

Multivariate classification of multichannel long-term electrophysiology data identifies different sleep stages in fruit flies

Sridhar R. Jagannathan^{1*,2†}, Rhiannon Jeans^{3†}, Matthew N. Van De Poll³, Bruno van Swinderen^{3*}

¹Department of Psychology, University of Cambridge, Cambridge, United Kingdom

²Institute of Neurophysiology, Charité Universitätsmedizin Berlin, Berlin, Germany

³Queensland Brain Institute, The University of Queensland, St Lucia, Australia

†These authors contributed equally to this work

*Corresponding authors: sridhar-rajan.jagannathan@charite.de, b.vanswinderen@uq.edu.au

ABSTRACT

Sleep is observed in most animals, which suggests it subserves a fundamental process associated with adaptive biological functions. However, the evidence to directly associate sleep with a specific function is lacking, in part because sleep is not a single process in many animals. In humans and other mammals, different sleep stages have traditionally been identified using electroencephalograms (EEGs), but such an approach is not feasible in different animals such as insects. Here, we perform long-term multichannel local field potential (LFP) recordings in the brains of behaving flies undergoing spontaneous sleep bouts. We developed protocols to allow for consistent spatial recordings of LFPs across multiple flies, allowing us to compare the LFP activity across awake and sleep periods and further compare the same to induced sleep. Using machine learning, we uncover the existence of distinct temporal stages of sleep and explore the associated spatial and spectral features across the fly brain. Further, we analyze the electrophysiological correlates of micro-behaviours associated with certain sleep stages. We confirm the existence of a distinct sleep stage associated with rhythmic proboscis extensions and show that spectral features of this sleep-related behavior differ significantly from those

27 associated with the same behavior during wakefulness, indicating a dissociation between behavior and
28 the brain states wherein these behaviors reside.

29

30

31 **Introduction**

32 Humans spend a third of their life engaged in sleep, wherein they become less responsive to external
33 stimuli. Most animals studied so far, starting from the tiny fruit fly to the large sperm whale (Miller et
34 al. 2008) display extended periods of quiescence, which are now categorized as sleep. Evolutionary
35 conservation of the sleep state in all animals suggests that its benefits outweigh the potential risks and
36 vulnerabilities brought on by losing awareness of one's external environment. Sleep deprivation has
37 been shown to produce deficits in learning and memory (Rasch and Born 2013), immune system
38 malfunction (Besedovsky, Lange, and Born 2012) and stress regulation (Paul J. Shaw et al. 2002).
39 However, the organization of sleep in relation to its potential functions remains unclear. Different
40 theories have been proposed for functions of sleep including those involving processes like neuronal
41 plasticity and synaptic downscaling (Cirelli and Tononi 2008) and metabolic waste clearance (Xie et al.
42 2013). However, sleep research methodology is largely driven by research in humans and other
43 mammals and the primary way of classifying sleep states has therefore been using electrophysiological
44 readouts, such as electroencephalography (EEG). By identifying distinct electrical signatures associated
45 with the different stages of sleep, different functional roles have been hypothesized for them. For
46 example, rapid eye movement (REM) sleep in mammals has been proposed to regulate motor learning
47 and memory consolidation (Siegel 2001; Walker and Stickgold 2004), while slow wave sleep (SWS) has
48 been proposed to regulate synaptic strength and homeostasis mechanisms (Tononi and Cirelli 2014).

49

50 One of the primary challenges for understanding sleep architecture has been developing a capacity to
51 record and assess brain-wide patterns of electrical activity across long time periods that encompass
52 several sleep-wake transitions. In this context, small animals such as the fruitfly *Drosophila*
53 *melanogaster* present as extremely challenging subjects, even though they could potentially provide a
54 wealth of molecular genetic tools to help better understand sleep biology. Previous sleep studies in flies
55 have either recorded from just a single LFP channel during spontaneous sleep bouts (Yap et al. 2017;

56 van Alphen et al. 2013; Nitz et al. 2002; B. van Swinderen, Nitz, and Greenspan 2004), or from multi
57 channel probes during short (~15min) bouts of genetically-induced sleep (Yap et al. 2017; Paulk et al.
58 2013). In other work, whole-brain calcium imaging in sleep-deprived flies revealed distinct stages of
59 spontaneous sleep (Tainton-Heap et al. 2021), although these recordings were rarely long enough to
60 display any revealing sleep architecture, and it remains unclear how these different sleep stages might
61 be manifested across the fly brain from the central complex to optic lobes.

