Research article

Does chronic systemic injection of the DREADD agonists clozapine-N-oxide or Compound 21 change behavior relevant to locomotion, exploration, anxiety, and depression in male non-DREADD-expressing mice?

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ARTICLE INFO

Keywords:
Open field
Elevated plus maze
Marble burying
Sucrose splash test
Clozapine back-metabolism
Chemogenetics

ABSTRACT

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are chemogenetic tools commonly-used to manipulate brain activity. The most widely-used synthetic DREADD ligand, clozapine-N-oxide (CNO), is back-metabolized to clozapine which can itself activate endogenous receptors. Studies in non-DREADD-expressing rodents suggest CNO or a DREADD agonist that lacks active metabolites, such as Compound 21 (C21), change rodent behavior (e.g. decrease locomotion), but chronic injection of CNO does not change locomotion. However, it is unknown if chronic CNO changes behaviors relevant to locomotion, exploration, anxiety, and depression, or if chronic C21 changes any aspect of mouse behavior. Here non-DREADD-expressing mice received i.p. Vehicle (Veh), CNO, or C21 (1 mg/kg) 5 days/week for 16 weeks and behaviors were assessed over time. Veh, CNO, and C21 mice had similar weight gain over the 16-week-experiment. During the 3rd injection week, CNO and C21 mice explored more than Veh mice in a novel context and had more open field center entries; however, groups were similar in other measures of locomotion and anxiety. During the 14th-16th injection weeks, Veh, CNO, and C21 mice had similar locomotion and anxiety-like behaviors. We interpret these data as showing chronic Veh, CNO, and C21 injections given to male non-DREADD-expressing mice largely lack behavioral effects. These data may be helpful for behavioral neuroscientists when study design requires repeated injection of these DREADD agonists.

1. Introduction

The preclinical use of chemogenetics, such as designer receptors exclusively activated by designer drugs (DREADDs), has enhanced manipulation of the brain activity in awake and behaving rodents [1–3]. The prototypical DREADD activator, Clozapine-N-oxide (CNO), was initially thought to be biologically-inactive [3]. However, CNO is now presumed to cause off-target endogenous receptor activation due to its back-metabolism to clozapine [4–7]. Clozapine is a clinical antipsychotic [8] and its acute administration to non-DREADD-expressing rodents decreases locomotion in a dose- and time-dependent manner [9–12] and can be either anxiolytic: [11–13] or anxiogenic-like [10,14] depending on dose. Thus, it is important to understand if the behavioral changes documented in studies that have used CNO are due to specific activation of DREADDs or to off-target effects via the non-DREADD-specific actions of clozapine. To avoid potential off-target

Abbreviations: CNO, clozapine-N-oxide; C21, compound 21; DREADD, designer receptor exclusively activated by designer drugs; h, hour; i.p., intraperitoneal; min, minutes; Veh, vehicle.
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https://doi.org/10.1016/j.neulet.2020.135432
Received 17 May 2020; Received in revised form 9 October 2020; Accepted 13 October 2020
Available online 17 October 2020
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effects, other designer drugs have been developed to interact with DREADDs [15,16], such as Compound 21 (C21) which has no back-metabolism to clozapine [16]. Notably, it is unknown if chronic administration of CNO or C21 to non-DREADD-expressing rodents changes behavior relevant to locomotion, exploration, anxiety, or depression. This is an important knowledge gap, as many studies administer DREADD agonists repeatedly [17,18]. Identifying the behavioral effect - or lack thereof - of chronic CNO or C21 in non-DREADD-expressing rodents would enable researchers to best adhere to the principles of the 3R’s (replacement, reduction, refinement) [19].

