Effects of clozapine-N-oxide and compound 21 on sleep in laboratory mice

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Abstract

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are chemogenetic tools that modulate a targeted cell population using chemical ligands which bind specifically to modified receptors. Despite the common use of DREADDs in neuroscience and sleep research, potential effects of the DREADD actuator clozapine-N-oxide (CNO) on sleep, which could arise from back-metabolism to clozapine or binding to endogenous neurotransmitter receptors, have never been systematically tested. Here we show that intraperitoneal injections of commonly used doses of CNO alter sleep in wild-type male laboratory mice. Using electroencephalography (EEG) and electromyography (EMG) to analyse sleep, we found that CNO suppresses rapid eye-movement (REM) sleep, changes EEG spectral power during non-REM (NREM) sleep, and alters sleep architecture in a pattern previously reported for clozapine. Interestingly, we found that the novel DREADD actuator, compound 21 (C21), similarly modulates sleep despite not being able to back convert to clozapine. Our results demonstrate that CNO and C21 can affect the sleep of wild-type mice, which do not express DREADD receptors. This implies that back-metabolism to clozapine is not the sole mechanism underlying side effects of chemogenetic actuators and therefore any chemogenetic experiment should include a DREADD-free control group injected with the same CNO, C21 or newly developed actuator. We suggest that electrophysiological sleep assessment could serve as a sensitive tool to test the biological inertness of novel chemogenetic actuators.

49 # Introduction

50 Chemogenetics is an important and commonly used experimental approach in sleep research
51 (Weber and Dan 2016; Varin and Bonnavion 2019) and could serve as a novel therapeutic
52 strategy in sleep medicine (Venner et al. 2019). Designer Receptors Exclusively Activated by
53 Designer Drugs (DREADDs) enable non-invasive and cell-type specific remote control of
54 neuronal activity in freely moving animals on a time scale of minutes to hours (Roth 2016). In
55 the context of sleep research, these characteristics make DREADD technology a powerful tool
56 with which to probe the contribution of selected neuronal populations in controlling vigilance
57 states (Hayashi et al. 2015; Varin, Luppi, and Fort 2018; Yu et al. 2019; Mondino et al. 2021),
58 sleep-state specific network oscillations (Funk et al. 2017; Vaidyanathan et al. 2021), as well
59 as sleep-related physiology (Harding et al. 2018; Fleury Curado et al. 2018) and behaviour
60 (Eban-Rothschild et al. 2016; Tossell et al. 2020).

61 Typically, intraperitoneal injections of clozapine-N-oxide (CNO) are used to activate excitatory
62 hM3Dq or inhibitory hM4Di DREADDs (Campbell and Marchant 2018). Early work suggested
63 that CNO is pharmacologically inert (Armbruster et al. 2007) and not back metabolized to its
64 parent drug clozapine in mice (Guettier et al. 2009). However, more recent studies
65 demonstrated relevant conversion of CNO to pharmacologically active metabolites including
66 clozapine (Gomez et al. 2017; Manvich et al. 2018; Jendryka et al. 2019). Clozapine is an
67 atypical antipsychotic drug used in the treatment of schizophrenia with a high affinity for
68 dopamine D2 and serotonin 5-HT2A coupled with a broad binding profile to cholinergic,
69 adrenergic, histaminergic and serotonergic receptors (Wenthur and Lindsley 2013), which
70 may account for its high efficacy compared to other antipsychotics (Kane et al. 1988). In
71 addition, CNO itself was found to present off-target binding at a broad range of

neurotransmitter receptors (Gomez et al. 2017; Jendryka et al. 2019) and to elicit behavioural effects (Gomez et al. 2017; Manvich et al. 2018; MacLaren et al. 2016) at doses commonly used for DREADD experiments.

It is widely thought that CNO does not affect sleep (Harding et al. 2018; Mondino et al. 2021; Naganuma et al. 2018; Erickson et al. 2019) and many chemogenetic sleep studies include convincing control experiments with DREADD-free animals that demonstrate the absence of relevant effects of the chosen CNO preparations and doses on the assessed sleep parameters (Erickson et al. 2019; Mondino et al. 2021; Takata et al. 2018; Yu et al. 2019; Anaclet et al. 2015; Venner et al. 2016). A comprehensive assessment of putative dose-dependent effects of CNO on sleep in wild-type mice has never been systematically conducted, however a recent study reported that high CNO doses affected sleep in DREADD-free control animals (Varin, Luppi, and Fort 2018). This is an important omission, given that clozapine is known to modulate sleep in humans (Hinze-Selch et al. 1997; Monti, Torterolo, and Pandi Perumal 2017; Riemann and Nissen 2012) and laboratory rodents (Spierings, Dzoljic, and Godschalk 1977; Sorge, Pollmächer, and Lancel 2004; Grønli et al. 2016; Coward et al. 1989). Many of the endogenous neurotransmitter receptors, which are drug targets of clozapine (Wenthur and Lindsley 2013) and to which CNO presents off-target binding affinity (Jendryka et al. 2019; Gomez et al. 2017), are also involved in the regulation of arousal and sleep (Saper and Fuller 2017).

Initially intended as a control experiment, we here tested whether commonly used doses of CNO and the novel DREADD agonist compound 21 (C21), which does not convert to clozapine (Thompson et al. 2018), but has an off-target binding profile similar to CNO (Jendryka et al. 2019), affect sleep in wild-type C57BL/6J mice under laboratory conditions. We find dose-

95 dependent effects of CNO on the duration, architecture, and electrophysiological signature

96 of sleep. In addition, we observed sleep-modulatory effects of C21.

97

98 # Results

99 **Clozapine-N-oxide suppresses REM sleep**

100 We first assessed the time spent in wakefulness, non-rapid eye movement (NREM) and rapid
101 eye-movement (REM) sleep in hourly intervals over the first six hours of the light period
102 following injections of CNO or saline at light onset (Figure 1a). There was no significant main
103 effect of the treatment condition on the proportion of time spent awake or in NREM and no
104 interaction effect between treatment condition and time point. However, we observed a
105 significant main effect of the treatment condition on the percentage of REM sleep ($F_{(2, 30)}$ =
106 5.601, p = 0.009), but no interaction between the time point and the treatment condition
107 (Figure 1a).

108 CNO concentrations in blood plasma, cerebrospinal fluid (CSF) and brain tissue of mice peak
109 within the first 15-30 minutes after both intraperitoneal and subcutaneous injections
110 (Jendryka et al. 2019; Manvich et al. 2018), and behavioural side effects are typically tested
111 within the first two hours following drug administration (MacLaren et al. 2016; Manvich et al.
112 2018; Gomez et al. 2017). Yet, CNO effects might persist for much longer. To investigate this
113 possibility more closely, we next analysed the effect of CNO on vigilance states in an acute (0-
114 2 hours) and prolonged (0-6 hours) time window (Figure 1b). There was a significant effect of
115 CNO treatment on REM sleep both in the acute ($F_{(2, 22)}$ =8.951, p = 0.002) and prolonged time
116 window ($F_{(2, 23)}$ = 7.525, p = 0.004). Effect size calculations for the post-hoc comparisons
117 between the individual CNO conditions and the saline condition indicated medium to large
118 effects of CNO on REM sleep (Supplementary Table 1).

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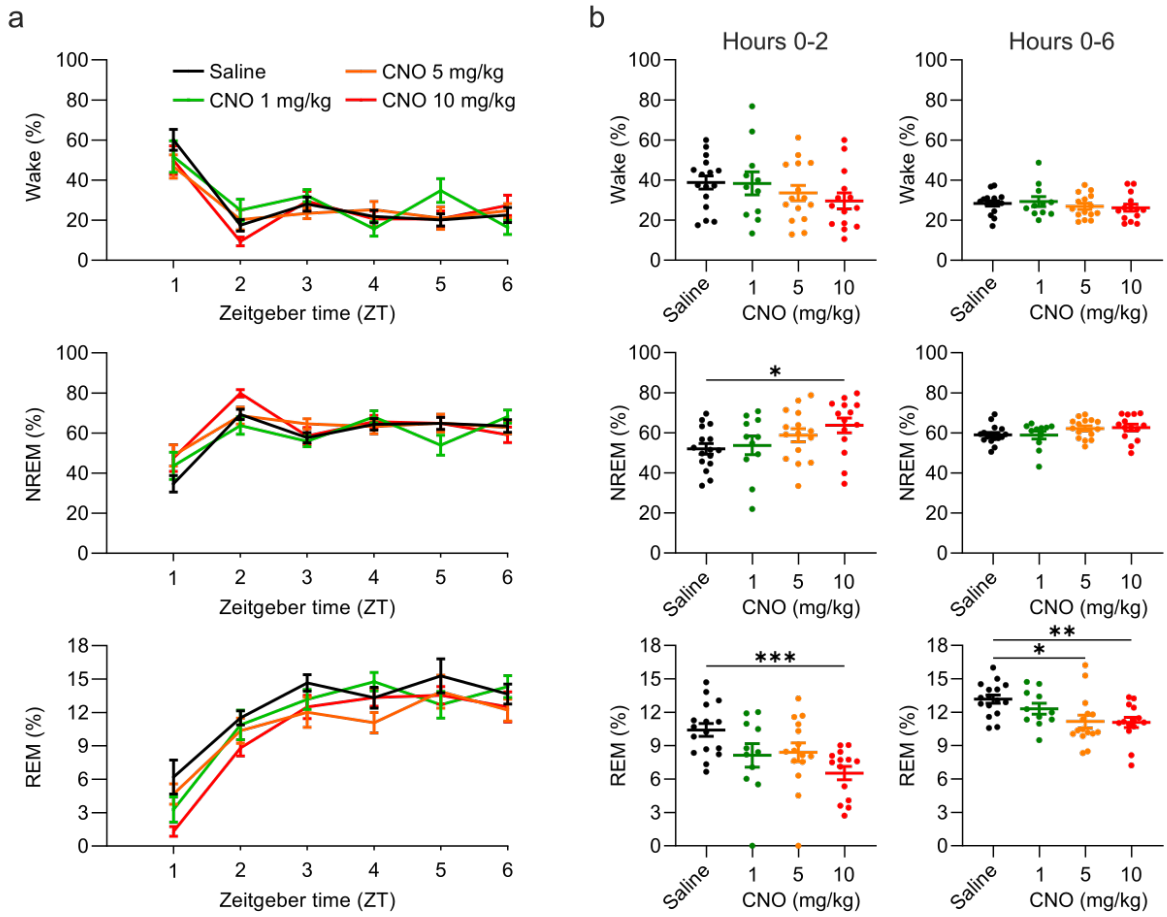


