

STATE-RELATED DISCHARGE OF NEURONS IN THE BRAINSTEM OF FREELY MOVING BOX TURTLES, *TERRAPENE* *CAROLINA MAJOR*

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INTRODUCTION

REM sleep was discovered by Aserinsky and Kleitman in studies of sleeping humans (1). The somewhat surprising discovery that the “dream state” also exists in animals followed in 1958 (3). Soon it became apparent that most if not all mammals had REM sleep. In the early 1970’s work by Allison and colleagues led to the suggestion that REM sleep, while ubiquitous in placental and marsupial mammals, might be absent in the monotreme mammals and hence might have evolved relatively recently in the mammalian line. However, more recent studies of monotremes (20, 21) suggested that, in fact, these animals do have REM-like polygraphic, behavioral, and brainstem unit activity. Since these discoveries suggested a much earlier origin for REM than previously believed, it was logical to look back even farther in the evolutionary line, to reptiles, to determine whether REM-like activity may have been a part of the prototypical sleep state.

Numerous polygraphic studies have examined the sleep of reptiles. In general, these studies demonstrated that the EEG of turtles and lizards does not fluctuate throughout the same range across states as in mammals, often declining in frequency and amplitude during sleep. Distinct spikes and sharp waves were recorded in the EEG during behavioral sleep, with increasing spike rates associated with increasing depth of sleep without any other changes in the background EEG (5, 6, 8, 23). In crocodylian reptiles, however, the lower frequencies in the EEG increase in amplitude from waking to sleep. Spiking activity and sharp waves are also associated with behavioral sleep in these species (7, 14).

Evidence for REM in reptiles is uncertain. Some studies concluded that reptiles have REM (12, 23) while others found no features of REM (5, 25). Since all of these studies focussed exclusively on polygraphic and behavioral signs of REM – without reference to unit firing in the brainstem or subcortical structures – it is unclear whether REM sleep-like patterns of brain activity might occur in some reptiles without the polygraphic signs seen in most mammals.

In mammals, state-specific neuronal discharge occurs throughout the brain, particularly in the brainstem. Unit discharge in both locus coeruleus and raphe is greatest in wake, decreases in sleep, and ceases in REM, a general pattern docu-

mented by many studies in rats, cats, and dogs (13, 17). Two populations of cholinergic cells contribute to producing tonic and phasic components of REM-sleep, showing burst discharge only in wake or in REM (or transition to REM sleep) (17, 22). Comparisons with the firing patterns of these populations in reptiles would provide the first data at the cellular level bearing on the evolution of sleep in the vertebrate line.

To this end, we chose to record brainstem units from turtles, which are phylogenetically more ancient than the modern mammals (10). Here we report preliminary results and analysis of neuronal discharge of 23 brainstem units in freely-moving turtles across different behavioral stages. To the best of our knowledge, unit recording has never been performed in freely-moving reptiles prior to our study.

METHODS

Experiments were performed on 4 adult box turtles (700-1000 g), of the species *Terrapene carolina major*. Under pentobarbital anesthesia turtles were surgically implanted for chronic recording of EEG, electrooculogram (EOG), electrocardiogram (ECG), neck electromyogram (EMG) and neuronal activity. All turtles were administered an initial dose of pentobarbital (20-25 mg per kg) intraperitoneally (i.p.) followed by 2 to 4 additional injections (20-25% of the initial dosage every 45-60 min) to reach and maintain the surgical plane of anesthesia. In order to provide the turtle's head sufficient freedom of movement once the probe was in place, a section of shell behind the head was surgically removed using a fiberglass cutting wheel. The size of this section, approximately 1 cm wide and 5 cm long, was limited by the necessity of not breaching the body cavity.

Two pairs of 1 mm diameter screw electrodes were placed in the skull on both sides 1-2 mm lateral to the sagittal suture to record EEG. One additional screw, serving as an indifferent electrode, was implanted in the skull along the midline line above the nasal cavity. Postmortem examination confirmed that in all turtles caudal electrodes were implanted over the occipital regions of cerebral hemispheres while the rostral electrodes were positioned above the frontal cortex close to the olfactory bulbs. EEG recordings were performed bipolarly from rostral and caudal electrodes of the same hemisphere. Pairs of screws were implanted in the bone above the orbit to record the EOG of both eyes. Two to four Teflon-coated multistranded stainless steel wires (Cooner Wire, Chatsworth, CA, U.S.A.) were placed in the dorsal neck musculature for EMG recordings. To record ECG, we implanted screw electrodes into the rostral-most scute on one side and the caudal-most scute on the opposite side of the shell. This placement of ECG electrodes also allowed us to observe EMG from the leg musculature.