The behavioral relevance of back-metabolized clozapine from acute CNO remains unclear [9-14,20]. Some data support an off-target behavioral effect of acute CNO [5,7]; for example, non-DREADD-expressing mice given acute CNO (1 mg/kg) locomote less than control mice when examined 2–3 h post-injection, the predicted time point when back-metabolized clozapine concentration is highest [7]. However, other data do not support a behavioral or physiological effect of back-metabolized clozapine [6,21,22]; for example, in non-DREADD expressing animals, CNO (<5 mg/kg) does not substitute for clozapine. To understand this apparent discrepancy - 1 mg/kg acute CNO decreases locomotion, but <5 mg/kg CNO is not discriminated - it is important to directly assess the influence of CNO on fundamental behaviors (e.g. locomotion) in non-DREADD-expressing rodents, and to examine these behaviors at the interval post-CNO when back-metabolized clozapine is thought to be highest. Also, since clozapine’s effect in both humans and mice is greater after chronic vs. acute administration [8,23], it is important to assess the behavioral effect of chronic CNO injections in non-DREADD-expressing rodents.

Here we assessed the behavioral effect of giving male mice chronic injections of Veh or the DREADD agonists CNO and C21. In line with best practices [5,6], non-DREADD-expressing mice were given Veh or a DREADD agonist at an experimentally-relevant dose (1 mg/kg) [4,16] to discern off-target effects unrelated to DREADD activation. We hypothesized that relative to chronic Veh, chronic (similar to acute [6,7]) CNO would result in back-metabolized clozapine and thus decrease locomotion and exploration, while chronic C21 injections would result in similar performance in tasks relevant to locomotion, exploration, anxiety, and depression.

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 NIH’s Guide for the Care and Use of Laboratory Animals.

2.2. Animals and genotyping

Fifteen-week-old, experimentally-naive and non-DREADD-expressing male B6.FVB-Tg (CamK2a-cre) 2Gsc/Cnrm mice (CamKII\alpha-cre, congenic C57BL/6 J, n = 24, MGI: 3694800 [18,24]) were bred, housed, and weighed weekly at CHOP’s AAALAC-accredited, specific-pathogen-free conventional vivarium (Supplementary materials).

2.3. Drugs

Each cage (2-4 mice) was randomly-assigned to the Veh, CNO, or C21 group (n = 8/group). At 15-weeks-of-age (Fig. 1A), mice began receiving daily (Monday-Friday, 11:00am-1:00pm) i.p. injections of Veh (5 mL/kg 0.5 % dimethyl sulfoxide, 0.9 % Saline), CNO (NIMH Chemical Synthesis and Drug Supply Program; 0.2 mg/mL in Veh, 5 mL/kg for final 1 mg/kg dose), or C21 (Hello Bio, #HB4888; 0.2 mg/mL in 0.9 % Saline, 5 mL/kg for final 1 mg/kg dose). Injections were given between 11:00am-1:00pm and in counterbalanced-order by cage 3 h prior to most behavior tests, 90 minutes (min) prior to locomotion testing on Day1, and 60 min into activity monitoring.

2.4. Behavior

Behavior testing proceeded as shown (Fig. 1A). Since clozapine back-metabolized from CNO peaks 2–3 h after CNO injection, and behavior changes 3–4 h post-injection [6,7,18,25], most behaviors were tested 3 h post-injection.

2.5. Statistics

Main effects and interactions were considered significant at P < 0.05, and Bonferroni post-hoc tests were then performed. Results and Supplementary Table 1 provide effect sizes (omega-squared, \(\omega^2\); partial omega-squared, \(\omega_p^2\) and P-values to four significant digits. All data are presented in the Results, but conclusions are not stated when subject numbers fell below the predetermined threshold (Supplementary materials).

3. Results

3.1. Body Weight, Weeks 0–16

Veh, CNO, and C21 groups gained a similar amount of weight at a similar rate (Fig. 1B; Mixed Measure Analysis, main effects of Treatment (F\(_{2,21}\) = 1.036, P = 0.3722, \(\omega^2 = 0.00\)) and Week (F\(_{16,326}\) = 51.77, ****P < 0.0001), and Interaction of TreatmentXWeek F\(_{32,326}\) = 0.6287, P = 0.9438).

