Chronic chemogenetic slow-wave-sleep enhancement in mice.

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Abstract

While epidemiological associations and brief studies of sleep effects in human disease have been conducted, rigorous long-term studies of sleep manipulations and in animal models are needed to establish causation and to understand mechanisms. We have previously developed a mouse model of acute slow-wave-sleep (SWS) enhancement using chemogenetic activation of parafacial zone GABAergic neurons (PZ^{GABA}) in the parvicellular reticular formation of the pontine brainstem. However, it was unknown if SWS could be enhanced chronically in this model.

In the present study, mice expressing the chemogenetic receptor hM3Dq in PZ^{GABA} were administered daily with one of three chemogenetic ligands, clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21), and sleep-wake phenotypes were analyzed using electroencephalogram (EEG) and electromyogram (EMG).

We found that SWS time is increased for three hours, and at the same magnitude for at least six months. This phenotype is associated with an increase of slow wave activity (SWA) of similar magnitude throughout the 6-month dosing period. Interestingly, at the end of the 6-month dosing period, SWA remains increased for at least a week.

This study validates a mouse model of chronic SWS enhancement that will allow mechanistic investigations into how SWS promotes physiological function and prevents diseases. The approach of a rotating schedule of three chemogenetic ligands may be broadly applicable in chemogenetic studies that require chronic administration.

Introduction

A growing number of studies show that long-term sleep disruption affects many physiological functions and is a risk factor for numerous chronic diseases such as Alzheimer's disease (Shen *et al.*, 2016; Minakawa *et al.*, 2019). While improved sleep may therefore be an intervention of choice to promote healthy aging and reduce the burden of chronic conditions, the use of animal models of disease is necessary to rigorously demonstrate causal impacts and explore mechanisms. Before advancing to the translational and clinical stages of investigating this treatment avenue, we need to consider whether sleep enhancement in general, and possibly slow-wave sleep enhancement in particular, is able to provide beneficial effects over the long term. We have previously developed a mouse model of slow-wave-sleep (SWS) enhancement. We showed that SWS can be increased and enhanced by acute chemogenetic activation of parafacial zone GABAergic neurons (PZ^{GABA}) (Anaclet *et al.*, 2014). We have also previously shown that the oral route of administration is as effective as the traditional intraperitoneal injections (Ferrari *et al.*, 2022). It has remained unclear, however, if SWS can be chronically enhanced in this mouse model.

In mice, two major sleep stages are conventionally distinguished, non-rapid eye movement sleep (NREMS) and REM sleep (REMS). In humans, NREMS is further subdivided into three stages. Stage 1 is drowsiness, stage 2 is light sleep and stage 3 is deep sleep, also called slow-wave-sleep (SWS). SWS is characterized by high amplitude slow waves at the delta (0.5-4 Hz) frequency band, also called slow wave activity (SWA). Of NREMS stages, SWS has particularly importance. Specifically, SWS has been involved in clearing the metabolites accumulated during wakefulness and intense neuronal activity (Grubb & Lauritzen, 2019), and is important for the processing and consolidation of learning(Malkani & Zee, 2022). As relates to metabolites, a significant number of animal studies provide evidence that the pathological hallmarks of Alzheimer's disease, amyloid β and tau protein, accumulate in the interstitial fluid during wakefulness and are cleared during SWS (Holth *et al.*, 2019) acutely, suggesting that long-term sleep disruption will accelerate the accumulation of the amyloid β and tau protein in the brain and increase the risk of developing Alzheimer's disease. And this process precedes cognitive decline (Chen *et al.*, 2023). Extrapolating from acute sleep deprivation studies and epidemiological evidence suggesting chronic sleep deprivation during mid-life increases the risk of developing Alzheimer's, it has been hypothesized that chronic sleep enhancement can prevent neurodegeneration and promote cognitive function. Similarly, chronic deficits of sleep have been involved in other disorders, such as epilepsy (Garg *et al.*, 2022), cardiovascular disorders (Chasens *et al.*, 2021), cognitive decline (Dzierzewski *et al.*, 2022) and others (Shen *et al.*, 2016), and sleep promotion has been suggested as a potential interventional strategy. To test this hypothesis and study the underlying mechanisms, it was first necessary to develop an animal model of chronic sleep enhancement.

A number of chemogenetic studies have found either antagonistic or null effects of repeated and/or chronic dosage of chemogenetic agonists, possibly due to receptor desensitization or neuroadaptive changes (Claes *et al.*, 2022). In this study, to limit metabolite accumulation, we used three previously validated

chemogenetic ligands: clozapine N-oxide (CNO), deschloroclozapine (DCZ) and compound 21 (C21) (Ferrari *et al.*, 2022).We show that chemogenetic activation of PZ^{GABA} results in SWS enhancement of same magnitude, daily, during at least 6-month.

Methods

Animals. A total of 6 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 (S*lc32a1tm2(cre)Lowl*/J, Jackson stock #028862), *EGFP-L10A* (Jackson stock #024750) and B6.CgTg (APPswe,PSEN1dE9)85Dbo/Mmjax (Jackson stock #5864). The study includes 3 males and 3 females, 3 Vgat-Cre+/GFP/APP,PS1+ mice and 3 Vgat-Cre+/GFP/APP,PS1- mice, age 10-21 month old (14.1 ± 0.9 months). Mice were bred at our animal facility and underwent genotyping both before and after experiments, as previously described (Jankowsky *et al.*, 2005; Vong *et al.*, 2011). 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.

