

Research report

Rapid Eye Movement Sleep induction by microinjection of the GABA-A antagonist bicuculline into the dorsal subcoeruleus area of the rat

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Abstract

In cats, rapid eye movement sleep (REMS) can be induced rapidly and reliably by injections of the cholinergic agonist carbachol into the anterodorsal pontine tegmentum, also recognized as the perilocus coeruleus alpha, and designated the REMS Induction Zone (RIZ). In rats, the RIZ has been ascribed to a much larger and more ventral region within the entire oral pontine reticular formation. However, carbachol injections throughout this area produce only small, unreliable, and long latency REMS enhancements. The present study investigated whether REMS induction in the rat is possible by microinjection into the dorsal subcoeruleus nucleus (SubCD), a region with similarities to the cat RIZ. In freely moving unanaesthetized rats, microinjection of the GABA-A antagonist bicuculline significantly increased the amount and reduced the latency to REMS during a 2-h recording in the mid-light period. However, at effective doses, bicuculline usually also produced intermittent ipsiversive circling behavior that disrupted REMS maintenance. Attempts at eliminating this side-effect by: (i) coinjection of bicuculline with the NMDA antagonist, APV, (ii) lower bicuculline doses, or (iii) injection of the GABA-B antagonist, phaclofen, were unsuccessful. Other drugs injected into this area did not induce REMS; these included carbachol, the acetylcholinesterase inhibitor neostigmine, the glutamate agonist kainate, and vasopressin. In the rat, the SubCD is a highly sensitive region for both REMS induction and locomotor effects.

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1. Introduction

A periodic state during sleep characterized by the rapid movement of the eyes beneath closed eyelids and associated with intense dreaming, was first discovered and named Rapid Eye Movement Sleep (REMS) in the 1950s [2,15]. It has also been termed ‘paradoxical sleep’ by Jouvet [22] due to its paradoxical nature of high, waking-like cortical activity concurrent with a state of deep, muscle-atonic sleep. The function of this sleep state remains contentious. A large body of research has impli-

cated REMS in learning and memory [20,21,25,31,41,43], but the interpretation of these results is complicated because the principal technique used to assess a causal relationship between REMS and memory—namely, the REMS deprivation technique—also produces changes in non-mnemonic functions that might impact learning performance [39,48]. The development of a REMS-enhancing technique as an experimental method for testing sleep function would be useful for circumventing some of these confounds. However, this method has yet to be exploited because a reliable technique for inducing large increases in REMS in rats and mice, the species of choice in studies of sleep and memory, has not yet been developed.

In the cat, the anterodorsal pontine tegmentum is considered to contain a ‘REM sleep induction zone’ (RIZ)

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because injection of the cholinergic agonist carbachol into this region induces immediate and prolonged REMS [51], and injections outside this area show diminishing efficacy with distance. This same region has been identified as the RIZ under other anatomical labels. For example, it corresponds to the *rostral gigantocellular tegmental field* (FTG) [1,34], the *rostradorsal pontine tegmentum* [4], the *medial pontine reticular formation* (mPRF) [5,9,38], and the *perilocus coeruleus alpha* (peri-LCa) [37,46]. An exception that should be noted, though, is one study that obtained the opposite result, i.e. induction of REMS by carbachol in the ventral portion but not the anterodorsal region of the cat pontine tegmentum [35].

A carbachol-sensitive RIZ in the rat has been sought throughout the pontine reticular formation (PRF) [11], but with limited success [17]. In a recent evaluation of this technique [17], most carbachol injections did not alter sleep–wake states and, even when carbachol was effective, it was as likely to increase waking. Also, in the effective cases, REMS was increased by only ~70% (4 min) during the first 2 h after treatment, and the latency to REMS (66 min) was not significantly different from controls. In contrast, carbachol injections into the RIZ of the cat [9] reliably induce very large REMS increases in the first hour (e.g. 3700%, ~50 min), with a much shorter REMS latency (~9 min). However, results similar to the rat have been obtained when carbachol is injected outside the cat's primary RIZ [4]. One interpretation of these comparative data is that the region of the rat brainstem that is truly homologous to the cat RIZ has not yet been identified.

Here we report on studies of REMS following microinjection into the dorsal subcoeruleus nucleus (SubCD) of the rat. This region was investigated as a potential RIZ for several reasons. First, this area has recently been identified as the PGO wave generation site in the rat, based on a mapping study of carbachol injections in anaesthetized rats [14]. The effective region has been described as being 0.7 mm in diameter, similar in size to the cat's RIZ (0.8 mm diameter). The mechanism of PGO wave generation itself has been hypothesized to be actively involved in the generation of REMS, primarily because PGO waves always immediately precede and are continuous with REMS (reviewed in Ref. [14]).

