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Electrophysiological Correlates of Rest and Activity in *Drosophila melanogaster*

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Summary

Extended periods of rest in *Drosophila melanogaster* resemble mammalian sleep states in that they are characterized by heightened arousal thresholds and specific alterations in gene expression [1, 2]. Defined as inactivity periods spanning 5 or more min, amounts of this sleep-like state are, as in mammals, sensitive to prior amounts of waking activity, time of day, and pharmacological intervention [1–3]. Clearly recognizable changes in the pattern and amount of brain electrical activity accompany changes in motor activity and arousal thresholds originally used to identify mammalian sleeping behavior [4–6]. Electroencephalograms (EEGs) and/or local field potentials (LFPs) are now widely used to quantify sleep state amounts and define types of sleep. Thus, slow-wave sleep (SWS) is characterized by EEG spindles and large-amplitude delta-frequency (0–3.5 Hz) waves. Rapid-eye movement (REM) sleep is characterized by irregular gamma-frequency cortical EEG patterns and rhythmic theta-frequency (5–9 Hz) hippocampal EEG activity [7]. It is unknown whether rest and activity in *Drosophila* are associated with distinct electrophysiological correlates. To address this issue, we monitored motor activity levels and recorded LFPs in the medial brain between the mushroom bodies, structures implicated in the modulation of locomotor activity, of *Drosophila* [8]. The results indicate that LFPs can be reliably recorded from the brains of awake, moving fruit flies, that targeted genetic manipulations can be used to localize sources of LFP activity, and that brain electrical activity of *Drosophila* is reliably correlated with activity state.

Results and Discussion

LFP recordings were made from the differential activity between one electrode placed between the mushroom bodies at approximately the level of the base of the calyces versus one placed in the lamina or medulla of the optic lobes (Figure 1). The most striking and consistent feature of our LFP recordings was the presence of spike-like potentials. Such potentials occurred against a background of low-amplitude, irregular activity. Typically, the potentials were 5–50 ms in duration, 0.05–4.0 mV in amplitude, and tended to occur in bursts. Spike-like potentials were sometimes positive going but were more often negative going (Figure 2). The summed amplitude of all negative potentials was 76% greater than that for positive potentials ($n = 8$). Particular frequency ranges did not typically stand out against others in power spectral analyses of the LFP. Rather, overall power varied as a function of the amount of spike-like activity observed in particular recording segments.

Most LFP spike-like potentials appeared to be generated in the vicinity of the electrode placed in the medial protocerebrum (mpc) as opposed to the electrode placed within the optic lobe. This was shown in two ways: by differential recording and by localized genetic manipulation of neural activity. First, spike-like potentials were much more numerous in differential recordings between mpc and optic lobe placements than in recordings made differentially between the right and left optic lobes. In support of this, integrated power spectral density (3–50 Hz) was, on average, 96% ($\pm 0.22\%$, $n = 5$) higher for mpc-optic lobe recordings than for optic lobe-optic lobe recordings. Second, spike-like potentials were significantly suppressed in experiments during which synaptic transmission of mushroom body neurons was reversibly interrupted in transgenic animals (Figure 3A). These flies expressed a thermolabile dynamin protein, a product of the *shibire* gene (shi^{ts1}) [9, 10] involved in synaptic vesicle endocytosis, targeted to the mushroom bodies by using the *GAL4* system [11] with strain *c309* [12]. *c309* drives expression in a large subset of mushroom body Kenyon cells. In these flies, spike-like potentials were greatly suppressed despite continued movement during heat application (Figures 3 and 4D, $n = 4$). Activity recorded differentially between the right and left optic lobes was not altered under the same conditions (Figure 4D, $n = 3$), further suggesting a central localization of the signal. In a converse experiment, we tested mutant flies in which expression of the shi^{ts1} gene product was targeted to motoneurons by using the *GAL4* strain *D42* [13, 14] (see the Experimental Procedures). The presence of spike-like potentials in the brain of such flies was unaffected by heat-induced paralysis (Figures 3B and 4D, $n = 3$). Control heating experiments on wild-type (CS, $n = 3$) and parental strains (*c309*/+, *D42*/+, *UAS-shibire*/+, $n = 2$ for each) did not cause paralysis or attenuate brain activity (data not shown).

