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Sleep in invertebrates: crayfish

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1. ABSTRACT

Although sleep is a very conspicuous behavior in all animals that we are frequently in contact with and possibly in many others, its scientific study was for many years restricted to very few of them. However, since the end of the XX century there have been studies about sleep in several animals and currently many of them attempt to found, first, if all animals sleep and second, if their sleep is similar to that of other animals. An important objective of this search is to identify the animal species in which sleep originated, which might give us clues about the need that was fulfilled by such behavior. The search started with insects, among the most developed arthropods, but has now been expanded to include other invertebrates, among them crustaceans. In this work we review some aspects of sleep in invertebrates, focusing on the crustacean crayfish, animals in which both, behavioral and electrophysiological studies have been conducted and whose results show surprising similarities with sleep in mammals.

2. INTRODUCTION

Sleep is a very conspicuous behavior that has attracted the attention of humans for thousands of years. This can be illustrated with the paintings in the walls of the Lascaux caves, depicting a human next to a buffalo and a bird, a scene that has been interpreted as a human dreaming about hunting (1).

However, despite numerous efforts we do not know why we sleep. We certainly know that sleep is "by, from, and to the brain" (2), but we ignore what is the function subserved by this activity. Nevertheless, we assume that sleep has an adaptive value, as otherwise it is difficult to explain its persistence and wide distribution among vertebrates and invertebrates (3,4,5).

The first definition of sleep included three main behavioral components, motor rest, increased sensory thresholds and easy reversibility (6). Some other behavioral characteristics have been added later, including a stereotyped posture, specific rest sites, circadian organization, homeostatic regulation, and closing of eyes (7,8,9,10).

The scientific study of sleep is barely more than 50 years old, as it was in 1953 that Aserinsky and Kleitman (11) performed its first careful description, characterizing it with a group of behavioral observations which included: a) a stereotyped posture; b) quiescence; c) loss of muscle tonus; d) low reactivity to sensory stimulation, and; e) rapid awakening.

Later on, records of the brain electrical activity were added to that characterization and formed the basis for dividing sleep in its two main periods, NREM and REM (11,12). Currently, reactivity to drugs such as caffeine has also being added, as well as a phenomenon labeled homeostasis, which describes the rebound that occurs after a period of sleep deprivation (9).

The initial observations about sleep were made in humans and although there were previous experiments in animals to study the effects of their deprivation (13), was Dement (12) who first performed experiments in cats to search for the biological basis of such behavior; he found that sleep in animals met the criteria defining the same behavior in humans. These experiments provided a very useful animal model of sleep, where the biological basis and descriptions of the brain regions and neuronal circuits involved in sleep have been studied.

Experiments have also been conducted on uncommon animals, such as echidna and platypus (14, 15, 16) and sloths (17), and results show that they also seem to follow the general pattern of sleep seen in mammals.

The initial experimental studies were all conducted in mammals, endotherm animals that regulate to a great extent their internal temperature and have not only a well developed nervous system, but also a brain similar to our own. Furthermore, as it was found that sleep depends to a great extent on structures present on the brain stem, the most conserved and primitive part of the central nervous system, it was asked if vertebrate animals which lack brain cortex but maintain that old part of the nervous system also sleep. Thus, experiments on reptiles and birds soon followed, and although results are not as clear cut as those from mammals (18), it seems that sleep is also a property of all those animals and brains.

Experiments and observations on reptiles showed that many of the characteristics of sleep are to be found in these animals (19,20), but there is some controversy about the presence of REM-sleep periods. Some reptiles seem to have a 'reversed' electroencephalogram, in which the awake period is characterized by waves of slow frequency and large amplitude, sometimes interrupted briefly by periods of desynchronized activity. Furthermore, the amplitude of the signals diminishes with the level of activity of the animal (21, 22), and it has been suggested that turtles have a single sleep period that mixes the properties of REM and NREM periods of mammals (23); a similar proposition has been made for sleep in Echidna (14).