Figure 1: Suppression of REM sleep following CNO injection

a) Time course of wakefulness, NREM, and REM sleep in the six hours following subcutaneous injections of CNO or saline at light onset (ZT 0). b) Percentage of time spent in the three vigilance states during the first two hours (left column) and over the entire six-hour observation period after saline and CNO injections. Note that there is no main effect of the treatment condition on NREM sleep in the acute time window ($F_{(2,24)} = 3.054$, $p = 0.068$). Therefore, the significant post-hoc contrast for the 10 mg/kg condition has to be interpreted with caution. Percentage of REM sleep expressed as proportion of total sleep time. n=16 for saline, n=11 for 1 mg/kg, n=15 for 5 mg/kg, n=14 for 10 mg/kg for vigilance state analysis. Asterisks indicate post-hoc contrasts with significant differences (*P < 0.05, **P < 0.01, ***P < 0.001). CNO: clozapine-N-oxide. EEG: electroencephalogram. NREM: non-rapid eye movement sleep. REM: rapid eye movement sleep. ZT: Zeitgeber time.

Clozapine-N-oxide alters sleep architecture

Physiological sleep in mammals is typically entered through NREM sleep and characterised by the alternation between NREM and REM sleep episodes. The average timing, duration, and frequency of NREM and REM episodes in mice vary slightly depending on the genetic

138 background but are kept within tight limits for individual strains (Franken, Malafosse, and
139 Tafti 1999; Huber, Deboer, and Tobler 2000). The effects of psychotropic drugs on sleep are
140 often most prominent in sleep architecture parameters (Riemann and Nissen 2012). For
141 example, clozapine evokes characteristic changes in sleep architecture in humans (Monti,
142 Torterolo, and Pandi Perumal 2017), rats (Sorge, Pollmächer, and Lancel 2004; Spierings,
143 Dzoljic, and Godschalk 1977), and mice (Grønli et al. 2016). Hence, the analysis of sleep
144 architectural parameters is of paramount importance in assessing whether a pharmacological
145 compound modulates sleep.
146 Characteristics of both NREM and REM episodes were altered by CNO injections (Figure 2a).
147 CNO injections were followed by longer (main effect NREM episode duration: $F_{(2, 30)} = 11.64$,
148 $p < 0.001$) but fewer (main effect NREM episode number: $F_{(2, 31)} = 7.796$, $p = 0.001$) NREM
149 sleep episodes. These effects appeared to be dose-dependent, with higher doses eliciting
150 stronger effects (Figure 2b). The latency between the injection and the onset of NREM sleep
151 was also significantly affected by CNO injections ($F_{(2,26)} = 3.380$, $p = 0.046$, Figure 2b).
152 However, the reduction of the NREM sleep latency was only statistically significant in the
153 1 mg/kg condition. REM sleep episodes were significantly shorter following CNO injections
154 ($F_{(2, 40)} = 4.509$, $p = 0.013$) and the latency between sleep onset and the first transition to REM
155 sleep was significantly changed ($F_{(2, 28)} = 6.985$, $p = 0.002$) with delayed REM onset in the
156 1 mg/kg and 10 mg/kg condition (Figure 2b). In contrast to the effects on sleep states, neither
157 the duration nor the number of wake episodes was modulated by CNO injections
158 (Supplementary Table 2). In summary, we observed longer but fewer NREM episodes
159 following CNO injections in a dose-dependent fashion, and a similar but less pronounced
160 change in the duration and frequency of REM episodes. Furthermore, CNO injections
161 accelerated sleep onset, but delayed the transition to REM sleep, particularly at low doses.

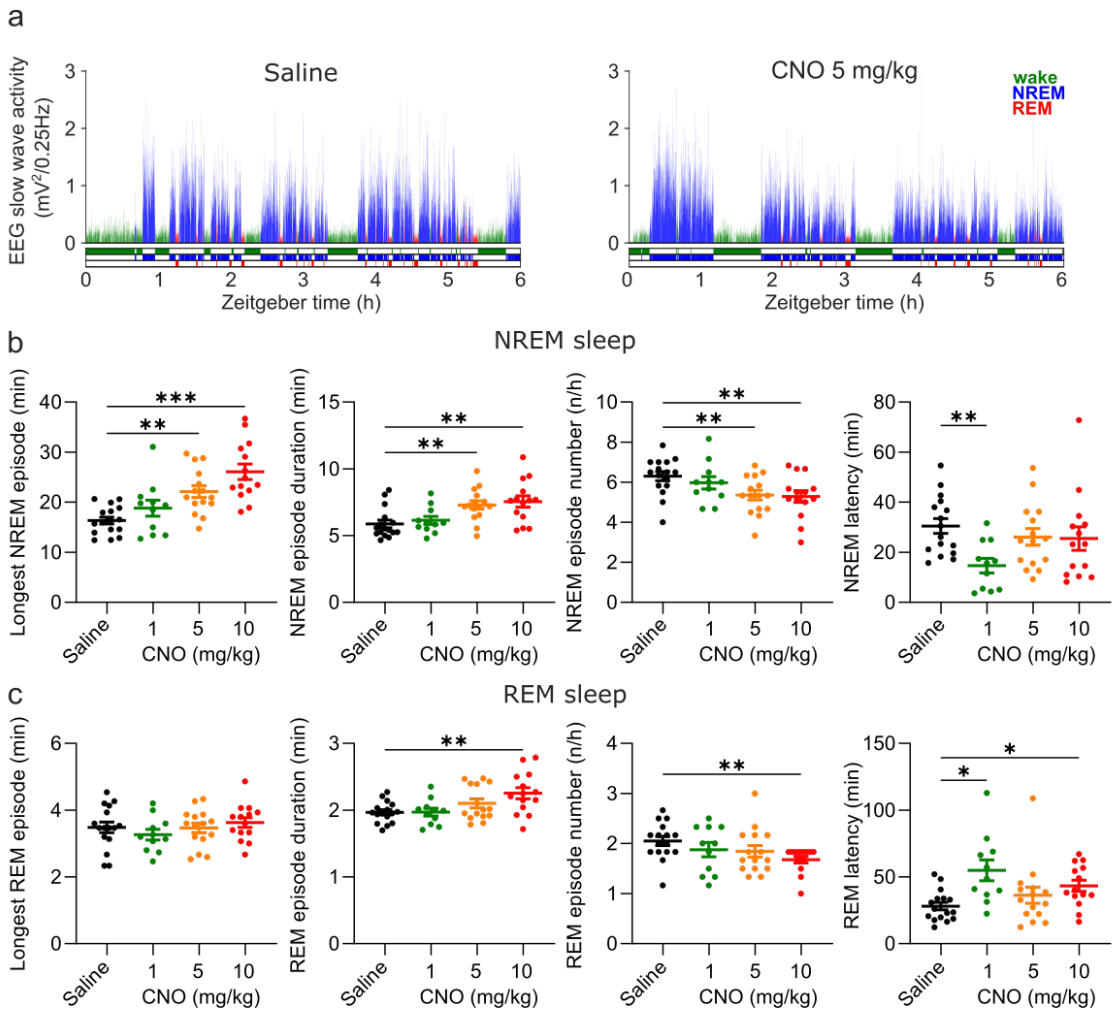


Figure 2: Altered sleep architecture following CNO injections

a) Representative hypnograms and EEG slow wave activity (0.5- 4.0 Hz, 4-s epochs) from one individual mouse after injection of saline (left panel) and 5 mg/kg CNO (right panel). Note the reduced latency to NREM sleep, the suppression of REM sleep, and the increased duration of individual NREM sleep episodes. b) NREM sleep architecture following saline and CNO injections. c) REM sleep architecture following saline and CNO injections. Note that there is no main effect of CNO treatment on the number of REM sleep episodes ($F_{(3,33)} = 2.680$, $p = 0.069$). Therefore, the significant post-hoc contrast for the 10 mg/kg condition has to be interpreted with caution. n=16 for saline, n=11 for 1 mg/kg, n=15 for 5 mg/kg, n=14 for 10 mg/kg for vigilance state analysis in panels b and c. Asterisks indicate post-hoc contrasts with significant differences (*P < 0.05, **P < 0.01, ***P < 0.001). CNO: clozapine-N-oxide. EEG: electroencephalogram. NREM: non-rapid eye movement sleep. REM: rapid eye movement sleep.

