Title: Chronic systemic injection of DREADD agonists Clozapine-N-oxide and Compound 21 does not change behavior relevant to locomotion, exploration, anxiety, or affect in male mice

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Abstract

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are a chemogenetic tool commonly-used to manipulate rodent brain circuit activity. The most widely-used synthetic ligand for DREADDs is Clozapine-N-oxide (CNO). However, CNO is back-metabolized to clozapine, which itself activates numerous endogenous receptors and therefore may influence rodent behavior. To eliminate potential off-target effects of CNO, a new DREADD agonist, Compound 21 (C21), has been proposed as an alternative as it lacks active metabolites. The literature is mixed on whether acute administration of CNO or C21 changes mouse behavior. In contrast, there is no substantial literature on whether chronic administration of CNO or C21 changes mouse behavior. Here we tested whether chronic injections of these two distinct DREADD agonists change key behaviors in non-designer receptor-expressing mice relative to Vehicle (Veh)-injected control mice. Mice (CamKIIα-icre males) were injected i.p. with Veh (0.5% dimethyl sulfoxide in 0.9% saline, 5ml/kg), CNO (0.2mg/ml, 1mg/kg), or C21 (0.2mg/ml, 1mg/kg) 5 days a week for 16 weeks. All three groups had similar weight gain over the 16 week-experiment, and showed similar measures in behaviors assessed during week 3 (beam breaks in a 30-min locomotion task, time in center of open field or open arms of elevated plus maze) and week 14-16 (ambulatory distance during 240-min activity monitoring, percent marbles buried, grooming time during the sucrose splash test). These data show chronic injections of CNO or C21 do not affect key behaviors as compared to chronic injections of Veh, and may be helpful for behavioral neuroscientists when study design requires repeated injection of these DREADD agonists.

Keywords: open field; elevated plus maze; marble burying; sucrose splash test; clozapine retroconversion; chemogenetics

Abbreviations: CNO = clozapine-n-oxide, C21 = compound 21, DMSO = dimethyl sulfoxide, DREADD = designer receptor exclusively activated by designer drugs, h = hour, i.p.= intraperitoneal, mins = minutes, Veh= vehicle

Highlights

- Acute injection of CNO changes behavior of non-DREADD-expressing mice
- It's not known if chronic CNO or alternative agonist C21 also changes mouse behavior
- DREADD agonists or Veh were given chronically to non-DREADD-expressing mice
- Mice given Veh, CNO, or C21 were similar in regard to locomotion and other behaviors
- Thus CNO and C21 can be injected repeatedly without non-specific behavior effects

1. Introduction

The preclinical use of chemogenetics, such as designer receptors exclusively activated by designer drugs (DREADDs), has greatly expanded our ability to manipulate the activity of neural circuitry in the awake and behaving rodent [1–3]. DREADD technology is applied across many research areas, including models for neuropsychiatric and neurological disorders [1]. The prototypical DREADD activator Clozapine-N-oxide (CNO) was initially thought to be biologically inactive [4]. However, CNO is now presumed to cause off-target endogenous receptor activation due to its back-metabolism to biologically-active clozapine [4–7]. Clozapine is a clinical antipsychotic [8] and its acute administration to laboratory rodents causes dose- and time-dependent decreases in locomotor activity [9–12], and anxiolytic [11–14] or anxiogenic behaviors [10,15]. Thus, it is important to understand whether the behavioral changes documented in the many studies that have used CNO are due to the specific action of DREADDs or to the non-specific actions of clozapine. This is particularly true with chronic injection of CNO, since such repeated injections may be necessary in some behavioral paradigms and may exacerbate off-target clozapine effects. To address these potential issues with CNO, other designer drugs have been developed to interact with engineered human muscarinic receptors [16,17]. Compound 21 (C21), an alternative DREADD agonist to CNO, has no reported back-metabolism to clozapine, but does interact with other endogenous receptors *in vitro*, including serotonin and dopamine receptors [17]. While CNO and C21 are widely-used tools in neuroscience, neither designer drug has been examined for its potential ability to cause behavioral side effects following

chronic injection. It is important to fill this knowledge gap since many studies administer DREADD agonists repeatedly [18–20] and identifying the behavioral effect - or lack thereof - of chronic CNO or C21 would enable researchers to best adhere to the principles of the 3R's (replacement, reduction, refinement)[21].

The behavioral relevance of retroconverted clozapine from CNO remains unclear [9–15]. When mice are given acute clozapine they appear mildly sedated and less anxious than control mice [9,11–14]. In support of the retroconverted clozapine from CNO having a behavioral effect, mice given acute CNO (1mg/kg) locomote less than control mice when examined 2-3 hours (h) after injection, the time point when retroconverted clozapine concentration is predicted to be highest [22]. Further indirect support for a behavioral effect of retroconverted clozapine comes from rats given CNO having reduced acoustic startle response and blunted psychostimulant-induced locomotion [6]. However, indirect support against a behavioral effect of retroconverted clozapine also exists. For example, in a drug discrimination study, mice do not discriminate CNO from Vehicle (Veh) at doses <5mg/kg [7]. To understand this apparent discrepancy - 1mg/kg CNO decreases locomotion, but <5mg/kg CNO is not discriminated - it is important to directly assess the influence of CNO on fundamental behaviors, such as locomotion, and to examine behaviors over time to enable potential off-target action of retroconverted clozapine. Finally, as clozapine has the greatest effect when administered chronically in both humans and mice [8,23], it is important to assess any potential behavioral effect of chronic injection of CNO.

