



Research report

Microinjection of neostigmine into the pontine reticular formation of the mouse: further evaluation of a proposed REM sleep enhancement technique

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Abstract

Microinjections of cholinergic agonists into the pontine reticular formation (PRF) powerfully induce rapid eye movement sleep (REMS) in cats but have comparatively weaker effects in rats. Recently, the cholinomimetic neostigmine has been reported to strongly enhance REMS following microinjection into the PRF of the mouse. That study used behavioral assessments of locomotion in lieu of electrophysiological measures of muscle tone to identify REMS. We sought to confirm that the behavioral state induced in mice by PRF injections of neostigmine meets standard electroencephalogram (EEG) and electromyogram (EMG) criteria for defining REMS. Cortical EEG, nuchal muscle EMG, and PGO waves were recorded from male C57BL/6N mice with chronic indwelling cannulae for the delivery of neostigmine to the PRF. Recordings were made during midday following injections of neostigmine (8.8 mM, 50 nl), 2 h after lights on (LD 12:12). Neostigmine induced a behavioral state characterized by low amplitude, highly desynchronized cortical EEG with little theta, no PGO waves, and a sustained high muscle tone. Behavioral states meeting standard criteria for slow-wave sleep (SWS) and REMS were significantly suppressed compared to baseline recordings, and REMS onset was delayed by 3 h. Consistent with earlier reports, neostigmine did strongly suppress locomotor activity in open field tests and in the home cage. Due to the failure to meet criteria for defining REMS, we conclude that neostigmine microinjection into the PRF of the mouse induces an abnormal waking state rather than REMS.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Rapid eye movement sleep; Paradoxical sleep; Desynchronized sleep; Acetylcholine; Neostigmine; Pontine reticular formation

1. Introduction

A method for rapid and extended induction of rapid eye movement sleep (REMS) in the cat, using microinjection of

various compounds into the pontine reticular formation (PRF), has been known for several decades. Of these compounds, cholinergic agonists, such as carbachol, are among the most effective in terms of the rapidity and magnitude of the response (see the cat section of Table 1), with up to 80% of the first two hours spent in REMS following a single microinjection in cats [7].

Rapid and prolonged induction of REMS by drugs or other techniques could be exploited to test hypotheses concerning the function of REMS, which remains controversial. For example, REMS-enhancing techniques have recently been shown to improve memory consolidation in rats [69]. Could other manifestations of neuroplasticity,

Abbreviations: REMS, rapid eye movement sleep; SWS, slow-wave sleep; TS, transitional sleep; LS, low-amplitude sleep; NREMS, non-REM sleep; W, waking; PS, paradoxical sleep; PFR, pontine reticular formation; PnO, oral pontine reticular nucleus; LC, locus coeruleus; EEG, electroencephalogram; EMG, electromyogram; EOG, electrooculogram; PGO waves, ponto-geniculo-occipital waves

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t1.1 Table 1

t1.2 REMS enhancement by microinjection of carbachol at effective sites within the pontine reticular formation of the unanaesthetized rat versus cat, with control subjects' values shown in curly brackets {}

t1.3	Species	Ref.	Dose (μg)	Volume (μl)	REMS Bout		REMS latency (min)	REMS% of TRT (%)	TRT (min)
t1.4					Frequency	Duration			
t1.5	Rat	[9]	0.001	0.05	+	—	57.6 {72.1}	15 {9}	360
t1.6		[9]	0.0025	0.05	+	—	65 {72.1}	16 {9}	360
t1.7		[17]	0.01	0.1	?	?	? {69}	14 {10.4}	240
t1.8		[9]	0.01	0.05	+	—	55.8 {72.1}	16 {9}	360
t1.9		[37]	0.012	0.06	+	—	56.3 {58.8}	11.6 {5.2}	480
t1.10		[10]	0.02	0.1	+	—	53.9 {74.6}	17.4 {10.6}	480
t1.11		[9]	0.05	0.05	+	—	65.3 {72.1}	13 {9}	360
t1.12		[9]	0.1	0.05	—	—	62.2 {72.1}	11 {9}	360
t1.13		[17]	0.1	0.1	?	?	? {69}	16.5 {10.4}	240
t1.14		[69]	0.1	0.1	+	—	79.5 {97.4}	8 {4}	240
t1.15		[25]	0.1	0.1	—	—	? {69}	10 {7}	360
t1.16		[17]	0.5	0.1	?	?	? {69}	15.1 {10.4}	240
t1.17		[25]	0.5	0.1	+	—	? {69}	15 {7}	360
t1.18		[29]	0.5	0.1	?	?	?	16.8 {11}	360
t1.19		[9]	0.5	0.05	—	—	95.1 {72.1}	10 {9}	360
t1.20		[12]	1	0.5	—	+	?	13 {8}	480
t1.21		[42]	1	0.5	+	—	60 {47}	14.6 {11.4}	508
t1.22		[17]	1	0.1	+	—	66 {69}	17.7 {10.4}	240
t1.23		[25]	1	0.1	+	—	39 {69}	14 {7}	360
t1.24		[25]	5	0.1	—	—	? {69}	13 {7}	360
t1.25	Cat	[4]	0.01	0.25	—	—	77.4 {87}	6 {9}	240
t1.26		[4]	0.04	0.25	—	—	47 {87}	12 {9}	240
t1.27		[24]	0.04	0.02	+	+	7.6 {107.6}	49.3 {12.2}	240
t1.28		[24]	0.08	0.02	+	+	4.9 {107.6}	48.7 {12.2}	240
t1.29		[24]	0.8	0.02	+	+	4.7 {107.6}	57.7 {12.2}	240
t1.30		[52]	0.8–4	0.02–0.03	?	+	4.7 {?}	44.6 {4.3}	240
t1.31		[4]	0.1	0.25	—	+	23.5 {87}	45 {9}	240
t1.32		[57]	0.1	0.25	+	+	10 {39}	50 {10}	120
t1.33		[4]	0.4	0.25	+	+	15 {87}	47 {9}	240
t1.34		[67]	0.4	0.2	+	+	5.5 {44.9}	36.8 {12}	360
t1.35		[7]	0.5	0.5	?	+	9.2 {65.6}	57.7 {9.7}	300
t1.36		[4]	1	0.25	+	+	16 {87}	60 {9}	240
t1.37		[1]	3 or 9	3	+	+	10.7 {43.2}	42.2 {9.4}	120–240
t1.38		[68]	3.6	0.1	?	?	18 {57.5}	45 {10}	300
t1.39		[60]	4	1	?	—	23.4 {59.2}	46.5 {5.2}	180
t1.40		[2]	4	0.5	+	+	?	34.4 {5.2}	240
t1.41		[3]	4	0.25–0.5	+	+	19.4 {50.5}	55.2 {9.2}	240
t1.42		[4]	4	0.25	+	+	10.4 {87}	85 {9}	240
t1.43		[5]	4	0.25	+	+	11 {161.4}	55.8 {5.3}	120 or 240
t1.44		[60]	4	0.25	?	+	7.4 {59.2}	71.5 {5.2}	180
t1.45		[71]	4	0.25	?	?	9.2 {50.2}	50.9 {?}	>40
t1.46		[16]	4	0.25	+	+	26.9 {89.9}	43.5 {8.6}	240
t1.47		[51]	4	0.25	?	?	9 {71.2}	47.4 {12.2}	240
t1.48		[24]	4	0.02	+	+	6.5 {107.6}	52.8 {12.2}	240
t1.49		[18]	8	1	—	+	165.9 {45.2}	25.5 {13.8}	480
t1.50		[52]	16	0.02–0.03	?	+	4.7 {?}	50.5 {4.3}	240

t1.51 Ref.=reference number; +=increase; —=no change; ?=unknown; TRT=total recording time.