Second, the location of this PGO-wave generation site in the SubCD is similar to that of the cat RIZ. The cat RIZ has been described as being centered 2.0 mm lateral to the midline, just lateral to the medial longitudinal fasciculus (mlf) and ventromedial to the dorsal peribrachial region (PB) [51]. The rat SubCD PGO wave generation site is centered ~1.3 mm lateral to the midline, also just lateral to the mlf and ventromedial to the dorsal PB [14]. Both structures are located 1 mm ventral to the locus coeruleus (LC), for which reason the SubCD got its name, and the cat RIZ has in some studies been localised to the locus coeruleus alpha and peri-LCa structures [46]. The cat RIZ is dorsal to the FTG while the SubCD is dorsal to what

appears to be the rat equivalent: the caudal pontine reticular nucleus (PnC). Finally, the cat RIZ has been described as beginning at the same anterior–posterior plane as the ventral tegmental nucleus (VTg) and shown to extend as far posterior as the trigeminal motor nucleus (Mo5), ending roughly before the appearance of the facial nerve (7n) [4,46,51]. The sensitive region for PGO-wave generation in the SubCD begins just slightly more posterior to the VTg plane, but does run parallel with the Mo5 and ends before transection of the SubCD area by the 7n.

A third reason for investigating the SubCD as a RIZ is that ablation of this structure [36], but not other pontine tegmental sites [18], completely abolishes the occurrence of REMS in the rat without affecting slow wave sleep (SWS). Similarly, lesion and single unit recording studies have suggested that the SubCD area of the cat RIZ is more essential to REMS generation than is the FTG region (reviewed in Ref. [46]).

Finally, there is one preliminary report that iontophoretic application of the GABA-A antagonist, bicuculline, can induce a short latency (2–3 min) and long-lasting (15–90 min) episode of REMS in unanaesthetized but head-restrained rats [10]. Bicuculline significantly reduced the latency to REMS and increased the duration of the REMS episodes, which are uncommon in rat REMS-induction studies. Bicuculline has similar effects following injection into the RIZ of the cat [49,50].

To confirm and extend these results, we tested whether bicuculline and other compounds known to increase REMS following injection into the cat RIZ can induce REMS following injection into the SubCD of freely moving, unanaesthetized rats.

2. Materials and methods

2.1. Subjects

Sixteen male Long–Evans hooded rats (Charles River, Quebec, Canada) weighing 250–300 g at the time of surgery were used in this experiment. Food and water were available ad libitum throughout the experiment. Rats were housed in pairs in standard polypropylene cages (47×26×20 cm) before surgery and housed individually for the remainder of the experiment. To control for circadian factors, all experimental procedures took place 3–7 h following the time of light onset (LD 12:12) and each individual subject was always retested at the same time within this 4-h period. Rats were handled each day throughout the experiment except during the first 4 days after surgery.

2.2. Surgery

Animals were anaesthetized with Somnotol (sodium pentobarbital, 60 mg/kg) supplemented with halothane as

necessary. Rats were placed in a stereotaxic apparatus with the skull level. Body temperature was maintained at 37 ± 2 °C using a digital rectal thermometer and a heating pad.

For drug injections, a 22-gauge guide cannula (Plastics One, Inc.) with a 26-gauge internal blocker ('dummy cannula') extending 1 mm beyond the tip of the guide cannula was implanted into the left SubCD, at the following coordinates: 1.5 mm lateral from midline, 0.8 mm posterior of the lambdoid suture, and 8 mm ventral from the skull surface of lambda.

To record sleep/waking states, four EEG and two EMG electrodes (Plastics One, Inc.) were also implanted. EEG electrodes were stainless-steel jeweller's screws threaded into holes drilled through locations on the skull as previously described [26,27]. Briefly, a lateral EEG was obtained by differential recording from two electrodes positioned 1.0 mm posterior and 2.5 mm lateral to bregma, and 1.0 mm anterior and 3.5 mm lateral to lambda, respectively. A midline EEG was obtained from electrodes positioned 1 mm lateral to the midline, and 3 mm anterior to bregma and midway between bregma and lambda. EMG was recorded from two subcutaneous wire electrodes implanted behind the occipital bone and underneath the neck muscle. The pins from all six electrodes were connected to a protective plastic headcap. Three additional anchor screws were implanted and the entire assembly was insulated and bonded to the skull with dental acrylic. A topical antibiotic steroid, Topagen, was applied to the wound. Finally, a subcutaneous injection of Butorphanol (0.2 cc/kg) was given for postsurgical analgesia.

2.3. Drug test procedure

Beginning 7 days after surgery, the rats were placed in an electrically shielded chamber (dimensions: 38 cm long $\times$ 38 cm wide $\times$ 45 cm high) and connected to an EEG recording cable for 15 min/day for 5 days, for habituation. The recording chambers were housed in a temperature- and light-controlled isolation room with a video camera and a white noise generator (Lafayette Instrument Co.). EEG and video signals were monitored remotely in a separate room.

Following this adaptation period, all rats were given two 2-h baseline recording sessions on consecutive days. For drug tests, rats were taken from their home cage, connected to the recording cable, given a drug injection and then placed in the recording chamber for a 2-h recording session. Drug injections were made via a stainless-steel injection cannula (26 gauge) inserted into the guide cannula. The injection cannula was attached to a 1- μ l Hamilton syringe by polyethylene tubing (PE-20). The Hamilton syringe was mounted in a manual microdrive (Kopf), and 0.25 μ l of saline or drug solution were delivered over 1 min. To control for possible order effects, drug tests were scheduled using a Latin square design with two subjects per row. At least 4 days were allowed between drug injections in the same rat.

