

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.

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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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Table 1

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 {}

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

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

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

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

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 ($47 \times 26 \times 20$ 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 $\times$ 38 cm wide $\times$ 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

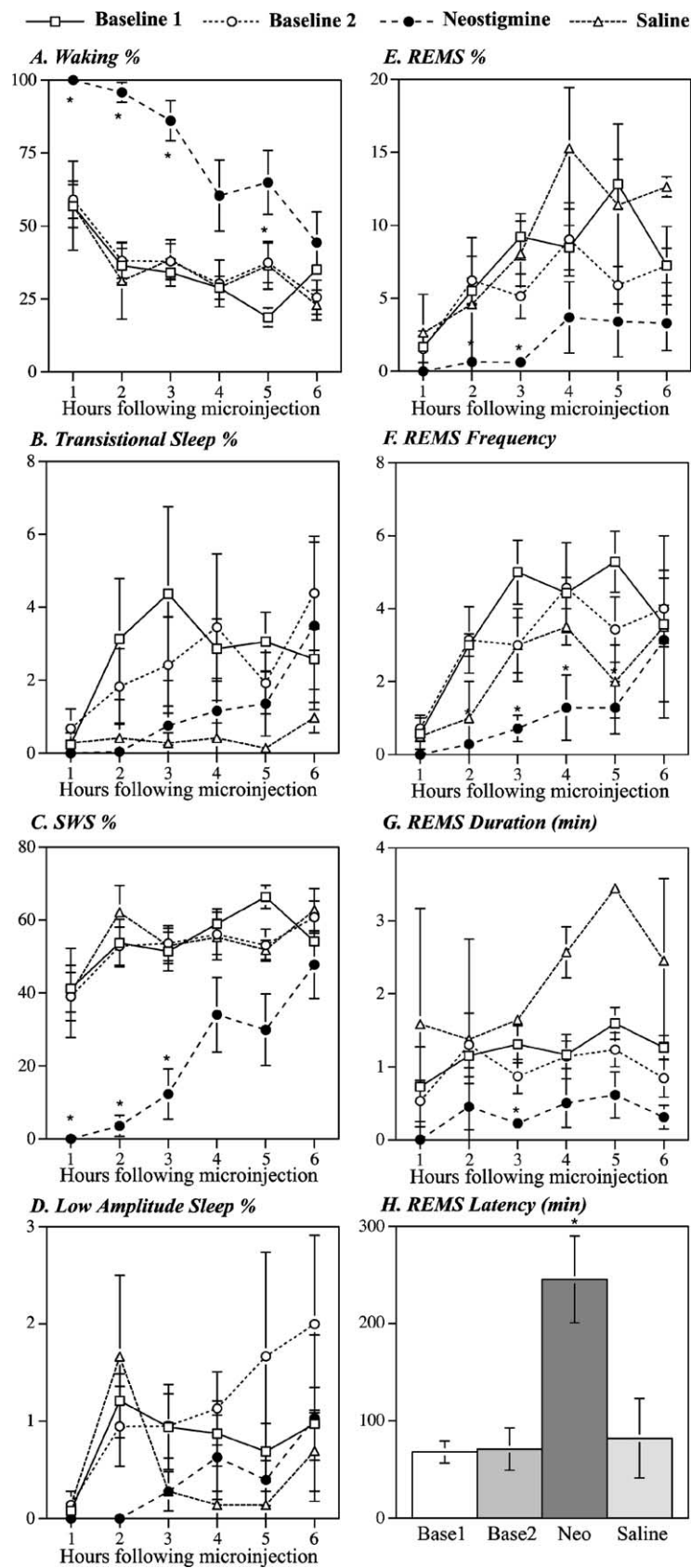


Fig. 1. Changes across recording time in each of the sleep parameters for baseline versus neostigmine conditions. Error bars represent the standard error of the mean. Asterisks represent statistical significance at the alpha 0.01 level between neostigmine and the average baseline value.