44 such as experience-dependent plasticity in development,
 45 recovery from brain damage, and other categories of
 46 learning and memory, be similarly affected by the
 47 stimulation of REMS? One limitation of studies using
 48 PRF carbachol injections and other methods to induce
 49 REMS in rats is that the magnitude of the induction is small
 50 by comparison with the effects observed in cats. For
 51 example, cats and rats show similar percentages of total
 52 recording time in REMS following vehicle control injections
 53 (both averaging ~9%), but cats show a much more

dramatic response to intra-PRF microinjections of carba-
 54 chol: REMS latency is short, REMS bout duration is
 55 lengthened, and total time occupied by REMS averages
 56 nearly 50% across studies (see Table 1). By contrast, rats
 57 show a delayed onset of REMS, no lengthening of bout
 58 duration, and total time occupied by REMS averaging
 59 ~14% across studies. However, despite the obvious
 60 advantage of the cat for REMS induction, cats are not the
 61 species of choice for behavioral studies of learning and
 62 memory. Ideally, a powerful REMS induction technique
 63

with rats would be preferred, given the excellent behavioral models of neuroplasticity they provide.

Mice also provide good behavioral models and other advantages of a small mammal with an experimentally tractable genome. Recently, microinjection of the cholinesterase inhibitor, neostigmine, into the PRF of mice has been reported to powerfully induce REMS [36], with a short latency (<5 min) and long bout duration (>60 min) much more comparable to the effects previously reported in cats. To utilize this newly proposed model of REMS induction for studies of REMS mechanisms and functions, it will be important to further establish homology with natural REMS. A first step is at the descriptive level: How closely does the neostigmine-induced behavioral state match the consensus behavioral and electrophysiological criteria for defining REMS? In rodents, REMS is defined by low-amplitude, desynchronized cortical electroencephalogram (EEG), moderate amplitude hippocampal theta activity, and atonia in antigravity muscles (e.g., neck muscles; [13]). The REMS state is also associated with spontaneous ‘ponto-geniculo-occipital spikes’ (PGO waves), which can be recorded from the pons, in addition to other sites. In the first report of neostigmine-induced REMS in mice [36], video recordings of locomotor activity were used to assess REM sleep, in lieu of electrophysiological recordings of muscle tone. We used electrophysiological methods to record cortical EEG, PGO waves, and neck muscle electromyogram (EMG) in mice and report here that PRF neostigmine microinjections produce a behavioral state that fails to meet important consensus criteria for REMS.

2. Methods

2.1. Subjects

Adult male C57BL/6N mice ($n=7$, Charles River Quebec) were used in this study. Food and water were made available ad libitum throughout the experiment. Mice were group housed in standard polypropylene cages (47×26×20 cm) before surgery and housed individually for the remainder of the experiment in a climate-controlled room [12-h light:12-h dark (LD) cycle; $22^{\circ}\pm 1^{\circ}\text{C}$]. Mice were handled each day throughout the experiment, except during the first week after surgery. The principles for the care and use of laboratory animals in research, as outlined by the Canadian Council on Animal Care, were strictly followed.

2.2. Surgery

Animals were anaesthetised with isoflurane, 5% for induction and 1–2% for maintenance, in 100% O_2 . Mice were placed in a David Kopf stereotaxic apparatus with a mouse gas anaesthesia mask (Harvard Apparatus), and the skull was levelled. Body temperature was maintained at

$37\pm 2^{\circ}\text{C}$ using a digital rectal thermometer and electrical heating pad.

For drug infusions, a 26-gauge guide cannula (Plastics One) was implanted so that a 32-gauge internal cannula extending 3 mm beyond the tip of the guide cannula would reach the left oral pontine reticular nucleus (PnO) according to the mouse stereotaxic atlas [48]. Patency of the guide cannula was protected between injections by a 32-gauge internal blocker also extending 3 mm beyond the tip of the guide cannula.

To identify the sleep–wake states, differential recordings were made using two ipsilateral cortical EEG electrodes and two EMG electrodes (Plastics One). EEG electrodes were stainless-steel jeweller’s screws threaded into holes drilled through the skull, 1.5 mm lateral from the midline and 1 mm anterior to the bregma (frontal cortex) or 2.5 mm posterior to the bregma (parietal cortex, over the hippocampus). EMG was recorded from subcutaneous wire electrodes implanted behind the occipital bone and underneath the neck muscle.

To record PGO waves, in three of the mice, a bipolar electrode (twisted 127 μm diameter nichrome wire), insulated except at the tips, was implanted into the left locus coeruleus (LC) of each of these mice, according to the mouse stereotaxic atlas [48]. The LC is a site that consistently yields PGO waves in the rat [19,39].

The pins from all six electrodes were connected to a protective plastic headcap (Plastics One). Three additional anchor screws were implanted, and the entire assembly was insulated and bonded to the skull with dental acrylic. Lactate ringier’s solution was given (0.5 cc, s.c.) to prevent dehydration following surgery. Finally, daily subcutaneous injections of meloxicam (Metacam, 1 mg/kg, s.c.) were provided for analgesia on the first three post-surgical days.

2.3. Sleep recording

Beginning at least 7 days after surgery, each mouse was placed in an electrically shielded chamber (Faraday cage dimensions: 38 cm long×38 cm wide×45 cm high) and habituated for 6 h/day for a minimum of 2 days to an EEG recording cable/swivel system that allowed free movement. The chambers were housed in an isolation room with controlled temperature and light, a video camera, and a white noise generator (Lafayette Instrument). EEG and video signals were monitored remotely in a separate room.

Following these adaptation days, electrophysiological recordings were made in the following conditions, over consecutive days: two baseline days, one drug injection day, and one recovery day. Injection and baseline conditions involved identical handling procedures, with the mice connected to the recording cables immediately before the beginning of each recording session. In addition to the baseline conditions controlling for handling effects upon subsequent sleep, to ensure that the behavioral state effects