In a series of preliminary acute and chronic implantations we had failed to reliably record units using the previously described microwire technique (13). Others (16; Joyce Keifer, personal communication; Kristyna Hartse, personal communication) were also not able to record units in acute turtles using 300-500 k Ω impedance microwires. Therefore, tungsten microelectrodes (5 M Ω , 8 tip, A-M Systems, Carlsborg, WA, U.S.A.) were used for unit recording in turtles. One tungsten microelectrode was glued using cyanoacrylate glue into the inner movable cannula of the type of microdrive that we developed for microwire recordings in freely moving animals (13). The microdrive was implanted with the outer cannula lowered to a depth of approximately 1-2 mm below the surface of the optic tectum or cerebellum. The microdrive, EEG and EOG electrodes, and the exposed skull surface were covered with dental acrylic. The EEG, EOG, EMG, ECG and microelectrode leads were soldered to 7-pin connectors which were cemented to the turtle's shell.

Within 5-7 days after the surgery, turtles were placed in a sound-attenuated recording chamber (60 x 60 x 90 cm) to which they were adapted for the next 2-3 days. Recordings were performed mostly between 7 am and 12 pm with occasional data acquisition throughout the night. In addition to fluorescent light coming through the chamber window, a supplementary light in the chamber was turned on during the day (7 am-7 pm). Temperature during different recordings varied between 23° and 29°C. We observed the behavior of turtles during recording via a video camera mounted in the ceiling of the chamber.

During recordings turtles were free to move around the chamber. The microelectrode was advanced in steps of 20 μ m until one or more well-isolated units were seen. For most units the signal-to-noise ratio varied between 2:1 and 3:1 but in some cells it was more than 10:1. The extracellular signal from the microelectrode was recorded monopolarly with reference to the indifferent electrode. The signal was amplified, digitized and filtered at a bandpass of 300 Hz to 5 KHz (A-M Systems, model 1700) and stored on disk for subsequent off-line analysis using Spike 2 Software (Cambridge Electronic Design 1401 plus, Cambridge, U.K.). EEG, EOG, EMG and ECG signals were amplified by a Grass polygraph, digitized and stored on disk.

Cells were held from 0.5 to 5 hours (10 of these units for >4 hours, 6 units from 2-4 hours, 7 units from 0.5-2 hours). The number of spikes per epoch was counted for each period during which the unit was stable (consistent amplitude and uniform waveform of spikes). The mean unit discharge rate was calculated for every behavioral stage present during the recording. For each unit, we attempted to record several episodes during which the turtle moved from active wakefulness to unambiguous quiescence. Five behavioral stages were identified on the basis of the criteria described below. Polygrams were scored in 10, 30 or 60 sec epochs. A total of 39 hours of unit recordings and their accompanying polygraphic data were analyzed.

At the end of the recordings turtles were sacrificed with an overdose of pentobarbital (60-80 mg/kg, i.p.). Recording locations were marked by passing an electric current through the tip of microelectrode. Turtles were perfused and the positions of the lesions were histologically identified in sections stained using Nissl stains and immunohistochemical stains for tyrosine hydroxylase and serotonin. Nuclei were identified on the basis of published atlases and publications on closely related turtle species (15, 24).

RESULTS

Five behavioral stages were scored in turtles on the basis of neck and leg EMG and behavior:

Active wakefulness (AW) was characterized by high tonic and phasic activity in neck and leg EMG and was scored when it was present for more than 50% of the epoch. In AW the turtle was mostly walking in the chamber. *Quiet wakefulness (QW)* was scored when the same pattern was present in the EMG and ECG for less than 50% of the epoch (Fig. 1, A). In QW the turtle stayed in place and occasionally moved its head. The shell was positioned on the floor and the limbs were mostly extended. In both stages, eyes were open. *Transitional stage (T)* was scored when neck and leg EMG had no high amplitude phasic components. Neck muscle tone in this state was lower than in both stages of waking. Muscle tone increased for a short time or steadily and slowly decreased during several consecutive minutes (Fig. 1, A). The shell was always positioned on the floor. Forelimbs could be extended or withdrawn, but hindlimbs were always withdrawn. The head was partially extended and rested on the edge of the plastron. Eyes could be either opened or closed.

