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 for remote control of targeted cell populations using chemical actuators that bind to modified receptors. Despite the popularity of DREADDs in neuroscience and sleep research, potential effects of the DREADD actuator clozapine-N-oxide (CNO) on sleep have never been systematically tested. Here we show that intraperitoneal injections of commonly used CNO doses (1, 5, and 10 mg/kg) alter sleep in wild-type male laboratory mice. Using electroencephalography (EEG) and electromyography (EMG) to analyse sleep, we found a dose-dependent suppression of rapid eye-movement (REM) sleep, changes in EEG spectral power during non-REM (NREM) sleep, and altered sleep architecture in a pattern previously reported for clozapine. Effects of CNO on sleep could arise from back-metabolism to clozapine or binding to endogenous neurotransmitter receptors. Interestingly, we found that the novel DREADD actuator, compound 21 (C21, 3 mg/kg), similarly modulates sleep despite a lack of back-metabolism to clozapine. Our results demonstrate that both CNO and C21 can modulate sleep of mice not expressing DREADD receptors. This implies that back-metabolism to clozapine is not the sole mechanism underlying side effects of chemogenetic actuators. 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.

50 # Introduction

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

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

73 neurotransmitter receptors (Gomez et al. 2017; Jendryka et al. 2019) and to elicit behavioural
74 effects (Gomez et al. 2017; Manvich et al. 2018; MacLaren et al. 2016) at doses commonly
75 used for DREADD experiments.
76 It is widely thought that CNO does not affect sleep (Harding et al. 2018; Mondino et al. 2021;
77 Naganuma et al. 2018; Erickson et al. 2019) and many chemogenetic sleep studies include
78 convincing control experiments with DREADD-free animals that demonstrate the absence of
79 relevant effects of the chosen CNO preparations and doses on the assessed sleep parameters
80 (Erickson et al. 2019; Mondino et al. 2021; Takata et al. 2018; Yu et al. 2019; Anaclet et al.
81 2015; Venner et al. 2016). However, a comprehensive assessment of putative dose-
82 dependent effects of CNO on sleep in wild-type mice has never been systematically
83 conducted, although a recent study reported that high CNO doses affected sleep in DREADD-
84 free control animals (Varin, Luppi, and Fort 2018). This is an important omission, given that
85 clozapine is known to modulate sleep in humans (Hinze-Selch et al. 1997; Monti, Torterolo,
86 and Pandi Perumal 2017; Riemann and Nissen 2012) and laboratory rodents (Spierings,
87 Dzoljic, and Godschalk 1977; Sorge, Pollmächer, and Lancel 2004; Grønli et al. 2016; Coward
88 et al. 1989). Many of the endogenous neurotransmitter receptors, which are drug targets of
89 clozapine (Wenthur and Lindsley 2013) and to which CNO presents off-target binding affinity
90 (Jendryka et al. 2019; Gomez et al. 2017), are also involved in the regulation of arousal and
91 sleep (Saper and Fuller 2017).
92 Initially intended as a control experiment, we here tested whether commonly used doses of
93 CNO (1, 5, and 10 mg/kg) and the novel DREADD agonist compound 21 (C21), which does not
94 convert to clozapine (Thompson et al. 2018) but has an off-target binding profile similar to
95 CNO (Jendryka et al. 2019), affect sleep in wild-type C57BL/6J mice under laboratory
96 conditions. We find dose-dependent clozapine-like effects of CNO on the duration of rapid

97 eye movement (REM) sleep, sleep architecture parameters, and frontal EEG power spectra of
98 non-rapid eye movement (NREM) sleep. In addition, we observed a similar pattern of sleep
99 modulation after injections of a 3 mg/kg dose of C21 resulting in effect sizes comparable to
100 those of the 5 mg/kg CNO condition.
101

102 # Results

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

104 We first assessed the time spent in wakefulness, non-rapid eye movement (NREM) and rapid
105 eye-movement (REM) sleep in hourly intervals over the first six hours of the light period
106 following injections of CNO or saline at light onset (Figure 1a). There was no significant main
107 effect of the treatment condition on the proportion of time spent awake or in NREM and no
108 interaction effect between treatment condition and time point. However, we observed a
109 significant main effect of the treatment condition on the percentage of REM sleep ($F_{(2, 30)}$ =
110 5.601, p = 0.009), but no interaction between the time point and the treatment condition
111 (Figure 1a).