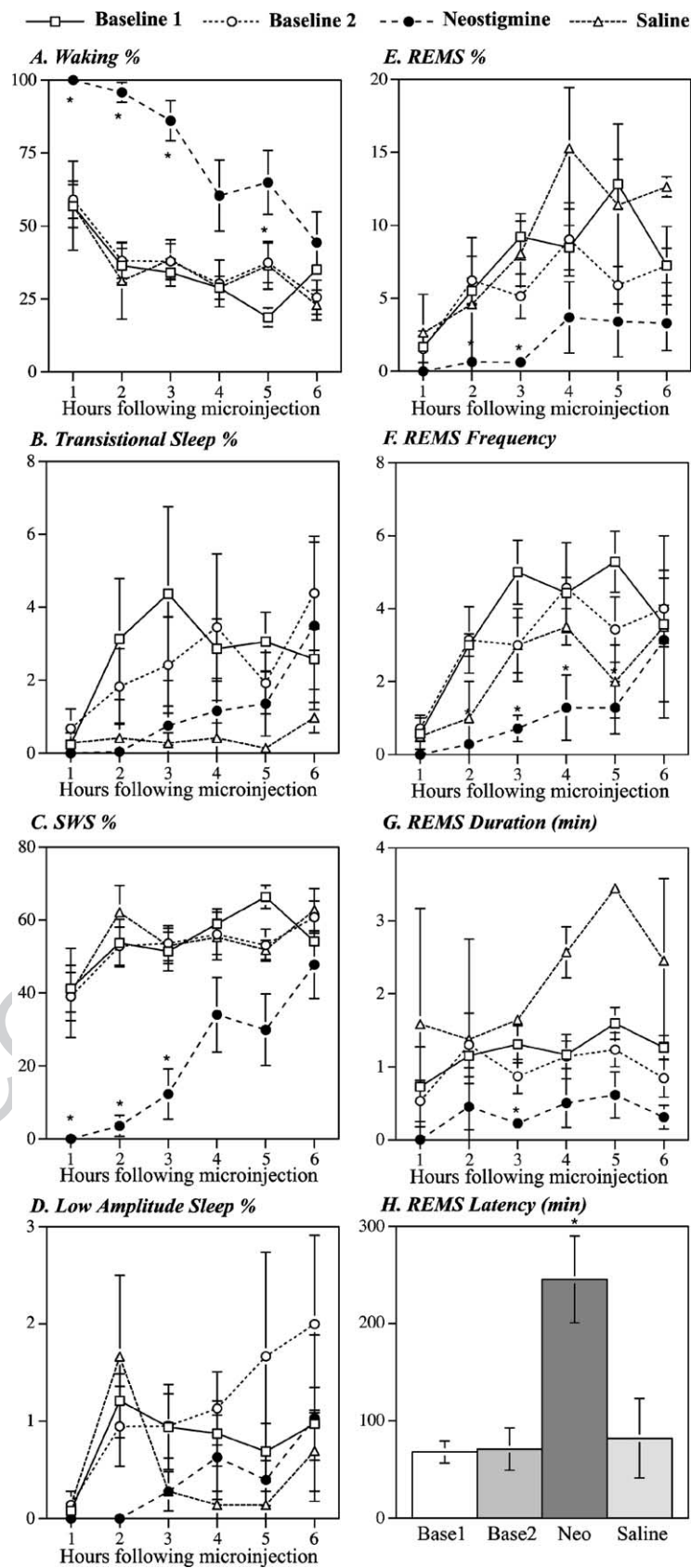


Fig. 1. Changes across recording time in each of the sleep parameters for baseline versus neostigmine conditions. Error bars represent the standard error of the mean. Asterisks represent statistical significance at the alpha 0.01 level between neostigmine and the average baseline value.

of neostigmine were not due to nonspecific effects of the bolus drug injections, two mice received a saline vehicle injection on one additional day. Injections were given 2 h after lights on, and recordings were continuous for 6 h. At the end of each recording period, the animal was untethered from the cable and left in the cage overnight.

EEG and EMG signals were amplified (Grass model 79D, Grass Instruments), digitized at a sampling rate of 250 Hz, and stored on computer using AcqKnowledge

data collection software (Biopac). Behavioral states were visually scored off-line in 10-s epochs on the basis of four electrographic measures: cortical EEG broadly filtered (0.3 to 50 Hz), cortical EEG filtered for delta waves (1–3 Hz), cortical EEG filtered for bandwidths (5–15 Hz) that include theta (5–9 Hz), and EMG (high pass filtered at 50 Hz). Each epoch was classified as whichever state was predominant. As previously described [49], the following five sleep/wake stages were recognised:

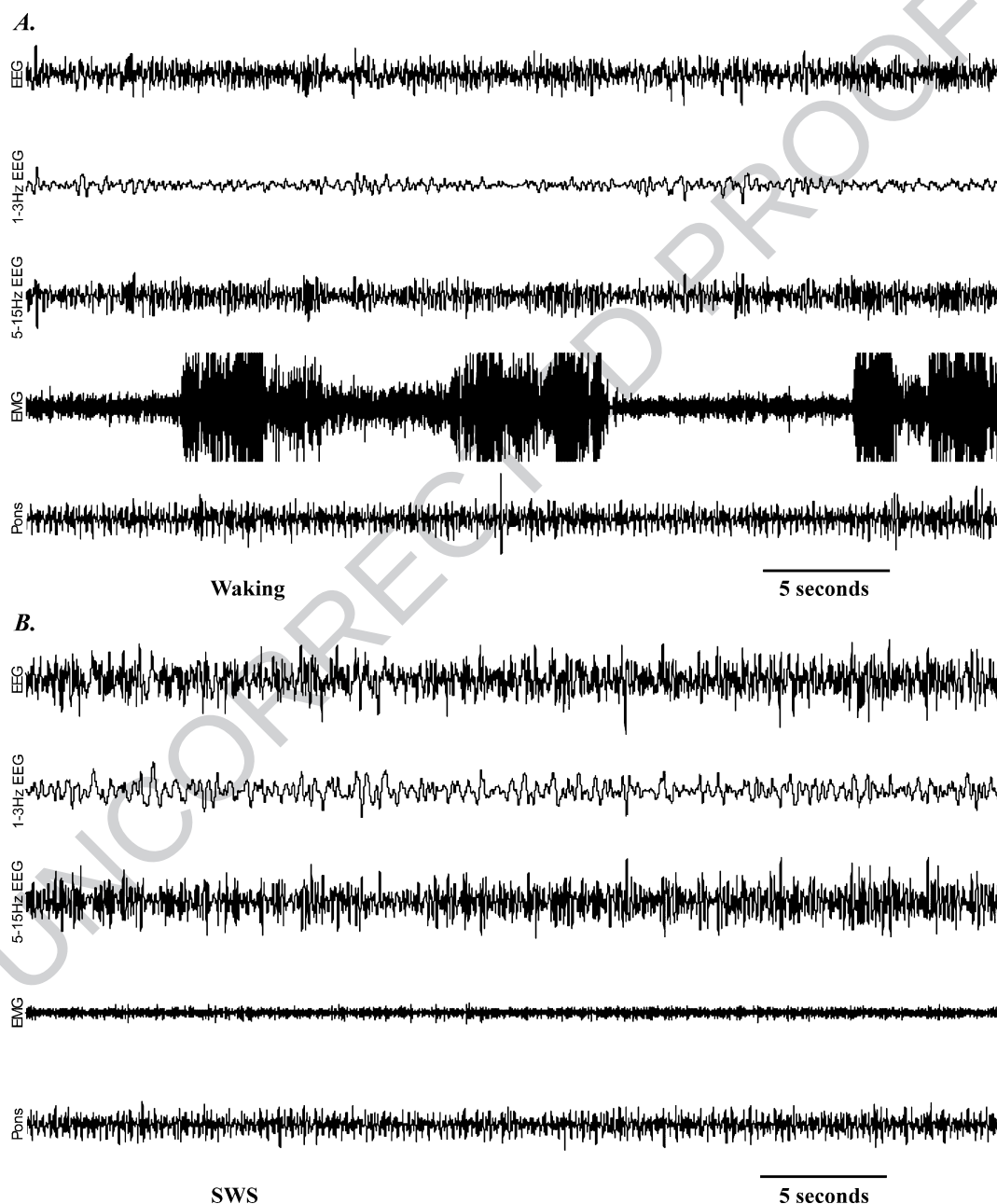


Fig. 2. Polygraphic recording examples of normal behavioral states and the neostigmine-induced state from a single mouse. Each example shows in order: cortical EEG broadly filtered (0.3 to 50 Hz) scaled ± 0.6 mV, cortical EEG filtered for delta waves (1–3 Hz) scaled ± 0.6 mV, cortical EEG filtered for bandwidths (5–15 Hz), including theta (5–9 Hz), scaled ± 0.3 mV, EMG (high pass filtered at 50 Hz) scaled ± 0.8 mV, and pontine EEG (filtered 8 to 16 Hz) scaled ± 0.3 mV. Panels A–E represent the following behavioral states taken from baseline recordings: (A) W, (B) SWS, (C) TS, (D) REMS, and (E) LS. Panel F represents the neostigmine-induced state at 15 min following neostigmine microinjection. Normal amplitude SWS was typically not seen until ~ 3 h after neostigmine microinjection, and clear REMS, not until near the end of the 6 h recording sessions.

