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Uncoupling of Brain Activity from Movement Defines Arousal States in *Drosophila*

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Summary

Background: An animal's state of arousal is fundamental to all of its behavior. Arousal is generally ascertained by measures of movement complemented by brain activity recordings, which can provide signatures independently of movement activity. Here we examine the relationships among movement, arousal state, and local field potential (LFP) activity in the *Drosophila* brain.
Results: We have measured the correlation between local field potentials (LFPs) in the brain and overt movements of the fruit fly during different states of arousal, such as spontaneous daytime waking movement, visual arousal, spontaneous night-time movement, and stimulus-induced movement. We found that the correlation strength between brain LFP activity and movement was dependent on behavioral state and, to some extent, on LFP frequency range. Brain activity and movement were uncoupled during the presentation of visual stimuli and also in the course of overnight experiments in the dark. Epochs of low correlation or uncoupling were predictive of increased arousal thresholds even in moving flies and thus define a distinct state of arousal intermediate between sleep and waking in the fruit fly.
Conclusions: These experiments indicate that the relationship between brain LFPs and movement in the fruit fly is dynamic and that the degree of coupling between these two measures of activity defines distinct states of arousal.

Introduction

Brain activity and movement are causally intertwined. Although movements are directed by brain mechanisms, they are also sensed as stimuli, thereby closing a loop between brain and body. Theories of brain evolution have suggested that motility was critical to the evolution of the brain as an anticipation device and that other more sophisticated brain functions such as attention evolved from the predictive mechanisms needed by self-moving creatures [1, 2, 3]. The coordination of attention-like mechanisms among diverse sensory stimuli, including memory, may have given rise to mentation processes dissociated from ongoing movement. Nonetheless, the connection between bodily movement and brain activity is necessarily tight in all animals.

It is often assumed, however, that movement is the only measure of arousal in less-sophisticated animals such as insects [4]. Nevertheless, arousal can be dissociated from movement in predatory arthropods, which can be immobile while stalking prey. Similarly, fruit flies that spend most of their natural life relatively inactive under a leaf are not necessarily sleeping.

Sleep in fruit flies shares key characteristics with sleep in all other animals [5, 6]. In *Drosophila melanogaster*, sleep is homeostatically regulated and is defined by increased arousal thresholds, and molecular changes correlated with sleep mirror similar changes occurring in mammals. Sleep measures, which include rest/activity data, arousal thresholds, and sleep rebound following deprivation, are most often quantified behaviorally by locomotor activity; an awake fly shows a high probability of walking within a 5 min period.

Sleep in *Drosophila* is accompanied by decreased brain activity (10–100 Hz), as measured by local field potentials (LFPs) in the medial protocerebrum (mpc). In the preparation developed for these studies, Nitz et al. [7] defined sleep by lack of fly movement for periods of more than 5 min and an increased arousal threshold. In contrast to the methods of Shaw et al. [6], movement was not monitored in freely moving flies. Instead, flies were tethered by their head and thorax, and movement was monitored by an infrared beam directed across their legs. Alternatively, a wire implanted into their thorax served as a movement detector. By these techniques, gross movements from the fly's wings, legs, and abdomen were monitored continuously to determine sleep states and arousal thresholds, and these were correlated with simultaneous brain activity recordings. The central finding in Nitz et al. [7], that sleep in *Drosophila* is correlated with significantly decreased brain LFP activity, also indicated, by the same token, that waking is correlated with increased brain LFP activity in the mpc. However, waking LFP activity was not well correlated with fly movement on a short time scale; moment-to-moment correlation between brain activity and movement potentials was insignificant and increased only moderately for longer (5 s) correlation bins ($r = 0.2$). This observation was important because it uncoupled the waking levels of brain activity from every movement and suggested that increased brain activity is truly a correlate of waking rather than a correlate of just the movement that accompanies most waking states. Similarly, B.v.S. and R.J.G. [8] showed that 20–30 Hz brain activity in response to visual salience can be uncoupled from flight or movement behavior in the fruit fly. Increased arousal, as measured by increased responsiveness to a visual or mechanical stimulus, can therefore be manifested in the fly brain without necessarily being accompanied by gross behavioral changes. Behavior is usually correlated with states of arousal, especially over circadian time scales, but changes in arousal, as evidenced by neural signatures in the brain, can occur without changes in behavior. In the current study, we explore more closely the dynamic relationship between brain activity, movement, and arousal in the fruit fly *Drosophila melanogaster*. We seek to define how arousal is manifested over short time scales by examining the