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

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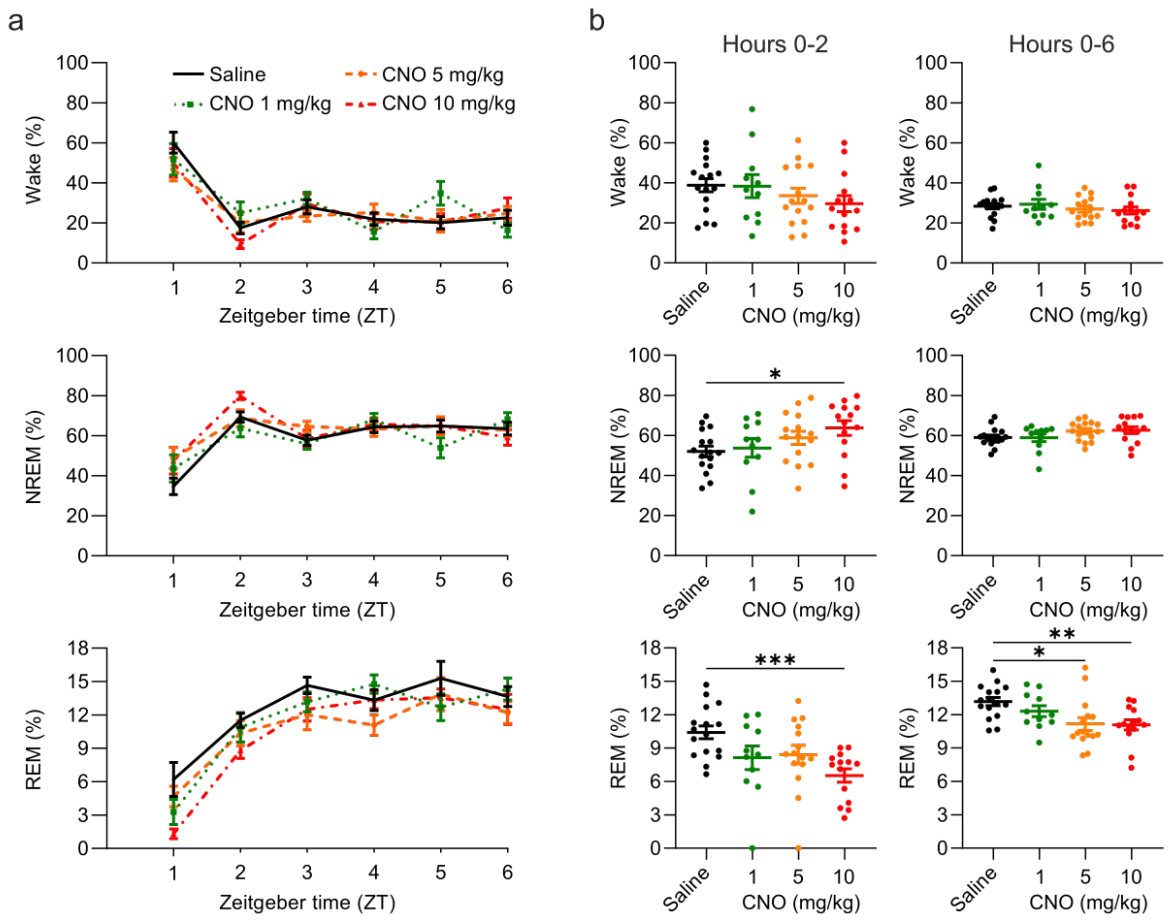