Surgery. At the age of 8 weeks, naïve mice were subjected to two independent surgeries separated by four 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, as previously described (Anaclet *et al.*, 2014). 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 & Franklin, 2001). The AAV (200nl) was injected into the PZ of mice using a 10 µl Hamilton syringe (Hamilton Co., Reno, NV) at a rate of 1nl/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 [1mm 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., United States, Catalog #8403) and two flexible electromyogram (EMG) wire electrodes in the neck muscles (Protech International, United States, catalog #E363/76/SPC), previously soldered to a 6-pin connector (Heilind Electronics, United States, 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).

Sleep-Wake Recording. Following the second surgery, 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*. Following 10 days for post-surgical recovery, mice were connected to flexible recording cables and habituated

to the recording conditions for 5 days before starting polygraphic recording. 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 (Biopac Systems Inc, United States).

Drug Administration. During the habituation period, mice were trained to the voluntary oral administration (Jelly) (Mahoney *et al.*, 2019; Ferrari *et al.*, 2022). Vehicle Jelly (control-Jelly) was prepared as follows: gelatine (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, United States) 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 clozapine N-oxide (CNO, 0.3 mg/kg, CNO-Jelly), deschloroclozapine (DCZ, 0.5 mg/kg, DCZ-Jelly) or compound 21 (C21, 3 mg/kg, C21-Jelly), was pipetted into the cap of an Eppendorf tube and placed at 4°C until it forms a jelly again. Before giving 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 within < 1 min following presentation. CNO, DCZ and C21 were obtained from the NIMH Chemical Synthesis and Drug Supply Program.

Experimental timeline. Following the 24-hr baseline recording period, the mice were given a control-Jelly at 10:00 [Zeitgeber time 3 (ZT3)]. Then the mice were given CNO-Jelly (0.3 mg/kg), DCZ-Jelly (0.5 mg/kg) or C21-Jelly (3 mg/kg), daily (ZT3) for a 6-month period. At the end of the drug treatment, mice were recorded for an additional 8-day washout period (Fig. 1). The mice remained connected to the recording cable and were recorded for 2 weeks per month and were left free from the cable for the rest of the month to relieve them from the cable constraints. Cable connection/disconnection as well as the weekly cage change were performed between ZT11-12, when the mice show an increase in wake time in anticipation of the active period, to minimize the sleep-wake disruptions due to the mouse manipulation and to the clean cage.

Sleep Scoring and Analysis. The 24-hr baseline recording, the 24-hr post control-Jelly and the 24-hr post CNO-Jelly, DCZ-Jelly or C21-Jelly from the 2 first administrations of each month were analyzed.

Using SleepSign for Animal (Biopac Systems Inc, United States) assisted by spectral analysis using fast Fourier transform (FFT), polygraphic records were visually scored in 10 s epochs for wakefulness, SWS, and REMS. 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. REMS 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 REMS were summarized for each group and each condition. The SWS and

REMS latencies are defined as the time between the placement of the Jelly on the floor of the cage and the onset of the first SWS episode, lasting >20 s, and the onset of the first REMS 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 containing two vigilance states were visually identified and omitted from the spectral analysis. Re-scoring with a shorter epoch length allows us to provides a greater time resolution and thus minimizes the proportion of total time 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)/0.5–120 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.

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 length of drug dosing on sleep-wake amount and power bands; and 2) one-way ANOVA followed by a post hoc Bonferroni test was used to compare the effect of the length of drug dosing on 24-hr sleep-wake amounts as well as on the SWS and REMS latencies.

Results

To examine whether repeated daily oral administration of chemogenetic agonists produces antagonistic or null effects on sleep measures over time, sleep/wake stages were quantified for the first two 24-hour periods of each month. The duration of daily drug dosing had a significant effect on the percentage of daily wakefulness when CNO [$F (3.145, 15.73) = 3.844$, $P = 0.0292$] was administrated but not DCZ [$F (2.592, 12.96) = 2.698$, $P = 0.0949$] and C21 [$F (2.896, 14.48) = 2.312$, $P = 0.1205$]. The length of drug dosing had a significant effect on the percentage of daily SWS percentages when CNO [$F (3.089, 15.44) = 4.948$, $P = 0.0129$] and C21 [$F (2.921, 14.60) = 10.55$, $P = 0.0006$] were administrated but not DCZ [$F (2.679, 13.40) = 3.041$, $P = 0.0702$]. Similar effects are seen on daily REMS percentages [CNO, $F (2.648, 13.24) = 9.902$, $P = 0.0014$; C21, $F (3.025, 15.13) = 11.41$, $P = 0.0004$; and DCZ, $F (2.853, 14.26) = 3.324$, $P = 0.0520$]. Daily REMS percentages tend to decrease over the 6-month treatment period (**Fig. 2A3,B3,C3**). Overall, a strengthening of the response (i.e. more enhancement of SWS) to chemogenetic agonists was seen in those recording periods that significantly deviated from the initial recording period. This suggests that desensitization is not occurring in our paradigm.

