

Extraocular muscle activity, rapid eye movements and the development of active and quiet sleep

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Abstract

Rapid eye movements (REMs), traditionally measured using the electrooculogram (EOG), help to characterize active sleep in adults. In early infancy, however, they are not clearly expressed. Here we measured extraocular muscle activity in infant rats at 3 days of age (P3), P8 and P14–15 in order to assess the ontogeny of REMs and their relationship with other forms of sleep-related phasic activity. We found that the causal relationship between extraocular muscle twitches and REMs strengthened during the first two postnatal weeks, reflecting increased control of the extraocular muscles over eye movements. As early as P3, however, phasic bursts of extraocular muscle twitching occurred in synchrony with twitching in other muscle groups, producing waves of phasic activity interspersed with brief periods of quiescence. Surprisingly, the tone of the extraocular muscles, invisible to standard EOG measures, fluctuated in synchrony with the tone of other muscle groups; focal electrical stimulation within the dorsolateral pontine tegmentum, an area that has been shown to contain wake-on neurons in P8 rats, resulted in the simultaneous activation of high tone in both nuchal and extraocular muscles. Finally, when state-dependent neocortical electroencephalographic activity was observed at P14, it had already integrated fully with sleep and wakefulness as defined using electromyographic criteria alone; this finding is not consistent with the notion that active sleep in infants at this age is 'half-activated.' All together, these results indicate exquisite temporal organization of sleep soon after birth and highlight the possible functional implications of homologous activational states in striated muscle and neocortex.

Introduction

Sleep and wakefulness are discriminated in adult placental mammals using measures of eye movements, muscle tone and neocortical activity. This trio of indicators is thought to provide necessary and sufficient information for categorizing sleep–wake states in adults, but its usefulness for categorizing these states in infants is limited (Rechtschaffen & Kales, 1968). Indeed, although sleep predominates during infancy (Roffwarg *et al.*, 1966; Jouvet-Mounier *et al.*, 1970), the idiosyncratic nature of infant sleep poses unique challenges (Blumberg *et al.*, 2005). For example, before the end of the second postnatal week, infant rats do not exhibit state-dependent electroencephalographic (EEG) activity (Gramsbergen, 1976; Corner & Mirmiran, 1990; Frank & Heller, 1997), a feature of infant sleep that has contributed to its characterization as 'disorganized', 'diffuse' and produced by distinct neurophysiological mechanisms (Adrien & Lanfumey, 1984; Frank & Heller, 1997, 2003).

Work from our laboratory has shown that nuchal electromyography (EMG) alone is sufficient for defining sleep–wake states during early infancy (Karlsson & Blumberg, 2002; Karlsson *et al.*, 2004; Seelke & Blumberg, 2005). Most recently, it was shown in week-old rats that sleep and wake states identified in this way are governed by neural mechanisms that are similar to those that govern sleep–wake states in adults (Karlsson & Blumberg, 2005; Karlsson *et al.*, 2005). Thus, of the trio of established indicators of adult sleep–wake states, only the

mechanisms underlying rapid eye movements (REMs) have yet to be examined adequately in infants.

Although work in rats, cats and guinea pigs (Shimizu & Himwich, 1968; Jouvet-Mounier *et al.*, 1970; McGinty *et al.*, 1977; Van Someren *et al.*, 1990) has addressed the development of REMs using electrooculographic (EOG) techniques, such techniques may not be adequate for assessing the developmental precursors of REMs in very young animals. Thus, although it was reported that rats, in which the eyelids open on postnatal day (P)15, first exhibit REMs around P6 (Jouvet-Mounier *et al.*, 1970), it is unclear whether the extraocular muscles that control REMs are active during sleep at earlier ages. If, however, REMs are produced by twitches of the extraocular muscles, as has been suggested (Chase & Morales, 1983, 1990), and if these extraocular muscle twitches are phenomenologically similar to the twitches produced by other striated muscles, then direct measurement of extraocular muscle activity might reveal ontogenetic precursors of REMs that have heretofore gone unnoticed.

Materials and methods

All experiments were performed under National Institutes of Health guidelines for the care of animals in research and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Subjects

A total of 21 rats from 21 litters were used: 18 P3, P8 and P14–15 rats for behavioural experiments and three P7–9 rats for electrical

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stimulation. Weights ranged from 8.6 to 10.1 g at P3, 19.8–20.7 g at P7–9 and 37.0–41.1 g at P14–15. All pups were born to Harlan Sprague-Dawley rats housed in the animal colony at the University of Iowa. The pups were raised in litters that were culled to eight pups within 3 days of birth (day of birth was day 0). Litters and mothers were raised in standard laboratory cages (48 cm long \times 20 cm wide \times 26 cm high), in which food and water were available *ad libitum*. All rats were maintained on a 12-h light–dark schedule with lights on at 07.00 h.

Behavioural recording

Test environment

Pups were tested inside an electrically shielded double-walled glass chamber (height, 17.0 cm; i.d., 12.5 cm) with a Plexiglas lid. Air temperature inside the chamber was regulated using a temperature-controlled water circulator. Access holes in the side and lid of the chamber allowed for passage of air through the chamber as well as the passage of EMG and EEG electrodes. A round platform constructed of polyethylene mesh was fitted inside the chamber and a perforated felt pad was placed on top of the mesh.

Procedure

Eighteen P3, P8 and P14–15 rats from 18 litters were used. On the day of testing a P3 or P8 rat with a visible milkband was removed from the litter, weighed and anaesthetized with isoflurane. An incision was made in the scalp, which was then retracted to expose the top half of each eyeball. One unipolar stainless steel hook electrode (50 μ m diameter; California Fine Wire, Grover Beach, CA, USA) was inserted between the eyeball and orbit on both the nasal and temporal sides of the eye with the aim of implanting in the medial and lateral rectus muscles, respectively. This procedure was repeated for the other eye. The electrodes were secured to the skull with cyanoacrylate adhesive and the scalp was closed. Two bipolar stainless steel hook electrodes (California Fine Wire) were then placed bilaterally in the nuchal muscle and secured with collodion. The subject was lightly restrained on a felt-covered platform, placed in an incubator maintained at thermoneutrality (i.e. 35 °C) and allowed to recover for 1 h. It should be noted that restraint does not interfere with sleep–wake cycling at this age; Karlsson *et al.* (2004). The subject was then infused with warm milk (3% body weight, commercial half-and-half) and allowed to acclimatise to the testing chamber (also maintained at 35 °C) for 1 h. Testing consisted of collecting 1 h of both behavioural (using digital video) and electrographic data. After testing the subject was killed using an overdose of Nembutal.

