzo[b,e][1,4]diazepine (14)

To a solution of compound **11** (0.102 g, 0.326 mmol) and DIPEA (87 µl, 0.907 mmol) in 10 mL of CH_2Cl_2, MsCl (27.8 µl, 0.359 mmol) was added at 0°C. After 1 h, the reaction was completed. The reaction mixture was diluted with 50 mL of CH_2Cl_2 and washed with 10 mL of aqueous $NaHCO_3$. The organic layer was dried over Na_2SO_4, and the filtrate was concentrated and the residue was purified by flash column chromatography with 50% EtOAc in hexanes to give the desired product **14** (0.118 g) in 93% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 (t, J = 7.7 Hz, 1H), 7.22 (d, J = 7.8 Hz, 1H), 7.08 – 6.96 (m, 2H), 6.87 – 6.78 (m, 2H), 6.61 (d, J = 8.3 Hz, 1H), 4.90 (s, 1H), 3.57 (br s, 4H), 3.29 (br s, 4H), 2.79 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.44, 154.11, 142.23, 141.48, 132.24, 129.80, 126.69, 125.58, 122.88, 122.64, 122.40, 120.69, 120.40, 46.33, 45.14 (2C), 33.85 (2C); HPLC purity 100%, RT 4.752 min; MS (ESI) 391.1 $[M+H]^+$; HRMS (ESI) calcd for $C_{18}H_{20}ClN_4O_2S^+$ $[M+H]^+$: 391.0990, Found: 391.0994.

Compounds **17**, **18**, **19**, **20**, and **21** were prepared according to the previously reported procedures.[48]

11-(Piperazin-1-yl)-5*H*-dibenzo[b,e][1,4]diazepine (21) ^{1}H NMR (400 MHz, MeOH-d4) δ 7.39 – 7.24 (m, 1H), 7.06 – 6.95 (m, 3H), 6.94 – 6.82 (m, 3H), 3.34 (br s, 1H), 2.96 – 2.85 (m, 2H); ^{13}C NMR (101 MHz, MeOH-d4) δ 165.39, 156.04, 145.04, 141.55, 133.31, 131.47, 127.85, 125.34, 124.88, 124.66, 123.78, 121.26, 120.71, 46.47; HPLC purity 99%, RT 2.772 min; MS (ESI) 279.2 $[M+1]^+$; HRMS (ESI) calcd for $C_{17}H_{19}N_4^+$ $[M+H]^+$: 279.1604, Found: 279.1610.

11-(4-Ethylpiperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine (22)

A solution of compound **20** (0.420 g, 1.84 mmol) and 1-ethylpiperazine (1.5 mL, 11.81 mmol) in toluene (20 mL) was heated under reflux conditions for 2 h. After cooling down to room temperature and concentration, the residue was purified by flash column chromatography with 0-10% MeOH in CH_2Cl_2 to give the desired product **22** (0.409 g) in 72% yield: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.18 (m, 2H), 7.06 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.00 – 6.90 (m, 2H), 6.84 (tt, *J* = 7.3, 3.7 Hz, 1H), 6.80 (dd, *J* = 7.4, 1.1 Hz, 1H), 6.67 (dd, *J* = 7.7, 1.4 Hz, 1H), 4.89 (s, 1H), 3.45 (br s, 4H), 2.53 (br s, 4H), 2.46 (q, *J* = 7.2 Hz, 2H), 1.10 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.52, 153.35, 141.97, 140.73, 131.82, 130.54, 127.40, 124.46, 123.86, 123.82, 122.93, 120.16, 119.48, 53.06 (2C), 52.65 (2C), 47.56, 12.18; HPLC purity 100%, RT 3.122 min; MS (ESI) 307.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{23}N_4^+$ $[M+H]^+$: 307.1917, Found: 307.1918.