All drugs used in this experiment were obtained from Sigma (St Louis, MO, USA) and were dissolved in sterile saline fresh on the test days. Concentrations of the drugs used were as previously reported to be effective in the cat RIZ (Table 1). Upon completion of all planned single drug tests, five additional tests were conducted, each again using a 0.25- μ l volume: (i) coinjection of bicuculline (8 mM) with the NMDA antagonist, APV (50 mM; same as Ref. [24]); (ii) injection of bicuculline at lower concentrations (1 and 2 mM); (iii) injection of the GABA-B antagonist, phaclofen (20 mM; same as Ref. [50]); (iv) coinjection of bicuculline (2 mM) with carbachol (5.5 mM); and (v) coinjection of phaclofen (20 mM) with carbachol (5.5 mM).

2.4. Sleep recording

EEG and EMG signals were amplified (Grass model 79D, Grass Instruments Co.), then digitised (sampling rate of 250 Hz) and stored on a computer using data collection software (AcqKnowledge, Biopac Inc.). Behavioral states were visually scored off-line in 10-s epochs on the basis of four electrographic measures: unfiltered lateral EEG, lateral EEG filtered for delta waves (1–3 Hz), midline EEG filtered for bandwidths including theta (5–15 Hz) and EMG (high pass filtered at 50 Hz). Each epoch was

Table 1
Drug concentrations used in the present experiment, previously reported to be effective in the cat REMS Induction Zone

Reference number	Drug	Amount delivered in 0.25 μ l saline solution	Molar concentration
[9]	Carbachol	0.25 μ g	5.5 mM
[3]	Neostigmine	20 μ g	264 mM
[29]	Kainate	5.3 ng	0.1 mM
[50]	Bicuculline (methobromide)	0.72 μ g	8 mM
[45]	Propranolol	4 μ g	54 mM
[33] ^a	Vasopressin	0.024 and 2.4 ng	0.09 and 9 μ M

^a The dosages of vasopressin used here are higher than that originally reported by Pompeiano to induce muscle atonia [33], but within the lower range reported to be capable of producing physiological effects in other parts of the brain [6,7,47]. Different, even opposite, reactions can occur at low (nM) versus high (μ M) concentration levels of vasopressin [12,19], thus low and high doses were tested.

classified as whichever state was predominant. The following five sleep/wake stages were recognised:

2.4.1. Wakefulness (W)

Low amplitude lateral EEG, absence of delta waves, only occasional regularity in midline theta EEG, and high amplitude EMG.

2.4.2. Transitional sleep (TS)

High amplitude lateral EEG, absence of delta waves, spikiness in midline theta EEG, and low amplitude EMG. With its low frequency and high amplitude EEG activity, TS is distinguished chiefly by EEG spindles. This stage encompasses both the light sleep (SWS-1) and the transitional state between SWS and REMS (tS-R) previously described [22]. Due to the lack of a pontine wave measurement, it was not possible in the present study to differentiate between these two specific types of transitional states. This stage may be homologous with stage 2 sleep of humans.

2.4.3. Slow-wave sleep (SWS)

High amplitude lateral EEG, presence of delta waves, spikiness in midline theta EEG, and low amplitude EMG.

2.4.4. REMS

Low amplitude lateral EEG, absence of delta waves, sinusoidal midline theta rhythms, and muscle atonia.

2.4.5. Low-amplitude sleep (LS)

Low amplitude cortical EEG, absence of delta waves, spikiness in midline theta EEG, and low amplitude EMG. Without a midline theta measure, 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 REMS before entry into SWS (our unpublished observations). Under some criteria for scoring rat sleep/wake stages (e.g. Datta and Hobson [13]), this state would be classified as a type of quiet W. However, Bergmann et al. [8] have argued convincingly that LS more closely resembles the remainder of Non-REM (NREM) sleep in spontaneous behavior, response threshold, and incidence of PGO-type waves. Accordingly, we consider it to be a substage of NREM sleep rather than W.

2.5. Histology

To mark the injection site, 0.25 μ l of a 1% Pontamine blue dye was injected through the cannula. The subjects were then euthanized with an overdose of sodium pentobarbital and perfused transcardially with 9% saline followed by 10% formalin. The brains were removed and post-fixed for 5 h in 10% formalin before being stored in a 20% sucrose-formalin solution overnight. Coronal sections (50 μ m) were taken through the pons using a cryostat and

stained with cresyl violet. Injection sites were located by reference to the rat brain atlas of Paxinos and Watson [30].

2.6. Data analysis

REMS latency, duration, frequency, percentages during the 1st and 2nd hours, and overall amounts of W, TS, SWS, REMS, and LS in the total recording time (TRT) were evaluated by repeated measures ANOVA and post-hoc Bonferroni *t*-tests at the $P < 0.05$ criterion level (SigmaStat for Windows Version 1.0, Jandel Corporation, 1993). REMS latency was calculated from the end of the injection to the first 10-s epoch of REMS. Recording sessions that did not display REMS were assigned the maximum recording time (120 min) for REMS latency. 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 total REMS time by REMS frequency.

