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Validation of DREADD agonists and administration route in a murine model of sleep enhancement

Loris L. Ferrari [a], Oghomwen E. Ogbeide-Latario [a,b], Heinrich S. Gompf [a,c], Christelle Anaclet [a,c,1,*]

[a] Department of Neurobiology, University of Massachusetts Chan Medical School, USA
[b] Morningside Graduate School of Biomedical Sciences, University of Massachusetts Chan Medical School, USA
[c] Department of Neurological Surgery, University of California Davis School of Medicine, USA

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ABSTRACT

Background: Chemogenetics is a powerful tool to study the role of specific neuronal populations in physiology and diseases. Of particular interest, in mice, acute and specific activation of parafacial zone (PZ) GABAergic neurons expressing the Designer Receptors Activated by Designer Drugs (DREADD) hM3Dq ($PZ^{GABA\text{-}hM3Dq}$) enhances slow-wave-sleep (SWS), and this effect lasts for up to 6 h, allowing prolonged and detailed study of SWS. However, the most widely used DREADDs ligand, clozapine N-oxide (CNO), is metabolized into clozapine which has the potential of inducing non-specific effects. In addition, CNO is usually injected intraperitoneally (IP) in mice, limiting the number and frequency of repeated administration.

New methods: The present study is designed to validate the use of alternative DREADDs ligands—deschloroclozapine (DCZ) and compound 21 (C21)—and a new administration route, the voluntary oral administration.

Results: We show that IP injections of DCZ and C21 dose-dependently enhance SWS in $PZ^{GABA\text{-}hM3Dq}$ mice, similar to CNO. We also show that oral administration of CNO, DCZ and C21 induces the same sleep phenotype as compared with IP injection.

Comparison with existing methods and conclusion: Therefore, DCZ and C21 are powerful alternatives to the use of CNO. Moreover, the voluntary oral administration is suitable for repeated dosing of DREADDs ligands.

1. Introduction

During the past decade chemogenetic tools have permitted extraordinary progress in neuroscience knowledge. Chemogenetics allows specific activation or inhibition of neuronal populations over a long time frame (hours) to study the effect of these populations on behavior, including sleep-wake cycle control (Anaclet et al., 2014; Roth, 2016; Smith et al., 2021; Song et al., 2022). A category of the chemogenetic receptors, the Designer Receptors Activated by Designer Drugs (DREADDs), are modified G protein coupled receptors. They are expressed by the selected neuronal population using various strategies, including viral vector transfection and cre/lox system (Fuller et al., 2015). The DREADDs bind pharmacologically inert drugs such as clozapine N-oxide (CNO). However, metabolites of CNO, such as clozapine, are not pharmacologically inert and may induce non-specific effects that can confound the results (Jendryka et al., 2019). Most studies have used appropriate controls, such as CNO injection in wild-type (WT) mice or mice expressing DREADDs treated with vehicle (Anaclet et al., 2014) and the lack of non-specific action of the DREADD agonist/receptor support the trustworthiness of these results. However, other studies have used dramatically higher doses of CNO and lack appropriate controls. As a result, the DREADD tool has become controversial (Baerentzen et al., 2019; MacLaren et al., 2016; Mahler and Aston-Jones, 2018) and more specific DREADD agonists have been developed (Nagai et al., 2020; Thompson et al., 2018).

In a series of studies, we showed that DREADD activation of parafacial zone (PZ) GABAergic (PZ^{GABA}) neurons powerfully enhances slow-wave-sleep (SWS), the deepest stage of sleep (Anaclet et al., 2014, 2018b). We showed that CNO injection in mice expressing the DREADD hM3Dq in PZ^{GABA} neurons ($PZ^{GABA\text{-}hM3Dq}$) induces SWS with a short

* Corresponding author at: Department of Neurological Surgery, University of California Davis School of Medicine, USA.
E-mail address: canaclet@ucdavis.edu (C. Anaclet).
[1] Senior Author

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latency, induces long lasting SWS bouts and enhances cortical electroencephalogram (EEG) slow frequencies, called slow wave activity (SWA), a marker of SWS quality. Therefore, these studies provided the first mouse model of SWS-enhancement, permitting induction of long lasting episodes of deep SWS. This mouse model is of particular interest given that deep sleep, believed to be high sleep quality, is hypothesized to actively promote other physiological functions and protect from diseases, as for instance neurodegenerative diseases such as Alzheimer's. Yet this inference is based on studies using sleep deprivation or long-term sleep restriction. Therefore, DREADD activation of PZ^{GABA} will allow researchers to study the role of SWS in physiology and diseases using, for the first time, gain of sleep experiments, in mice with multiple genetic backgrounds.

Our studies used the DREADD agonist CNO and confirmed the absence of non-specific action in WT mice at the dose used (0.3 mg/kg) following acute administration (Anaclet et al., 2014; Erickson et al., 2019). However, the study of SWS in physiology and diseases requires the use of chronic SWS enhancement which might result in accumulation of metabolites and non-specific effects. Therefore, in the present study we investigate the SWS enhancement potential of other DREADD ligands believed to produce inert metabolites, such as deschloroclozapine (DCZ; 11-(4-methyl-1-piperazinyl)− 5*H*-dibenzo(*b*,*e*)(1,4) diazepine) (Nagai et al., 2020) and compound 21 [C21; 11-(1-piperazinyl)− 5 H-dibenzo[*b*,*e*][1,4]diazepine)] (Thompson et al., 2018).

In our previous studies, we administered CNO via intraperitoneal (IP) injections (Anaclet et al., 2018a, 2018b, 2014, 2015; Erickson et al., 2019; Todd et al., 2020; Venner et al., 2016; Venner et al., 2019). Though this technique is easy and safe for the mice acutely, repeated IP injection is not recommended. Therefore, in the present study, we investigate a less stressful noninvasive administration route, the voluntary oral administration (Mahoney et al., 2019). This technique consists of placing a small disc of jelly containing the drug, pre-prepared in a mold, in the mouse cage and letting the mouse eat the jelly. Therefore, no mouse handling is required and dosing can be repeated often.

2. Materials and methods

2.1. Animals

A total of 28 pathogen-free mice, on the C57BL/6J genetic background, were used in this study. The mice resulted from the cross of the following mouse lines: *Vgat*-IRES-Cre (*Slc32a1tm2(cre)Lowl*/J, Jackson stock #028862), *EGFP-L10A* (Jackson stock #024750) and B6. CgTg (APPswe,PSEN1dE9)85Dbo/Mmjax (Jackson stock #5864). The slow-wave-sleep (SWS) enhanced mouse groups included 5 males and 12 females, 7 Vgat-Cre+ /GFP/APP,PS1 + mice and 10 Vgat-Cre+ /GFP/APP,PS1- mice, age 10–21 months old (14.1 ± 0.9 months). The control group included littermate mice that do not express Cre (Cre-), 8 males and 3 females, 7 APP,PS1 + mice and 4 APP,PS1- mice, and 12–22 months old (17.1 ± 1.1 months). Mice were bred at our animal facility and underwent genotyping both before and after experiments, using the Jackson Laboratory PCR protocols. Care of these animals met the National Institutes of Health standards, as set forth in the *Guide for the Care and Use of Laboratory Animals* and all protocols were approved by the University of Massachusetts Chan Medical School Institutional Animal Care and Use Committees.

2.2. Surgery

Naïve mice were subjected to two independent surgeries separated by at least two weeks. Mice were anesthetized with ketamine/xylazine [100 and 10 mg/kg, respectively, intraperitoneally (IP)] and then placed in a stereotaxic apparatus. During the first surgery mice received bilateral injections of an adeno-associated viral (AAV; serotype 2) vector expressing the hM3Dq receptor and mCherry (reporter gene) in a cre-dependent configuration (hSyn-DIO-hM3Dq-mCherry-AAV, UMASS Vector Core, titer: 3.0E+12 Viral particles/ml) into the PZ to specifically express hM3Dq receptors in PZ^{GABA} neurons (Fig. 1A-C), as previously described (Anaclet et al., 2014). Cre- control mice did not express GFP or hM3Dq-mCherry (Fig. 1D-F). Coordinates from Bregma were − 5.6 mm Antero-posterior, ± 1.0 mm Lateral, − 4.2 mm Dorso-ventral, as per the mouse atlas of Paxinos and Franklin (Paxinos and Franklin, 2001). The AAV (200 nl) was injected into the PZ of mice using a 1 µl Hamilton syringe (Hamilton Co., Reno, NV) at a rate of 30 nl/min driven by an UMP2 microinfusion pump with a SMARTouch Controller (World Precision Instruments, Inc., Sarasota, Florida). During the second surgery, mice were implanted with four EEG screw electrodes (2 frontal [1 mm frontal, 1 mm lateral from bregma] and 2 parietal [mid-distance between bregma and lambda and 1 mm lateral from the mid-line] electrodes; Pinnacle Technology Inc., Catalog #8403) and two flexible electromyogram (EMG) wire electrodes (in the neck muscles; Plastics One, catalog #E363/76/SPC), previously soldered to a 6-pin connector (Heilind Electronics, catalog #853–43–006–10–001000) and the assembly was secured to the skull with dental cement. After completing the surgery, mice were kept in a warm environment until resuming normal activity as previously described (Anaclet et al., 2014).

2.3. Sleep-wake recording

Following a minimum of 10 days for post-surgical recovery, the mice were subjected to sleep-wake recordings (Ogbeide-Latario et al., 2022) and behavioral testing (unpublished) before entering the protocol of the present study. This allowed us to reduce the number of animals used in our studies. The mice were housed individually in transparent barrels in an insulated sound-proofed recording chamber maintained at an ambient temperature of 22 ± 1 °C and on a 12 h light/dark cycle (lights-on at 07:00, Zeitgeber time: ZT0) with food and water available ad libitum. Mice were connected to flexible recording cables and habituated to the recording conditions for 5 days before starting polygraphic recordings. One cortical EEG (bipolar, fronto-parietal, ipsilateral) and the EMG signals were amplified (A-M System 3500, United States) and digitalized with a resolution of 256 Hz using Vital Recorder (Kissei, Japan). Mice were recorded for a 24 h baseline period followed by drug administration.

2.4. Drug administration

During the habituation period, mice were trained to the voluntary oral administration (jelly) (Mahoney et al., 2019). Vehicle jelly (control-Jelly) was prepared as follows: gelatin (7 g, original unflavored gelatin, Knox) and Splenda (10 g) were mixed in 49 ml ddH2O + 1 ml natural food flavor (strawberry flavor, Frontier Co-op, USA) and stirred at 50 °C until the mix was clear. The stock solution was then placed in 4 °C for 1–2 months of storage. To prepare the jellies, the stock solution was stirred at 50 °C until completely melted, permitting dilution of the drugs. Then the appropriate volume of stock solution (control-Jelly) or of stock solution containing CNO (CNO-Jelly), DCZ (DCZ-Jelly) or C21 (C21-Jelly), was pipetted into the cap of an Eppendorf tube and placed at 4 °C until it forms a jelly again. Before giving it to the mice, the jelly was removed from the cap of the Eppendorf tube and placed in the cap of a 15 ml falcon tube. In order to train the mice to eat the jelly, mice were food deprived overnight (12–14 hr) before the first control-Jelly presentation. The following morning, mice were given control-Jelly, placed in a 15 ml falcon tube cap and on the floor of the cage. Mice were left without food until they ate the jelly (5–10 min). Once the mice had eaten the jelly, they were given regular chow ad libitum. Mice were then given jelly daily without food deprivation. After a 5-day habituation period, the mice were eating the jelly in < 1 min following presentation.