To address these questions, here we assessed the behavioral effect of giving mice chronic (5 days/week, 3-16 weeks) intraperitoneal (i.p.) injections of Veh or the DREADD agonists CNO and C21. In line with best practices recommended for DREADD studies [6,7], non-DREADD expressing mice were given Veh or a DREADD agonist at an experimentally-relevant dose (1 mg/kg)[5,17] to discern off-target effects unrelated to DREADD activation. For CNO, we hypothesized that, as with

acute injection, chronic injection would result in retroconverted clozapine and thus depress locomotion and perhaps be anxiolytic relative to chronic injection of Veh. For C21, we hypothesized chronic Veh and chronic C21 injections would result in the same performance in a battery of mouse neurobehavioral tasks relevant to locomotion, exploration, anxiety, and affect.

2. Materials and Methods

2.1 Ethics

Experiments were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia (CHOP) and performed in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were group-housed by treatment in an AAALAC-accredited, specific-pathogen free conventional vivarium at the CHOP.

2.2 *Animals*

Fifteen-week-old experimentally-naive B6.FVB-Tg (Camk2a-cre) 2Gsc/Cnrm mice (CamKIIα-icre, n=24 with 8 mice per group, MGI: 3694800) were bred in-house for this study. Each cage of 2-4 mice/cage was randomly-assigned to the Veh, CNO, or C21 group. Individual mice were identified using ear tags at four weeks of age (National Band and Tag Company, #1005-1L1). Mice were kept on a 12-h light/dark cycle (lights on at 6:15am) with *ad libitum* access to food (Lab Diets 5015 #0001328) and water. Room environments were maintained according to *Guide* standards (20-23°C and 30-70% humidity). Home cages consisted of individually ventilated polycarbonate microisolator cages (Lab Products Inc., Enviro-Gard™ III, Seaford, DE) with HEPA filtered air, corncob (Bed-o' Cobs® ¼") bedding, with provision of one nestlet (Ancare) and a red plastic hut (Bio-Serv, #K3583 Safe Harbor).

2.3 Genotyping

Only hemizygous CamKIIα-icre male mice were used, and genotype was verified by PCR of genomic DNA. Ear punch samples were heated in 0.05M NaOH at 95°C for 10 minutes. The NaOH/genomic DNA was neutralized with 0.5M TRIS HCl (pH 8.0), centrifuged and the supernatant used for PCR. PCR was performed using primers (TGG TGC CCA AGA AGA AGA GGA A and CAT TCT TTC TGA TTC TCC TCA TCA) for Cre recombinase and GoTaq® Green Master Mix (Promega, M7123).

2.4 Drug Administration

At 15 weeks of age, mice began receiving daily (Monday-Friday, between 11:00am to 1:00pm) i.p. injections of Vehicle (5ml/kg 0.5% DMSO, 0.9% Saline), CNO (National Institute of Mental Health Chemical Synthesis and Drug Supply Program; made 0.2mg/ml in Veh, given as 5ml/kg for final dose of 1mg/kg), or C21 (Hello Bio, #HB4888; made 0.2mg/ml in 0.9% Saline, given as 5ml/kg for final dose of 1mg/kg). On days when mice underwent behavior testing (**Fig. 1A**), injections were given in counterbalanced order by cage 3-h prior to testing, unless stated otherwise.

2.5 Weight Monitoring

Weight gain is a clinical side effect of chronic clozapine administration (given at 388-477mg/day/person) [8,24,25]. Therefore, mouse weight was tracked from baseline and throughout the four-month study. Mice were weighed every Monday using an OHAUS™ Valor® 2000 scale (#V22PWE1501T). Due to unexpected construction, mice were moved to a different CHOP animal facility during the 7th week of injections and remained in the new facility for the duration of the experiment. Following this move, one mouse from the C21 group was removed from study due to unexpected weight loss.

2.6 Behavior Tests

Overview of behavior testing and timeline. Behavior testing began during the 3rd week of injections **(Fig. 1A)**. Each day, testing started between 11:00am to 3:00pm, mice were habituated to the testing room for ~1-h prior to testing, and testing was performed under red light (lux 35-50). In the 3rd week of injections, mice underwent locomotor testing, open field, and elevated plus maze. Activity monitoring occurred during the 14th week of injections. Marble burying was tested during the 15th week of injections and the sucrose splash test was the final behavior measure, performed during the 16th week of injections. Initial study design included the forced-swim test, but due to the loss of three mice from the Veh group during the first round of forced-swim. No additional mice underwent the forced-swim test.

Rationale for choice and timing of behavior tests. Behavior tests were chosen that are sensitive to clozapine and affected by CNO in the absence of DREADDs, including tests relevant to locomotion, exploration, anxiety, and affect [6,9,11–13,15,22]. Locomotion and exploration were assessed in two distinct tests run in the 3rd and 14th week of injection to gauge these metrics early vs. later in the experiment. Anxiety effects were examined using the open field and elevated plus maze since, relative to typical antipsychotics, atypical antipsychotics (like clozapine) are uniquely anxiolytic, though often only at high doses in rodents [9–11,14,26] and low dose clozapine may actually be anxiogenic [10,15]. Measures relevant to affect, such as marble burying and the sucrose splash test [13,27–30], were in the final injection weeks (15th and 16th) to gauge potential antidepressant activity of retroconverted clozapine.

2.6.1 Locomotor Behavior

Locomotor testing was performed in total darkness on two consecutive days during the 3rd week of injections. For the locomotor testing, individual mice were transferred to a Bussey-Saksida Touch System Chamber for Mice (Lafayette Instruments, Cat. #: 80614) where locomotion was automatically monitored for 30 mins. ABET II software (Lafayette Instruments) recorded the total number of infrared

beam breaks during each five-minute bin throughout the session. On the first day of locomotor testing, injections were given 90 minutes (mins) prior to being placed in the chamber for 30 mins total. On the next and second day of locomotor testing, no injections were given, allowing for a 24-h wash out period prior to the 30 mins total assessment. Data are presented as total beam breaks and as beam breaks per 5-min bins across the 30-min period.