3. Results

3.1. Sleep during control conditions

All rats exhibited some sleep in one or more of the three control conditions (i.e. two baseline and one saline condition). Comparisons between the first two baseline recordings (Fig. 1) showed that the second baseline recording

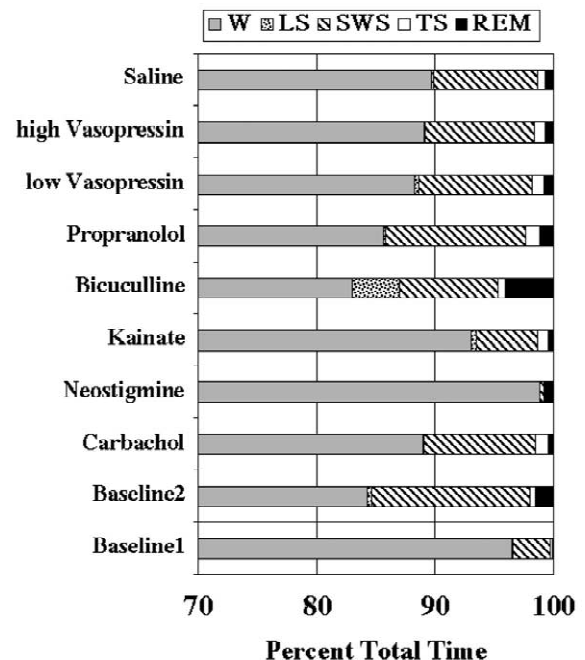


Fig. 1. Percentage of total recording time spent in each behavioral state across drug conditions without regard for cannula placements. Dark filled column, REMS; unfilled column, TS; diagonal column, SWS; dotted column, LS; shaded grey column, W.

was associated with significantly more SWS (3.22 vs. 13.34% of TRT), and significantly more REMS during the 2nd hour (0.13 vs. 1.54% of hour 2). This may reflect a habituation process over these consecutive days of recording.

3.2. Drug effects on behavioral state

The percentage of TRT spent in each behavioral state across conditions is illustrated in Fig. 1. There was a significant effect of condition on waking ($F_{(15, 9)}=3.47$, $P<0.001$), SWS ($F_{(15, 9)}=3.83$, $P<0.0005$), and REMS ($F_{(15, 9)}=2.93$, $P<0.005$). Post-hoc analyses revealed that these effects were due to three factors: more sleep in the baseline2 condition as noted above, less sleep following neostigmine, and more REMS following bicuculline.

In the neostigmine condition, SWS was reduced to 0.32% of TRT, and REMS was evident in only two subjects, albeit with relatively short latencies (15 and 26 min). The drug also produced prolonged theta rhythms during wakefulness (sometimes nearly continuous throughout the entire recording session) and occasional lachrymation.

Enhanced REMS following bicuculline occurred at the expense of W, and there was no significant effect on the percentage of TRT spent in other sleep states. Other REMS parameters were also significantly affected by bicuculline. There was a significant main effect of drug condition on REMS latency ($F_{(15, 9)}=3.76$, $P<0.0005$), REMS frequency ($F_{(15, 9)}=3.41$, $P<0.001$), and 1st hour percentage ($F_{(15, 9)}=3.94$, $P<0.0002$) (Table 2), and post-hoc comparisons revealed that these effects were attributable entirely to a REMS-enhancing effect of bicuculline. Compared to baseline1, bicuculline was associated with reduced REMS latency, increased REMS episode frequency,

and increased REMS% of TRT. Compared to baseline2, bicuculline significantly elevated REMS% only during the 1st hour and not during the 2nd hour.

The occurrence and duration of REMS for each trial of bicuculline and control injections are illustrated in Fig. 2. Only 32.5% of the rats showed REMS during both of these trials, thus paired comparisons of REMS duration were not always possible. The mean REMS duration following bicuculline infusions was not significantly different from control conditions, but the longest REMS bouts occurred in the bicuculline condition ($t_{(5)}=2.02$, $P=0.049$; Table 2).

3.3. Locomotor side-effects of bicuculline

Bicuculline-induced REMS was often interrupted by periods of W accompanied by ipsiversive circling behavior. This locomotor activity ranged in severity from mild stepping behavior (19% of cases) to wild-running seizures (31% of cases). In some of the animals that displayed wild-running reactions to bicuculline, repeated applications of bicuculline (described below) were associated with afterdischarges in the cortical EEG suggesting a kindling effect.