Those experimental results are not surprising, as the central nervous system of reptile and monotremes differs substantially from that of mammals, particularly in relation to the cortex, which may even be absent. Nonetheless, the observations of sleep in those primitive animals suggest that sleep did not originated in land animals and, although in a limited fashion, sleep has also been searched for in some aquatic animals.

Although experiments about sleep in aquatic mammals and fishes are surprisingly few, results seem to show two types of patterns. One of them pertaining to animals that became aquatic mammals after being terrestrial ones, such as whales, dolphins, etc., and other developed in fishes (24, 25) and mollusks (cuttlefish, 26). Aquatic mammals have alternating hemispherical sleep and although this form of sleep is well known, it has not been completely characterized. Thus, it is not know if these animals have NREM and REM periods.

Sleep in fishes has not been well studied, and although there are numerous behavioral observations on these animals, few have performed experiments on them. However, some fishes have been suspected of not sleeping at all (27).

These observations suggests that all vertebrates, whether land or aquatic borne, have periods of sleep with characteristics that are shared with mammals and although there are also significant differences, these are more about form than of substance. The observation that all vertebrates sleep suggests that this behavior has its evolutionary roots in simpler animals, such as invertebrates.

2. DO INVERTEBRATES SLEEP?

Observations that many invertebrates, particularly insects, have periods of quiescence during which there is also low responsiveness, suggesting that they sleep, were made early in the century (13). Experiments to study sleep were performed until late in the XX century and among the first ones were observations on bees, in which the antenna position was used to detect the decrease of muscle tonus that characterizes sleep in vertebrates (28, 29, 30). These experiments showed that bees pass through periods during which they become quiescent and loose muscle tonus, with a consequent fall of antennae and it was concluded that they sleep. However, not all bees show a similar behavior, as during the night some solitary bees clamp their mandibles to a stick and with the body free-born remain in this position, to release the grip and fly away when the sun lights up (29).

Figure 1. Crayfish in communal aquarium. In the first plane is a crayfish lying on one side at the water surface. The left walking legs are supported by another crayfish; the right walking legs on the wall of the aquarium. At the bottom there are three standing up animals.

Those observations were followed by a more extensive study of the behavior of several arthropods (31, 32), where it was found that these animals become quiescent and limp during the day and active at night. Further observations on scorpions and cockroaches (33, 34) led to the conclusion that this sleep-like behavior is common to most arthropods.

Obviously, a sleep-like behavior is not full sleep. Therefore, attempts were also made to record the electrical activity of some organs, to strengthen this way the probability of detecting real sleep during resting periods. Thus, records were made of the electrical activity of the heart and muscles during resting periods in bees (35,36), and results showed that there was a decrease in the activity of these organs during the quiescent periods, strongly suggesting that those resting periods of invertebrates were periods of real sleep.

About the same time two independent groups characterized several behavioral, pharmacological and genetic aspects of the sleep-like behavior of *Drosophila* (37, 38). These and several other studies reinforced the presumption that flies sleep (39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49). However, if we accept the postulate that "sleep is for, from and to the brain" (2), many of those results still seem inconclusive. The certainty of real sleep requires measuring some brain activity, whether electrical, biochemical or other.

Attempts to measure the electrical activity of the brain during sleep have been made with electrodes placed on brain regions of two types of flies, wild ones during the day when their activity peaks, and mutants that show extensive periods of quiescence, that were equated to sleep (50,51). More recently records have been made from the mushroom bodies of flies, involving them in sleep (52, 53, 54, 49). Results show that there is a difference in the amount of neuronal activity recorded, leading to the conclusion that during sleep flies have a decreased neuronal activity.

Animals in which it is difficult to obtain all signs for sleep have nonetheless been studied and the tentative conclusion that they also sleep has been reached. For example, the common nematode *C. elegans* was found to have a period of larval stage transition during which it shows a behavioral quiescence that was interpreted as sleep (55, 56).