All the mice received the following: 1) intraperitoneal (IP) injections of saline (control-IP), CNO (CNO-IP; NIMH Chemical Synthesis and Drug

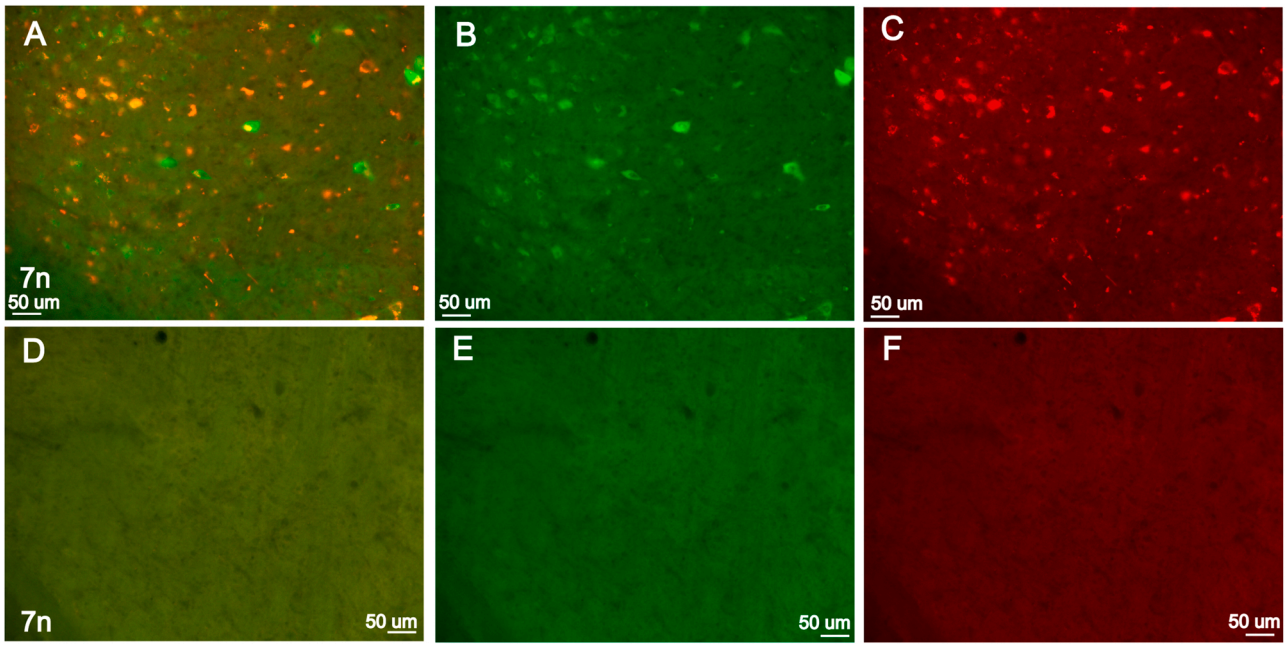

Fig. 1. Photomicrographs at the parafacial zone (PZ) level. (**A**) Co-localization of GFP (green, B) and mCherry (red, C) in an APP/PS1/Vgat-GFP mouse injected into PZ with AAV-hM3Dq-mCherry. (D) A Cre- control mouse did not express GFP (E) or hM3Dq-mCherry (F). Scale bar: 50 μm. 7 n, seventh nerve.

Supply Program; 0.1, 0.3 and 1 mg/kg, 0.01, 0.03 and 0.1 mg/ml in saline respectively), Deschloroclozapine (DCZ) Dihydrochloride (DCZ-IP; NIMH Chemical Synthesis and Drug Supply Program; 0.1, 0.5, 1, and 5 mg/kg, 0.01, 0.05, 0.1, and 0.5 mg/ml in saline respectively), and C21 (C21-IP; NIMH Chemical Synthesis and Drug Supply Program; 1, 3 and 10 mg/kg, 0.1, 0.3 and 1 mg/ml in saline respectively), injection volume: 0.1 ml / 10 g of mouse; and 2) voluntary oral administration of control-Jelly, CNO-Jelly (0.3 mg/kg), DCZ-Jelly (0.5 mg/kg) and C21-Jelly (3 mg/kg), administration volume: 0.05 ml / 10 g of mouse. Injections and administrations were performed at 19:00 (ZT12, beginning of the dark period, at a time of high wake-drive), in a randomized cross-over design, with each injection/administration separated by a 2 – 3 day washout period. To allow for comparative analysis between different agonists and routes of administration, the mice in this study received all injections/administrations except DCZ 5 mg/kg that was injected in only 11 mice. For analysis, mice were removed from a drug/administration group if any one of the conditions was missing due to a technical problem.

2.5. Sleep scoring and analysis

Using SleepSign for Animal (Kissei, Japan) assisted by spectral analysis using fast Fourier transform (FFT), polygraphic records were visually scored in 10 s epochs for wakefulness, SWS, and rapid eye movement (REM) sleep. Wakefulness is characterized by low amplitude fast frequency EEG associated with EMG activity. SWS is characterized by high amplitude, low frequency EEG, and low EMG activity. REM sleep is characterized by an EEG dominated by hippocampal theta rhythm and no EMG activity (Anaclet et al., 2015). The percentage of time spent in wakefulness, SWS, and REM sleep were summarized for each group and each condition. The SWS and REM sleep latencies are defined as the time between the end of the IP injection, or when the mouse had eaten the entire jelly, and the onset of the first SWS episode, lasting > 20 s, and the onset of the first REM sleep episode, lasting > 10 s, respectively.

Recordings were scored again in 4 s epochs to allow for performance of the cortical EEG power spectral analysis. Based on visual and spectral analysis, epochs containing artifacts occurring during active wakefulness (with large movements) or those containing two vigilance states were visually identified and omitted from the spectral analysis. Re-scoring with a shorter epoch length allows us to minimize the number of the recording epochs omitted from the analysis. Recordings containing artifacts during more than 20% of the recorded time were removed from the spectral analysis. Cortical EEG power spectra were computed for consecutive 4 s epochs within the frequency range of 0.5 – 60 Hz using a fast Fourier transform (FFT) routine. The data were collapsed into 0.5 Hz bins. The data were standardized by expressing each frequency bin as a percentage relative to the total power of the same epochs [for example, (bin power * 100)/0.5–60 Hz total power]. To analyze the EEG frequency bands, standardized power bins were summed in delta (δ, 0.5 – 4.5 Hz), theta (θ, 4.5 – 10 Hz), sigma (α, 10 – 15 Hz), beta (β, 15 – 30 Hz) and gamma (γ, 30 – 60 Hz) bands.

2.6. Statistical analysis

Statistical analysis was performed using Prism v8 (GraphPad Software, San Diego, CA, United States). Following confirmation that the data met the assumptions of the ANOVA model, 1) two-way ANOVA followed by a post hoc Bonferroni test was used to compare the effect of the dose, administration route, agonist, or genotype on sleep-wake amount and power bands; 2) one-way ANOVA followed by a post hoc Bonferroni test was used to compare the effect of the dose or administration route on SWS and REM sleep latencies; and 3) t-test was used to compare the effect of DCZ 5 mg/kg with respective control-IP mice.

3. Results

In order to validate the use of alternative DREADD agonists in our mouse model of SWS enhancement, we first performed a dose response study of DCZ and C21 and compared with different doses of CNO. In the following study, both SWS-enhancement and control mouse groups include APP,PS1 + and APP,PS1- mice. Before including these two genotypes in the same experimental groups, the effect of each agonist and each dose was compared between genotypes. According to our previous study comparing the SWS enhancement effect of CNO between APP/PS1 and littermate control mice (Ogbeide-Latario et al., 2022), none of the doses or agonists significantly affect sleep-wake parameters in a genotype as compared with the other genotype.

3.1. Dose dependent effect of CNO, DCZ and C21 on sleep-wake amount and sleep latencies

We had previously shown that at the dose of 0.3 mg/kg, CNO enhances SWS in mice expressing the DREADD hM3Dq in PZ^{GABA} neurons ($PZ^{GABA\text{-}hM3Dq}$), while no vigilance state alterations were found at that dose in control mice, not expressing hM3Dq (Anaclet et al., 2014). In the present study, we tested a lower (0.1 mg/kg) and a higher (1 mg/kg) dose. Results show that IP injection of CNO dose dependently increases the percentage of SWS in $PZ^{GABA\text{-}hM3Dq}$ mice [interaction: time x dose, $F(69, 897) = 4.532$, $p < 0.0001$, two-way ANOVA; Fig. 2A2]. As compared with control injection, SWS percentage is significantly increased during the first hour post injection at 0.1 mg/kg and during the three hours post injection at both 0.3 and 1 mg/kg (Fig. 2A2). Analysis of the 3-hr post injection period shows that the percentage of SWS is dose dependently increased (Fig. 2A2 insert). Meanwhile, both wakefulness and REM sleep percentages are dose dependently inhibited (Fig. 2A1, A3). However, though the percentage of wakefulness and SWS move in opposite directions in the first 3 h, REM sleep remains significantly inhibited for up to 9 hrs and 12 hrs at the dose of 0.3 and 1 mg/kg, respectively (Fig. 2A3 insert). SWS latency is dose dependently decreased [$F(1.369, 17.80) = 17.26$, $p = 0.0002$, one-way ANOVA; Fig. 2A4], while REM sleep latency is dose dependently increased [$F(1.981, 25.76) = 8.645$, $p = 0.0014$, one-way ANOVA; Fig. 2A5]. At the three tested doses, CNO does not affect sleep-wake amount and sleep latencies in Cre- control mice (Fig. 3A1–5).

Doses of 0.1, 0.5 and 1 mg/kg, DCZ each induced a similar increase in SWS percentage and decreases in both wakefulness and REM sleep percentages. During the first hour post-DCZ injection, the mice spent nearly 100% of their time in SWS (Fig. 2B2), indicating that DCZ action is faster (SWS latency < 3 min) and more powerful (nearly 100% of SWS during the first hour post DCZ injection) than CNO action (SWS latency ~8–14 min; 90% of SWS during the 2-hr post CNO injection at the highest dose). This is consistent with a previous study showing faster brain penetration for DCZ than CNO (Nagai et al., 2020). However, while CNO is able to sustain SWS for multiple hours, the amount of SWS rapidly decreases during the second hour post DCZ injection and is similar to control injection during the third hour post DCZ injection. These results suggest a ceiling effect of the doses. To test this hypothesis, we tested a higher DCZ dose (5 mg/kg) and showed that SWS amount was significantly increased during the three hours post DCZ injection (Fig. 2B2), as compared with control injection, confirming that DCZ action can last longer than 1–2 hr, similar to CNO. REM sleep is inhibited during 3-hr following DCZ (0.1 mg/kg) and 6-hr following DCZ (0.5 & 1 mg/kg; Fig. 2B3 insert). At the dose of 5 mg/kg, DCZ appears to inhibit REM sleep during 12-hr post injection, however, the two-way ANOVA did not reach significance. Same dose injections in Cre- control mice confirm that DCZ does not affect sleep-wake amount and sleep latencies in mice not expressing hM3Dq receptors in PZ^{GABA} (Fig. 3B1–5).

At the dose of 1, 3 and 10 mg/kg, C21 induces similar phenotypes as CNO at the dose of 0.1, 0.3 and 1 mg/kg (Fig. 2C1–5). SWS amount is dose dependently increased [interaction: time x dose, $F(69, 759) = 3.828$, $p < 0.0001$, two-way ANOVA; Fig. 2C2]. The percentage of SWS is significantly increased, as compared with control injection, during 1-hr, 2-hr and 3-hr post injection period following C21 at the dose of 1, 3 and 10 mg/kg, respectively (Fig. 2C2). The percentage of wakefulness is significantly decreased during the first hour post injection for both 1 and 3 mg/kg of C21, and during the 3-hr post injection of C21 at 10 mg/kg (Fig. 2C1). REM sleep is significantly inhibited during the 6 hr following C21 (3 mg/kg), 9 hr following C21 (1 mg/kg) and 12 hr following C21 (10 mg/kg; Fig. 2C3 insert). SWS latency is dose dependently decreased [$F(1.711, 18.82) = 16.49$, $p = 0.0001$, one-way ANOVA; Fig. 2C4]. Finally, REM sleep latency is dose dependently increased [$F(1.756, 19.32) = 13.05$, $p = 0.0004$, one-way ANOVA; Fig. 2C5]. Similar to CNO and DCZ, at the three tested doses, C21 does not significantly affect sleep-wake amount and sleep latencies in Cre- control mice (Fig. 3C1–5). However, in these control mice, REM sleep latency shows a trend to increase dose-dependently (Fig. 3C5). This trend is not significant due to the high variability of the REM sleep latency between mice indicated by the large error bar. Nevertheless, non-specific effects of C21 on REM sleep need to be considered.