Similarly, we examined sleep onset latency. The length of drug dosing had a significant effect on SWS latency when CNO [$F (1.603, 8.013) = 5.565$, $P = 0.0352$] and DCZ [$F (1.248, 6.242) = 10.44$, $P = 0.0144$] were

administrated but not C21 [F (1.621, 8.105) = 4.415, P = 0.0560]. Even if SWS latencies are about 10 mins following drug administration and 30 mins after control-Jelly, after correction for multiple comparisons (Bonferroni) there were no significant differences between control-Jelly and drug-Jelly (**Fig. 2D1-3**). The length of drug dosing had a significant effect on REMS latency when DCZ [F (2.806, 14.03) = 3.752, P = 0.0382] was administrated but not CNO [F (2.693, 13.46) = 2.561, P = 0.1027] or C21 [F (2.241, 11.20) = 2.143, P = 0.1601]. REMS latencies were not significantly affected by drug administration (**Fig. 2E1-3**).

Analysis of the sleep-wake percentages at all timepoints revealed significant variations between control-Jelly and drug-Jelly (**Fig. 3A1,B1,C1**). The percentage of wakefulness was significantly decreased during the 3-hr following CNO and C21 administration from month 2-6, but only from month 5-6 when DCZ was administrated. Interestingly, the percentage of wakefulness shows an increase over the months of administration, during the 3-hr period preceding the presentation of the drug-Jelly, indicating anticipatory behavior. In fact, the mice were awake at the time of the drug presentation (ZT3), a time the mice are usually sleeping the most. The percentage of SWS was significantly increased during the 3-hr following CNO and C21 administration from the first two administrations of each drug to month 6, but only from month 1-6 when DCZ was administrated (**Fig. 3A2,B2,C2**). Interestingly, the percentage of SWS shows a trend to increase over the 6-month administration. The percentage of REMS was significantly decreased during the entire light period following drug administration (ZT3-6 & ZT6-12; **Fig. 3A3,B3,C3**), from the first two administration of each drug to month 6. No rebound is seen during the following dark period or during the 3-hr light period preceding the next day administration, as was also the case with acute administration in our sleep enhancement paradigm.

The increase in SWS amount is associated with a significant increase in SWA, and the hallmark of PZ-mediated SWS enhancement is a particularly prominent increase in SWA beyond what is seen during normal NREMS. The proportion of delta (0.5-4.5 Hz) frequency band is significantly increased at the same level throughout the 6-month dosing period (**Fig. 4A-C**). This increase is associated with a significant decrease in the proportion of the theta (4.5-10 Hz) frequency band. Interestingly, the proportion of the delta frequency band remains increased during the 8-day washout period following the 6-month dosing (**Fig. 4D**). However, the amount of SWS during this whole 8 -day period was at pre-administration baseline (**Fig. 5**).

Discussion

This study validates a method of chronic SWS enhancement in mice. Our results show that daily administration of a chemogenetic ligand in mice expressing the chemogenetic receptor hM3Dq in PZ^{GABA} increases SWS amount and enhances SWA at the same magnitude throughout a 6-month dosing period. These results validate an approach to specifically promoting a sleep stage over long durations of murine lifespans and are therefore critical to supporting further research into the effects of long-term sleep promotion.

Chronic administration of drugs associated with sleep-wake recording are accompanied with challenges, such as the stress of daily administration, the wake-promoting effect of the weekly cage change and the

constraint of the recording cable. To minimize these effects, we used the voluntary oral administration technique (Jelly) (Ferrari *et al.*, 2022) to administer the chemogenetic ligands. This is particularly important given the potential morbidity (skin breakdown, infection, adhesions, perforated viscus) that can occur with multiple intraperitoneal injections. Daily, the Jelly was placed on the floor of the cage, therefore the mouse was not touched by the experimenter, reducing the stress associated with the administration. The weekly cage changes were performed between ZT11-12, when the mice usually show more wakefulness in anticipation of the upcoming dark period. Finally, the mice were connected to the cable and recorded for 2 weeks, followed by 2 weeks free from the cable. Cable connection/disconnection, were also performed between ZT11-12 to minimize the sleep deprivation due to the wake-promoting effect of the mouse handling.

For daily SWS enhancement, we used three previously validated chemogenetic agonists: CNO, DCZ and C21 (Ferrari et al 2022), given sequentially, to limit potential build-up of metabolites and non-specific actions. The known metabolite of CNO is clozapine which binds multiple endogenous receptors (Meltzer 1994), including the muscarinic receptor from which hM3Dq is derived. Our previous studies showed that acute administration of CNO did not have non-specific effects on the sleep-wake cycle in control mice that do not express hM3Dq in PZ^{GABA} (Anaclet et al 2014). However, we cannot exclude non-specific effects on other physiological functions. Higher doses of CNO induce sleep-wake effects in mice (Traut *et al.*, 2023). DCZ metabolites are DCZ N-oxide and C21 which seem to be pharmacologically inert on endogenous receptors (Nagai et al 2020). Finally, the metabolites of C21 are unknown (Thompson et al 2018). Three-week chronic administration of CNO and C21 in wild-type mice showed a significant but limited beneficial effect on exploration and anxiety (Chen *et al.*, 2023). However, this effect was abolished after a 14-week treatment. These results are consistent with the antipsychotic action of clozapine (Baldessarini & Frankenburg, 1991) and suggest that C21 displays off target effects even at a lower dose. It has been shown that, at the dose used in our study (3 mg/kg), C21 significantly reduces the amount or REMS during the 6-hr post-injection period (Traut *et al.*, 2023). CNO-mediated activation of PZ^{GABA} results a significant reduction of REMS amount lasting about 8-9 hrs (Anaclet *et al.*, 2014), while the SWS enhancement lasts only 3-hr on average. In the present study, REMS amounts are also reduced during the 9-hr period following the administration of the three chemogenetic ligands. Of note, at the dose of 0.3 mg/kg, CNO did not affect REMS amounts in wild-type mice, in our previous study (Anaclet *et al.*, 2014). However, we cannot exclude that the inhibition of REMS amount is at least in part due to non-specific effects of the chemogenetic ligands. Further study will require the use of wild-type control mice to assess the off-target effects of our chronic administration protocol.