Records of the brain electrical activity continuously during periods of days have been possible using the crustacean crayfish, which shows periods of rest and it is large enough to carry implanted electrodes during its daily errants. In experiments conducted during these periods we have measured all parameters of significance for sleep, such as: a characteristic body position; quiescence; high threshold for awakening with sensory stimulation; a pattern of brain electrical activity typical of these periods and different from that recorded during the awake periods, and; lack of processing of the cognitive signals characteristic of the awake periods. These results indicate that real sleep, with all signs showed in complex mammals, are also present in the simpler arthropods, further indicating that sleep must have been originated early on during the evolution of animals (57).

In this paper we will present the data obtained from crayfish that led to the conclusion that these animals

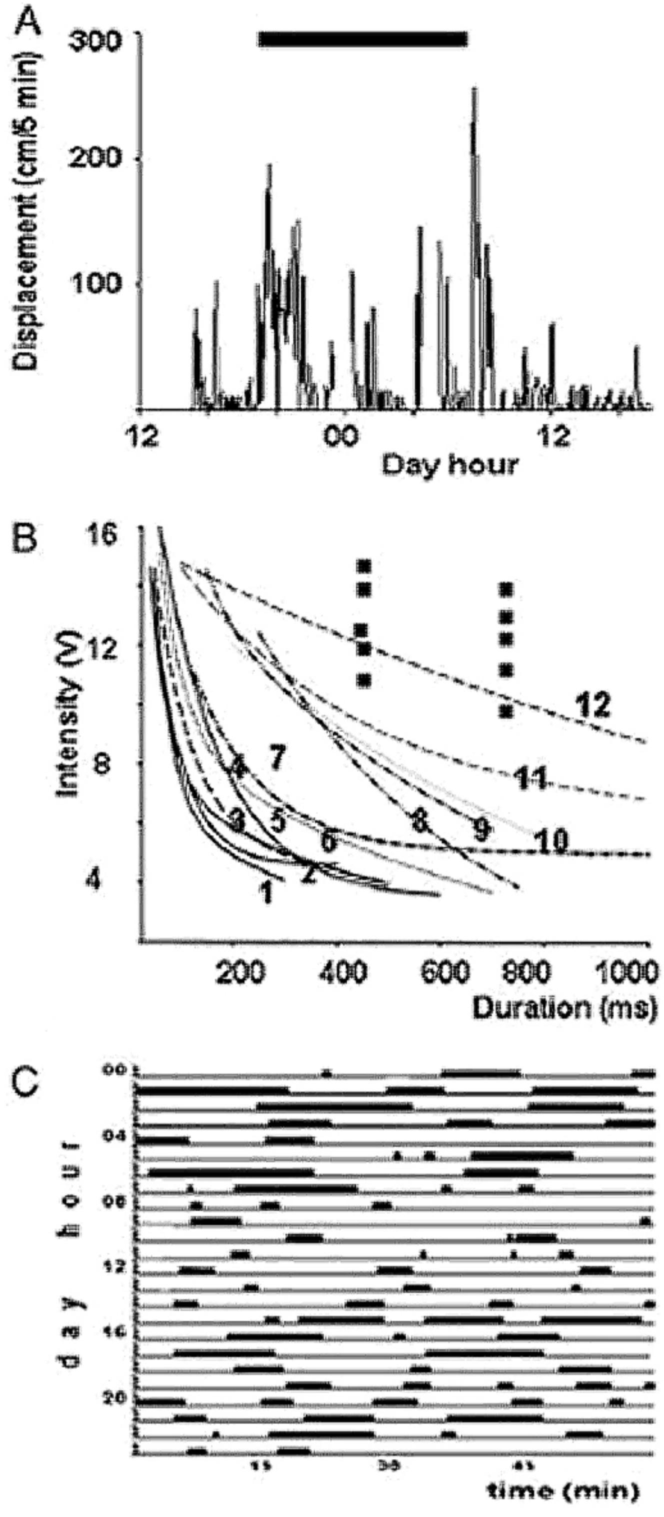

Figure 2. Parameters of crayfish behaviors. (A) Time hours at which a crayfish walks around its aquarium. The black bar indicates night hours. (B) Intensity-duration threshold curves of crayfish while active (curves 1-7), standing up motionless (curves 8-11), and lying on one side against the surface of the water (curve 12 and isolated squares). We defined mechanical threshold as the stimulus value (intensity and duration) at which we observed a just noticeable movement of either left or right third pereiopod after each mechanical pulse.(C) Time hours at which a crayfish lies on a side against the surface of the water. A and C show examples of 12 recorded cases (Reproduced with permission from 57).