3.2. Dose dependent effect of CNO, DCZ, and C21 on SWS power distribution

IP administration of CNO, DCZ, and C21 at increasing doses, in $PZ^{GABA\text{-}hM3Dq}$ mice, resulted in an EEG visually indistinguishable from normal SWS (Fig. 4A-D). The cortical EEG power distribution (Fig. 4E-G) suggests an increase in the proportion of slow frequencies and that was confirmed by statistical analysis of the power bands. CNO dose dependently affects the cortical EEG power bands [interaction: frequency band x dose, $F(12, 132) = 12.47$, $p < 0.0001$, two-way ANOVA; Fig. 4E]. Interestingly, the proportion of delta (0.5–4.5 Hz) power is not only significantly increased following injection of CNO (0.1 mg/kg) as compared with control injection but both of the higher doses of CNO (0.3 and 1 mg/kg) significantly increase the proportion of delta power as compared with the low dose (0.1 mg/kg; Fig. 4E). Given that delta power, also called SWA, is a marker of SWS depth/quality, our results indicate that increasing doses of CNO not only lengthen SWS duration but also induce increasing SWS quality. The increase in the proportion of the delta band was compensated by a significant decrease of the proportion of the theta (4.5–10 Hz) band at the dose of 0.3 and 1 mg/kg (Fig. 4E).

Similar results are seen with DCZ and C21. Both DCZ [interaction: frequency band x dose, $F(12, 108) = 23.86$, $p < 0.0001$, two-way ANOVA; Fig. 4F] and C21 [interaction: frequency band x dose, $F(12, 108) = 36.17$, $p < 0.0001$, two-way ANOVA; Fig. 4G] dose dependently affect the cortical EEG power bands. The proportion of the delta band is significantly increased by all doses. Interestingly, not only the proportion of the theta band is significantly decreased by all doses but also the proportion of the beta (15–30 Hz) band is significantly decreased by all DCZ doses (Fig. 4F) and by C21 (3 and 10 mg/kg; Fig. 4G).

As previously shown (Anaclet et al., 2014), at the dose of 0.3 mg/kg, CNO does not affect the cortical EEG power distribution in mice not expressing the DREADD hM3Dq (Cre- mice, Fig. 5A). Here, we further show that a lower CNO dose (0.1 mg/kg) and, more importantly, a higher CNO dose (1 mg/kg) also do not affect the cortical EEG power distribution in control mice (Fig. 5A). Similar results are obtained with DCZ (Fig. 5B) and C21 (Fig. 5C). None of the doses tested affect the cortical EEG power distribution in Cre- mice (Fig. 5).

3.3. CNO (0.3 mg/k), DCZ (0.5 mg/kg), and C21 (3 mg/kg) have similar effects on sleep-wake phenotypes

The three DREADD agonists, CNO (0.3 mg/kg), DCZ (0.5 mg/kg), and C21 (3 mg/kg) significantly affect the hourly distribution of wakefulness [interaction: time x drug, $F(69, 690) = 2.496$, $p < 0.0001$; Fig. 6A1], SWS [interaction: time x drug, $F(69, 690) = 3.003$, $p < 0.0001$; Fig. 6A2] and REM sleep [interaction: time x drug, $F(69, 690) = 1.633$, $p = 0.0014$; Fig. 6A3]. During the first hour post injection, similar to CNO, C21 significantly increased the percentage of SWS and significantly decreased the percentage of wakefulness (Bonferroni's multiple comparisons test: CNO vs C21, $p > 0.05$; Fig. 6A2 & 6A1) as compared with control injection. However, DCZ further increased the percentage of SWS as compared with both CNO (Bonferroni's multiple comparisons test: CNO vs DCZ, $p < 0.01$; Fig. 6A2) and C21 (Bonferroni's multiple comparisons test: DCZ vs C21, $p < 0.01$; Fig. 6A2). In addition, DCZ but not CNO or C21, significantly increased the percentage of SWS during the second hour post injection (Bonferroni's multiple comparisons test: control injection vs DCZ, $p < 0.001$; Fig. 6A2). Opposite effects are seen on the percentage of wakefulness (Fig. 6A1).

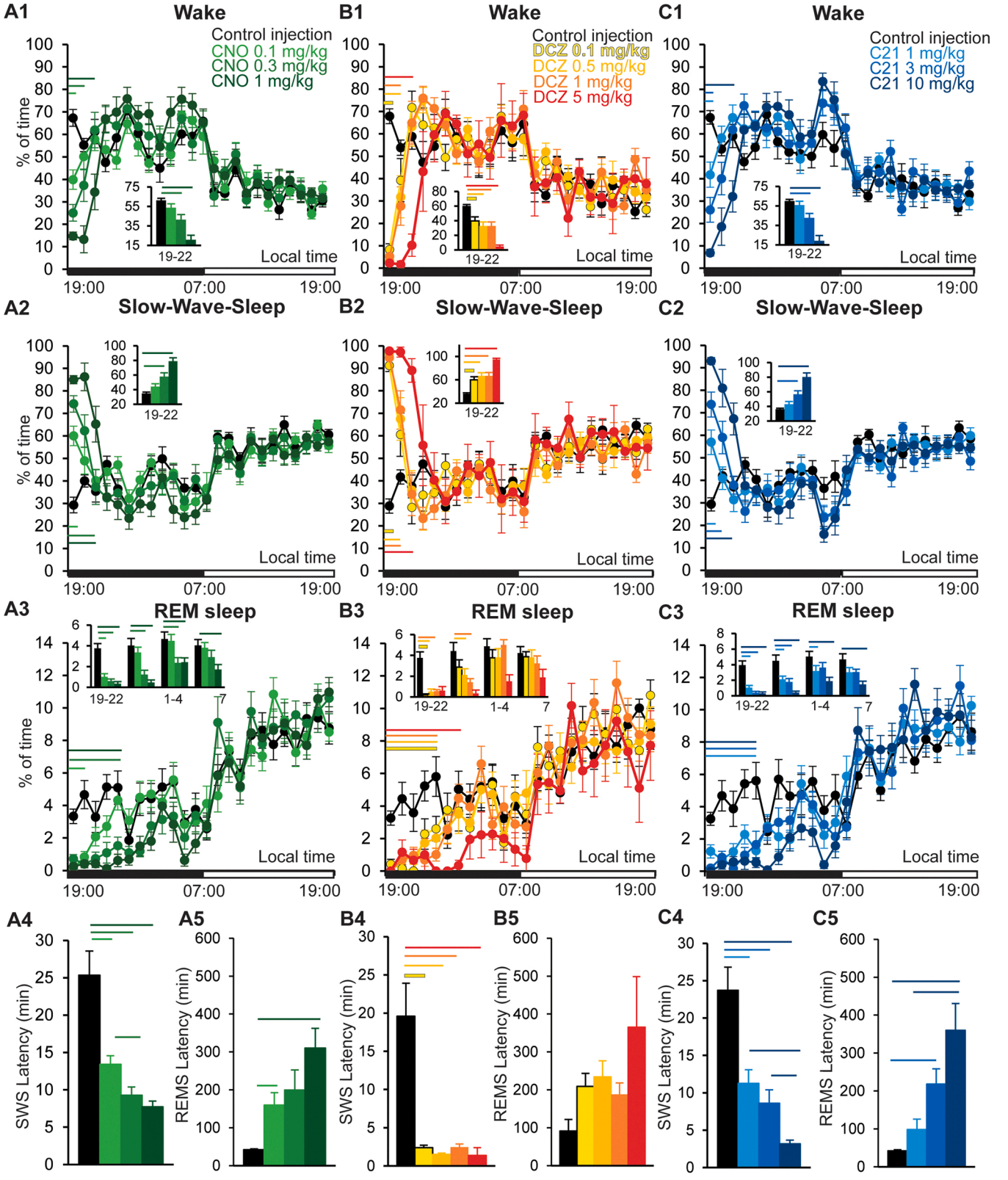

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Fig. 2. Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) increase slow-wave-Sleep (SWS) amount and display similar dose response in mice expressing hM3Dq receptor in parafacial zone GABAergic neurons. (**A**) Wakefulness (A1), SWS (A2) and REM sleep (REMS; A3) hourly percentage following injection of CNO at the dose of 0.1, 0.3 and 1 mg/kg (green, darker shades denote higher concentration), at lights-off (19:00; n = 14). (A4–5) CNO dose-dependent effect on SWS and REMS latencies. (**B**) Wakefulness (B1), SWS (B2) and REMS (B3) hourly percentage following injection of DCZ at the dose of 0.1 (yellow), 0.5 (light orange), 1 (dark orange, n = 11) and 5 (red, n = 5) mg/kg, at lights-off (19:00). (B4–5) DCZ dose-dependent effect on SWS and REMS latencies. (**C**) Wakefulness (C1), SWS (C2) and REMS (C3) hourly percentage following injection of C21 at the dose of 1, 3 and 10 mg/kg (darker shades of blue denote higher doses), at lights-off (19:00; n = 12). (C4–5) C21 dose-dependent effect on SWS and REMS latencies. (A1–2, B1–2, C1–2) Inserted histograms show the percentage of the vigilance stage during the 0–3-hr period following injection. (A3, B3, C3) Inserted histograms show the percentage of REM sleep during the 0–3, 3–6, 6–9 and 9–12-hr period following the injection. (A1–3, B1–3, C1–3) Horizontal, colored lines show significant differences of the color-coded dose vs control injection, $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test. (A4–5, B4–5, C4–5) Color lines demarcate the significant differences between conditions, $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test. (B) DCZ (5 mg/kg, n = 5) significant difference as compared with respective control condition (n = 5), $p < 0.05$, paired t-test.

CNO and C21 similarly decreased SWS latency (Bonferroni's multiple comparisons test: CNO vs C21, $p > 0.05$; Fig. 6B). SWS latency is further decreased by DCZ and is significantly shorter as compared with CNO (Bonferroni's multiple comparisons test: CNO vs DCZ, $p < 0.001$; Fig. 6B).

The three DREADD agonists, CNO (0.3 mg/kg), DCZ (0.5 mg/kg), and C21 (3 mg/kg) significantly increase REM sleep latency as compared with control injection (Fig. 6 C), to a similar extent. The percentage of REM sleep is decreased during the 5-hr post CNO, DCZ, and C21 injection period (Fig. 6A3), as compared with control injection. This result suggests that C21 inhibits REM sleep to the same extent as CNO and DCZ, indicating that the potential non-specific effects suggested earlier are not pharmacological and might instead be the result of large REM sleep latency variabilities observed between mice.

The three DREADD agonists, CNO (0.3 mg/kg), DCZ (0.5 mg/kg) and C21 (3 mg/kg) significantly affect the cortical EEG power distribution and bands during SWS [interaction: power band x drug, $F(12, 108) = 24.47$, $p < 0.0001$; Fig. 6D]. The proportion of the delta (0.5–4.5 Hz) band is significantly increased and the percentage of the theta (4.5–10 Hz) band is significantly decreased. Interestingly, DCZ further increases the proportion of the delta band as compared with both CNO (Bonferroni's multiple comparisons test: CNO vs DCZ, $p < 0.0001$) and C21 (Bonferroni's multiple comparisons test: DCZ vs C21, $p < 0.0001$). This indicates that DCZ induces a deeper SWS as compared with the two other DREADD agonists.