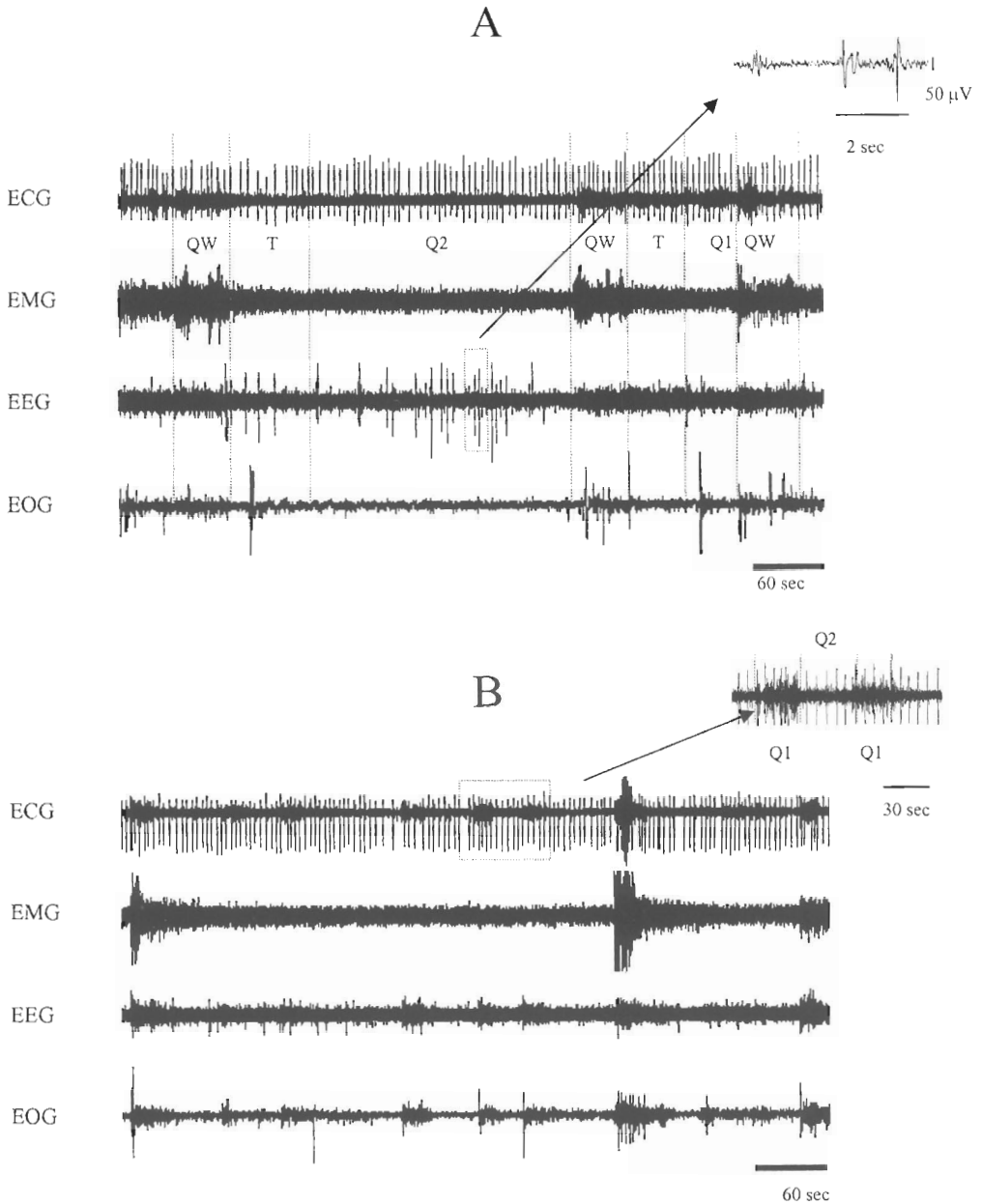


Fig. 1. - Examples of low speed polygrams from two turtles, showing how behavioral stages were identified.

ECG - electrocardiogram; EMG - neck musculature electromyogram, EEG - electroencephalogram; EOG - electrooculogram; QW - quiet wakefulness; T - transitional stage; Q1 and Q2 - two stages of quiescence. A: Recording at 28°C. Inset: the waveform of EEG spikes. Note disappearance of EEG spikes in QW. B: Recording at 23°C. Inset: Leg EMG components in ECG allowed discrimination of two stages of quiescence. Note the difference in the rate and amplitude of EEG spikes in (A) versus (B).

Two stages of quiescence, Q1 and Q2, were scored when neck EMG was at its lowest level during the entire epoch. The turtle's head was partly extended forward and lay on the edge of the plastron during both stages of quiescence and eyes could be either closed or opened during Q1 and Q2. High amplitude deflections in the EOG usually correlated well with eyelid movements (openings or closing of eyes; Fig. 1). Low amplitude deflections occasionally occurred in the EOG when both eyes appeared to be closed. Up to 3 limbs were partly extended backward in turtles during Q1 and Q2. We never saw turtles extend all 4 limbs as described by Flanigan et al. (8). On rare occasions high frequency components in neck EMG were recorded in Q1 and Q2, which could be considered muscle jerks. However, we never visually observed jerks in turtles. *Stage Q1* was scored when any signs of leg EMG were seen in the ECG derivations. *Stage Q2* was scored when there were no leg EMG components at all in the ECG background (Fig 1 A,B).