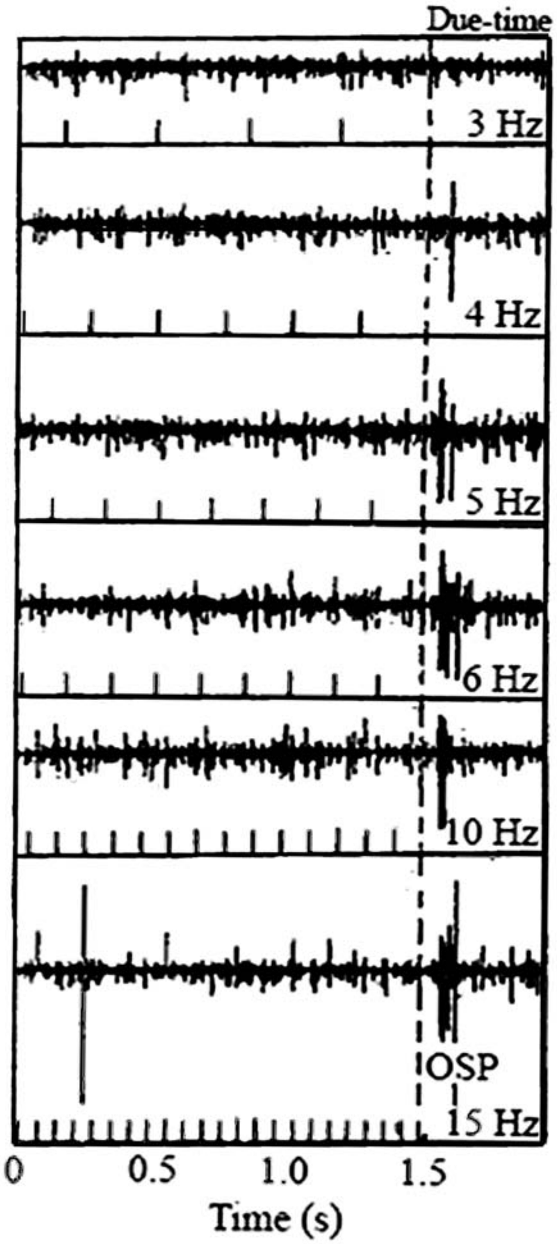

Figure 3. Omitted Stimulus Potentials. Records from the protocerebrum of an alert crayfish, showing single sweeps during trains of flashes at different rates. Responses (OSPs) aligned by the due-time (dotted line) of the first missing flash show a near constancy in latency, independently of the stimuli frequency. At 3 Hz no OSP is seen, and a single spike occurs in some trials at 4 Hz. Above 15 Hz some preparations emit a burst at the end of the train due to the flicker-fusion-frequency effect. The constant latency from a given locus is the hallmark of the OSP (Reproduced with permission from 62).

show real sleep, and also other data that allow us to identify the brain region where it is generated and the neuronal steps preceding it.

3. THE SLEEPING CRAYFISH

3.1. Behavior

In an aquarium and under laboratory conditions, crayfish display a variety of behaviors and the most common are two, alert interacting with conspecifics, motionless, and lying on one side on the surface of the water (Figure 1).