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:

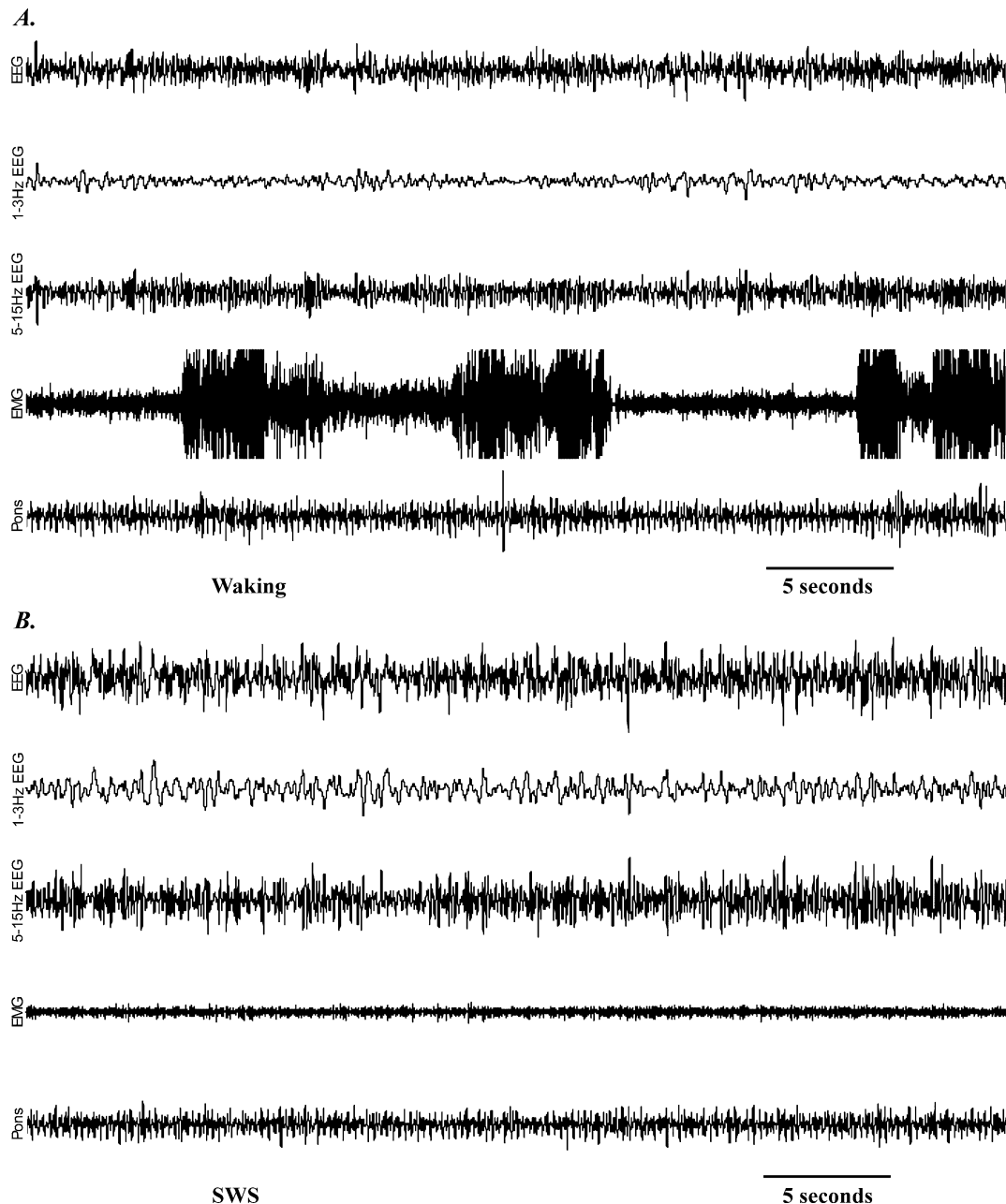


Fig. 2. Polygraphic recording examples of normal behavioral states and the neostigmine-induced state from a single mouse. Each example shows in order: cortical EEG broadly filtered (0.3 to 50 Hz) scaled ± 0.6 mV, cortical EEG filtered for delta waves (1–3 Hz) scaled ± 0.6 mV, cortical EEG filtered for 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.