Figure 1: Suppression of REM sleep following CNO injection

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

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

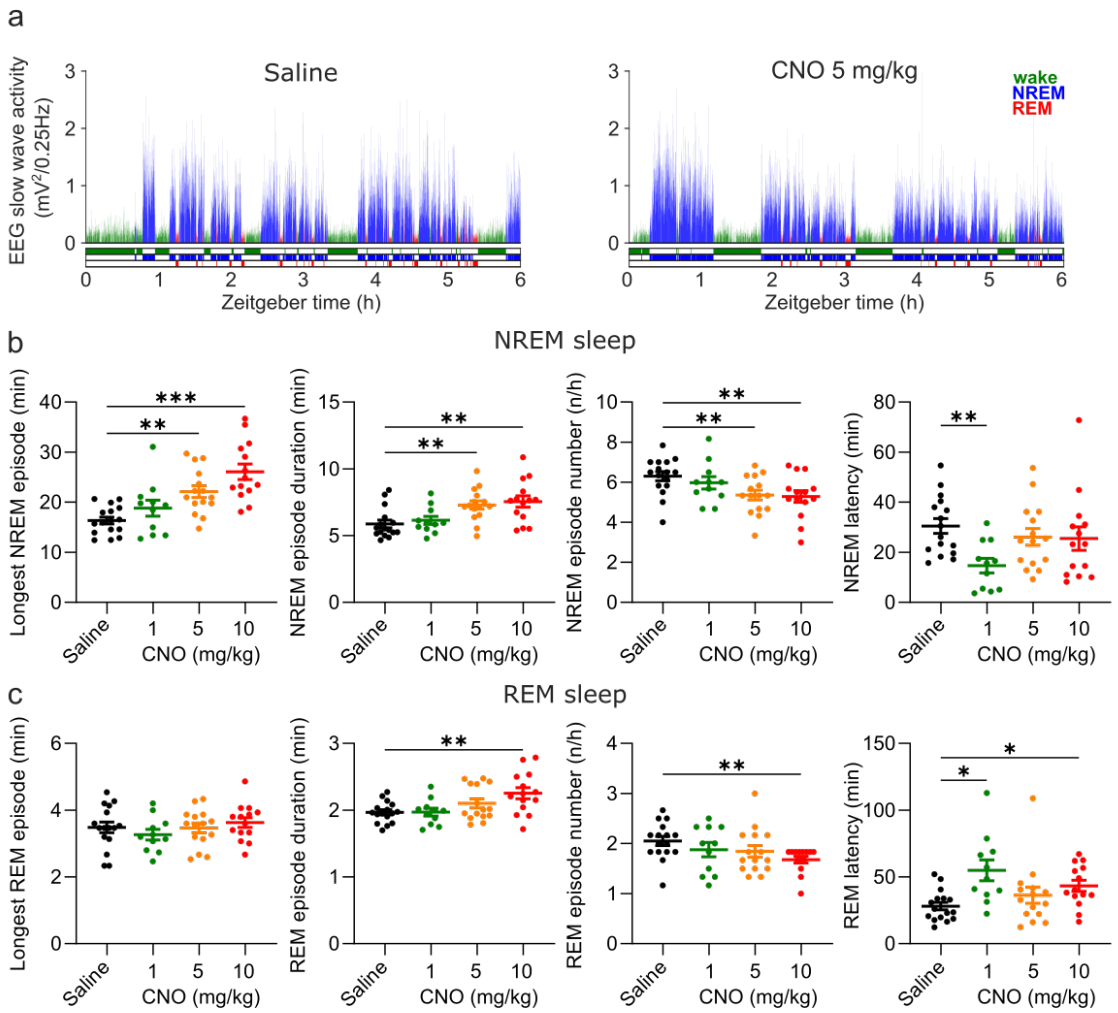


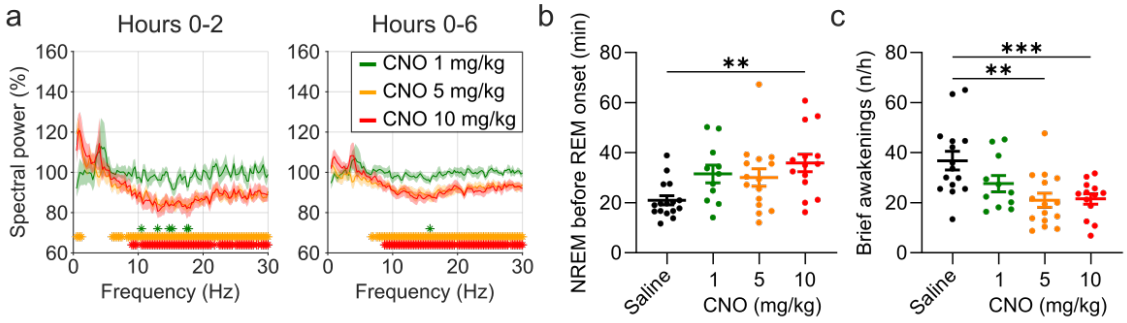
Figure 2: Altered sleep architecture following CNO injections

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

184 spectral analysis was on the comparison between the medium dose (5 mg/kg) of CNO and
185 saline during NREM sleep. We observed that CNO injections were followed by a small but
186 significant increase in spectral power in the range between 0.5 and 1.25 Hz and suppression
187 of spectral power in nearly all frequency bins between 6 and 30 Hz during NREM sleep over
188 the first two hours (Figure 3a and Supplementary Figure 1). While the increase in slow
189 frequency bins during NREM sleep appeared to be temporary, the suppression of power
190 above 6 Hz was significant for the entire six-hour observation period. Spectral analysis of
191 wakefulness and REM sleep did not reveal any systematic effects of CNO (Supplementary
192 Figure 1). A comparison of all three CNO conditions with saline injection indicated similar
193 effects of CNO on the NREM sleep spectrogram in the high dose (10 mg/kg CNO), but not in
194 the low dose (1 mg/kg CNO) condition (Figure 1).
195 Elevated spectral power in slow frequencies, longer but fewer NREM episodes, and less REM
196 sleep are typically observed during the initial recovery sleep following sleep deprivation,
197 when sleep is more consolidated (Huber, Deboer, and Tobler 2000). To explore whether the
198 stability of sleep is affected by CNO, we assessed the cumulative amount of NREM sleep
199 before the first REM episode (1 min or longer of REM sleep), as well as the frequency of brief
200 awakenings (4-16 second intrusions of wake-like EMG and EEG during sleep), which is a
201 behavioural marker of sleep continuity (Franken et al. 1991). Both measures were significantly
202 changed after CNO injection (NREM before REM: $F_{(3, 33)} = 5.071$, $p = 0.007$; brief awakenings:
203 $F_{(2, 24)} = 10.38$, $p < 0.001$). Effect size calculations indicated a medium to strong increase in
204 NREM sleep before REM onset and reduction in brief awakenings for all CNO doses
205 (Supplementary Table 2).



206

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

208 a) Frontal EEG spectra during NREM sleep relative to saline injections for the acute (first 2
209 hours) and prolonged (6 hours) observation period. b) Cumulative amount of NREM sleep
210 before the first REM sleep episode. c) Frequency of brief awakenings (4-16 sec) per hour of
211 sleep for the first 2 hours after injections. n=10 for saline, n=6 for 1 mg/kg, n=10 for 5 mg/kg,
212 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,
213 n=14 for 10 mg/kg for vigilance state analysis. n=15 for saline, n=11 for 1 mg/kg, n=15 for 5
214 mg/kg, n=13 for 10 mg/kg for analysis of brief awakenings. Asterisks in panels b and c
215 indicate post-hoc contrasts with significant differences (*P < 0.05, **P < 0.01, ***P < 0.001).
216 Asterisks in panel a indicate frequency bins with significant differences in post-hoc
217 comparison (P < 0.05). Data in a are presented as the mean ± s.e.m. (shaded areas). CNO:
218 clozapine-N-oxide. EEG: electroencephalogram. NREM: non-rapid eye movement sleep.