Clozapine-N-oxide affects the NREM spectrogram and sleep consolidation

In addition to sleep time and architecture, EEG spectra are typically analysed in sleep studies. We therefore assessed whether CNO affects EEG spectral power. The focus of the EEG

180 spectral analysis was on the comparison between the medium dose (5 mg/kg) of CNO and
181 saline during NREM sleep. We observed that CNO injections were followed by a small but
182 significant increase in spectral power in the range between 0.5 and 1.25 Hz and suppression
183 of spectral power in nearly all frequency bins between 6 and 30 Hz during NREM sleep over
184 the first two hours (Figure 3a and Supplementary Figure 1). While the increase in slow
185 frequency bins during NREM sleep appeared to be temporary, the suppression of power
186 above 6 Hz was significant for the entire six-hour observation period. Spectral analysis of
187 wakefulness and REM sleep did not reveal any systematic effects of CNO (Supplementary
188 Figure 1). A comparison of all three CNO conditions with saline injection indicated similar
189 effects of CNO on the NREM sleep spectrogram in the high dose (10 mg/kg CNO), but not in
190 the low dose (1 mg/kg CNO) condition (Figure 1).
191 Elevated spectral power in slow frequencies, longer but fewer NREM episodes, and less REM
192 sleep are typically observed during the initial recovery sleep following sleep deprivation,
193 when sleep is more consolidated (Huber, Deboer, and Tobler 2000). To explore whether the
194 stability of sleep is affected by CNO, we assessed the cumulative amount of NREM sleep
195 before the first REM episode (1 min or longer of REM sleep), as well as the frequency of brief
196 awakenings (4-16 second intrusions of wake-like EMG and EEG during sleep), which is a
197 behavioural marker of sleep continuity (Franken et al. 1991). Both measures were significantly
198 changed after CNO injection (NREM before REM: $F_{(3, 33)} = 5.071$, $p = 0.007$; brief awakenings:
199 $F_{(2, 24)} = 10.38$, $p < 0.001$). Effect size calculations indicated a medium to strong increase in
200 NREM sleep before REM onset and reduction in brief awakenings for all CNO doses
201 (Supplementary Table 2).

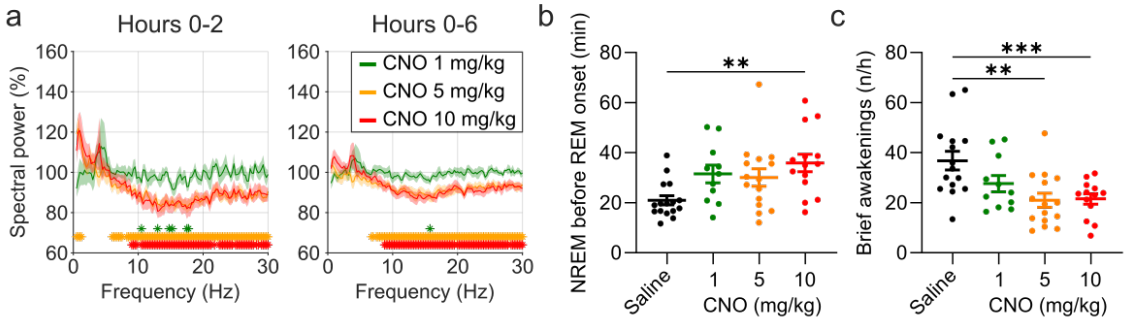


Figure 3: EEG spectral changes and increased sleep continuity following CNO injections

a) Frontal EEG spectra during NREM sleep relative to saline injections for the acute (first 2 hours) and prolonged (6 hours) observation period. b) Cumulative amount of NREM sleep before the first REM sleep episode. c) Frequency of brief awakenings (4-16 sec) per hour of sleep for the first 2 hours after injections. n=10 for saline, n=6 for 1 mg/kg, n=10 for 5 mg/kg, n=8 for 10 mg/kg for spectral analysis. n=16 for saline, n=11 for 1 mg/kg, n=15 for 5 mg/kg, n=14 for 10 mg/kg for vigilance state analysis. n=15 for saline, n=11 for 1 mg/kg, n=15 for 5 mg/kg, n=13 for 10 mg/kg for analysis of brief awakenings. Asterisks in panels b and c indicate post-hoc contrasts with significant differences (*P < 0.05, **P < 0.01, ***P < 0.001). Asterisks in panel a indicate frequency bins with significant differences in post-hoc comparison (P < 0.05). Data in a are presented as the mean ± s.e.m. (shaded areas). CNO: clozapine-N-oxide. EEG: electroencephalogram. NREM: non-rapid eye movement sleep.

Compound 21 has sleep-modulatory effects similar to clozapine-N-oxide

Based on recent reports indicating that back-metabolism of CNO to clozapine causes behavioural effects in rodents (Ilg et al. 2018; Gomez et al. 2017; Manvich et al. 2018; MacLaren et al. 2016) and might affect sleep (Varin, Luppi, and Fort 2018), we postulated that the effects of CNO injections on sleep could be avoided by using alternative DREADD ligands. However, another possibility, is that it is the off-target binding of CNO to endogenous receptors that causes or contributes to the change in sleep patterns. In this scenario, the effects of CNO on sleep would be, at least in part, mediated by direct action of the DREADD actuator at neurotransmitter receptors, which are involved in the regulation of sleep and could not be overcome by minimising the conversion to clozapine. To discriminate between these two possibilities, we investigated whether the next generation DREADD actuator C21 (Chen et al. 2015) has sleep-modulatory effects similar to those observed after CNO

228 injections. C21 does not back-convert to clozapine *in vivo* (Thompson et al. 2018) but has an
229 almost identical profile of binding affinities to endogenous neurotransmitter receptors as
230 CNO (Jendryka et al. 2019).
231 Intraperitoneal injections of C21 at a dose of 3 mg/kg modulated sleep compared to saline
232 (Figure 4 and Figure 5). REM sleep was significantly reduced over the six-hour observation
233 period ($t_{(6)} = 3.234$, $p = 0.009$, Cohen's d = -1.22). The numerical reduction of REM sleep in the
234 acute, two-hour, time window following C21 injections did not reach statistical significance in
235 this small sample of seven mice ($t_{(6)} = 1.829$, $p = 0.059$, Cohen's d = -0.70; Figure 4b), but had
236 an effect size comparable to that of the statistically significant REM sleep reduction in the
237 same time window after 5 mg/kg CNO injections.