4-(5*H*-Dibenzo[*b*,*e*][1,4]diazepin-11-yl)-1-ethylpiperazine *N*-oxide (23)

A solution of compound **22** (0.095 g, 0.31 mmol) in CH_2Cl_2 (10 mL) was treated with mCPBA (0.069 g) at room temperature. After 10 min, the reaction mixture was concentrated and the residue was purified with 0-10% C (5% NH_4OH in MeOH) in CH_2Cl_2 to afford the desired product **23** (0.078 g) in 78% yield: ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.39 – 7.29 (m, 2H), 7.07 – 6.97 (m, 3H), 6.96 – 6.82 (m, 3H), 3.84 (br s, 2H), 3.79 – 3.66 (m, 2H), 3.56 – 3.43 (m, 2H), 3.35 (q, *J* = 7.0 Hz, 2H), 3.18 – 3.07 (m, 2H), 1.37 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (101 MHz, MeOH-d_4) δ 163.66, 155.93, 144.50, 141.44, 133.64, 131.23, 127.98, 125.69, 125.05, 124.16, 124.13, 121.44, 120.80, 66.90 (2C), 63.79 (2C), 43.32, 7.70; HPLC purity 100%, RT 3.312 min; MS (ESI) 323.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{19}H_{23}N_4O^+$ $[M+H]^+$: 323.1866, Found: 323.1863.

Biological Assays. hM3Dq and hM3 FLIPR assays were performed according to the protocols reported previously.[11, 50, 51] Protocols for 5-HT_{2A}, 5-HT_{2C}, α_{1A} and H_1 radioligand binding assays are available at the National Institute of Mental Health – Psychoactive Drug Screening Program website (http://pdsp.med.unc.edu/UNC−CH%20Protocol%20Book.pdf).

AUTHOR INFORMATION

Corresponding Authors: *J.J.: Phone: 212-659-8699. E-mail: jian.jin@mssm.edu. B.L.R.: Phone: 919-966-7535. E-mail: bryan_roth@med.unc.edu

Author Contributions: X.C., H.C., X.P.H., X.Y., and O.S. performed the experiments. J.J. and B.L.R. designed the studies. J.J., B.L.R., and X.C. wrote the manuscript.

†These authors contributed equally to this work.

Funding: This work was supported by the NIH grant U01MH105892 (to B.L.R. and J.J.).

Notes: The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. H. Ümit Kaniskan for checking synthetic procedures and NMR spectra of novel compounds.

REFERENCES

1. Sternson, S. M., and Roth, B. L. (2014) Chemogenetic Tools to Interrogate Brain Functions, *Annu. Rev. Neurosci. 37*, 387-407.

2. Urban, D. J., and Roth, B. L. (2015) DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility, *Annu. Rev. Pharmacol. Toxicol. 55*, 399-417.

3. Penfield, W., and Jasper, H. H. (1954) Epilepsy and the Functional Anatomy of the Human Brain (First Edition), Little Brown, Boston.

4. Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., and Deisseroth, K. (2005) Millisecond-timescale, genetically targeted optical control of neural activity, *Nat. Neurosci. 8*, 1263-1268.

5. Armbruster, B. N., and Roth, B. L. (2005) Mining the receptorome, *J. Biol. Chem. 280*, 5129-5132.

6. Zhang, F., Wang, L. P., Brauner, M., Liewald, J. F., Kay, K., Watzke, N., Wood, P. G., Bamberg, E., Nagel, G., Gottschalk, A., and Deisseroth, K. (2007) Multimodal fast optical interrogation of neural circuitry, *Nature 446*, 633-639.

7. Airan, R. D., Thompson, K. R., Fenno, L. E., Bernstein, H., and Deisseroth, K. (2009) Temporally precise in vivo control of intracellular signalling, *Nature 458*, 1025-1029.

8. Berndt, A., Yizhar, O., Gunaydin, L. A., Hegemann, P., and Deisseroth, K. (2009) Bi-stable neural state switches, *Nat. Neurosci. 12*, 229-234.

9. Li, X., Gutierrez, D. V., Hanson, M. G., Han, J., Mark, M. D., Chiel, H., Hegemann, P., Landmesser, L. T., and Herlitze, S. (2005) Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin, *Proc. Natl. Acad. Sci. U. S. A. 102*, 17816-17821.

10. Zhang, F., Prigge, M., Beyriere, F., Tsunoda, S. P., Mattis, J., Yizhar, O., Hegemann, P., and Deisseroth, K. (2008) Red-shifted optogenetic excitation: a tool for fast neural control derived from Volvox carteri, *Nat. Neurosci. 11*, 631-633.

11. Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S., and Roth, B. L. (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand, *Proc. Natl. Acad. Sci. U. S. A. 104*, 5163-5168.

12. Alexander, G. M., Rogan, S. C., Abbas, A. I., Armbruster, B. N., Pei, Y., Allen, J. A., Nonneman, R. J., Hartmann, J., Moy, S. S., Nicolelis, M. A., McNamara, J. O., and Roth, B. L. (2009) Remote Control of Neuronal Activity in Transgenic Mice Expressing Evolved G Protein-Coupled Receptors, *Neuron 63*, 27-39.

13. Rogan, S. C., and Roth, B. L. (2011) Remote control of neuronal signaling, *Pharmacol. Rev. 63*, 291-315.

14. Sternson, S. M., and Roth, B. L. (2014) Chemogenetic tools to interrogate brain functions, *Annu. Rev. Neurosci.37*, 387-407.

15. Farrell, M. S., and Roth, B. L. (2012) Pharmacosynthetics: Reimagining the pharmacogenetic approach, *Brain Res*. *1511*, 6-20.

16. Jennings, J. H., and Stuber, G. D. (2014) Tools for Resolving Functional Activity and Connectivity within Intact Neural Circuits, *Curr. Biol. 24*, R41-50.

17. Lee, H. M., Giguere, P. M., and Roth, B. L. (2013) DREADDs: novel tools for drug discovery and development, *Drug Discovery Today 19*, 469-473.

18. Giguere, P. M., Kroeze, W. K., and Roth, B. L. (2014) Tuning up the right signal: chemical and genetic approaches to study GPCR functions, *Curr. Opin. Cell Biol. 27*, 51-55.

19. Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S., and Roth, B. L. (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand, *Proc. Natl. Acad. Sci. U. S. A. 104*, 5163-5168.

20. Nawaratne, V., Leach, K., Suratman, N., Loiacono, R. E., Felder, C. C., Armbruster, B. N., Roth, B. L., Sexton, P. M., and Christopoulos, A. (2008) New insights into the function of M(4) muscarinic acetylcholine receptors gained using a novel allosteric modulator and a DREADD (designer receptor exclusively activated by a designer drug), *Mol. Pharmacol. 74*, 1119-1131.

21. Ray, R. S., Corcoran, A. E., Brust, R. D., Kim, J. C., Richerson, G. B., Nattie, E., and Dymecki, S. M. (2011) Impaired Respiratory and Body Temperature Control Upon Acute Serotonergic Neuron Inhibition, *Science 333*, 637-642.

22. Garner, A. R., Rowland, D. C., Hwang, S. Y., Baumgaertel, K., Roth, B. L., Kentros, C., and Mayford, M. (2012) Generation of a synthetic memory trace, *Science 335*, 1513-1516.

23. Atasoy, D., Betley, J. N., Su, H. H., and Sternson, S. M. (2012) Deconstruction of a neural circuit for hunger, *Nature 488*, 172-177.

24. Krashes, M. J., Shah, B. P., Madara, J. C., Olson, D. P., Strochlic, D. E., Garfield, A. S., Vong, L., Pei, H., Watabe-Uchida, M., Uchida, N., Liberles, S. D., and Lowell, B. B. (2014) An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger, *Nature 507,* 238-242.

25. Kuhlman, S. J., Olivas, N. D., Tring, E., Ikrar, T., Xu, X., and Trachtenberg, J. T. (2013) A disinhibitory microcircuit initiates critical-period plasticity in the visual cortex, *Nature 501*, 543-546.

26. Carter, M. E., Soden, M. E., Zweifel, L. S., and Palmiter, R. D. (2013) Genetic identification of a neural circuit that suppresses appetite, *Nature 503*, 111-114.

27. Vrontou, S., Wong, A. M., Rau, K. K., Koerber, H. R., and Anderson, D. J. (2013) Genetic identification of C fibres that detect massage-like stroking of hairy skin in vivo, *Nature 493*, 669-673.