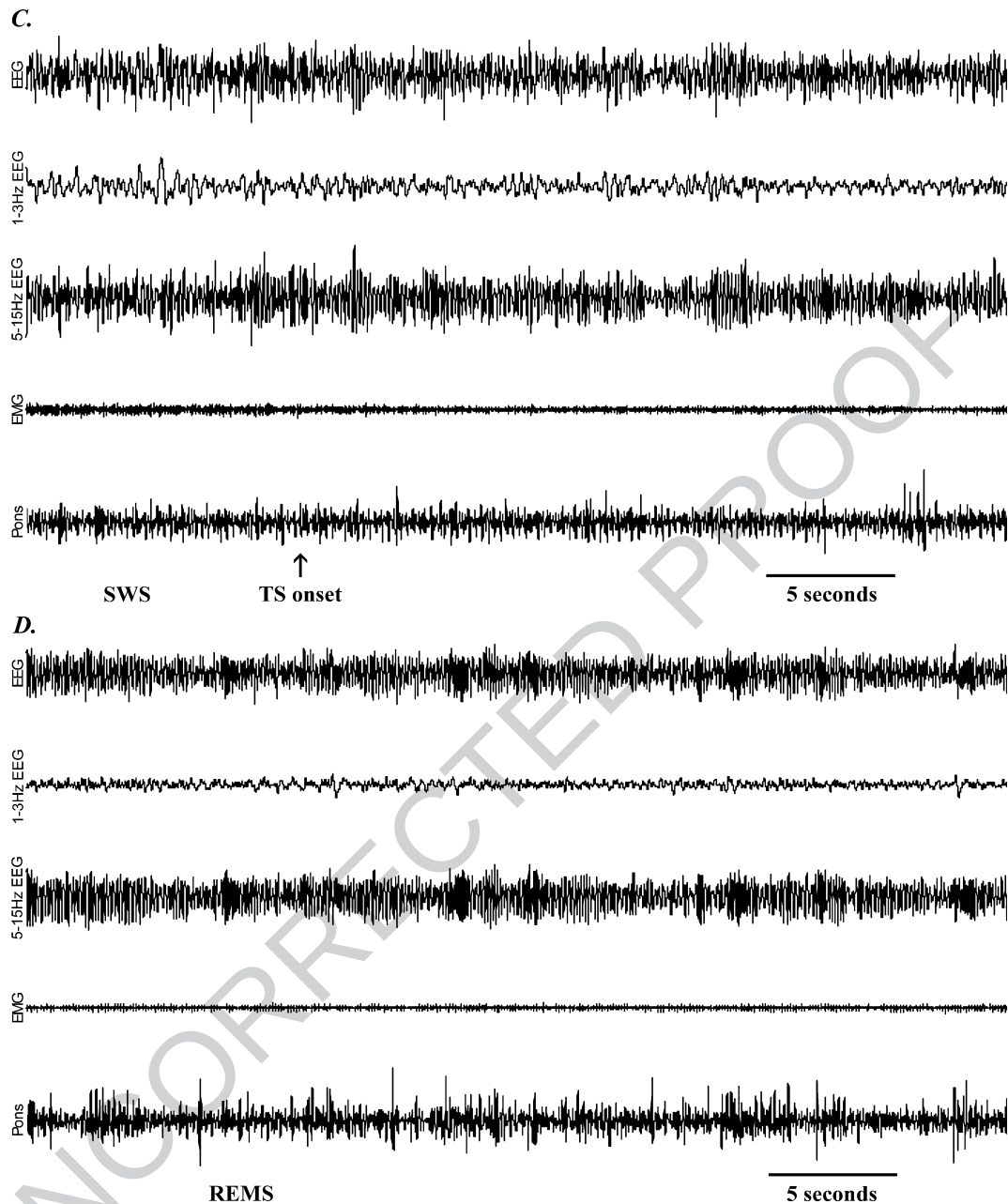


Fig. 2 (continued).

186

187 *Wake (W)*: low-amplitude cortical EEG with an absence
 188 of delta waves, low theta activity, and high-amplitude
 189 EMG.

190 *Transitional sleep (TS)*: high-amplitude cortical EEG
 191 with an absence of delta waves, irregularity in amplitude
 192 in the theta range, and low-amplitude EMG. With its low
 193 frequency and high-amplitude EEG activity, TS is chiefly
 194 distinguished by EEG spindles. This stage encompasses
 195 both the light sleep (SWS-1) and the transitional state
 196 between SWS and REMS (tS-R) previously described
 197 [13]. Due to the lack of a PGO wave recording in some
 198 mice, it was not always possible to differentiate between
 199 these two more specific types of transitional states.

200 *Slow-wave sleep (SWS)*: high-amplitude cortical EEG
 201 with delta waves and no regular amplitude theta activity,
 202 and low-amplitude EMG.

203 *REMS*: low-amplitude cortical EEG with regular, sinus-
 204 oidal theta rhythms and no delta waves and minimum
 205 amplitude EMG (muscle atonia). REMS is also identified
 206 by spontaneous high-density PGO wave spiking [13].

207 *Low-amplitude sleep (LS)*: low-amplitude cortical EEG
 208 with no regular amplitude theta activity and no delta
 209 waves, and low-amplitude EMG. Without a theta rhythm
 210 or PGO wave measurement, this stage could easily be
 211 misidentified as REMS. LS typically occurs for a brief
 212 period of time (10–20 s) following a mini-arousal from