219

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

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

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

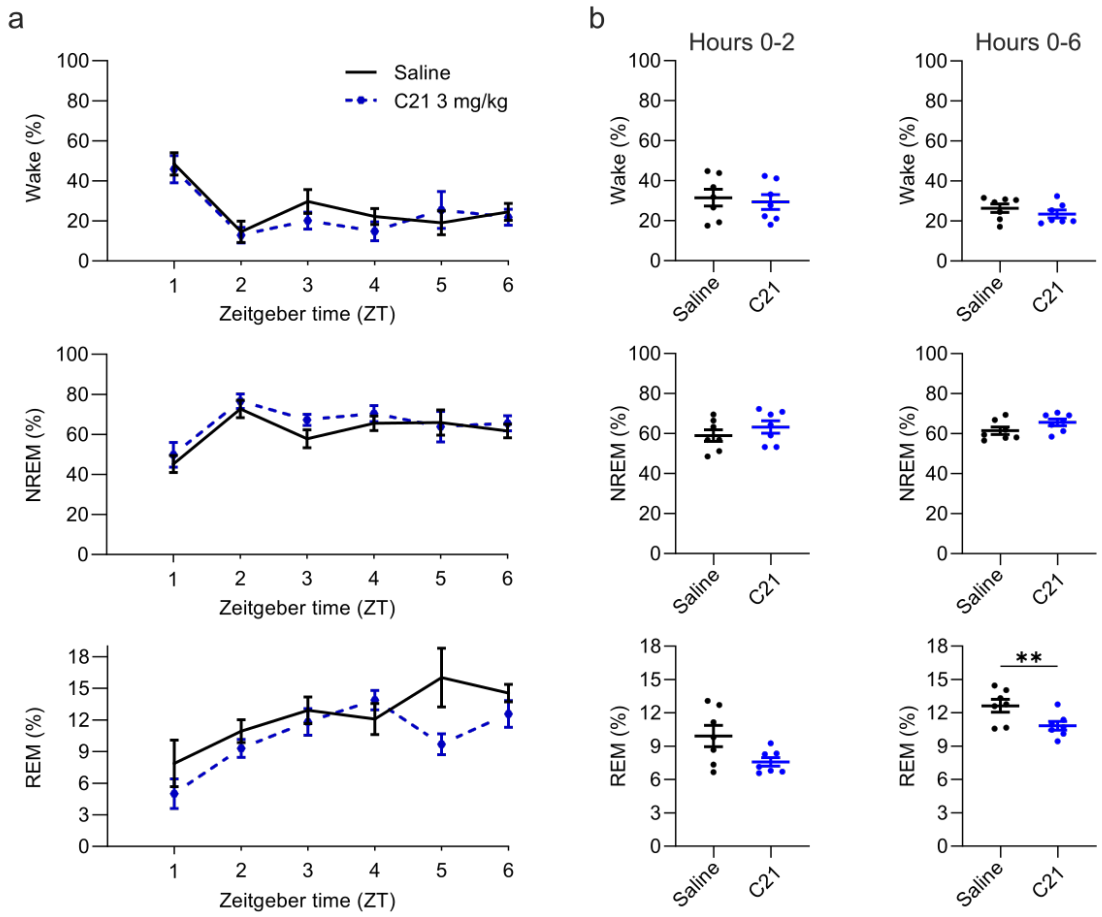


Figure 4: Suppression of REM sleep following C21 injections

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

258 d = 0.84; Figure 5b). For all NREM and REM architecture parameters that were significantly
259 changed after CNO injections, effect sizes of 3 mg/kg C21 were similar to that of 5 mg/kg CNO
260 compared to saline, even if the post-hoc comparisons did not reach significance in this small
261 sample (*Supplementary Table 3*). NREM spectra showed an increase in the very slow
262 frequency bins and a suppression of frequencies above 6 Hz (Figure 5c) – a pattern which
263 resembled that of the medium and high dose CNO injections (Figure 3a). Markers of sleep
264 consolidation were also significantly altered as a result of C21 injections. The amount of NREM
265 sleep before the first occurrence of REM sleep was increased ($t_{(6)} = 2.252$, $p = 0.033$, Cohen's
266 d = 0.85) and brief awakenings were reduced ($t_{(5)} = 2.164$, $p = 0.041$, Cohen's d = -0.52; Figure
267 5d,e).