As previously reported (6, 7, 8), EEG in turtles was of low amplitude and did not change significantly across stages, with the exception of EEG spikes and sharp waves. EEG spikes were highly variable in shape and amplitude and were recorded

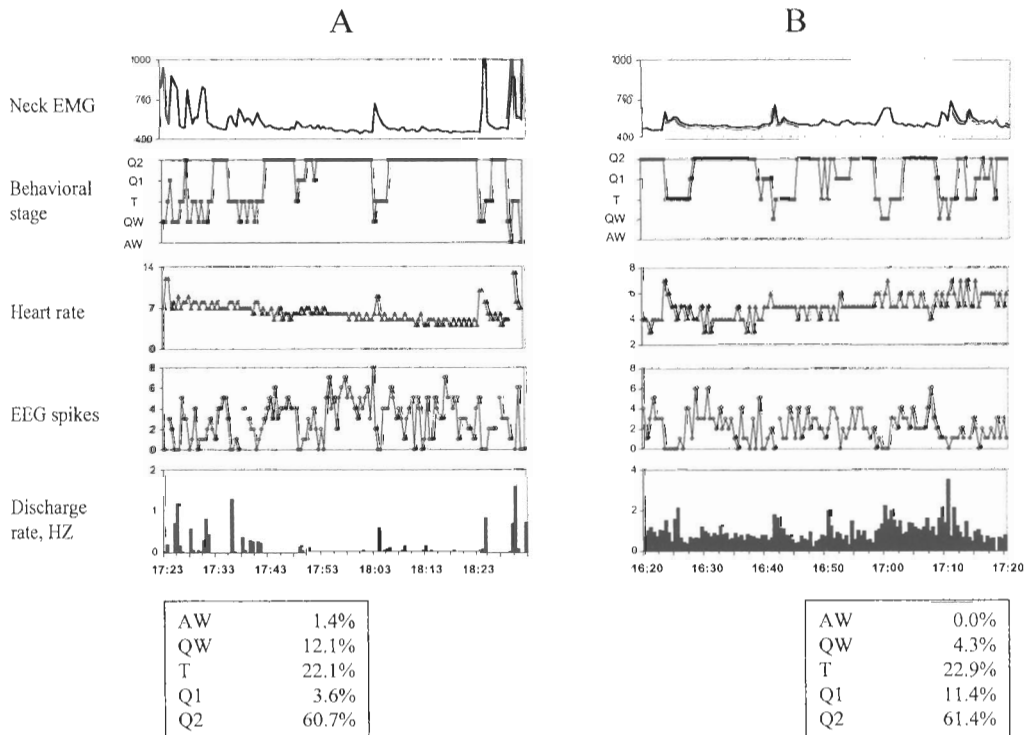


Fig. 2. - Discharge pattern of two units, recorded at 28°C.

Insets: The percentage of time spent in different behavioral stages during recordings. Neck EMG integrated values presented in relative units; heart rate, in beats per 30 sec epoch; EEG spikes, in spikes per 30 sec epoch.

in all behavioral stages (Fig. 1). We counted spikes when they were more than twice the background amplitude, but we excluded rare large amplitude spikes (7-10 times the background level), which were presumably movement-related artifacts. The number of EEG-spikes per 30 sec epoch varied between 0 to 8 (Fig. 2). In general, spike rate increased when turtles moved from waking to quiescence but the difference between stages was not always significant. EEG spike rates in all stages decreased when the environmental temperatures dropped below 24°C. (Fig 1 A, B).

Heart rate in turtles was the highest and the most irregular during AW (10-30 beats/min) and steadily decreased, becoming more regular as turtles moved from QW (10-18 beats/min) to T (8-12 beats/min), Q1 (6-12 beats/min) and Q2 (4-8 beats/min). A more detailed description of polygraphic characteristics of behavioral stages in turtles will be presented elsewhere.

A total of 23 units were recorded from the brainstem area at the level between the roots of nerve V and VIII (Fig. 3). In 3 of 4 turtles, units were recorded close the midline (15 units, Fig. 3A). In one turtle the units (2 of those 15 midline units) were recorded within the dorsal-most portion of a serotonin-positive area (immunostaining data not shown). In one turtle the recording area was located more rostral and lateral to the midline, in the medial reticular nucleus (8 units, Fig. 3B). The small size of the turtle brain creates unique difficulties in the reconstruction of recording tracks, and therefore we presently cannot be more anatomically specific.