Because flies were tethered but not completely immobilized in our preparation, it was important to demonstrate that LFPs had their origin in brain activity and were not merely an artifact of movement or electromyographic activity. LFPs were found to reflect brain activity by two approaches. First, close visual observation of fly movements and LFP recordings revealed the presence of spike-like activity during short (seconds long) periods of motor inactivity (see the right side of Figure 2B). Point-to-point correlations between the movement recording and rectified LFP were low ($r = 0.04$, $n = 12$), as were correlations between total recorded movement versus integrated LFP spectral power calculated in 5-s segments over long recording periods ($r = 0.19$, $n = 12$).

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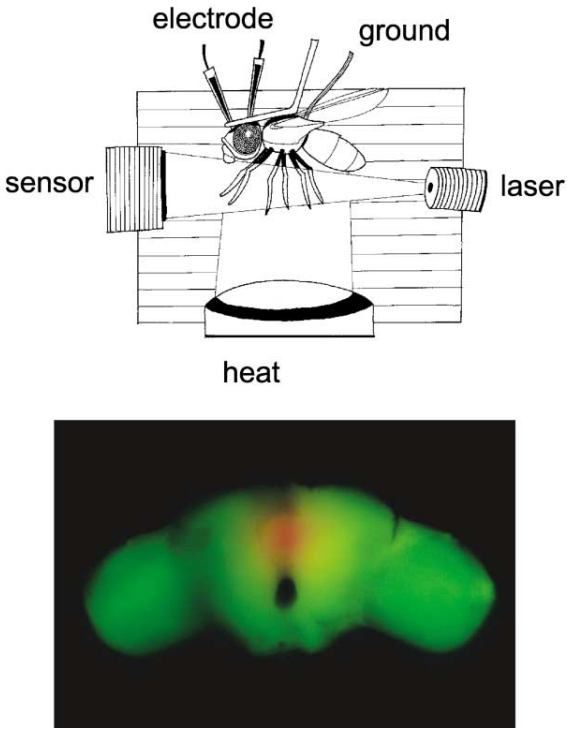

Figure 1. Schematic of Experimental Procedures and Histological Identification of Recording Site

(Top) A diagram of the recording setup. The fly is glued to a wire at the dorsal surface of its head and thorax. An electrode is inserted through the dorsal cuticle of the head to a depth of ~100 μm between the mushroom body calyces. Another is placed in the lamina or medulla of the optic lobes. An infrared laser casts a shadow of the fly's legs and abdomen onto a sensor to record movement, and a heat lamp transiently raises the animal's temperature to 38°C. (Bottom) Whole mount of the brain from a fly viewed from the anterior pole after recording followed by injection of Evans Blue dye through the recording electrode placed in the central brain and processing for fluorescence microscopy (see the Experimental Procedures). The red area corresponds to the dye, the green area corresponds to autofluorescence of the brain (250× magnification). The dye staining is diffuse due to its extracellular mode of injection. The actual recording site corresponds to the point of most intense staining in the middle of the red area.

While greater activity is associated with greater overall LFP amplitude, the relationship is not linear on short (3.33 ms and 5 s) time scales, and movement on such time scales accounts for only a small fraction of the variability of the LFP. Second, the results of experiments employing targeted disruption of synaptic activity (see above) indicate that LFP spiking activity can, under certain circumstances, occur in the complete absence of movement for extended time periods (as in *D42/UAS-shi^{ts1}*) and can also be suppressed for extended time periods despite continuous movement (as in *c309/UAS-shi^{ts1}*) (Figures 3 and 4D).

The classical *Drosophila* mutant affecting spiking activity in the nervous system is *paralytic-temperature-sensitive* (*para^{ts1}*) [15, 16], which becomes immobilized at 32°C due to a mutation in the gene encoding a major voltage-sensitive sodium channel in the fly. The spontaneously occurring LFP of *para^{ts1}* flies at their permissive temperature was like that of wild-type flies. When heat was applied to the animal for a period of 30 s, *para^{ts1}* flies were paralyzed and LFP spiking activity was suppressed or eliminated in all flies tested (n = 5, see Figure 4D). The simultaneously recorded movement detector correctly showed suppressed activity during the heating periods. As expected, reversible suppression of neural activity in *para^{ts1}* flies is associated with suppression of brain spike-like potentials, leaving only a background of low-amplitude, irregular activity (Figure 3C).