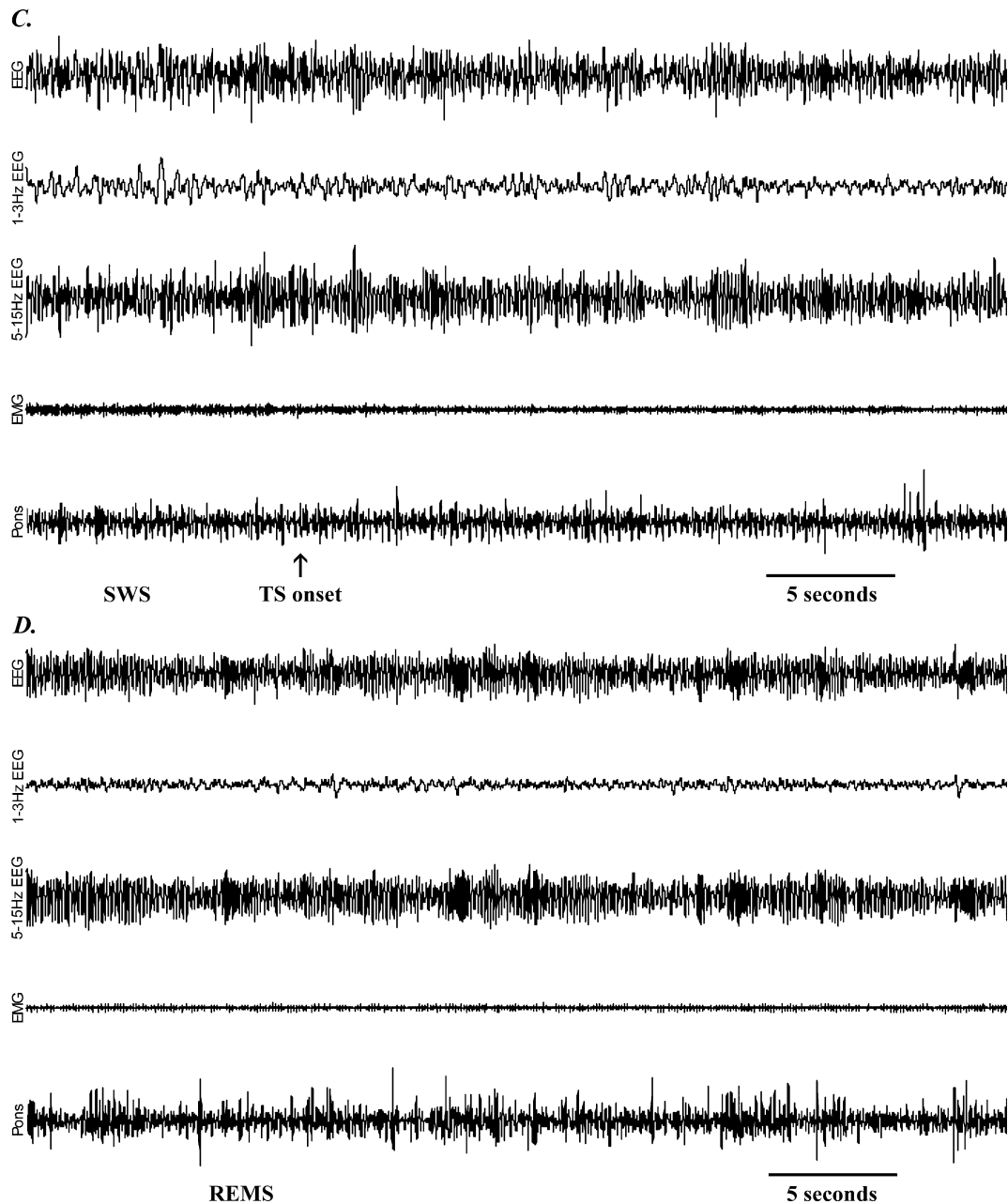


Fig. 2 (continued).

