Multichannel brain recordings in behaving *Drosophila* reveal oscillatory activity and local coherence in response to sensory stimulation and circuit activation.

Running head: Multichannel brain recordings in behaving flies.

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Abstract

Neural networks in vertebrates exhibit endogenous oscillations that have been associated with functions ranging from sensory processing to locomotion. It remains unclear whether oscillations may play a similar role in the insect brain. We describe a novel “whole-brain” readout for *Drosophila melanogaster*, using a simple multichannel recording preparation to study electrical activity across the brain of flies exposed to different sensory stimuli. We recorded local field potential (LFP) activity from >2000 registered recording sites across the fly brain in >200 wild type and transgenic animals to uncover specific LFP frequency bands that correlate with: 1) brain region; 2) sensory modality (olfactory, visual, or mechanosensory); 3) activity in specific neural circuits. We found endogenous and stimulus-specific oscillations throughout the fly brain. Central (higher order) brain regions exhibited sensory modality-specific increases in power within narrow frequency bands. Conversely, in sensory brain regions such as the optic or antennal lobes, LFP coherence, rather than power, best defined sensory responses across modalities. By transiently activating specific circuits via expression of TrpA1, we found that several circuits in the fly brain modulate LFP power and coherence across brain regions and frequency domains. However, activation of a neuromodulatory octopaminergic circuit specifically increased neuronal coherence in the optic lobes during visual stimulation while decreasing coherence in central brain regions. Our multichannel recording and brain registration approach provides an effective way to track activity simultaneously across the fly brain *in vivo*, allowing investigation of functional roles for oscillations in processing sensory stimuli and modulating behavior.

KEYWORDS: Local field potential, electrophysiology, *Drosophila*, behavior, genetics, coherence, vision, olfaction
INTRODUCTION

Networks of neurons in the mammalian brain produce repeating waves of activity or oscillations (Berger 1929; Buzsáki 2006; Gray 1994). Particular oscillation frequencies have been associated with behavioral function (Bragin et al. 1995; Klimesch 1999) and neural connectivity (Buzsáki et al. 2012; Nunez and Srinivasan 2006). For example, low frequency (< 20 Hz) oscillations are associated with synchronizing activity across longer distances in the mammalian brain, while high frequency oscillations (>30 Hz) are thought to be generated by local neural networks (Buzsáki 2006; Nunez and Srinivasan 2006). The behavioral functions tied to these separate frequency bands include slow-wave sleep, which is associated with low frequency “delta” oscillations (0.5-4 Hz) occurring throughout the brain in mammals and birds (Borbely 2001; Lesku et al. 2011). Middle frequency bands, such as “theta” (5-8 Hz) and “alpha” (9-13 Hz), have been linked to temporal processing (Buzsáki 2002) and inhibition (Jensen et al. 2012), respectively. Higher frequency bands (i.e. “gamma”, 30-90 Hz) have been associated with attention and visual sensory processing in mammals (Buzsáki et al. 2012; Fries et al. 2001; Gray and Singer 1989). An increasing number of studies suggest that oscillations play important roles in controlling action potential firing and timing (Harvey et al. 2009), synaptic homeostasis (Tononi and Cirelli 2003), and organizing distant neurons into functional groups (Fries 2009).

Although correlations between brain oscillations and behavior have been observed across nervous systems from mammals to invertebrates, the function of oscillatory activity in invertebrate brains has been debated (Christensen et al. 2003; Daly et al. 2011; Kirschfeld 1992; Laurent and Naraghi 1994; Martin et al. 2011). In locusts, endogenous oscillations in olfactory neurons are involved in encoding odor cues (Cassenaer and Laurent 2012a; Laurent and Naraghi 1994). In the fruit fly, Drosophila melanogaster, visual salience has been linked to increased 20-30 Hz activity in the brain (van Swinderen and Greenspan 2003), and these oscillations appear to have behavioral relevance for stimulus selection and suppression (Tang and Juusola 2010; van Swinderen 2007). Several studies have demonstrated that endogenous oscillations in insects are induced during sensory stimulation (Christensen et al., 2003; Daly...
et al., 2011), suggesting that oscillations may be a result of transiently induced synchronous network activity in response to stimuli. Given that Drosophila flies display attention-like behavior ((Sareen et al. 2011; Tang and Juusola 2010; van Swinderen and Greenspan 2003), endogenous oscillations in the insect brain may perform similar functional roles for neural processing as in vertebrates.

The majority of past electrophysiological studies in insects involve recordings from single, focal points within the brain, such as intracellular recordings (Maimon et al., 2010; Wilson et al., 2004), so it is unclear whether coherent, endogenous oscillations perform specific roles in behavior and sensory processing in the insect brain. Using a novel whole-brain recording and registration preparation, we find frequency-specific local field potential (LFP) responses to different sensory stimuli. Transient activation of different neuronal circuits modulates oscillation power, while octopamine activity appears to specifically control LFP coherence between different regions of the fly brain. Finally, we show that our multichannel recording paradigm works for walking flies, and find that LFP power and coherence across the fly brain changes significantly in this altered behavioral context.

Materials and Methods

Animals

Female laboratory-reared fruit flies (3-10 days past eclosion; Drosophila melanogaster) were collected under cold anesthesia and positioned for tethering. Flies were glued dorsally to a tungsten rod using dental cement (Coltene Whaledent synergy D6 FLOW A3.5/B3). In addition, dental cement was applied to their necks to stabilize the heads which was cured with blue light (Radii Plus, Henry Scheinn Dental).

Canton-S (CS) flies were used as our wild-type control flies. The following lines were from the Bloomington Drosophila stock center: UAS-mCD8::GFP, Tdc2-Gal4, Gad1-Gal4, Cha-Gal4, c309-Gal4, 7B-Gal4, vGlut-Gal4, and Th-Gal4. Trh-Gal4 was provided by Olga Alekseenko. UAS-TrpA1 was provided by Paul Shaw. GMR-Gal4 was provided by Sean Millard.
Electrode preparation and insertion

We painted the back of a 16-electrode linear silicon probe (model number A1x16-3mm50-177, Neuronexus Technologies) with Texas Red fluorescent dye conjugated to 10000 MW dextran dissolved in distilled water (Invitrogen). The probe was inserted into the flies' eyes laterally, perpendicular to the curvature of the eye, with the aid of a micromanipulator (Fig. 1A,B; Merzhauser). We inserted the probe such that the electrode sites faced posteriorly within the brain. A sharpened fine tungsten wire (0.01 inch, A-M Systems) acted as a reference electrode and was placed superficially in the thorax. Recordings were made using a Tucker-Davis Technologies multichannel data acquisition system at 25 kHz unless otherwise specified (Tucker-Davis Technologies).

Locomotion analysis

Flies were tethered as above and placed on an air-supported ball to observe their walking behavior, prior to probe insertion. The air-supported ball was a 45.5 mg Styrofoam ball (PioneerCraft) painted with a black and red pattern using a Sharpie® pen. The ball was held in a mold made of Plaster of Paris (Prep) and a 50 mL Falcon tube with a small tube providing the air flow leading from the side to the base of the hollow where the ball was placed. Airflow to the ball was approximately 1 L/min, as regulated using an air flow meter (EZI-FLOW).

The flies' movements were filmed using a A602f-2 Basler firewire camera (Basler) at 30 frames per second and a 1-6010 Navitar 12x Zoom lens (Navitar) to magnify the fly seven times. The view of the fly from above allowed us to track the movement of the ball during experiments as the flies walked on the surface of the ball. Following 10-20 minutes of walking, a single 16 channel probe was inserted laterally with the aid of a moveable dissecting microscope and micromanipulator (Merzhauser). The recordings were then performed as listed above for 20 minutes during which we filmed the ball’s movements along with recording the neural data. A small red LED (peak wavelength, 640 nm, Unity Opto...
Technology) was placed in the view of the camera behind the fly and was lit briefly at the start and end of the video to compare the timing of the video with the electrophysiological recordings. The flies were surrounded by a lit white LED arena to improve visibility (Zhou et al., 2012). To compare walking patterns with flies without their heads, flies were tethered, allowed to walk. Their heads were then removed and the neck area was sealed with dental cement. The headless flies were then placed on the ball to walk. See Table 1 for comparisons of locomotion before and after electrode insertion, and comparisons to locomotion in headless flies.

Visual stimuli

The visual stimuli comprised of 270 blue (peak 470 nm, 30 nm half-peak width), green (peak 525 nm, 30 nm half-peak width), and ultraviolet (peak 360 nm, 20 nm half-peak width) light emitting diodes (LEDs) arranged into three 90-LED panels around the flies. Each panel subtended 30 by 34 degrees of the visual field. We flickered all the panels on and off (all panel flicker) at 1, 6, 15, 20, 30, and 70 Hz to test visual responses. The luminance levels of the LEDs were controlled using a stimulator within the recording setup and voltage levels were acquired at 6 kHz along with the brain activity data.