Having demonstrated that neuronal activity can be recorded in behaving fruit flies, we sought to determine whether brain activity in *Drosophila* changes during extended periods of quiescence as it does in mammals. To answer this, we made 4- to 12-hr recordings in eight Canton-S wild-type flies and made comparisons between LFPs recorded during active and quiescent periods. Each record was divided into 5-s segments. For each segment, we applied a power spectral analysis to the recorded LFP and integrated total movement (Figure 4A).

We defined extended rest periods as those for which 5 min or more of immobility was observed. This 5-min cutoff was derived from work by Shaw et al. [2], in which periods of quiescence longer than 5 min in duration were clearly associated with heightened thresholds to motor arousal. In mammals, such heightened thresholds to arousal are closely related to the presence of EEG-defined sleep states. We tested the validity of this criterion as applied to our recording paradigm. In two flies, the motor response to automated taps was greatly reduced for trials in which such taps were preceded by 5-min of immobility as compared to trials in which at least 30 s, but not more than 1 min, of immobility preceded the tap. In a second pair of flies, the same result was achieved by using a dim-light flash as the arousing stimulus (Table 1).

From each of the eight extended recording sessions, we were able to find four or more periods of rest greater than 5 min in duration (range 4–31, mean = 11.3 ± 10.4, n = 8). The percentage of recording time spent in such extended rest was 20.64% ± 12.56% across all animals and ranged between 3.4% and 41.2%. The mean duration of rest bouts was 525.5 (±50.5) s. For comparison, duration-matched periods during which animals were active were sampled at random.

As in mammals and birds, periods of extended rest in *Drosophila* were associated with consistent changes in LFP activity (Figure 4). Such periods were associated with decreased incidence of spike-like LFP activity in differential recordings between the mpc and optic lobe. The decreased incidence of spike-like activity was reflected in spectral analyses in which power across all frequencies was reduced during sleep-like periods as compared with active periods (Figures 4B and 4C). Decreases in power averaged 60.5% (range 39.5%–77.6%). Decreased power (25.5% and 35.3%) during extended rest was also observed in two overnight experiments in which differential recordings between the left and right optic lobes were obtained. However, we did not find evidence for the emergence of unique LFP events such as spindles. Instead, these results are more comparable to brainstem unit recordings during SWS in mammals and periods of extended rest in turtles [17]. Extracellular