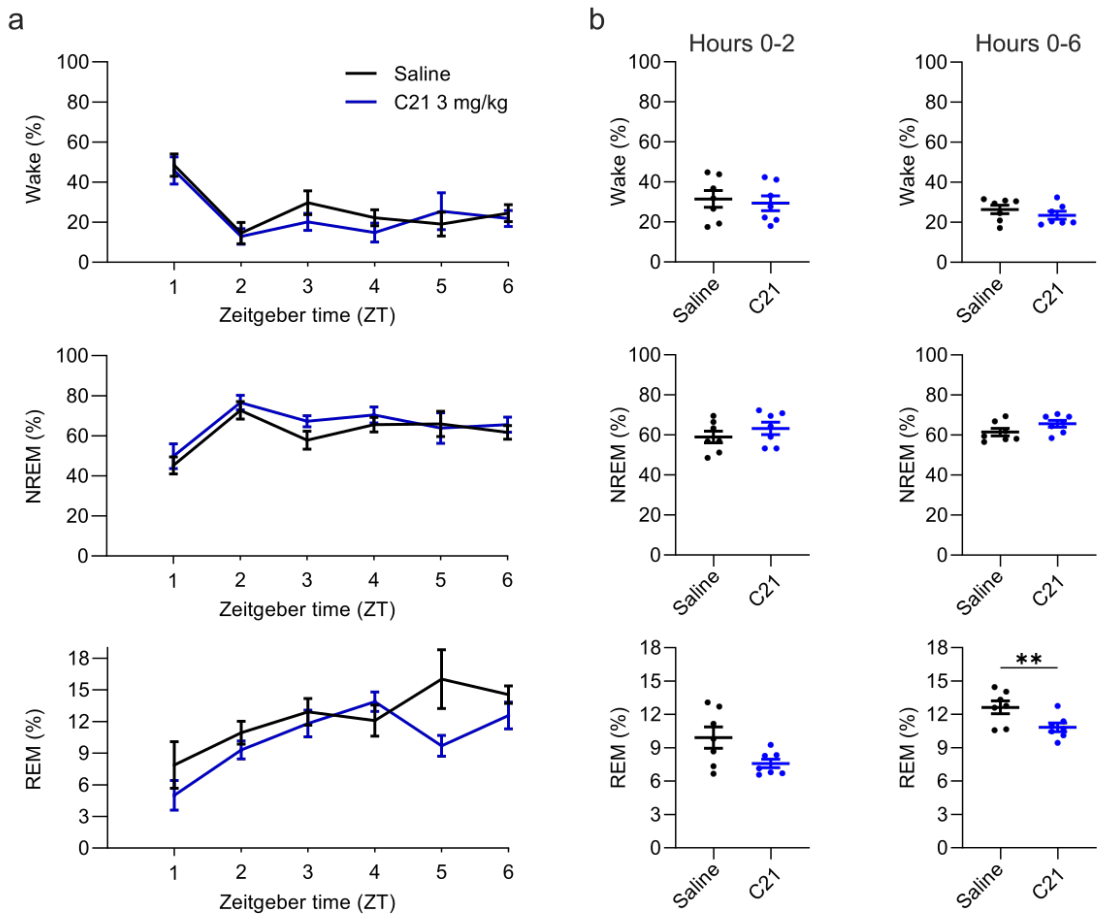


Figure 4: Suppression of REM sleep following C21 injections

a) Time course of wakefulness, NREM, and REM sleep in the six hours following injection of C21 or saline at light onset (ZT 0). b) Percentage of time spent in the three vigilance states during the first two hours (left column) and over the entire six-hour observation period after saline and C21 injections. Percentage of REM sleep expressed as proportion of total sleep time. n=7. Asterisks indicate post-hoc contrasts with significant differences (*P < 0.05, **P < 0.01, ***P < 0.001). C21: compound 21. EEG: electroencephalogram. NREM: non-rapid eye movement sleep. REM: rapid eye movement sleep. ZT: Zeitgeber time.

In addition to the suppression of REM sleep, C21 elicited significant changes in sleep architecture (Figure 5a,b). The maximum and average duration of NREM sleep episodes was increased (maximum duration: $t_{(6)} = 2.551$, $p = 0.022$, Cohen's d = 0.96; average duration: $t_{(6)} = 2.462$, $p = 0.025$, Cohen's d = 0.93) while the number of NREM sleep episodes in the first six hours following C21 injection was reduced ($t_{(6)} = 2.809$, $p = 0.015$, Cohen's d = -1.06; Figure 5a). The average duration of REM episodes was also increased ($t_{(6)} = 2.229$, $p = 0.034$, Cohen's

254 d = 0.84; Figure 5b). For all NREM and REM architecture parameters that were significantly
255 changed after CNO injections, effect sizes of 3 mg/kg C21 were similar to that of 5 mg/kg CNO
256 compared to saline, even if the post-hoc comparisons did not reach significance in this small
257 sample (*Supplementary Table 3*). NREM spectra showed an increase in the very slow
258 frequency bins and a suppression of frequencies above 6 Hz (Figure 5c) – a pattern which
259 resembled that of the medium and high dose CNO injections (Figure 3a). Markers of sleep
260 consolidation were also significantly altered as a result of C21 injections. The amount of NREM
261 sleep before the first occurrence of REM sleep was increased ($t_{(6)} = 2.252$, $p = 0.033$, Cohen's
262 d = 0.85) and brief awakenings were reduced ($t_{(5)} = 2.164$, $p = 0.041$, Cohen's d = -0.52; Figure
263 5d,e).

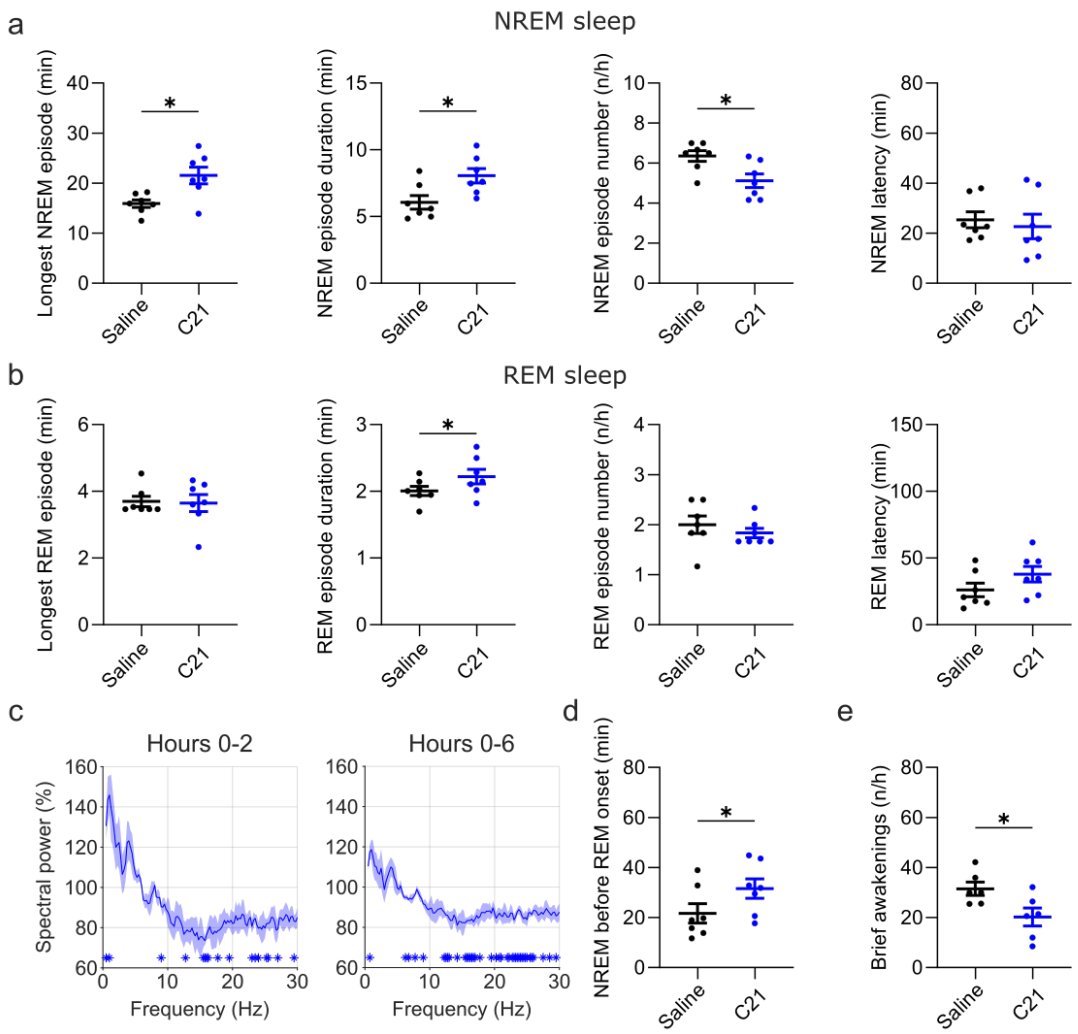


Figure 5: Changes in sleep architecture, NREM sleep spectra, and sleep continuity measures following C21 injections

a) NREM sleep architecture. b) REM sleep architecture. c) Frontal EEG spectra during NREM sleep relative to saline injections for the acute (2 hour) and full (6 hour) observation period following C21 injections. d) Cumulative amount of NREM sleep before the first REM sleep episode. e) Frequency of brief awakenings (4-16 sec) per hour of sleep for the first 2 hours after injections. Number of animals n=7 mice for vigilance state analysis in panels a, b and d. For spectral analysis in panel c: n=3. For analysis of brief awakenings in panel e: n=6 mice. Asterisks in panels a, b, d and e indicate post-hoc contrasts with significant differences (*P < 0.05, **P < 0.01, ***P < 0.001). Asterisks in panel c indicate frequency bins with significant differences in post-hoc comparison (P < 0.05). Data in c are presented as the mean ± s.e.m. (shaded areas). C21: compound 21. EEG: electroencephalogram. NREM: non-rapid eye movement sleep. REM: rapid eye movement sleep.