Olfactory and mechanosensory stimuli

An olfactory stimulus controller was used to regulate the air flow to produce both a continuous air stream and a second air stream through which we could inject odorants (Reinhard et al. 2010). Olfactory stimuli were injected into the continuous air stream at 0.6 liters per minute. Three odorants, previously used in Drosophila learning and memory assays (Hallem et al. 2004; Wang et al. 2003), were presented to the flies separately: 3-octanol, ethyl acetate, and isoamyl acetate diluted to 1:10 in ethanol. Each odorant was pipetted onto clean filter paper and placed in a 1 mL syringe through which the humidified air stream passed on the way to the fly. The stimuli consisted of several 1 second pulses of odorants separated by 30 second intervals. Controls included 70% ethanol pipetted onto filter paper and a clean,
odorant free filter paper in a syringe. Mechanosensory airborne stimuli were produced by
turning on and off both the continuous and injected air stream simultaneously.

UAS-TrpA1 activation: behavioral and electrophysiological effects

Behavioral effects of UAS-TrpA1 activation in different Gal4 circuits was examined
by placing at least 15 flies in test tubes and filming their movements as they were heated from
22 to 31°C. Changes in behavior were divided into six main categories: 1) increased
locomotor activity; 2) paralysis, where the flies move very little and are twitching when
examined closely; 3) tremors or shaking while still walking; 4) seizure-like, with high
frequency rhythmic movements producing a near-seizure like state and the flies unable to
walk; 5) wing flicking while still able to walk; 6) abdominal extension while walking. Flies
were then allowed to recover to determine whether these induced behavioral states persisted
after circuit activation. Gal4/+ and Gal4/UAS-TrpA1 were placed in separate tubes side by
side to examine changes in behavior. In all cases, control wild type, Gal4/+ and UAS-
TrpA1/+ flies simply walked faster and moved more quickly in the tubes while heated, unlike
many of the Gal4/UAS-TrpA1 flies, which displayed categories 2-7.

To study the electrophysiological effects of TrpA1 activation, we suspended two flies
side by side above a heat source, which was a 100 W halogen lamp (Zeiss) with an infrared
long wavelength-pass filter (long pass: 800 nm). During each experiment, one genetic control
(whether wild type, Gal4/+, or UAS-TrpA1/+) and one fly which expressed TrpA1
(Gal4/UAS-TrpA1) were placed side by side above the heat lamp. Therefore, control flies
could be compared simultaneously with flies expressing TrpA1 during the same temperature
shifts.

Histology and immunohistochemistry

Fly heads were fixed in 4% paraformaldehyde (Electron Microscopy Services) in a
phosphate buffer solution (PBS). After 8 hours in fixative, they were transferred and washed
in PBS. Brains were embedded in 6% low melting point agarose to be able to align the heads
on the slides, then placed between two coverslips to view the location of the electrode entry
on both eyes (Fig. 1). These alignments were verified by embedding flies (N=10) in agarose,
sectioning sagittally at 100 µm, slide mounting in Vectashield (Vector Labs), and imaging
using an LSM 510 microscope (Zeiss). Of the 220 flies from which we recorded using this
preparation, none died during the recordings. In addition, when we examined the locations of
the probes in the brain, we could rarely see the location of the electrode path, which was
likely due to the fact that it is only 15 µm thick. For this reason, we painted the probe with
the Texas Red fluorescent dye (Invitrogen) and imaged the heads. Without the use of
fluorescent dye, we could not locate the electrode insertion points in the majority of the cases.

To confirm our Gal4 fly lines expressed within specific structures, we crossed the
Gal4 fly lines to UAS-mCD8::GFP and examined the brains of the progeny by performing
immunohistochemistry. Briefly, the tissue was washed with 0.2% Triton-X in PBS with
0.01% sodium azide (Sigma), blocked in 5% bovine serum albumin (Jackson
ImmunoResearch), and washed overnight in a primary antibody solution. The next day, the
brains were washed in PBST and placed overnight in secondary solution. The primary
solution was: 1:250 mouse anti-nc82 (labeling bruchpilot, a synaptic active zone marker;
Wagh et al., 2006; Developmental Studies Hybridoma Bank); 1:500 rabbit anti-GFP
(Invitrogen) in 5% block with PBST and sodium azide. The secondary was: 1:250 goat anti-
mouse Alexa® 647 (Invitrogen); 1:250 goat anti-rabbit Alexa® 488 (Invitrogen). The brains
embedded in Vectashield and imaged using a Zeiss confocal.

Registration of electrode locations in the brain

To examine the location of the electrode sites, we created a standard brain map
registered to the facets of the eyes. This three dimensional map formed the basis for mapping
the location of the linear probe with the 16 electrode sites inserted into the fly eye (Fig. 1).
The method involved outlining each brain region at each z-stack level, moving through the
brain at 1 micron steps, labeled with the presynaptic protein nc82 (Wagh et al. 2006) using the
Art Studio application on an iPad 2 (Apple, Inc.; Fig. 1C). These outlines were merged
across optical sections to form specific brain regions, such as the mushroom bodies (yellow) or lobula (magenta) (Fig. 1C).

To be able to map the inserted electrodes to this three dimensional representation of the brain, the reconstructed brain was aligned to the facets of the two eyes. The facets were pinpointed by detecting the brightest point through a series of confocal stacks of three partially dissected head capsules, where the eyes are kept intact while the brain was exposed. By aligning the facets to the visible brain, we could use the eye as a coordinate system to map electrode locations to specific brain regions. This mapping was made possible since the fly eye is a relatively rigid structure and the electrodes are linear, with each electrode site 50 µm apart. As the insertion sites of the multielectrode probe was easily visible on the eye surface (Fig. 1D), a line from one insertion site on one eye to the exit site on the other eye was mapped. The electrode was estimated to be along this line without dissecting the head capsule and damaging the brain (Fig. 1D). Given that the brain regions could be distinguished as separate volumes based on synaptic staining, we could then assign electrode sites to brain regions (Fig. 1E,F, and see Movie1 for a 3-dimensional model of registered recording sites). To test the accuracy of this mapping system using the eye coordinates, ten fly head capsules were embedded in 8% agarose to allow sagittal sectioning of whole heads (Fig. 1E). These locations were compared with the mapping protocol involving only the eyes (Fig. 1E,F).

Electrophysiological analysis: local field potentials

The electrophysiological data were analyzed as local field potentials (LFPs). The LFPs included data filtered to below 300 Hz and down sampled to 6 kHz. Some channels in the multielectrode were either not in the brain or were blocked by residual tissue. As these channels often had no signal or were dominated by 50 Hz line noise, we did not analyze the resultant data.

To remove movement deflections in the recordings due to muscle potentials, we performed two types of analyses. First, we subtracted one channel from a neighboring channel, called a differential signal. Second, we utilized a method used in
electroencephalogram recordings wherein we performed independent components analysis (ICA) using the fastICA function (Hyvärinen et al. 2001) in the sigTOOL Matlab toolbox (Lidierth 2009; Fig. 1G-I). This approach used ICA to separate the signal common across all the channels from that seen in individual channels (Hyvärinen et al. 2001). We compared the results of these two methods with the baseline LFP by applying the analyses to recordings made in saline while stimulating with a common electrode placed in the bath.

To validate and compare these noise-elimination techniques, we placed the silicon probes in 1M KCl and introduced current pulses ranging from -50 to 50 nA. To remove these current pulses across all channels, we performed both differential and ICA analysis. In the case of differential analysis, subtracting the channels from each other resulted in deflections in the recording due to subtle differences in the electrodes, particularly as each electrode could range in impedance from 0.5-1.5 MΩ due to manufacturing differences of the electrodes (Neuronexus Technologies). ICA, on the other hand, could take into account variation in electrode impedance. ICA was able to remove the shared signal, which resulted in significantly lower variance in the voltage fluctuations (234.0 ± 34.9) than in the baseline voltages (7636.6 ± 821.0 µV; Wilcoxon rank sum test; $\chi^2 = 232.3$; $p = 1.8 \times 10^{-52}$) and in the differential signal (1401.1 ± 373.1 µV; Wilcoxon rank sum test; $\chi^2 = 124.0$; $p = 8.4213 \times 10^{-29}$). However, even though differential subtraction did decrease variance significantly compared to baseline (Wilcoxon rank sum test; $\chi^2 = 95.4$; $p = 1.53 \times 10^{-22}$), we used LFP data which had been analyzed with ICA due to the fact that it removed the majority of the common signal. After performing ICA analysis and filtering the data, we divided it into time bins throughout the recording. We divided the data into 3 second time bins throughout the recording.

Power was calculated using the multitaper method with the use of the Chronux Matlab toolbox (http://chronux.org/; Bokil et al. 2010)). We set the tapers at 3, with a bandwidth of 0.5 Hz. Line noise (50 Hz) was removed by band-pass filtering the LFP to 49-51 Hz, and subtracting this 50 Hz filtered signal from the remaining channels. We normalized the power spectra by dividing each spectrum per electrode recording by the average of the
entire power spectrum from 0 to 255 Hz. Power spectra were converted to dB using the
following formula: power (in dB) = 20*log10(power) to normalize the differences between
low and high frequencies.

Clustering analysis using k-means clustering was performed on the power spectra
data to determine if there was clustering of power spectral domains within the endogenous
brain activity. This algorithm involved combining all channels in 25 wild-type flies and
performing cluster analysis, comparing across each 0.5 Hz frequency step from 0 to 255 Hz.