62

63 The primary reasons for the lack of whole-brain or multichannel sleep data in *Drosophila* are technical
64 in nature: a) it is difficult to perform long-term electrophysiological recordings with multiple electrodes
65 in such small brains; the survival rate is low; and the recording tools used do not yet allow for consistent
66 spatial positioning of multiple electrodes across different flies. b) calcium imaging on the other hand,
67 which lacks in temporal precision compared to LFPs, does allow for consistent spatial locations of
68 recordings (with image registration tools), however concerns with photobleaching and phototoxicity
69 have made it difficult to achieve the long-term recordings to acquire spontaneous sleep data.
70 Subsampling provides one solution: for example, in a recent study 24 hr recordings were conducted by
71 recording for only 1 sec after every minute (thus recording for only 1.6% of the overall time) (Flores-
72 Valle and Seelig 2022). However, this subsampling approach might miss important sleep transitions or
73 longer-lasting sleep phenomena. To best compare the brain activity during sleep in flies with similar
74 data from other animals would ideally involve similar readouts akin to a whole-brain EEG, which in
75 *Drosophila* would necessarily involve miniaturized multichannel probes such as used previously for
76 visual studies (Paulk et al. 2013, 2015) as well induced sleep (Yap et al. 2017) and anesthesia
77 experiments (Leung et al. 2021; Cohen, van Swinderen, and Tsuchiya 2018; Cohen et al. 2016; Cohen
78 and Tsuchiya 2018; Muñoz et al. 2020). Additionally, such recordings would ideally be supplemented
79 by detailed behavioral analysis beyond the simple locomotory determinants that have traditionally
80 defined sleep in flies (P. J. Shaw et al. 2000; Hendricks et al. 2000). Mammalian sleep stages involve
81 distinct micro-behaviors in addition to electrophysiological correlates (Dement and Kleitman 1957;
82 Fulda et al. 2011), and this seems to be true for invertebrates as well (van Alphen et al. 2021; Rößler et
83 al. 2022; Iglesias et al. 2019).

84

85 In this study, we optimized a multichannel LFP recording preparation for *Drosophila* flies, to track
86 long-term neural activity in 16 channels across one hemisphere of the fly brain, in a transect from the
87 retina to the central complex. The flies underwent spontaneous sleep bouts while walking/resting on
88 an air-supported ball, and survived long enough to provide 20 hrs of data over one circadian cycle. We
89 developed calibration tools to consistently record from similar spatial locations in different flies. We
90 used machine learning based methods (support vector machines and random forest classifiers) to first
91 investigate the structure of sleep bouts, and further explored the spectral features across multiple brain
92 channels. We also employed machine learning techniques (pose tracking and identification) to identify
93 fly micro-behaviors during these long-term recordings, to determine their potential association with
94 different sleep stages. Taken together our analyses identify distinct sleep stages in the fly central brain,
95 with rhythmic proboscis extensions being a key behavioral feature. We find that the LFP features
96 associated with proboscis extensions during wake and sleep are dissimilar, suggesting that a distinct
97 brain state is driving the sleep functions associated with this rhythmic micro-behavior.