Administrations were performed at ZT3, a time when the mice are usually mostly asleep. Nevertheless, the time in SWS was significantly increased during the 3-hr post administration as compared with control administration. Importantly, SWA was significantly enhanced, indicating a deeper stage of sleep. Therefore, SWS can be promoted chronically during the habitual sleeping period. As previously shown in the acute SWS enhancement model (Anaclet *et al.*, 2014), chronic administrations did not significantly increase the total 24-hr SWS amounts either. Similar results are obtained when PZ^{GABA} are activated during the dark period, a time of

high waking drive, the SWS enhancement period is followed by a wake rebound (Anaclet *et al.*, 2014), indicating that the sleep needs were fulfilled and suggesting a potential homeostatic regulation of wakefulness. Eventhough the daily amount of SWS is unchanged when SWS is enhanced during the normal sleep period, the mice experience deeper, more consolidated sleep during the normal sleep time, and this extended and intensified restorative activity may lead to therapeutic benefits (Greene & Frank, 2010; Lee *et al.*, 2020). Interestingly, it was observed that when the experimenter opens the sleep chamber, daily at ZT3, all the mice were awake and waiting for the Jelly delivery. This anticipatory behavior is reflected by the increase in wake time during the ZT0-3 period (Fig. 3A1, B1, C1). These visual observations are reinforced by the short latency to consume the Jelly. In fact, mice immediately approached the Jelly and began eating it within a few seconds after the Jelly was placed in the cage. This indicates that the mice have a fondness for the Jelly itself and quite possibly for the subsequent deep sleep experience. Possibly, this model could be used to further study the interplay between sleep and reward pathways (Hasegawa *et al.*, 2022).

The increase in SWS amount is associated with an increase in the proportion of SWA, as previously shown (Anaclet *et al.*, 2014). The increase in SWA is associated with a decrease of the proportion of theta rhythm, indicating a deeper stage of SWS. This phenotype remains of the same magnitude throughout the 6-month dosing period, indicating no compensatory mechanisms, receptor desensitization or neuronal circuitry fatigue. In fact, SWA tends to increase over the 6-month dosing period, as does the amount and duration of PZ-mediated SWS. Interestingly, SWA remains significantly increased even after the end of the 6-month treatment period when the mice are no longer receiving chemogenetic agonist-infused jelly. This may indicate a plastic effect on the neuronal circuitry regulating SWA during SWS. We hypothesize that high amplitude SWA prompts the circuitry involved in regulating SWA and as a result, deep consolidated sleep will promote further deep consolidated sleep while sleep fragmentation will promote sleep fragmentation. There might be similarities to human sleep hygiene and cognitive behavioral therapy coaching for patients with insomnia, whereby small initial changes build upon each other and result in long-lasting beneficial sleep pattern changes. The mechanisms underlying these observations could also be studied using this model.

SWS enhancement is accompanied by a reduction of REMS time. Therefore, our mouse model could open the door to specifically studying the role of SWS in various phenomena including memory, neurodegeneration, and other neurologic diseases. This could be paired with methods of REM sleep enhancement (Weber *et al.*, 2015; Stucynski *et al.*, 2022) to disentangle the role of these sleep states. The reduction of REMS following the administration lasts longer than the SWS enhancement period and is not compensated by a rebound, indicating that the reduction in REMS did not induce a dramatic lack requiring homeostatic rebound. In fact, following sleep deprivation, the REMS rebound never fully compensates for the lack of REMS (Achermann & Borbely, 2011), indicating that REMS duration is not strictly regulated. Whereas REMS is necessary for various neurological functions (Mukai & Yamanaka, 2023), excessive REMS can be associated with mood disorders and depression (Yasugaki *et al.*, 2023). REMS deprivation elicits positive effects on mood and is used as a treatment in patients suffering from severe depression (Hemmeter *et al.*, 2010). We

also observed a trend to decreased REMS daily amounts during the course of the 6-month period, suggesting a negative entrainment of the neuronal circuitry regulating REMS. Similar to the chronic SWA enhancement, it is possible that chronic REMS inhibition modifies the circuitry leading to a new, reduced baseline amount. And on the opposite, increased amount of REMS would promote the REM-on neuronal circuitry. Because REMS amounts are increased in patients suffering from depression (Yasugaki *et al.*, 2023), and REMS deprivation positively impacts the mood (Hemmeter *et al.*, 2010), this mechanism might be involved in mood control. This hypothesis remains to be studied.