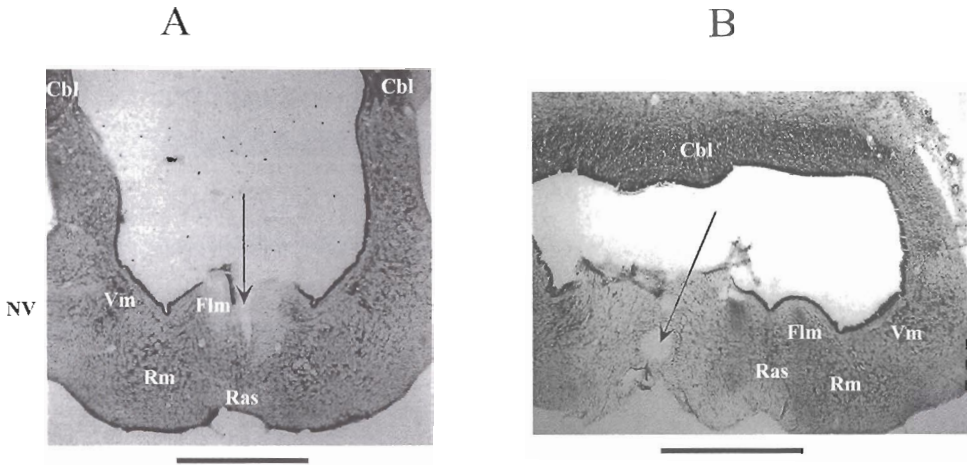


Fig. 3. - Recording locations in coronal view, in two turtles.

A: Midline recording location, most likely in the medial longitudinal fasciculus (FIm) or serotonergic cells of raphe in turtle 47. Arrow: damage left by the microelectrode tip. B: Arrow: electrolytic lesion in Rm in turtle 22, where 5 of 6 low spontaneous active cells (Group I) were recorded. Scale bar = 1 mm. NV: the root of 5th nerve, Ras: superior nucleus of raphe, Rm: medial reticular nucleus, Vm: motor nucleus of 5th nerve, FIm: fasciculus longitudinalis medialis, Cbl: cerebellum.

22 of 23 recorded cells (95%) clearly increased discharge during active movement in association with any leg or neck movements and corresponding changes in the EMG (Fig. 2, 4, 5). Most cells discharged during movements of the head or limbs. In a few cells the discharge was found to be more closely linked to either head or limb movements but we saw no units whose discharge related exclusively to one or the other. In one cell, discharge also coincided with EOG deflections. One unit discharged exclusively in relation to the cardiac cycle when the turtle was quiescent (Fig. 4).

In 13 neurons, responses to various stimuli were tested. 8 cells (62%) responded to touching of the shell and 5 (38%) of them showed differential responses to stimulation of different parts of the shell (Fig. 5 A, B). At least 5 (38%) of 13 tested units responded both to visual stimulation (movement, flashing) and to touching. Two cells responded to knocking at the chamber door (Fig. 5B). This may have been a response to vibration rather than sound because none of the tested cells responded to other auditory stimuli.

In order to determine the effect of epoch length on estimation of mean discharge rate, we scored several recordings and calculated the discharge rate of the same units using 10, 30 and 60 sec epochs. There was no significant difference between mean discharge rates calculated using 10, 30 and 60 sec epochs in any stage except AW, which under our conditions was recorded very rarely.

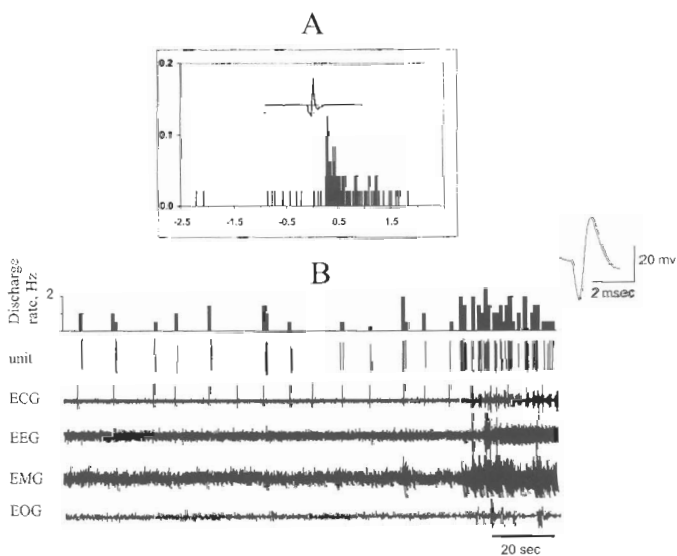


Fig. 4. - An example of cardiac-related discharge in one unit.

A: A frequency histogram of spikes in a cardiac-cycle related unit from (B), correlating unit spikes with heartbeats during an 8 min period of quiescence which included 48 heartbeats. Inset: Heartbeat averaged ECG waveform. X axis: Timing of unit spike in relation to heartbeat in seconds. Bin size: 0.01 sec. Y axis: Averaged number of spikes per bin ($n = 48$ heartbeats). B: Unit discharge during quiescence and quiet wakefulness. ECG - electrocardiogram; EMG - neck musculature electromyogram; EEG-electroencephalogram; EOG - electrooculogram. Inset: Average spike waveform.