A similar protocol was followed for P14–15 subjects. On the day of testing a pup was removed from the litter, weighed and anaesthetized using isoflurane. An incision was made in the scalp, which was then retracted to expose the skull. Holes were drilled over the cerebellum, left parietal and left frontal cortices, and electrodes, consisting of skull screws (00–96 \times 1/16; Plastics One, Roanoke, VA, USA) attached to insulated silver wires (AG 10T; Medwire, Mt. Vernon, NY, USA), were placed in each of the holes to a depth of 1.5 mm. The cerebellar electrode served as the ground. The scalp was then retracted further, exposing the top half of each eyeball, and electrodes were implanted into the extraocular muscles as described above. The scalp was then closed and bipolar stainless steel hook electrodes were implanted bilaterally into the nuchal muscle and secured with collodion. The subject was placed into the testing chamber, maintained at thermoneutrality (i.e. 32 °C), for 2–4 h to recover and acclimatise. At this age, pups were tested while freely moving. Electrographic (including extraocular and

nuchal EMG and cortical EEG) data were collected for 2 h, including 1 h in which behaviour was recorded to digital videotape. After testing, the subject was killed using an overdose of Nembutal.

Nuchal EMG electrodes were connected to differential amplifiers (A-M Systems, Carlsborg, WA, USA) and their signals were amplified ($\times 10\,000$) and filtered (300–5000 Hz). Extraocular EMG electrodes were filtered from 1 to 5000 Hz. EEG electrodes were connected to a separate differential amplifier (A-M Systems) and the signal was amplified ($\times 10\,000$) and filtered (1–5000 Hz). EMG and EEG data were digitized at 1 kHz using a data acquisition system (BioPac Systems, Santa Barbara, CA, USA) and simultaneously visualized by the experimenter during the test. A microcamera was placed above the chamber lid for monitoring and recording of behaviour. EMG and video data were recorded to digital videotape using a data recorder (DV8; WinTron Technologies, Rebersberg, PA, USA).

Data analysis

Using Spike2 software (CED, Cambridge, UK), extraocular EMG data were filtered from 1 to 35 Hz to examine gross eye movements (similar to EOG) and from 300 to 5000 Hz to examine high-frequency myoclonic twitches.

Peri-event histograms were used to examine the relationship between events in different muscle groups. Two types of comparisons were made. First, we examined the relationship between fluctuations in nuchal and extraocular muscle tone. The nuchal and extraocular EMG data were integrated and full-wave rectified, and the onset of high tone in the nuchal EMG was determined using criteria described previously (Karlsson *et al.*, 2004). In the extraocular EMG trace, the onset of tone in the nuchal muscle was set as the trigger, the 5-s period before and after the trigger was divided into 1-s bins and the mean amplitude for each bin was determined. Five high-tone onset periods were examined for each subject. The same procedure was repeated for atonia onset. Paired *t*-tests were used to compare adjacent 1-s bins.

Second, we examined the relationship between phasic activity in the extraocular and nuchal muscles. The first twitch of an atonia period in the right extraocular muscle was identified. A twitch was defined as a spike in the EMG that was at least 2 \times the noiseband. In the left extraocular muscle trace, the 5-s period before and after the onset of twitching in the EMG of the extraocular muscles of the right eye was divided into 1-s bins and the number of twitches in each bin was determined. The same procedure was used when quantifying twitching in the nuchal muscle. Ten atonia periods from each subject were analysed. Paired *t*-tests were used to compare the amount of twitching in adjacent bins within an age.

The characteristics of rapid eye movements were examined across development. In each subject, 20 eye movements were chosen based upon the following criteria: eye movements were discrete events exhibiting a deflection from baseline of at least 4 \times the noiseband and exhibiting clear onset and end times. Once an eye movement was identified, its amplitude (defined from peak to trough) and duration were determined. Values were averaged for each animal and compared across ages using ANOVA.

The relationship between extraocular muscle twitches and eye movements was also examined. In each subject, 20 extraocular muscle twitches, each preceded by at least 2 s of quiescence, were selected and the timing of the corresponding eye movement was examined. The number of extraocular muscle twitches that corresponded to the beginning of a rapid eye movement was determined for each animal at each age and tested using ANOVA. An identical procedure was used to examine the number of rapid eye movements that were immediately preceded by an extraocular twitch.

A separate analysis examined the development of simultaneous twitching of the left and right extraocular muscles. A twitch in the extraocular muscle of the right eye was selected as a trigger and the extraocular muscle activity of the left eye was examined for evidence of a simultaneous twitch. One hundred twitches were examined for each subject. The percentage of simultaneous twitches was compared across age using ANOVA.

In order to assess limb twitches, 30-min segments of behavioural data from each P3 and P8 pup, and 60-min segments of data from each P14–15 pup, were digitized. As the pup's behaviour was observed on a monitor, an event recorder was activated when a myoclonic twitch or high-amplitude movement was detected. In this way, a synchronized digital record of the pup's behaviour and EMG activity was created. As described elsewhere (Gramsbergen *et al.*, 1970; Blumberg & Lucas, 1994), twitching was defined as phasic, rapid and independent movements of the limbs and tail. High-amplitude movements, indicative of wakefulness, include locomotion, stretching, and yawning.

The relationship between multiple limb, nuchal and extraocular muscle twitches was investigated. Five atonia periods from each P3 and P8 subject, and two atonia periods from each P14–15 subject, were chosen and divided into 1-s bins. For each subject, data were organized such that each row represented a 1-s bin and each column represented a category of twitching (i.e. limb, extraocular or nuchal). Each cell was assigned a 1 or a 0 depending on whether a particular category of twitch did or did not, respectively, occur

during that 1-s bin. Next, in order to determine whether twitching within the three muscle groups was more synchronized than would be expected by chance, the following protocol was used. First, for each subject, the selected atonia periods were divided into quiet sleep (QS) and active sleep (AS) periods, as described previously (Seelke & Blumberg, 2005) and the QS periods were disregarded. Second, the 1s and 0s were summed across columns to produce a phasic activity index (PAI) for each row of data. Third, observed PAI values were derived for each subject by determining the proportion of rows with PAI values of 0, 1, 2 or 3. Fourth, each of the three columns was shuffled 10 times and the proportion of rows with PAI values of 0, 1, 2 or 3 was again calculated; this produced the PAI values expected by chance. Finally, at each age, differences in the observed and expected PAI values were tested using paired *t*-tests.

A spectral analysis of the EEG of P14–15 subjects was conducted using Spike2. AS and QS periods, at least 100 s in duration and uninterrupted by movement artifact, were chosen for analysis; four of the six P14–15 subjects provided adequate EEG data. One sample of each sleep state was taken from each subject. A Hanning window with 2048 samples was used (bin size 0.5 Hz).

For all tests, alpha was set at 0.05. Where appropriate, the Bonferroni correction procedure was used. When ANOVA indicated a significant effect, the *post hoc* test was Fisher's protected least significant difference.