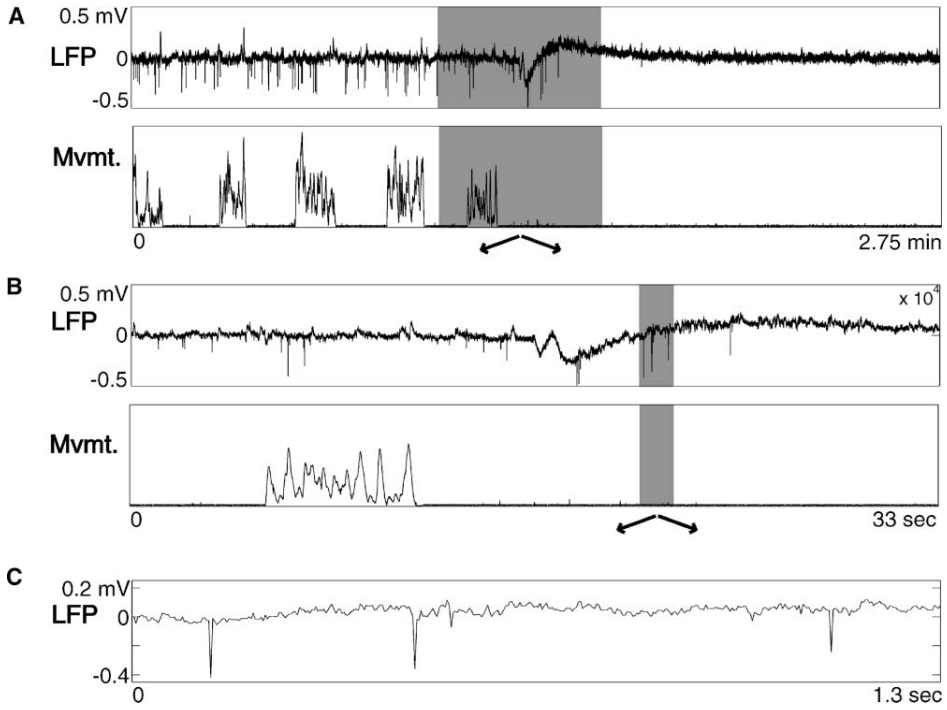

Figure 2. Typical Recording of Raw LFPs and Movement at Different Timescales

(A–C) The time period of recording with the gray background in (A) (2.75 min) is expanded below in (B). The time period with the gray background within (B) is expanded in (C) (movement is not depicted for this expansion). Note that LFP spikes occurred in the presence and absence of movement but were observed more often during time periods associated with frequent movement.

unit recordings in the optic lobes of the honeybee, *Apis mellifera*, also revealed decreased activity in response to visual stimuli, correlated with their nocturnal quiescent period [18].

Notably, the LFP of extended rest periods resembles that of *para*^{ts1} flies during heat-induced suppression of neural activity as well as the heat-induced suppression of mushroom body synaptic transmission in *c309/UAS-shi*^{ts1} flies. Thus, it is possible that the suppression of spike-like potentials during extended rest in flies represents a decrease in neuronal activity in the mpc or a decrease in synaptic transmission from afferents to this region. During SWS in mammals, neuronal discharge in many brain regions is reduced relative to discharge during active waking states. Thus, a common electrophysiological correlate of mammalian SWS sleep and resting states in insects appears to be the occurrence of periods of decreased neural activity. Such interruptions in activity could represent the electrophysiological context necessary for biochemical processes central to the purpose of sleep.

Conclusions

In summary, spontaneously occurring brain activity in the form of LFPs can be reliably recorded from the brains of awake, moving flies. Genetic targeting of a temperature-sensitive mutation eliminating synaptic transmission demonstrated that LFP activity does occur in the absence of movement and can be eliminated in the presence of movement. The heat-induced, reversible suppression of the LFP signal recorded between the mushroom bodies in mutant flies also suggests that localization of LFP sources in other brain regions may be possible to discern by using targeted expression of this sort. Finally, the sleep-like state exhibited by *Drosophila* is indeed accompanied by changes in brain activity as measured by LFPs. The LFPs observed during extended immobility periods, when the arousal threshold is elevated, appear to reflect a generalized decrease in activity inasmuch as it resembles the pattern of activity observed when sodium channel activity is suppressed during heating of *para*^{ts1} flies. The importance of reduced brain activity to the function of sleep-like states in *Drosophila* remains to be determined.

Experimental Procedures

Fly Cultures and Crosses

Flies were cultured at 25°C, 50%–60% humidity, 12hr:12hr light:dark cycle on brewer's yeast, dark corn syrup, and agar food (modified from Bennett and van Dyke [19]). Flies were aged 2–7 days prior to preparation for physiological recording experiments described below. Wild-type flies are from the Canton-S strain. Targeted expression of the temperature-sensitive *shi*^{ts1} mutant form of dynamin was produced by crossing a strain homozygous for insertions of *UAS-shi*^{ts1} on the *X* and *3*rd chromosomes (generously provided by T. Kitamoto, City of Hope) to flies homozygous for the *GAL4* insertion *c309* (generously provided by T. Tully, Cold Spring Harbor Laboratory) or to *D42/TM3* (generously provided by G. Boulianne, Hospital for Sick Children). Expression patterns were confirmed by crossing the *GAL4* strains to *UAS-GFP* (obtained from the Bloomington *Drosophila* Stock Center) and visualizing these strains under fluorescence microscopy. *c309* targets *GAL4* expression to the mushroom