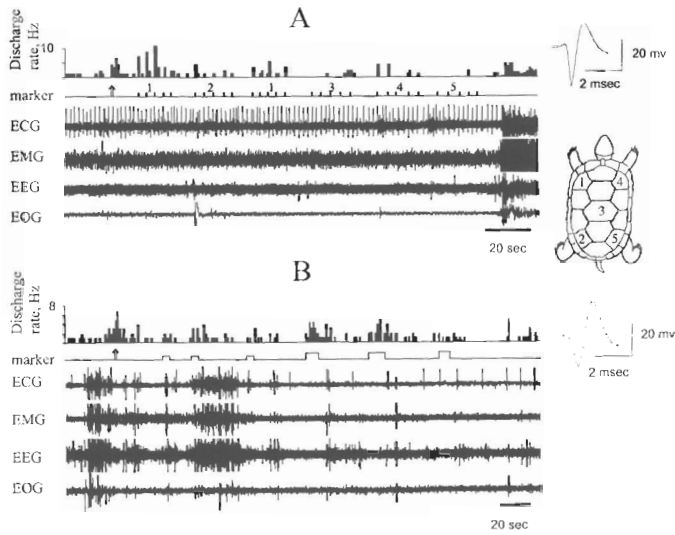


Fig. 5. - Examples of responses of 2 cells to stimulation.

A: Responses to visual and tactile stimulation. Arrow: An object was placed in front of the turtle. Each mark in the numbered series (1-5) corresponds to a single touch on the shell. The average waveform of this unit and the location of the stimulated location on the shell are shown on left side. B: Response to visual stimulation and knocking at the chamber door. Arrow: An object was placed in front of the turtle's head. Three short marks: Flashing of the light in the chamber. Three long marks: Knocking at the chamber door. Insets: Average spike waveforms.

The mean discharge during QW, T-stage, Q1 and Q2 was calculated in 30 sec epochs for 17 units. Since not every unit was observed during Q1, we calculated mean discharge for only 13 of these 17 units in Q1. The mean discharge rate was 0.90 ± 0.18 Hz ($n = 17$; 0.11-2.73 Hz,) in QW; 0.45 ± 0.13 Hz ($n = 17$; 0.02-1.97 Hz) in T-stage; 0.49 ± 0.21 Hz ($n = 13$; 0.01-2.81 Hz) in Q1; 0.31 ± 0.12 Hz ($n = 17$; 0.01-1.99 Hz) in Q2. The instantaneous discharge during movements could be as high as 180 Hz.

The mean discharge rate in most neurons steadily decreased when the turtle moved from QW to Q2 (Fig. 6A). The difference between mean discharge rates was significant in QW versus T-stage (Wilcoxon matched pairs test; $p < 0.01$), in T stage versus Q1 ($p < 0.05$) versus in T-stage and Q2 ($p < 0.01$). Mean discharge rates did not differ between Q1 and Q2. The variability of mean discharge rate in all units also decreased with lengthening quiescence. This follows from Figure 6B, in which we plotted T, Q1 and Q2 standard errors against QW standard errors for all recorded units. A larger proportion of symbols representing standard errors in QW versus Q2 in Figure 6 are shifted to the right, compared to symbols representing QW versus T (in both cases $p < 0.01$), suggesting that variability of discharge decreased steadily as turtles move from QW to Q2. There was also a significant difference in the variability of discharge between stages QW and Q1 ($p < 0.05$), QW and T-stage ($p < 0.01$), Q1 and Q2 ($p < 0.01$) but not between T-stage and Q1.