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

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

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

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

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

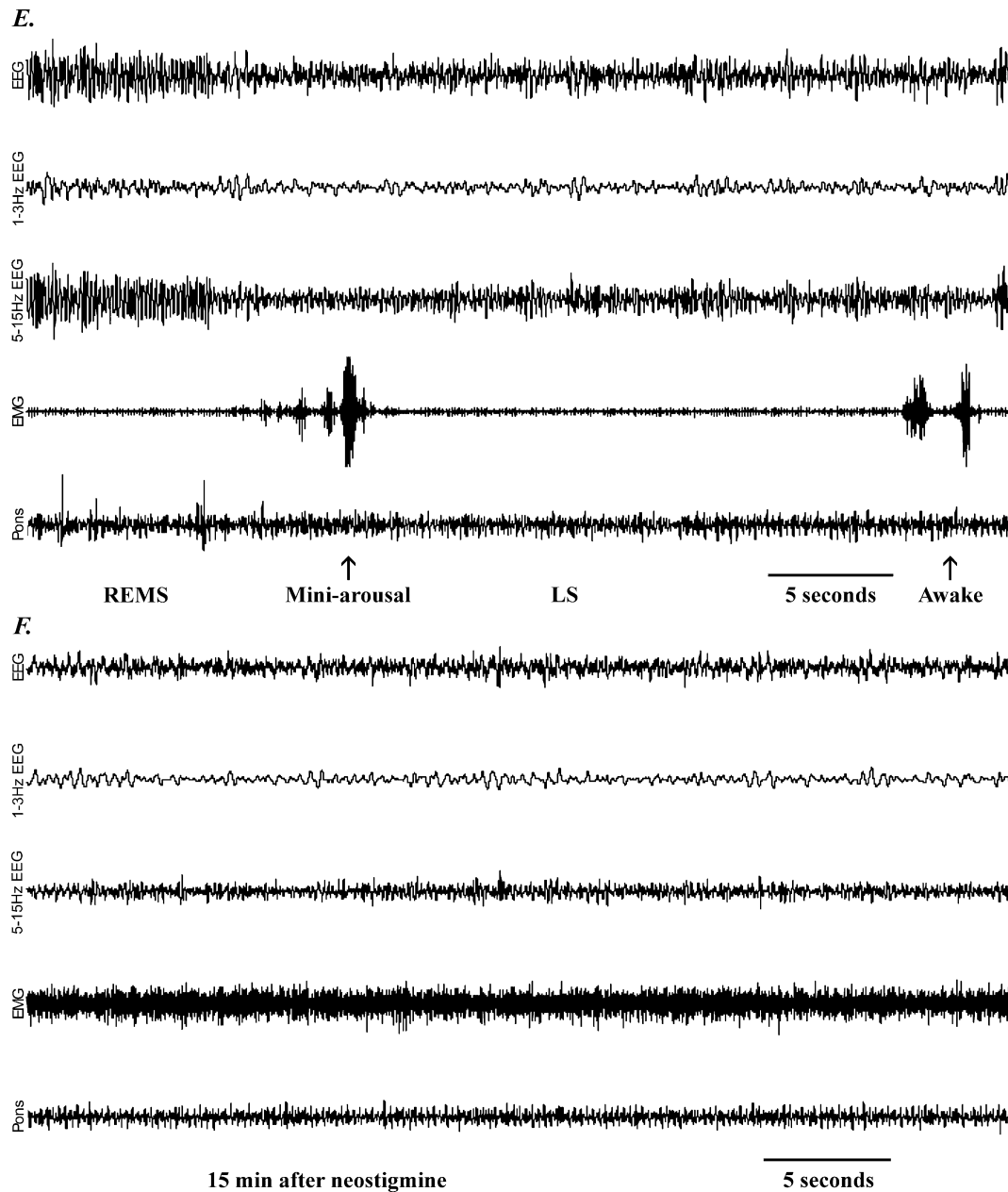


Fig. 2 (continued).