98

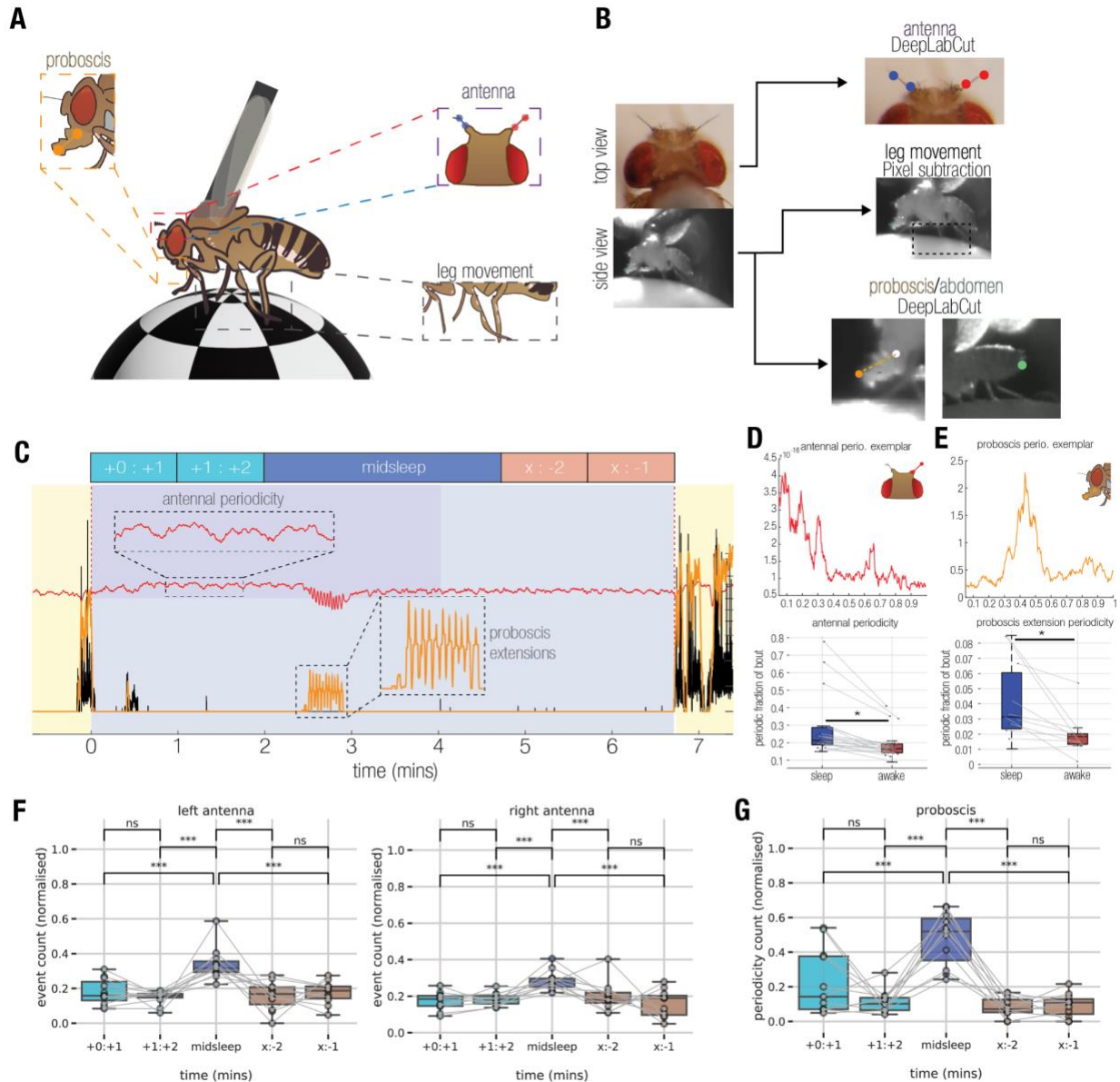
99 **Results**

100 *Behavioral analysis of tethered flies during sleep and wake.*

101 Prior to conducting any electrophysiological recordings, we first investigated how flies slept when
102 tethered to a rigid metal post while being able to walk on an air-supported ball (*Figure 1A*). Flies were
103 filmed overnight under infrared illumination, and locomotory behavior was quantified using a pixel
104 subtraction method (Yap et al. 2017) to identify sleep epochs, defined by the absence of locomotion or
105 grooming behavior for 5 minutes or more (P. J. Shaw et al. 2000; Hendricks et al. 2000; van Alphen et
106 al. 2013; Yap et al. 2017). We also tracked the movement of different body parts, including the
107 proboscis, antennae, and abdomen to detect potential micro-behaviors during sleep. For this, we used
108 machine learning (DeepLabCut (Mathis et al. 2018)) to train a classifier to track micro-behavioral
109 movements through wake as well as sleep (*Figure 1B*). As shown previously (Yap et al. 2017) tethered
110 flies were able to sleep in this context (*Figure 1C; Figure S1A*). As described recently (van Alphen et
111 al. 2021), we also observed regular proboscis extensions (PEs) during wake, as well as during sleep bouts
112 (*Figure S1B*), which often occurred in rhythmic succession (*Figure 1C, orange trace*). We also observed
113 antennal movements and were surprised to discover that these were oscillatory in a subset of flies

114 (*Figure 1C, red trace*). Since PEs were also often rhythmic during sleep, we characterized both micro-
115 behaviors in the frequency domain (*Figure 1D,E, top*) to determine if these were different between
116 sleep and wake. We found that a greater proportion of the sleeping states displayed both antennal
117 periodicity as well as PE periodicity, compared to the waking states (*Figure 1D,E, bottom*), and that
118 antennal periodicity occurred at a small but significantly lower frequency during wake (*Figure S1G*).
119 However, the time course and presence of individual proboscis extensions (*Figure S1B/C*), as well as
120 the dynamics (e.g., periodicity, frequency) of periodic proboscis extensions were not different between
121 sleep and wake (*Figure S1F*), even if this presence varied across sleep and wake.

122
123 A previous study suggested that PEs during sleep are accomplishing a specific function in flies linked
124 to waste clearance, and that these might be specific to a deeper sleep stage (van Alphen et al. 2021).
125 We therefore next examined if PE and antennal periodicity varied throughout a sleep bout. For this,
126 we segmented all >5 min sleep bouts into 5 distinct epochs, as done previously for spontaneous sleep
127 experiments in tethered flies (Yap et al. 2017; Tainton-Heap et al. 2021) (*Figure 1C, top schema*). The
128 first 2 and last 2 minutes of sleep (flanked by locomotory behavior) were analyzed separately for micro-
129 behaviors, and compared to ‘midsleep’ epochs which could be of different durations. To understand if
130 likelihood of periodicity for both antennae and proboscis vary based on the sleep epochs we used
131 multilevel modeling instead of traditional repeated measures of analysis of variance (as different flies
132 had varying numbers of sleep epochs). For details refer to the methods section (Multilevel models -
133 models for antennal, proboscis periodicity). For all the micro-behaviors, the ‘epoch’ model (where the
134 periodicity depends only on the sleep epoch) emerged as the winning model and a reliable main effect
135 of epoch was found ($p < 0.001$) in all cases. Further, we performed post-hoc tests using tukey adjustment
136 (for multiple comparisons) to identify differences between pairs that are significant. Thus, we found an
137 apparent increase in the likelihood of periodicity for both antennae and proboscis during the middle
138 segments of sleep bouts (*Figure 1F,G*). This suggested physiological differences which might be detected
139 in the fly brain, so we then performed electrophysiological recordings in a similar context.