3.4. Voluntary oral administration (jelly) of CNO, DCZ or C21 has similar effect on sleep-wake quantity as compared with IP injection

We then tested an alternative administration route that will allow chronic SWS enhancement. We used the following: CNO 0.3 mg/kg, DCZ 0.5 mg/kg and C21 3 mg/kg. IP injections indicate that DCZ 0.5 mg/kg is inducing SWS faster, increasing SWS longer and enhancing SWA to a greater extent than CNO 0.3 mg/kg and C21 3 mg/kg. However, at a lower dose (0.1 mg/kg), DCZ displays similar effects on sleep-wake phenotypes as compared with the dose of 0.5 mg/kg and 1 mg/kg (Figs. 2B, 4F). Therefore, we decided to use the middle dose, 0.5 mg/kg, to test the voluntary oral administration (jelly).

When CNO was administered in jelly (CNO-Jelly), mice display a significant decrease in the percentage of wakefulness (Fig. 7A1) and increase in the percentage of SWS (Fig. 7A2), as compared with both IP control injection (control-IP) and jelly control administration (control-Jelly), during the first two hours following the injection/administration. These phenotypes are similar to IP injection of CNO (CNO-IP; Bonferroni's multiple comparisons test: CNO-IP vs CNO-Jelly, $p > 0.05$) during the first hour following the injection/administration. Interestingly, during the second hour post injection/administration, CNO-Jelly significantly increased the percentage of SWS as compared with CNO-IP (Bonferroni's multiple comparisons test: CNO-IP vs CNO-Jelly, $p < 0.0001$; Fig. 7A2).

Although not reaching statistical significance (Bonferroni's multiple comparisons test: control-IP vs control-Jelly, $p > 0.05$; Fig. 6A4), the voluntary oral administration (control-Jelly) seems to decrease the latency to SWS as compared with control IP injection (control-IP). This observation is consistent with the absence of stress induced by the mouse handling during the IP injection. As a result, the SWS latency following control-Jelly is similar to the SWS latency following both CNO-IP and CNO-Jelly. However, both CNO-IP (Bonferroni's multiple comparisons test: control-IP vs CNO-IP, $p < 0.01$) and CNO-Jelly (Bonferroni's multiple comparisons test: control-IP vs CNO-Jelly, $p < 0.05$) significantly decrease SWS latency as compared with control-IP, at a similar extent (Bonferroni's multiple comparisons test: CNO-IP vs CNO-Jelly, $p > 0.05$).

REM sleep is similarly inhibited by CNO-IP and CNO-Jelly during the 6 hr and 9-hr following injection/administration, as compared with both control-Jelly and control-IP, respectively (Fig. 7A3 insert). REM sleep latency is significantly increased by CNO-IP and CNO-Jelly as compared with both control-IP and control-Jelly (Fig. 7A5).

Similar results were obtained with DCZ-Jelly. Following DCZ-Jelly the mice spent similar time in SWS as compared with DCZ-IP (Bonferroni's multiple comparisons test: DCZ-IP vs DCZ-Jelly, $p > 0.05$; Fig. 7B2). This is associated with similar percentage of both wakefulness (Bonferroni's multiple comparisons test: DCZ-IP vs DCZ-Jelly, $p > 0.05$; Fig. 7B1) and REM sleep (Bonferroni's multiple comparisons test: DCZ-IP vs DCZ-Jelly, $p > 0.05$; Fig. 7B3) between the two administration routes. In addition, SWS and REM sleep latencies are similarly decreased and increased, respectively, after DCZ-IP and DCZ-Jelly (Bonferroni's multiple comparisons test: DCZ-IP vs DCZ-Jelly, $p > 0.05$; Fig. 7B4–5).

C21-Jelly also induced identical phenotypes compared to C21-IP (Fig. 7C1–5). Both C21-IP and C21-Jelly significantly increase the percentage of SWS during the 2-hr post injection/administration period, as compared with control-IP and control-Jelly, to a similar extent (Bonferroni's multiple comparisons test: C21-IP vs C21-Jelly, $p > 0.05$; Fig. 7C2). Wakefulness and REM sleep percentages are also similar between C21-IP and C21-Jelly. Finally, both C21-IP and C21-Jelly decrease the latency to SWS and increase the latency to REM sleep in a similar manner (Fig. 7C4–5).

Importantly, control-Jelly, CNO-Jelly, DCZ-Jelly and C21-Jelly have no significant effects on sleep-wake amount and latency when administrated to Cre- control mice (Fig. 8), confirming the absence of non-specific effects. Interestingly, C21-IP seems to increase REM sleep latency, something that is not observed following C21-Jelly. This result reinforces the conclusion that there are no non-specific actions of C21 on REM sleep latency. Rather, any differences seen are the result of an analysis artifact due to high variability of REM sleep latency between mice.

3.5. Voluntary oral administration (jelly) of CNO, DCZ and C21 have similar effects on SWS quality as compared with IP injection

One of the most significant phenotypes induced by the activation of PZ^{GABA} neurons is the enhancement of the cortical EEG slow frequencies, delta (0.5–4.5 Hz) band, also called SWA, which is routinely used as a marker of sleep quality. In the present study, we show that CNO-Jelly, DCZ-Jelly and C21-Jelly significantly increase the proportion of delta power during SWS in a similar manner as CNO-IP, DCZ-IP and C21-IP

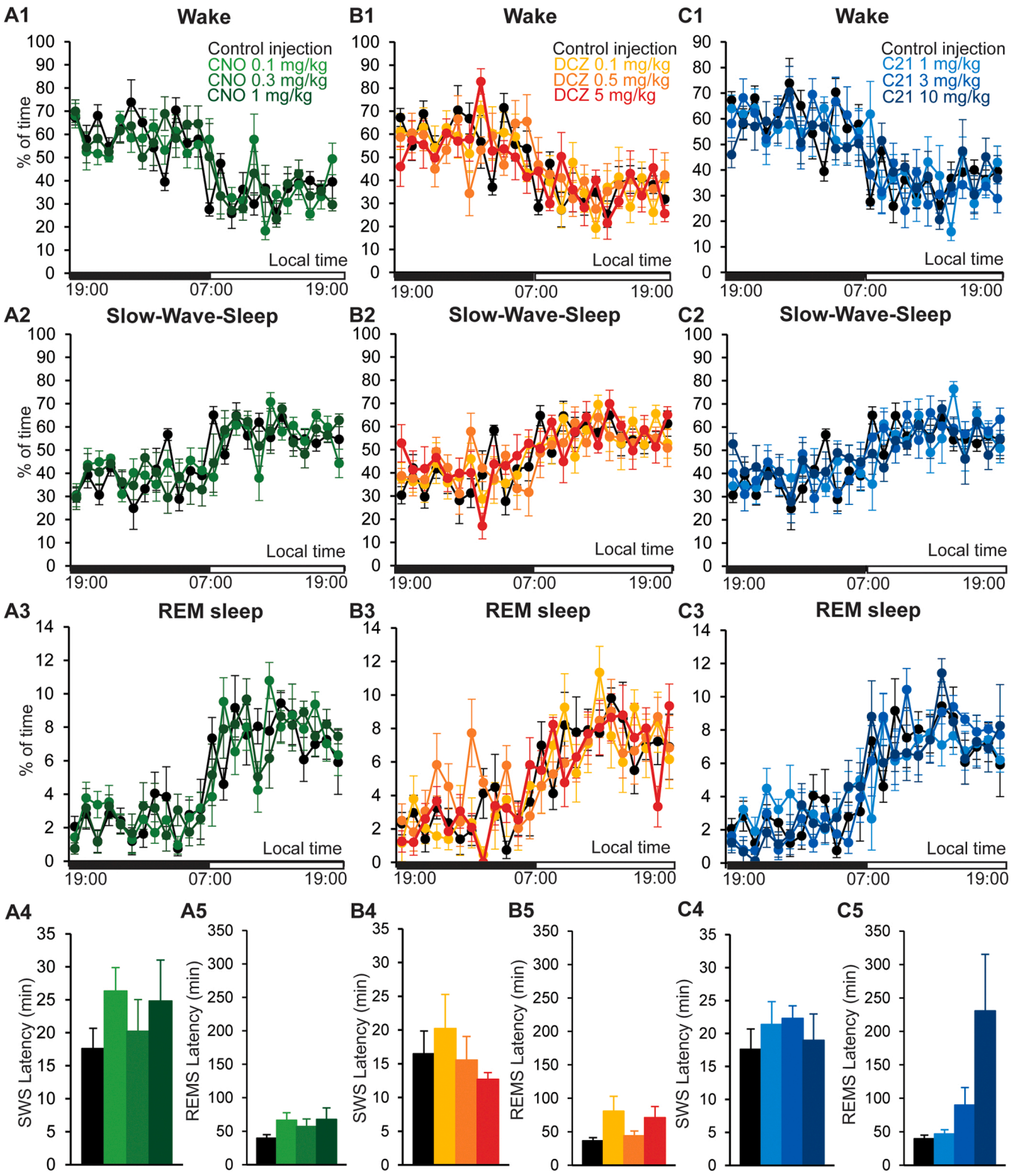

Fig. 3. Increasing doses of Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) do not affect sleep-wake amount in wild-type mice. (**A**) Wakefulness (A1), slow-wave-sleep (SWS) (A2) and REM sleep (REMS; A3) hourly percentage following injection of CNO at the dose of 0.1, 0.3 and 1 mg/kg (green, darker shades denote higher concentration), at lights-off (19:00; n = 7). (A4–5) CNO dose-dependent effect on SWS and REMS latencies. (**B**) Wakefulness (B1), SWS (B2) and REMS (B3) hourly percentage following injection of DCZ at the dose of 0.1 (yellow), 0.5 (light orange), 1 (dark orange) and 5 (red) mg/kg, at lights-off (19:00; n = 6). (B4–5) DCZ dose-dependent effect on SWS and REMS latencies. (**C**) Wakefulness (C1), SWS (C2) and REMS (C3) hourly percentage following injection of C21 at the dose of 1, 3 and 10 mg/kg (darker shades of blue denote higher doses), at lights-off (19:00; n = 7). (C4–5) C21 dose-dependent effect on SWS and REMS latencies. (A4–5, B4–5, C4–5) No significant difference ($p > 0.05$) compared with control injection, two-way ANOVA followed by a post hoc Bonferroni test. (A4–5, B4–5, C4–5) No significant difference ($p > 0.05$), one-way ANOVA followed by a post hoc Bonferroni test.