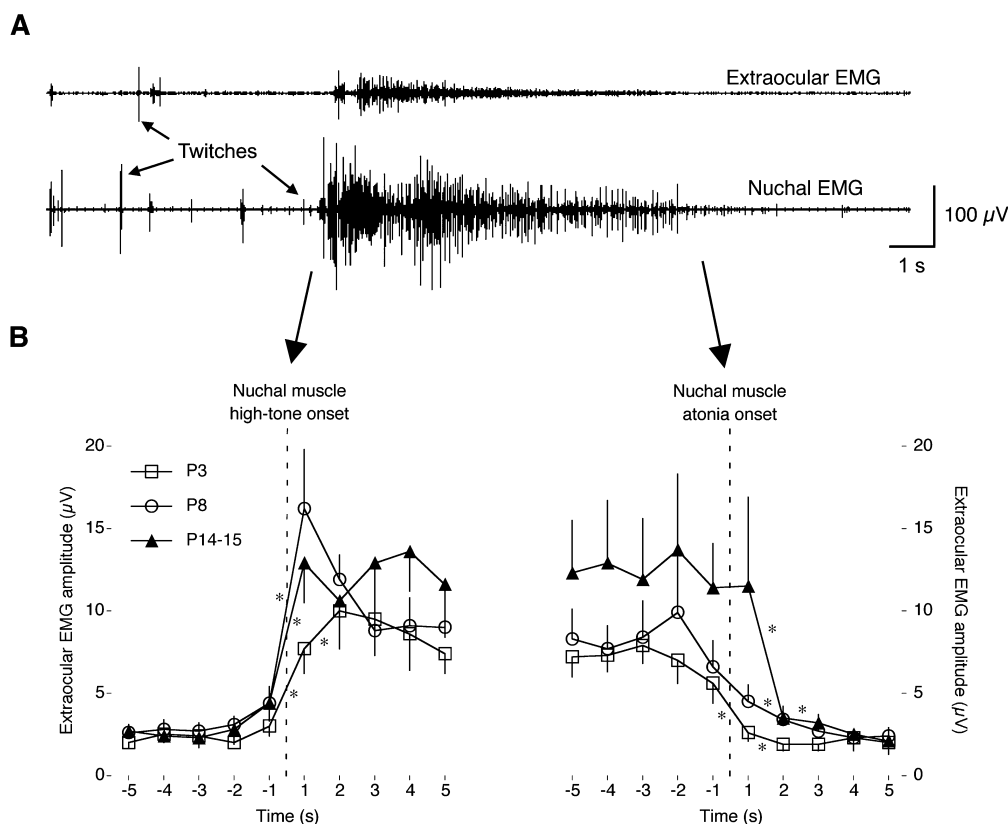


FIG. 1. Extraocular muscle activity fluctuated in synchrony with nuchal muscle activity. (A) Representative EMG activity for the extraocular (upper trace) and nuchal (lower trace) muscles in a P8 rat. Two periods of atonia (indicative of sleep) separated by a period of high muscle tone (indicative of wakefulness) can be seen. Myoclonic twitches (indicative of active sleep) are also indicated. (B) Mean changes in EMG amplitude for the extraocular muscles in relation to the onset of high nuchal muscle tone (left panel) and the onset of nuchal atonia (right panel) for P3, P8 and P14–15 rats; $n = 6$ pups/group. $*P < 0.05$ vs. the previous time point. Mean \pm SEM.

Electrical stimulation

Surgery

Three P7–9 rats from three litters were used. Under isoflurane anesthesia the pup was prepared for testing using a procedure similar to that described previously (Karlsson *et al.*, in press). Briefly, to strengthen the skull, it was bleached, dried, and then coated with VetBond (3M, St Paul, MN, USA). Next, a custom-built T-shaped stainless steel head-plant, designed to fit the earbar and nosebar holders of a stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA), was attached to the skull. Finally, the pup was wrapped in gauze to inhibit movements and induce sleep (Corner & Kwee, 1976).

Procedure

After recovering for 1 h in an incubator maintained at 35 °C, subjects were transferred to a stereotaxic instrument where the pup's body temperature was maintained at 37 °C. Subjects were allowed to acclimatise to the stereotaxic instrument for 1 h, at which point they cycled rapidly between high muscle tone and atonia.

Stimulations were performed using concentric bipolar tungsten electrodes (1 M Ω , 3–4 μ m at the tip, Model TM33CCINS; World Precision Instruments, Sarasota, FL, USA) connected to a stimulus isolator (Model A395; World Precision Instruments) and a stimulus generator (Model 48; Grass, Quincy, MA, USA). As the electrode was lowered into the pons, trains of electrical pulses (0.2 ms, 50–80 μ A, 100 Hz) were delivered for 500 ms every 5 s. When a site was identified that produced high tone in both the extraocular and nuchal muscles, 5 min of EMG data were collected to hard disk. Following data collection, the recording site was marked by passing 50 mA anodal current through the electrode for 5 s.

Data analysis

Data were analysed off-line using Spike2 software. Nuchal and extraocular EMG recordings were rectified and amplitudes were measured over three consecutive 500-ms periods: before, during and after stimulation. Averages from 10 stimulations were calculated for each pup, and paired *t*-tests were performed within each subject comparing mean amplitude during stimulation to the mean amplitude before and after stimulation. For these tests, alpha was set at 0.05 and the Bonferroni correction procedure was used.

Results

Synchronous fluctuations in extraocular and nuchal muscle tone

The nuchal EMG fluctuates rapidly in infants between periods of high muscle tone, indicative of wakefulness, and atonia, indicative of sleep (Karlsson *et al.*, 2004). Surprisingly, during normal cycling between sleep and wakefulness in P3, P8 and P14–15 rats, the tone of the extraocular muscles fluctuated in lock-step with the tone of the nuchal muscle (Fig. 1A). Specifically, the mean amplitude of extraocular muscle tone increased significantly with the onset of high nuchal muscle tone, and decreased significantly with the onset of nuchal atonia (Fig. 1B).

Stimulation of the pontine tegmentum increased muscle tone in extraocular and nuchal muscles

It was demonstrated recently that neurons that increase their discharge rates during periods of high nuchal muscle tone are found in the laterodorsal tegmental (LDT) and parabrachial (PB) nuclei (Karlsson

et al., 2005); furthermore, lesions of the dorsolateral pontine tegmentum that include these nuclei result in substantial increases in atonia bout durations. Thus, in order to determine whether the near-simultaneous changes in extraocular and nuchal muscle tone are produced by activity within this region, we stimulated the LDT and PB in three P7–9 rats. If a common neural mechanism regulates the changes in both extraocular and nuchal muscle tone, then it should be possible to identify sites where stimulation produces simultaneous increases in the tone of both muscle groups.