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

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

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

2.4. Microinjection procedure

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

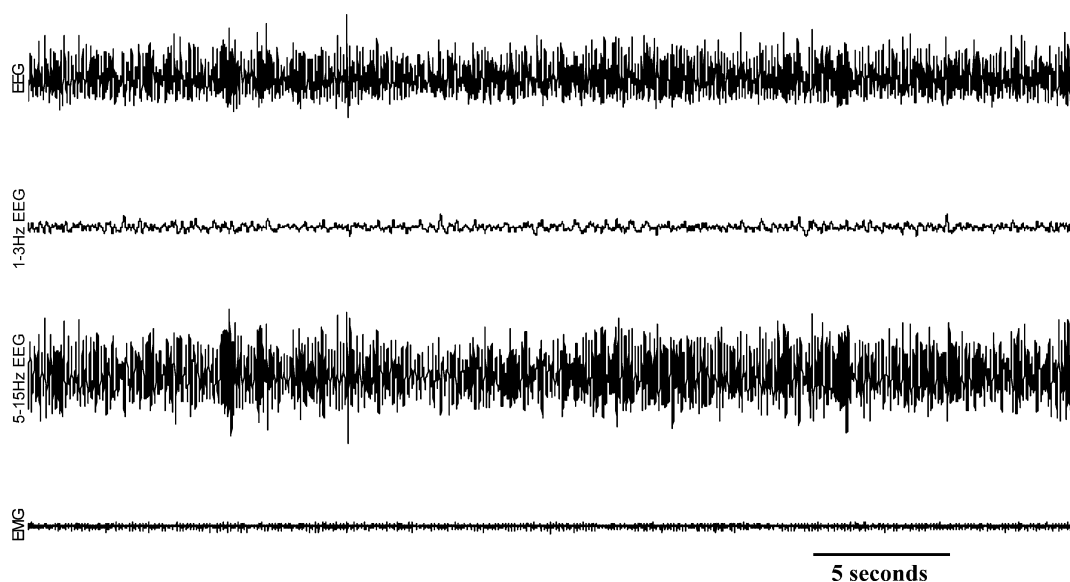


Fig. 3. Polygraphic recording example of REM sleep following within the first hour after saline injection alone. This example shows in order: cortical EEG broadly filtered (0.3 to 50 Hz) scaled ± 0.6 mV, cortical EEG filtered for delta waves (1–3 Hz) scaled ± 0.6 mV, cortical EEG filtered for bandwidths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

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

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

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

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

All of the above described characteristics were especially pronounced in the first 2 h following neostigmine microinjection and then gradually dissipated over the next several

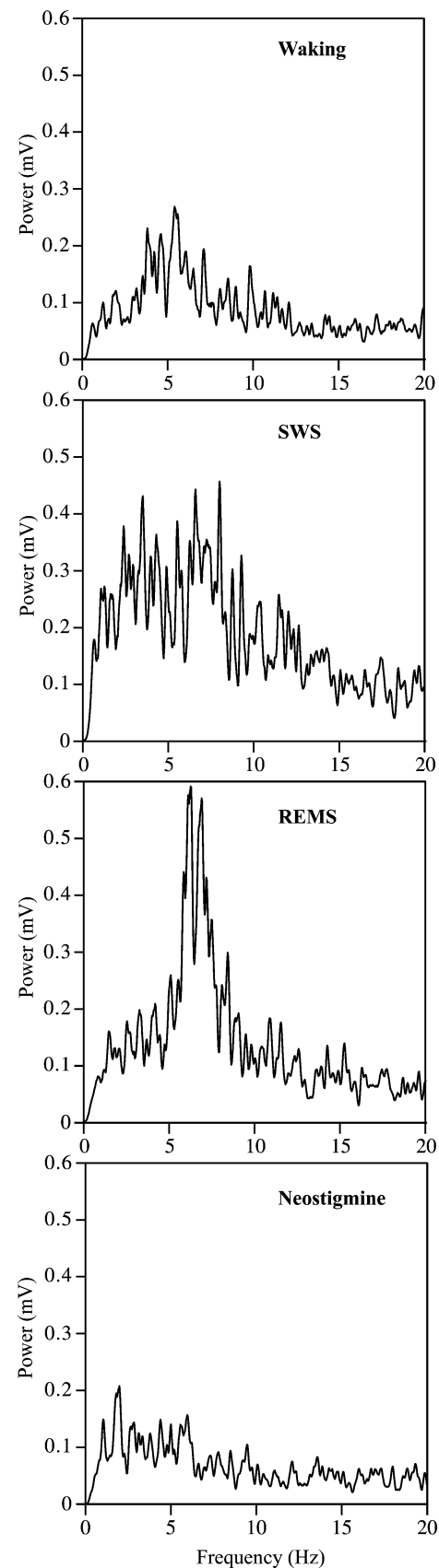


Fig. 4. Spectral distribution of cortical EEG power during wakefulness, SWS, REMS, and after neostigmine PRF microinjection, as revealed by Fast Fourier Transformation (FFT). Data for the FFT analyses were all taken from a single animal within the first hour of recordings following either saline injection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