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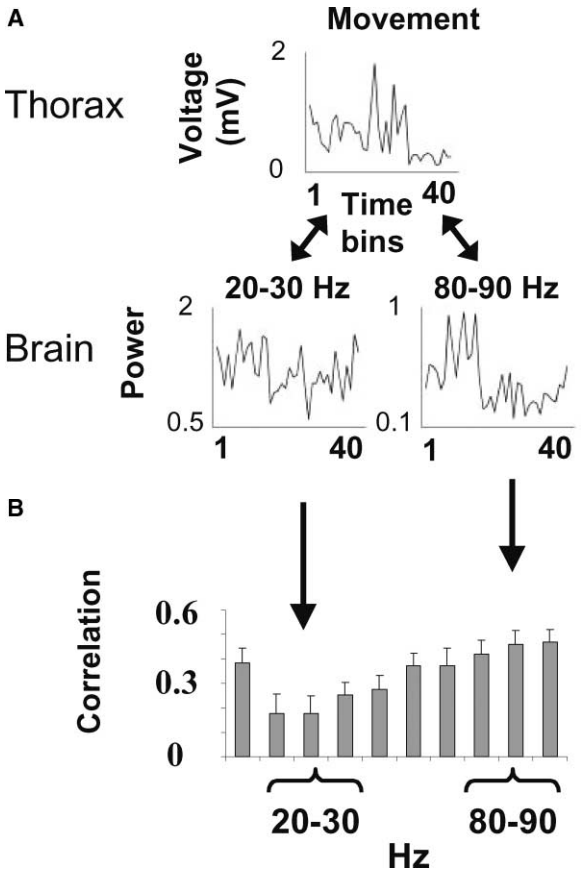

Figure 1. Correlation of Brain Activity with Spontaneous, Waking Movement

(A) 200 s of recording in a sample fly yields forty 5 s bins of averaged movement data. These data are correlated to the corresponding forty 5 s bins of average brain activity power. The data for the 20–30 Hz and the 80–90 Hz range are shown for the same sample fly.

(B) Average correlation (± standard error of the mean) between movement and brain activity for all frequencies 1–100 Hz, in batches of 10 Hz. Brain recordings were performed with electrodes fixed at 75–100 μm depth in the medial protocerebrum (N = 15 flies, 7 with glass electrodes and 8 with silicon. Correlation values were not significantly different for either preparation).

ongoing correlation between two parameters central to describing arousal: brain activity and movement.

Results

LFP-Movement Coupling

We monitored spontaneous fly movement in 5 s bins with an electrode implanted into the thorax (see Experimental Procedures and [7]) and simultaneously recorded brain activity from electrodes inserted 75–100 μm into the medial protocerebrum (mpc) with a reference electrode in the eye. Dye released from the mpc electrode tip showed that this brain-recording position in adult CS females is level with the base of the mushroom bodies, above the esophagus and in the vicinity of the central complex [7]. The simultaneous recordings of spontaneous movement from the thoracic electrode and brain activity from the mpc revealed a correlation profile for this recording position (Figure 1). The correlation level is not equal for all frequencies (1–100 Hz) of brain activity. The higher frequencies (60–100 Hz) are more strongly correlated to movement than the lower frequencies in the 10–50 Hz range (average $r = 0.42 \pm 0.03$ versus 0.22 ± 0.03, respectively, $P = 0.00005$ for the comparison between frequency ranges, $N = 15$ flies). The very lowest frequency range examined, 1–10 Hz, also shows a stronger correlation to movement than the 10–50 Hz bracket does ($P = 0.03$). Among the lower frequencies, the 20–30 Hz bracket shows the lowest correlation to movement during spontaneous, daytime waking activity at this recording position.

Visual Arousal Effects on Coupling

In a separate study of *Drosophila* LFP responses to visual stimuli, we have shown 20–30 Hz brain activity in the fly to be associated with salience effects evoked by novelty, conditioning, and selective discrimination of visual stimuli [8]. In that study, the 20–30 Hz effects were found to be independent of spontaneous, gross movement. Such movement-independent changes in 20–30 Hz activity may account for some of the 10–50 Hz trough in the profile correlating brain activity and movement (Figures 1A and 1B). Two distinct frequency ranges therefore emerge from these results: the lower range centered around 20–30 Hz, which is less correlated with unstimulated, waking movement but which is associated with salience-related arousal, and the higher frequencies, which are more strongly correlated with unstimulated, waking movement but less associated with salience effects [8].