2.6.2 Open Field

The open field arena used for testing during the 3rd week of injections measured 42 x 42 x 42 cm (opaque white Plexiglas, custom design, Nationwide Plastics). For open field testing, a single mouse was placed in the arena and allowed free movement of the novel environment during the 5-min recording period. The parameters of open field (total movement distance, entries and duration in the center zone, entries and duration in the periphery zones) were scored via EthoVisionXT software (Noldus Information Technology) using nose-center-tail tracking to determine position. Via the software, the center zone was established at 14 x 14 cm and corner periphery zones 5 x 5 cm each.

2.6.3 Elevated Plus Maze

The elevated plus maze apparatus used in the 3rd week of injections consisted of two open arms (L 67 x W 6 cm) and two closed arms with walls (L 67 x W 6 x H 17 cm, opaque grey Plexiglas walls and black Plexiglas floor, Harvard Apparatus, #760075). At the start of the test, mice were placed on the far end of the open arms and allowed free movement throughout the maze for 5 min. The parameters of the elevated plus maze (total distance of movement, entries and duration in the open arms, entries and duration in the closed arms) were scored via EthoVisionXT software (Noldus Information Technology) using nose-center-tail tracking to determine position.

2.6.4 Activity Monitoring

Activity chambers (L 27 x W 27 x H 20 cm, Med Associates Inc., #ENV-510) were used during the 14th week of injections to measure activity via infrared beam breaks. A single, transparent activity chamber was positioned inside of an opaque sound-attenuating chamber. A single mouse was allowed free movement of this novel environment for 240 mins (4-h). After the first 60 mins, mice were quickly removed, injected with Veh, CNO, or C21, and immediately placed back into the chamber for the final 180 mins. The parameters of activity monitoring (total ambulatory distance, ambulatory duration, number of movement events, total number of jumps) were scored with Activity Monitoring 5 software (Med Associates Inc., #SOF-811).

2.6.5 Marble Burying

Transparent polycarbonate cages (L 25.7 x W 48.3 x H 15.2 cm, with filter-top lids; Allentown Inc. #PC10196HT) were used during the 15th week marble burying test. After distributing ~5 cm of Beta Chip Bedding (Animal Specialties and Provisions, #NOR301) to cover the bottom of the cage, 20 glass marbles (13mm diameter, cat's eye design, mixed primary colors) were placed on top of the bedding (4 x 5 rows). Mice were placed in the marble burying arena for 20 mins. After the test and mouse removal, the number of marbles buried (criteria: covered ⅔ or more in bedding) was scored by two independent observers, and the final score was an average of the two values.

2.6.6 Sucrose Splash Test

During the 16th week of injections, mice were singly-housed in new microisolator cages the day prior to the sucrose splash test. On the next day and 1-h prior to the sucrose splash test, the nestlet and red plastic hut were removed from the home cage. Freshly made 10% sucrose was then sprayed onto the back of each mouse. Mice were recorded via video camera (Panasonic, HC-V270) for the duration of the behavior. Video recordings were manually scored by a single observer unaware of the treatment group. Measures reported are total duration of grooming, latency to begin grooming, and total number of grooming events.

2.7 Statistics

Data was analyzed using Prism 8 (GraphPad Software, San Diego, CA). One-way ANOVAs were performed for locomotor behavior, open field, elevated plus maze, marble burying, sucrose splash test. For beam breaks over time in locomotor behavior testing, a repeated measure two-way ANOVA was performed. Due to variation in group size, body weight and activity monitoring were evaluated via a mixed measure analysis. For all data, differences among treatment groups were considered significant at $P<0.05$ (**Table S1**). Bonferroni post-hoc tests were performed when treatment differences met criteria for significance. Data are reported as the mean ± SEM.

3. Results

3.1. Body weight, Weeks 0-16.

Over the 16-week duration of the experiment, Veh, CNO, and C21 groups gained a similar amount of weight (**Fig. 1B**, **Table S1**).

3.2 Locomotion, Week 3.

When tested during the 3rd week of injections in the 30-min locomotion test, Veh, CNO, and C21 mice had a similar number of total beam breaks (**Fig. 2A**, **2C**) and decline in total movement as they acclimated to the chamber (**Fig. 2B**, **2D**, **Table S1**).

3.3 Anxiety-relevant tests, Week 3.

When tested during the 3rd week of injections in the open field test, Veh, CNO, and C21 mice had similar measures in total distance moved (**Fig. 3A**), frequency to enter the center zone (**Fig. 3B**), and time spent in the center zone (**Fig. 3C**, **Table S1**). When tested during the 3rd week of injections in the elevated plus maze, Veh, CNO, and C21 mice had similar measures in total distance moved (**Fig.**

3D) and open arm exploration when reported as either entries into open arms (**Fig. 3E**) or total time in open arms (**Fig. 3F**, **Table S1**).

3.4 Activity monitoring, Week 14.

When tested during the 14th week of injections in the 240-min activity monitoring test, Veh, CNO, and C21 mice showed similar activity during the 1-h prior to injection as well as during the 3-h post-injection (**Fig. 4, Table S1**).

3.5 Affect-relevant tests, Weeks 15 and 16.

When tested during the 15th week of injections for marble burying, Veh, CNO, and C21 mice buried a similar percentage of marbles (**Fig. 5A**, **Table S1**). Finally, when tested during the 16th week of injections on sucrose splash test-induced grooming, Veh, CNO, and C21 mice had similar measures on percent of time spent grooming (**Fig. 5B**), latency to the first grooming event (**Fig. 5C**), and total number of grooming events (**Fig. 5D**, **Table S1**)

4. Discussion

In line with our hypothesis, our data show chronic injections of Veh, CNO, or C21 given to non-designer receptor-expressing mice results in similar performance in a range of behavioral tests. This lack of behavioral effect suggests CNO or C21 can be given repeatedly as a DREADD agonist in studies that examine behaviors relevant to locomotion, exploration, anxiety, or affect when analyzed alongside the appropriate control group. Below we speculate why we found no behavioral influence of CNO or C21, in contrast with previously-published work which, in light of the behavioral impact they report of CNO [7,22] or C21 [16,31], cautioned against future use of these DREADD agonists.