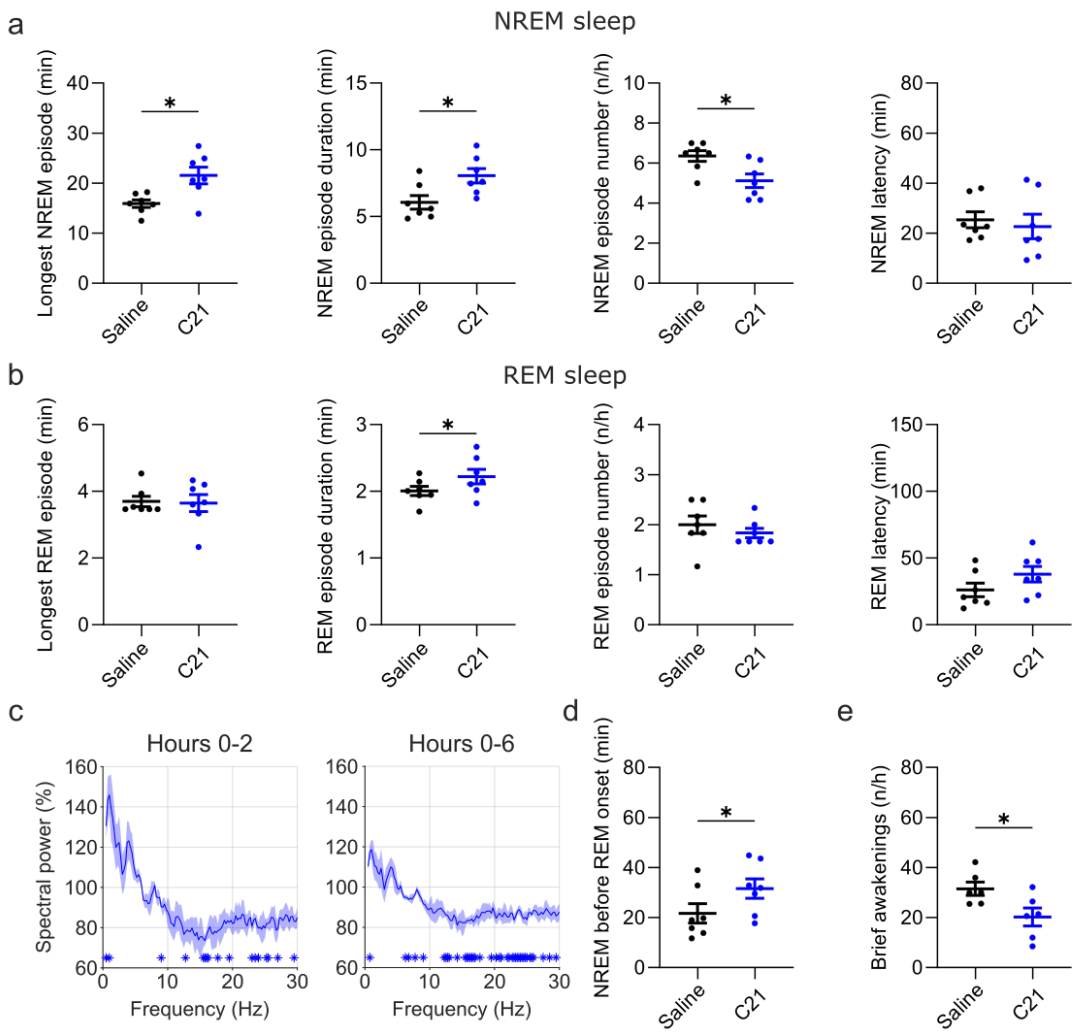


Figure 5: Changes in sleep architecture, NREM sleep spectra, and sleep continuity measures following C21 injections

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.

284 # Discussion

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

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

307

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

309 In our view, the most parsimonious explanation for the observed sleep-modulatory effects of
310 CNO and C21 is off-target binding at endogenous neurotransmitter receptors. It has been
311 shown that CNO is a competitive inhibitor of several neurotransmitter receptors, including
312 histaminergic H1, serotoninergic 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, muscarinic M1, M2, M3,
313 M4, adrenergic α1A and α2A, and dopaminergic D1 and D2 receptors (Gomez et al. 2017;
314 Jendryka et al. 2019). C21 has an off-target binding profile similar to CNO (Jendryka et al.
315 2019). In addition, a recent study reported increased firing rates of nigral dopaminergic
316 neurons in wild-type rats, indicating that C21 can elicit direct neuromodulatory effects in
317 rodents (Goutaudier et al. 2020). Among several endogenous receptors relevant for sleep
318 regulation, CNO and C21 induce a strong competitive inhibition at histamine H1-receptors
319 (Jendryka et al. 2019). Tested against a panel of g-protein coupled receptors (GPRCs), C21 had
320 a greater affinity for histamine H1-receptors than for muscarinic DREADDs (Thompson et al.
321 2018). H1-receptor knockout and pharmacological antagonism of the H1 receptor in mice
322 both result in a reduced number of brief awakenings, fewer but longer NREM sleep episodes,
323 and a reduced latency to NREM sleep (Huang et al. 2006). In addition, antihistamines which
324 induce drowsiness, are known to strongly suppress REM sleep, but can be acutely NREM-
325 promoting at low doses and wake-promoting at high doses, respectively (Ikeda-Sagara et al.
326 2012). Therefore, we speculate that the shared sleep-modulatory effects of CNO and C21
327 might be due to direct antihistaminergic action. However, a contribution of other shared off-
328 target sites of CNO and C21 such as the 5-HT_{2A} receptors, which are thought to mediate the
329 locomotor suppression after high doses of clozapine (McOmish et al. 2012), should also be
330 taken into consideration.