We combined both visual and movement paradigms in order to test the effect of visually induced arousal [8] on the correlation profile coupling brain activity and ongoing movement. We found that introducing a visual stimulus (a rotating dark bar layered onto an unchanged lit background) to the fly uncoupled brain activity from movement (Figure 2). That this uncoupling was most significant in the higher frequency range (60–100 Hz, $P = 0.0057$) was surprising because these higher frequencies are not coupled to the visual response either [8]. Neither low (10–50 Hz) nor high (60–100 Hz) frequencies by themselves changed significantly in average power for the duration of the experiment in comparison to the imageless control, and average movement was unchanged as well. Rather, it appears that visual salience (which has a characteristic 20–30 Hz response [8]) uncouples most brain LFP activity (at this medial recording position) from ongoing movement activity. The correlation level between movement and brain LFP activity appears to depend on the fly's arousal state as well as on the frequency bracket examined. In the following experiments we examine the relationship between the correlation phenotype and arousal more closely by focusing on movement activity and two LFP frequency ranges: 20–30 Hz because of its association to salience effects and 80–90 Hz as a contrasting range that is more correlated to movement.

Overnight Coupling Dynamics

The preceding correlation studies were all performed for short time periods (200 s, or 40 five-second bins)

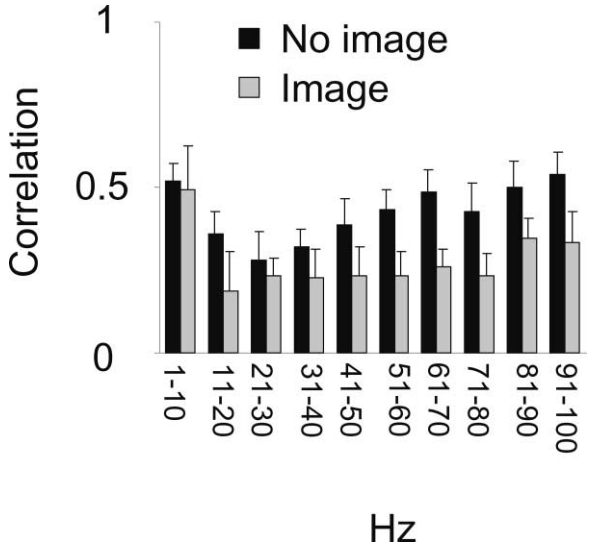

Figure 2. A Visual Stimulus Uncouples Brain LFP Activity from Movement

The correlation profile between LFPs and movement was lower for flies exposed to a moving visual stimulus (gray bars) than for the same flies (N = 6) exposed to the featureless background alone (black bars). Error bars indicate standard error of the mean. Aggregate correlation values (1–100 Hz) were significantly different between conditions (mean correlation without image = 0.42 ± 0.02; with image 0.28 ± 0.03; P = 0.0004).

during the day. In the absence of salient, rotating, visual stimuli, the correlation between brain LFP activity and movement was consistent for these daytime experiments ($r = 0.17 \pm 0.07$ for the 20–30 Hz range, and $r = 0.46 \pm 0.05$ for 80–90 Hz at the middle mpc recording position, N = 15 flies). We questioned whether such consistency persisted throughout much longer recording sessions (12 hr) extending through the animal's night time, when characteristic changes in arousal state are endogenously generated. We have previously shown in overnight experiments that epochs without movement more than 5 min long are associated with decreased power for all frequencies in the brain [7]. However, such immobile epochs do not constitute the majority of a tethered fly's night time behavior, in contrast to the behavior of a freely walking fly [5, 6]. In fact, tethered flies move most of the time, even at night, and will be rendered immobile by sleep for only about 20% of the night, not necessarily contiguously [7]. We investigated whether the correlation profile (between movement and brain LFP activity) changed during spontaneous night time movement.

The correlation was analyzed for six flies kept in sealed and humidified chambers during overnight (12 hr) experiments performed in complete darkness. All animals were still alive and moving in the morning after lights were turned back on. Average movement activity during the first daylight hour after the experiments was not significantly different from pre-experiment levels (0.90 ± 0.35 compared to levels set at 1.0 for the prior daylight session). For each hour of the night, the correlation coefficient between brain activity (20–30 Hz, 80–90 Hz) and movement was calculated from averages of 5 s activity data (data from a sample fly are shown in the left panel of Figure 3A). In all six flies, the correlation between brain activity and movement decreased during several consecutive hours of the night compared to the first two hours of the night (Figure 3A, right panels display collapsed averages). Both frequency ranges showed a proportionally similar decrease in correlation to movement. These decreases did not necessarily occur during the same contiguous hours in all animals. This "correlation trough" was maximal in hours 6–7 after dark for four flies and hours 3–4 for two flies. Average correlation levels increased later in the night (Figure 3A, hours 11–12) to pre-trough levels, before the lights were turned back on.