A second analysis was used to determine whether the oscillations grouped based on amplitude
according to frequency bands per brain region. For both algorithms, the data were clustered
into 2-10 clusters. To determine the optimal cluster number, the distance between points was
calculated by measuring the squared Euclidean distance between each point for within versus
outside the cluster, giving a measure of how well the clusters overlapped.

Coherence between channels within brain structures was calculated to determine the
effects of sensory stimulation and neural circuit activation. Neighboring channels within
neural structures were identified through three dimensional mapping of channels to optic lobe,
antennal lobe, and central brain regions (Fig. 1C-F). Coherence between channels within
these regions was calculated for the same 3 second time windows as was used in power
spectral analysis. Coherence analysis was performed using the following equation:

\[ C_{xy}(f) = \frac{|S_{yx}(f)|}{\sqrt{(S_x(f) \cdot S_y(f))}}; \] where \( S_x(f) \) and \( S_y(f) \) were the multitapered power spectrum
estimates of recordings from channels \( x_n \) and \( y_n \) over time \( t \) and \( S_{yx}(f) \) gave the cross-spectral
power of these two channels during that same time period (Roach and Mathalon 2008).

Electrophysiological analysis: spike detection

To detect spikes, data was filtered to between 300 Hz and 5000 Hz. Spike detection
proceeded by calculating the standard deviation of the entire recording (signal and noise) then
applying a threshold above which any signal is a candidate spike. The peak of the threshold
crossing is found (i.e. the peak of the signal between the upward crossing of the threshold and
the subsequent downward crossing back below threshold). Then, 0.5 ms before and 1 ms after
the peak is extracted and this waveform is added to the list of potential spikes for spike
sorting. While 1-2 units could be clearly identified from 4 standard deviations above or below the mean via traditional spike sorting algorithms in many flies, spikes were not consistently present in all flies. Therefore, we treated spikes as multiunit activity in our data analysis.

Statistical comparisons

Data were tested for normality using the Lilliefors test (Lilliefors 1967). As the majority of the data set was nonparametric, we used the Kruskal-Wallis test for multiple comparisons or the Wilcoxon rank sum test for paired comparisons. In figures and the text, N indicates the number of flies, n indicates number of electrode sites.

RESULTS

LFP recordings reveal distinct oscillation patterns across the fly brain

Local field potential (LFP) recordings represent summed synaptic activity across networks of neurons and often exhibit voltage oscillations in both mammals and invertebrates (Buzsáki et al. 2012; Daly et al. 2011). To determine whether these oscillations correspond to specific functions, such as sensory processing, we examined how neural oscillations varied across brain structures while flies were exposed to sensory stimuli (visual, olfactory, or mechanosensory), and during transient activation of neural circuits in transgenic animals. To detect neural oscillations across multiple brain structures simultaneously, we developed a simple, robust multichannel recording preparation for tethered flies that involves penetrating the fly head from eye to eye with a re-usable, commercially available probe (Neuronexus Technologies; Fig. 1A and B), which did not require dissection or the introduction of foreign solutes. The quasi-linear layout of the fly brain made it possible to map the approximate path of our linear electrode array in 51 wild-type and 169 transgenic flies and genetic controls. This yielded >2000 recordings that could be assigned to distinct neural structures in a three-dimensional model of the brain registered to facet positions on the compound eyes (Fig. 1C-F; Movie 1 (Strausfeld 1976)).
Since all brain recordings are referenced to a common ground electrode in the thorax (Fig. 1A), we removed thoracic and movement artifacts before analyzing brain activity. To do this, we applied a noise elimination method commonly used in human electroencephalograms (EEG), independent component analysis (ICA; Fig. 1G-I; (Brown et al. 2001; Makeig et al. 2004)). In the resultant signal, we could observe clear differences among channels across the fly brain (Fig. 1I). The cleaned signal allowed us to identify two different forms of neural activity, local field potentials (0-100Hz, Fig. 2 A-C), and action potentials, or spikes (300-5000 Hz, Fig. 2 D-F). Our brain registration method revealed decreased spike intervals (or increased spike rates) in the central brain, with the most spiking in the antennal lobes (Fig. 2 G). This observation is consistent with previous studies recording focally from the antennal lobes in Drosophila (Wilson et al. 2004), and shows that our multichannel preparation can be used for comparing regional electrical activity across the fly brain. For the purposes of the current study on oscillations in the fly brain, we focus on LFP activity.

Not all oscillatory activity in the fly brain reflects the summed activity of neurons. One major source of oscillatory activity is the fly’s heartbeat, which beats at approximately 3 Hz (Miller 1950; Robbins et al. 1999). As in all insects, the fly brain surrounds the esophagus and associated muscles, such as muscle 16, which runs through the middle of the brain to pulse haemolymph (insect blood) into the head (Fig. 2 B). As predicted, we observed a 2-4 Hz waveform localized to a small group of central brain channels, corresponding to the fly’s heartbeat (Fig. 2 A). Localization of the 2-4Hz waveforms to a central, ventral location in the head near the esophagus confirmed our electrode registration method (Fig. 2 B), but also allowed us to remove these non-neural oscillations from our analysis. In subsequent analyses, we therefore only examined flies with the heartbeat channels removed, so the remaining LFP activity likely reflected summed neuronal activity in the brain (Fig. 2 C).

We classified our recording sites as belonging to three general areas (Fig. 2 H): the central brain (a higher-order region), antennal lobes (the primary olfactory sensory regions),
and optic lobes (the primary visual sensory areas). We found that the three regions dominated different spectral frequency domains (Fig. 2 H). There was significantly increased power in the higher frequencies above 15 Hz in the antennal lobes compared to the central brain, while central brain channels displayed increased low frequency oscillations (Fig. 2 H). In addition, significant differences between the optic lobes and antennal lobes were within a bounded frequency range (6-40 Hz). There were no significant differences between averaged spectra from the optic lobes and the central brain. These LFP effects suggested the existence of distinct endogenous oscillations across the fly brain, which may serve some functional role.

We used k-means clustering analysis of the LFP power spectrum (0-100 Hz; (Fellous et al. 2004; MacQueen 1967) to determine whether LFP activity exhibited separable oscillations across the fly brain. We found six distinct frequency clusters across the entire brain (Fig. 3 A-C), and a similar set of six clusters was also observed when brain regions were subdivided into three broad regions (central brain, optic lobes, and antennal lobes, Fig. 3D). This analysis therefore allowed us to define six distinct oscillatory domains to better frame our subsequent investigations of LFPs in the fly brain: 0-2 Hz, 2-6 Hz, 6-15 Hz, 15-30 Hz, 30-50 Hz, and 50-100 Hz (see Materials and Methods).

**Sensory stimulation induces global changes in brain activity**

Frequency effects in the optic lobes, antennal lobes, and central brain (Fig. 2H) suggest a functional role for oscillations in processing different sensory stimuli. We investigated whether sensory stimulation could induce regional responses in the fly brain, and whether these could be linked to different sensory modalities. We therefore exposed flies to pulsed visual or olfactory stimuli that should evoke responses in the optic and antennal lobes, respectively, since these are the primary sensory processing regions for visual and olfactory information. To localize these sensory effects to brain regions, we first analyzed the voltage responses in the temporal domain (as evoked potentials; Fig. 4; (Kirschfeld 1992; Kloppenburg and Heinbockel 2000). In response to a 1 Hz flicker of green/blue/ultraviolet LEDs, an on/off response was observed throughout the brain, with a reduced response in the
antennal lobes (N=31; Fig. 4 A and B). Similarly, in response to olfactory stimulation, voltage changes were observed throughout the brain, with the largest olfactory responses in the antennal lobes and central brain regions (N=12; Fig. 4 C-E). Together, these data indicated that: 1) our brain registration technique localized sensory effects to relevant brain tissue, and 2) sensory stimuli induce changes in LFP activity in both peripheral and central brain regions.

We next examined whether sensory stimuli also evoked responses within the distinct frequency bands characteristic of each region that we previously categorized in non-stimulated trials.

**Oscillations are modality-specific**

We found that sensory stimuli altered oscillatory activity across the fly brain, in addition to producing distinct event-related effects. For each sensory manipulation, we compared effects to the baseline activity seen in non-stimulated animals (Fig. 5). More specifically, we found that stimulus-induced oscillations in the central brain reflected the frequency ranges that predominated in the periphery, for each sensory modality. Thus, olfactory stimuli (4 different odors were tested) evoked high frequency activity (30-100 Hz) in the central brain (Fig. 5 A and D), the same broad range that predominated in the antennal lobes in non-stimulated animals (Fig. 2H). Mechanosensory stimulation (in the form of an air stream) also increased power in high frequencies in the central brain, but increased low frequency power in the antennal lobes (Fig. 5B and E). Flickering visual stimuli evoked oscillatory activity in the central brain as well (Fig. 5C and F), predictably centered on the frequency of the stimulus (6 different frequencies were tested). Whereas visual stimuli produced a narrow range of LFP frequency effects in the central brain, effects in the periphery spanned a much broader range of frequencies (Fig. 5F). There appears to be spread of oscillatory effects in the optic and antennal lobes: visual flicker also increased LFP activity (0-100Hz) in the antennal lobes (Fig. 5F), some odors evoked high frequency (50-100 Hz) activity in the optic lobes (Fig. 5D), and air puffs induced oscillations in both the optic and antennal lobes (Fig. 5E).
Oscillations are induced with circuit-specific TrpA1 activation

Oscillations in vertebrates have been associated with specific neural structures (Buzsáki 2002) and often involve local inhibitory feedback networks (Buzsáki and Wang 2012). To test what neural circuits might be involved in generating oscillations in the fly brain, we performed brain recordings while activating distinct cell types. To transiently control activity of different neurons, we used the UAS/Gal4 system (Brand and Perrimon 1993) to restrict expression of TrpA1 channels in different classes of neurons (Fig. 6A).