331

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

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

348

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

350 Previous work has highlighted that several factors such as age, sex and strain of the
351 experimental animals (Manvich et al. 2018), as well as differences in the activity of the
352 cytochrome P450 enzymes converting CNO to clozapine (Mahler and Aston-Jones 2018),
353 might contribute to the variability of side effects of DREADD actuators. In addition, the galenic
354 formulation and use of solvents can alter the pharmacokinetic properties of DREADD

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

379

380 **Limitations**

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

401

402 **Conclusions**

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

416

417 # Materials and Methods

418 **Animals**

419 Sixteen young adult male C57BL/6J mice (age: 113±6 days, weight: 25.2±0.5 g) were used in
420 this study. All animals were sourced internally from the Biomedical Services at the University
421 of Oxford. As this study was originally designed as a control experiment, we did not intend to
422 implant animals exclusively for this project. In order to reduce the number of animals used in
423 laboratory research, 13 animals were implanted for combined sleep experiments involving
424 procedures for this project and related work. All 16 animals used in this study were implanted
425 with a right frontal EEG screw, a reference EEG screw above the cerebellum, and EMG wires
426 in the neck muscles as described previously (Fisher et al. 2016). In addition to this EEG/EMG
427 configuration, which provided the electrophysiological signals analysed in this manuscript,
428 five of the animals were implanted with a frontal left and bilateral occipital EEG screws as well
429 as with an anchor screw in the midline anterior to the frontal EEG screws, which served as a
430 socket for a detachable light-emitting diode (LED) device (Figure 6a); four animals were
431 implanted with a right occipital EEG screw and a cannula (C315I; PlasticsOne) targeted to layer
432 5 of the primary somatosensory cortex (Figure 6b); four animals received an additional frontal
433 and occipital EEG screw over the left hemisphere as well as a left cerebellar ground screw and
434 a right occipital 16-channel laminar probe as well as a midline frontal anchor screw for the
435 detachable LED device (Figure 6c); three animals received a left frontal and occipital EEG
436 screw (Figure 6d). All animals had a rest interval of at least three days between the previous
437 experiment and this study.

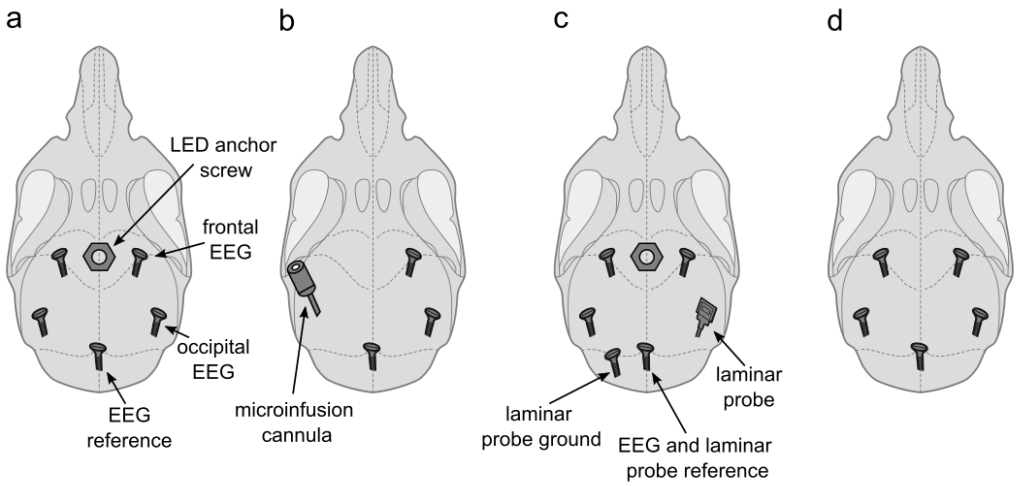


Figure 6: Implant configurations

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

460 under which our experiments were conducted, these animals could only be subjected to three
461 and two i.p. injections of different CNO doses and saline. The three animals implanted with
462 bilateral frontal and occipital EEG screws were not used for any additional experiments. Only
463 seven animals, the four animals implanted with laminar probes and the three animals used
464 exclusively for this study, were used for C21 injections as this condition was added to the
465 experimental protocol after the pilot data from CNO injections had been obtained.

466

467 **Electrophysiological signal acquisition, data processing, and sleep scoring**

468 EEG/EMG recordings were performed using the 128 Channel Neurophysiology Recording
469 System (Tucker-Davis Technologies Inc., Alachua, FL, USA) and the electrophysiological
470 recording software Synapse (Tucker-Davis Technologies Inc., Alachua, FL, USA). Raw data was
471 stored on a local computer in 24-h recording blocks. During the continuous recordings EEG
472 and EMG signals were filtered between 0.1 - 100 Hz, and stored at a sampling rate of 305 Hz.
473 After transfer to an analysis desktop computer, the raw signals were resampled at a sampling
474 rate of 256 Hz using custom-made code in Matlab (The MathWorks Inc, Natick,
475 Massachusetts, USA, version v2020a) and converted into the European Data Format (EDF) as
476 previously described (McKillop et al. 2018). The EDF files were visually scored in individual 4-s
477 epochs by blinded experimenters using the software package Sleep Sign for Animals
478 (SleepSign Kissei Comtec Co., Ltd., Nagano, Japan). If EEG signals contained temporary
479 artefacts due to electrical noise, movements, or chewing, the respective vigilance state was
480 assigned to the respective epoch but the EEG signals were not included in the spectral
481 analysis. Using the Sleep Sign for Animals software, fast Fourier transform routine (Hanning
482 window) with a 0.25 Hz resolution was computed in the frequency range between 0 and 30 Hz
483 for each individual 4-s epoch.