Electrical stimulation of the PB (Fig. 2A) and LDT (Fig. 2B) produced marked effects on nuchal and extraocular muscle activity. Overall, in all three subjects at a total of four sites, stimulation of the LDT or PB produced rapid, robust and significant increases in extraocular and nuchal muscle tone (all $t_9 > 5.5$, $P < 0.0005$); muscle tone also decreased significantly after termination of the stimulation at three of the four stimulation sites (all $t_9 > 9.4$, $P < 0.0001$). Stimulation at nearby locations did not produce these effects. These results, in conjunction with the synchronous fluctuations in extraocular and nuchal muscle tone described earlier (Fig. 1), indicate that our

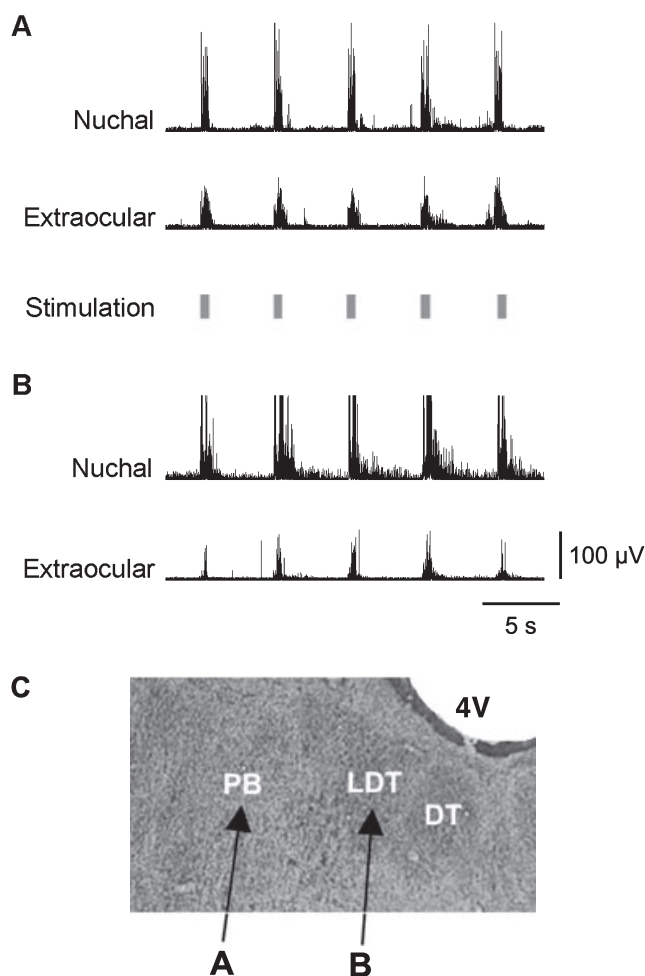


FIG. 2. Stimulation within the dorsolateral pontine tegmentum simultaneously activated high tone in the nuchal and extraocular muscles. (A) Nuchal and extraocular EMG activity in response to stimulus trains of electrical pulses (vertical bars) delivered to the PB of a P9 rat. Each pulse was delivered for 500 ms (60 μ A, 100 Hz). (B) Same as (A) except the stimulation was applied to the LDT of a P9 rat. (C) Coronal section from an infant rat indicating the locations of the stimulation electrodes in A and B. DT, dorsal tegmental nucleus; 4V, 4th ventricle.

method for measuring extraocular EMG provides a valid and reliable measure of extraocular muscle activity.

Developmental relationship between extraocular muscle twitches and REMs

In addition to indicating changes in tone, nuchal EMG records can reveal the occurrence of myoclonic twitches (Karlsson & Blumberg, 2002; Seelke & Blumberg, 2005), as illustrated in the lower trace of Fig. 1A. In light of the hypothesis that REMs represent twitches of the extraocular muscles (Chase & Morales, 1983), we hypothesized that myoclonic twitches would appear in the extraocular EMG record in infants and would occur in synchrony with REMs at those ages when REMs are detectable.

Examples of extraocular muscle twitches and REMs are depicted in Fig. 3A, produced by filtering the extraocular EMG to reveal high- and

low-frequency activity, respectively. Although REMs were easiest to identify at P14–15, at or near the day of eye opening, twitches of the extraocular muscles were apparent as early as P3. As shown in Fig. 3B, the duration and amplitude of the eye movements increased significantly with age (duration, $F_{2,15} = 12.8$, $P < 0.001$; amplitude, $F_{2,15} = 58.5$, $P < 0.0001$).

A tight relationship between extraocular muscle twitches and REMs developed over the first two postnatal weeks. This was demonstrated by calculating the conditional probabilities associated with extraocular muscle twitches and REMs. Specifically, as shown in the left panel of Fig. 3C, the occurrence of a twitch increasingly signaled the subsequent occurrence of a REM between P3 and P14–15 ($F_{2,15} = 22.1$, $P < 0.0001$). Conversely, as shown in the right panel of Fig. 3C, the occurrence of a REM increasingly signaled the prior occurrence of a twitch between P3 and P14–15 ($F_{2,15} = 74.7$, $P < 0.0001$). Thus, these data indicate that eye movements are