3.2. Activity (Novel Context 1), Week 3

The measure of Day 1 exploration (Fig. 2A,C) showed threshold main effects of Treatment in both total beam breaks (F\(_{2,21}\) = 2.949, P = 0.0744, \(\omega^2 = 0.14\), “large”) and 5 min-bin breaks (F\(_{2,21}\) = 3.35, P =
Activity in Novel Context 1 (during 3rd week of injections)

Fig. 2. Activity (Novel Context 1) after three weeks of injection of Veh, CNO, and C21: exploration and locomotion. (A–D) Day 1: Activity in Novel Context 1 90 min after injection of Veh, CNO, or C21 as measured by indices of exploration (A, C) and locomotion (B, D) on total beam breaks (A–B) and beam breaks/5 min (C–D). (E–H) Day 2: 24 h after most recent injection of Veh, CNO, and C21, activity as measured by indices of exploration (E, G) and locomotion (F, H) on total beam breaks (E–F) or beam breaks/5 min (G–H). Mean ± SEM. n = 8/group.

3.3. Anxiety-relevant Tests, Week 3

In the open field (Fig. 3A–C), Veh, CNO, and C21 mice moved a similar total distance (F2, 21 = 0.5440, P = 0.5884, \( \omega^2 = -0.92 \)) but no post-hoc significance. There was a threshold main effect on center zone duration (F2, 21 = 3.308, P = 0.0564, \( \omega^2 = 0.06 \)). In the elevated plus maze (Fig. 3D–F), Veh, CNO, and C21 mice had similar total distance moved (F2, 21 = 0.3409, P = 0.715, \( \omega^2 = -0.06 \)) and open arm entries (F2, 21 = 0.9192, P = 0.414, \( \omega^2 = 0.01 \)) and duration (F2, 21 = 1.235, P = 0.311, \( \omega^2 = -0.02 \)). Thus, during the 3rd injection week, CNO and C21 had mixed effects, lowering anxiety/raising exploration in the open field but not changing elevated plus maze behavior.

3.4. Activity (Novel Context 2), Week 14, and after acute CNO

Veh, CNO, or C21, ambulatory distance, duration, and movement events (Fig. 4A–C) showed a main effect of Time (distance: F7, 140 = 68.45, \( \omega^2 < 0.0001, \omega^2 = 0.68 \)); subject: F20, 140 = 3.597, \( \omega^2 < 0.0001 \); duration: F7, 140 = 61.56, \( \omega^2 < 0.0001, \omega^2 = 0.7 \); subject: F20, 140 = 3.429, \( \omega^2 < 0.0001 \); treatment: F7, 140 = 61.56, \( \omega^2 < 0.0001 \); treatment and subject: F20, 140 = 3.429, \( \omega^2 < 0.0001 \), a threshold effect of Treatment only in distance (F2, 140 = 3.014, P = 0.0718, \( \omega^2 = 0.08 \)) but not in duration (F2, 20 = 2.298, P = 0.1263, \( \omega^2 = 0.06 \)) or movement (F2, 20 = 1.900, P = 0.1756, \( \omega^2 = 0.05 \)) and no TimeXTreatment interaction (distance: F14, 140 = 0.5073, P = 0.507, \( \omega^2 = 0.00 \)); duration: F14, 140 = 0.8441, P = 0.62, \( \omega^2 = 0.00 \); movement: F14, 140 = 0.8441, P = 0.62, \( \omega^2 = -0.01 \). Total jumps did not show a Treatment effect (F2, 20 = 0.5659, P = 0.576, \( \omega^2 = -0.04 \)). Thus, during the 14th injection week, Veh, CNO, and C21 result in similar activity. To complement these chronic data, we assessed if acute 1 mg/kg CNO changed activity in non-DREADD-expressing mice as reported by some [7,26] but not others [22]. Naive mice given a single injection of Veh or
0.3 or 1 mg/kg CNO appeared to have similar activity over the next 16 h (Supplementary Fig. 1), but subject numbers were too low to state a conclusion.