140
 141 **Figure 1: Micro-behaviors of tethered flies. A)** Schema for the setup used to record micro-behaviors of sleeping
 142 and waking flies. A tethered fly stands on an air-supported ball. **B)** The fly is filmed by two cameras. Footage from
 143 these cameras is fed through a preprocessing pipeline that tracks movements of the antennae (Top), legs (Middle),
 144 abdomen and proboscis (Bottom). **C)** An example sleep bout from a fly. Locomotive activity (Black) has ceased
 145 for long enough that the period of inactivity is classified as a sleep bout. The movement of the right antenna (Red
 146 trace) shows an apparent low frequency periodicity (See inset) across the 6 minute bout, interrupted in the middle
 147 by a series of proboscis extensions (Orange trace; See inset). **D)** Top, FFT of antennal activity during an exemplar
 148 sleep bout containing antennal periodicity. Bottom, Comparison of the fraction of sleep and wake that consisted
 149 of periodic antennal activity (* $p < 0.05$; Student's T-test). **E)** As with D, for proboscis periodicity. **F)** Proportions
 150 of antennal periodicity (left and right antennae) across different sleep segments: +0:+1 indicates 1 mins after start
 151 of sleep, +1:+2 indicates 2 mins after start of sleep, x:-2 indicates 2 mins before end of sleep, x:-1 indicates 1 mins

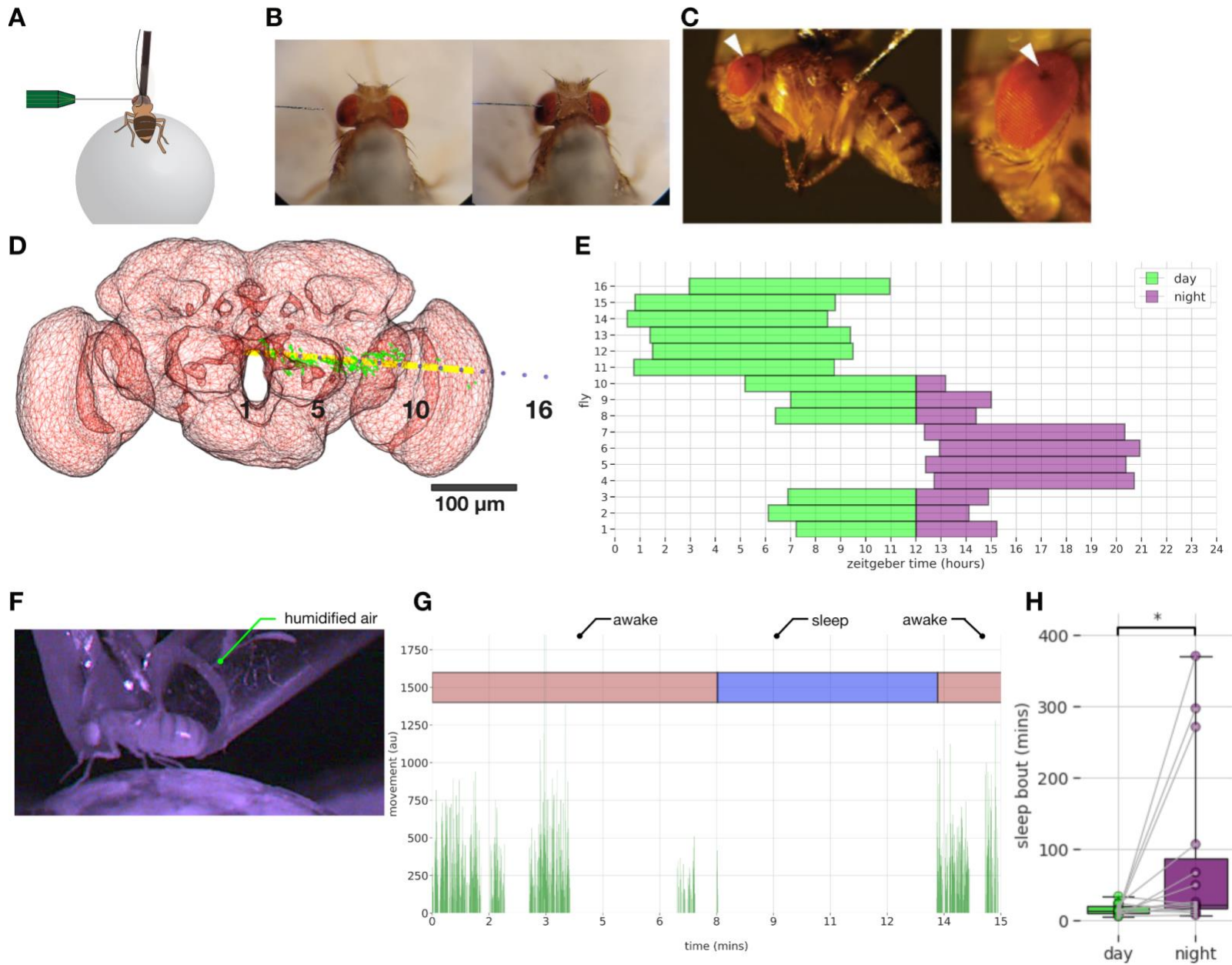
152 before end of sleep. The normalized count is significantly higher in the midsleep segments compared to other
153 segments. **G)** As with F, but for periodic extensions of the proboscis*** $p < 0.001$, ns indicates not significant.
154