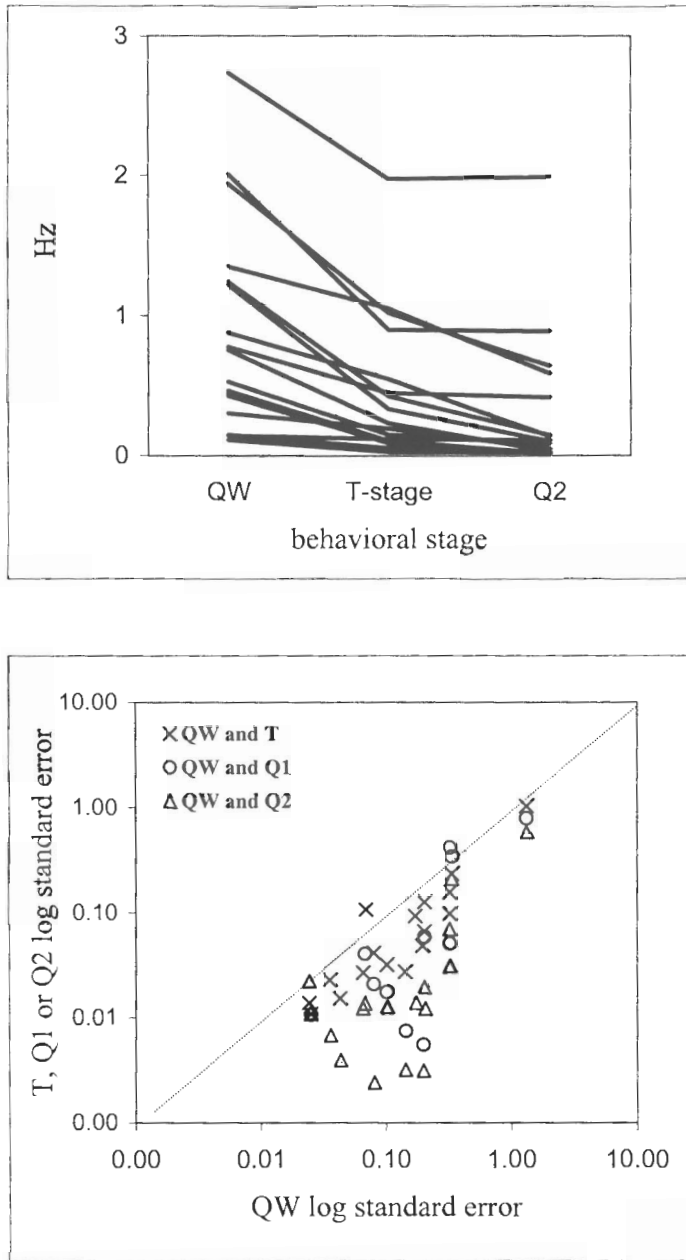


Fig. 6. - Firing characteristics of recorded units in turtles during different behavioral stages.

A: Discharge profiles of 17 recorded units during quiet wakefulness (QW), transitional stage (T-stage) and quiescence 2 substage (Q2). Mean discharge rates in quiescence 1 substage (Q1) are not shown on this diagram, since not every unit was observed during Q1. B: QW standard error of the mean discharge rate of recorded cells plotted against T, Q1 and Q2 standard errors. Values were calculated for 30 sec epochs.

Monoaminergic cells of the locus coeruleus and raphe decrease firing throughout sleep and virtually cease during REM in mammals (17). In the turtle, 10 of 19 (52%) units showed pauses longer than 1 min during Q1, Q2 and some part of T-state. They were silent more than 1 min from 9-54% of epochs scored as Q1 and Q2 and from 3-56% of epochs scored as Q1, Q2 or T. We have divided units into two groups based on the presence of pauses longer than 5 min during periods of quiescence (Group I) or the absence of such pauses (Group II). The mean discharge rate during stage Q2 of group I cells was significantly lower than that of group II cells: 0.02 ± 0.01 Hz ($n = 6$; 0.01-0.05 Hz) and 0.47 ± 0.17 Hz ($n = 11$; 0.02-2.00 Hz; Mann-Whitney test, $p < 0.05$). Group I units exhibited lower mean firing rates during QW than group II units (0.43 ± 0.08 vs 1.17 ± 0.24 Hz, respectively) but the difference between two groups of cells was not significant (Mann-Whitney test, $p > 0.05$). Group I cells had a higher QW/Q2 discharge ratio (23.0 ± 6.0 , $n = 6$ in group I cells and 4.5 ± 1.0 , $n = 11$ in group II cells) and T/Q2 discharge ratio (5.8 ± 1.0 and 1.8 ± 0.3 , respectively). Therefore, the mean discharge rate of Group I units was lower in all stages and showed a higher degree of contrast between stages than Group II units.

We investigated whether the pattern of unit discharge was correlated with the incidence of EEG spikes. 10 cells were recorded during episodes of quiescence in which turtles displayed more than 3 EEG spikes per epoch. For these intervals of time we calculated the mean discharge rate of units during Q2 in epochs with low (0-2 spikes per 30 sec) and high (more than 2 spikes per epoch) numbers of EEG-spikes per epoch. Neither the mean discharge rate nor the level of variability of discharge differed significantly in these units during epochs with low versus high numbers of EEG spikes per epoch (Wilcoxon matched pairs test; $p > 0.05$). The integrated neck EMG values for these two groups of Q2 epochs also did not differ.