Average hourly movement and average LFP amplitude at both 20–30 and 80–90 Hz also decrease during the night (Figures 3B and 3C, right panels; cf. [7]), but the decrease of either is not significant during the respective "correlation trough" hours compared to the first two hours of the experiments. Thus, the loss of correlation between brain activity and movement cannot be accounted for by any significant changes in the hourly averages of either brain activity or movement. The epoch in which both average hourly movement and LFP power do attenuate significantly, during the last hours of the night (Figures 3B and 3C, right panels), exhibits a correlation between the two statistics that is as high as pre-trough levels.

Closer inspection of hourly averages through the night reveals that average movement can increase while average brain activity does not (e.g., hours 4–6 for the sample shown in the left panels of Figure 3). Indeed, combined data from all six flies show that the variance in 20–30 Hz activity decreases significantly during the respective trough hours compared to the first hours of the night (0.61 ± 0.09 versus a baseline set at 1, P = 0.007). Variance in 80–90 Hz brain activity was also significantly decreased ($P < 0.05$) during the "correlation trough" hour. In comparison, the variance of movement activity was not significantly decreased during the trough hours compared to hours 1–2. Such unmatched changes in variance may partially explain why a decreased correlation with brain activity is seen during consecutive hours of the night.

Because our records also show evidence of sleep, we investigated the relationship between the LFP/movement correlation dynamics and epochs of extended immobility embedded throughout our overnight records. We found that long bouts of quiescence (>5 min) were immediately preceded by significantly lower levels of correlation to movement (for both 20–30 Hz and 80–90 Hz), compared to the correlation levels seen immediately after the resumption of movement (Table 1). Brain activity and movement are thus more uncoupled immediately before quiescence as well as during contiguous hours of the night.

Arousal Thresholds and Coupling Dynamics

Sleep is typically associated with immobility, but determining sleep in an animal is also contingent on testing arousal thresholds by measuring behavioral responsiveness to a stimulus [4]. Because we now find that