280 # Discussion

281 **CNO and C21 injections have clozapine-like effects on sleep**

282 Our study demonstrates that the chemogenetic actuators CNO and C21 can modulate sleep
283 in wild-type laboratory mice which do not express DREADD receptors. Both substances led to
284 a suppression of REM sleep and affected sleep architecture and NREM sleep spectra in a
285 pattern consistent with more consolidated sleep. The sleep changes following CNO and C21
286 injections in wild-type mice bore striking similarities with those previously reported for
287 clozapine in rats (Sorge, Pollmächer, and Lancel 2004; Spierings, Dzoljic, and Godschalk 1977),
288 and humans (Hinze-Selch et al. 1997). In particular, the initial suppression of REM sleep and
289 the occurrence of longer but fewer NREM episodes were the most consistent dose-dependent
290 effects of clozapine on sleep in male Wistar rats, leading the authors to conclude that
291 clozapine has sedative effects, suppresses REM initiation and increases sleep maintenance in
292 rats (Sorge, Pollmächer, and Lancel 2004). Interestingly, in rats low doses of clozapine (2.5
293 mg/kg) had immediate sleep-promoting effects while high doses of clozapine (7.5 mg/kg)
294 initially promoted wakefulness before a sustained increase NREM sleep after the first two
295 hours (Sorge, Pollmächer, and Lancel 2004). This might explain why in our study NREM and
296 REM sleep latency were most strongly affected by the lowest dose (1 mg/kg) of CNO. Our
297 findings of sleep modulatory effects of CNO are in line with previous reports of behavioural
298 side effects of CNO resulting from back-metabolism to clozapine (Gomez et al. 2017; Manvich
299 et al. 2018). However, the fact that we observed similar changes in sleep after injections with
300 C21, a DREADD actuator that does not convert to clozapine (Thompson et al. 2018), suggests
301 that *in vivo* metabolism to clozapine conversion is not the sole mechanism through which
302 DREADD actuators can elicit unwanted effects.

303

304 **Possible mechanisms underlying sleep-modulatory effects of CNO and C21**

305 In our view, the most parsimonious explanation for the observed sleep-modulatory effects of
306 CNO and C21 is off-target binding at endogenous neurotransmitter receptors. It has been
307 shown that CNO is a competitive inhibitor of several neurotransmitter receptors, including
308 histaminergic H1, serotoninergic 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, muscarinic M1, M2, M3,
309 M4, adrenergic α1A and α2A, and dopaminergic D1 and D2 receptors (Gomez et al. 2017;
310 Jendryka et al. 2019). C21 has an off-target binding profile similar to CNO (Jendryka et al.
311 2019). In addition, a recent study reported increased firing rates of nigral dopaminergic
312 neurons in wild-type rats, indicating that C21 can elicit direct neuromodulatory effects in
313 rodents (Goutaudier et al. 2020). Among several endogenous receptors relevant for sleep
314 regulation, CNO and C21 induce a strong competitive inhibition at histamine H1-receptors
315 (Jendryka et al. 2019). H1-receptor knockout and pharmacological antagonism of the H1
316 receptor in mice both result in a reduced number of brief awakenings, fewer but longer NREM
317 sleep episodes, and a reduced latency to NREM sleep (Huang et al. 2006). In addition,
318 antihistamines which induce drowsiness, are known to strongly suppress REM sleep, but can
319 be acutely NREM-promoting at low doses and wake-promoting at high doses, respectively
320 (Ikeda-Sagara et al. 2012). Therefore, we speculate that the shared sleep-modulatory effects
321 of CNO and C21 might be due to direct antihistaminergic action. However, a contribution of
322 other shared off-target sites of CNO and C21 such as the 5-HT_{2A} receptors, which are thought
323 to mediate the locomotor suppression after high doses of clozapine (McOmish et al. 2012),
324 should also be taken into consideration.

325

326 **Previous indications for sleep-modulatory effects of CNO**

327 Many chemogenetic sleep studies provide adequate control data, which excludes relevant
328 effects of CNO on sleep in the given experimental paradigms (Erickson et al. 2019; Mondino
329 et al. 2021; Takata et al. 2018; Yu et al. 2019; Anaclet et al. 2015; Venner et al. 2016). Many
330 other studies do not present or analyse data of CNO-injected controls but instead only
331 compare CNO versus saline conditions in DREADD-expressing animals. However, occasionally
332 authors have mentioned that the use of CNO injections of up to 10 mg/kg may have affected
333 the sleep of animals in DREADD-free control groups (Funk et al. 2017) and recent work
334 focussing on REM sleep regulation presented statistically significant effects of CNO doses
335 higher than 5 mg/kg in the supplementary data (Varin, Luppi, and Fort 2018). Another well-
336 controlled study found a slight increase in NREM sleep bout duration of control mice injected
337 with 0.3 mg/kg CNO, while all other analysed parameters were unaffected by this small CNO
338 dose (Liu et al. 2021). This finding supports our effect size analysis indicating that NREM sleep
339 bout duration is the sleep architectural parameter most strongly affected by CNO and C21
340 and that CNO can cause sleep-modulating effects at doses of 1 mg/kg CNO, and possibly
341 below.

342

343 **Implications for future use of chemogenetics in sleep research**

344 Previous work has highlighted that several factors such as age, sex and strain of the
345 experimental animals (Manvich et al. 2018), as well as differences in the activity of the
346 cytochrome P450 enzymes converting CNO to clozapine (Mahler and Aston-Jones 2018),
347 might contribute to the variability of side effects of DREADD actuators. In addition, the galenic
348 formulation and use of solvents can alter the pharmacokinetic properties of DREADD
349 actuators (Campbell and Marchant 2018). The hydrochloride salt preparations of CNO used

350 here and in other recent sleep studies (Fernandez et al. 2018; Stucynski et al. 2021) have
351 reduced back-metabolism to clozapine and an improved water solubility and bioavailability
352 compared to equivalent doses of CNO-DMSO preparations (D. C. Allen et al. 2019). The
353 increased bioavailability and thereby supposedly amplified off-target receptor binding might
354 explain that we find sleep-modulating effects of CNO already at doses where other studies
355 have shown no effects. Considering the difficulty to predict behavioural effects of DREADD
356 actuators in a specific experimental paradigm, our study supports the proposal that it is vital
357 to minimise the injected doses of DREADD actuators and to include CNO-injected non-
358 DREADD-expressing control animals in each individual experiment (MacLaren et al. 2016;
359 Mahler and Aston-Jones 2018; Campbell and Marchant 2018). More importantly, our work
360 shows that avoiding clozapine back-metabolism of CNO by using novel DREADD actuators
361 such as C21 does not prevent behavioural side effects. Interestingly, another next generation
362 DREADD agonist, perlapine, which is structurally similar to C21 (Chen et al. 2015; Thompson
363 et al. 2018), is a REM-suppressing sedative and muscle relaxant used in the treatment of
364 insomnia (Ando et al. 1970; S. R. Allen and Oswald 1973; Stille et al. 1973). Chemogenetic
365 approaches that do not require actuators with high chemical similarity to clozapine might
366 provide an alternative for sleep research (Magnus et al. 2019). Considering the complexity of
367 neurotransmitter systems regulating sleep (Saper and Fuller 2017) and the sensitivity of sleep
368 architecture to pharmacological intervention (Riemann and Nissen 2012), testing the effects
369 of new chemogenetic actuators, such as deschloroclozapine (Nagai et al. 2020) or JHU37152
370 and JHU37160 (Bonaventura et al. 2019), on sleep might prove not only useful for sleep
371 research but could become a valuable tool in evaluating the biological inertness of newly
372 developed chemogenetic actuators.
373

374 **Limitations**

375 We would like to highlight that this study was initiated as a control experiment and was not
376 designed to assess dose-dependent effects of CNO. Instead of counterbalancing all
377 experimental conditions, we counterbalanced the saline and the 5 mg/kg CNO condition for
378 the first two sessions to enable a direct comparison between saline and a medium dose of
379 CNO avoiding potential adaptation effects resulting from repeated CNO injections. The
380 decision to conduct a full study and to include C21 injections as an additional condition was
381 only made once visual inspection of pilot data from four animals had indicated relevant
382 effects of CNO on sleep. Because injections of 1 and 10 mg/kg CNO and 3 mg/kg C21 were
383 always performed after the initial two injections, sequence effects for those conditions
384 cannot be excluded. This should particularly be considered in the interpretation of the results
385 on NREM and REM sleep latency, which do not show a dose-dependency. In order to reduce
386 the number of animals used in laboratory research, most of the animals (13 out of 16) were
387 used for combined experiments including other procedures such as light presentation with
388 light emitting diodes (LEDs) or local intracortical microinfusions. The type of experiments, the
389 duration of the rest interval, and the within-subject design of our study make it unlikely that
390 our findings were confounded by previous experiences but this cannot be fully excluded. It
391 should further be noted that the sample size of C21-injected animals was very small (n=7) and
392 all analyses concerning C21 effects should be considered exploratory. To avoid
393 misinterpretation of one sided null-hypothesis testing we provided effect sizes for all pairwise
394 comparisons (*Supplementary Table 3*).

395

396 **Conclusions**

397 In conclusion, our study suggests that the DREADD actuators, CNO and C21, have sleep-
398 modulatory effects, which cannot be explained by back-metabolism to clozapine alone but
399 might result from off-target binding to endogenous receptors. While our results require
400 replication in an optimised experimental design, our findings have important implications for
401 the future application of chemogenetics in sleep research and neuroscience. Our study
402 highlights the need to use non-DREADD-expressing controls, even when novel actuators are
403 used that cannot convert to clozapine. Considering the sensitivity of sleep architectural
404 parameters to CNO and C21 demonstrated here, our work reveals a new opportunity of using
405 simple EEG/EMG sleep screening to assess the pharmacological inertness of novel
406 chemogenetic actuators *in vivo*. We are confident that these experimental refinements of the
407 DREADD approach, plus novel technological improvements, will unleash the full potential of
408 this powerful tool in behavioural neuroscience and help pave the way for its clinical
409 application.