In alert animals the proximity of the experimenter triggers a clear response, elevation of chelae, walking backwards or even escaping using strong tail flips. On the contrary, lying on one side animals do not seem aware of other animals nor the experimenter (even if they pass close by or touch them). Indeed, it is possible to touch them lightly without inducing a response. However, a

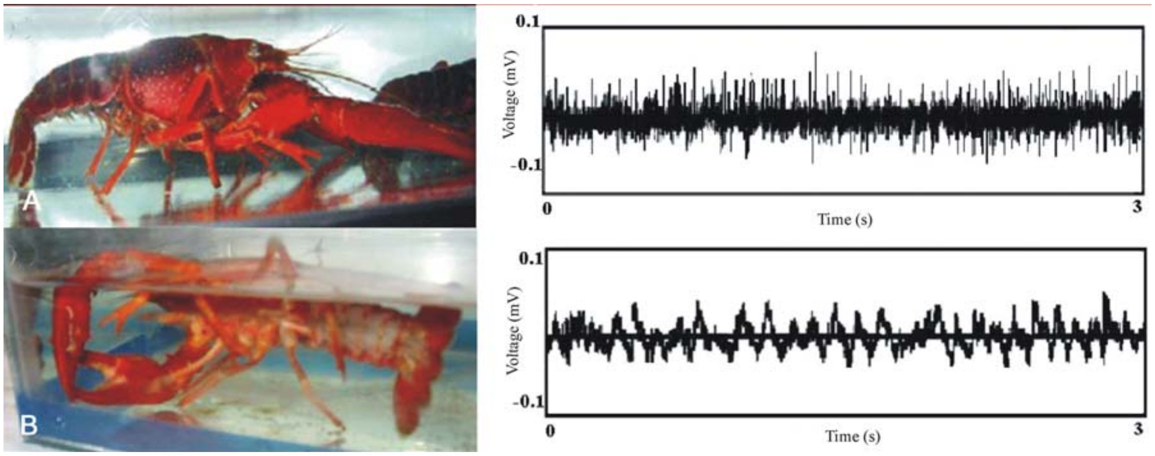

Figure 4. Crayfish behavior (left) is associated to a characteristic brain electrical activity (right). (a) In an alert animal the brain electrical activity is comprised of numerous spikes on an almost flat baseline. (b) When the animal lies on one side against the surface of the water the brain activity pattern changes to slow waves.