CNO is reported to cause off-target receptor activation through retroconversion to biologically-available clozapine [7,22]. In non-DREADD-expressing mice, an acute, low dose of CNO (1mg/kg)

decreases ambulatory distance in an open field 2-3 h after injection, when retroconverted clozapine is at its peak [22]. In the present work, mice chronically injected for either 3 weeks or 14 weeks with Veh, CNO, or C21 had similar locomotion when assessed 1.5-h post-injection (3rd week locomotor test) or 0-3-h post-injection (14th week activity monitoring). While our study was not designed to assess the amount of retroconverted clozapine in the brain after DREADD agonist injection, our study clearly shows no change in locomotion or activity after 3 or 14 weeks of CNO or C21. Why did prior work see CNO-induced locomotor depression [22] while we did not? One possibility is that a single CNO injection given immediately prior to testing acts via endogenous receptors to change behavior, while the chronic injection paradigm used here decreases the sensitivity of endogenous receptors, thus preventing any effect on locomotion. Support for this possibility comes from the fact that acute clozapine is sedative in humans and mice, and that humans become tolerant to the sedative effects over time [8]. However, it is also notable that in humans clozapine is only anxiolytic and able to normalize affect after repeated dosing [8,25]. In our present study, 3-16 weeks of CNO or C21 have no effect on anxiety- or affect-related behaviors. Direct assessment of the influence of chronic CNO or C21 on the sensitivity of endogenous receptors is outside the scope of this work, but will be an important question for future research. Other possible contributors to this discrepancy (behavioral effect seen in the prior work and the lack of behavioral effect seen in the present work) include differences in mouse genotype (Drd1-cre$^{-/-}$ vs. CamKIIα-icre), sex (mixed sex vs. male), and parameters of locomotor and activity monitoring (returned to the home cage between 60-120 mins, and only saw an activity change after the return to testing vs. continuous 240 mins in the chamber). While we did not see any locomotor effect of chronic CNO, it is also notable that in male mice the back-conversion rate of CNO to clozapine is low: 7.4% [7]. Given that DREADDs can be activated *in vivo* by as low as 0.5-1mg/kg CNO [17], the even lower concentration of resulting retroconverted clozapine concentration is unlikely to activate off-target endogenous receptors [5]. Our study design also considered the ability of clozapine to influence anxiety- and affect-relevant behaviors in humans [12,14,30] as well as in rodents. As clozapine is reported to have both anxiolytic effects (increase time

spent in the open field center and open arms of the elevated plus maze [12], decrease number of marbles buried [13,30]) and anxiogenic effects (decrease overall locomotor activity) [9], it is notable that our present work shows mice given chronic injection of CNO (1mg/kg) or Veh performed similarly on all anxiety- and affect-related tasks.

CNO and C21 are relatively short-acting drugs that are rapidly metabolized by mice and unlikely to accumulate in the body even with chronic injections [5]. Both CNO and C21 cross the blood brain barrier and enter the cerebrospinal fluid of mice [5]. However, the concentration of CNO detectable in brain tissue rapidly diminishes 30-60 mins post-injection (3.5mg/kg, i.p.) [5]. Therefore, even with chronic injections, CNO is not expected to have a lasting effect due to its relatively rapid clearance. In keeping with this prediction, the present work shows no behavioral impact of chronic injections of CNO. As for C21, it has a slightly longer lasting presence in brain tissue, lasting ~1-h after i.p. injection (3.0mg/kg) [5]. This persistence in brain tissue could potentially make C21 a better designer drug for studies requiring longer duration of DREADD activation. While C21 binds to a wide range of G-protein coupled receptors (including dopamine D1 and D2 and histamine H4 receptors) at high doses (>3mg/kg), it appears to be a reliable alternative (at doses of 0.3-3mg/kg i.p.) to the known limitations of CNO, as it has no detectable conversion to either clozapine or CNO [5,17].

While in the present study CNO and C21 were given via i.p. injections, CNO is also available for oral administration via food pellets or drinking water [19]. When given orally (via food, water, or gavage), some N-oxide compounds are subject to a first-pass effect in the liver which actually results in greater back-metabolism to their parent compounds [32,33]. It is plausible that when N-oxide compounds are administered orally, off-target effects of back-metabolized clozapine will be exacerbated. While food- or water-administration of N-oxide compounds prevents the stress of handling that accompanies injections, it also has limitations. For example, in group-housed mice, it is difficult to determine the exact dose per mouse and time of last dose, and continuous exposure to CNO in food or water could

also desensitize DREADD receptors [1]. To accurately calculate a dose for oral administration, the animal may need to be singly housed, which is itself an additional stressor. To avoid these pitfalls, CNO should be given intraperitoneally as consistent with the published literature on CNO metabolism [5,7]. Alternatively, some concerns with the route of administration can be ameliorated by using a direct infusion of CNO to the brain area of interest via a cannula [1,34–36]. This affords regional specificity, allows an even lower dose of CNO to be used (3 □M, 50–100 nl), and delays retroconversion [1,36,37]. Direct infusion of CNO in the terminal area of specific neurons will be useful for circuit-specific functional studies; of course, this is more technically challenging and may not be compatible with all models.