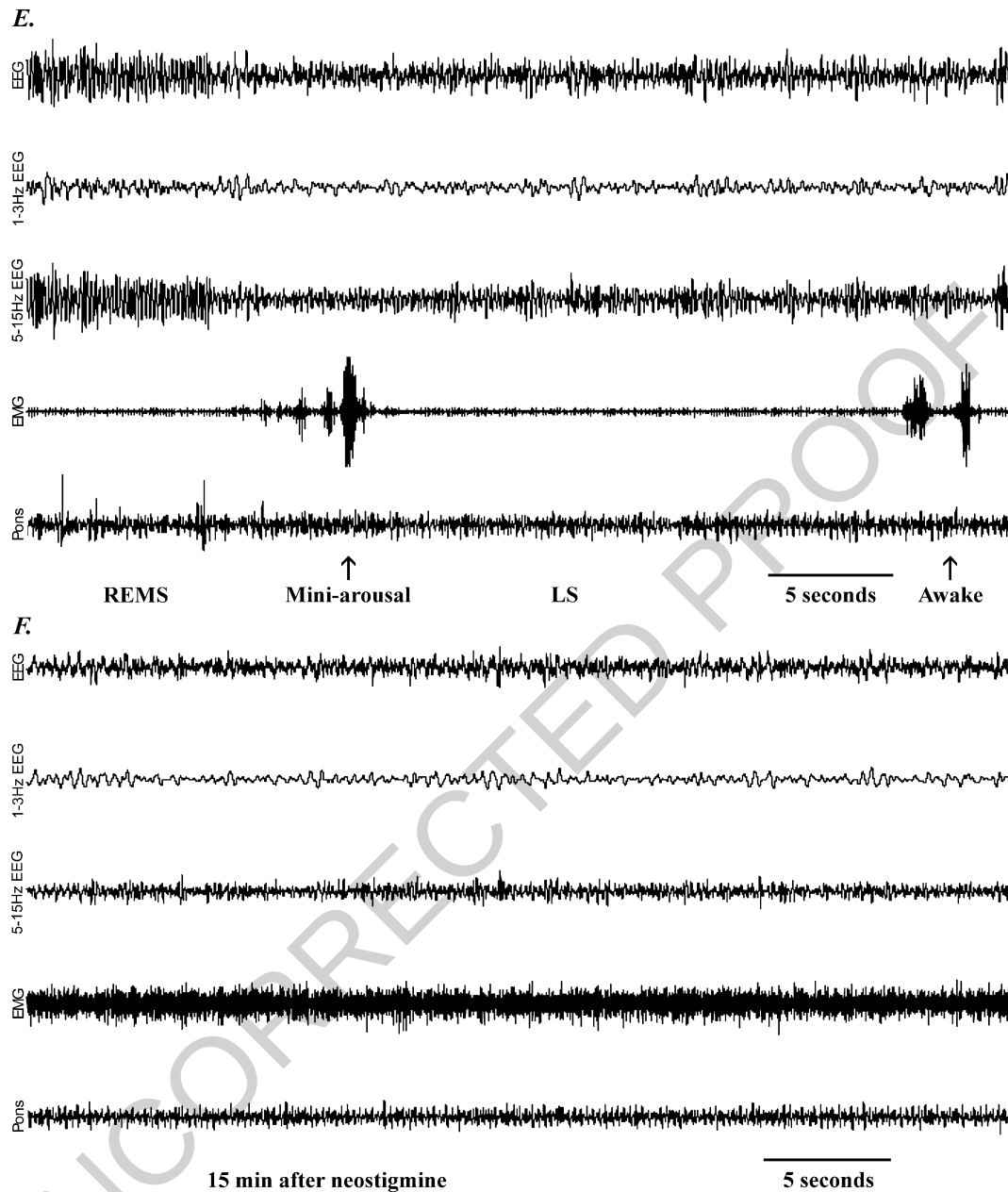


Fig. 2 (continued).

213 REMS before entry into SWS. Under some criteria for
 214 scoring rat sleep/wake stages [13], this state would be
 215 classified as a type of quiet W. However, Bergmann et al.
 216 [6] have convincingly argued that LS more closely
 217 resembles the remainder of non-REM sleep (NREMS) in
 218 its lack of spontaneous behavior and high response
 219 threshold. Accordingly, we consider it to be a substage of
 220 NREMS rather than of W.

221 For the analysis of pontine EEG, we used the same
 222 criteria used previously to identify PGO waves in the rat
 223 [34,40], as follows: the waves had to (i) be present in every
 224 REM sleep episode; (ii) occur at least 80% of the time in
 225 REM sleep and the 2 min of slow-wave sleep preceding

226 either REM sleep or a spontaneous arousal; (iii) show single
 227 spike durations of between 60 and 120 ms (which was
 228 accomplished by filtering the pontine EEG at 8 to 16 Hz
 229 bandwidths); and (iv) exceed background amplitude by a
 230 ratio of at least 2:1.

2.4. Microinjection procedure

231
 232 Neostigmine bromide was obtained from Sigma–Aldrich
 233 (Canada) and was dissolved in sterile saline fresh on the test
 234 days. The concentration of the drug was as previously
 235 reported [36] to be effective: 8.8 mM. Infusions were made
 236 via a stainless-steel injection cannula (32 gauge) inserted
 237 into the guide cannula. The injection cannula was attached

to a 1 μ L Hamilton syringe by polyethylene tubing (PE-20). The syringe was mounted in a manual microdrive (Kopf), and 50 nL of saline or drug solution was delivered over 1 min. The injector was left in place for an additional 1 min before removal, to ensure absorption of the drug. Both prior to and following microinjection, the injection system was tested for steady flow rate.

In each animal, one neostigmine microinjection was conducted for polygraphic recording, and another for locomotor recording. At least 5 days separated the neostigmine microinjections. For locomotor recordings, saline and neostigmine injections were given on consecutive days of testing in counterbalanced order. Saline injections for polygraphic recording were given 4 days following locomotor recording.

2.5. Locomotor recording

At least 4 days after the last EEG recording session, the mice received injections of vehicle or neostigmine 2 h after lights on and were placed in a 1-m square open field apparatus for 30 min. Order was counterbalanced on consecutive days. An overhead camera coupled to a computer by an image analyzer (Chromotrack, San Diego Instruments) was used to track movements. Data were sampled every 0.055 s. The minimum displacement necessary for movement detection was set at 4 cm.

2.6. Histology

To mark the injection site, 50 nL of India ink was microinjected through the cannula. The mice were then euthanized with an overdose of sodium pentobarbital and perfused transcardially with 9% saline, followed by 10%

formalin. The brains were removed and postfixed for 5 h in 10% formalin before being stored overnight in 20% sucrose–formalin. Coronal sections (50 μ m) were taken through the pons using a cryostat, mounted on slides, and stained with cresyl violet. Injection sites were located by reference to the mouse stereotaxic atlas [48].

2.7. Data analysis

The following dependent variables were quantified: REMS latency, REMS bout duration, REMS frequency, percentage of total recording time spent in each behavioral state, and total locomotor activity in the open field. REMS latency was calculated from the end of the microinjection to the first 10-s epoch of REMS. For calculating REMS frequency, REMS episodes were considered complete only following (but not including) an additional 60 s without REMS. REMS duration was calculated by dividing the total REMS time by the REMS frequency. Differences in dependent variables between baseline and drug conditions were subjected to analyses with repeated measures ANOVA and paired *t*-tests corrected for multiple comparisons by Bonferroni adjustment of alpha (acceptable *p*-value set at <0.01).

3. Results

3.1. Drug effects on behavioral state

The effects of neostigmine on behavioral states and REMS parameters are illustrated in Fig. 1. During the 6-h recording period, significant differences between baseline 1, baseline 2, and neostigmine conditions were found for the

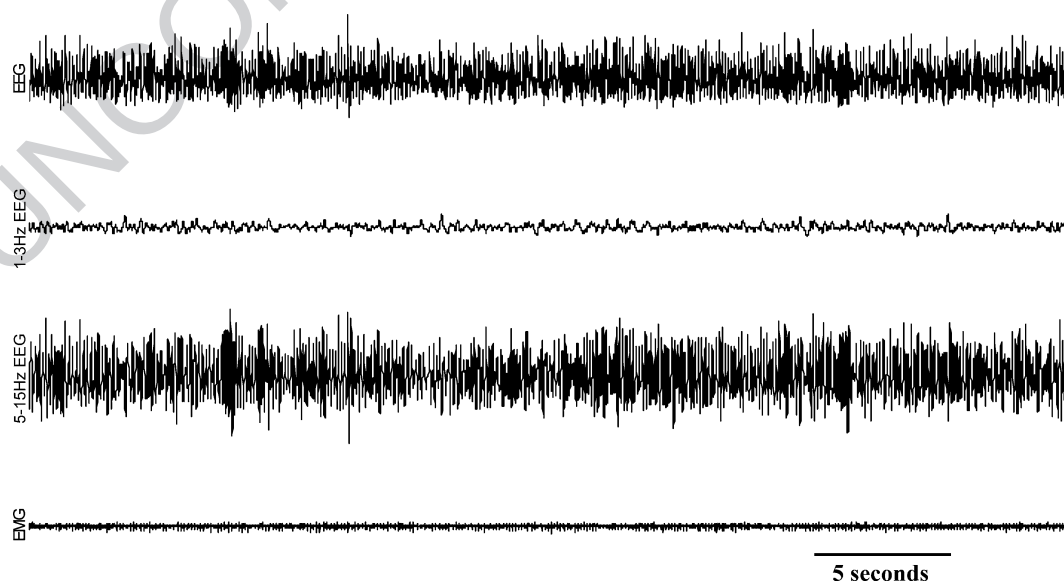


Fig. 3. Polygraphic recording example of REM sleep following within the first hour after saline injection alone. This example shows in order: cortical EEG broadly filtered (0.3 to 50 Hz) scaled ± 0.6 mV, cortical EEG filtered for delta waves (1–3 Hz) scaled ± 0.6 mV, cortical EEG filtered for bandwidths (5–15 Hz), including theta (5–9 Hz), scaled ± 0.3 mV, and EMG (high pass filtered at 50 Hz) scaled ± 0.8 mV.