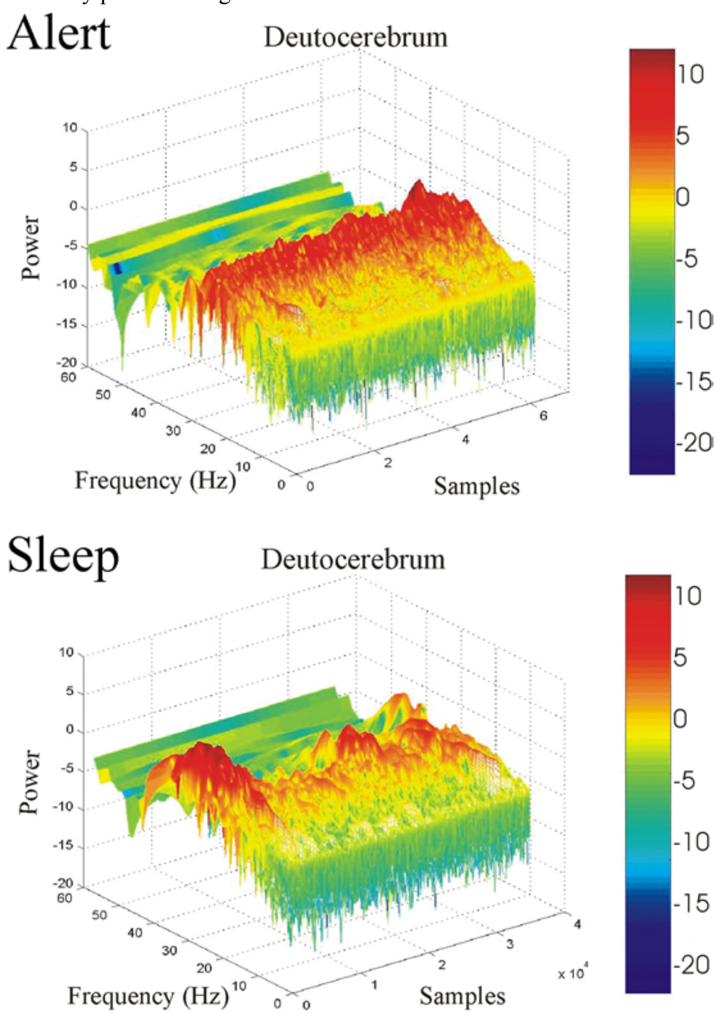

Figure 5. Wavelet Transform of the deutocerebral electrical activity. 3D graphs show time (s), frequency (Hz, only 0-60 Hz are shown), and power (normalized and color coded; the higher the redder) for 200 seconds of recording. In the alert animal (upper part) the WT analysis shows high power at frequencies between 20-45 Hz. In the sleeping crayfish there is a large decrease in power in the 30-45 Hz range.