The world is still waiting for the perfect DREADD agonist. Other candidate agonists such as JHU37152, JHU37160, and [^{18}F]JHU37107, show higher binding affinity to DREADDs than CNO, C21, or clozapine [13]. Interestingly, compound [^{18}F]JHU37107 provides a unique imaging advantage in the pairing of DREADD ligands to a fluorine radiochemical tracer allowing for *in vivo* imaging via positron emission tomography to confirm DREADD location and activation. Current techniques rely on *post mortem* confirmation via immunohistochemistry to confirm the presence of DREADD receptors. Though current DREADD activator criteria are biological inactivity and binding only to the designer receptors [1], an alternative recent proposal is to use low dose clozapine or perlapine in a narrow dose range intended to limit off-target effects [15,38]. Until any alternative DREADD ligands can be proven, it is necessary to think critically on study design to include appropriate control groups.

Conclusion

In summary, mice given either CNO or C21 (both 1mg/kg i.p) for 5 days/week for 3-16 weeks perform indistinguishably from mice given Veh in tests relevant to locomotion, exploration, anxiety, and affect. These data suggest that, as long as appropriate dose and control groups are used, both CNO and

C21 can be employed as DREADD agonists for studies that require long-term, repeated injection of these compounds without concern for non-specific behavioral or physiological effects.

Declaration of Competing Interests:

Authors have no conflicts of interest to declare.

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Author Contributions:

Sanghee Yun: Conceptualization, Funding Acquisition, Methodology, Project Administration, Resources, Writing - review and editing, Supervision; **Kyung J. Ahn**: Investigation,Writing - original draft; **Amelia J. Eisch**: Conceptualization, Funding Acquisition, Resources, Supervision, Writing - review and editing; ***Stella L. Spears***: Formal Analysis, Visualization, Writing - original draft, Writing - review and editing; ***Fionya H. Tran***: Data curation, Investigation, Visualization, Writing - original draft, Writing - review and editing

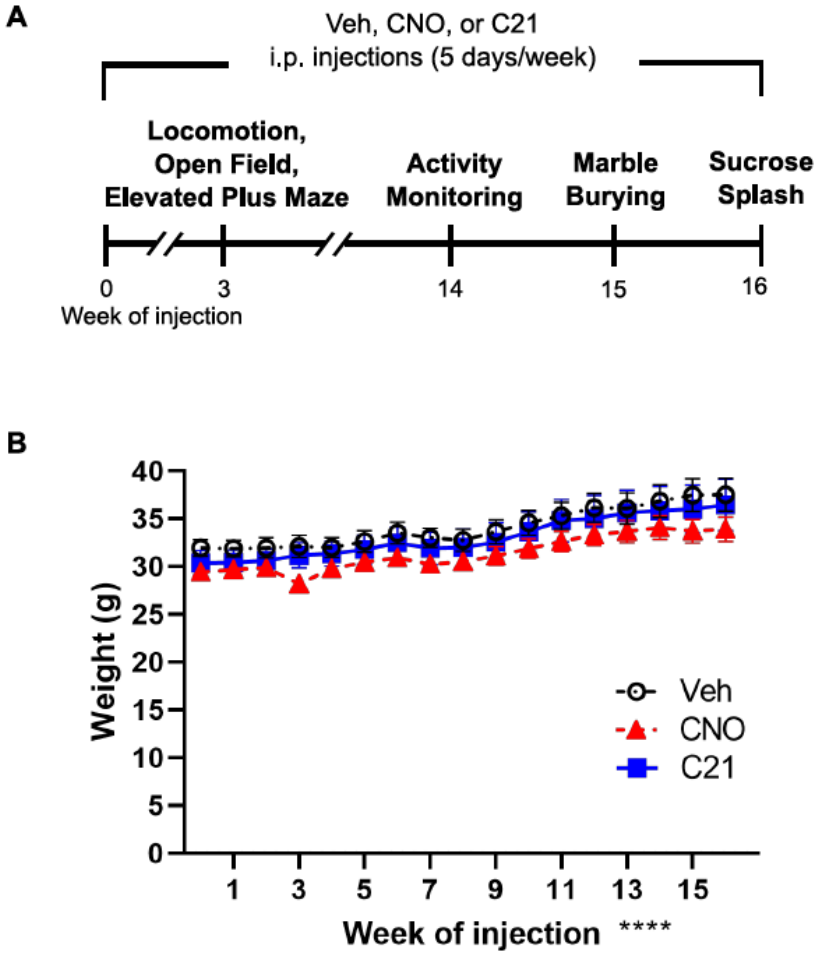

Figure 1. Timeline of behavior tests and data on body weight. (A) Injections began at 15 weeks of age. Injections (i.p.) were given between 11:00am to 1:00pm each day from Monday to Friday. Behavioral tests were begun after three weeks of injections. **(B)** Weights of the mice did not differ among treatment groups. Mean±SEM. Week 0-Week 7 n=8/group. Week 7-Week 16 Veh and CNO: n=8, C21: n=7. Mixed Measure Analysis, Main effect of Weeks $F_{(16, 326)}$=51.77, ****P<0.0001, Treatment $F_{(2,21)}$=1.036, P>0.05, Treatment x Weeks $F_{(32,326)}$=0.6287, P>0.05. i.p.=Intraperitoneal, CNO = Clozapine-N-oxide, C21 = Compound 21, g = grams Veh = Vehicle