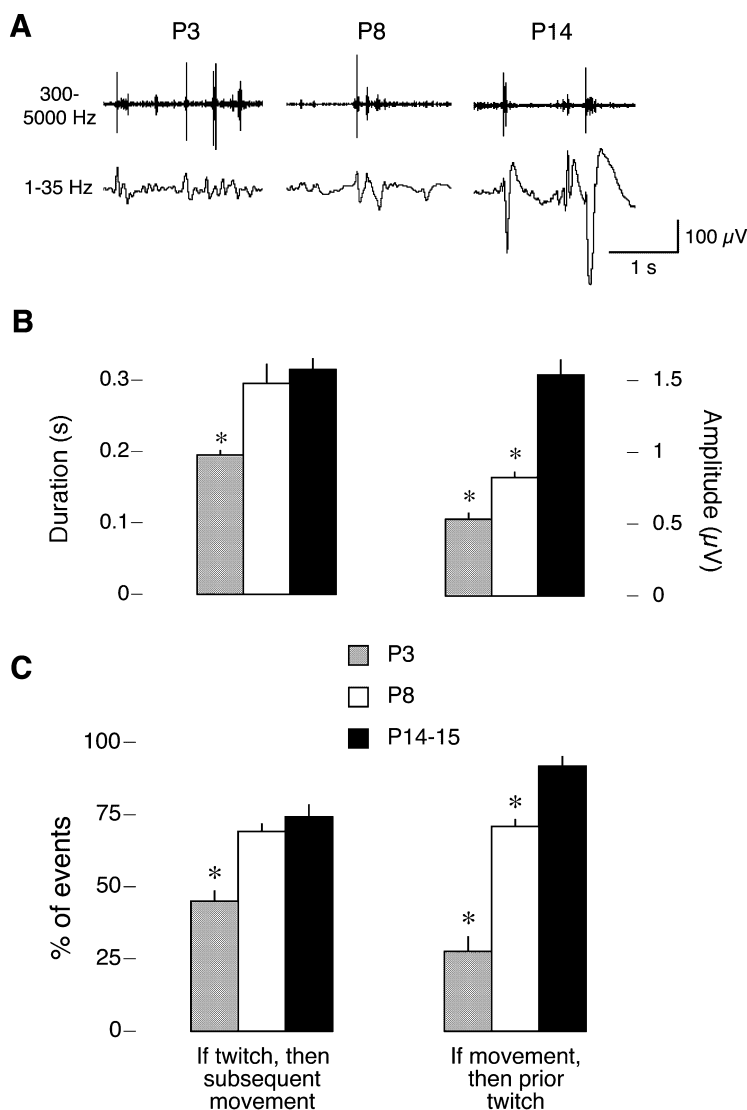


FIG. 3. Coupling between extraocular muscle twitches and REMs increases during the first two postnatal weeks. (A) Representative extraocular EMG activity and eye movements at P3, P8 and P14. Top row: high-pass (300–5000 Hz) filtering to reveal myoclonic twitching. Bottom row: low-pass (1–35 Hz) filtering to reveal REMs. (B) Analysis of the mean duration (left panel) and amplitude (right panel) of REMs at P3, P8 and P14–15; $n = 6$ pups/group, $*P < 0.001$ vs. the other two groups. (C) Analysis of conditional probabilities relating extraocular muscle twitches to REMs at P3, P8 and P14–15. Left panel, percentage of events in which an extraocular muscle twitch occurred and was followed by a REM. Right panel, percentage of events in which a REM occurred which was preceded by an extraocular muscle twitch; $n = 6$ pups/group, $*P < 0.0005$ vs. the other two groups. Mean + SEM.

gradually brought under the control of the extraocular muscles over the first two postnatal weeks.

Age-related changes in the proportion of simultaneous twitches of the extraocular muscles of the two eyes were also determined. The percentage of simultaneous twitches increased significantly from $57.5 \pm 7.1\%$ at P3 to $74.5 \pm 2.8\%$ at P8 to $84.8 \pm 2.9\%$ at P14–15 ($F_{2,15} = 8.5$, $P < 0.005$). Thus, although significant coordination of activity of the extraocular muscles of both eyes already exists at P3, this coordination increases significantly over the next 2 weeks and may form the foundation for conjugate eye movements.

Synchronized onset of sleep-related phasic activity in the extraocular and nuchal muscles

For each atonia period, the first twitch of the extraocular muscles of the right eye was designated as a trigger for a peri-event histogram; then, in relation to this trigger, the number of twitches exhibited by the extraocular muscles of the left eye and the nuchal muscle was determined (Fig. 4). At P3, P8 and P14–15, bouts of twitching were initiated in synchrony in the extraocular muscles of both eyes and the nuchal muscle. These data demonstrate that, during any given atonia period, phasic activity in the form of myoclonic twitching begins synchronously across multiple muscle groups.

Waves of phasic activation during sleep

If infant sleep is truly 'diffuse' and 'disorganized', as has been argued (Adrien & Lanfumey, 1984; Frank & Heller, 1997, 2003), we would expect twitching in various muscle groups to be randomly distributed throughout each atonia period. We tested this by dividing representative atonia periods into 1-s bins and calculating a PAI that reflects, for each bin, the presence or absence of twitching of the limbs, of the extraocular muscles of the right eye, and of the nuchal muscle (see Materials and methods).

Representative atonia periods from P3, P8 and P14 subjects are shown in Fig. 5A. Each atonia period begins with a brief period of quiescence in all three categories of twitching (PAI = 0) followed by the onset of phasic activity, which often occurs in synchronized bursts separated by brief periods of quiescence. To determine whether the waxing and waning of phasic activity was more organized than expected by chance, the number of PAIs of 0 and 3 that was experimentally observed was compared with the number of PAIs of 0 and 3 expected by chance (see Materials and methods). At each age tested, the number of observed PAIs of 0 and 3 significantly exceeded the number expected by chance (Fig. 5B; all paired $t_5 > 3.6$, $P < 0.05$). Therefore, phasic activity was produced by a mechanism that synchronizes activity across multiple muscle groups.

At P14, state-dependent neocortical activity mapped onto EMG-defined sleep–wake states

As mentioned above, infant rats do not exhibit state-dependent changes in neocortical EEG until approximately P11 (Gramsbergen, 1976; Frank & Heller, 1997). Therefore, we next addressed the following question: when slow-wave activity emerges, does it map onto periods of QS defined using measures of nuchal EMG alone?

Figure 6A shows one representative sleep–wake cycle for a P3 rat. The cycle begins with a period of high extraocular and nuchal muscle tone (wakefulness; W), followed by a transition to a 6-s period of muscle atonia coupled with behavioural quiescence (QS). Then, a 20-s period of phasic activity commences in the limbs and extraocular and

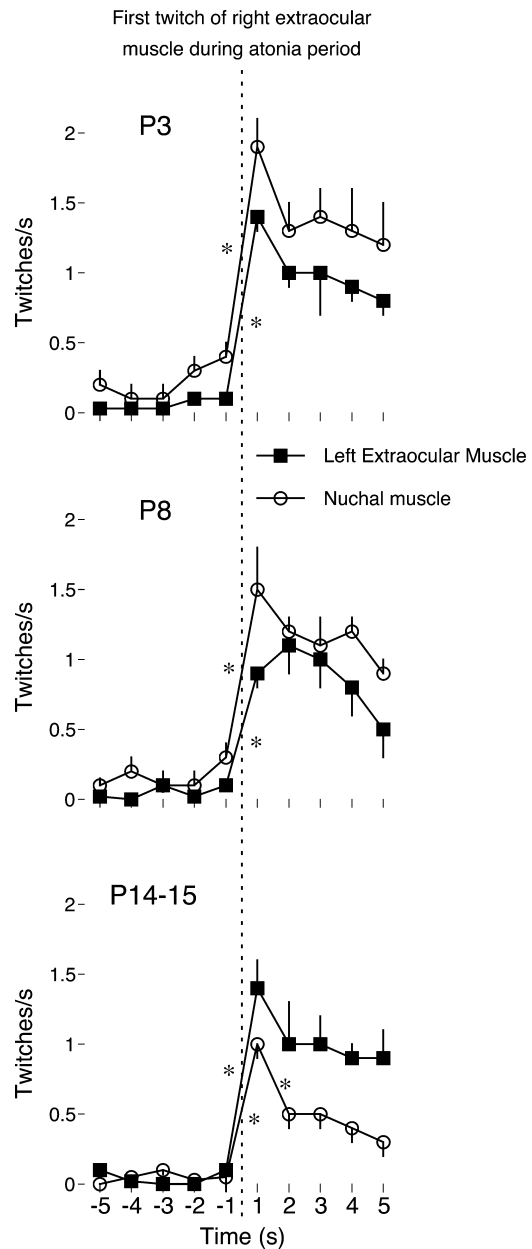


FIG. 4. Synchronized onset of phasic activity in extraocular and nuchal muscles during sleep at P3, P8 and P14–15. The mean number of myoclonic twitches/s determined from the left extraocular and nuchal EMGs in relation to the first twitch of the right extraocular muscle during a period of atonia; $n = 6$ pups/group, $*P < 0.05$ vs. the previous time point. Twitches were determined from the extraocular and nuchal EMGs. Mean \pm SEM.

nuchal muscles (AS). After this period of phasic activity, the animal arouses and muscle tone again increases (W).