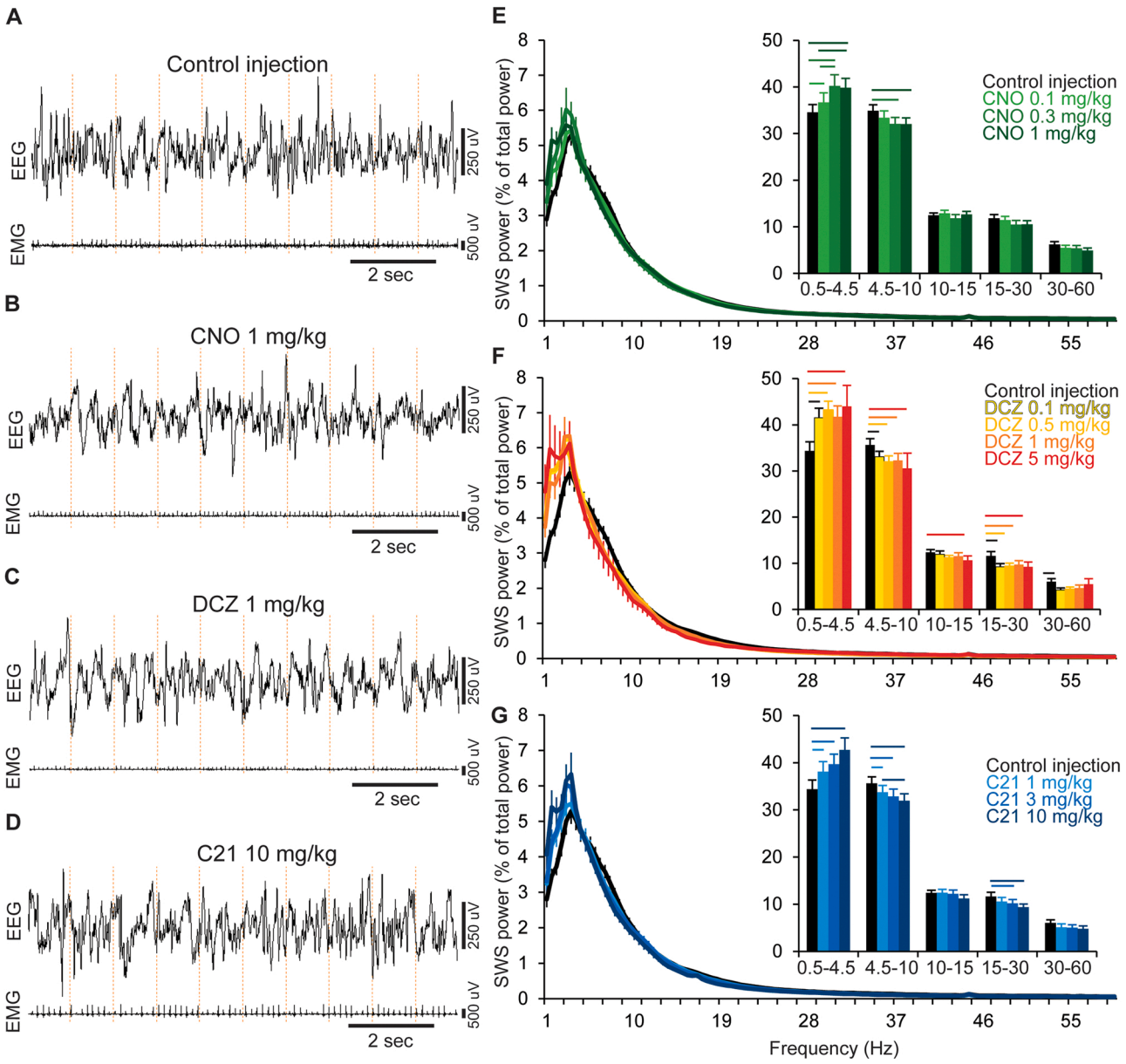

Fig. 4. Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) enhance slow wave activity (SWA) and display similar dose response in mice expressing hM3Dq receptor in parafacial zone GABAergic neurons. (**A-D**) EEG/EMG examples, from the same mouse, of slow-wave-sleep (SWS) from the first hour following injection of vehicle (control injection, **A**), CNO (1 mg/kg, **B**), DCZ (1 mg/kg, **C**) and C21 (10 mg/kg, **D**). (**E-F**) Cortical EEG power spectral distribution and bands (inserted histogram), expressed in percentage of total (0.5–60 Hz) power, from the first hour of SWS following injection of CNO (0.1, 0.3 and 1 mg/kg, darker shades of green denote higher concentration, n = 12; **E**), DCZ [0.1 (yellow), 0.5 (light orange), 1 (dark orange) and 5 (red) mg/kg, n = 10 or 4 for the highest dose; **F**], and C21 (1, 3 and 10 mg/kg, darker shades of blue denote higher concentration, n = 10; **G**) at lights-off (19:00). Horizontal, colored lines show significant differences between conditions, two-way ANOVA followed by a post hoc Bonferroni test. (F) DCZ (5 mg/kg, n = 4) significant difference as compared with respective control condition (n = 4), red horizontal line $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test.

respectively (Fig. 9A2, B2, C2). At the same time, frequency bands used to measure cortical activation and cognitive functions, such as theta (4.5–10 Hz), beta (15–30 Hz) and gamma (30–60 Hz) are decreased. Importantly, during SWS, the cortical EEG power distribution of Cre-control mice was not affected by control-Jelly, CNO-Jelly, DCZ-Jelly and C21-Jelly, as compared with control-IP, CNO-IP, DCZ-IP and C21-IP (Fig. 10), confirming the absence of non-specific effects.

All together these results show that voluntary oral administration of DREADD agonists is as effective as IP injection and can be used as a non-invasive and stress-attenuating administration route.

4. Discussion

Here we show that DCZ and C21 are equally potent DREADD agonists that do not affect sleep/wake architecture or EEG signatures on their own, at the doses used. We have also identified voluntary oral administration of these compounds to be equally effective as the traditional IP route of administration. Our data also suggest that this route of administration is less stressful in mice, due to the reduced sleep onset times following control-Jelly compared to control-IP, and therefore is superior for sleep studies.

CNO was initially thought to not reverse-metabolize to its parent compound clozapine in mice and therefore became the agonist of choice in functional brain circuit mapping, including in sleep research (Anaclet

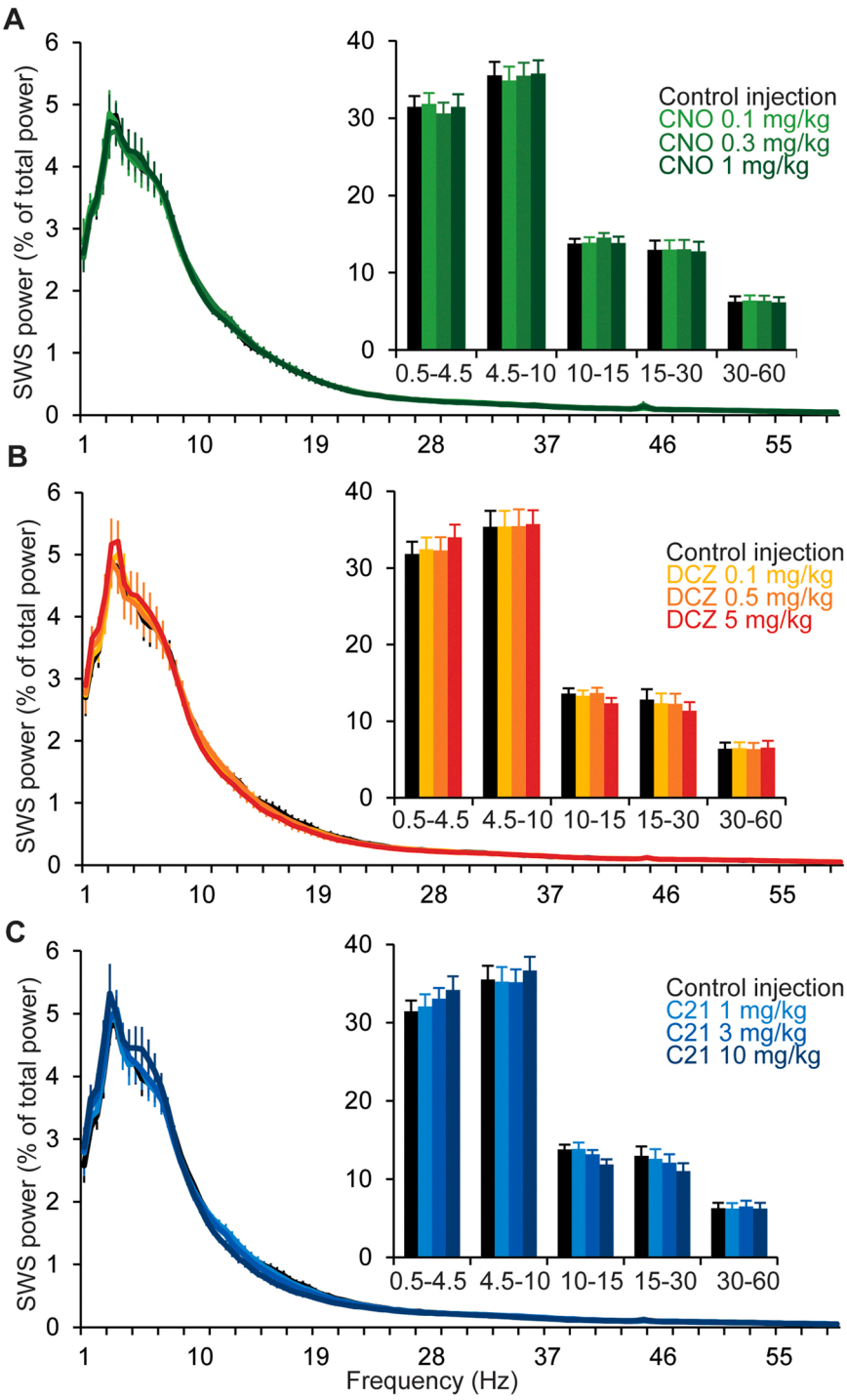

Fig. 5. Increasing doses of Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) do not affect slow-wave-Sleep (SWS) power distribution in wild-type mice. Cortical EEG power spectral distribution and bands (inserted histogram), expressed in percentage of total (0.5–60 Hz) power, from the first hour of SWS following injection of CNO (0.1, 0.3 and 1 mg/kg, darker shades of green denote higher concentration, n = 7; **A**), DCZ [0.1 (yellow), 0.5 (light orange), 1 (dark orange) and 5 (red) mg/kg, n = 6; **B**], and C21 (1, 3 and 10 mg/kg, darker shades of blue denote higher concentration, n = 7; **C**) at lights-off (19:00). No significant difference ($p > 0.05$) compared with control injection, two-way ANOVA followed by a post hoc Bonferroni test.

et al., 2014; Fuller et al., 2015; Roth, 2016; Smith et al., 2021; Song et al., 2022). More recent data, generated from CNO administration of doses multiple factors higher than the one used here and in our previous studies, show that reverse metabolism can occur and lead to brain clozapine concentrations high enough to occupy central serotonin and dopamine receptors, or even the DREADD receptors themselves (Jendryka et al., 2019). The combined effect of these observations has made the use of alternatives to CNO not only desirable but necessary. Thus far, however, the effects of these CNO alternatives on sleep have not been studied. Moreover, with multiple DREADD agonists now available and in use by various groups, it is important to have a metric by which to compare the different agonists. To begin to provide such a metric, we have therefore tested each of these agonists in the same animals at varying concentrations. Here we provide evidence using 3 different concentrations of CNO, DCZ and C21 in a known SWS-enhancing model that all three agonists are capable of eliciting SWS and these agonists do not on their own interfere with sleep in control (not expressing DREADDs) mice.

The present study shows that, in mice expressing the excitatory DREADD hM3Dq in parafacial zone GABAergic ($PZ^{GABA\text{-}hM3Dq}$) neurons, DCZ induces SWS significantly faster than CNO, suggesting a more powerful mechanism of action. This is reinforced by the fact that a very