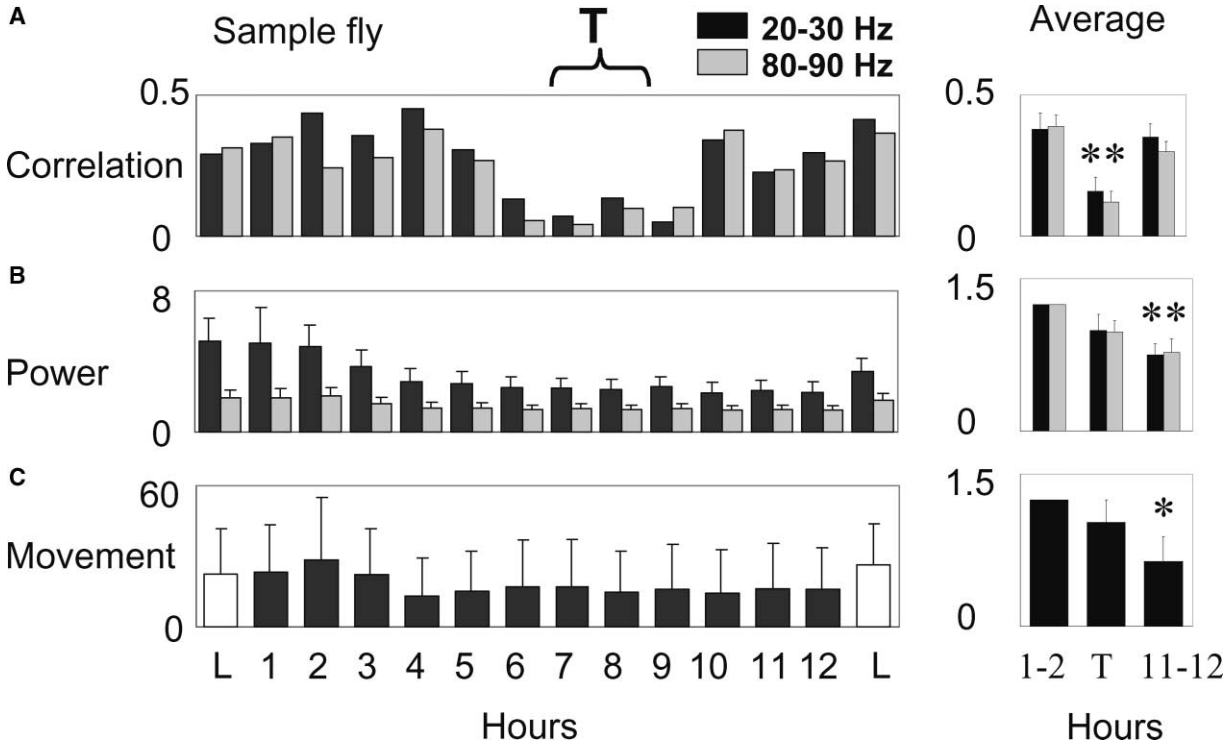

Figure 3. Overnight Coupling Dynamics

Overnight (12 hr) change in correlation between brain activity (20–30 Hz, black bars; 80–90 Hz, gray bars) and movement. Hourly data are shown for a sample fly in the left panels (± standard deviation in [B] and [C]), and averages for all flies (n = 6) are shown in the right panels (± standard error of the mean.). For each fly, two contiguous hours of lower-than-average correlation were found, and these defined the correlation "trough" (T). Averaged data compared the trough values with the correlation values for the first 2 hr of the night, and significance (*, $P < 0.05$) was calculated by a t test. Average values for the last 2 hr were also tested for significance against hours 1–2.

(A) Average correlation between brain activity and movement for the sample individual (left panel) and for all flies (right panel). The first and last hours were recorded with lights on (L) immediately prior and immediately after the 12 hr overnight experiment.

(B) 20–30 Hz and 80–90 Hz activity for the same hours as in (A). In the averaged data, the first 2 hr average defined a baseline against which all subsequent hours were normalized. The "trough" hours are the same as in (A) for each fly.

(C) Average movement activity for the data in A, normalized as in B.

the average correlation between movement and brain activity is dynamic during the night and that sleep is preceded by uncoupling, we questioned whether the loss of correlation between brain and body was similarly associated with altered behavioral responsiveness to a test stimulus, as was shown for sleep [7].

Six additional flies were prepared for testing arousal thresholds throughout the night, as in Nitz et al. [7]. Responsiveness to arousing stimuli (measured by increased movement following mechanical taps or light flashes) was analyzed in terms of the preceding level of correlation between movement and brain activity. Using an automated online paradigm (See Figure 4A and Experimental Procedures), we periodically scanned the thoracic channel for brief epochs (5 s) of immobility before delivering a stimulus, so that all subsequent behavioral responses were compared with this baseline immobility. We found that responsive animals displayed, on average, a greater correlation between movement and 20–30 Hz brain activity during the 3 min preceding the test stimulus (Figure 4B). The level of correlation between 80–90 Hz brain activity and movement was also predictive of responsiveness to the test stimulus (Figure 4C). By the same token, unresponsive flies displayed a significantly decreased correlation between brain activity at both frequencies and ongoing movement in the 3 min preceding the test stimulus.

As in the overnight "correlation troughs" shown in Figure 3, correlation level in these arousal experiments was not dependent on the amount of movement displayed by the animal. Different levels of movement showed similar

Table 1. LFP/Movement Correlations Flanking Quiescence

	20–30 Hz	80–90 Hz
Prequiescence	0.10 ± 0.05	0.11 ± 0.07
Postquiescence	0.48 ± 0.07	0.40 ± 0.07

Epochs of extended quiescence are preceded by significantly lower levels of correlation between brain activity and movement when they are compared to the matching periods that follow extended quiescence ($P = 0.0003$ and $P = 0.01$ by paired t test for 20–30 Hz and 80–90 Hz, respectively. $N = 10$ sleep epochs distributed among eight flies, including data from [7]). Random "islands" of extended quiescence (>5 min), which were flanked by extended periods of movement (>5 min), were sought. Correlation between frequency ranges and movement was determined, as described in the Experimental Procedures, for the 200 s immediately preceding and immediately following the epoch of extended quiescence.