28. Kozorovitskiy, Y., Saunders, A., Johnson, C. A., Lowell, B. B., and Sabatini, B. L. (2012) Recurrent network activity drives striatal synaptogenesis, *Nature 485*, 646-650.

29. Ferguson, S. M., Eskenazi, D., Ishikawa, M., Wanat, M. J., Phillips, P. E., Dong, Y., Roth, B. L., and Neumaier, J. F. (2011) Transient neuronal inhibition reveals opposing roles of indirect and direct pathways in sensitization, *Nat. Neurosci. 14*, 22-24.

30. Silva, B. A., Mattucci, C., Krzywkowski, P., Murana, E., Illarionova, A., Grinevich, V., Canteras, N. S., Ragozzino, D., and Gross, C. T. (2013) Independent hypothalamic circuits for social and predator fear, *Nat. Neurosci. 16*, 1731-1733.

31. Bock, R., Shin, J. H., Kaplan, A. R., Dobi, A., Markey, E., Kramer, P. F., Gremel, C. M., Christensen, C. H., Adrover, M. F., and Alvarez, V. A. (2013) Strengthening the accumbal indirect pathway promotes resilience to compulsive cocaine use, *Nat. Neurosci. 16*, 632-638.

32. Parnaudeau, S., O'Neill, P. K., Bolkan, S. S., Ward, R. D., Abbas, A. I., Roth, B. L., Balsam, P. D., Gordon, J. A., and Kellendonk, C. (2013) Inhibition of mediodorsal thalamus disrupts thalamofrontal connectivity and cognition, *Neuron 77*, 1151-1162.

33. Brancaccio, M., Maywood, E. S., Chesham, J. E., Loudon, A. S., and Hastings, M. H. (2013) A gq-ca(2+) axis controls circuit-level encoding of circadian time in the suprachiasmatic nucleus, *Neuron 78*, 714-728.

34. Kong, D., Tong, Q., Ye, C., Koda, S., Fuller, P. M., Krashes, M. J., Vong, L., Ray, R. S., Olson, D. P., and Lowell, B. B. (2012) GABAergic RIP-Cre neurons in the arcuate nucleus selectively regulate energy expenditure, *Cell 151*, 645-657.

35. Bender, D., Holschbach, M., and Stocklin, G. (1994) 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.

36. Chang, W. H., Lin, S. K., Lane, H. Y., Wei, F. C., Hu, W. H., Lam, Y. W. F., and Jann, M. W. (1998) Reversible metabolism of clozapine and clozapine N-oxide in schizophrenic patients, *Prog. Neuro-Psychoph. 22*, 723-739.

37. Massey, C. A., Kim, G., Corcoran, A. E., Haynes, R. L., Paterson, D. S., Cummings, K. J., Dymecki, S. M., Richerson, G. B., Nattie, E. E., Kinney, H. C., and Commons, K. G. (2013) Development of brainstem 5HT(1A) receptor-binding sites in serotonin-deficient mice, *J. Neurochem. 126*, 749-757.

38. Jann, M. W., Lam, Y. W. F., and Chang, W. H. (1994) Rapid Formation of Clozapine in Guinea-Pigs and Man Following Clozapine-N-Oxide Administration, *Arch. Int. Pharmacodyn. Ther. 328*, 243-250.

39. Jann, M. W., Lam, Y. W., and Chang, W. H. (1994) Rapid formation of clozapine in guinea-pigs and man following clozapine-N-oxide administration, *Arch. Int. Pharmacodyn. Ther. 328*, 243-250.

40. Chang, W.-H., Lin, S.-K., Lane, H.-Y., Wei, F.-C., Hu, W.-H., Lam, Y. F., and Jann, M. W. (1998) Reversible metabolism of clozapine and clozapine N-oxide in schizophrenic patients, *Prog. Neuro-Psychopharmacol. and Biol. Psychiatry 22*, 723-739.

41. Loffler, S., Korber, J., Nubbemeyer, U., and Fehsel, K. (2012) Comment on "Impaired Respiratory and Body Temperature Control Upon Acute Serotonergic Neuron Inhibition", *Science 337*, 646.