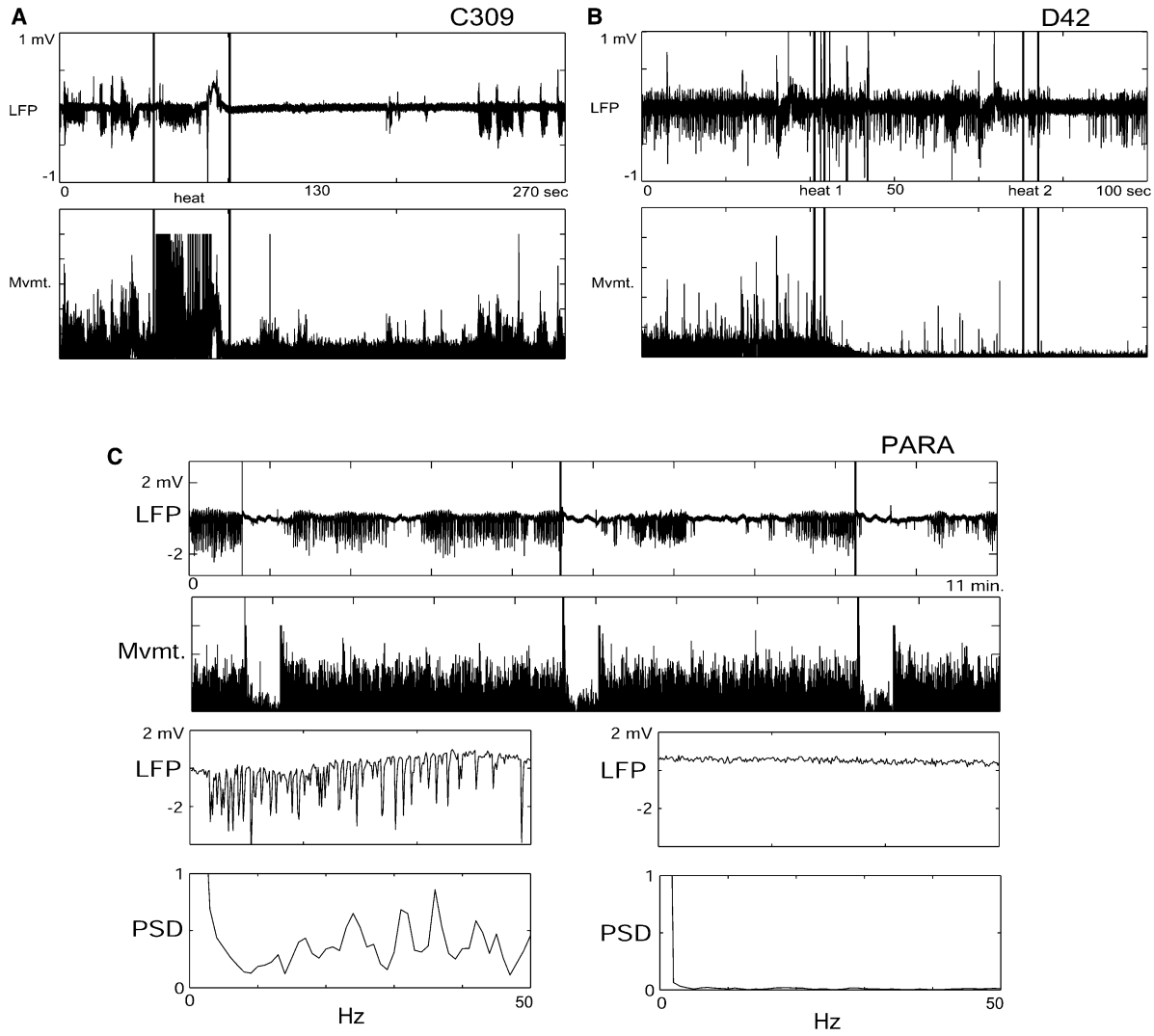

Figure 3. LFP Responses to Heat Application

(A) Raw LFP (central brain versus optic lobe) and movement recording in a *c309/UAS-shi^{ts1}* fly before, during, and after heat application. Application of heat (region of record bracketed by vertical lines) resulted in an initial period of motor activation followed by a period (~75 s) during which LFP spiking activity was suppressed.

(B) Raw LFP and movement recording in a *D429/UAS-shi^{ts1}* fly before, during, and after two separate heat applications. In this genotype, heat application resulted in paralysis; nevertheless, LFP spiking activity persisted.