296 following dependent variables: W% [$F(6,2)=44.46$,
 297 $p<0.001$], SWS% [$F(6,2)=34.14$, $p<0.001$], REMS%
 298 [$F(6,2)=28.66$, $p<0.001$], REMS episode frequency
 299 [$F(6,2)=21.90$, $p<0.001$], REMS episode duration
 300 [$F(6,2)=14.22$, $p<0.001$], and REMS latency [$t(6)=4.62$,
 301 $p<0.002$]. As expected, the two baseline conditions were
 302 nearly identical for each of these variables, with no
 303 differences approaching statistical significance (average
 304 $p=0.44$, S.D.=0.17). Thus, for subsequent comparisons with
 305 the neostigmine condition, baseline 1 and 2 values were
 306 averaged and will be referred to as baseline.

307 Neostigmine significantly increased W and suppressed
 308 both SWS and REMS during the first 3 h following
 309 injection. REMS onset was delayed by 3 h, on average,
 310 and REMS episode frequency did not return to normal
 311 levels for at least 6 h following injection.

312 Representative examples of the electrophysiological
 313 correlates of behavioral states during baseline (no injection)
 314 and following neostigmine injection are illustrated in Fig. 2.
 315 A representative example of REMS following a saline
 316 injection is illustrated in Fig. 3. The neostigmine-induced
 317 state was characterized by an extremely low-amplitude
 318 cortical EEG that was lacking delta waves and regular theta
 319 rhythms. Vehicle injection alone did not reproduce this state.
 320 Fast Fourier Transformation (FFT) of 30-s episodes of
 321 wakefulness, NREMS, REMS, and the neostigmine-induced
 322 state are displayed in Fig. 4. It has previously been reported
 323 [36] that the neostigmine-induced state shows a lower
 324 predominant frequency compared to naturally occurring
 325 REMS. However, sampling the data using EEG filtering
 326 parameters of 4–7 or 6–9 Hz did not reveal regular theta
 327 rhythms in the neostigmine-induced state, as were evident
 328 during natural REMS (Fig. 5). In the delta frequency band,
 329 power was lower than that characteristic of SWS and more
 330 similar to waking.

331 EMG recordings following neostigmine injection (Fig.
 332 2F) showed a moderately high continuous muscle tone
 333 similar to levels observed during quiet wakefulness in
 334 baseline recordings (e.g., as evident between periods of
 335 active wakefulness in Fig. 2A). Complete muscle atonia, on
 336 the other hand, was evident only in REMS, occasionally in
 337 SWS, and in our ‘low-amplitude’ substage of NREMS
 338 (based on the Bergmann et al. [6] scoring system) and was
 339 not observed in our mice during the neostigmine-induced
 340 state. In addition, pontine recordings showed a lack of PGO
 341 waves following neostigmine until normal EEG- and EMG-
 342 defined REMS episodes reemerged several hours later.

343 All of the above described characteristics were especially
 344 pronounced in the first 2 h following neostigmine micro-
 345 injection and then gradually dissipated over the next several

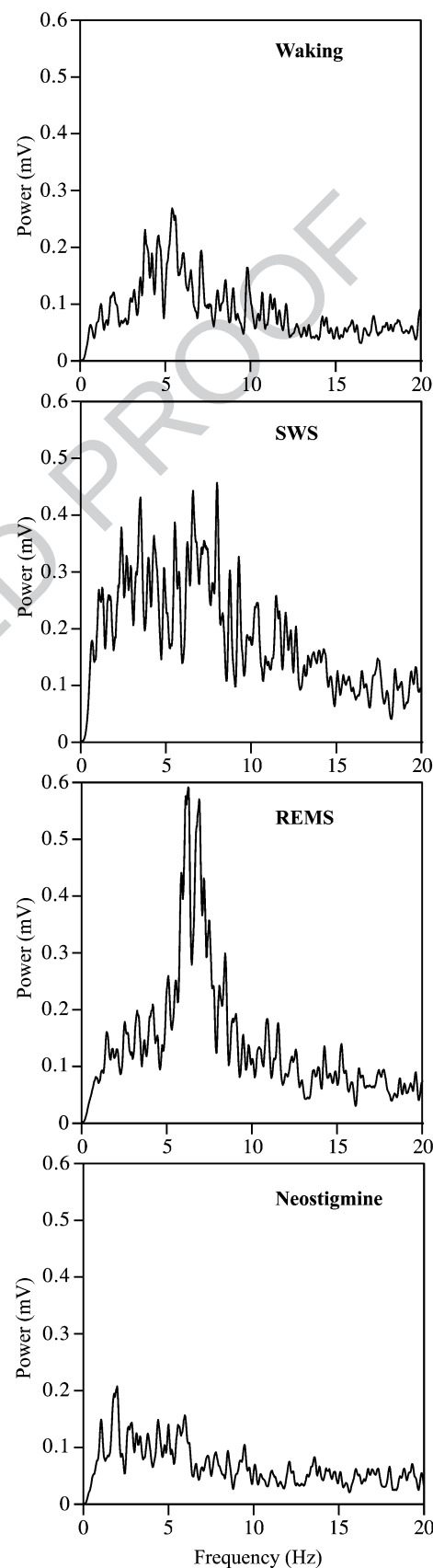


Fig. 4. Spectral distribution of cortical EEG power during wakefulness, SWS, REMS, and after neostigmine PRF microinjection, as revealed by Fast Fourier Transformation (FFT). Data for the FFT analyses were all taken from a single animal within the first hour of recordings following either saline injection or neostigmine injection. AcqKnowledge 3.5 (Biopac Systems) software was used to perform the FFT analyses.