As shown in Fig. 6B, the basic pattern of sleep–wake activity seen at P3 is also seen at P14, although the duration of each period is elongated. Focusing first on the EMG measures, the cycle begins with a period of high extraocular and nuchal muscle tone (W), followed by a 240-s period of muscle atonia coupled with behavioural quiescence (QS). Then, a 250-s period of phasic activity commences in the limbs and extraocular and nuchal muscles (AS). After this period of phasic activity, the animal arouses and muscle tone increases (W). When neocortical EEG activity is considered, it is seen that high-amplitude slow-wave activity overlaps precisely

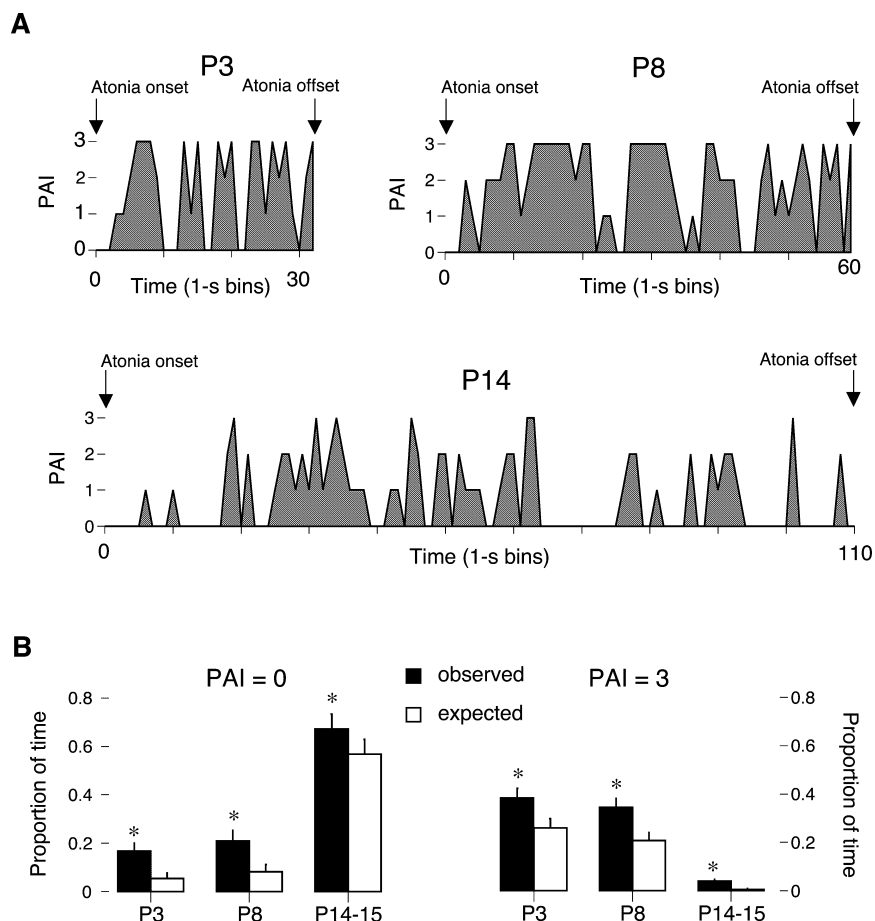


FIG. 5. Synchronized phasic activity in multiple muscle groups during sleep at P3, P8 and P14–15. (A) Representative depictions of phasic activity during atonia periods at each age. The PAI was determined for each 1-s bin by assessing myoclonic twitching of the limbs (based on behavioral observation) and of the nuchal and extraocular muscles (based on EMG activity). Four categories were identified ranging from PAI = 0 (no phasic activity) to PAI = 3 (phasic activity detected in the limbs and nuchal and extraocular muscles). (B) Mean proportion of observed 1-s bins in which PAI = 0 (left panel) and PAI = 3 (right panel) in relation to the expected values expected by chance; $n = 6$ pups/group, $*P < 0.05$ vs. the expected value. Mean \pm SEM.

with the period of QS; spectral analysis of this slow-wave activity reveals a frequency peak between 2 and 4 Hz, which characterizes delta activity (Fig. 6C and D). Thus, by P14, a tight link between sleep–wake states defined using EEG and EMG criteria has already been established.

Discussion

The direct measurement of extraocular muscle activity in the present study has revealed features of oculomotor activity during early postnatal life that are invisible to the conventional EOG measure of eye movements. First, and perhaps most surprisingly, fluctuations in the tone of the extraocular muscles mirrored fluctuations in the tone of the nuchal muscle. Second, as early as P3, synchronized waves of phasic activity were seen in the extraocular muscles, nuchal muscle and limbs; these waves were separated by brief periods of quiescence. Finally, twitches of the extraocular muscles became increasingly associated with robust REMs that resemble those detected in adults using standard EOG techniques. In other words, the extraocular EMG provides a more sensitive measure than the EOG of both tonic and phasic oculomotor activity. The refined organizational structure of infant sleep revealed here contrasts with the perspective of infant sleep as diffuse, primitive, and dissociated (Adrien & Lanfumeu, 1984; Frank & Heller, 1997, 2003).

The present study depended upon the development of a reliable method for measuring extraocular EMG activity and minimizing movement artifact. This method included a technique whereby the extraocular muscles were approached for implantation of the hook electrode by retracting the scalp and exposing the top half of each eyeball. It was also helpful to secure the electrodes to the skull near their point of exit from the orbits. In this way, the majority of movement artifact was eliminated. Similar techniques have been used by previous investigators working with infant rats (Jouvet-Mounier *et al.*, 1970; Adrien & Lanfumeu, 1984), although these investigators did not use hook electrodes and filtered their data to reveal only slow-wave activity.

In a recent report (Karlsson *et al.*, 2005), we identified state-dependent neurons within medullary and mesopontine regions of week-old rats that are also considered important for the expression of sleep in adults. For example, neurons were identified within the dorsolateral pontine tegmentum, including the LDT, that increase their discharge rates during periods of elevated nuchal muscle tone. Lesions within this area nearly eradicated the expression of high muscle tone. We extended these results in the present study through electrical stimulation of this region to show that extraocular muscle tone is elevated simultaneously with nuchal muscle tone. Finally, we recently found that carbachol infusion into this region is sufficient to activate high tone in these muscles (A. J. Gall & M. S. Blumberg, unpublished data).