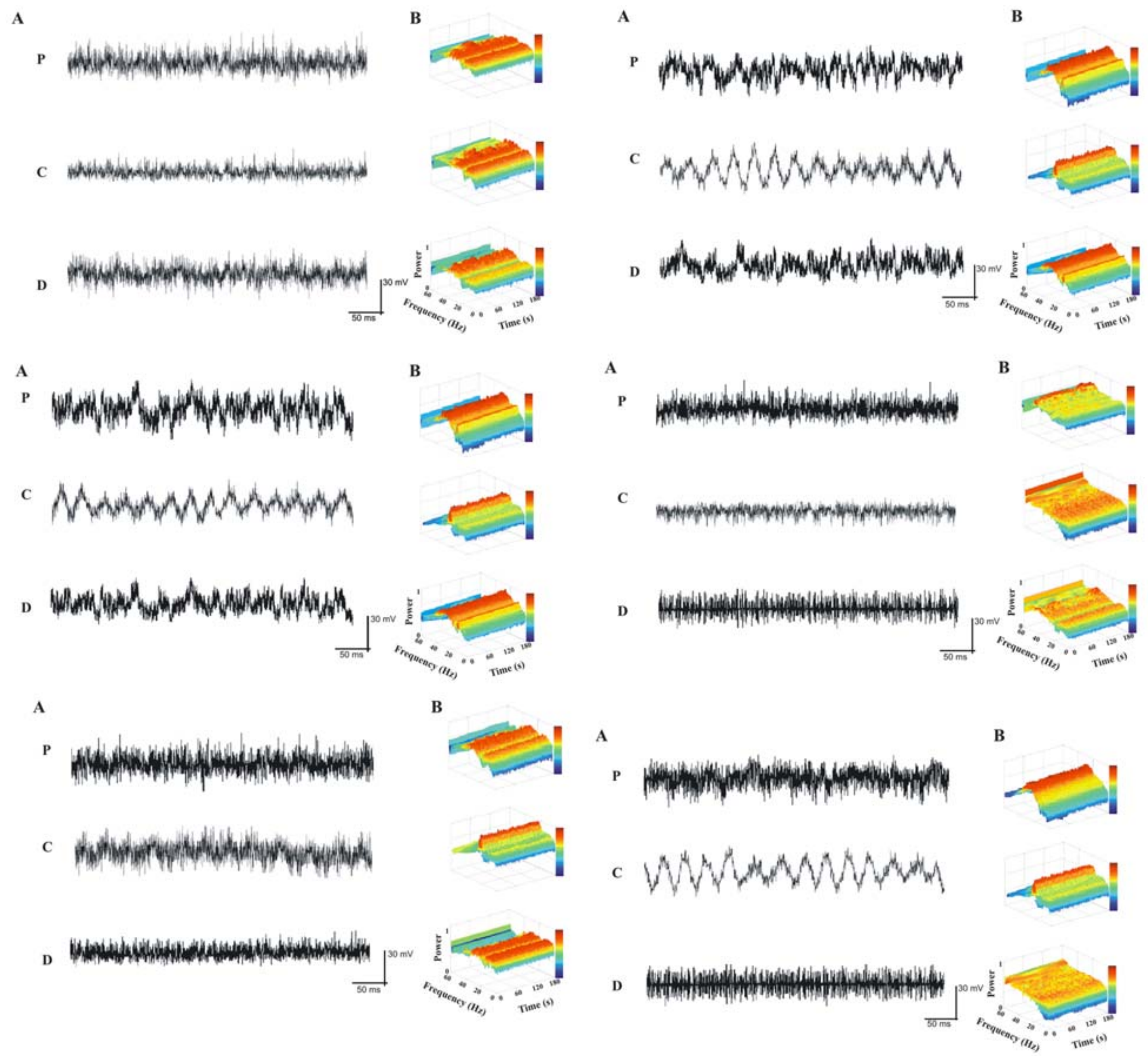

Figure 6. Slow waves spread from the central complex of the brain. Left side of each column shows raw data of simultaneous recording of electrical activity from lateral protocerebrum (P), central complex of the brain (C), and deutocerebrum (D) while one region is chilled. Right side shows the wavelet transform of the records. In alert animals, chilling the lateral protocerebrum (A) or the deutocerebrum (C) reduces its activity, while abolishes the slow waves from the central complex of the brain (B). In sleeping animals, cooling the central complex of the brain (E) abolishes the slow waves in the whole brain, while chilling other areas (D & F) only abolishes the slow waves in the cooled area.

stronger stimulus (tactile or visual) is immediately followed by straightening and walking away. That is, lying on one side animals have the three behavioral characteristics that comprise the early definitions of sleep: motionless, increased sensory thresholds and rapid reversibility (6). Therefore, to study sleep in crayfish, we initially measured its hourly displacement around the aquarium (Figure 2A), reaction threshold to a vibratory stimulus (provided by a small DC motor glued to the dorsal carapace, Figure 2B), and frequency with which an animal lies on one side during a 24-hour cycle (Figure 2C). These data are similar to those measured from other animals during sleep and strongly suggested that crayfish also sleep (57).

3.2. Brain electrical activity

Because the brain electrical activity is arguably the most important sign characterizing sleep (2), we also recorded the electrical activity from the brain of crayfish under a variety of conditions. In these animals it is possible to implant chronic electrodes on the surface of the brain to record its electrical activity for long periods of time and under different experimental conditions (58, 59, 60). It is also possible to show that alert crayfish possess simple cognitive functions, such as detecting the absence of a stimulus within a short train of stimuli, named expectation. Expectation was studied in crayfish with an electrode implanted on the surface of the brain, stimulating with 1 second trains of 10 μsec duration flashes at various rates. Then we searched for a spike discharge time-locked to the due-time (the expected time of the stimulus after the last one, if it had been applied) (Figure 3; Omitted Stimulus Potential, 61).

As it is clear in Figure 4, the brain electrical activity of alert unrestrained animals is comprised of numerous spikes with frequencies of 100-300 Hz, riding on an almost flat base line (62). However, when the animal lies on the surface of the water (Figure 4B), the number of spikes decreases markedly and the record becomes dominated by slow waves.

A few words on nomenclature will clarify our use of the term slow-waves. The brain electrical activity of vertebrates has frequencies ranging from 0.1-80 Hz, with mostly stationary slow waves and in the range 0.1-4 Hz. Therefore, it is possible to identify their main components using the Fourier Transform (3). In contrast, in crayfish brain waves range from 15-300 Hz. High frequency signals are observed in alert animals, but since during sleep frequencies decrease to 15-20 Hz, we use the words 'slow waves' to describe them, without any intention to equate them with those of vertebrates.

3.3. Slow waves

The crayfish brain structure and architecture are completely different from those of vertebrates. In those animals there are no cortices or cortex-like structures, nuclei or an organization that resembles the vertebrate brain. The crayfish brain results from the fusion of three main ganglia, protocerebrum, deutocerebrum and tritocerebrum, and the neuropiles have multiple arborizations and interconnections (63; 64). The electrical activity recorded from the brain is comprised by many spikes that mask the underlying synaptic activity. In contrast, the electrical activity from the vertebrate brain is mainly the result of multiple synaptic interactions. These differences result in a non-stationary activity for the crayfish brain and, therefore, the Fourier Transform is not the most suitable technique to analyze it.