Heating flies to 31°C (Fig. 6B and Fig. 7A) rapidly increases the excitability of neurons where this cation channel is expressed (Pulver et al. 2009). Since neurotransmitters, such as GABA, and neuromodulators, such as dopamine, have been shown to have a role in neural oscillations in vertebrates (Buzsáki et al. 2012) and invertebrates (Calcagno et al. 2013; Stopfer et al. 1997), we investigated effects of activation of three neurotransmitter circuits (acetylcholine, Cha-GAL4; GABA, GAD1-GAL4; glutamate, vGlut-GAL4) and three neuromodulator circuits (dopamine, Th-GAL4; octopamine, Tdc2-GAL4; serotonin, Trh-GAL4) expressed throughout the brain (Fig. 6A). In addition, we tested three lines that express in central brain structures such as the mushroom bodies (c309-GAL4, 7B-GAL4), antennal lobes (7B-GAL4), and optic lobes (GMR-GAL4). The transient nature of the assay made it possible to compare experiments to baseline and recovery controls.

Transient activation of these neural circuits resulted in a range of both muscle and neural activity changes. Changes in muscle activity were reflected behaviorally, as several of the activated Gal4/UAS-TrpA1 flies displayed distinct actions, such as wing flicking, seizures, or paralysis (Fig. 6B, left columns). Since ICA allowed us to separate neural activity from general muscle activity (see Materials and Methods), we were able to isolate neural oscillations in the LFP voltage traces (Fig. 6B and Fig. 7B), which we could then assign to different frequency domains (Fig. 8A and B).
While it was clear from the genetic controls that mild heat already increased LFP activity (Fig. 8A), these effects were small compared to the effect of TrpA1 activation in Gal4 circuits, which modulated a variety of frequency domains. Activating GABAergic neurons, the major inhibitory system in the insect brain, increased higher frequency oscillations (15-100 Hz) throughout the brain (Fig. 7C and Fig. 8A). Activating glutamatergic neurons also increased high frequency activity (50-100Hz), but only in the optic lobes and central brain (Fig. 8A). In addition, glutamate activity produced high-amplitude slow oscillations (0-6 Hz) in the optic lobes (Fig. 7D and Fig. 8A). More specific 50-100 Hz effects in the central brain were produced by 7B-Gal4, which expresses in the mushroom bodies as well as the antennal lobes (Fig. 7E and F and Fig. 8A), as well as by neuromodulatory circuits such as octopamine and dopamine (Fig. 7G and H and Fig. 8A). Some frequency-specific LFP effects persisted after the heat-induced activation period (e.g. octopamine effects in the optic lobes; Fig. 7G and Fig. 8B). Some neurotransmitter-induced LFP effects were significantly attenuated after transient activation, compared to baseline (Fig. 8B).

How do these induced oscillatory effects correspond with any known functions in the fly brain? Olfaction studies provide some grounding to validate our findings. Two Gal4 circuits would be predicted to induce effects in the antennal lobes, namely GABA and 7B-Gal4, since both have a high level of expression in that region, and GABA has been shown to be involved in producing high frequency oscillations in the antennal lobes of bees (Stopfer et al. 1997). Remarkably, only these two lines increased high-frequency (50-100Hz) activity in the antennal lobes of the fly in our preparation (Fig. 8A, left panel). In contrast, all Gal4 circuits produced some specific oscillatory effect in the optic lobes, both in the high frequency and low frequency range (Fig. 8A, middle panel). This observation was consistent with our previous finding that higher frequency oscillations dominate in the antennal lobes, whereas, in the optic lobes, lower frequency (0-6 Hz) oscillations dominate (Fig. 2H). Furthermore, the contribution of every Gal4 circuit tested to oscillatory effects in the optic lobes matches the fact that all of these Gal4s express to some extent in that broadly defined
region (Fig. 6A; (Kołodziejczyk et al. 2008). One conclusion from our focused screen of 9
different neural networks is that transient activation of any Gal4 circuit is likely to produce
strong oscillatory activity somewhere in the fly brain, but especially in the optic lobes. This
suggests that different patterns of neural oscillations are constantly occurring in behaving
flies, which raises the question of how circuit-specific oscillations might affect
communication across the fly brain during sensory processing.

Coupled oscillatory activity within brain regions increases with sensory input

Since the specific frequency of oscillations in the mammalian brain has been linked to
local versus global communication (Buzsáki et al. 2012), we next examined interactions
between recording sites in the fly brain. Increased oscillatory activity in the central brain as a
result of sensory stimulation (Fig. 5) may result from synchronized neuronal activity in the
peripheral sensory tissues that process these stimuli. LFP oscillations less than 100 Hz are
likely to reflect post-synaptic potentials driven by coherent presynaptic input (Lindén et al.
2010; Ray and Maunsell 2011). Coherence analysis provides a measure of the correlation
between recording sites, in the frequency domain (Roach and Mathalon 2008). In other
words, coherence calculates how oscillations in different channels correlate with one another
within a given window of time. Importantly, coherence is a normalized value from 0 to 1 and
can therefore be used to examine the relationship between channels in the frequency domain
across brain structures independent of estimated power (Roach and Mathalon 2008). For
example, in data from a sample fly (Fig. 9A), the power spectra were similar in two
neighboring channels in the antennal lobes, which was reflected by the high coherence values
between the two channels from 0-100 Hz (Fig. 9B). We also observed high coherence
between neighboring channels in the optic lobes (Fig. 9B), associated with similar power
across frequencies (Fig. 9A). As expected, coherence between more distant channels (e.g.
optic lobe and antennal lobe) was lower than between the neighboring channels within each
structure (Fig. 9B). As sensory processing involves local computation and should affect
coherence within brain regions, we first investigated how sensory stimulation affected coherence within each brain region.

We found that coherence between neighboring channels in primary sensory regions, namely the optic lobes and antennal lobes, increased with modality-specific stimuli. Olfactory stimuli increased coherence from 15-100 Hz specifically in the antennal lobes, the primary olfactory regions of the fly brain, but not elsewhere (Fig. 9C). Mechanosensory stimuli (air puffs) also increased local coherence between channels in the antennal lobes, within a more narrow frequency range (Fig. 9C). A 1 Hz visual flicker increased coherence in the lowest frequencies (0-2 Hz) between neighboring channels in the optic lobes, but nowhere else (Fig. 9C). Coherence between neighboring channels in the central brain remained unaffected by visual, olfactory, or mechanosensory stimuli. Instead the central brain shows modality-specific changes in frequency power (Fig. 5), while the peripheral regions become more internally coherent in the matched frequency domain.

Circuit-based modulation of coherence during 1 Hz flicker

We next asked which neural circuits might be involved in regulating LFP coherence, both in the presence or absence of flickering (1 Hz) visual stimulation. We therefore focused our analyses on effects in the optic lobes and central brain. Activation of GABA neurons (Gad1-Gal4) and photoreceptors (GMR-Gal4) increased high-frequency coherence in the optic lobes (Fig. 9D, first panel from the left). GABA activation also increased coherence in the low frequencies (0-2Hz) in the optic lobe, whereas cholinergic activation (Cha-Gal4) decreased coherence in the same low frequency range (Fig. 9D, first panel). Adding a visual stimulus abolished the effect of GABA activation on local coherence in the optic lobes. Notably, with the 1 Hz visual stimulus, octopaminergic activation (Tdc2-Gal4) increased coherence within the optic lobe in the lower frequency range (0-15 Hz) (Fig. 9D, second panel). In addition, glutamatergic (vGlut-Gal4) and dopaminergic (Th-Gal4) activity decreased coherence within narrowly defined frequency ranges. All of these circuits express
strongly in the optic lobes (Fig. 6A) and primarily form either the input (GMR-GAL4) or are
major neurotransmitter (Cha-Gal4, Gad-Gal4, vGlut-Gal4) or neuromodulatory (Th-Gal4,
Tdc2-Gal4) networks in the visual system, and are therefore likely to be involved in visual
processing (Kolodziejczyk et al. 2008).