In conclusion, we showed that our mouse model permits long-term SWS enhancement and could be an appropriate tool to study the beneficial role of SWS in chronic conditions such as aging, Alzheimer's disease and epilepsy.

References

Achermann, P. & Borbely, A.A. (2011) Sleep Homeostasis and Models of Sleep Regulation. In Kryger, M.H., Roth, T., Dement, W.C. (eds) *Principles and Practice of Sleep Medicine*. Saunders, W.B., pp. 431-444.

Anaclet, C., Ferrari, L., Arrigoni, E., Bass, C.E., Saper, C.B., Lu, J. & Fuller, P.M. (2014) The GABAergic parafacial zone is a medullary slow wave sleep-promoting center. *Nat Neurosci*, **17**, 1217-1224.

Anaclet, C., Pedersen, N.P., Ferrari, L.L., Venner, A., Bass, C.E., Arrigoni, E. & Fuller, P.M. (2015) Basal forebrain control of wakefulness and cortical rhythms. *Nat Commun*, **6**, 8744.

Baldessarini, R.J. & Frankenburg, F.R. (1991) Clozapine. A novel antipsychotic agent. *N Engl J Med*, **324**, 746-754.

Chasens, E.R., Imes, C.C., Kariuki, J.K., Luyster, F.S., Morris, J.L., DiNardo, M.M., Godzik, C.M., Jeon, B. & Yang, K. (2021) Sleep and Metabolic Syndrome. *Nurs Clin North Am*, **56**, 203-217.

Chen, C.W., Kwok, Y.T., Cheng, Y.T., Huang, Y.S., Kuo, T.B.J., Wu, C.H., Du, P.J., Yang, A.C. & Yang, C.C.H. (2023) Reduced slow-wave activity and autonomic dysfunction during sleep precede cognitive deficits in Alzheimer's disease transgenic mice. *Scientific Reports*, **13**, 1-17.

Claes, M., De Groef, L. & Moons, L. (2022) The DREADDful Hurdles and Opportunities of the Chronic Chemogenetic Toolbox. *Cells*, **11**.

Dzierzewski, J.M., Perez, E., Ravyts, S.G. & Dautovich, N. (2022) Sleep and Cognition: A Narrative Review Focused on Older Adults. *Sleep Med Clin*, **17**, 205-222.

Ferrari, L.L., Ogbeide-Latario, O.E., Gompf, H.S. & Anaclet, C. (2022) Validation of DREADD agonists and administration route in a murine model of sleep enhancement. *J Neurosci Methods*, **380**, 109679.

Garg, D., Charlesworth, L. & Shukla, G. (2022) Sleep and Temporal Lobe Epilepsy - Associations, Mechanisms and Treatment Implications. *Front Hum Neurosci*, **16**, 849899.

Greene, R.W. & Frank, M.G. (2010) Slow wave activity during sleep: functional and therapeutic implications. *Neuroscientist*, **16**, 618-633.

Grubb, S. & Lauritzen, M. (2019) Deep sleep drives brain fluid oscillations. *Science*, **366**, 572-573.

Hasegawa, E., Miyasaka, A., Sakurai, K., Cherasse, Y., Li, Y. & Sakurai, T. (2022) Rapid eye movement sleep is initiated by basolateral amygdala dopamine signaling in mice. *Science*, **375**, 994-1000.

Hemmeter, U.M., Hemmeter-Spernal, J. & Krieg, J.C. (2010) Sleep deprivation in depression. *Expert Rev Neurother*, **10**, 1101-1115.

Holth, J.K., Fritschi, S.K., Wang, C., Pedersen, N.P., Cirrito, J.R., Mahan, T.E., Finn, M.B., Manis, M., Geerling, J.C., Fuller, P.M., Lucey, B.P. & Holtzman, D.M. (2019) The sleep-wake cycle regulates brain interstitial fluid tau in mice and CSF tau in humans. *Science*, **363**, 880-884.

Jankowsky, J.L., Slunt, H.H., Gonzales, V., Savonenko, A.V., Wen, J.C., Jenkins, N.A., Copeland, N.G., Younkin, L.H., Lester, H.A., Younkin, S.G. & Borchelt, D.R. (2005) Persistent amyloidosis following suppression of Abeta production in a transgenic model of Alzheimer disease. *PLoS Med*, **2**, e355.

Lee, Y.F., Gerashchenko, D., Timofeev, I., Bacskai, B.J. & Kastanenka, K.V. (2020) Slow Wave Sleep Is a Promising Intervention Target for Alzheimer's Disease. *Front Neurosci*, **14**, 705.

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

Malkani, R.G. & Zee, P.C. (2022) Brain Stimulation for Improving Sleep and Memory. *Sleep Med Clin*, **17**, 505-521.

Minakawa, E.N., Wada, K. & Nagai, Y. (2019) Sleep Disturbance as a Potential Modifiable Risk Factor for Alzheimer's Disease. *Int J Mol Sci*, **20**.

Mukai, Y. & Yamanaka, A. (2023) Functional roles of REM sleep. *Neurosci Res*, **189**, 44-53.

Paxinos, G.T. & Franklin, K. (2001) *The mouse brain in stereotaxic coordinates*. Academic, San Diego.

Shen, X., Wu, Y. & Zhang, D. (2016) Nighttime sleep duration, 24-hour sleep duration and risk of all-cause mortality among adults: a meta-analysis of prospective cohort studies. *Sci Rep*, **6**, 21480.