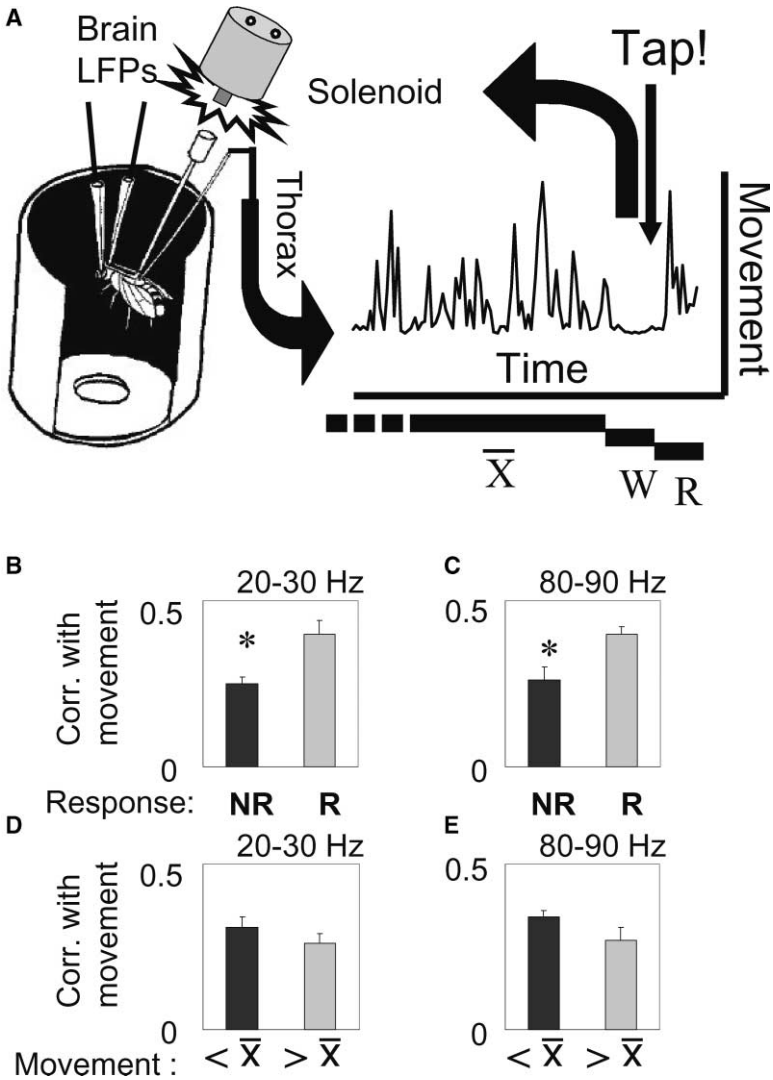

Figure 4. Testing the Effect of Correlation Levels between Brain Activity and Movement on Behavioral Responsiveness

(A) Fly brain LFPs and movement activity were recorded overnight as described (Experimental Procedures). Simultaneously, movements (Thorax) were averaged online (every 5 s) with a LABVIEW platform that continuously monitored the animal's movement and automatically delivered a stimulus. Two types of stimuli were used to test arousal responses as described previously [7]. One was a dim 25 ms light flash, delivered by an automatic shutter. The second was a light tap, delivered by a solenoid, to the metal post used to hold the tungsten loop to which the fly was affixed. Delivery of arousal stimuli (Tap!) was contingent upon a preceding period of inactivity lasting at least 5 s. Such a window (W) for testing arousal was sought for 60 s every 5 min. For analyzing the data, the threshold for arousal was not fixed at one value; rather, it was defined by the average movement shown by the fly in the 3 min preceding the stimulus (Xbar). The data were thus divided into two categories: above average and below average response (R) following the stimulus. Corresponding calculations could therefore be made for other parameters according to these categories.

(B) Arousal samplings (N = 624, distributed fairly evenly among six flies) were placed into a "not responding" category (NR) or a "responding" category (R). A response was detected by movement greater than the set threshold in the 5 s following the stimulus. The two categories of response (NR and R) were compared with respect to the average level of correlation between 20–30 Hz brain activity and movement in the 3 min preceding each stimulus (* = significantly different, $P < 0.05$, by paired t test.

(C) The same analyses performed for 80–90 Hz correlation to movement.

(D–E) Average movement during the 3 min preceding each stimulus was categorized as being below (<) or above (>) the grand mean (Xbar) for movement in these flies. The correlation level between movement and 20–30 Hz (D) or 80–90 Hz (E) brain activity was not significantly dependent on whether prestimulus movement was greater or less than the grand mean. All correlation values are given ± standard error of the mean.

levels of correlation to brain activity when considered irrespectively of responsiveness to stimuli (Figures 4D and 4E). Unresponsiveness to a test stimulus is therefore predicted by a decreased correlation between brain LFPs and movement as well as by prolonged immobility (cf. [7]) in our tethered preparation. These predictors of arousal level are distinct but complementary. As in Nitz et al. [7], unresponsiveness was also associated with less average movement in the minutes preceding a test stimulus because these cases included instances of extended (> 5 min) immobility. The predictive value for arousal level of the correlation between movement and LFPs was significant, however, even when these few cases were removed from our analysis.