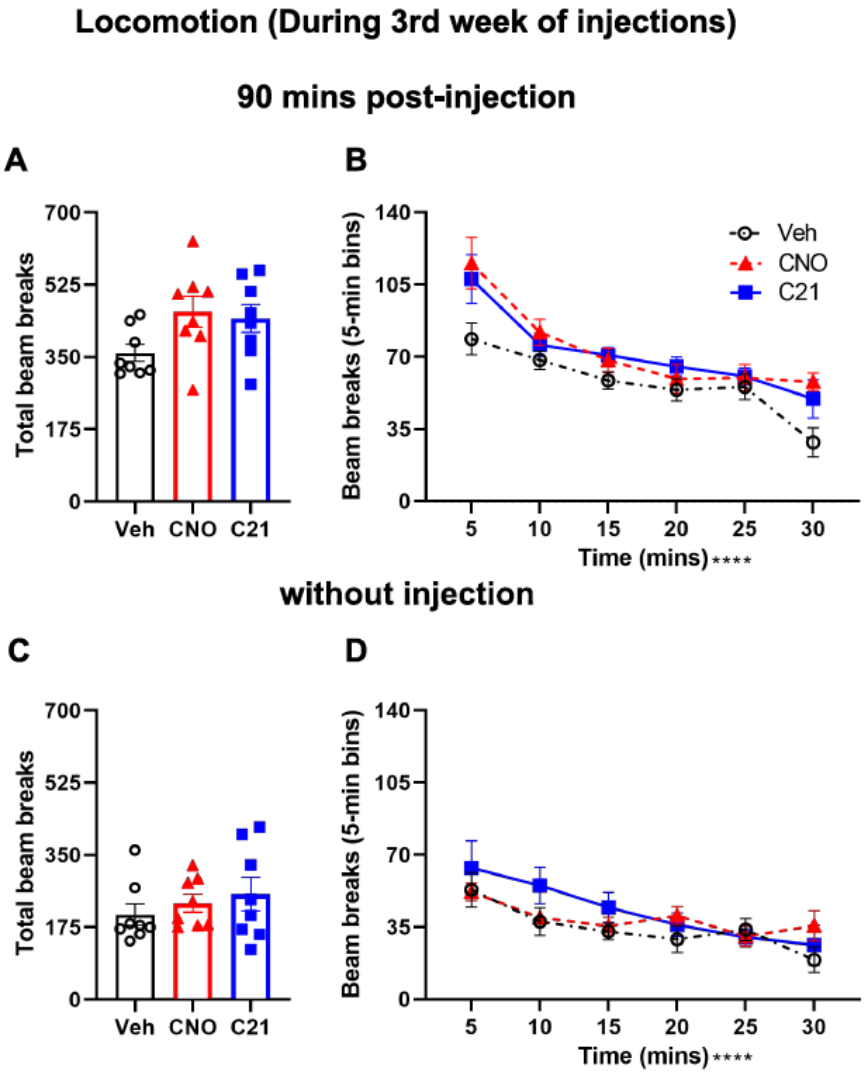

Figure 2. After three weeks of injections, locomotion was similar among mice given Veh, CNO, or C21. (A,B) On day one of locomotor behavior testing, mice were given Veh, CNO or C21 and placed in the testing apparatus 90 mins later. Veh, CNO, and C21 mice had a similar number of total beam breaks **(A)** and beam breaks in 5-minute bins **(B)**. **(C,D)** On day two of locomotor behavior testing, mice were placed into the testing chamber having received no injection for 24-h. Veh, CNO, and C21 mice had a similar number of total beam breaks **(C)** and beam breaks in 5-minute bins **(D)**. Mean±SEM. n=8/group. One-way ANOVA, Treatment $F_{(2, 21)}$=2.900, P>0.05 **(A)**, Repeated Measures [RM] Two-way ANOVA, Main effect of Time $F_{(5, 105)}$=39.09, ****p<0.0001, Main effect of Subject $F_{(21, 105)}$=5.414, **** p<0.0001, Treatment $F_{(2, 21)}$=3.248, p>0.05, Time x Treatment $F_{(10, 105)}$=1.793, P>0.05 **(B)**, One-way ANOVA, Treatment $F_{(2, 21)}$=0.7004, P>0.05 **(C)**, RM Two-way ANOVA, Main effect of

Time $F_{(5, 105)}$=13.05, ****P<0.0001, Main effect of Subject $F_{(21, 105)}$=6.060, ****P<0.0001, Treatment $F_{(2, 21)}$=0.7004, P>0.05, Time x Treatment $F_{(10,105)}$=1.380, P>0.05 **(D)**. CNO=Clozapine-N-oxide, C21 = Compound 21, Veh = Vehicle, mins = Minutes