(C) Raw LFP and movement recording during three separate applications of heat to a *para^{ts1}* mutant. Heat was applied for 30 s at time points indicated by vertical lines. (Below) LFP activity and associated power spectral density for 1-s time periods preceding (left) and subsequent to (right) the second heat application.

bodies in adults [13]. *D42* targets *GAL4* expression to motor neurons in larvae [14] and to thoracic motor neurons and scattered cells in the brain in adults [14]. Mutant *para^{ts1}* flies were obtained from the Bloomington *Drosophila* Stock Center.

Electrophysiology

Flies were first fixed at the dorsal surfaces of the head (between the eyes) and thorax to a loop of tungsten rod [20]. Although flies were unable to move their head or thorax, this procedure left the antennae, limbs, proboscis, wings, and abdomen free to move (Figure 1). Under anesthesia induced by placing flies over a cold block, recording and ground electrodes were then inserted. Recording electrodes were placed under stereotaxic control into the medial protocerebrum (mpc) between the mushroom bodies and into one or both optic lobes. A ground electrode (25 μm tungsten) was placed into the thorax. Cyano-acrylate glue (Loctite) quickly cured by short exposure to ultraviolet light was used to fix all electrodes in place and to the loop of tungsten rod holding the fly.

Recording electrodes were either 12 μm nichrome wire cut flat at the tip or glass electrodes filled with artificial cerebrospinal fluid (ACSF) composed of (in mM) 125 NaCl, 3 KCl, 10 HEPES, 25 $NaHCO_3$, 1 NaH_2PO_4, 10 D-glucose, 2.4 $CaCl_2$, and 1.2 $MgCl_2$. When glass electrodes were used, recordings were made from 12 or 25 μm wire inserted into the ACSF. Resistance between the mpc and optic lobe electrodes was similar for both electrode types (0.2–0.8 MΩ). The recorded waveforms were indistinguishable for the two electrode types.

Signals were fed to a headstage connector containing field effect transistors (FETs) (NBLabs) in which signals were current amplified. Output from the headstage led to a Grass DP-304 amplifier in which