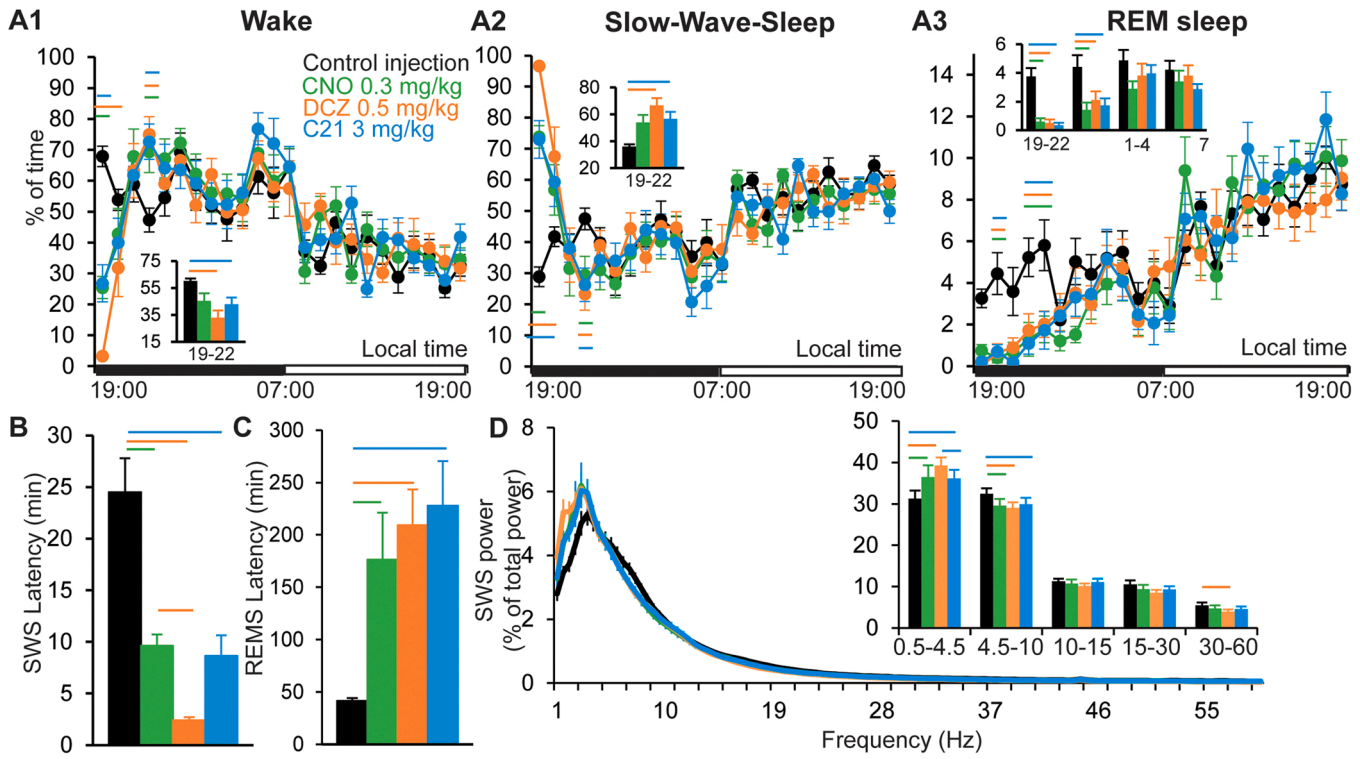

Fig. 6. Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) similarly promote slow-wave-sleep (SWS) and enhance slow wave activity (SWA) in mice expressing hM3Dq receptor in parafacial zone GABAergic neurons. (**A**) Wakefulness (A1), SWS (A2) and REM sleep (REMS; A3) hourly percentage following injection of CNO (0.3 mg/kg, green), DCZ (0.5 mg/kg, orange) and C21 (3 mg/kg, blue), at lights-off (7 P.M.; n = 11). (A1–2) Inserted histograms show the percentage of the vigilance stage during the 0–3-hr period following injection. (A3) Inserted histogram shows the percentage of REM sleep during the 0–3, 3–6, 6–9 and 9–12-hr period following the injection. (**B-C**) SWS and REMS latencies following injection of CNO (0.3 mg/kg, green), DCZ (0.5 mg/kg, orange) and C21 (3 mg/kg, blue), at lights-off (7 P.M.; n = 11). (**D**) Cortical EEG power spectral distribution and bands (inserted histogram) from the first hour of SWS following injection of CNO (0.3 mg/kg, green), DCZ (0.5 mg/kg, orange) and C21 (3 mg/kg, blue) at lights-off (7 P.M.; n = 10). (A1–3) Horizontal, colored lines show significant differences of that agonist (green = CNO, orange = DCZ, blue = C21) vs control injection, $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test. (B-C) Horizontal colored lines show significant differences between conditions, $p < 0.05$, one-way ANOVA followed by a post hoc Bonferroni test. (D) Horizontal colored lines show significant differences between conditions, $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test.

low dose (0.1 mg/kg) powerfully enhances SWS. Our results are in accordance with previous studies showing that DCZ displays a higher affinity and more selectivity for muscarinic-based DREADDs than CNO and C21 (Nagai et al., 2020). Moreover, the pharmacokinetics of DCZ action appears to be short, following IP injection of DCZ (0.1 mg/kg) in mice, DCZ concentration in the plasma and in the brain is reduced 10 fold after an hour and is undetectable after two hours (Nagai et al., 2020). Interestingly, following IP injection of DCZ (0.1 mg/kg), C21 is detected in the plasma of mice during 2-hr post-injection period (Nagai et al., 2020), the result of metabolism of DCZ into C21. Here, we found that only the relatively high dose of DCZ (5 mg/kg) is able to extend the duration of the SWS enhancement. This result could be explained by the metabolism of DCZ into C21 at a concentration high enough to excite $PZ^{GABA\text{-}hM3Dq}$ neurons, thereby extending the SWS enhancement duration. The high dose of DCZ (5 mg/kg) could lead to non-specific effects even though DCZ has been shown to be metabolized into two pharmacologically inert molecules, C21 and DCZ-N-oxide (Nagai et al., 2020). DCZ itself displays low affinity for muscarinic and serotoninergic receptors (Nagai et al., 2020). Here we show that at 5 mg/kg, DCZ does not affect sleep-wake quantity or quality in control mice, not expressing DREADDs, suggesting that 5 mg/kg does not induce non-specific sleep-wake effects. No other physiological functions were assessed in the present study and the possibility that the DREADD agonists affects other systems cannot be disregarded. For example, a recent study showed that at 0.3 mg/kg DCZ alters the brain-wide resting-state functional connectivity in WT non-human primates not expressing DREADDs (Fujimoto et al., 2022). Nevertheless, the risk of DCZ non-specific actions appears to be more limited than for CNO. However, further studies using DCZ should include a negative control group.

In the present study, C21 displays similar dose dependent SWS enhancement as compared with CNO, but at doses 10-fold higher. Following C21 administration neither clozapine nor CNO are found in the brain or plasma of mice (Jendryka et al., 2019; Thompson et al., 2018), suggesting that C21 is not metabolized into clozapine or CNO. However, as far as we are aware, the metabolic products of C21 are not known and we cannot exclude the existence of non-specific effects of C21. The trend to dose dependently increase REM sleep latency in control mice suggested non-specific effects of C21 on sleep-wake cycle. However, it is important to note that C21 does not extend REM sleep latency as compared with CNO and DCZ in $PZ^{GABA\text{-}hM3Dq}$ mice. Moreover, C21-Jelly did not increase REM sleep latency as compared with both Control-IP and Control-Jelly in control mice, indicating that C21 does not pharmacologically inhibit REM sleep in mice. One explanation for the trend to increase REM sleep latency in control mice is the high variability of REM sleep latencies across mice and days. It is likely that increasing the number of mice will result in REM sleep latency close to control conditions. Nevertheless, the use of appropriate negative controls remains necessary in chemogenetic studies.

The present study compares the SWS promoting action of three DREADD agonists, using the excitatory hM3Dq receptors. DREADD agonists are also high affinity ligands for the inhibitory hM4Di receptors and both DCZ and C21 show high affinity for the hM4Di receptor

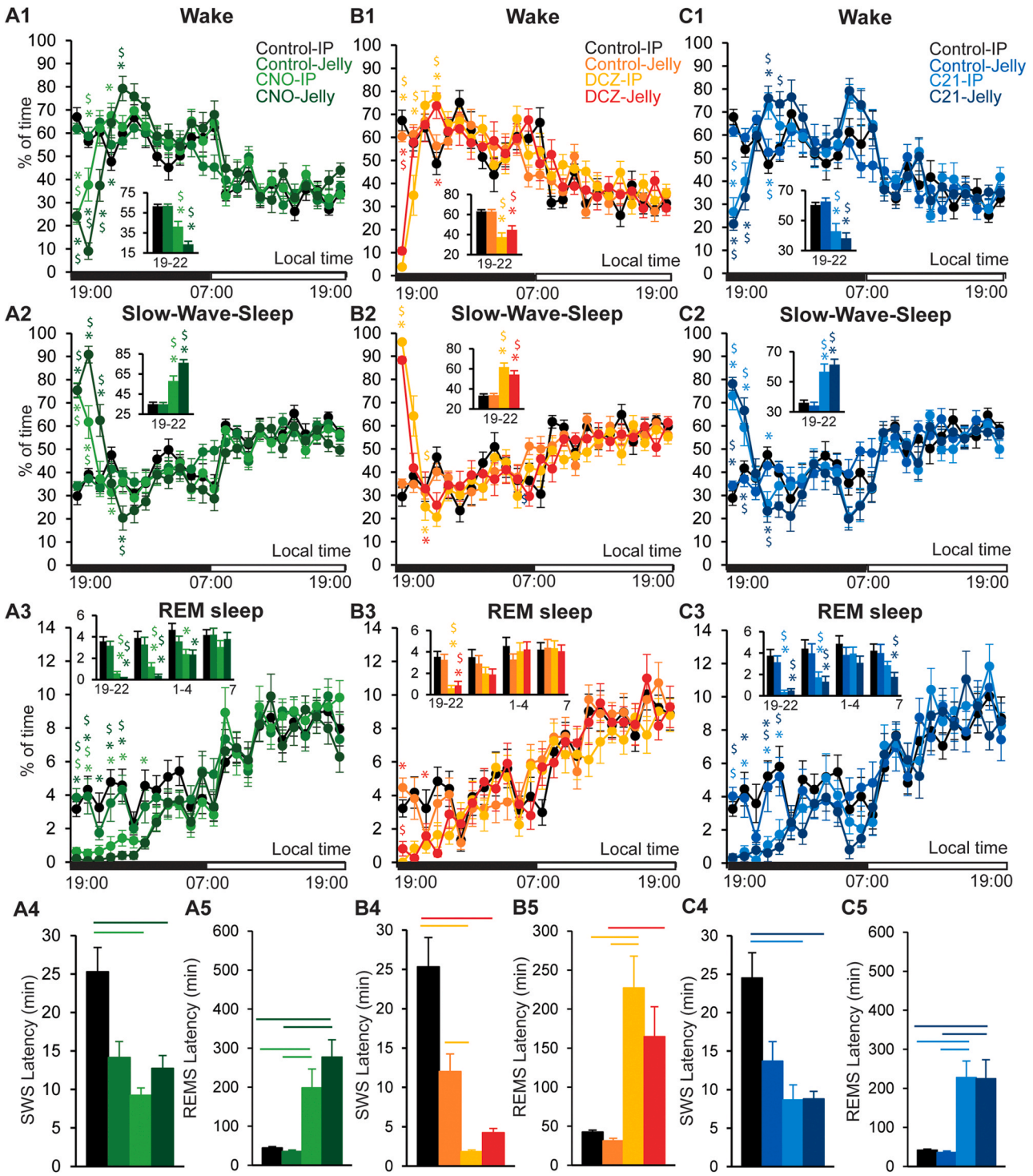

Fig. 7. Voluntary oral administration (jelly) of Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) increases slow-wave-sleep (SWS) amount similar to intraperitoneal (IP) injection in mice expressing hM3Dq receptor in parafacial zone GABAergic neurons. (**A**) Wakefulness (A1), SWS (A2) and REM sleep (REMS; A3) hourly percentage following IP injection of CNO (0.3 mg/kg; light green, CNO-IP) or administration of CNO (0.3 mg/kg) in jelly (dark green, CNO-Jelly), compared with control IP injection (control-IP, black) and control jelly administration (control-Jelly, green), at lights-off (19:00; n = 16). (A4–5) Effect of CNO administration route on SWS and REMS latencies. (**B**) Wakefulness (B1), SWS (B2) and REMS (B3) hourly percentage following IP injection of DCZ (0.5 mg/kg; yellow, DCZ-IP) or administration of DCZ (0.5 mg/kg) in jelly (red, DCZ-Jelly), compared with control-IP (black) and control-Jelly (orange), at lights-off (19:00; n = 12). (B4–5) Effect of DCZ administration route on SWS and REMS latencies. (**C**) Wakefulness (C1), SWS (C2) and REMS (C3) hourly percentage following IP injection of C21 (3 mg/kg; light blue, C21-IP) or administration of C21 (3 mg/kg) in jelly (dark blue, C21-Jelly), compared with control-IP (black) and control-jelly (blue), at lights-off (19:00; n = 11). (A4–5) Effect of C21 administration route on SWS and REMS latencies. (A1–2, B1–2, C1–2) Inserted histograms show the percentage of the vigilance stage during the 0–3-hr period following injection. (A3, B3, C3) Inserted histograms show the percentage of REM sleep during the 0–3, 3–6, 6–9 and 9–12-hr period following the injection. (A1–3, B1–3, C1–3) colored stars (*) show significant differences as compared with control-IP; colored dollar signs ($) show significant differences as compared with control-Jelly; $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test. (A4–5, B4–5, C4–5) Horizontal, colored lines show significant differences between routes of administration, $p < 0.05$, one-way ANOVA followed by a post hoc Bonferroni test.