In summary, the correspondence between movement and brain LFP activity in the mpc decreases significantly during overnight recordings, and such uncoupling is characterized by increased arousal thresholds. These results demonstrate that ongoing changes in arousal levels in the fly do not necessarily parallel spontaneous movement activity. Rather, changes in arousal are marked by changes in the coupling dynamics between brain activity and movement.

Discussion

By simultaneously monitoring brain LFPs and gross movement in a tethered *Drosophila* preparation, we have demonstrated that brain activity can be uncoupled from the body activity typically used as the measure of arousal state in nonhuman animals [4, 7, 11]. We were thus able to examine arousal in terms of the correlation between two relevant yet distinct measures of fly activity. Our results suggest that ongoing changes in arousal in the fly can be effectively studied as a function of the degree of coupling between brain LFP activity and movement.

In humans and other mammals, most states of heightened arousal (waking, attention), as well as "paradoxical" (REM) sleep, are accompanied by increased high-

frequency (40–80 Hz) field activity in the brain, whereas deep sleep is associated with slow (0.5–4 Hz) field activity [12, 13]. Such brain signatures can be uncorrelated to bodily movement, as in paradoxical sleep and in sleep disorders such as narcolepsy/catalepsy or somnambulism [14], although most sleep is indeed accompanied by quiescence. In fruit flies, extended immobility also correlates with sleep and associated changes in brain activity [5, 6, 7]. Yet, as we have shown here and previously [7, 8], changes in fly brain LFP activity are not always associated with movement on shorter time scales. Because behavioral changes, evidenced by movement of some kind, are the primary way of ascertaining arousal states in nonhuman animals, the relationship between arousal and movement can be difficult to disentangle. Brain activity may be closer to reflecting ongoing changes in arousal in the fruit fly, and the uncoupling between brain activity and movement appears to be a useful indicator of a change in arousal state.

We have shown previously that 20–30 Hz brain activity plays a crucial role in visually directed arousal [8]. In our present study, we find that 20–30 Hz brain activity is less coupled to spontaneous, waking movement than are other frequencies, including the 80–90 Hz range contrasted throughout this study. The higher frequencies (of which 80–90 Hz is just representative) may represent a variety of stimuli coming from the body, whereas the 20–30 Hz signature might represent the fly's version of a "spotlight." In support of this idea, the level of correlation between movement and 20–30 Hz brain activity increased up to 80–90 Hz correlation levels during the initial hours of our overnight experiments (Figure 3A), immediately after epochs of extended quiescence (Table 1), and also throughout our overnight arousal-testing experiments (Figures 4D and 4E). This contrasts with the lower correlation (approximately 0.2) found during the day for the 20–30 Hz range in spontaneously moving flies. We have demonstrated previously that 20–30 Hz activity can be selectively correlated with visual stimuli [8]. For an awake fly in complete darkness at night, when visual (as well as auditory and vibrational) stimuli are lacking, this signal may associate with a different set of stimuli, such as those engendered by the fly's own movement.

The most surprising outcome of our overnight studies is the finding that fruit flies display a distinct behavioral state intermediate between sleep and waking; this state is defined by heightened arousal thresholds and is characterized by the loss of correlation between ongoing movement and LFP activity. In unperturbed flies during the course of overnight experiments, such loss of correlation is consolidated during several contiguous hours of the night. Additionally, periods of low correlation between brain activity and movement immediately precede epochs of extended quiescence. During sleep, already well characterized in this organism [5, 6, 7], animals become immobile, and all brain frequencies attenuate to equal extents [7]. The uncoupled state in moving animals may enable subsequent sleep or may itself accomplish certain key sleep functions. Beyond increased arousal thresholds, both behavioral states are also similar in the uniformity of their effects on the different frequency ranges. In the low-arousal, moving state, correlation to movement is decreased proportionally for both 20–30 Hz and 80–90 Hz frequency ranges (this study), and during sleep, both frequency ranges decrease proportionally in terms of overall power [7]. In contrast, in awake flies, frequencies between 1 and 100 Hz are partitioned by salience effects in the 20–30 Hz range [8] and movement effects in the higher frequencies (e.g., 80–90 Hz, this study). When flies are in either of the two states characterized by increased arousal threshold, there is a corresponding decrease in the variance or amount of information in the entire LFP signal.