In the cat brainstem, the mesencephalic locomotor region (MLR) overlaps with the RIZ [24]. Electrical stimulation of the MLR in the rat has been shown to induce effects similar to what we observed with bicuculline injections: violent running, explosive jumps, and ipsiversive circling [16]. Locomotor activation via the MLR may be mediated by NMDA receptors [24]. Therefore, in an attempt to eliminate the circling behavior and isolate REMS-enhancing effects, bicuculline was given in combination with the anti-convulsant NMDA antagonist, APV ($N=16$). However, locomotor side-effects were observed in 25% of these cases. Notably, REMS-enhancement was evident in three rats that previously did not show

Table 2
Effects of drugs upon REMS latency, duration, frequency, and 1st hour percentage without regard for cannula placements

Condition	REMS latency (min) ^a Mean ($\pm$ S.E.M.)	REMS episode duration (min)			REMS frequency Mean ($\pm$ S.E.M.)	REMS% 1st hour Mean ($\pm$ S.E.M.)
		Mean ($\pm$ S.E.M.)	Mean Max ^b	Range		
Baseline1	119.4 $\pm$ 0.4	1.3 $\pm$ 0.3	2	0.5–2	0.1 $\pm$ 0.1	0 $\pm$ 0
Baseline2	98.8 $\pm$ 5.8	1.9 $\pm$ 0.13	2.3	0.8–2.8	1.1 $\pm$ 0.4	0.1 $\pm$ 0.1
Carbachol	111.3 $\pm$ 4.4	0.6 $\pm$ 0.1	0.8	0.2–1.8	0.6 $\pm$ 0.3	0 $\pm$ 0
Neostigmine	107.8 $\pm$ 8.4	2.9 $\pm$ 0.7	3.5	0.9–6	0.3 $\pm$ 0.2	1.7 $\pm$ 1.5
Kainate	117.3 $\pm$ 2.2	2.7 $\pm$ 0.3	3.5	1.8–3.7	0.3 $\pm$ 0.2	0 $\pm$ 0
Bicuculline	77.2 $\pm$ 13.6 ^c	1.7 $\pm$ 0.4	6.7 ^d	0.5–20.2	2.9 $\pm$ 1.1 ^c	7 $\pm$ 3.1 ^e
Propranolol	103.3 $\pm$ 6	1.6 $\pm$ 0.2	2.1	0.8–3	1.0 $\pm$ 0.3	0.3 $\pm$ 0.3
Low dose vasopressin	109.8 $\pm$ 4.7	1.6 $\pm$ 0.2	2.2	0.6–2.8	0.8 $\pm$ 0.4	0.1 $\pm$ 0.1
High dose vasopressin	113.9 $\pm$ 2.6	2.0 $\pm$ 0.2	2.33	1.3–3	0.5 $\pm$ 0.2	0 $\pm$ 0
Saline	106.6 $\pm$ 5.3	1.4 $\pm$ 0.2	2	0.8–3.2	0.8 $\pm$ 0.3	0.1 $\pm$ 0.1

^a Recording sessions that did not show any REMS were given the maximum recording time (120 min) as their REMS Latency.

^b The group average of the longest REMS bouts for each subject in each condition.

^c Different from baseline1 and saline; $P<0.05$, Bonferroni *t*-test.

^d Different from baseline2; $P<0.05$, Bonferroni *t*-test.

^e Different from baseline1, baseline2, and saline; $P<0.05$, Bonferroni *t*-test.

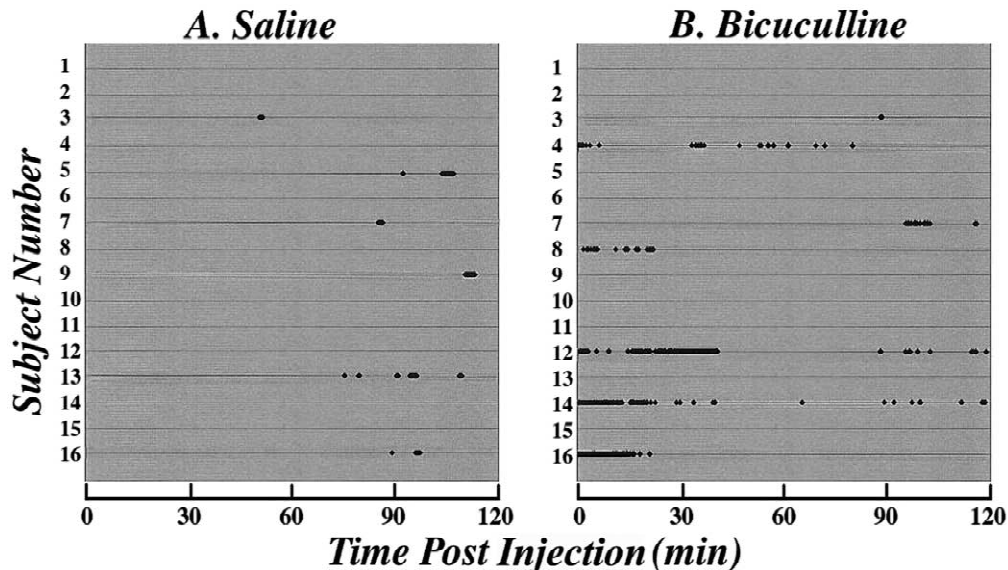


Fig. 2. Hynograms illustrating the temporal distribution of REMS. Each black bar represents one REMS episode. All subjects (ordinate) are shown from the time of injection (abscissa, Time 0) to the end of the 2nd hour post-injection; A, saline; B, bicuculline.

a response to bicuculline alone. Three other rats that showed REMS induction to bicuculline showed only locomotor side-effects to the bicuculline-APV cocktail.