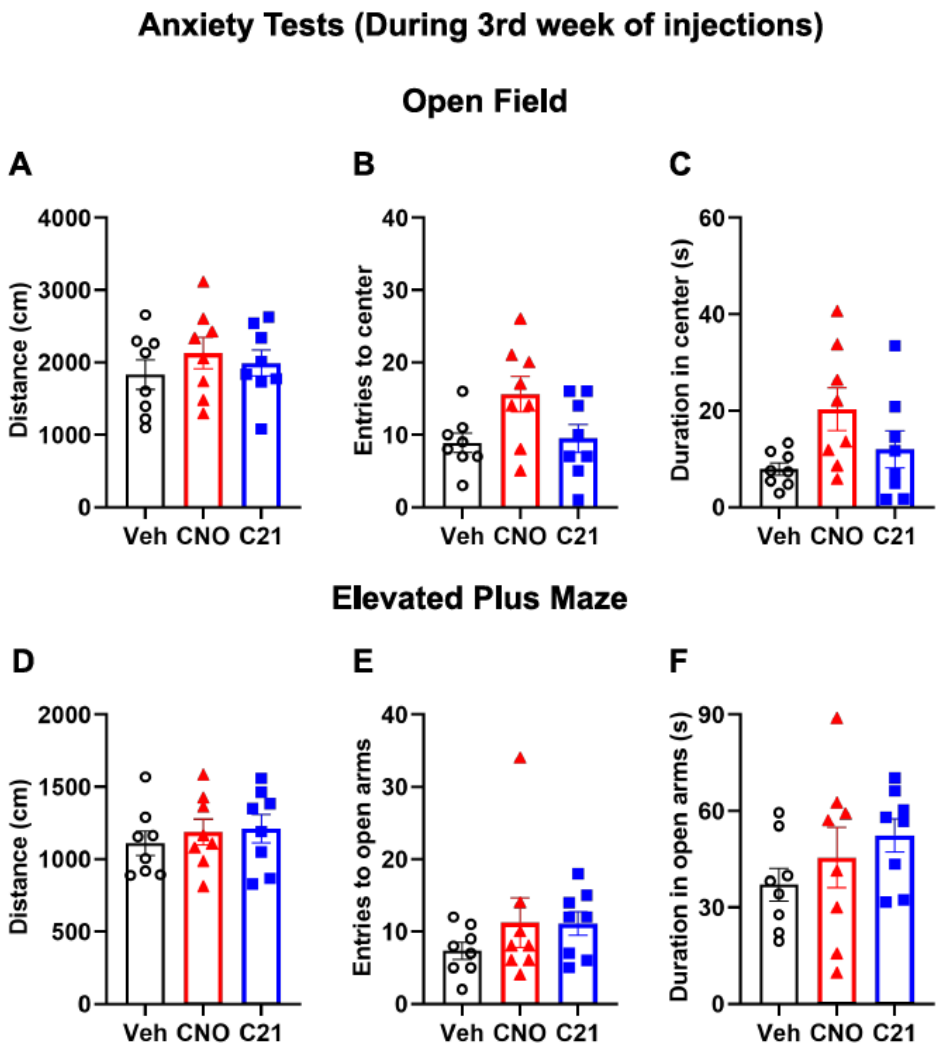

Figure 3. After three weeks of injections, performance on two tests relevant to anxiety - the open field and elevated plus maze - was similar among mice given Veh, CNO, or C21. (A-C) Mice given Veh, CNO or C21 had similar performance in the open field, as based on measures of total distance moved **(A)**, number of entries into the center zone **(B)**, and duration of time spent in the center zone **(C)**. **(D-F)** Mice given Veh, CNO, or C21 had similar performance in the elevated plus maze, as based on measures of total distance moved **(D)**, number of entries into the open arms **(E)**, and duration of time spent in the open arms **(F)**. Mean±SEM. n=8/group. One-way ANOVA, Treatment $F_{(2, 21)}$=0.5440, P>0.05 **(A)**, One-way ANOVA, Treatment $F_{(2, 21)}$=3.363, P<0.05, Bonferroni post-hoc test P>0.05 **(B)**, One-way ANOVA, Treatment $F_{(2, 21)}$=3.308, P>0.05 **(C)**, One-way ANOVA, Treatment $F_{(2, 21)}$=0.3409, P>0.05 **(D)**, One-way ANOVA, Treatment $F_{(2, 21)}$=0.9192, P>0.05 **(E)**, One-

way ANOVA, Treatment $F_{(2, 21)}$=1.235, P>0.05 **(F)**. CNO = Clozapine-N-oxide, C21 = Compound 21, Veh = Vehicle, cm = Centimeters, s = Seconds