Because those characteristics of the crayfish brain, we used an alternative method to the Fourier Transform to analyze its electrical activity, the Wavelet Transform. With this transform we avoided the non-stationarity problem and studied the changes in the spectrum of the brain waves. Our analysis showed that during sleep there is a shift in the dominant frequencies, from 35-45 Hz in the alert animal to 15-20 Hz, and a decrease of power in the 30-45 Hz band (Figure 5; 65, 66). Thus, the electrical activity of the sleeping crayfish brain is the result of a unique state at this time, completely different from that at rest and qualifying for the status of "real sleep".

When an animal takes a recumbent position, its brain electrical activity shows mostly slow waves of 15-20 Hz that start after the position change and last until few seconds before standing up. This is shown in Figure 5, where the results of the Wavelet Transform of records from the deutocerebrum are shown. Figure 5A shows that in an alert animal the dominant power at a frequency around 25 Hz throughout the sampling period. In contrast, in the sleeping animal (Figure 5B) the high power of the 20 Hz frequency lasts only for few seconds, to decrease markedly as soon as the animal goes to sleep.

To determine the origin and dynamics of the slow waves, we then developed a tethered preparation that allowed us to simultaneously record from several identified brain areas. We found that the slow waves are present in the central complex of the brain in both, alert and sleeping animals (Figure 6), and when the temperature of this area is reduced, the whole brain stops oscillating, a change that does not occur when other areas of the brain are chilled (Figure 6D,E,F). These results point to the presence of an oscillator in the central complex of the crayfish brain. When the animal sleeps, the slow wave activity of the central complex of the brain spreads to the rest of the brain, deutocerebrum and then protocerebrum. Tritocerebrum does not seem involved in this oscillatory activity, as it oscillates according to the respiratory (scaphognatites) activity (57).

Altogether, these data show that the crayfish brain is able to produce a state that can be described as sleep, as it fulfills all criteria described for such state in more complex animals. Therefore, it seems that the origin of sleep has to be searched for in animals even simpler than arthropods, perhaps at the level of worms (56).

The fact that it can be shown that arthropods sleep, has profound implications for understanding the neuronal basis of this behavior. Sleep has been demonstrated in animals with an enormous variety of brains, ranging from the very complex brain of mammals and their well developed reticular activating system, thalamus and encephalon (67), through simple vertebrates with only few of those structures (68), and finally to invertebrates with none of them (69). Therefore, it seems that such a complex behavior as sleep is not based on a particular brain region or neuronal circuit, as these have undergone many changes throughout evolution and it would not be expected that specific neuronal changes would have survived. Whether sleep can be generated by a variety of neuronal circuits or it depends on only the most basic elements of a neuronal circuit, such as very few neurons with specific properties, it is unknown.

Our assumption is that sleep is based on only few neurons with some characteristic properties, such as a specific bursting activity, which leads to unexpected predictions. For example, animals whose nervous system is not well organized or it is comprised by only networks without centralized elements, could possess those neurons to regulate their body behavior. In these animals activation of those neurons might not conduce to sleep, as they lack the appropriate target organs, but to a behavior such as what has been defined as 'rest'. This would explain the unexpected observation that cubic medusa (70), whose nervous system is comprised by only a neuronal network without a central ganglia, show activity during the day and 'rest' at night, a rhythmic behavior that resembles in many ways the sleep of other more complex animals.

3.4. Conclusion; future directions

Crayfish is a suitable animal in which to study sleep. Its characteristics from an evolutionary perspective would be useful to found out how this behavior originated and evolved to reach the complexity seen in humans. Besides, future work on circuits, neurotransmitters, receptors, mechanisms of regulation, structures involved, ontogeny, etcetera, might provide clues on the main problem faced by human sleep, why we sleep?

4. ACKNOWLEDGEMENTS

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