In a second attempt to reduce motor side-effects, lower doses of bicuculline were tested. A dose–response curve has previously been reported for bicuculline in the cat [50]. In that study, the lowest effective REMS-inducing dose was 1 mM, peak REMS induction was at 10 mM, and a ‘state of wakefulness accompanied by hyperexcitation’ was evident at 15 mM or higher. This ‘hyperexcitation’ appears to be comparable to the motor side-effects observed at 8 mM in the present study (M.C. Xi, personal communication, June, 2002). Doses of 1 ($N=16$) and 2 mM ($N=15$) were therefore tested but these did not induce REMS, yet did produce locomotor effects, including some wild-running seizures, in 19% of the subjects.

Like bicuculline, the GABA-B receptor antagonist, phaclofen, has been reported to induce REMS when injected into the cat RIZ [50], although with lower efficacy. Phaclofen also reduces seizures in some animal models of epilepsy [42]. Therefore, we tested whether phaclofen might have REMS-inducing properties but without locomotor side-effects. Six rats were tested, five of which had previously shown REMS in response to bicuculline. Although phaclofen did not induce motor effects, it also did not induce REMS at the dose tested.

Carbachol activates REMS-associated neurons in the SubCD in urethane anaesthetized rats, as indicated by PGO wave generation [14]. However, in the present study carbachol did not induce REMS in unanaesthetized freely-moving rats. Anaesthesia may attenuate the inhibition of REMS-on neurons that must be exerted during W, making these cells more sensitive to activation by carbachol. Given that bicuculline can induce REMS directly from W, GABA

may mediate the inhibitory effects of W on REMS-on cells. Therefore, we tested whether low-dose bicuculline (2 mM) might mimic anaesthesia, and increase the sensitivity of REMS-on cells to carbachol. Instead, we observed an intensification of the locomotor side-effects (five subjects with wild-running seizures and one with stepping behavior out of nine rats tested). A combination of carbachol and the GABA-B receptor antagonist, phaclofen, was also tested in two rats that had previously shown REMS-enhancement by bicuculline, but in neither case was REMS or locomotion induced.

3.4. Appearance of bicuculline-induced REMS

Under baseline conditions, REMS was always observed to follow SWS (e.g. Fig. 3a), usually with a brief bout of TS intermediate between the two behavioral states. Rats would often make several apparent attempts to enter REMS with awakenings out of TS, before finally entering fully into REMS towards the end of the recording session. However, following bicuculline injections, rats would fall into REMS directly out of W. In most of these cases, upon completion of the 1 min microinjection procedure and placement of the rat in its recording chamber, the rat would immediately walk to the back of the chamber, curl up in a ball, and go to sleep.

Bicuculline-induced REMS matched the polysomnographic criteria for the classification of REMS. These criteria include a continuous midline EEG theta rhythm that is uniform in appearance and lacking in the spikiness evident during other behavioral states. However, in ~50% of the cases, theta associated with bicuculline-induced REMS, while uniform in appearance, was about 30% lower in amplitude than theta associated with naturally-

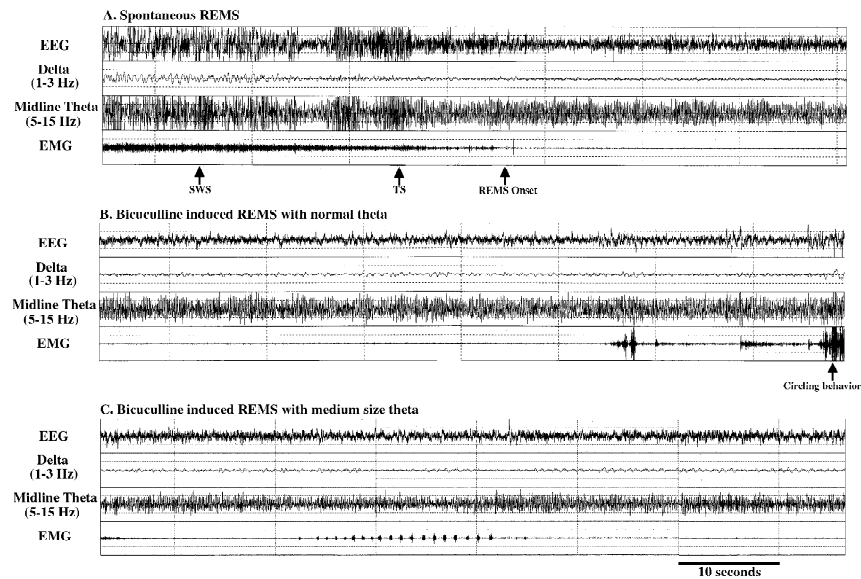


Fig. 3. Representative recordings of a spontaneous REMS episode (A) and two REMS episodes following microinjection of bicuculline, with normal midline EEG theta (B) and with medium-sized midline EEG theta (C).

occurring REMS (e.g. Fig. 3b vs. 3c). A similar effect was reported in the only cat REMS-induction study that included a direct measure of hippocampal theta rhythms [43].