42. Roth, B. L., Sheffler, D. J., and Kroeze, W. K. (2004) Magic shotguns versus magic bullets: selectively non-selective drugs for mood disorders and schizophrenia, *Nat. Rev. Drug Discovery 3*, 353-359.

43. Hunziker, F., Kuenzle, F., and Schmutz, J. (1966) *Helv. Chim. Acta 49*, 1433-1439.

44. Schmutz, J. (1975) Neuroleptic piperazinyl-dibenzo-azepines. Chemistry and structure-activity relationships, *Arzneimittelforschung 25*, 712-720.

45. Steiner, G., Franke, A., Hadicke, E., Lenke, D., Teschendorf, H. J., Hofmann, H. P., Kreiskott, H., and Worstmann, W. (1986) Tricyclic epines. Novel (E)- and (Z)-11H-dibenz[b,e]azepines as potential central nervous system agents. Variation of the basic side chain, *J. Med. Chem. 29*, 1877-1888.

46. Hunziker, F., Fischer, E., and Schmutz, J. (1967) 11-Amino-5h-Dibenzo[B,E]-1,4-Diazepine .10. Mitteilung Uber Siebengliedrige Heterocyclen, *Helv. Chim. Acta 50*, 1588-1599.

47. Wenthur, C. J., and Lindsley, C. W. (2013) Classics in Chemical Neuroscience: Clozapine, *ACS Chem. Neurosci. 4*, 1018-1025.

48. Kalhapure, R. S., Patil, B. P., Jadhav, M. N., Kawle, L. A., and Wagh, S. B. (2011) Synthesis of 11-(Piperazin-1-yl)-5H-dibenzo[b,e] [1,4]diazepine on Kilo Scale, *E-J. Chem. 8*, 1747-1749.

49. Ullmann, F., and Bielecki, J. (1901) Synthesis in the Biphenyl series. (I. Announcement), *Ber. Dtsch. Chem. Ges. 34*, 2174-2185.

50. Davies, M. A., Compton-Toth, B. A., Hufeisen, S. J., Meltzer, H. Y., and Roth, B. L. (2005) The highly efficacious actions of N-desmethylclozapine at muscarinic receptors are unique and not a common property of either typical or atypical antipsychotic drugs: is M1 agonism a pre-requisite for mimicking clozapine's actions?, *Psychopharmacology 178*, 451-460.

51. Besnard, J., Ruda, G. F., Setola, V., Abecassis, K., Rodriguiz, R. M., Huang, X. P., Norval, S., Sassano, M. F., Shin, A. I., Webster, L. A., Simeons, F. R., Stojanovski, L., Prat, A., Seidah, N. G., Constam, D. B., Bickerton, G. R., Read, K. D., Wetsel, W. C., Gilbert, I. H., Roth, B. L., and Hopkins, A. L. (2012) Automated design of ligands to polypharmacological profiles, *Nature 492*, 215-220.

52. Wiberg, K. B. (1955) The Deuterium Isotope Effect, *Chem. Rev. 55*, 713-743.

53. Elison, C., Rapoport, H., Laursen, R., and Elliott, H. W. (1961) Effect of Deuteration of N-CH_3 Group on Potency and Enzymatic N-Demethylation of Morphine, *Science 134*, 1078-1079.

54. Shao, L., Abolin, C., Hewitt, M. C., Koch, P., and Varney, M. (2006) Derivatives of tramadol for increased duration of effect, *Bioorg. Med. Chem. Lett. 16*, 691-694.

55. Korber, J., Loffler, S., Schollmeyer, D., and Nubbemeyer, U. (2013) Synthesis and Oxidant Properties of Phase 1 Benzepine N-Oxides of Common Antipsychotic Drugs, *Synthesis 45*, 2875-2887.

56. Bender, D., Holschbach, M., and Stocklin, G. (1994) Synthesis of Nca C-11 Labeled Clozapine and Its Major Metabolite Clozapine-N-Oxide and Comparison of Their Biodistribution in Mice, *Nucl. Med. Biol. 21*, 921-925.

For Table of Contents Use Only

The First Structure-activity Relationship Studies for Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

Xin Chen, Hyunah Choo, Xi-Ping Huang, Xiaobao Yang, Orrin Stone, Bryan L. Roth,* Jian Jin*