3.5. Tests Relevant to Anxiety and Depression, Weeks 15–16

In marble burying (Supplementary Fig. 2A), Veh, CNO, and C21 mice buried a similar percentage of marbles (F$_2$,20 = 0.09887, P = 0.906, $\eta^2_p$ = -0.09), indicating similar behavior relevant to anxiety and/or repetitive action. In the sucrose splash test (Supplementary Fig. 2B-C), data suggest Veh, CNO, and C21 mice had similar measures on latency to grooming and grooming events; however, the Veh subject number was too low to state a conclusion.

4. Discussion

Here we examined the behavioral effects of chronic CNO or C21 injections in non-DREADD-expressing mice. We administered 1 mg/kg based on the ability of this dose to change behavior in DREADD-expressing rodents when injected acutely [16,27] or chronically [18,28]. We assayed most behaviors 3 h post-injection, when CNO-to-clozapine back-metabolism peaks [4,7], and tested locomotor activity 0–3 h post-injection. For the non-DREADD-expressing mouse line, we selected CamKIIa-icre mice given the use of this forebrain glutamatergic neuron cre-expressing line in DREADD studies by us and others [1,18,24,29]. Data from the 3rd injection week show these non-DREADD-expressing mice given chronic CNO or C21 explored a novel context more (but had similar locomotion) relative to mice given Veh, and showed mixed responses in anxiety-relevant tests. In all other
tests during the 16-week experiment, Veh, CNO, and C21 mice had similar behavior; the effect of chronic CNO or C21 on depressive-like behavior (splash test) and acute CNO on locomotion was inconclusive due to loss of subjects. Overall, our data suggest that when appropriate control groups are used, non-DREADD-expressing mice can be injected repeatedly with either DREADD agonist without causing gross behavioral effects.

Our largely-negative data are consistent with reports that chronic 1 mg/kg CNO given to non-DREADD-expressing mice does not change behavior/physiology [28,30], but our data importantly show no effect on locomotion and a mixed effect on anxiety-relevant behavior. While the literature is unanimous that chronic 1 mg/kg CNO does not grossly change mouse behavior in non-DREADD-expressing mice, the literature is mixed on the effects of acute CNO given to non-DREADD-expressing rodents. Several studies report acute CNO or C21 does not change behavior in non-DREADD-expressing rodents (1 mg/kg [our data and 22] or 3 mg/kg [4,21] CNO, or 3.5 mg/kg C21 [4]). Of studies that do report a change in behavior in non-DREADD-expressing rodents after acute CNO, two saw behavioral effects in rats with acute 1 mg/kg CNO [7,26] and two in mice and rats with higher CNO doses than the present work [4,6]. Further work is needed to understand what factors (mouse strain, behavior parameters, etc.) underlie these divergent results with acute CNO.

CNO and C21 are both rapidly-metabolized in mice [4], but brain C21 levels last slightly longer than brain CNO levels [4]. Thus, C21 may be a better designer drug for studies requiring longer duration of DREADD activation. While C21 binds to a range of G-protein coupled receptors (including dopamine D1 and D2 and histamine H4 receptors) at doses >3 mg/kg, below this dose it appears to be a reliable activator since unlike CNO it has no detectable conversion to either clozapine or CNO [4,16].

5. Conclusion

While non-DREADD-expressing mice given CNO or C21 (1 mg/kg i.p) for 5 days/week for 3–16 weeks perform indistinguishably from Veh mice in tests relevant to locomotion, mice given CNO or C21 have increased exploration during the 3rd injection week; results on anxiety-relevant measures were mixed. These data suggest with appropriate dose and control groups, CNO and C21 can be used as DREADD agonists for studies that require long-term, repeated injection of these compounds without concern for gross non-specific behavioral or physiological effects.

CRediT authorship contribution statement

Fionya H. Tran: Data curation, Investigation, Visualization, Writing - original draft, Writing - review & editing. Stella L. Spears: Formal analysis, Visualization, Writing - original draft, Writing - review & editing. Kyung J. Ahn: Investigation, Writing - original draft. Amelia J. Eisch: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing. Sanghee Yun: Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.
Appendix A. Supplementary data

Supplementary data related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.neulet.2020.135432.

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