155 *Long-term multichannel recordings with spontaneous sleep bouts.*

156 We recorded local field potentials (LFPs) across the fly brain using a linear 16-channel electrode
157 inserted into the left eye of flies in a similar context as above, walking (or resting) on an air-supported
158 ball (*Figure 2A,B*). The electrode insertion location was positioned to sample LFPs from the retina to
159 the central brain (Paulk et al. 2013) (*Figure 2C*, white arrowheads). The depth of insertion of the
160 electrode was optimized by using a visual stimulus calibration protocol, based on a reliable LFP
161 polarity-reversal identified in the fly inner optic lobes (*Figure S2*; and see Methods for polarity
162 reversal). The change in polarity (positive to negative deflections in response to the visual stimulus)
163 was always positioned between electrodes 11-13 in all flies, before the start of the long-term LFP
164 recordings. This LFP polarity-based method allowed us to maintain a level of recording consistency
165 across flies in terms of spatial locations of the electrodes, thereby allowing us to compare and combine
166 LFP data across multiple flies. To further ensure reproducible recording locations, we also developed a
167 dye-based registration method (*Figure S3,4*; and see Methods for dye-based localization) and estimated
168 recording channel locations in the brain for two sample flies. Using this method we identified three
169 broadly-defined brain recording regions to simplify our subsequent analyses (*Figure 2D*): central
170 channels (1-5), middle channels (6-10) and peripheral channels (12-16); here assuming polarity reversal
171 in channel 11. Also for further analysis, as the polarity reversal channel is used for re-referencing, the
172 number of channels used in analysis becomes 15.

173
174 We utilized the above calibration steps and recorded LFP data from 16 flies over the course of a day
175 and night cycle (*Figure 2E*; and See Methods for data exclusion criteria). We designed our recordings
176 so that experiments were started at different times in different flies, to achieve complete coverage of a
177 full day-night cycle. We however only examined the first 8 hours of the LFP data in each fly (*Figure*
178 *2E*), to ensure we were always recording from active and responsive animals (all 16 flies were still alive
179 after 24 hours).

180



181

182 **Figure 2:** A) In vivo long-term electrophysiology recording setup: tethered flies were placed on an air supported
183 ball setup which served as a platform for walking/rest. B) Top view of electrode insertion process, with electrode
184 approaching from the left eye of an example fly. C) Side view of electrode insertion site on the dorsal part of the
185 left eye. D) Localization of electrodes using fluorescent dye: the electrode numbers (black) are displayed along
186 with the dye (green), eigenvector (yellow) indicating the main path of the probe, the *fafb14* neuropil (red). E)
187 Raster plot showing the used recording times (LFP) for the 16 flies. Only the first 8 hours of LFP recording were
188 used for analysis though all the flies survived for more than 24 hours. F) Fly movement is quantified using video
189 recorded in profile view with infrared lighting. Movement was quantified between adjacent frames with pixel

190 difference and contour thresholding. G) Movement area (activity pattern) plotted along with ‘awake’ and ‘sleep’
191 state labeling for an example sleep bout. H) Sleep bouts during day are significantly longer than night thus
192 confirming the occurrence of natural sleep in our setup. * indicates $p < 0.05$