410

411 # Materials and Methods

412 **Animals**

413 Sixteen young adult male C57BL/6J mice (age: 113±6 days, weight: 25.2±0.5 g) were used in
414 this study. All animals were sourced internally from the Biomedical Services at the University
415 of Oxford. As this study was originally designed as a control experiment, we did not intend to
416 implant animals exclusively for this project. In order to reduce the number of animals used in
417 laboratory research, 13 animals were implanted for combined sleep experiments involving
418 procedures for this project and related work. All 16 animals used in this study were implanted
419 with a right frontal EEG screw, a reference EEG screw above the cerebellum, and EMG wires
420 in the neck muscles as described previously (Fisher et al. 2016). In addition to this EEG/EMG
421 configuration, which provided the electrophysiological signals analysed in this manuscript,
422 five of the animals were implanted with a frontal left and bilateral occipital EEG screws as well
423 as with an anchor screw in the midline anterior to the frontal EEG screws, which served as a
424 socket for a detachable light-emitting diode (LED) device (Figure 6a); four animals were
425 implanted with a right occipital EEG screw and a cannula (C315I; PlasticsOne) targeted to layer
426 5 of the primary somatosensory cortex (Figure 6b); four animals received an additional frontal
427 and occipital EEG screw over the left hemisphere as well as a left cerebellar ground screw and
428 a right occipital 16-channel laminar probe as well as a midline frontal anchor screw for the
429 detachable LED device (Figure 6c); three animals received a left frontal and occipital EEG
430 screw (Figure 6d). All animals had a rest interval of at least three days between the previous
431 experiment and this study.

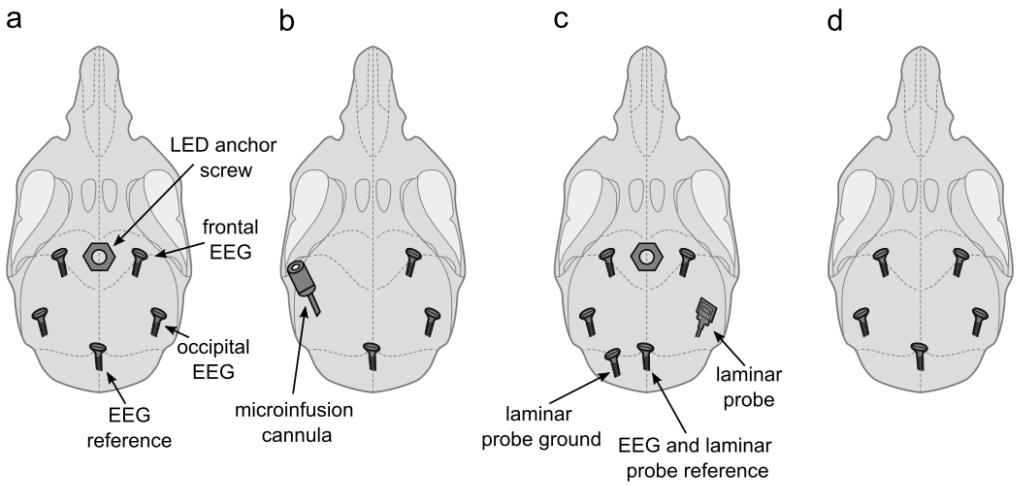


Figure 6: Implant configurations

a) LED anchor screw, bilateral frontal and occipital EEG screws, and cerebellar reference screw. Implant configuration of n=5 mice. b) microinfusion cannula, right frontal and occipital EEG screws, and a cerebellar reference screw. Implant configuration of n=4 mice. c) LED anchor screw, bilateral frontal and left occipital EEG screws, right occipital laminar probe, and cerebellar ground and reference screws. Implant configuration of n=4 mice. d) bilateral frontal and occipital EEG screws, and cerebellar reference screw. Implant configuration of n=3 mice. LED: light-emitting diode. EEG: electroencephalogram.

The nine animals implanted with a socket for the placement of a detachable LED device received flickering light stimulation to one of the eyes combined with a 4-h sleep deprivation on up to four experimental days before being used for our study. All animals had at least three rest days without experimental interventions before the first injections of CNO or saline for the study presented here. The four animals implanted with a cannula in the left primary motor cortex received intracortical microinfusions of bumetanide for a transient and localised blockade of the Na-K-2Cl cotransporter NKCC1 (Kahle and Staley 2008), as well as of VU0436271 for a transient and localised blockade of the chloride potassium symporter KCC2 (Sivakumaran et al. 2015). Two of these animals underwent two intracortical microinfusions and the other two animals underwent three intracortical microinfusions before inclusion in this study. The last infusion took place 12 days before the start of the study presented here. Due to the limit of five injection or infusion procedures on the UK Home Office project license

454 under which our experiments were conducted, these animals could only be subjected to three
455 and two i.p. injections of different CNO doses and saline. The three animals implanted with
456 bilateral frontal and occipital EEG screws were not used for any additional experiments. Only
457 seven animals, the four animals implanted with laminar probes and the three animals used
458 exclusively for this study, were used for C21 injections as this condition was added to the
459 experimental protocol after the pilot data from CNO injections had been obtained.
460
461 **Electrophysiological signal acquisition, data processing, and sleep scoring**
462 EEG/EMG recordings were performed using the 128 Channel Neurophysiology Recording
463 System (Tucker-Davis Technologies Inc., Alachua, FL, USA) and the electrophysiological
464 recording software Synapse (Tucker-Davis Technologies Inc., Alachua, FL, USA). Raw data was
465 stored on a local computer in 24-h recording blocks. During the continuous recordings EEG
466 and EMG signals were filtered between 0.1 - 100 Hz, and stored at a sampling rate of 305 Hz.
467 After transfer to an analysis desktop computer, the raw signals were resampled at a sampling
468 rate of 256 Hz using custom-made code in Matlab (The MathWorks Inc, Natick,
469 Massachusetts, USA, version v2020a) and converted into the European Data Format (EDF) as
470 previously described (McKillop et al. 2018). The EDF files were visually scored in individual 4-s
471 epochs by blinded experimenters using the software package Sleep Sign for Animals
472 (SleepSign Kissei Comtec Co., Ltd., Nagano, Japan). If EEG signals contained temporary
473 artefacts due to electrical noise, movements, or chewing, the respective vigilance state was
474 assigned to the respective epoch but the EEG signals were not included in the spectral
475 analysis. Using the Sleep Sign for Animals software, fast Fourier transform routine (Hanning
476 window) with a 0.25 Hz resolution was computed in the frequency range between 0 and 30 Hz
477 for each individual 4-s epoch.

478 **Clozapine-N-oxide and compound 21 products**

479 To avoid the use of the toxic solvent dimethyl sulfoxide (DMSO), which is typically used for
480 the preparation of CNO products in concentrations of up to 15% (Campbell and Marchant
481 2018), we opted for the use of a water-soluble salt preparation of CNO, clozapine-N-oxide
482 dihydrochloride (Tocris, Bio-Techne LTD, Abingdon, UK, catalog no.: 6329) dissolved in sterile
483 saline. The dihydrochloride preparation of CNO undergoes less back-metabolism to clozapine
484 but has a higher bioavailability compared to CNO-DMSO as indicated by pharmacokinetic
485 work in rhesus macaques (D. C. Allen et al. 2019). This product has previously been used in
486 sleep studies on mice at concentrations between 1 mg/kg and 5 mg/kg (Fernandez et al. 2018;
487 Stucynski et al. 2021). For C21 injections we used the water-soluble version of DREADD
488 agonist compound 21 (compound 21 dihydrochloride, Tocris, Bio-Techne LTD, Abingdon, UK,
489 catalog no.: HB6124). We chose a dose of 3 mg/kg because a detailed pharmacokinetic
490 assessment of this product at this specific concentration as well as behavioural testing in a
491 5-choice serial-reaction-time task, which did not reveal any behavioural effects at this dose
492 (Jendryka et al. 2019).