Stucynski, J.A., Schott, A.L., Baik, J., Chung, S. & Weber, F. (2022) Regulation of REM sleep by inhibitory neurons in the dorsomedial medulla. *Curr Biol*, **32**, 37-50 e36.

Traut, J., Mengual, J.P., Meijer, E.J., McKillop, L.E., Alfonsa, H., Hoerder-Suabedissen, A., Song, S.H., Feher, K.D., Riemann, D., Molnar, Z., Akerman, C.J., Vyazovskiy, V.V. & Krone, L.B. (2023) Effects of clozapine-N-oxide and compound 21 on sleep in laboratory mice. *Elife*, **12**.

Vong, L., Ye, C., Yang, Z., Choi, B., Chua, S., Jr. & Lowell, B.B. (2011) Leptin Action on GABAergic Neurons Prevents Obesity and Reduces Inhibitory Tone to POMC Neurons. *Neuron*, **71**, 142-154.

Weber, F., Chung, S., Beier, K.T., Xu, M., Luo, L. & Dan, Y. (2015) Control of REM sleep by ventral medulla GABAergic neurons. *Nature*, **526**, 435-438.

Yasugaki, S., Okamura, H., Kaneko, A. & Hayashi, Y. (2023) Bidirectional relationship between sleep and depression. *Neurosci Res*.

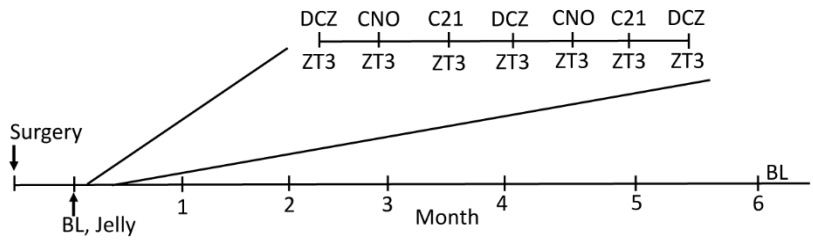


Figure 1: **Experimental timeline.** Two weeks after surgery, the mice were recorded in the baseline condition (BL, 24-hr) followed by control administration of Jelly at ZT3. Then the mice received daily administration of CNO, DCZ or C21, in Jelly, at ZT3 for 6 months. At the end of the administration period, the mice were recorded for 10-day wash-out period.