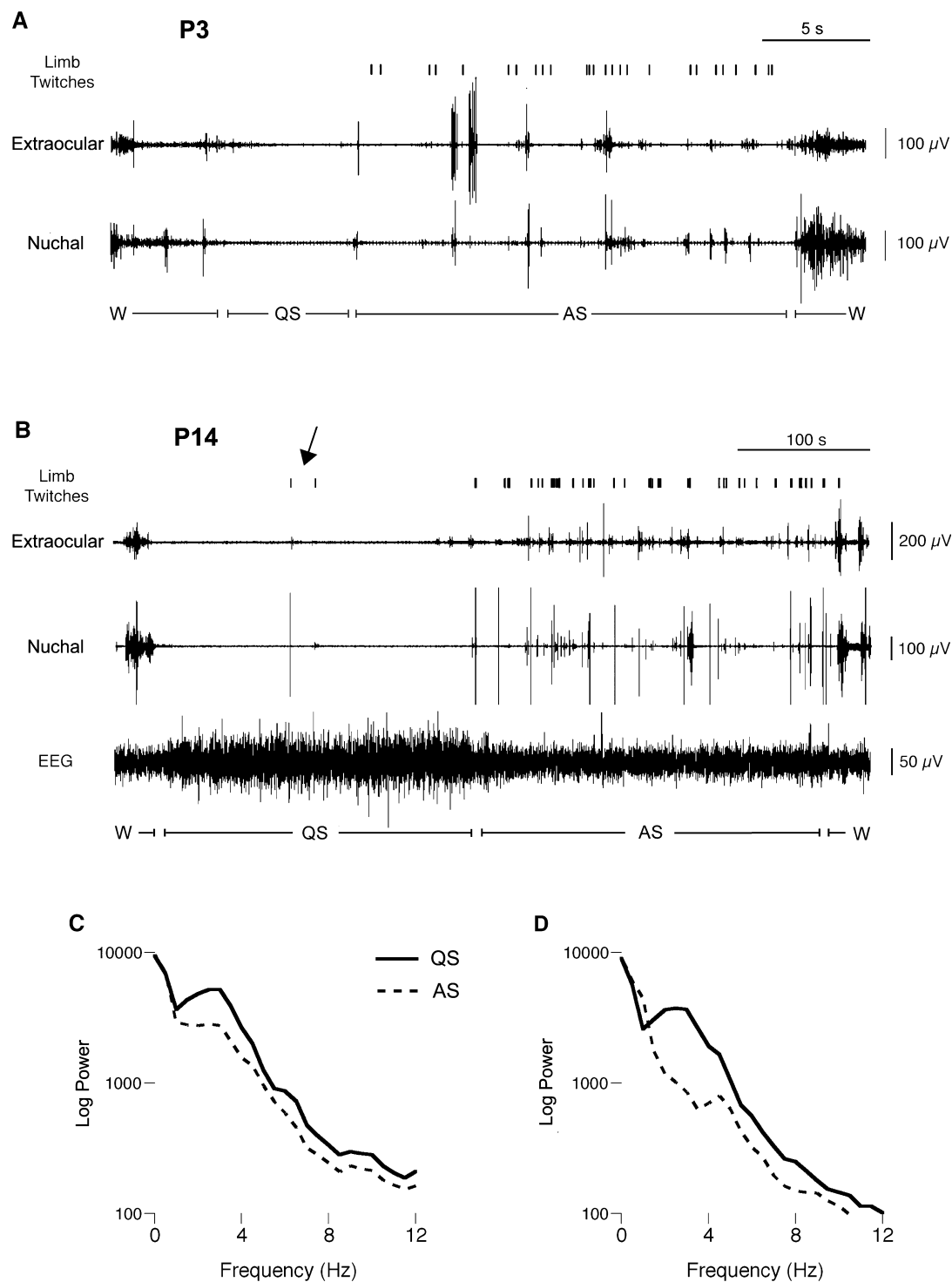


FIG. 6. Phasic and tonic behavioral and electrographic events across a complete sleep cycle in a P3 and a P14 rat. (A) Behaviorally scored limb twitches and extraocular and nuchal EMGs for a P3 rat. Periods of W, QS and AS are indicated. (B) Same as A except the subject is a P14 rat and neocortical EEG is also recorded. Arrow indicates two twitch-like events that were later identified as startles. Note the different time scales for A and B. (C) EEG power spectrogram of the QS and AS periods in B. (D) EEG power spectrogram of QS and AS periods across four P14–15 subjects (at least 100 s of continuous data per sleep state per subject).

We found that, at P14–15, neocortical EEG slow-wave activity coincided with the period of QS defined using EMG measures alone (see Fig. 6). This observation is interesting in light of the finding that, between P13 and P17, twitches have been reported to occur occasionally against a background of slow-wave EEG activity

(Jouvet-Mounier *et al.*, 1970). Jouvet-Mounier *et al.* (1970) referred to such episodes as ‘half-activated paradoxical sleep’ or ‘slow-wave sleep with muscular twitches’. These episodes were also reported by McGinty *et al.* (1977) in their study of sleep in kittens but, as with the study of Jouvet-Mounier *et al.* (1970), such events were relatively

infrequent. In their study of sleep development in rats beyond the age of P11, Frank & Heller (1997) also noted the occurrence of half-activated paradoxical sleep and, based largely on this phenomenon, posited their 'presleep theory' in which infant sleep is viewed as a single undifferentiated state that is an amalgam of the sleep states seen in adults.

We did not find evidence of a fragmented relationship between EMG and EEG sleep measures at P14. Consider, for example, the events indicated by the arrow in Fig. 6B. First, it can be seen that these two events are isolated from the cluster of phasic activity that occurs later during the same atonia period. Second, when we reexamined the video and EMG records, it was apparent that these isolated events were not twitches but, rather, startles. In contrast with twitches, startles are relatively rare, discrete behavioural events characterized by the simultaneous activation of multiple muscle groups (Gramsbergen *et al.*, 1970). We have confirmed the distinction between startles and twitches by monitoring EMG activity simultaneously in nuchal, limb and abdominal muscles (G. Viana di Prisco & M. S. Blumberg, unpublished data). Moreover, we have found that startles, but not twitches, are accompanied by hippocampal sharp waves in early infancy (Karlsson & Blumberg, 2003). It is apparent, then, that startles and twitches are phenomenologically distinct events with distinct patterns of neural activation. Thus, it is possible that the notion of a half-activated sleep state arose from a confusion between startles and twitches. Resolving this issue will require intensive examination of EMG activity and behaviour as EEG differentiation occurs around P11. Regardless, it is clear from Fig. 6 that myoclonic twitching in multiple muscle groups, beginning at P3, typically occurs in coherent clusters that clearly demarcate periods of active sleep.