493

494 **Experimental design**

495 All 16 animals received a medium dose of CNO (5 mg/kg, 0.25 mg/ml solution) and an
496 equivalent volume of saline in a counterbalanced order with a 72-h rest interval between the
497 first two experimental sessions. Of those 16 mice, fourteen also received a 10 mg/kg CNO
498 injection (0.5 mg/ml solution) and twelve a 1 mg/kg CNO (0.05 mg/ml solution). These two
499 injections were counterbalanced as third and fourth injections. Seven animals also received a
500 3 mg/kg (0.15 mg/ml) C21 injection in a fifth experimental session. This semi-counterbalanced
501 design was chosen to ensure that at the medium dose condition is counterbalanced with

502 saline injections to circumvent putative habituation or adaptation effects following repeated
503 injection of CNO.
504 Animals were on a regular light-dark cycle (lights on at 9 am) and intraperitoneal (i.p.)
505 injections were performed within 15 minutes after light onset following a brief health check.
506 The delay between light onset and injection was (mean±s.e.m.): saline: 4.19±0.87 min,
507 1 mg/kg CNO: 4.58±0.59, 5 mg/kg CNO: 3.52±1.04 min, 10 mg/kg CNO: 4.75±0.59 min,
508 3 mg/kg C21: 6.01±0.96 min. Individual recording sessions were separated by a rest interval
509 of at least 72 hours. The recording chambers were kept open for approximately 10-15 minutes
510 after the injection to monitor for potential adverse effects. The chambers were then closed
511 and the animals checked remotely at regular intervals for the first 6-12 hours after injection.
512 For data analysis, all recordings were aligned to the time point of injections.
513
514 **Sample size determination and power analysis**
515 Sample size and power calculations were performed using G*Power 3.1, an open-source
516 statistical power analysis program (Faul et al. 2007). The sample size was chosen based on
517 previous experiments in our lab investigating the effects of the sedative diazepam on sleep
518 (McKillop et al. 2021), which indicated an effect size of Cohen's d = 0.90 for the key outcome
519 parameter NREM sleep time. We therefore decided that our study should be sufficiently
520 powered to detect effects of sizes of d=1 designed between saline and individual CNO
521 treatment conditions with a power of 0.9 at the given α-error probability of 0.05. The
522 estimated sample size from this calculation was 12-13 animals per group. Based on
523 experiences from previous EEG studies in mice, we aimed to account for an attrition rate of
524 approximately 20% and decided to include 16 animals in this study.
525

526 **Statistical procedures**

527 Data were analysed using MATLAB (version R2020a; The MathWorks Inc, Natick, MA, USA),
528 SAS JMP (version 7.0; SAS Institute Inc. Cary, NC, USA) and IBM SPSS Statistics for Windows
529 (version 25.0; IBM Corp., Armonk, N.Y., USA). Reported averages are mean±s.e.m. For all main
530 effects and interactions an α-error probability of 0.05 was adopted. For the statistical
531 comparison of CNO and saline injections mixed-effect models were calculated using
532 GraphPad Prism (version 9.1.1 for Windows; GraphPad Software, San Diego, California, USA,
533 www.graphpad.com). Significant main effects of treatment conditions or interaction terms
534 were followed up with Dunnett's adjustment for post-hoc comparisons. In mixed-effect
535 models with multiple time points we analysed acute (0-2 hours post injection) and prolonged
536 (0-6 hours post injection) effects separately. For spectral analysis, EEG/LFP power spectra of
537 individual animals were log-transformed before hypothesis testing. Individual spectral bins
538 were compared between individual CNO treatments and saline used two-tailed t-tests. No
539 correction was applied for post-hoc comparison of spectral data because ANOVAs consisting
540 of 119 EEG spectral bins would be too conservative and reduce statistical power (Achermann
541 and Borbély 1998); thus, α was kept at 0.05 in these cases. Comparisons between the C21 and
542 saline condition were performed using one-tailed *t*-tests to assess whether sleep variables
543 were changed in the same direction as after CNO treatment. For analysis of the percentage
544 of time spent in the three vigilance states, wakefulness and NREM sleep were expressed as
545 the percentage of time in the respective time window, REM was expressed as percentage of
546 total sleep time in the respective time window as in previous work (Huber, Deboer, and Tobler
547 1999). For sleep architecture analysis, NREM and REM sleep episodes were defined as
548 intervals of at least one minute with allowing an interruption of 4-16 seconds. Brief
549 awakenings are defined as up to 16 second interruptions of sleep by wake-like EEG and EMG

patterns (Franken et al. 1991). In all figures, significance levels of post-hoc comparisons are indicated with black asterisks: '*' for 0.05 ≥ p > 0.01; '**' for 0.01 ≥ p > 0.001; '***' for 0.001 ≥ p. Effect sizes are reported as Cohen's d calculated using the MATLAB function computeCohen_d (Ruggero G. Bettinardi (2020). computeCohen_d(x1, x2, varargin) (https://www.mathworks.com/matlabcentral/fileexchange/62957-computecohen_d-x1-x2-varargin), MATLAB Central File Exchange. Retrieved October 4, 2020). Data from one animal had to be partially excluded because of a defective EEG headstage. This animal had to be excluded from the analysis of brief awakenings due to EMG artefacts, which made it difficult to identify a sudden increase of muscle tone during sleep. The same animal also had to be excluded from the analysis of the 1 mg/kg and 5 mg/kg CNO treatments because of technical issues. Six animals had to be excluded from spectral analysis due to occasional artefacts in the EEG signals.

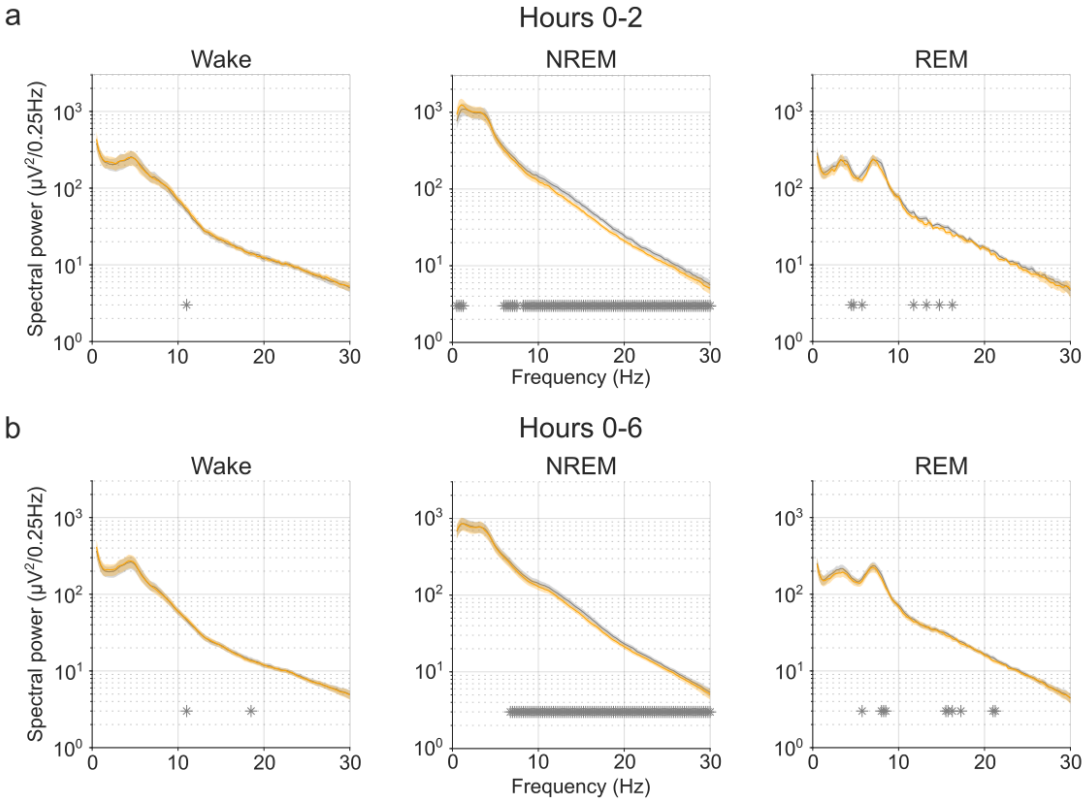
Ethical approval

All experiments were performed in accordance with the United Kingdom Animal Scientific Procedures Act 1986 under personal and project licences granted by the United Kingdom Home Office. Ethical approval was provided by the Ethical Review Panel at the University of Oxford. Animal holding and experimentation were located at the Biomedical Sciences Building (BSB) and the Behavioural Neuroscience Unit (BNU), University of Oxford.

Data and code availability

We invite readers to further explore the dataset used for this publication. Raw data and custom written MatLab code are available from the corresponding author upon reasonable request.

Supplementary materials



Supplementary Figure 1: EEG power spectra of wakefulness, NREM, and REM sleep following injections of saline and 5 mg/kg CNO

Frontal EEG spectra in 0.25 Hz bins between 0.5 and 30 Hz arranged by vigilance state and time window. Frequency bins with significant differences in uncorrected post-hoc testing are indicated with grey asterisks. Note the systematic increase of slow frequencies (0-1.25 Hz) in the first 2 hours and the suppression of spectral power in higher frequencies (6.25-30 Hz) in the NREM sleep spectrogram both in the first 2 hours and over the entire observation period. n=10 for saline (grey) and for 5 mg/kg CNO (orange) in spectral analysis. Data are presented as the mean ± s.e.m. (shaded areas). CNO: clozapine-N-oxide. EEG: electroencephalogram. NREM: non-rapid eye movement sleep. REM: rapid eye movement sleep.