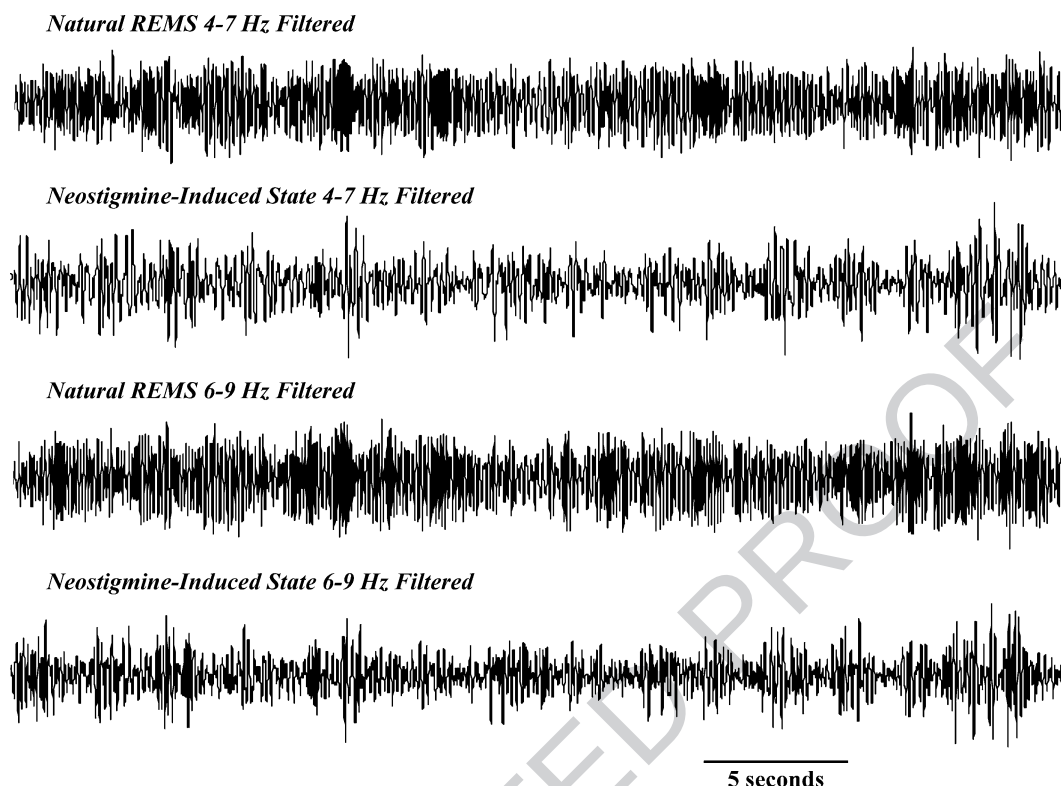


Fig. 5. Examples of filtering effects upon theta rhythms during natural REMS versus the neostigmine-induced state. Each example is scaled ± 0.4 mV. (Top) Cortical EEG activity filtered between the 4–7 Hz range previously reported [36] to be increased during the neostigmine-induced state. (Bottom) Cortical EEG activity now filtered between the 6–9 Hz range previously reported [36] to be increased only during natural REMS. Note that filtering by either cut-off frequency range still demonstrates regular sinusoidal activity only during natural REMS and not in the neostigmine-induced state.

346 hours. Saline injections did not affect any sleep parameter
347 by comparison with the baseline recordings, which were
348 matched for the brief disturbance required to connect the
349 recording cable.

350 3.2. Drug effects on locomotor activity

351 Neostigmine, by comparison with vehicle injections, was
352 associated with a significant suppression of locomotor
353 activity in the 30 min open field test in all mice
354 [$t(4)=4.00$, $p<0.01$; Fig. 6]. In a few cases, neostigmine
355 treatment was associated with occasional and mild ipsiver-
356 sive circling which contributed to the total locomotor score.
357 While this behavior was not observed during polygraphic
358 recording, since video surveillance was not continuous
359 during EEG recording, some ipsiversive circling cannot be
360 ruled out. In the home cage after the open field test,
361 neostigmine-treated mice could move about when prompted
362 by handling but otherwise remained in a still posture with
363 eyes open.

364 3.3. Location of cannulas

365 Injection sites were located from -4.72 to -5.20 mm
366 posterior of the bregma, 0.6 to 1.0 mm lateral of midline,
367 and 3.6 to 4.75 mm ventral of the horizontal plane passing
368 through the bregma and lambda on the surface of the skull

(Fig. 7). There was no apparent association between the
location of the cannula within the PRF and the behavioral or
electrophysiological effects of neostigmine injections.

372 4. Discussion

373 We sought additional descriptive evidence to further
374 establish the homology between natural REMS and the
375 neurobehavioral state induced by microinjections of neo-
376 stigmine into the PRF of the mouse. In agreement with what
377 has previously been reported [36], neostigmine induced a
378 behavioral state characterized by cortical EEG desynchroni-
379 zation and locomotor quiescence. However, additional
380 results do not support an interpretation that this state is
381 homologous with REMS. First, recordings from neck
382 muscles confirmed a very low resting EMG in REMS during
383 baseline recordings, but a tonic, moderate amplitude EMG
384 following neostigmine, comparable to muscle tone in quiet
385 waking. Second, REMS was associated with PGO waves,
386 whereas no PGO waves were observed following neo-
387 stigmine treatment. Finally, the regular EEG theta activity
388 characteristic of REMS was absent following neostigmine,
389 regardless of the filter settings used for quantifying theta.
390 The neostigmine-induced state thus failed to meet important
391 descriptive criteria for REMS. Although movement was
392 suppressed, and breathing rate is reported to be depressed

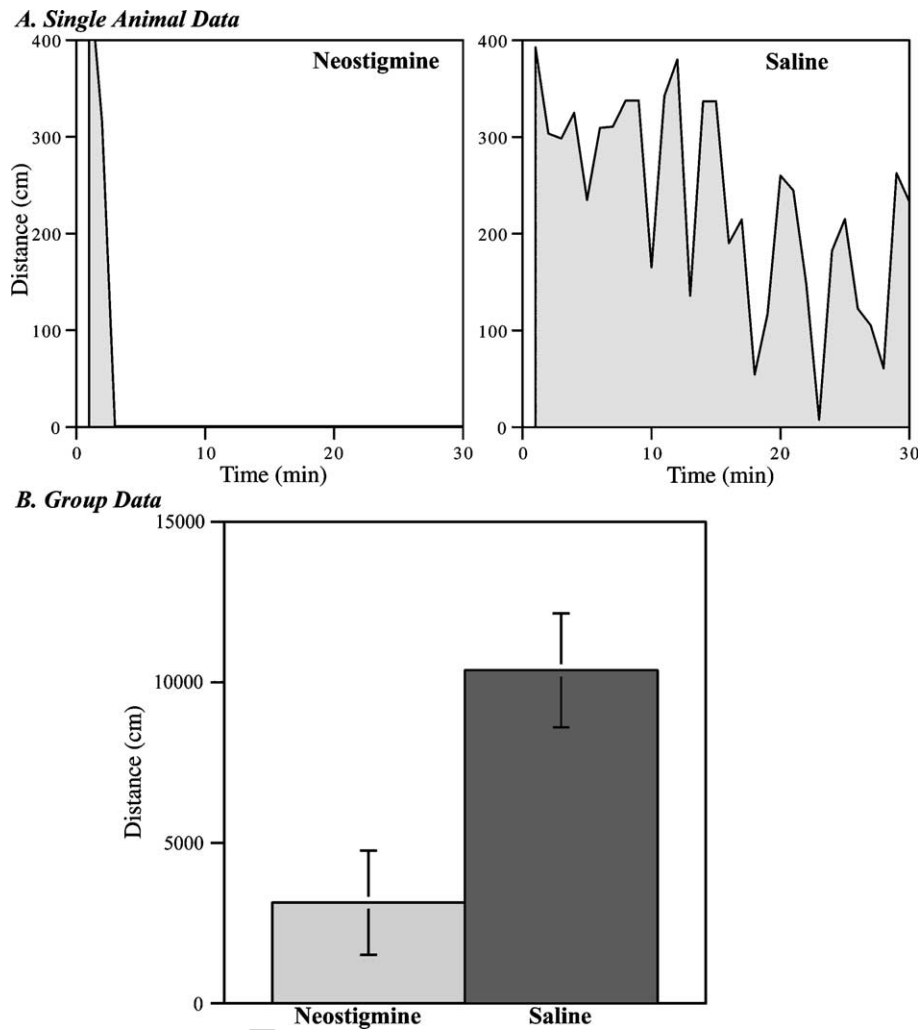


Fig. 6. Open field activity. (A) Single animal data of open field activity immediately following neostigmine versus saline microinjections. The samples shown here are taken from a single mouse whose electrophysiological data are displayed in Fig. 2, and that received, in order, neostigmine and then saline injections across consecutive days of locomotor recordings. (B) Group data of open field activity immediately following neostigmine versus saline microinjections. Error bars represent the standard error of the mean.