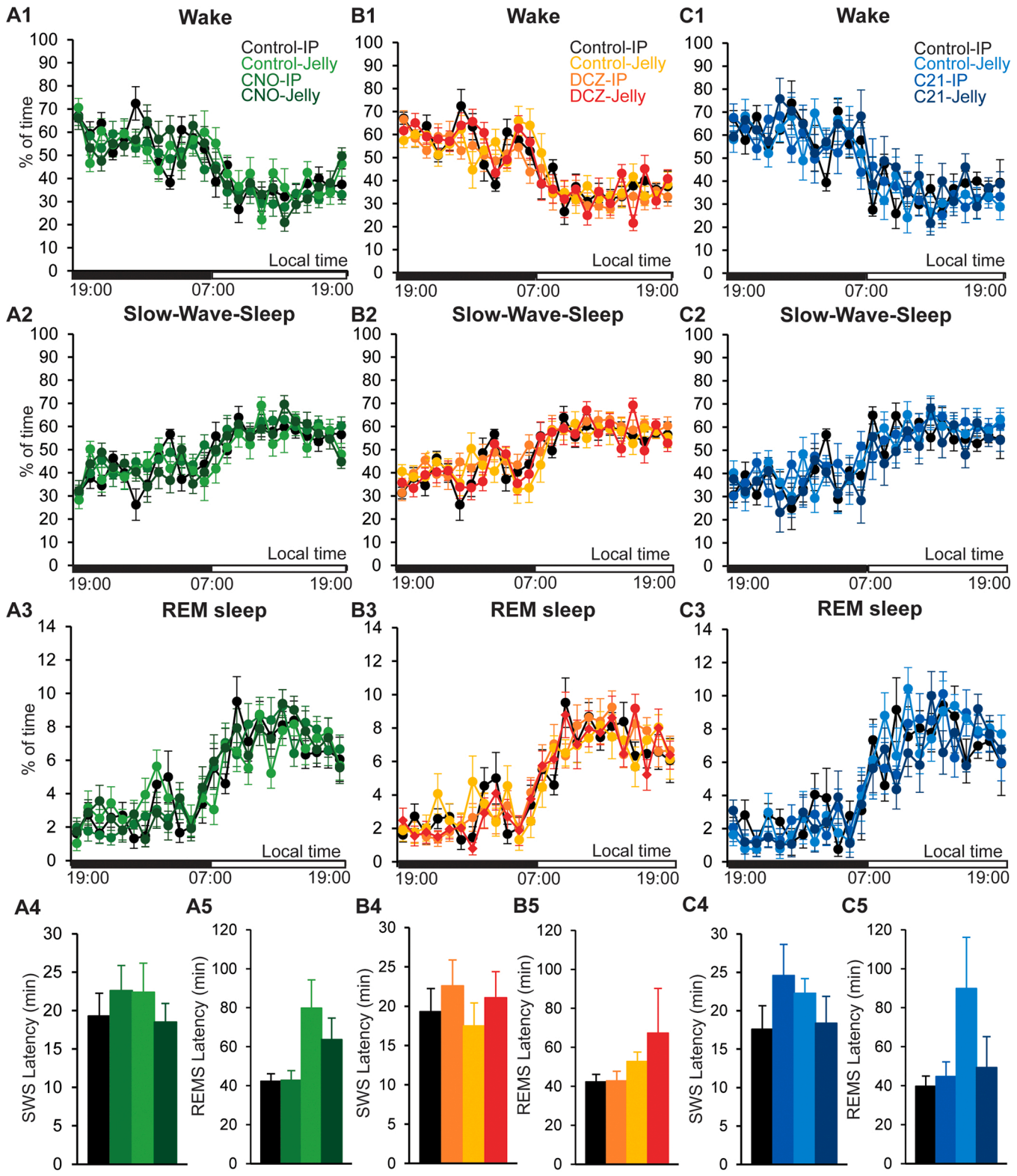

Fig. 8. Voluntary oral administration (jelly) of Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) do not affect sleep-wake amount in wild-type mice. (**A**) Wakefulness (A1), SWS (A2) and REM sleep (REMS; A3) hourly percentage following intraperitoneal (IP) injection of CNO (0.3 mg/kg; light green, CNO-IP) or administration of CNO (0.3 mg/kg) in jelly (dark green, CNO-Jelly), compared with control IP injection (control-IP, black) and control jelly administration (control-Jelly, green), at lights-off (19:00; n = 11). (A4–5) Effect of CNO administration route on SWS and REMS latencies. (**B**) Wakefulness (B1), SWS (B2) and REMS (B3) hourly percentage following IP injection of DCZ (0.5 mg/kg; yellow, DCZ-IP) or administration of DCZ (0.5 mg/kg) in jelly (red, DCZ-Jelly), compared with control-IP (black) and control-Jelly (orange), at lights-off (19:00; n = 11). (B4–5) Effect of DCZ administration route on SWS and REMS latencies. (**C**) Wakefulness (C1), SWS (C2) and REMS (C3) hourly percentage following IP injection of C21 (3 mg/kg; light blue, C21-IP) or administration of C21 (3 mg/kg) in jelly (dark blue, C21-Jelly), compared with control-IP (black) and control-Jelly (blue), at lights-off (19:00; n = 7). (A4–5) Effect of C21 administration route on SWS and REMS latencies. No significant difference ($p > 0.05$), two-way ANOVA followed by a post hoc Bonferroni test.

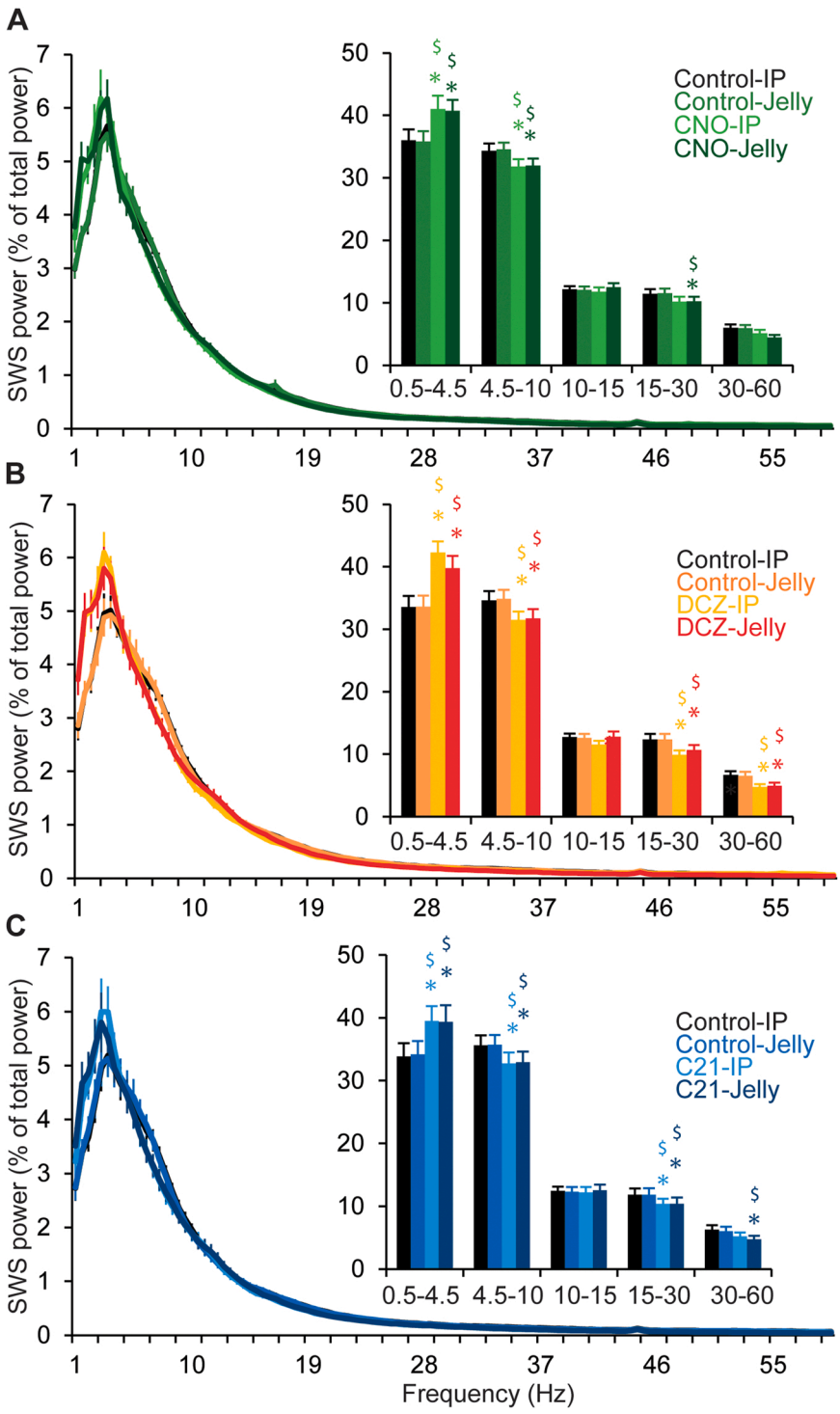

Fig. 9. Oral administration of Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) enhance slow wave activity (SWA) similar to intraperitoneal (IP) injection in mice expressing hM3Dq receptor in parafacial zone GABAergic neurons. Cortical EEG power spectral distribution and bands (inserted histogram), expressed in percentage of total (0.5–60 Hz) power, from the first hour of slow-wave-sleep (SWS) following IP injection of CNO (0.3 mg/kg; light green, CNO-IP, **A**) or administration of CNO (0.3 mg/kg; n = 14) in jelly (dark green, CNO-Jelly), IP injection of DCZ (0.5 mg/kg; yellow, DCZ-IP, **B**) or administration of DCZ (0.5 mg/kg; n = 10) in jelly (red, DCZ-Jelly), and IP injection of C21 (3 mg/kg; light blue, C21-IP, **C**) or administration of C21 (3 mg/kg; n = 9) in jelly (dark blue, C21-Jelly), compared with respective control IP injection (control-IP, black) and control jelly administration (control-Jelly, green, orange or blue) at lights-off (19:00). Colored stars (*) show significant differences compared with control-IP; colored dollar signs ($) show significant differences compared with control-Jelly; $p < 0.05$, two-way ANOVA followed by a post hoc Bonferroni test.

(Jendryka et al., 2019; Nagai et al., 2020; Thompson et al., 2018) that results in neurophysiological and behavioral changes (Nagai et al., 2020; Nentwig et al., 2022; Thompson et al., 2018). Therefore, DCZ and C21 are good DREADD agonist alternatives not only for chemogenetic activation but also for chemogenetic inhibition experiments. One limitation of the chemogenetic inhibition experiments comes from the targeted neuronal population. Indeed, intracellulary, hM4Di acts via a signal transduction cascade resulting in GIRK channel activation and subsequent membrane hyperpolarization, similar to the metabotropic receptor from which it was derived (Roth, 2016). A possible concern raised by this mechanism of action is whether the neurons expressing DREADDs also express sufficient numbers of GIRK channels. To address this concern, a recent study has shown that neurons in brain areas with low GIRK expression are still able to respond to hM4Di activation via GIRK channels to reduce membrane excitability (Shan et al., 2022).