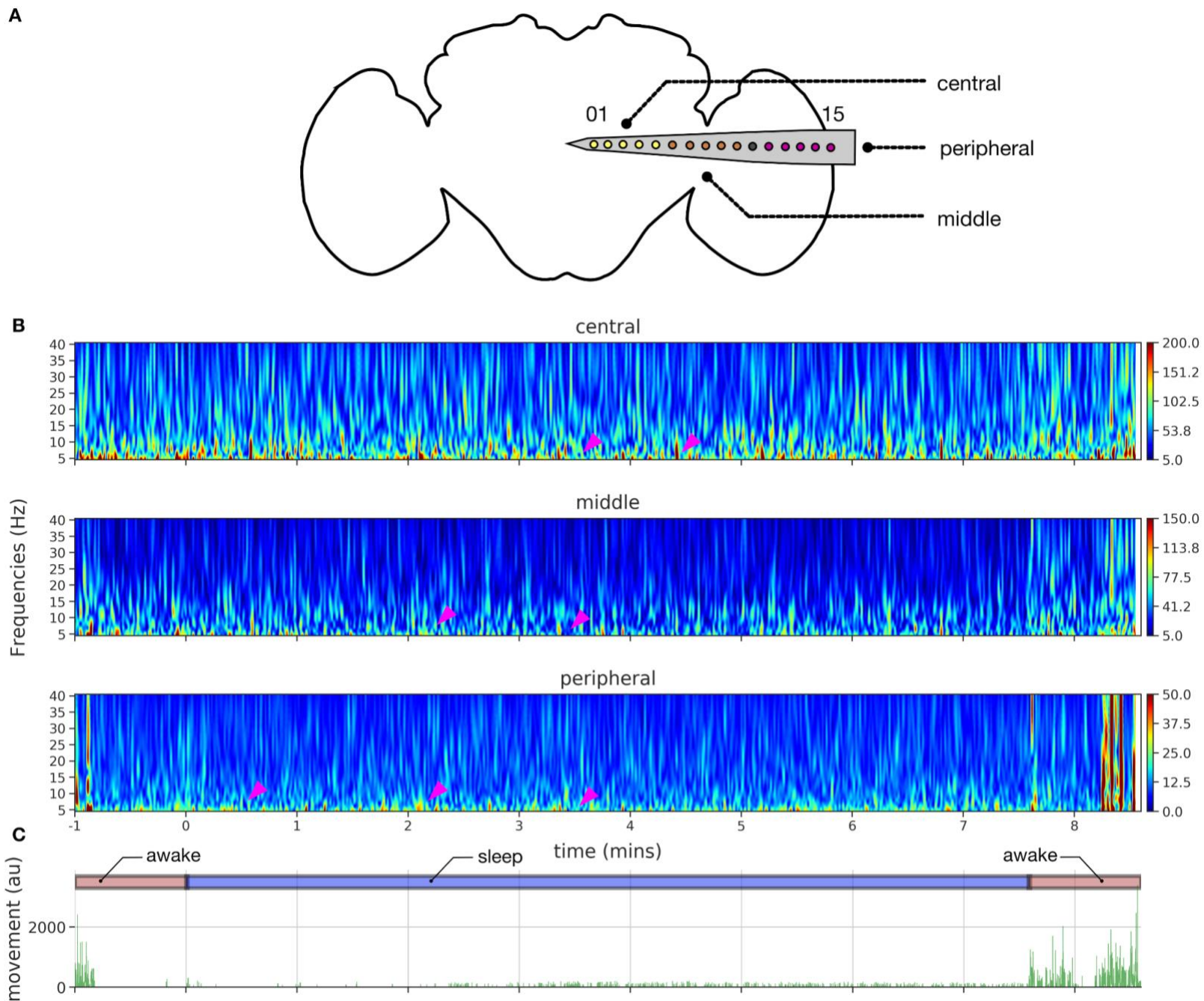
193

194 The behavior of the flies was recorded under infrared lighting (*Figure 2F*) and their movements were
195 quantified using a combination of pixel difference (van Alphen et al. 2013) and contour thresholding
196 between neighboring frames (See Methods for movement analysis). Sleep was defined by 5 min
197 immobility criteria, based on previous observations in unrestrained flies (P. J. Shaw et al. 2000;
198 Hendricks et al. 2000) as well as tethered flies (van Alphen et al. 2013; Yap et al. 2017). Fly mobility
199 along with classification of different behavioral states (‘awake’, ‘sleep’) for an example sleep bout is
200 shown in *Figure 2G*. Since it was unclear whether flies would even sleep in this multichannel recording
201 preparation, we tallied immobility bout durations across the day and the night for each fly (we used 16
202 hrs of video data for each fly - See Methods for data exclusion criteria), expecting that flies should be
203 sleeping more at night on average. We found that flies were able to sleep in this preparation, and that
204 nighttime sleep bouts were indeed longer than daytime sleep bouts (median = 22.42 min vs 13.99 min,
205 respectively; $t(13) = -2.32$, $p < 0.05$) (*Figure 2H*). This confirms that similar to single channel LFP
206 recordings (van Alphen et al. 2013; Yap et al. 2017) flies slept reliably in this multichannel recording
207 preparation, allowing us to assess changes in LFP activity across the fly brain during sleep and
208 wakefulness, and to relate these changes to sleep micro-behaviors.

209

210 *LFP differences across the brain during spontaneous sleep and awake.*

211 Next, we focused on the multichannel data to identify potential differences between sleep and wake
212 across the fly brain, separating our recordings into three broad regions: central, middle, and peripheral
213 (*Figure 3A*). An example sleep bout and its corresponding spectrograms across the central, middle, and
214 peripheral channels reveals increased activity during sleep in the central brain compared to the
215 periphery (*Figure 3B*). Additionally, we noted variegated effects in the lower frequencies (5-10 Hz)
216 within the sleep bout (*Figure 3B*, arrowheads) as well as significant LFP activity (5-40 Hz) associated
217 with locomotion.