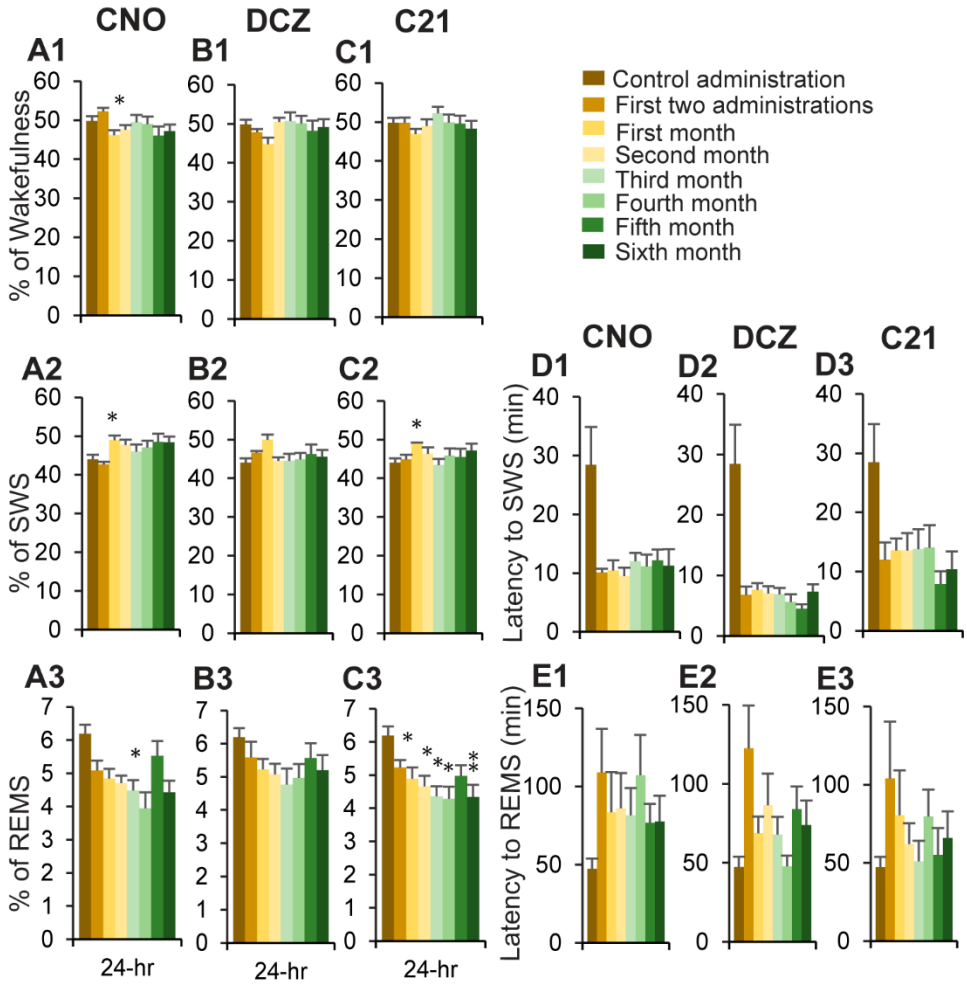
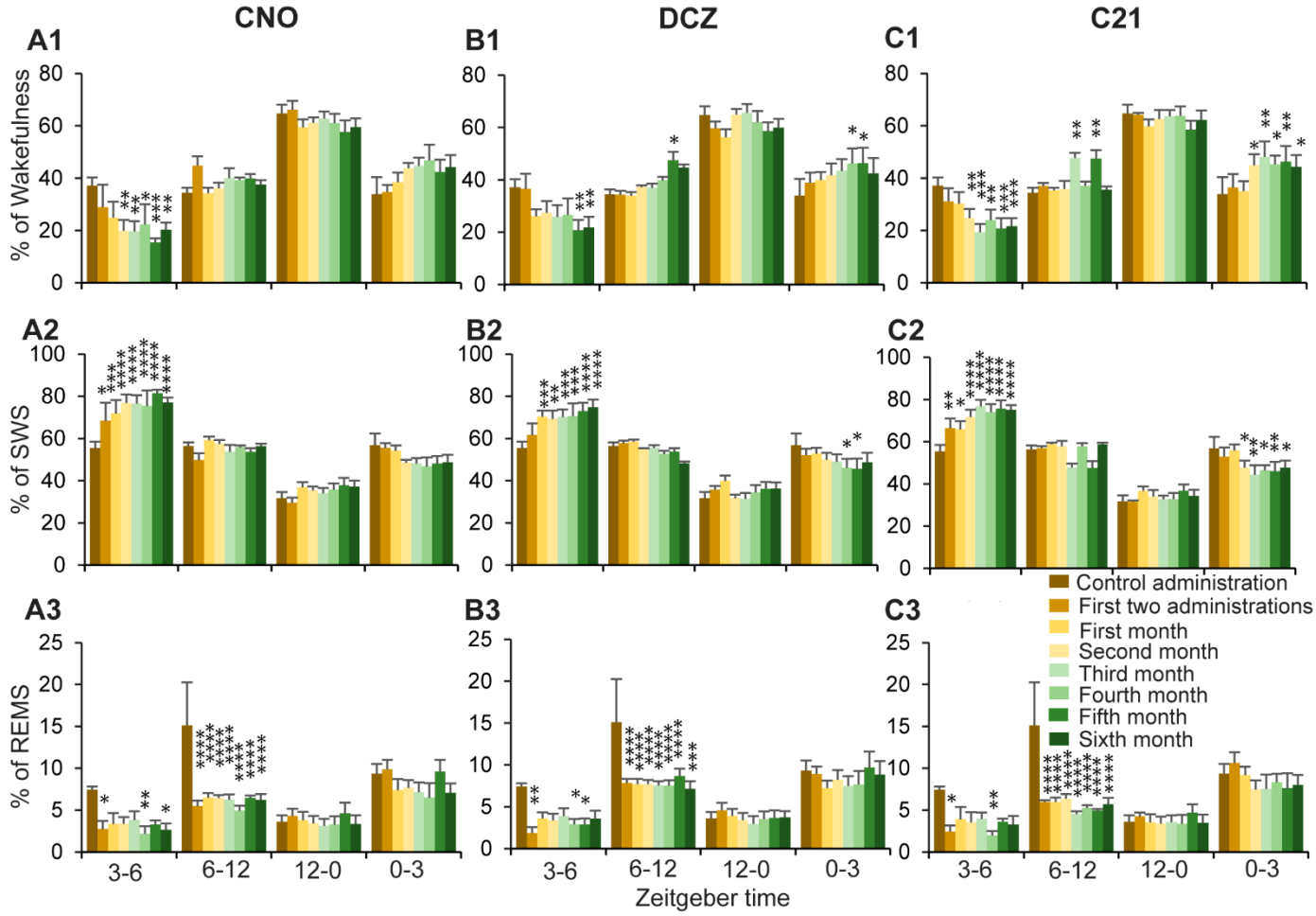


Figure 2: 24-hr sleep-wake percentages and sleep latencies. (**A-C**) Daily percentage (± SEM) of wakefulness (**A1,B1,C1**), SWS (**A2,B2,C2**) and REMS (**A3,B3,C3**) following administration of CNO (**A**), DCZ (**B**) or C21 (**C**) and from the two first administration of each month. (**D-E**) Latency to SWS (**D**) and to REMS (**E**) following placement of the CNO-Jelly (**D1,E1**), DCZ-Jelly (**D2,E2**) or C21-Jelly (**D3,E3**) in the cage. N = 6 mice. Significant differences compared with control administration, * $p < 0.05$, ** $p < 0.01$, one-way ANOVA followed by Bonferroni test.



<u>Figure 3:</u> Percentage of sleep-wake states during the 6-month dosing. (**A-C**) Percentage (± SEM) of wakefulness (**A1,B1,C1**), SWS (**A2,B2,C2**) and REMS (**A3,B3,C3**) following administration of CNO (**A**), DCZ (**B**) or C21 (**C**) and from the two first administrations of each month, between ZT3-6, ZT6-12, ZT12-0 and ZT0-3. N = 6 mice. Significant differences compared with control administration, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA followed by Bonferroni test.