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

485 To avoid the use of the toxic solvent dimethyl sulfoxide (DMSO), which is typically used for
486 the preparation of CNO products in concentrations of up to 15% (Campbell and Marchant
487 2018), we opted for the use of a water-soluble salt preparation of CNO, clozapine-N-oxide
488 dihydrochloride (Tocris, Bio-Techne LTD, Abingdon, UK, catalog no.: 6329) dissolved in sterile
489 saline. The dihydrochloride preparation of CNO undergoes less back-metabolism to clozapine
490 but has a higher bioavailability compared to CNO-DMSO as indicated by pharmacokinetic
491 work in rhesus macaques (D. C. Allen et al. 2019). This product has previously been used in
492 sleep studies on mice at concentrations between 1 mg/kg and 5 mg/kg (Fernandez et al. 2018;
493 Stucynski et al. 2021). For C21 injections we used the water-soluble version of DREADD
494 agonist compound 21 (compound 21 dihydrochloride, Tocris, Bio-Techne LTD, Abingdon, UK,
495 catalog no.: HB6124). We chose a dose of 3 mg/kg because a detailed pharmacokinetic
496 assessment of this product at this specific concentration as well as behavioural testing in a
497 5-choice serial-reaction-time task, which did not reveal any behavioural effects at this dose
498 (Jendryka et al. 2019).

499

500 **Experimental design**

501 All 16 animals received a medium dose of CNO (5 mg/kg, 0.25 mg/ml solution) and an
502 equivalent volume of saline in a counterbalanced order with a 72-h rest interval between the
503 first two experimental sessions. Of those 16 mice, fourteen also received a 10 mg/kg CNO
504 injection (0.5 mg/ml solution) and twelve a 1 mg/kg CNO (0.05 mg/ml solution). These two
505 injections were counterbalanced as third and fourth injections. Seven animals also received a
506 3 mg/kg (0.15 mg/ml) C21 injection in a fifth experimental session. This semi-counterbalanced
507 design was chosen to ensure that at the medium dose condition is counterbalanced with

508 saline injections to circumvent putative habituation or adaptation effects following repeated
509 injection of CNO.
510 Animals were on a regular light-dark cycle (lights on at 9 am) and intraperitoneal (i.p.)
511 injections were performed within 15 minutes after light onset following a brief health check.
512 The delay between light onset and injection was (mean±s.e.m.): saline: 4.19±0.87 min,
513 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,
514 3 mg/kg C21: 6.01±0.96 min. Individual recording sessions were separated by a rest interval
515 of at least 72 hours. The recording chambers were kept open for approximately 10-15 minutes
516 after the injection to monitor for potential adverse effects. The chambers were then closed
517 and the animals checked remotely at regular intervals for the first 6-12 hours after injection.
518 For data analysis, all recordings were aligned to the time point of injections.
519
520 **Sample size determination and power analysis**
521 Sample size and power calculations were performed using G*Power 3.1, an open-source
522 statistical power analysis program (Faul et al. 2007). The sample size was chosen based on
523 previous experiments in our lab investigating the effects of the sedative diazepam on sleep
524 (McKillop et al. 2021), which indicated an effect size of Cohen's d = 0.90 for the key outcome
525 parameter NREM sleep time. We therefore decided that our study should be sufficiently
526 powered to detect effects of sizes of d=1 designed between saline and individual CNO
527 treatment conditions with a power of 0.9 at the given α-error probability of 0.05. The
528 estimated sample size from this calculation was 12-13 animals per group. Based on
529 experiences from previous EEG studies in mice, we aimed to account for an attrition rate of
530 approximately 20% and decided to include 16 animals in this study.
531