DISCUSSION

Though in general our polygraphic data conformed to that of Flanigan, et. al (8), it departed from theirs in certain details. First, the behavioral quiescence we observed was often not accompanied by pronounced changes in limb extension, a key feature of Flanigan's classification of substages of quiescence. We also noted significant variations in muscle tone during behavioral quiescence, even when there was no visible movement. Furthermore, we observed limb EMG components in the ECG background that appeared independently from nuchal EMG. We used these nuchal and limb EMG variations to subdivide behavior into waking (AW and QW), transitional stage (T-stage), and unambiguous quiescence (Q1 and Q2). The gradual decrease of tonic nuchal EMG activity distinguished T-stage from both stages of quiescence. T-stage usually preceded quiescence, supporting its "transitional" character. Q1 episodes appeared mostly framed within Q2 episodes and represented periods of short-lasting activation in the limb EMG. Only in Q2 were turtles completely atonic.

As in Flanigan's studies (6, 8), we observed series of polymorphic EEG spikes in turtles. Usually the rate of EEG spikes increased during quiescence, most often when the environmental temperature exceeded 24°C. Also, the number of EEG spikes per epoch and their amplitude varied between turtles, as was described before (9). Variations of EEG spike rate during quiescence did suggest that quiescence is not homogeneous, in general agreement with Flanigan's data (6, 8).

Several authors have observed phasic events during reptilian quiescence (2, 11, 12, 23) which some interpreted as REM-sleep like behavior, while others considered them part of a transition to quiescence. In our experiments, we occasionally recorded single EMG potentials in quiescence that resembled REM-related jerks in mammals but we were not able to confirm them in simultaneous polygraphic and video records. EOG deflections during periods of quiescence were present in turtles even during the nighttime. While large amplitude EOG deflections usually coincided with eye opening and closing, low amplitude EOG deflections occurred when eyes were closed and did not coincide with any visually detected eyelid movements. Both phenomena lacked apparent cyclicity and need additional study.

In waking, 95% of the units recorded from the brainstem in turtles clearly increased discharge during movements (neck, leg, eye). Cells discharged during movements of the legs or neck movements, either phasically, at the beginning of movements, or tonically, during all active movements. These cells responded to various stimuli (tactile, visual, rotation, knocking). About 60% of the tested cells responded to tactile and 40% to visual stimulation. In several cases responses to tactile stimuli were somatotopic.

Cells were divided into two groups based on levels of spontaneous activity in quiescence. Group I cells (32%) showed very low spontaneous discharge when the turtle did not actively move. All were silent for more than 5 min and as long as 27 min in Q1, Q2 and T-stage. Except for the lack of firing during any state of quiescence, the firing pattern of these of cells resembles that of some of the reticular neurons in mammals (17, 18, 19). Most Group I cells (5 of 6) were recorded lateral to the midline in a caudal pontine area (Fig. 3B), in the medial reticular nucleus. In the echidna, irregular bursting activity was found in this area during sleep, showing characteristics intermediate between REM and SWS (20). However, we observed no irregular bursting activity during quiescence that was not correlated with changes in leg or neck EMG or in EOG. Group II cells (68%) steadily decreased discharge rate and variability as the turtle moved from waking to quiescence. They were spontaneously active throughout quiescence, though several cells had low spontaneous activity and occasionally did not discharge for periods of 1 min or more. This firing pattern resembles that of serotonergic and noradrenergic cells in mammals, which are tonically active throughout waking, reduce discharge during sleep, and cease in REM (13, 18). Group II units were located in the rostral medulla and in the caudal part of the reticular formation (Fig. 3A, B) In 3 of 4 turtles we recorded this neuronal activity close to the midline superior raphe (Fig. 3A), and in 1 turtle, the recording area contained serotonin-positive cells.

Reptilian EEG spikes have been considered by some investigators to be analogous to mammalian slow waves (2, 4-9, 11, 25). In general, one might predict that EEG spike rates in the turtle would show a negative correlation with unit firing. We saw no correlation between the incidence of EEG spikes during stage Q2 in turtles and the pattern of neuronal discharge, the mean discharge rate, or the variability of discharge.

To summarize, our preliminary data shows that most cells located in the caudal pons and rostral medullary reticular formation in turtles show decreasing discharge rate and increasing regularity with lengthening quiescence. However, despite having observed at least two distinct substages of behavioral quiescence the present data as a whole are consistent with the interpretation that turtles have only one stage of sleep, homologous to NREM sleep. Further unit recordings with more extensive sampling of brainstem units could reveal substage-specific firing characteristics and shed further light on the origins of REM sleep.

SUMMARY

We have performed the first study of neuronal activity in freely-moving reptiles. 23 brainstem units were recorded from areas throughout the reticular formation, during wakefulness and quiescence in the box turtle. These units responded to various sensory stimuli and increased firing rates in relation to motor activity during wakefulness. All but one unit showed significant decreases in discharge during quiescence. Group I cells (32%) fired mostly during active movements and exhibited silent periods of 5 min or longer during quiescence while group II cells (68%) maintained slow tonic activity in quiescence. Polygraphic data showed no consistent, cyclically occurring phasic events during quiescence.

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