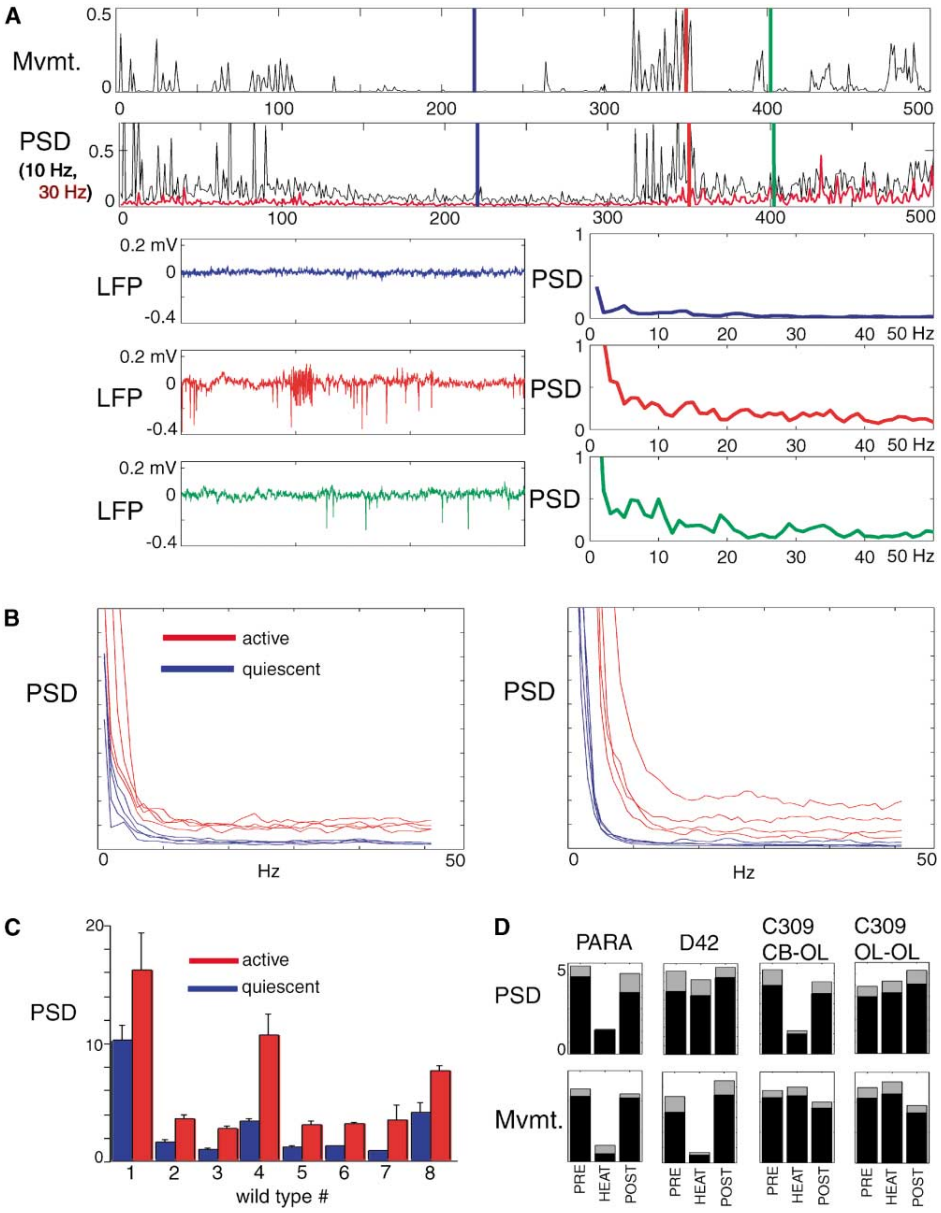

Figure 4. Summary of Comparisons for LFPs during Active and Quiescent States

(A) Depicted in the upper traces of this panel are total movement and power spectral density (PSD) across 500 contiguous 5-s time bins (~40 min). For clarity, only PSD at 10 and 30 Hz is shown. Note that power at each frequency tends to diminish during periods of inactivity. Blue, red, and green vertical lines correspond to 5-s bins for which pairs of LFPs and associated PSDs are depicted in the lower traces. The period of quiescence (blue) was associated with reduced spiking activity and overall PSD as compared with periods of high (red) and moderate (green) motor arousal.

(B) Representative PSDs for four active (red) and four quiescent (blue) time periods for two of the wild-type flies recorded. Quiescence was consistently associated with decreased overall PSD.

(C) Mean (±SEM) integrated power spectral density (3–50 Hz) for active (red) and quiescent (blue) periods for each Canton-S wild-type fly recorded across a behavioral state.

(D) Mean integrated LFP power spectral density (3–50 Hz) and total movement before (pre), during (heat), and after (post) application of heat for *para*^{ts1}, *D42*/*UAS*-shi^{ts1}, and c309/*UAS-shi*^{ts1} mutants. Heat both paralyzed and decreased LFP spectral power in *para*^{ts1} mutants. Heat paralyzed *D42*/*UAS*-shi^{ts1} mutants, but LFP spectral power was not affected. Heat did not produce paralysis in c309/*UAS-shi*^{ts1} mutants and did not affect LFP spectral power in recordings made differentially between the left and right optic lobes. Large decreases in power during heat application were observed in c309/*UAS-shi*^{ts1} mutants when recordings were made between the central brain and optic lobes.

signals were amplified 10,000 times and bandpass filtered between 1 and 100 Hz. Amplifier output was digitized (National Instruments, BNC2090) and then stored at a sampling rate of 300 Hz with custom-built software on a Labview platform. Recordings made for comparison of LFPs across arousal states began 4 hr following electrode implantation and continued for 8–12 hr. With one exception, all flies

Table 1. Mean Responses to Arousing Stimuli

Active	Quiescent	Stimulus	Percent Increase
0.418 (0.09)	0.068 (0.03)	tap	515
0.157 (0.05)	0.016 (0.06)	tap	900
0.359 (0.15)	0.109 (0.02)	light	226
0.133 (0.04)	0.055 (0.04)	light	142
0.267 (0.07)	0.062 (0.02)[a]		330

Mean absolute values (±SE) of baseline-subtracted voltages for the thoracic electrode during 1-s periods following stimuli. "Active" and "quiescent" refer to prestimulus behavioral states. See the Experimental Procedures for details.
[a] $p < 0.05$, $n = 4$.

survived the entire recording period. No data recorded less than 4 hr prior to death was analyzed. Four of the flies survived 6–24 hr after the end of recordings (or 18–36 hr total). LFP recordings and their relation to arousal states from these flies were indistinguishable from those obtained from more short-lived flies.