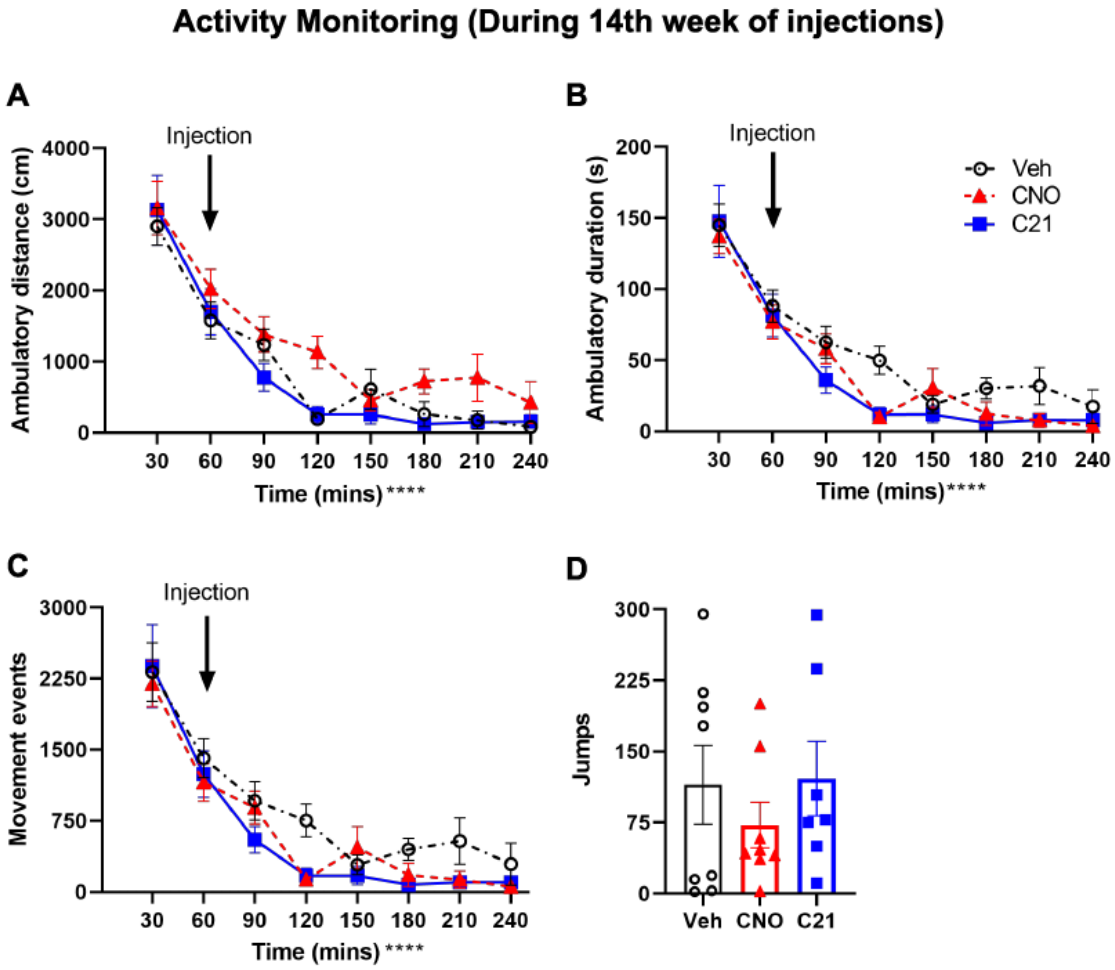

Figure 4. After fourteen weeks of injections, activity in a novel, dark environment was similar among mice given Veh, CNO, and C21. (A-D) Mice given Veh, CNO, or C21 had similar activity in a novel environment based on measures of ambulatory distance **(A)**, duration of ambulation **(B)**, total number of movement events **(C)**, and total number of jumps **(D)**. Mean±SEM. Veh and CNO: n=8, C21: n=7. Mixed Measure Analysis, Main effect of Time $F_{(7, 140)}$=68.45, ****P<0.0001, Main effect of Subject $F_{(20,140)}$=3.597, ****P<0.0001, Treatment $F_{(2, 20)}$=3.014, P>0.05, Time x Treatment $F_{(14, 140)}$=0.5073, P>0.05 **(A)**, Mixed Measure Analysis, Main effect of Time $F_{(7, 140)}$=71.28, ****P<0.0001, Main effect of Subject $F_{(20, 140)}$=3.043, ****P<0.0001, Treatment $F_{(2, 20)}$=2.298, P>0.05, Time x Treatment $F_{(14, 140)}$=1.013, P>0.05 **(B)**, Mixed Measure Analysis, Main effect of Time $F_{(7, 140)}$=61.56, ****P<0.0001, Main effect of Subject $F_{(20, 140)}$=3.429, ****P<0.0001, Treatment $F_{(2, 20)}$=1.900, P>0.05,

Time x Treatment $F_{(14, 140)}$=0.8441, P>0.05 **(C)**, One-way ANOVA, Treatment $F_{(2, 20)}$=0.5659, P>0.05

(D).CNO = Clozapine-N-oxide, C21 = Compound 21, Veh = Vehicle

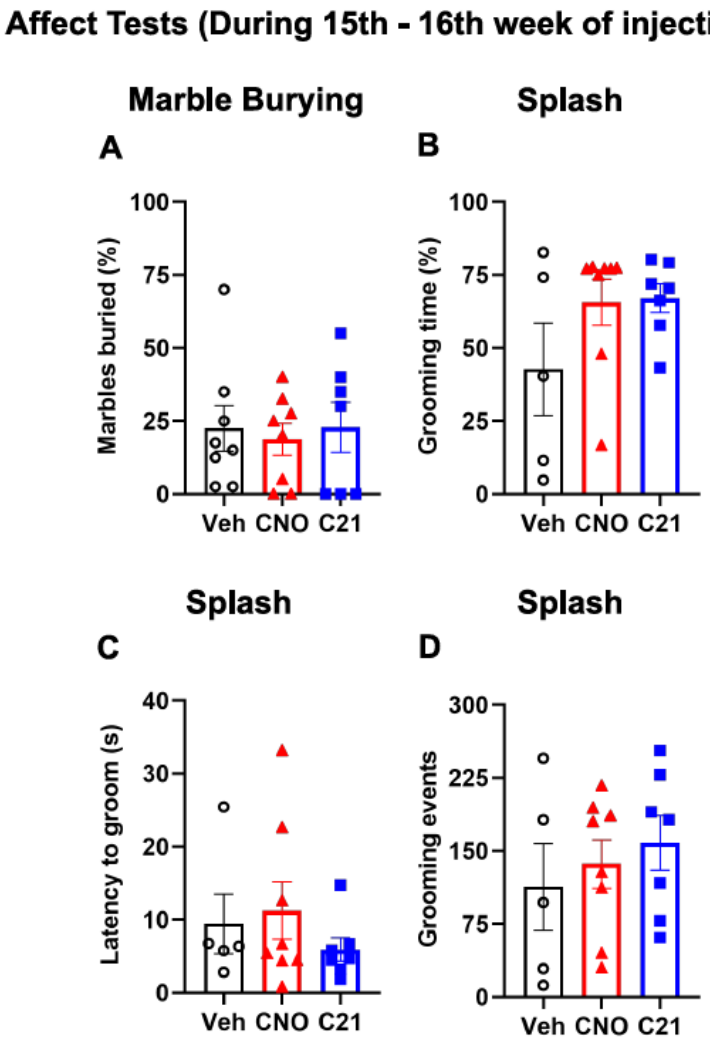

Figure 5. After fifteen and sixteen weeks of injections, respectively, behavior in two tests relevant to affect - marble burying and sucrose splash-induced grooming - was similar among mice given Veh, CNO, or C21. (A) In the marble burying test, mice given Veh, CNO, or C21 buried a similar percentage of marbles. **(B-D)** In the sucrose splash-induced grooming test, mice given Veh, CNO, or C21 performed similarly based on percent of time grooming **(B)**, the latency to begin grooming **(C)**, and the total number of grooming events **(D)**. Mean±SEM. Veh: n=8 **(A)**, n=5 **(B-D)**, CNO: n=8 **(A-D)**, C21: n=7 **(A-D)**. One-way ANOVA, Treatment $F_{(2, 20)}$=0.09887, P>0.05 **(A)**, One-way ANOVA, Treatment $F_{(2, 17)}$=1.878, P>0.05 **(B)**, One-way ANOVA, Treatment $F_{(2, 17)}$=0.7017, P>0.05 **(C)**, One-way ANOVA, Treatment $F_{(2, 17)}$=0.4806, P>0.05 **(D)**. CNO = Clozapine-N-oxide, C21 = Compound 21, Veh = Vehicle, Splash = Sucrose Splash Test

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