Of the nine Gal4 circuits that we tested, only octopaminergic (Tdc2-Gal4) activation
altered coherence levels between neighboring channels in the central brain, decreasing
coherence between neighboring channels, across all frequency domains (Fig. 9D, third panel).
This Tdc2-Gal4 effect on coherence persisted during visual stimulation, albeit only for the
higher frequencies (Fig. 9D, fourth panel). These results show that LFP coherence can change
within the central brain of the fly, but this seems to be a neuromodulatory effect rather than
sensory-driven. Notably, Tdc2-Gal4 activation has opposite effects on coherence in the optic
lobes and central brain when flies are presented with the visual stimulus: coherence increases
in the lower frequency ranges in the optic lobes, while simultaneously decreasing in the
higher frequency ranges in the central brain (Fig. 9D, second and fourth panels). This
suggests that octopamine may be playing crucial role in regulating neural coherence across
the fly brain.

Recording from walking flies

We next recorded from flies positioned on an air-suspended ball, to determine how
LFP power and coherence across the brain might be altered by substrate contact and walking
behavior (Fig. 10 A and B). We first confirmed that inserting the electrode did not
significantly compromise the flies’ ability to walk on the air-suspended ball (Table 1).
Interestingly, we found tarsal contact (walking or not) already dramatically changed brain
activity: LFP power in the optic lobes and the central brain was significantly increased for 15-
30 Hz when flies were placed on a substrate, but was decreased for higher frequencies, 50-
100 Hz (Fig. 10 C, black versus blue lines). For the middle 30-50Hz range, substrate contact
increased LFP activity in the retina and lamina but not in the medulla, lobula, or more central
brain structures (Fig. 10 C). These results point to a functional connection between specific
frequency domains, brain structures, and behavior: contact with a substrate increases power in
the lower frequencies (<50Hz), or alternatively, release from substrate contact increases high-
frequency (50-100Hz) oscillations throughout the brain. We also found a significant effect of
walking behavior: when we separated walking from stationary epochs on the ball (Fig. 10 B),
we found that that walking increased LFP power across most frequency ranges (2-100Hz),
compared to epochs when flies were also contacting the ball but not actively walking
(p<0.001; Fig. 10 C, red versus blue lines).

We next asked whether substrate contact and walking affected LFP coherence in the fly brain. We found that placing flies on a substrate significantly altered coherence between channels, and that this was brain region dependent (Fig. 10 D and E). Coherence between neighboring channels in the optic lobes increased significantly across all frequency domains when the flies were in contact with the ball (Fig. 10 D). The situation was reversed in the central brain: contact with the substrate decreased coherence between neighboring channels across all frequencies in that brain region (p<0.01; Fig. 10 E). When we compared walking versus stationary epochs for flies on the ball, we found no significant changes in coherence in any frequency domain (Fig. 10 D and E, red versus blue lines). Together, these results suggest that substrate contact produces a major reorganization of brain activity (changes in power and coherence in different brain regions), but active walking only increases LFP power.

DISCUSSION

Oscillations represent synchronized, cyclical patterns of activity of populations of neurons, possibly acting as a way to correlate the output of functional neural networks across the brain (Buzsáki 2006; Buzsáki et al. 2012; Nunez and Srinivasan 2006). Whereas oscillations have been associated with a variety of behavioral states in humans (Klimesch et al. 2007; Nunez and Srinivasan 2006), it is largely through research on models, such as cats and rodents, that hypotheses concerning their function have been tested (Buzsáki and Wang 2012; Fries 2001; Gray and Singer 1989). Key to this research strategy is the assumption that all brains are to a certain extent scale-free (Buzsáki 2006), and that a gamma oscillation (30-
90Hz) in a rat, for example, is similar in function to gamma in a human EEG (Buzsáki and Wang 2012). In the current study, we extend the scale-free hypothesis for oscillations to even smaller brains, and ask whether endogenously generated and behaviorally relevant oscillations are also present in the fruit fly brain, which has only approximately 100,000 neurons (Shimada et al. 2006). We demonstrate that oscillations in statistically distinct frequency bands appear to be generated endogenously, and not only as a consequence of external stimuli (Christensen et al. 2003; Daly et al. 2011; Kirschfeld 1992; Laurent and Naraghi 1994; Tang and Juusola 2010; van Swinderen and Greenspan 2003).

Understanding how the brain controls behavior requires a broad view of the simultaneous activity of populations of neurons, as well as a narrow focus on the activity of single neurons (Chiappe et al. 2010; Maimon et al. 2010; Seelig et al. 2011; Wilson et al. 2004). The development of a novel technique using multichannel LFP recordings across the fly brain in combination with three dimensional mapping of electrode locations opens up Drosophila research tools to a better understanding of brain function across entire neuronal networks. One of the key techniques we used to differentiate neural activity was ICA in combination with multichannel recording. Removal of common noise in all channels by ICA (Brown et al. 2001) allowed us to differentiate types of activity and extrapolate sources of signals across multiple channels, which we could then map to structures in the brain. Importantly, we could record neural activity in transgenic flies that were otherwise paralyzed or epileptic (Fig. 6B). Our multichannel recording therefore provides a valuable new paradigm with which to assess brain function, which can be associated with behavior (Figure 10), but which can also be treated as a “behavior” of the whole brain. Transient activation of neural circuits has become commonplace in Drosophila in order to dissect behaviors such as olfactory learning, sleep, or locomotion. Our results show that such manipulations produce global patterns of activity that can be studied as a complex phenotype. How these brain-wide oscillations modulate fly behavior remains to be seen, but it is clear that transient activation of Gal4 circuits that may seem quite narrowly defined can have far-ranging electrical effects across the entire Drosophila brain.
One caveat to this approach, however, is that the spread of voltage signals may not be
from highly specific locations, as the impedance of individual neural structures can vary
significantly (Zimmerman 1978). Flickering light increases LFP power significantly in the
antennal lobes, although visual responses there are not as strong as in the optic lobes or
central brain. Why are the antennal lobes responding to visual flicker? One possibility is that
we are observing a field effect from the eyes that is contaminating the entire brain. This is
however unlikely for a number of reasons. First, visual LFP effects in the central brain are
much more frequency-specific than in the antennal lobes, arguing for a circuit-level
integration of visual flicker in the central brain but not in feedback to the antennal lobes.
Second, of the six neurotransmitters and neuromodulators tested, only GABAergic activation
altered LFP activity in the antennal lobes, while other Gal4’s only had effects in the optic
lobes. If our LFP effects resulted only from field effect contamination, they would not have
revealed circuit-specific effects in defined regions. One possibility for the spread of visual
flicker effects to the antennal lobes could be feedback from the mushroom bodies (which
respond strongly to visual flicker in our paradigm), and for which there is neuroanatomical
evidence (Hu et al. 2010). However, higher resolution electrodes combined with careful
impedance measurements in the brain could help improve both the reliability and resolution
of detecting electrical activity, and will also allow these methods to be applied to tracking
individual unit activity in the fly brain through time. Future studies using these tools in
combination with the techniques presented here will help further determine the functional role
of these oscillatory patterns in different brain regions, and how these might relate to the firing
patterns of individual neurons in the fly brain (Fig. 2).

The distinct oscillatory patterns in the fly brain found in this study (0-2 Hz, 2-6 Hz, 6-
15 Hz, 15-30 Hz, 30-50 Hz, and 50-100 Hz) were uncovered by statistically separating
frequency domains. These analyses measure which frequencies group together across multiple
trials, and therefore are not identified only as separable peaks in a frequency power spectrum
(which is how human delta or alpha were first identified, for example; (Berger 1929; Buzsáki
2006). Although we are not proposing that the frequency domains are functionally equivalent
between flies and mammals, the similarity of the frequency bands to the subdivisions documented in mammalian brains is surprising in light of the enormous differences in brain architecture and size. However, scale-free similarities in the segregation of frequency effects in the brain may not be surprising considering that neural oscillations at high frequencies are thought to be produced through local circuit dynamics mediated by GABA in both vertebrates and invertebrates (Buzsáki and Wang 2012; Stopfer et al. 1997). Indeed, GABAergic activation increased high frequency activity in all brain regions in the fly. In addition, coherence at high frequencies also increased in the optic lobes with GABAergic activation, providing further support that this inhibitory neurotransmitter could be key to producing the high frequency oscillations required for local sensory processing in the fly.

Beyond the presence of endogenous oscillations which could be further induced with TrpA1 activation, we found that sensory input, such as odor cues, can induce frequency band specific changes in LFP activity. Our finding that higher frequencies predominate during olfactory stimulation in the antennal lobes is consistent with previous studies reporting such oscillatory activity in that region when recorded independently (Prieto-Godino and de Polavieja 2010; Tanaka et al. 2009). Responses in the central brain appear to follow these frequency and modality-specific inputs, as has already been documented in larger insects, such as locusts and bees (Cassenaer and Laurent 2012b; Denker et al. 2010; Laurent and Naraghi 1994). In locusts, a 20-30 Hz oscillation initiated in feedback circuits of the antennal lobes is perpetuated by feed-forward circuits to the mushroom bodies in the central brain (Cassenaer and Laurent 2012a). Oscillatory activity in the mushroom bodies, in this olfactory-driven frequency domain, is then thought to be involved in tuning the precise timing of action potentials involved in synaptic plasticity mechanisms (Cassenaer and Laurent 2007). Our high-throughput preparation in the vastly smaller Drosophila brain captures a similar frequency effect from the periphery to the center for olfaction.