3.5. Location of cannulas

Inspection of brain sections revealed that all cannula tips were located within a pontine region 0.3–0.8 mm posterior to the interaural line, 1–2 mm lateral to midline, and

6.8–8.8 mm ventral from the skull surface (Fig. 4; [30]). Cannulas were located in the SubCD ($N=8$), the locus coeruleus (LC; $N=2$), the mesencephalic trigeminal nucleus (Me5; $N=1$), the supratrigeminal nucleus (Su5; $N=1$), the caudal pontine reticular nucleus (PnC; $N=1$), and in an ambiguous area either just lateral to the subcoeruleus alpha (SubCA) or between the SubCA and the SubCD regions ($N=3$).

To determine whether the injection site could account for variability in REMS latencies seen following pontine

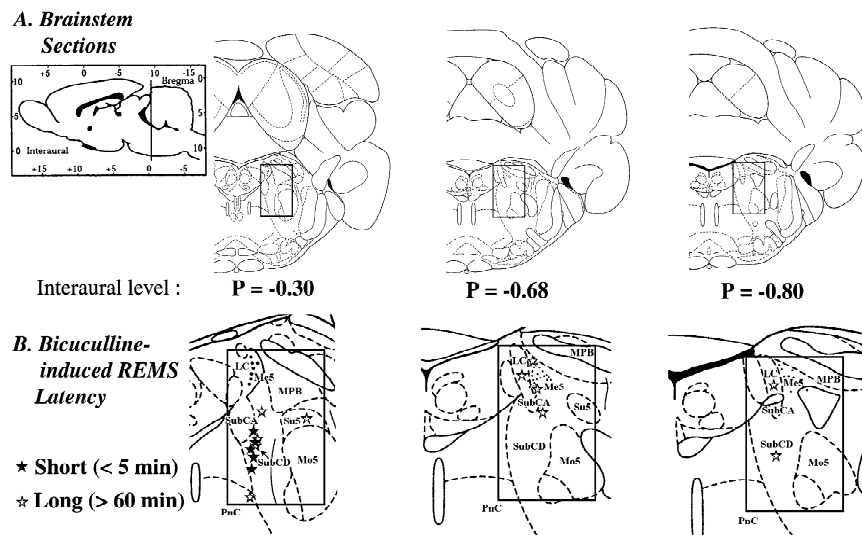


Fig. 4. Location of cannulas, coded for behavioral effects. Brainstem sections (A) showing pontine region explored roughly equivalent to 0.3–0.8 mm posterior from interaural line. The schematic drawings of the three coronal sections were taken from Ref. [30]. In each, the box corresponds to the area enlarged in the box below. REMS latency at each site following injection of bicuculline (B) shown by classifying the data into two groups: short latency sites (≤ 5 min) are indicated by filled stars and long latency sites (> 60 min) by unfilled stars. Abbreviations: LC, locus coeruleus; Me5, mesencephalic trigeminal nucleus; Mo5, motor trigeminal nucleus; MPB, medial parabrachial nucleus; PnC, caudal pontine reticular nucleus; Su5, supratrigeminal nucleus; SubCA, subcoeruleus nucleus alpha; SubCD, dorsal subcoeruleus nucleus.

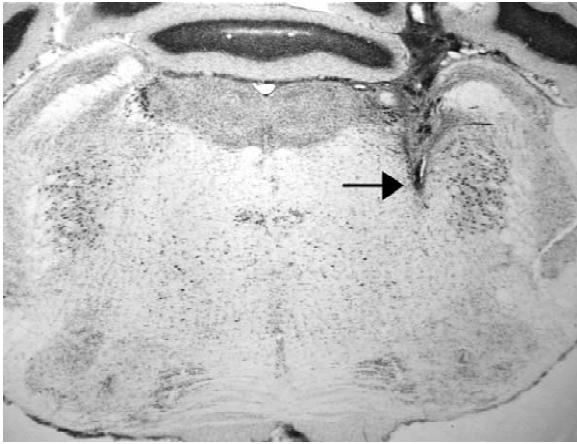


Fig. 5. Nissl-stained brain section illustrating the location of a cannula within the SubCD, through which short latency REMS-induction occurred in response to bicuculline. The arrow indicates the injection site.

carbachol infusions in cats, Baghdoyan et al. [4] and Yamamoto et al. [51] grouped REMS latencies into short, medium, and long categories. Baghdoyan et al. used categories of 0–20, 21–50, and >51 min, while Yamamoto et al. used 0–5, 5–10, >10 min. In the present study, a third of the REMS latencies following single bicuculline injections (five of 16 rats) fell within a 0–5 min range. For this group, the mean REMS latency was 0.6 min (compared to >60 min for the other 11 rats), and the percentage of time spent in REMS during the 1st hour of recording was 22.4% (13.43 min, compared to only 0.5 min following saline in the same rats). The injection sites that resulted in short REMS latencies were tightly clustered within the rostral SubCD (Figs. 4,5). Following bicuculline in combination with APV, short REMS latencies were obtained in those cases where the injection site was within the SubCD ($N=3$) or just lateral to the SubCA ($N=2$). In the two cases where intermediate REMS latencies were observed following neostigmine injections, one injection site was lateral to the SubCA and the other was in the Me5. Thus, sites associated with REMS induction appeared to be aligned mediolaterally with the Me5. This pattern matches the mediolateral alignment of the PGO wave generation site with the Me5 across coronal sections [14].