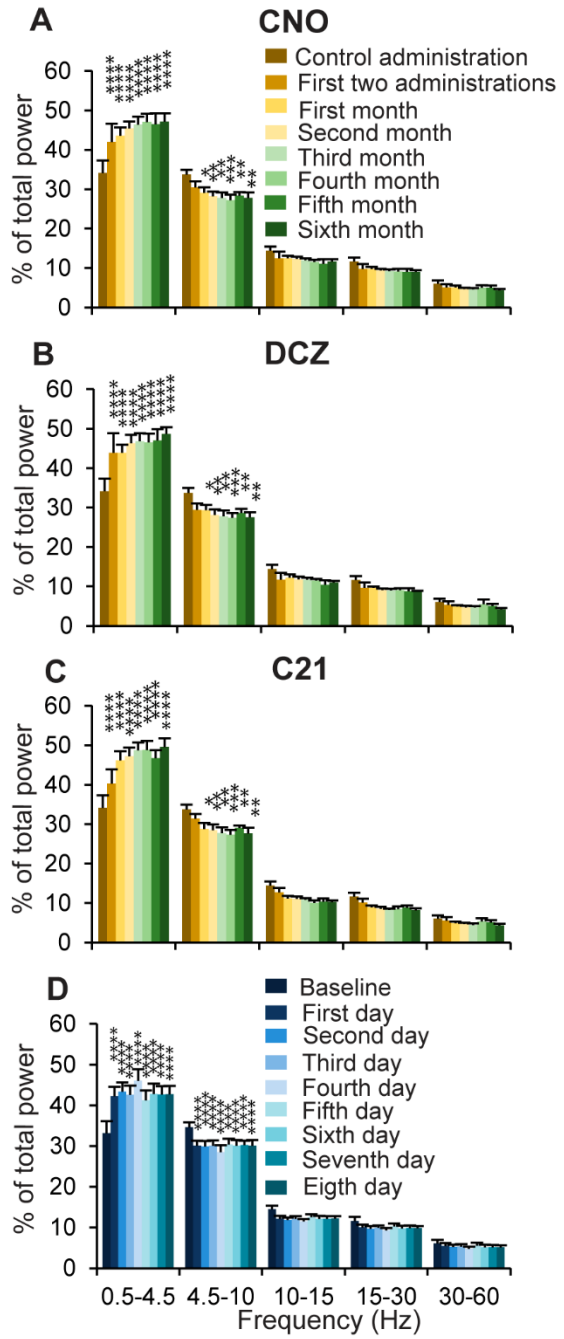
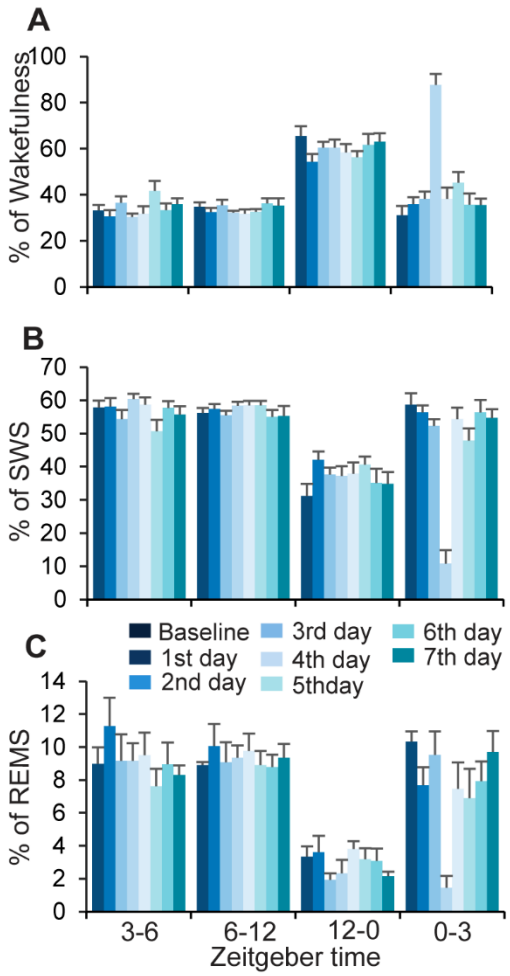


Figure 4: Cortical power bands. Percentage (± SEM) of total (0.5-60 Hz) power from the first hour of SWS following administration of CNO (**A**), DCZ (**B**) or C21 (**C**) and from the two first administrations of each month. N = 6 mice. Significant difference as compared with control administration, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA followed by Bonferroni test. (**D**) Percentage (± SEM) of total (0.5-120 Hz) power during SWS from ZT3-6, from the first baseline recording before the 6-month administration period and from the eight days following the end of the 6-month administration period. Significant difference as compared with the first baseline recording, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA followed by Bonferroni test.



<u>Figure 5:</u> Percentage of sleep-wake states during the washout period following the 6-month dosing. (**A-C**) Percentage (± SEM) of wakefulness (**A**), SWS (**B**) and REMS (**C**) during the 7-day following discontinuation of the chemogenetic agonist dosing, between ZT3-6, ZT6-12, ZT12-0 and ZT0-3. N = 6 mice. No significance differences, two-way ANOVA followed by Bonferroni test.