Visual flicker produces increased power of oscillations in the optic lobes as might have been expected (van Swinderen 2012), but surprisingly, these effects are more narrowly defined in the central brain. Thus, a visual flicker that significantly increased LFP activity
across most frequency domains in the periphery selectively increased power at the flicker frequency in the central brain of the fly (Fig. 5). This result was surprising, particularly since the visual response could be producing a field effect, with the visual response effects permeating through most of the brain (Fig. 4; (Weckström and Laughlin 2010; Zimmerman 1978)). Therefore, one would predict, based on the size of the optic lobes and the passage of current into the fly brain, that the central brain would produce similar changes in power as seen in the antennal lobes and optic lobes (Fig. 5). Yet, the specificity of the changes in power at certain frequencies in the central brain indicated that there are localized circuit effects which are not just the result of spread of current through extracellular space (Weckström and Laughlin 2010; Zimmerman 1978). Instead, changes in power centrally could be due to local computations within the fly brain, such as synchronized, frequency-specific inputs from sensory regions in the fly brain, or local neuromodulatory effects. The increased coherence in the periphery which correlated with the increased power centrally indicated to us that we could be recording the pre- and postsynaptic interactions between the periphery and the central brain (Fig. 9), giving us a glimpse of how multiple levels of the brain process, integrate, and produce responses to sensory cues simultaneously. Therefore, visual frequency-specific responses in the fly central brain promises to be a valuable approach for future studies using visual flicker to disambiguate brain-wide effects in response to competing sensory cues in visual attention studies (van Swinderen 2012). We have shown that it is possible to apply the multichannel recording preparation to behavioral experiments involving walking flies. This application will allow future studies to effectively connect our whole-brain phenotypes to behavior. Of particular interest for visual attention studies, for example, will be closed-loop walking paradigms, where fixation of visual stimuli should be possible together with multichannel brain recordings in flies. Although for technical reasons we did not manipulate neural activity of walking flies in this study, thermo- or optogenetic manipulation should in principle also be possible in this non-suspended context.

In conclusion, our multichannel brain-recording paradigm for *Drosophila* reveals a variety of oscillatory effects across the fly brain, as a consequence of sensory stimulation as...
well as of transient circuit activation. Sensory stimulation induces coupled, coherent oscillations in local circuits in the periphery, such as the antennal lobes or optic lobes. Coherent activity in the periphery correlates with increased power in central brain structures, within the modality-specific frequency range. Whereas a variety of neurotransmitter, neuromodulator, and other neural systems can alter the amplitude of oscillations in the central brain, octopaminergic activity appears to be involved in regulating local coherence within the central brain, which may be consistent with a central role for octopamine in regulating arousal in *Drosophila* (Crocker and Sehgal 2008) and in mediating sensory responses (Suver et al. 2012). It is possible that monoaminergic activity in general controls the oscillatory timing (or phase) across the central fly brain, to thereby regulate which sensory stimuli are attended to behaviorally. Finally, it is clear that the behavioral context, such as substrate contact, can produce as important changes in oscillatory activity and coherence across the fly brain as the transient manipulation of distinct neural circuits such as octopamine.
References


Table 1. Behavioral effects of electrode insertion.

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<tr>
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<th>No electrode</th>
<th>with electrode</th>
<th>with head</th>
<th>no head</th>
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<tbody>
<tr>
<td>Average walking bout length (sec)</td>
<td>22.6195 ± 12.6052</td>
<td>11.5064 ± 11.5064</td>
<td>18.2950 ± 6.3972</td>
<td>0.7800 ± 0.1756</td>
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<tr>
<td>Average walking frequency (bouts/sec)</td>
<td>0.0482 ± 0.0039</td>
<td>0.0563 ± 0.0055</td>
<td>0.0519 ± 0.0081</td>
<td>0.0077 ± 0.0020</td>
</tr>
<tr>
<td>Average walking bout activity (summed changes in pixels)</td>
<td>0.0285 ± 0.0039</td>
<td>0.0247 ± 0.0059</td>
<td>0.0311 ± 0.0084</td>
<td>0.0103 ± 0.0025</td>
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p values comparing:

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<tr>
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<th>p=0.5740</th>
<th>p=0.0003</th>
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<tr>
<td>Average walking bout length (sec)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average walking frequency (bouts/sec)</td>
<td>p=0.4102</td>
<td>p=0.0006</td>
</tr>
<tr>
<td>Average walking bout activity (summed changes in pixels)</td>
<td>p=0.6662</td>
<td>p=0.0059</td>
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Table 1. Behavioral effects of electrode insertion. We found no significant difference in average walking bout duration, walking frequency, and walking activity (see Materials and Methods) before and after inserting the electrodes (left columns, N=20). However, removal of the heads did significantly affect walking behavior for all three measures (right columns, N=10). This shows that spontaneous walking behavior depends on brain activity, but also that normal walking behavior is not compromised by the electrode insertion through the brain. Values are averages ± standard error.
Figure Legends

Figure 1. Multichannel recording and mapping across the fly brain. A: Recording from multiple sites in the fly brain with a multichannel silicon probe (Neuronexus), with all channels referenced to a common ground placed in the thorax (red line). B: Frontal dimensions of electrode and fly head. C: Three dimensional mapping of recording locations involved first creating a model brain from optical sections labeled with the synaptic marker, nc82 (Wagh et al., 2006) (top) which was then used to outline brain structures (bottom) to construct a standardized fly brain. D: The electrode entry and exit points (top) are mapped to a three dimensional model of the eye relative to brain structures (bottom). E: Accuracy of the model was checked in sagittally-sectioned fly heads (top panels indicate sample sections, below). White arrowheads show the locations of the electrode in the fly brain, based on Texas Red staining (left) and the mapped brain regions in the model (right). The brain is outlined with white dashed lines. F: The linear recording sites were mapped to the brain (dots) for each fly and combined for wild type flies. The recording sites were then assigned to specific neural structures (colored dots, and see Movie1). G: Left panel, electrode sites (black dots) mapped to a fly brain. Traces: raw activity recorded from all channels at the indicated sites. Bottom trace (blue, G): the summed, weighted independent component signal common to all channels, which was calculated from the individual independent components (H). Blue and green arrows in (G) indicate common muscle spikes which are then removed in the cleaned LFP signal (I) following noise removal by independent component analysis (ICA).

Figure 2. Localizing oscillations and spikes in the fly brain. A: A strong oscillatory signal (red traces) was present in central channels (red dots in model) for some experiments. Dashed line on the model brain indicates the sagittal section level for the sample fly in B). B: In the center of the brain (colored orange) is heart tissue and muscle 16, which runs along the esophagus. The fly head is facing left, and the electrode insertion path can be seen just below
the esophagus. Green is autofluorescence and orange is Texas Red labeling (see Materials and Methods). Scale bar = 100 µm. C: Average power spectrum for all combined channels (N=32 wild-type flies) with and without the heartbeat channels (red and gray, respectively). Inset is a close-up of the low-frequency spectra in the black box shown in C. Shaded areas represent standard error of the mean. Blue dots below the traces indicate significantly different values per frequency (1Hz), \( p<0.001 \), Wilcoxon rank sum test). D: Action potentials. When the signal is filtered from 300 -5000 Hz, spikes can be detected well above 4 standard deviations of the signal background, even after removing the primary independent components.

Recordings were taken from sites indicated on model brain, left. E: Close-up (red box) of spiking activity (arrowheads) from sample fly in D. Scalebar is 200µV. F) These repeatable spike waveforms, when overlaid on one another, can have different shapes and sizes indicating multiple neurons are likely producing these spikes (fly1-fly3, grey, orange, and green waveforms are single spikes, black is the average waveform). In fly 3, two different waveforms are visible (from the same recording site), one labeled in green, another in orange.

G: Average spike intervals (+SEM) for the large, detectable spikes occurring four standard deviations above the mean activity were shorter in the protocerebrum and antennal lobes, compared to other brain regions. The letters a, b, and c represent statistically distinct groups as tested via the Kruskal-Wallis multiple comparison test (\( p<0.001 \)). The locations of the recordings sites are mapped to brain structures in the standard brain (Fig. 1) and are color coded to these regions, as in the color scheme below. H: Average power spectra (+SEM) for three brain regions (central brain, purple; optic lobes, orange; antennal lobes, blue). Inset (gray box) shows a 0-10Hz close-up of the three average spectra. Colored dots indicate significant differences (green dots) between power spectra from the antennal lobes and the central brain (green dots), and between the antennal lobes and optic lobes (blue dots) (\( p<0.01 \); rank-sum test, N=32 wild-type flies). There were no significant differences between the average optic lobe and central brain spectra. al=antennal lobe; cb=central brain; ol=optic lobe.
Figure 3. Cluster analysis delineates frequency bands across power spectra. A: LFP power across the brains of 31 flies (361 total recordings) shows considerable variation, though there were clear trends across frequencies. B: To differentiate frequency bands, we performed iterative k-means clustering algorithms (see Materials and Methods) on all the power spectra where the number of clusters was assigned to 2, 3, 4, 5, 6, 7, 8, 9, or 10 clusters. In (B), every point in (A) was assigned to 6 clusters (represented by the different colors) using k-means cluster analysis. C: After classifying the power as separate clusters, we averaged the frequencies associated with these clustered power values. We found statistically separable bands based on power (represented by the different colors). D: To determine the optimal number of clusters to perform subsequent analyses, we calculated the average Euclidean distance between points within the cluster versus the distance to points outside of a cluster (which we term “cluster distance”). We found that the cluster distance increased up until around 5-6 clusters and leveled off for most brain regions, above 6 clusters. The error also decreased significantly above 5 clusters, indicating that classification of distinct power groups is close to optimal at 6 clusters. Shaded areas indicate standard error.