218

219

220 **Figure 3:** A) 16 electrodes color coded by location (purple - peripheral, gray - reference, brown - middle, yellow

221 - central) are illustrated on an outline of a standard *drosophila* brain. B) Spectrogram in different channels groups

222 across an example sleep bout shows variation (magenta arrowheads) in the lower frequency bands (5-10 Hz)

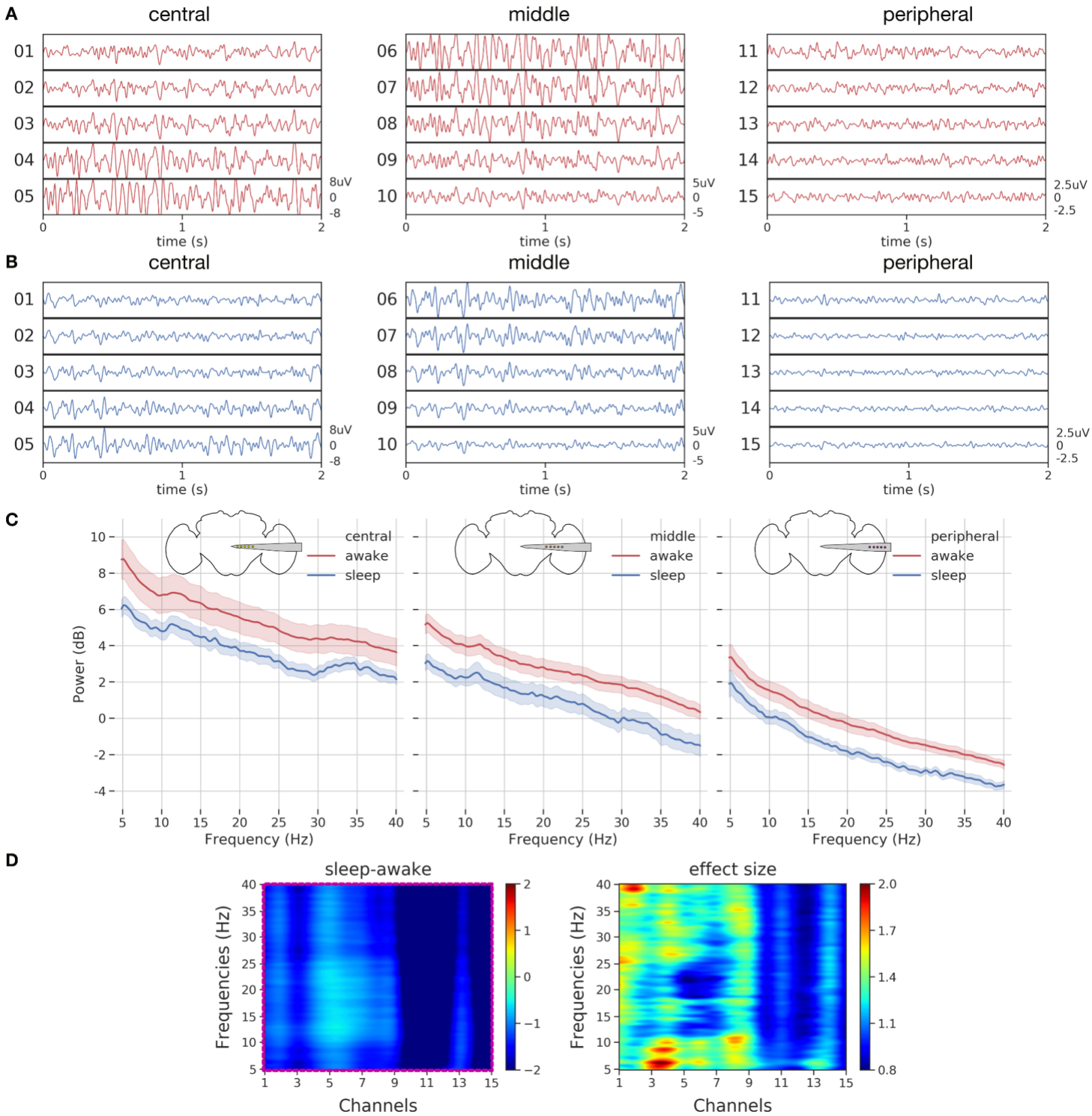
223 within the sleep bout, while activity across 5-40 Hz in the flanking awake period. C) Movement area (activity

224 pattern) plotted along with 'awake' and 'sleep' state labeling for this example sleep bout.

225 When we examined sample LFP data more closely across all channels (*Figure 4*), we observed higher
226 LFP amplitudes in the central and middle channels than in the peripheral channels, and more activity
227 during wake than during sleep (*Figure 4A,B*).

228
229 Interestingly, the fly brain is not necessarily quiet during sleep, with some channels (e.g., channels 5-
230 7) displaying increased activity compared to other channels. To substantiate our observations, we
231 performed spectral analysis on the data. For this purpose, we epoched the LFP data into 60 sec bins and
232 computed the power spectrum per epoch per channel (See Methods for LFP analysis - preprocessing,
233 power spectrum analysis). Since LFP data recorded from flies can be sensitive to physiological artifacts
234 such as heartbeat and body movements (Paulk et al. 2013), we employed a common referencing system
235 (based on a brain based signal) that allowed for removal of non-brain based physiological noise. Plotting
236 the power spectral density across the three different channel groupings for different frequency bands
237 (5-40 Hz), revealed consistently greater power in all flies (n=16) during wake than during sleep across
238 the entire recording transect (*Figure 4D*). Although decreased LFP power during sleep is consistent
239 with previous findings involving single channel recordings in flies (van Alphen et al. 2013; Yap et al.
240 2017; Nitz et al. 2002), it was surprising to see that even the fly optic lobes are significantly less active
241 during sleep compared to wake, suggesting a brain-wide effect.