Sites in the brainstem from which bicuculline induced circling and wild-running seizures overlapped with the REMS-inducing areas in 80% of the cases. Areas where bicuculline produced REMS with normal or medium amplitude theta showed no consistent pattern within the subcoeruleus area.

4. Discussion

This study confirms the preliminary report of Boissard et al. [10] that application of the GABA-A receptor antago-

nist bicuculline into the SubCD of intact, awake rats can significantly shorten the latency and increase the amount of REMS. Also consistent with that study, neither carbachol nor kainate induced REMS from this area. Other drugs which were ineffective in this area, but which can induce REMS following infusion into the cat RIZ, included neostigmine, propranolol, phaclofen, and vasopressin.

4.1. Methodological issues

The drug doses (Table 1) and volume (0.25 μ l) chosen for this study were based upon those previously reported to be effective in the cat RIZ. Consistent with our results, those studies reported anatomical specificity for REMS induction, such that injections of this volume anywhere outside a <1 mm effective region showed minimal efficacy [51]. Silberman et al. [40] estimated that this same volume affects a sphere of roughly 0.25 cubic mm of nervous tissue whereas larger volumes dramatically increase the spread of the drug solution. This would increase the risk of nonspecific drug effects that might interfere with sleep induction and maintenance.

Handling of the animals for microinjection is also an important methodological variable, as this may cause arousal that could delay the onset of sleep. This may account in part for the low REMS scores observed in most recordings. To control for the injection procedure, we used the standard method of comparing sleep following drugs with sleep following vehicle injections (for examples see Refs. [4,9,11,17]). The fact that bicuculline injections into the SubCD induced long REMS bouts with a short latency demonstrates that the injection procedure does not preclude REMS induction. On the other hand, it is possible that motor effects evident following bicuculline injection might have been reduced if a remote injection procedure was used to minimize arousal.

4.2. Comparison of results with previous work

We chose to record from freely-moving rats to avoid confounds related to physical restraint or anaesthesia. Boissard et al. [10] used unanaesthetized but head-restrained rats, which presumably explains why they did not report locomotor side-effects of bicuculline, despite observing some cases of ‘wakefulness with sustained muscle tonus’. However, they observed such effects only with drug injections outside the SubCD, whereas in the present study, motor effects were obtained from injection sites in the SubCD that in some cases also elicited REMS. The reason for this difference between studies is unclear, but could be related to procedural variables, including the use of head-restraint, and the different injection procedures (iontophoresis in Boissard et al.) and drug doses (25 mM in Boissard et al. vs. 8 mM here).

The REMS-inducing effects of bicuculline injections into the SubCD found in the present study were much greater than those previously reported for carbachol injections into the rat PnO. In those studies, REMS latencies were ~60 min and REMS increments were <200% above control levels in the first 2 h post-injection. In contrast, bicuculline injections into the SubCD produced mean REMS latencies of ~0.5 min and a REMS increment of >26 000% in the first 60 min post-injection. To our knowledge, this is the shortest drug-induced REMS latency yet reported in the unanaesthetized rat. These values are similar to what has been reported in cats following bicuculline injection into the RIZ [50], although the total amount of REMS in these recording periods is much greater in cats (13 vs. 45 min in rats vs. cats, respectively). The SubCD may therefore be an important region for REMS initiation. However, there appear to be considerable species differences in the sensitivity of this region to several drugs, particularly carbachol, that limit any claim that the SubCD is homologous to the cat RIZ. There would also appear to be species differences in the sensitivity of this region to locomotor activation in response to bicuculline. Why the rat is less responsive than cats to induction of REMS by cholinergic stimulation in the brainstem remains to be determined.

The principal aim of this study was to validate an experimental technique for rapid, robust and reliable REMS induction in the rat by which to test hypotheses about the function of REMS. Although rapid induction of REMS was achieved with bicuculline, the presence of disruptive locomotor effects would confound tests of learning and memory or other functions. Whether the problem of motor activation with bicuculline can be solved remains to be determined. Recent work has identified the hypocretin/orexin system as an important gating mechanism for REMS expression, thus it might be possible to lower the threshold for REMS induction by manipulations of this system [44]. Another peptide system that influences REMS expression in cats is nerve growth factor (NGF) [52]. This has not yet been explored in rats, although NGF immunoreactive cell body distributions in the rat brainstem are restricted almost entirely to the Me5, which is immediately dorsal to effective sites for REMS induction described in the present study [28,32]. Finally, recent *in vitro* work suggests that co-release of peptides and transmitters (e.g. substance P and acetylcholine) may have potent effects on activation of REMS-related neuronal populations [23], thus further work with coinjections in the rat *in vivo* may be profitable.

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