Figure 4. LFP responses to sensory cues reveal localized effects. A-B: Visual responses to light emitting diode light flashes at 1 Hz can be observed across the brain depending on electrode location (A), which is evident in the average visual responses in each structure (B). C: Olfactory responses, such as to isoamyl acetate, are also localized to brain structures compared to clean air controls (D). These olfactory responses are primarily visible in averaged traces from the antennal lobe and mushroom body and central complex regions (E). N indicates number of flies per preparation. Significance was determined by Kruskal-Wallis multiple comparisons test (*, p<0.001). Shaded area indicates standard errors. cc=central complex; mb=mushroom bodies.
Figure 5. The LFP frequency map across the brain changes with sensory input. A: Olfactory stimuli increase LFP power at high frequencies. B: Air stream stimulation also increases LFP power at high frequencies. C: 1 Hz visual flicker shifts oscillations from high to low frequencies in the central brain. Black line: no sensory input. Orange line: visual flicker. Blue line: odor cue, averaged power across four odorants (see below). Burgundy line: visual flicker. Shaded areas are SEM. All comparisons are between the same flies before and during stimulation. D: After subdividing power into specific frequency bands and brain regions, we observed increased oscillations in all three brain regions with the application of ethanol and 3-octanol, though all four odorants induced increased LFP power in high frequencies in the central brain. E: Air stream stimulation increased high frequency oscillations in the central brain and optic lobes, but increased low frequency oscillations in the antennal lobes. F: Visual flicker at six different frequencies increased power across all brain regions, though there was more frequency specificity in the central brain. Colored boxes indicate significant changes in LFP power within the defined frequency range, compared to unstimulated flies, \( p < 0.01 \), Kruskal-Wallis multiple comparisons test, white boxes = n.s. \( N \) indicates number of flies.

Figure 6. Behavioral and electrophysiological effects of TrpA1 activation in specific neural circuits. A: Expression patterns of nine Gal4 circuits in the brain as visualized by GFP expression (green). The synaptic marker nc82 highlights neuropil structures (magenta). B: Gal4 circuit activation (by increasing the temperature from 22 °C to 31 °C) revealed a series of different behavioral effects, ranging from paralysis to increased locomotion, as evident by filmed analysis of freely-walking flies and genetic controls in tubes (see Materials and Methods). Left column: behavioral paradigm. Flies exhibited a range of behavioral effects, represented by colored boxes. Behavioral effects were also reflected in the raw LFP (an optic lobe channel is shown for all, right). For example, large-scale TrpA1 activation of the
nervous system (i.e. the GABAergic (GAD1) or cholinergic (Cha) systems) results in increased muscle spikes (gray arrowheads, raw trace). Removing muscle spikes with ICA reveals the brain activity associated with circuit activation (ICA). Black vertical scale bar to the right of each set of traces = 300 µV. raw: raw LFP signal; ICA: LFP with main ICA components removed; Black: baseline, 22 °C; Red: activated, 31 °C; Blue: recovery, 22 °C.

Figure 7. TrpA1 activation of neural circuits induces circuit-specific changes in the fly brain. A: Flies were heated to 31°C from 22° for a period of 20 minutes. B: Brain activity was recorded continuously for the period before (black), during (red), and after heating (blue). LFP activity (with main ICA components removed) in a sample Cha-Gal4/UAS-TrpA1 fly is shown. C-H: LFP power spectra show frequency-specific changes with TrpA1 activation, resulting in increased high frequency oscillations with activating GABAergic circuitry (C), low and high frequency oscillations peaks with glutamatergic activation (D), and single oscillatory peaks with mushroom body activation (E). High frequency oscillations also increase in the central brain with 7B-Gal4 activation (F) and with the activation of both octopaminergic (G) and dopaminergic (H) circuits. However, circuit activation of neuromodulators, such as Tdc2-GAL4 and TH-Gal4, induced longer-term effects after TrpA1 activation (blue). Grey boxes indicate close-up views of the power spectra shown in the insets. N is the number of flies. Black: baseline, 22 °C; Red: activated, 31 °C; Blue: recovery, 22 °C. Shaded area represents SEM.

Figure 8. TrpA1 activation of neural circuits induces frequency band-specific changes in the fly brain. A: Comparisons between the heated Gal4/UAS-TrpA1 with baseline brain activity show frequency specific changes with activation of different Gal4 circuits. B: Comparisons between the recovery brain activity at 22°C activity (after heating to 31°C) with baseline shows frequency specific changes for different Gal4 circuits. Note that the color scheme indicating the change in power in A is not the same as in B. Significant changes are colored boxes while white compartments indicate no significant difference, with significance
Figure 9. Coherence effects depend on sensory modality and brain circuit.  

A: Coherence analysis involves correlating oscillations between two channels, such as between neighboring channels in the optic lobes (magenta and red circles) or between neighboring channels in the antennal lobes (blue and teal circles). Power spectra reveal subtle differences between channels in the same brain, such as in the antennal lobes (blue and teal spectra) or in the optic lobes (magenta and red spectra).  

B: Coherence between these channels is higher for neighboring brain channels (red and blue), than between distant brain channels (green, contrasted channels shown in A).  

C: Coherence increased between neighboring channels in the optic lobes with 1 Hz visual stimulation (left) and between neighboring channels in the antennal lobes with olfactory and mechanosensory stimulation (center). Sensory stimulation did not significantly alter coherence within the central brain (right).  

D: TrpA1 activation of 9 Gal4 circuits (see Fig. 6 for GFP labeling) induces changes in coherence between neighboring channels in the optic lobes (left panels) and central brain (right panels), in the absence and presence of visual stimulation. Significant coherence effects are indicated by colored boxes (p<0.001, Wilcoxon rank sum test), while white compartments indicate no significant effects compared to genetic controls. At least 8 flies were tested per strain.

Figure 10. Neural oscillations are altered with substrate contact and walking behavior.  

A: Recording from a fly positioned on an air-supported ball, with a multichannel silicon electrode inserted as in Fig. 1.  

B: Brain activity was recorded with the multichannel electrode as flies walked on an air-supported ball. Fly walking activity was quantified by video-tracking movement of the air-suspended ball (red trace, Δ pixels/s)). This behavioral readout was compared to the LFPs recorded simultaneously across the fly brain (black traces). The 14 recording positions for this experiment are indicated in the brain shown on the left.  

C: Average power (± sem) across different recording sites in the brain of wild-type flies in three
different behavioral contexts (black: suspended, blue: stationary on the ball, red: walking on the ball) for four different frequency ranges (2-15 Hz effects were not different than 15-30Hz). 

D: Average LFP coherence (± sem) between neighboring recording sites within the optic lobes, for the same three behavioral contexts as in C. E: Average LFP coherence (± sem) between neighboring recording sites within the central brain. *, p<0.001, Wilcoxon rank sum test for the indicated comparisons, N=20 walking flies tested compared to N=26 suspended flies.
Figure 1

A. Fly head with labeled structures:
- 15 μm recording electrode array
- Reference electrode

B. Fly head dimensions:
- 123 μm
- 33 μm

C. Section stained with nc82:
- Retina
- Brain
- Proboscis
- Antenna

D. Ocelli and proboscis highlighted:

E. Brain structures outlined:

F. Color-coded brain regions:
- Red: Medulla
- Orange: Retina
- Purple: Mushroom body
- Blue: Antennal lobe
- Pink: Lobula complex

G. Local field potential (0-300 Hz filtered signal):
- Ch 1
- 500 μV

H. Independent components:
- Weighted sum of major independent components

I. Local field potential with major independent components removed:
- 300 μV

N = 25
Figure 3

A

N=31 flies
n=361 recordings

B

100 replicates to separate power into 6 clusters

C

- cluster 1: 1.88 ± 1.05 Hz
- cluster 2: 6.00 ± 1.41 Hz
- cluster 3: 12.25 ± 2.27 Hz
- cluster 4: 23.5 ± 4.29 Hz
- cluster 5: 41.3 ± 6.86 Hz
- cluster 6: 78.75 ± 13.53 Hz

D

- Optic lobes
- Antennal lobes
- Central brain
- Entire brain

Number of clusters vs. cluster distance
Figure 4

A

channels

N=1

B

retina
lamina
medulla
lobula complex
protocerebrum
mb and cc
antennal lobe

N=31

light ON 500 ms
light OFF 500 ms

C

channels

N=1

isoamyl acetate

D

clean air control

E

retina
lamina
medulla
lobula complex
protocerebrum
mb and cc
antennal lobe

N=12

isoamyl acetate

100 μV

500 μV
Figure 5

A. Olfactory N=12
B. Air stream N=12
C. 1 Hz visual flicker N=31

D. Odorant matrix:
- Significant increase compared to unstimulated fly
- Significant decrease compared to unstimulated fly