Over 20 years ago in a paper examining the spinal mechanisms of twitching in adult cats, Chase & Morales (1983, p. 1198) suggested the possibility that 'central neural areas that give rise to myoclonic activation of the limb muscles during REM periods also initiate a generalized pattern of twitches and jerks that affect all striated muscles', including the extraocular muscles that produce REMs. The present study, building on our previous report detailing the neural substrates of sleep in week-old rats (Karlsson *et al.* in press), provides the most direct experimental support to date for this hypothesis and, moreover, demonstrates its validity soon after birth in an altricial species.

The widely used sleep manual of Rechtschaffen & Kales (1968) established criteria for assessing the stages of sleep that relied on three electrographic measures: EOG, EMG, and EEG (Fig. 7A). Our results, however, indicate that REMs, measured conventionally using the EOG, co-occur with other forms of phasic activity, including the phasic activity that is detectable from the nuchal EMG record. Moreover, extraocular EMG records provide information about muscle tone that is qualitatively and quantitatively similar to that provided by the nuchal EMG. In other words, the extraocular and nuchal muscles control their associated 'limbs', that is, the eyes and head, respectively, and provide redundant information concerning behavioural state. Accordingly, we can restructure the trio of state indicators in Fig. 7A such that, as shown in Fig. 7B, only two measures are now needed to identify behavioural states under normal conditions: EMG and EEG.

But perhaps even two measures are more than is needed. Specifically, it is becoming clear in adult rats that the forebrain exhibits global EEG patterns that are sufficiently distinct to discriminate between AS, QS and W (Gervasoni *et al.*, 2004). These authors suggest that these EEG patterns provide the basis for the 'classification of global states without reference to behavioural or electromyogram data' (p. 11141). Remarkably, as shown here and elsewhere, the EMG

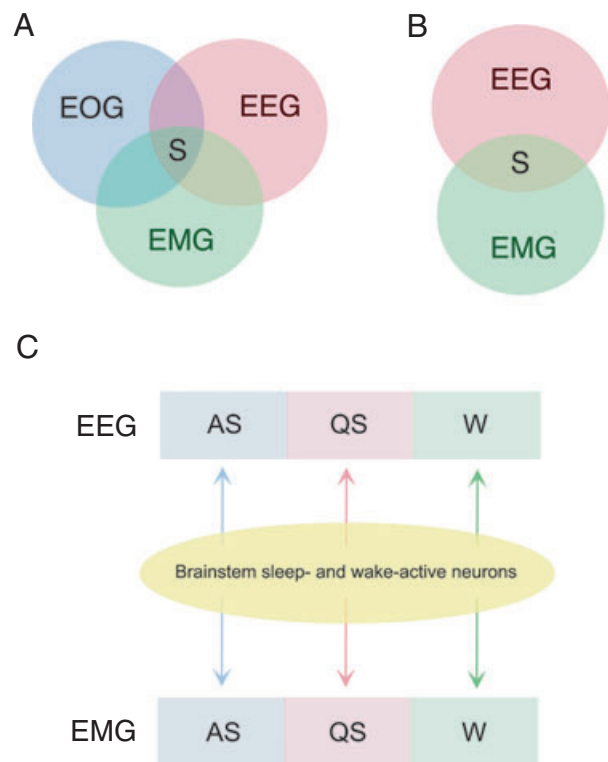


FIG. 7. Conceptual representations of sleep. (A) Venn diagram depicting conventional diagnostic criteria for assessing sleep-wake states in adults using three independent parameters: EOG, nuchal or chin EMG and neocortical EEG. S denotes the behavioral state (i.e., active or quiet sleep, wakefulness) defined using these three parameters. (B) Reorganization of the Venn diagram in A based on the notion that the EOG and EMG provide redundant information. Specifically, REMs can be viewed as phasic events that are analogous to myoclonic twitches that can be recorded using nuchal EMG; in addition, fluctuations in nuchal muscle tone are mirrored by fluctuations in extraocular muscle tone. Now, S denotes the behavioral state defined using only two parameters. (C) Alternative conceptualization that builds on the notion that either EEG (after P11) or EMG (as early as P2) is alone sufficient to define behavioral states in rats. According to this notion, individual homologous sleep-wake states (AS, QS, W) can be identified on the basis of either EEG or EMG activity alone. This homology arises because EEG- and EMG-defined states are generated by common brainstem mechanisms.

alone is also sufficient for differentiating these same basic sleep-wake states and for revealing neural mechanisms that have been implicated in adult sleep-wake states using the conventional trio of state indicators (Karlsson *et al.*, 2004; Karlsson & Blumberg, 2005; Seelke & Blumberg, 2005; Karlsson *et al.*, 2005). Thus, the reduced Venn diagram in Fig. 7B gives way to the qualitatively altered conceptualization of Fig. 7C, in which homologous states defined using either EEG or EMG measures alone are linked by their association with common neural sources within the brainstem, including the meso-pontine region.

The perspective captured by Fig. 7C indicates a mechanistic connection between the activational states of the forebrain (EEG) and striated muscle (EMG). Might there also be a functional connection? It has been suggested that infant sleep states, including myoclonic twitching, contribute to neural and neuromuscular development (Roffwarg *et al.*, 1966; Mirmiran, 1995; Blumberg & Lucas, 1996; Corner *et al.*, 2002) and recent reports have provided intriguing evidence in support of this perspective (Pettersson *et al.*, 2003; Khazipov *et al.*, 2004). For example, it has been suggested that

feedback from twitch-related movements of the limbs contributes to the self-organization of spinal circuits (Petersson *et al.*, 2003). Similarly, it has been suggested that twitches participate in the construction of topographic maps in somatosensory cortex (Khazipov *et al.*, 2004). Both of these reports fit neatly with the perspective being presented here: that the mechanistic link between forebrain and muscle activity derives from a functional linkage that reflects the development of topographic relationships throughout the neuraxis in infants and the modulation and maintenance of these relationships in adults (see Merzenich *et al.*, 1990). Accordingly, this hypothesized link between plasticity and sleep reflects the well-established observation that active sleep predominates during infancy and is retained, at reduced levels, in adulthood (Roffwarg *et al.*, 1966; Jouvet-Mounier *et al.*, 1970).

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Abbreviations

AS, active sleep; EEG, electroencephalogram/graphic; EMG, electromyogram/graphy; EOG, electrooculogram/graphic; LDT, laterodorsal tegmental nucleus; P, postnatal day; PAI, phasic activity index; PB, parabrachial nucleus; QS, quiet sleep; REM, rapid eye movement; W, wakefulness.

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