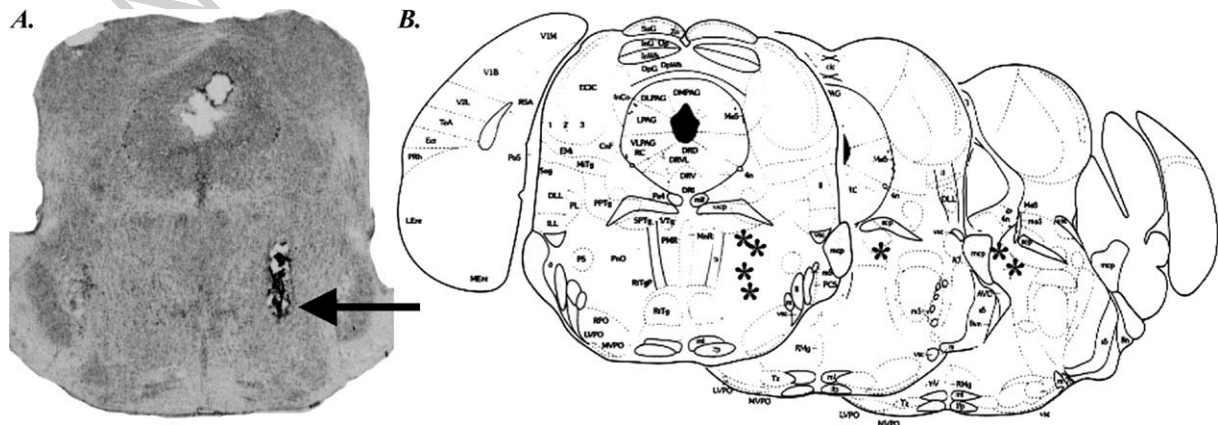


Fig. 7. Histology illustrating microinjection sites in the pons. (A) Digital image of a cresyl-violet-stained coronal section. To localize the injection site (arrow), 50 nl of India ink was microinjected through the cannula before the removal of the brain. (B) Mouse brainstem coronal sections along the anterior–posterior plane (from -4.72 to -5.20 mm posterior of bregma), with asterisks representing seven different PRF injection sites.

[36], this state did match polygraphic criteria for classification as waking, which include low-amplitude cortical EEG lacking delta activity and a high resting EMG amplitude.

In previous REMS induction studies, the absence of various REMS components following drug treatments has warranted the classification of additional dissociated states, as follows. When the amplitude and frequency of theta rhythms are reduced while all other criteria of REMS have been met, the state has been classified as a REMS-like state, also referred to as a “paradoxical sleep (PS)-like state” [8,67]. When more than one of the REMS components are missing, such as theta rhythms, PGO waves, and rapid eye movements, while the eyes are open with normal pupil contraction/dilation and eye-tracking reflex, the state has been labelled a “waking dissociated state” [35] or simply a “dissociated state” [67]. The components in common with REMS that remain in this dissociated state are cortical EEG desynchronization, muscle atonia, and sometimes, PGO waves. Since this state produces a condition of immobility but without the perceptual disengagement from the environment that characterizes sleep, it has been noted to bear resemblance to cataplexy [67]. However, when a drug-induced state displays cortical EEG desynchronization and a REMS-specific characteristic, such as PGO waves, while muscle tone is still present, it has customarily been scored as a waking state with that additional characteristic, e.g., waking with PGO waves [28,38,70]. Due to the presence of muscle tone and the lack of any REMS-specific characteristics in mice receiving neostigmine, a designation of ‘REMS-like’ for this drug-induced state is not compelling. Rather, the available evidence suggests a classification of ‘waking with suppressed locomotor activity and breathing’.

Previously, microinjection of neostigmine into the PRF of the rat has also been shown to be sleep suppressing [49]. In addition, while a number of studies have reported REMS-enhancing effects of carbachol PRF microinjection in rats (listed in Table 1), some studies with the rat have shown that the same technique is equally capable of producing an increase in wakefulness [17]. In humans, cholinergic stimulation—via the systemic administration of cholinomimetics—also leads to increases in both waking [26,62,63] and REMS episode frequency [62,63,64]. It has long been known that cholinergic stimulation can produce the cerebral arousal that characterizes both waking and REMS, and it has been hypothesized that the cholinergic pathways of the ascending reticular activating system mediate this effect (reviewed in Ref. [58]).

The suppression of locomotor activity by neostigmine injections in mice is consistent with the known depressant effects of cholinergic stimulation in rodents. Systemic injections of cholinesterase inhibitors [27,41,45–47,50,54–56,59,61,65] and intrapontine injections of cholinergic agonists [11,23,43] have been shown to decrease locomotor activity via muscarinic receptors, while the opposite effect may occur by injections of cholinomimetics elsewhere in the brain [20,33,72,73]. In humans, intravenous injection of

physostigmine produces acute psychomotor retardation [15,21,22,30–32,53,66], also via a muscarinic mechanism [21,32]. This behavioral effect is characterized by “withdrawal, lethargy, apathy and reduced energy, slowed thoughts, decreased thoughts, motor retardation, and decreased speech” [66]. Of relevance to the results of the present study, physostigmine did not produce either sedation or ataxia [31]. These results indicate that behavioral suppression by cholinomimetics has broad species generality.

The present study was designed as a replication and extension of the initial report of apparent REMS induction by neostigmine injections into the PRF of mice [36]. Therefore, dose, time, and injection site were selected to replicate the methods described in that study. By these methods, we did replicate the reported effects of neostigmine in mice, i.e., the induction of a state characterized by a very low voltage EEG with a paucity of both delta and regular theta, concurrent with behavioral suppression. Thus, we successfully replicated prior results. Our interpretation is based on the extension part of our study, i.e., the inclusion of additional electrophysiological measures, namely, EMG and PGO waves. These measures, taken together with the EEG and video analysis used in both studies, lead to a different interpretation, i.e., that neostigmine induces not REMS, but an abnormal wake state. The fact that we fully replicated the effects on the subset of measures that were previously reported indicates that our results cannot be dismissed as a false negative due to inadequate sampling of injection doses, times, or sites. Indeed, given the consistency of the results both across subjects in this study and between the two studies, despite typical variations in the precise placement of the cannulas, we do not anticipate that further mapping studies will produce radically different results (e.g., induction of a more REMS-like state in this region of the PRF). Whether neostigmine can produce REMS at other sites in the brainstem is a separate issue and not relevant to the results at hand. These results speak only to the interpretation of the behavioral state induced by injections into the PRF sites sampled in our study and the same sites sampled in the one previous study [36]. It also remains to be determined whether other cholinergic agonists, such as carbachol, that would directly activate cholinergic receptors would also fail to produce REMS in mice.

We also do not anticipate that tests at other times of day would produce different results. The effect of cholinergics on behavioral state can depend on the behavioral state at the time of the injection; for example, in cats, full REMS induction by carbachol is more effective when the drug is administered during sleep than during wake [35], and in rats, carbachol immediately induces phasic REMS events in anaesthetized [14] but not in unanaesthetised preparations [44]. Thus, it could be that arousal and/or endogenous circadian phase interacts with the REMS-enhancing effects of cholinomimetics. However, the time of drug administration used in the present study would seem to be most propitious for promoting REMS, as it was during the sleep

phase of the daily sleep–wake cycle and after the major peak of delta-enriched SWS that predominate early in the daily sleep phase.

For electrophysiological measurements, injections of neostigmine and saline were not counterbalanced for order. However, differential habituation to handling is unlikely to account for the differences seen between saline and neostigmine conditions, for two reasons: (1) all mice were habituated to the handling procedure prior to the first injection test, and (2) for locomotor recordings, the drug and saline conditions were counterbalanced for order, and it was the drug condition, not the order of testing, that was associated with the dramatic differences in locomotor activity.

In conclusion, the present results do not provide support for the proposal that neostigmine microinjection into the PRF induces REMS or a REMS-like state in mice. The data are consistent with results from rat studies demonstrating that cholinomimetics microinjected into the PRF are less effective enhancers of REMS in rodents, by comparison with cats. The reasons for this species difference remain to be elucidated, but may provide insight into the mechanisms that control REMS and may suggest new approaches to developing a powerful REMS enhancement model in rodents by which to test hypotheses about the functions of REMS.

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