Our experiments with visual stimuli show that a form of arousal directed to salient images (evidenced by increased 20–30 Hz response [8]) also uncouples brain activity from movement, even at the higher frequencies not associated with visual salience. This brings up the possibility that during the uncoupled state at night flies may still be partially aroused (as suggested, after all, by their ongoing movement), despite their higher arousal thresholds. This paradox may be partially understood if one considers some common features between sleep and selective attention, both arousal states with behavioral and neural correlates in the fruit fly [6, 7, 8]. Although humans perceive sleep and attention as clearly different states of arousal, both are defined to a certain extent by uncoupling. During sleep, most external stimuli are rendered less accessible, thereby uncoupling the brain from those sensory modalities. During selective attention, an animal may be seen as having partitioned its arousal between a high level directed at the salient stimulus and a low level directed at everything else. The current demonstration of uncoupling between brain activity and movement is consistent with *Drosophila*'s ability to suppress brain responses to simultaneous unattended stimuli [8]. Similarly, we have previously shown that responses to visual stimuli persist in the optic lobes during sleep, whereas the 20–30 Hz response in the medial brain is attenuated [8]. Here, we extend these ideas to propose that such uncoupling is a common feature of different arousal states and that fly brain activity might be uncorrelated from movement at night by a similar mechanism as that which suppresses visual stimuli. Altogether, our findings suggest that arousal states in the fly are a function of the degree of coupling within the nervous system and that changes in arousal can be defined more accurately by such criteria in the fly when considered in conjunction with the standard behavioral measures of responsiveness.

Arousal in *Drosophila*, like consciousness in humans [15], is unlikely to be localized to a unique set of cells in the brain. Rather, arousal probably recruits dynamic networks extending throughout the brain, a phenomenon that may be accessible to a combined genetic and electrophysiological approach in *Drosophila*.

Experimental Procedures

Recordings

Strains, culture conditions, and recording setup were as described previously [7, 8]: one electrode in the left optic lobe, one in the medial protocerebrum (mpc), the head and thorax fixed, appendages able to move. Electrodes were glass or silicon (Center for Neural Communication Technology, University of Michigan, Ann Arbor, MI), at a site approximately 75–100 μm below the top of the head. In order

to draw a correlation between fly movement and brain activity, we monitored spontaneous movements by means of a low-impedance, 25 μm tungsten ground electrode in the thorax [7]. The thoracic signal is a mixture of large deflections induced by body movements (legs, wings, and abdomen), sharper thoracic muscle potentials, and constant noise. We verified its accuracy by videotaping the preparation and observing that increases in thoracic signal amplitude coincided with fly movement, then quantifying this observation by monitoring fly movement continuously with a motion detector (an infrared beam interrupted by leg movement [7]). Simultaneous recordings from the thoracic electrode and the infrared monitor allowed us to correlate thoracic recordings with actual leg movement in several flies ($n = 8$). These were well correlated for 500 s experiments partitioned into 100 bins of 5 s (average $r = 0.39 \pm 0.05$, $P < 0.0001$), although the thoracic electrode was more sensitive to movement than the infrared system. Thus, ongoing changes in movement activity were best monitored by the thoracic electrode for 5 s bins, which is our standard bin size throughout this work.

Data Analysis

Each channel (brain and movement detectors) was sampled at 300 Hz as described previously [7, 8] and partitioned into 5 s bins, and average activity was calculated for each bin offline with MATLAB software as power for all frequencies 1–100 Hz (FFT length = 300, overlap = 150). Frequencies were generally collapsed into 10 Hz bins (e.g., 1–10 Hz, 11–20 Hz, etc.) by summing the power of each of the ten frequency components. Movement activity was calculated as the absolute value for voltage from either the thorax channel or from the infra-red detector [7]. All correlation analyses between brain activity and movement were performed on 5 s bin averages.

Visual Stimuli

Flies were exposed to a lit, featureless panorama for 200 s, as described previously [8]. The same set of flies was subsequently exposed to a dark bar rotating around the flies clockwise at 0.33 Hz for another 200 s, as described previously [8]. Correlation analysis between brain LFP activity and movement was performed for each experiment, as described above, and data were averaged.

Arousal Thresholds

Flies were monitored by the thorax electrode referenced to the optic lobe and quantified online (every 5 s) in LABVIEW, with movement continuously monitored and a stimulus periodically delivered to an inactive fly. "Inactivity" was defined empirically as the baseline of summed voltage in the movement channel when each videotaped fly was motionless. This was usually background noise for the channel, whereas movements produced large voltage deflections (>1.0 mV, often >100 ms). Arousal responses were evoked either by a dim 25 ms light flash from an automatic shutter [7] or by a light tap from a solenoid to the fly's tethering post. A 5 s baseline of inactivity was sought for 60 s every 5 min for testing responses throughout the night. For analysis, the arousal threshold was not fixed at one value but was defined by the average movement in the 3 min preceding the stimulus. The data were thus categorized as above or below average movement following the stimulus for all subsequent calculations.

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