E. Air stream matrix

F. Light flicker frequency matrix
### Figure 6

#### A

<table>
<thead>
<tr>
<th>Neuron Type</th>
<th>Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine (Cha-GAL4/UAS-GFP)</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>GABA (GAD1-GAL4/UAS-GFP)</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Glutamate (vGlut-GAL4/UAS-GFP)</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>Dopamine (TH-GAL4/UAS-GFP)</td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Octopamine (TDC2-GAL4/UAS-GFP)</td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>Serotonin (TRH-GAL4)/UAS-GFP</td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>Mushroom bodies (c309-GAL4/UAS-GFP)</td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
<tr>
<td>Antennal lobes (7B-GAL4/UAS-GFP)</td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
<tr>
<td>Optic lobes (GMR-GAL4/UAS-GFP)</td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>

#### B

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Behavior during heating</th>
<th>22°C</th>
<th>31°C</th>
<th>22°C</th>
<th>100 msec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Canton S)</td>
<td><img src="graph1.png" alt="Graph" /></td>
<td><img src="graph2.png" alt="Graph" /></td>
<td><img src="graph3.png" alt="Graph" /></td>
<td><img src="graph4.png" alt="Graph" /></td>
<td><img src="graph5.png" alt="Graph" /></td>
</tr>
<tr>
<td>UAS-TrpA1/+</td>
<td><img src="graph6.png" alt="Graph" /></td>
<td><img src="graph7.png" alt="Graph" /></td>
<td><img src="graph8.png" alt="Graph" /></td>
<td><img src="graph9.png" alt="Graph" /></td>
<td><img src="graph10.png" alt="Graph" /></td>
</tr>
<tr>
<td>Acetylcholine (Cha-GAL4/UAS-TrpA1)</td>
<td><img src="graph11.png" alt="Graph" /></td>
<td><img src="graph12.png" alt="Graph" /></td>
<td><img src="graph13.png" alt="Graph" /></td>
<td><img src="graph14.png" alt="Graph" /></td>
<td><img src="graph15.png" alt="Graph" /></td>
</tr>
<tr>
<td>GABA (GAD1-GAL4/UAS-TrpA1)</td>
<td><img src="graph16.png" alt="Graph" /></td>
<td><img src="graph17.png" alt="Graph" /></td>
<td><img src="graph18.png" alt="Graph" /></td>
<td><img src="graph19.png" alt="Graph" /></td>
<td><img src="graph20.png" alt="Graph" /></td>
</tr>
<tr>
<td>Glutamate (vGlut-GAL4/UAS-TrpA1)</td>
<td><img src="graph21.png" alt="Graph" /></td>
<td><img src="graph22.png" alt="Graph" /></td>
<td><img src="graph23.png" alt="Graph" /></td>
<td><img src="graph24.png" alt="Graph" /></td>
<td><img src="graph25.png" alt="Graph" /></td>
</tr>
<tr>
<td>Dopamine (TH-GAL4/UAS-TrpA1)</td>
<td><img src="graph26.png" alt="Graph" /></td>
<td><img src="graph27.png" alt="Graph" /></td>
<td><img src="graph28.png" alt="Graph" /></td>
<td><img src="graph29.png" alt="Graph" /></td>
<td><img src="graph30.png" alt="Graph" /></td>
</tr>
<tr>
<td>Octopamine (TDC2-GAL4/UAS-TrpA1)</td>
<td><img src="graph31.png" alt="Graph" /></td>
<td><img src="graph32.png" alt="Graph" /></td>
<td><img src="graph33.png" alt="Graph" /></td>
<td><img src="graph34.png" alt="Graph" /></td>
<td><img src="graph35.png" alt="Graph" /></td>
</tr>
<tr>
<td>Serotonin (TRH-GAL4)/UAS-TrpA1</td>
<td><img src="graph36.png" alt="Graph" /></td>
<td><img src="graph37.png" alt="Graph" /></td>
<td><img src="graph38.png" alt="Graph" /></td>
<td><img src="graph39.png" alt="Graph" /></td>
<td><img src="graph40.png" alt="Graph" /></td>
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<tr>
<td>Mushroom bodies (c309-GAL4/UAS-TrpA1)</td>
<td><img src="graph41.png" alt="Graph" /></td>
<td><img src="graph42.png" alt="Graph" /></td>
<td><img src="graph43.png" alt="Graph" /></td>
<td><img src="graph44.png" alt="Graph" /></td>
<td><img src="graph45.png" alt="Graph" /></td>
</tr>
<tr>
<td>Antennal lobes (7B-GAL4/UAS-TrpA1)</td>
<td><img src="graph46.png" alt="Graph" /></td>
<td><img src="graph47.png" alt="Graph" /></td>
<td><img src="graph48.png" alt="Graph" /></td>
<td><img src="graph49.png" alt="Graph" /></td>
<td><img src="graph50.png" alt="Graph" /></td>
</tr>
<tr>
<td>Optic lobes (GMR-GAL4/UAS-TrpA1)</td>
<td><img src="graph51.png" alt="Graph" /></td>
<td><img src="graph52.png" alt="Graph" /></td>
<td><img src="graph53.png" alt="Graph" /></td>
<td><img src="graph54.png" alt="Graph" /></td>
<td><img src="graph55.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

**Behavioral categories during heating**

- Increased locomotion
- Paralysis
- Tremors or shaking
- Seizure-like behavior
- Wing flicking
- Abdominal extension
Figure 7

A) Electrode array

B) TrpA1 activation

C) Antennal lobes
   - GAD1-GAL4
   - N=11

D) Optic lobes
   - vGlut-GAL4
   - N=19

E) Central brain
   - 7B-GAL4
   - N=18

F) 7B-GAL4
   - N=8

G) TDC2-GAL4
   - N=9

H) TH-GAL4
   - N=8
Figure 8

A

Strain name
GAL4/+ controls
UAS-TrpA1/+ controls

neurotransmitters
acetylcholine (Cha-GAL4/UAS-TrpA1)
GABA (GAD1-GAL4/UAS-TrpA1)
glutamate (vGlut-GAL4/UAS-TrpA1)
dopamine (TH-GAL4/UAS-TrpA1)
octopamine (TDC2-GAL4/UAS-TrpA1)
serotonin (TRH-GAL4/UAS-TrpA1)
mushroom bodies (c309-GAL4/UAS-TrpA1)
antennal lobes (7B-GAL4/UAS-TrpA1)
optic lobes (GMR-GAL4/UAS-TrpA1)

neuromodulators
dopamine (TH-GAL4/UAS-TrpA1)
serotonin (TRH-GAL4/UAS-TrpA1)

neural structures
antennal lobe optic lobes central brain

B

Strain name
GAL4/+ controls
UAS-TrpA1/+ controls

neurotransmitters
acetylcholine (Cha-GAL4/UAS-TrpA1)
GABA (GAD1-GAL4/UAS-TrpA1)
glutamate (vGlut-GAL4/UAS-TrpA1)
dopamine (TH-GAL4/UAS-TrpA1)
octopamine (TDC2-GAL4/UAS-TrpA1)
serotonin (TRH-GAL4/UAS-TrpA1)
mushroom bodies (c309-GAL4/UAS-TrpA1)
antennal lobes (7B-GAL4/UAS-TrpA1)
optic lobes (GMR-GAL4/UAS-TrpA1)

neuromodulators
dopamine (TH-GAL4/UAS-TrpA1)
serotonin (TRH-GAL4/UAS-TrpA1)

neural structures
antennal lobe optic lobes central brain
Figure 9

A

B

C

D

![Graph showing power distribution](image)

**Figure 9**

**A**

Power (dB) vs frequency (Hz) for different conditions.

**B**

Coherence between channels for different frequency bands.

**C**

Comparative coherence matrices for different conditions.

**D**

Matrix showing differences in coherence between neighboring channels for different strains and conditions.
Figure 10

A walking fly tethered to an air-supported ball

B walking activity (change in pixels)

C

<table>
<thead>
<tr>
<th>Power</th>
<th>0-2 Hz</th>
<th>15-30 Hz</th>
<th>30-50 Hz</th>
<th>50-100 Hz</th>
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<tbody>
<tr>
<td>retina</td>
<td>n.s.</td>
<td>*</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>lamina</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>medulla</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>lobula complex</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>protocerebrum</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>MB and CC</td>
<td>*</td>
<td>*</td>
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</table>

D Coherence

<table>
<thead>
<tr>
<th>Coherence</th>
<th>0.55</th>
<th>0.95</th>
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</thead>
<tbody>
<tr>
<td>optic lobe vs optic lobe, neighboring channels</td>
<td>*</td>
<td>***</td>
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</tbody>
</table>

E central brain versus central brain neighboring channels

<table>
<thead>
<tr>
<th>Coherence</th>
<th>0.55</th>
<th>0.95</th>
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</thead>
<tbody>
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<td>central brain vs central brain, neighboring channels</td>
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