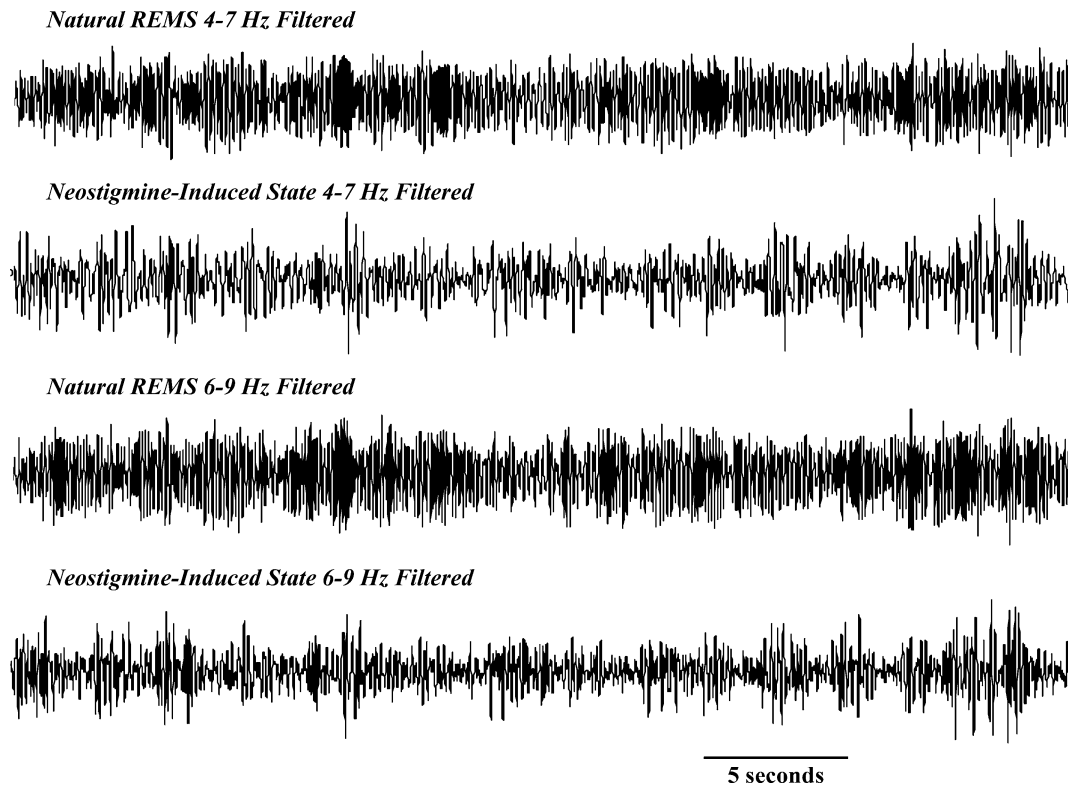


Fig. 5. Examples of filtering effects upon theta rhythms during natural REMS versus the neostigmine-induced state. Each example is scaled ± 0.4 mV. (Top) Cortical EEG activity filtered between the 4–7 Hz range previously reported [36] to be increased during the neostigmine-induced state. (Bottom) Cortical 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.

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

3.2. Drug effects on locomotor activity

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

3.3. Location of cannulas

Injection sites were located from -4.72 to -5.20 mm posterior of the bregma, 0.6 to 1.0 mm lateral of midline, and 3.6 to 4.75 mm ventral of the horizontal plane passing 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.