591 **Supplementary Table 1: Time spent in wakefulness, NREM, and REM sleep after CNO and**
592 **saline injections**

		Treatment condition (mean values ± standard error)				Mixed-effect analysis		Effect size for post-hoc comparisons (Cohen's d)		
Vigilance state	Parameter	Saline (n=16)	CNO 1 mg/kg (n=11)	CNO 5 mg/kg (n=15)	CNO 10 mg/kg (n=14)	*F*	*p*	low dose	medium dose	high dose
Wake										
	2-h time window (%)	38.7813 ±3.2743	38.3636 ±5.7692	33.5444 ±3.8399	29.6429 ±3.9910	F (1.939, 23.91) = 1.468	0.2504	-0.0234	-0.3393	-0.4815
	6-h time window (%)	28.3530 ±1.3114	29.3232 ±2.5037	26.9321 ±1.5278	26.2553 ±1.7923	F (1.730, 21.34) = 0.6888	0.4928	0.1467	-0.1715	-0.2633
NREM										
	2-h time window (%)	51.9896 ±2.6391	53.7020 ±4.6576	58.7741 ±3.2742	63.7103 ±3.7117	F (1.911, 23.57) = 3.054	0.0682	0.1157	0.4941	0.6961
	6-h time window (%)	59.0799 ±1.1641	58.9074 ±1.9870	62.1914 ±1.2667	62.6111 ±1.6784	F (1.531, 18.88) = 2.028	0.1661	-0.0361	0.4130	0.4630
REM										
	2-h time window (% of TST)	10.4160 ±0.5877	8.1286 ±1.0534	8.4071 ±0.8351	6.5371 ±0.5937	F (1.783, 21.99) = 8.951	0.0019	-0.5802	-0.5594	-1.2743
	6-h time window (% of TST)	13.1740 ±0.3774	12.3119 ±0.4884	11.1629 ±0.5722	11.0742 ±0.4606	F (1.839, 22.68) = 7.525	0.0038	-0.4697	-0.7724	-0.9333

593
594

Supplementary Table 2: Sleep architecture after CNO and saline injections

		Treatment condition (mean values ± standard error)				Mixed-effect analysis		Effect size for post-hoc comparisons (Cohen's d)		
Vigilance state	Parameter	Saline (n=16)	CNO 1 mg/kg (n=11)	CNO 5 mg/kg (n=15)	CNO 10 mg/kg (n=14)	*F*	*p*	low dose	medium dose	high dose
Wake										
	Longest episode (min)	31.3292 ±2.3901	32.0000 ±3.9528	31.6089 ±3.2855	31.7952 ±3.5150	F (2.108, 26.00) = 0.02110	0.9825	0.0663	0.0189	0.0298
	Episode duration average (min)	11.6562 ±0.9793	9.6981 ±0.6636	11.0855 ±0.8599	11.0939 ±0.9275	F (2.578, 31.80) = 0.8177	0.4777	-0.4160	-0.1221	-0.1355
	Episode number (n/h)	1.5313 ±0.1429	1.7727 ±0.1107	1.4889 ±0.1254	1.4286 ±0.0984	F (2.338, 40.52) = 1.241	0.3036	0.3046	-0.0592	-0.1694
NREM										
	Longest episode (min)	16.3458 ±0.6994	18.8061 ±1.6012	22.1067 ±1.1553	26.0905 ±1.5547	F (2.881, 35.53) = 13.24	<0.000 1	0.3562	0.9438	1.5129
	Episode duration average (min)	5.8746 ±0.2822	6.1381 ±0.2998	7.2894 ±0.3127	7.5503 ±0.4268	F (2.436, 30.04) = 11.64	<0.000 1	0.1810	1.1430	1.1883
	Episode number (n/h)	6.3021 ±0.2318	5.9697 ±0.3113	5.3556 ±0.2444	5.2857 ±0.2915	F (2.476, 30.54) = 7.796	0.0010	-0.2938	-1.0628	-1.1989
	Latency (min)	30.4958 ±2.9506	14.6485 ±2.9015	26.1378 ±3.3676	25.4762 ±4.6781	F (2.146, 26.47) = 3.380	0.0463	-1.2021	-0.3744	-0.2424
REM										
	Longest episode (min)	3.4833 ±0.1631	3.2667 ±0.1585	3.4622 ±0.1445	3.6286 ±0.1480	F (2.720, 33.55) = 1.101	0.3583	-0.3224	-0.0272	0.1800
	Episode duration average (min)	1.9684 ±0.0396	1.9699 ±0.0574	2.0995 ±0.0662	2.2542 ±0.0835	F (2.330, 40.39) = 4.509	0.0132	0.0049	0.3941	1.0034
	Episode number (n/h)	2.0521 ±0.0922	1.8788 ±0.1441	1.8444 ±0.1167	1.6786 ±0.0664	F (2.665, 32.87) = 2.680	0.0690	-0.2975	-0.3630	-0.9286
	Latency (min)	28.1375 ±2.8814	54.8970 ±7.7841	36.1778 ±6.0069	43.3667 ±4.1811	F (2.308, 28.46) = 6.985	0.0024	0.9798	0.3517	0.8288
Sleep consoli-dation										
	NREM before REM onset (min)	20.9500 ±1.8146	31.4970 ±3.5462	30.0711 ±3.5065	35.9095± 3.5029	F (2.709, 33.41) = 5.071	0.0066	0.7346	0.5683	1.0475
	Brief awakenings (n/h)*	36.8033 ±3.7206	27.6169 ±3.2139	20.9897 ±2.7844	21.5082± 2.0680	F (1.968, 23.62) = 10.38	0.0006	-0.6271	-1.0691	-1.5760

*animal numbers for analysis of brief awakenings: n=15 for saline, n=11 for 1 mg/kg, n=15 for 5 mg/kg, n=13 for 10 mg/kg

599 **Supplementary Table 3: Sleep parameters after saline and compound 21 injections**

Vigilance state	Parameter	Saline (n=7)	C21 3 mg/kg (n=7)	*t*	*p*	Effect size (Cohen's d)
Wake						
	2-h time window (%)	31.4841 ±4.1581	29.3571 ±3.7073	t=0.2993, df=6	0.3874	-0.1131
	6-h time window (%)	26.4153 ±2.1399	23.5106 ±1.9486	t=0.9230, df=6	0.1958	-0.3489
	Longest episode (min)	27.6286 ±2.5082	30.3524 ±4.1173	t=0.8184, df=6	0.2222	0.3093
	Episode duration average (min)	10.4205 ±0.9875	10.6269 ±1.8085	t=0.1339, df=6	0.4489	0.0506
	Episode number (n/h)	1.5714 ±0.2463	1.4286 ±0.1776	t=0.4680, df=6	0.3281	-0.1769
NREM						
	2-h time window (%)	58.9444 ±2.9443	63.2302 ±3.0788	t=0.8081, df=6	0.2249	0.3054
	6-h time window (%)	61.4603 ± 1.8489	65.5873 ±1.6922	t=1.623, df=6	0.0779	0.6133
	Latency (min)	25.3619 ±3.2217	22.6571 ±4.8946	t=0.3844, df=6	0.3570	-0.1453
	Longest episode (min)	15.9143 ±0.7453	21.5429 ±1.6727	t=2.551, df=6	0.0217	0.9641
	Episode duration average (min)	6.0655 ±0.5040	8.0568 ±0.5341	t=2.462, df=6	0.0245	0.9307
	Episode number (n/h)	6.3571 ±0.2708	5.1190 ±0.3426	t=2.809, df=6	0.0154	-1.0617
REM						
	2-h time window (% of TST)	9.9182 ± 0.9511	7.5927 ±0.3919	t=1.829, df=6	0.0585	-0.6915
	6-h time window (% of TST)	12.6278 ±0.5749	10.8374 ±0.3969	t=3.234, df=6	0.0089	-1.2223
	Latency (min)	26.0095 ±5.0918	37.8952 ±5.8049	t=1.474, df=6	0.0954	0.3718
	Longest episode (min)	3.6952 ±0.1539	3.6476 ±0.2573	t=0.2047, df=6	0.4223	-0.0774
	Episode duration average (min)	2.0054 ±0.0678	2.2206 ±0.1093	t=2.229, df=6	0.0337	0.8425
	Episode number (n/h)	2.0000 ±0.1744	1.8333 ±0.0962	t=1.000, df=6	0.1780	-0.3780
Sleep consoli-dation						
	NREM before REM onset (min)	21.6762 ±3.8776	31.5714 ±3.9007	t=2.252, df=6	0.0326	0.8511
	Brief awakenings (n/h)*	31.5102 ±2.6435	20.1852 ±2.6435	t=2.164, df=5	0.0414	-0.8836

600 *animal numbers for analysis of brief awakenings: n=6

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602

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873 ## Author contributions

874 JT, AHS, ZM, CJA, VVV, and LBK designed the study. JPM, EM, LEM, HA, and LBK performed
875 surgeries. JT, JPM, EM, LEM, LBK conducted the experiments. EM, AHS, and LBK prepared
876 the drug solutions. JT, JPM, EM, LEM performed the sleep scoring. JT, LEM, SMS, VVV, and

LBK analysed the data. JT, ZM, CJA, VVV, and LBK wrote the manuscript with input from all authors. All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no competing interests.