532 **Statistical procedures**

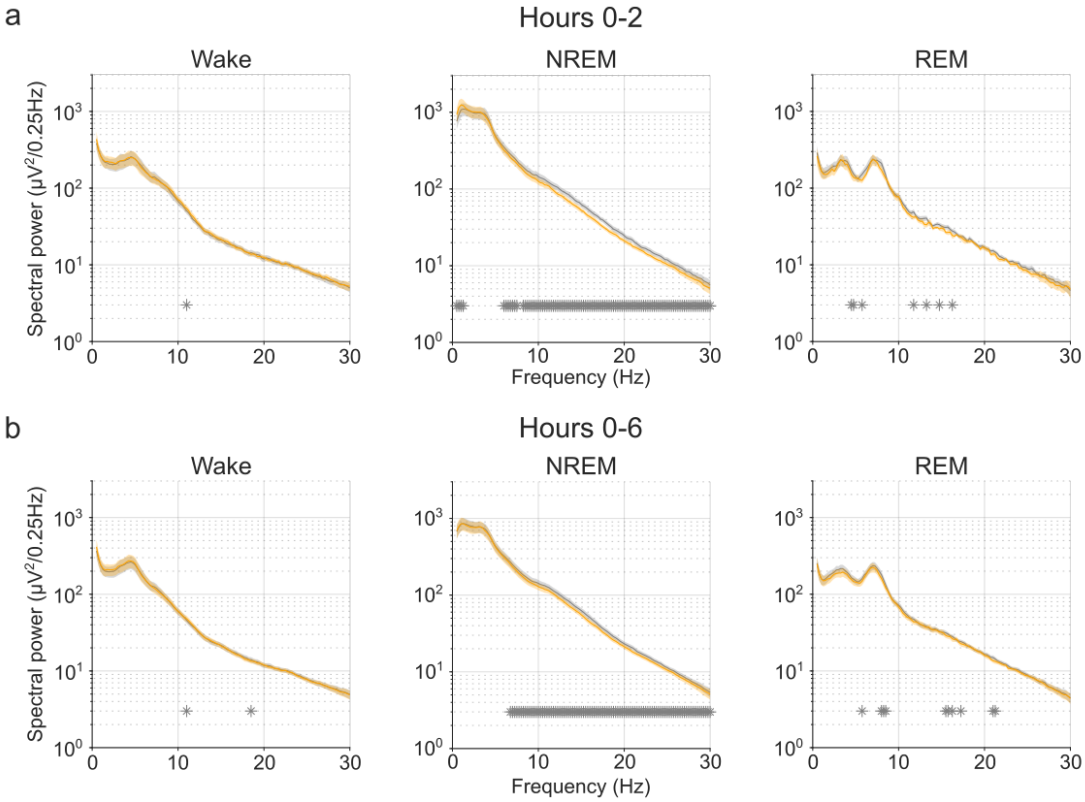
533 Data were analysed using MATLAB (version R2020a; The MathWorks Inc, Natick, MA, USA),
534 SAS JMP (version 7.0; SAS Institute Inc. Cary, NC, USA) and IBM SPSS Statistics for Windows
535 (version 25.0; IBM Corp., Armonk, N.Y., USA). Reported averages are mean±s.e.m. For all main
536 effects and interactions an α-error probability of 0.05 was adopted. For the statistical
537 comparison of CNO and saline injections mixed-effect models were calculated using
538 GraphPad Prism (version 9.1.1 for Windows; GraphPad Software, San Diego, California, USA,
539 www.graphpad.com). Significant main effects of treatment conditions or interaction terms
540 were followed up with Dunnett's adjustment for post-hoc comparisons. In mixed-effect
541 models with multiple time points we analysed acute (0-2 hours post injection) and prolonged
542 (0-6 hours post injection) effects separately. For spectral analysis, EEG/LFP power spectra of
543 individual animals were log-transformed before hypothesis testing. Individual spectral bins
544 were compared between individual CNO treatments and saline used two-tailed t-tests. No
545 correction was applied for post-hoc comparison of spectral data because ANOVAs consisting
546 of 119 EEG spectral bins would be too conservative and reduce statistical power (Achermann
547 and Borbély 1998); thus, α was kept at 0.05 in these cases. Comparisons between the C21 and
548 saline condition were performed using one-tailed *t*-tests to assess whether sleep variables
549 were changed in the same direction as after CNO treatment. For analysis of the percentage
550 of time spent in the three vigilance states, wakefulness and NREM sleep were expressed as
551 the percentage of time in the respective time window, REM was expressed as percentage of
552 total sleep time in the respective time window as in previous work (Huber, Deboer, and Tobler
553 1999). For sleep architecture analysis, NREM and REM sleep episodes were defined as
554 intervals of at least one minute with allowing an interruption of 4-16 seconds. Brief
555 awakenings are defined as up to 16 second interruptions of sleep by wake-like EEG and EMG

556 patterns (Franken et al. 1991). In all figures, significance levels of post-hoc comparisons are
557 indicated with black asterisks: '*' for 0.05 ≥ p > 0.01; '**' for 0.01 ≥ p > 0.001; '***' for 0.001
558 ≥ p. Effect sizes are reported as Cohen's d calculated using the MATLAB function
559 computeCohen_d (Ruggero G. Bettinardi (2020). computeCohen_d(x1, x2, varargin)
560 (https://www.mathworks.com/matlabcentral/fileexchange/62957-computecohen_d-x1-x2-
561 varargin), MATLAB Central File Exchange. Retrieved October 4, 2020). Data from one animal
562 had to be partially excluded because of a defective EEG headstage. This animal had to be
563 excluded from the analysis of brief awakenings due to EMG artefacts, which made it difficult
564 to identify a sudden increase of muscle tone during sleep. The same animal also had to be
565 excluded from the analysis of the 1 mg/kg and 5 mg/kg CNO treatments because of technical
566 issues. Six animals had to be excluded from spectral analysis due to occasional artefacts in the
567 EEG signals.
568
569 **Ethical approval**
570 All experiments were performed in accordance with the United Kingdom Animal Scientific
571 Procedures Act 1986 under personal and project licences granted by the United Kingdom
572 Home Office. Ethical approval was provided by the Ethical Review Panel at the University of
573 Oxford. Animal holding and experimentation were located at the Biomedical Sciences Building
574 (BSB) and the Behavioural Neuroscience Unit (BNU), University of Oxford.
575

Data and code availability

We encourage readers to further explore the dataset acquired for this publication. Raw data, custom written MATLAB code, and data tables for all figures will be made available on Figshare upon acceptance of the manuscript.

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.

597 **Supplementary Table 1: Time spent in wakefulness, NREM, and REM sleep after CNO and**
598 **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

599
600

601 **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

602 *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

603

604

605 **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

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

607

608

609 # References

610

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899

900 ## Author contributions

901 JT, AHS, ZM, CJA, VVV, and LBK designed the study. JPM, EM, LEM, HA, and LBK performed
902 surgeries. JT, JPM, EM, LEM, LBK conducted the experiments. EM, AHS, and LBK prepared
903 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.