Though most pharmacological agents, including CNO, used in murine sleep studies have a rapid response onset, mice typically do not immediately show a sleep response upon IP administration. Thus, the relatively rapid drug absorption afforded by IP injection—critical for certain sleep parameters such as sleep onset time—is masked by the arousing effect of the route of administration itself. Here we show that the voluntary oral administration (Mahoney et al., 2019) and IP

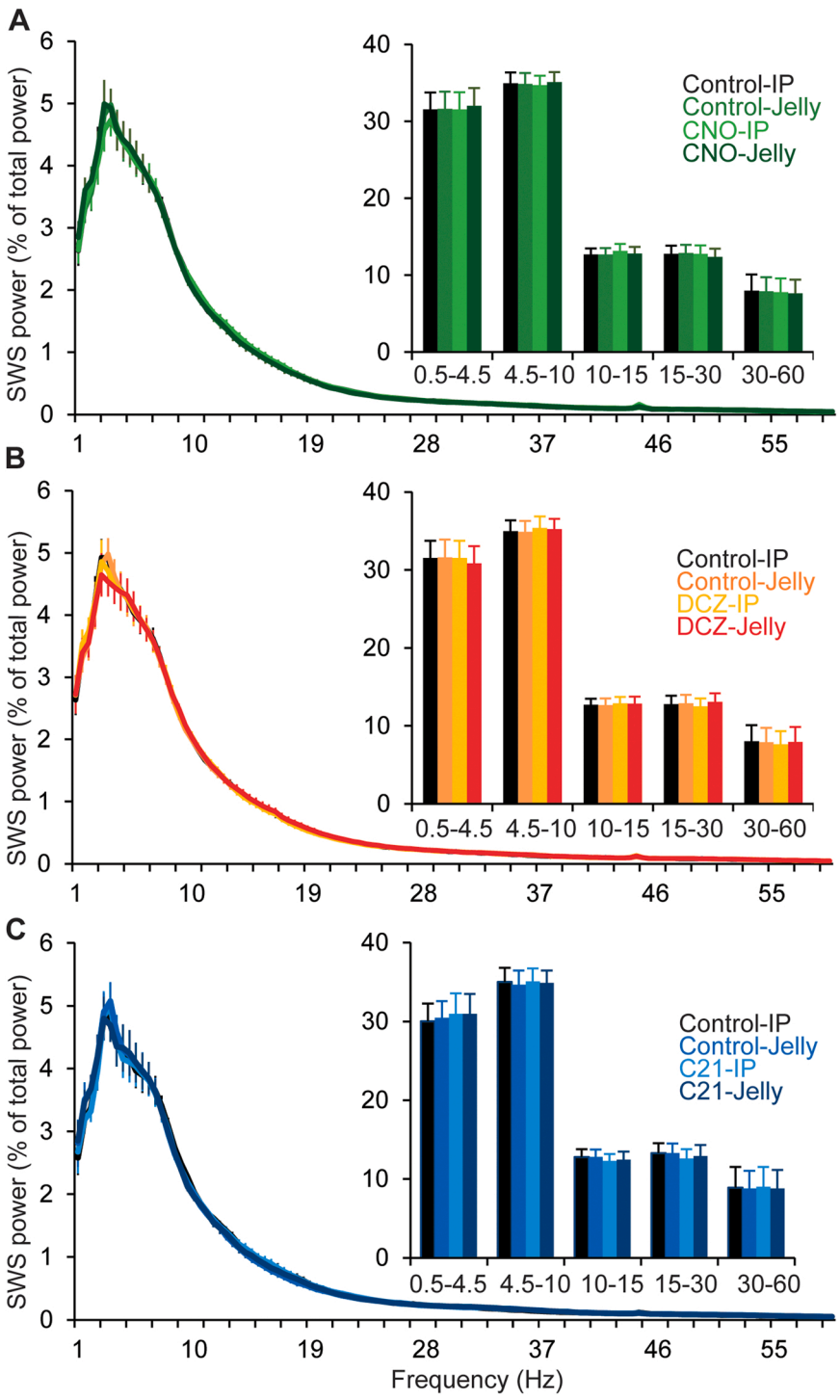

Fig. 10. Oral administration of Clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) do not affect slow-wave-Sleep (SWS) power distribution in wild-type mice. Cortical EEG power spectral distribution and bands (inserted histogram), expressed in percentage of total (0.5–60 Hz) power, from the first hour of SWS following injection of CNO (0.3 mg/kg; light green, CNO-IP, **A**) or administration of CNO (0.3 mg/kg; n = 9) in jelly (dark green, CNO-Jelly), IP injection of DCZ (0.5 mg/kg; yellow, DCZ-IP, **B**) or administration of DCZ (0.5 mg/kg; n = 9) in jelly (red, DCZ-Jelly), and IP injection of C21 (3 mg/kg; light blue, C21-IP) or administration of C21 (3 mg/kg; n = 7, **C**) in jelly (dark blue, C21-Jelly), compared with respective control IP injection (control-IP, black) and control jelly administration (control-Jelly, green, orange or blue) at lights-off (19:00). No significant difference ($p > 0.05$), two-way ANOVA followed by a post hoc Bonferroni test.

injection result in similar sleep-wake phenotypes and timelines in the three DREADD agonists tested, CNO, DCZ and C21. Therefore, the voluntary oral administration is an administration route as effective as IP injection. Besides the immediate advantages that voluntary self-administration of DREADD agonists provides, the opportunity for repeated or chronic administration over days/weeks/months can be explored using this administration route.

In a previous study (Ogbeide-Latario et al., 2022), we showed that CNO enhances SWS to the same extent in APP/PS1 + and in littermate WT control mice. Here, we extend the study to two additional DREADD agonists and to a non-invasive administration route that permits repetitive administrations. Taken together, these studies validate new methods to increase and enhance SWS in an Alzheimer's disease mouse model and provide ways for studying the role of SWS in the progression and manifestations of the disease (Ju et al., 2014; Wang and Holtzman, 2020).

Data availability

Data will be made available on request.

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none.

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none.

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References

Anaclet, C., De Luca, R., Venner, A., Malyshevskaya, O., Lazarus, M., et al., 2018a. Genetic activation, inactivation, and deletion reveal a limited and nuanced role for somatostatin-containing basal forebrain neurons in behavioral state control. J. Neurosci. 38, 5168–5181.

Anaclet, C., Ferrari, L., Arrigoni, E., Bass, C.E., Saper, C.B., et al., 2014. The GABAergic parafacial zone is a medullary slow wave sleep-promoting center. Nat. Neurosci. 17, 1217–1224.

Anaclet, C., Griffith, K., Fuller, P.M., 2018b. Activation of the GABAergic parafacial zone maintains sleep and counteracts the wake-promoting action of the psychostimulants armodafinil and caffeine. Neuropsychopharmacology 43, 415–425.

Anaclet, C., Pedersen, N.P., Ferrari, L.L., Venner, A., Bass, C.E., et al., 2015. Basal forebrain control of wakefulness and cortical rhythms. Nat. Commun. 6, 8744.

Baerentzen, S., Casado-Sainz, A., Lange, D., Shalgunov, V., Tejada, I.M., et al., 2019. The chemogenetic receptor ligand clozapine N-oxide induces in vivo neuroreceptor occupancy and reduces striatal glutamate levels. Front Neurosci. 13, 187.

Erickson, E.T.M., Ferrari, L.L., Gompf, H.S., Anaclet, C., 2019. Differential role of pontomedullary glutamatergic neuronal populations in sleep-wake control. Front Neurosci. 13, 755.

Fujimoto, A., Elorette, C., Fredericks, J.M., Fujimoto, S.H., Fleysher, L., et al., 2022. Resting-state fMRI-based screening of deschloroclozapine in rhesus macaques predicts dosage-dependent behavioral effects. J. Neurosci. 42 (29), 5705–5716. https://doi.org/10.1523/JNEUROSCI.0325-22.2022.

Fuller, P.M., Yamanaka, A., Lazarus, M., 2015. How genetically engineered systems are helping to define, and in some cases redefine, the neurobiological basis of sleep and wake. Temp. (Austin) 2, 406–417.

Jendryka, M., Palchaudhuri, M., Ursu, D., van der Veen, B., Liss, B., et al., 2019. Pharmacokinetic and pharmacodynamic actions of clozapine-N-oxide, clozapine, and compound 21 in DREADD-based chemogenetics in mice. Sci. Rep. 9, 4522.

Ju, Y.E., Lucey, B.P., Holtzman, D.M., 2014. Sleep and Alzheimer disease pathology–a bidirectional relationship. Nat. Rev. Neurol. 10, 115–119.

MacLaren, D.A., Browne, R.W., Shaw, J.K., Krishnan Radhakrishnan, S., Khare, P., et al., 2016. Clozapine N-oxide administration produces behavioral effects in long-evans rats: implications for designing DREADD experiments. eNeuro 3 (5). https://doi.org/10.1523/ENEURO.0219-16.2016.

Mahler, S.V., Aston-Jones, G., 2018. CNO Evil? considerations for the use of DREADDs in behavioral neuroscience. Neuropsychopharmacology 43, 934–936.

Mahoney, C.E., Mochizuki, T., Scammell, T.E., 2019. Dual orexin receptor antagonists increase sleep and cataplexy in wild type mice. Sleep 43 (6), zsz302.

Nagai, Y., Miyakawa, N., Takuwa, H., Hori, Y., Oyama, K., et al., 2020. Deschloroclozapine, a potent and selective chemogenetic actuator enables rapid neuronal and behavioral modulations in mice and monkeys. Nat. Neurosci. 23, 1157–1167.

Nentwig, T.B., Obray, J.D., Vaughan, D.T., Chandler, L.J., 2022. Behavioral and slice electrophysiological assessment of DREADD ligand, deschloroclozapine (DCZ) in rats. Sci. Rep. 12, 6595.

Ogbeide-Latario, O.E., Ferrari, L.F., Gompf, H.S., Anaclet, C., 2022. Two novel mouse models of slow-wave-sleep enhancement in aging and Alzheimer's disease. Sleep. Adv. 3, 1–21.

Paxinos, G.T., Franklin, K., 2001. The Mouse Brain In Stereotaxic Coordinates. Academic, San Diego.

Roth, B.L., 2016. DREADDs for neuroscientists. Neuron 89, 683–694.

Shan, Q., Fang, Q., Tian, Y., 2022. Evidence that GIRK channels mediate the DREADD-hM4Di receptor activation-induced reduction in membrane excitability of striatal medium spiny neurons. ACS Chem. Neurosci. 13 (14), 2084–2091. https://doi.org/10.1021/acschemneuro.2c00304.

Smith, K.S., Bucci, D.J., Luikart, B.W., Mahler, S.V., 2021. Dreadds: Use and application in behavioral neuroscience. Behav. Neurosci. 135, 89–107.

Song, J., Patel, R.V., Sharif, M., Ashokan, A., Michaelides, M., 2022. Chemogenetics as a neuromodulatory approach to treating neuropsychiatric diseases and disorders. Mol. Ther. 30, 990–1005.

Thompson, K.J., Khajehali, E., Bradley, S.J., Navarrete, J.S., Huang, X.P., et al., 2018. DREADD agonist 21 is an effective agonist for muscarinic-based DREADDs in vitro and in vivo. ACS Pharm. Transl. Sci. 1, 61–72.

Todd, W.D., Venner, A., Anaclet, C., Broadhurst, R.Y., De Luca, R., et al., 2020. Suprachiasmatic VIP neurons are required for normal circadian rhythmicity and comprised of molecularly distinct subpopulations. Nat. Commun. 11, 4410.

Venner, A., Anaclet, C., Broadhurst, R.Y., Saper, C.B., Fuller, P.M., 2016. A novel population of wake-promoting GABAergic neurons in the ventral lateral hypothalamus. Curr. Biol. 26, 2137–2143.

Venner, A., Mochizuki, T., De Luca, R., Anaclet, C., Scammell, T.E., et al., 2019. Reassessing the role of histaminergic tuberomammillary neurons in arousal control. J. Neurosci. 39, 8929–8939.

Wang, C., Holtzman, D.M., 2020. Bidirectional relationship between sleep and Alzheimer's disease: role of amyloid, tau, and other factors. Neuropsychopharmacology 45, 104–120.