Motion and Detection

Fly movements were monitored by either of two different methods. For initial experiments, the infrared (IR) laser and sensor system originally devised for monitoring wing beat [21] was aimed laterally in order to detect leg movements (Figure 1). Subsequently, an electrode placed in the animal's thorax was used to monitor vibrations resulting from movement. This signal was quantified as the mean absolute voltage value for 5-s bins. The correlation between both movement detection techniques was high for animals in which both systems were assayed simultaneously ($n = 3$, $r = 0.45, 0.70, 0.82$ for 1-min bins).

Heat Induction

Controlled heating of temperature-sensitive strains was accomplished by a modified version of an apparatus described by Wolf and Heisenberg [20] in which an IR beam is focused on the fly and is regulated by means of a shutter (Figure 1). In our case, the shutter was controlled manually by means of a UniBlitz electronic shutter (Vincent Associates). The beam was produced by a Zeiss 12V 100 W halogen lamp and was filtered through an IR longpass filter (Edmund Scientific). The intensity of the focused infrared beam was fine tuned to the correct temperature by a variable voltage power supply (LEP Products). The temperature of the fly was monitored in two ways: directly by a 25 μm diameter thermocouple placed in the fly's abdomen and read from an Omega Type T Thermocouple Thermometer model 650 (Omega Engineering), and empirically by calibrating the voltage of the light source so that it was just sufficient to paralyze specific mutants with known temperatures of paralysis (*para*^{ts1} and *shi*^{ts1}, see below for references and explanation of mutant phenotypes).

Dye Injection and Histology

Marking the position of the electrode tip was accomplished by filling glass microelectrodes with filtered 2% Evans Blue (Fluka) in ACSF. After recording LFPs, the Evans Blue was iontophoresed into the recording site with a negative current of 2 μA at 1.6V with impedance of 0.8 MΩ for 10 min. The microelectrode was withdrawn with an equivalent positive retaining current. Upon electrode withdrawal, the fly was immediately immersed in 4% paraformaldehyde in 0.1 M phosphate buffer and was fixed overnight at 4°C. CNS dissections were done in PBS + 0.1% Triton X-100, followed by additional fixation in 2% paraformaldehyde + 1.25% glutaraldehyde for 1–2 hr. Brains were dehydrated in an ethanol series with 5 min steps each of 30%, 50%, 70%, 90%, and 100% (2×) ethanol, then coverslipped with DPX. Staining was visualized with both Nomarski optics and FITC fluorescent filters, and photographs were recorded digitally and processed using Adobe PhotoShop 5.0 (Figure 1).

Arousal Thresholds

Four flies were prepared exactly as for electrophysiological recordings. Fly movements were monitored by the thorax electrode referenced to the optic lobe (see above) and quantified online (Labview). A threshold for rest was determined empirically for each fly by finding the baseline values produced by this channel when the videotaped fly was observed not to be moving. Typically, this baseline amounted to the level of background noise for the channel, whereas movements produced large voltage deflections (over 1.0 mV) that often lasted 100 ms or more. Custom-built software in a Labview platform was used to continuously monitor such fly movement and automatically deliver a stimulus if the mean movement was below threshold. Two types of stimuli were used to test arousal responses. The first was a dim 25 ms light flash, delivered in the same manner as heat, described above. 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 was contingent upon a preceding period of inactivity lasting at least 5 s. Such stimuli were followed by up to 10 additional stimuli if no movement responses to the stimuli were detected. Such stimulus "packets" were constrained to be delivered no more than once every 5 min. Quantification of response was made by integrating total movement in poststimulus 1-s bins following subtraction of baseline values. Comparisons were made between responses preceded by long (>5 min) versus short (>30 s, but <1 min) periods of quiescence.

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