4. Discussion

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

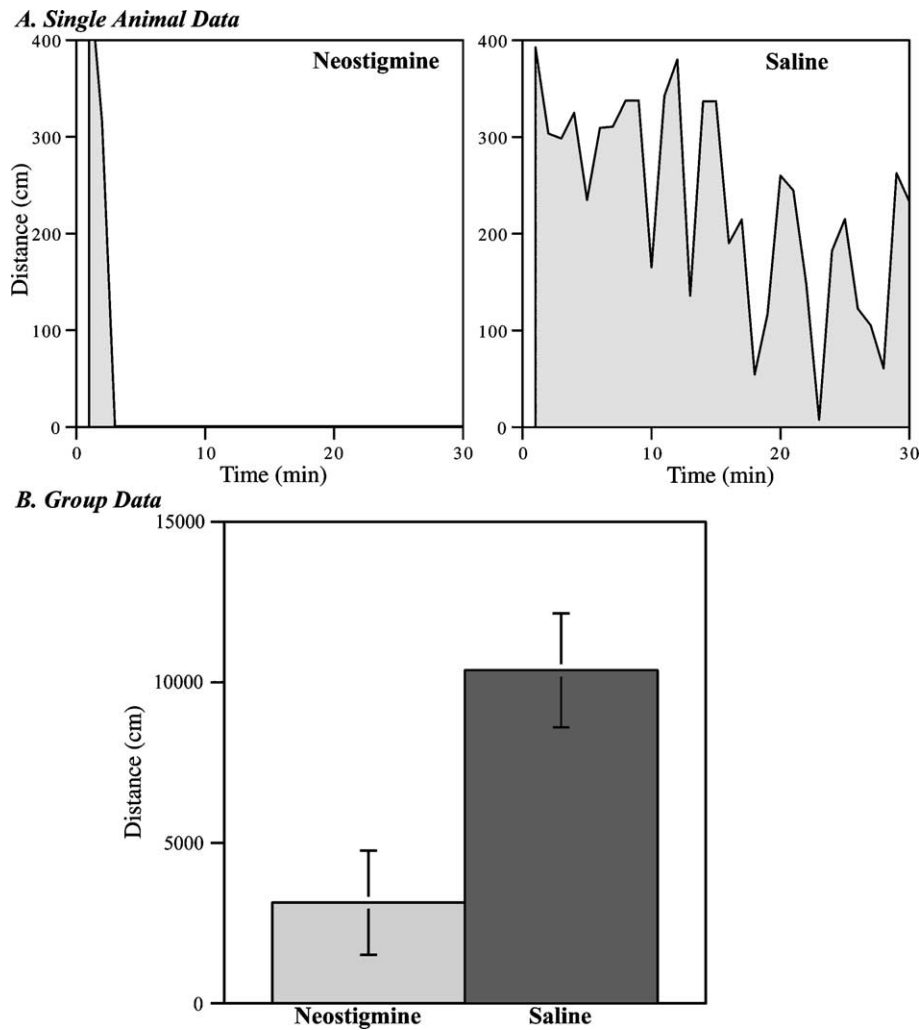


Fig. 6. Open field activity. (A) Single animal data of open field activity immediately following neostigmine versus saline microinjections. The samples shown here are taken from a single mouse whose electrophysiological data are displayed in Fig. 2, and that received, in order, neostigmine and then saline 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.

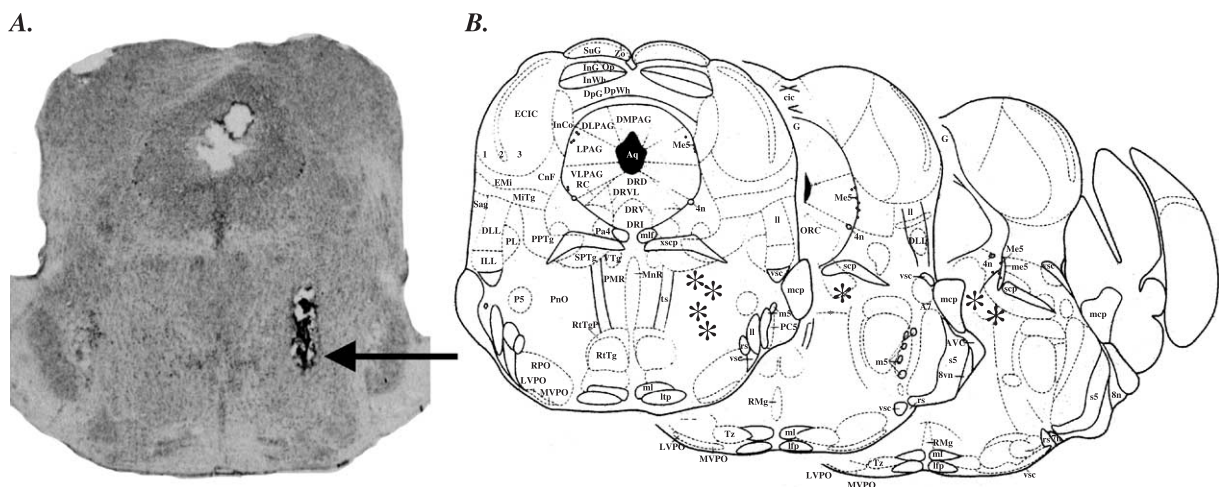


Fig. 7. Histology illustrating microinjection sites in the pons. (A) Digital image of a cresyl-violet-stained coronal section. To localize the injection site (arrow), 50 nl of India ink was microinjected through the cannula before the removal of the brain. (B) Mouse brainstem coronal sections along the 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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