242
243 We next examined more closely the relationship between individual channels and LFP spectral
244 frequency between sleep and wake states. We employed non-parametric resampling tools to identify
245 the precise patterns (frequency x channel pairs) differing across awake and sleep at the group level. For
246 this purpose, we first computed the difference in mean spectral data across awake and sleep for
247 individual flies. Then, we performed a cluster permutation test (flies x frequencies x channels) on the
248 difference between awake and sleep data (*Figure 4D - left panel*) to reveal a significant cluster
249 (frequency x channel pair). The significant cluster as indicated by the magenta box (*Figure 4D - left*
250 *panel*) covered all frequencies (5-40 Hz) and channels (1-15), thereby confirming the spectral results
251 in *Figure 4C* that found a brain-wide decrease in power during sleep compared to wake. As we had a
252 single significant cluster (magenta box), we then sought to identify subclasses of frequencies and
253 channels within this cluster which might be more specifically associated with sleep.



255 **Figure 4:** A,B) Average LFP across a sample awake and sleep bout in an example fly. C) Mean power spectrum of
256 LFP (5-40 Hz) across 'awake' and 'sleep' states in the central, middle, peripheral channels. Across all channels,
257 'sleep' periods have lower LFP power compared to the 'awake' periods. D) Spectrogram showing the mean
258 difference across 'sleep' and 'awake' periods, while clustering analysis reveals a single significant cluster (magenta
259 box) across all channels and frequencies. Effect sizes are also plotted to identify the individual effect values for
260 every frequency and channel pair.

261
262 We computed the effect sizes for every channel x frequency combination (*Figure 4E - right panel*).
263 This revealed an interesting frequency structure distinguishing sleep from wake. This included areas of
264 interest in the 5-10 Hz and 25-40 Hz range in the central channels (1-3). The 5-10 Hz frequency domain
265 was identified in a previous study as being relevant to sleep in *Drosophila* (Yap et al. 2017), and the
266 higher 25-40 Hz range overlaps with the frequencies associated with attention-like behavior in flies
267 (Bruno van Swinderen and Greenspan 2003; Grabowska et al. 2020). Consistent with previous work, it
268 is however clear that LFP activity is mostly decreased during all of sleep compared to wake, even in
269 the 7-10 Hz range that has been associated with certain sleep stages (*Figure S5*).

270
271 *LFP differences across induced sleep and awake.*

272 Sleep can be acutely induced in *Drosophila* by using optogenetic or thermogenetic activation of sleep-
273 promoting neurons (Shafer and Keene 2021). We were curious whether induced sleep revealed similar
274 effects across the fly brain, following the same statistical approaches employed above for spontaneous
275 sleep. For this, we focused on whole-brain recordings taken from 104y-Gal4 / UAS-TrpA1 flies, a sleep-
276 promoting circuit (*Figure S6A*) that expresses a temperature sensitive cation channel in the fan-shaped
277 body in the central brain (Donlea et al. 2011). As shown in a previous study (Yap et al. 2017) as well as
278 other *Drosophila* sleep studies (Dag et al. 2019), activating these neurons with heat (temperature ~
279 29°C) results in behavioral quiescence and induced sleep, whereas control strains remain awake and
280 active. In these recordings, a different multichannel probe was employed (*Figure S6B*), with 16
281 recording sites that spanned the entire brain from eye to eye (Paulk et al. 2013). We preprocessed the
282 induced sleep LFP data (See Methods for thermogenetic sleep induction) in a similar fashion to the
283 spontaneous sleep LFP data. We first contrasted the mean power spectra per fly under two conditions:
284 baseline and sleep induction (*Figure S6C*). As above, we then performed a cluster permutation test (flies
285 x frequencies x channels) on the difference between baseline wakefulness and induced sleep, to reveal

286 a significant cluster (frequency x channel pair). Thus, we uncovered a significant cluster (*Figure S6D*)
287 in the central brain channels across all (5-40Hz) frequency bands, whereas the 104y-Gal4/+ control
288 flies did not reveal such a cluster (*Figure S6E,F*). It is interesting to note that sleep induction using this
289 strain yielded an opposite effect to what we found during spontaneous sleep: LFP activity during
290 induced sleep is on average higher than during baseline wakefulness (*Figure S6D*), while it was lower
291 during spontaneous sleep (*Figure S5*). Additionally, the effect observed during induced sleep was only
292 observed in the central channels whereas the spontaneous sleep effects appear to at least cover the
293 entire hemisphere from center to periphery. This shows that genetically-induced sleep in flies can
294 produce strikingly different electrophysiological signatures than spontaneous sleep, consistent with
295 several previous similar observations (Tainton-Heap et al. 2021; Yap et al. 2017; Anthoney et al. 2023;
296 Lin, Panula, and Passani 2015; Troup et al. 2018). For the rest of this current study, we focus on
297 spontaneous sleep.

298

299 *Machine learning identifies distinct sleep stages in multichannel data.*

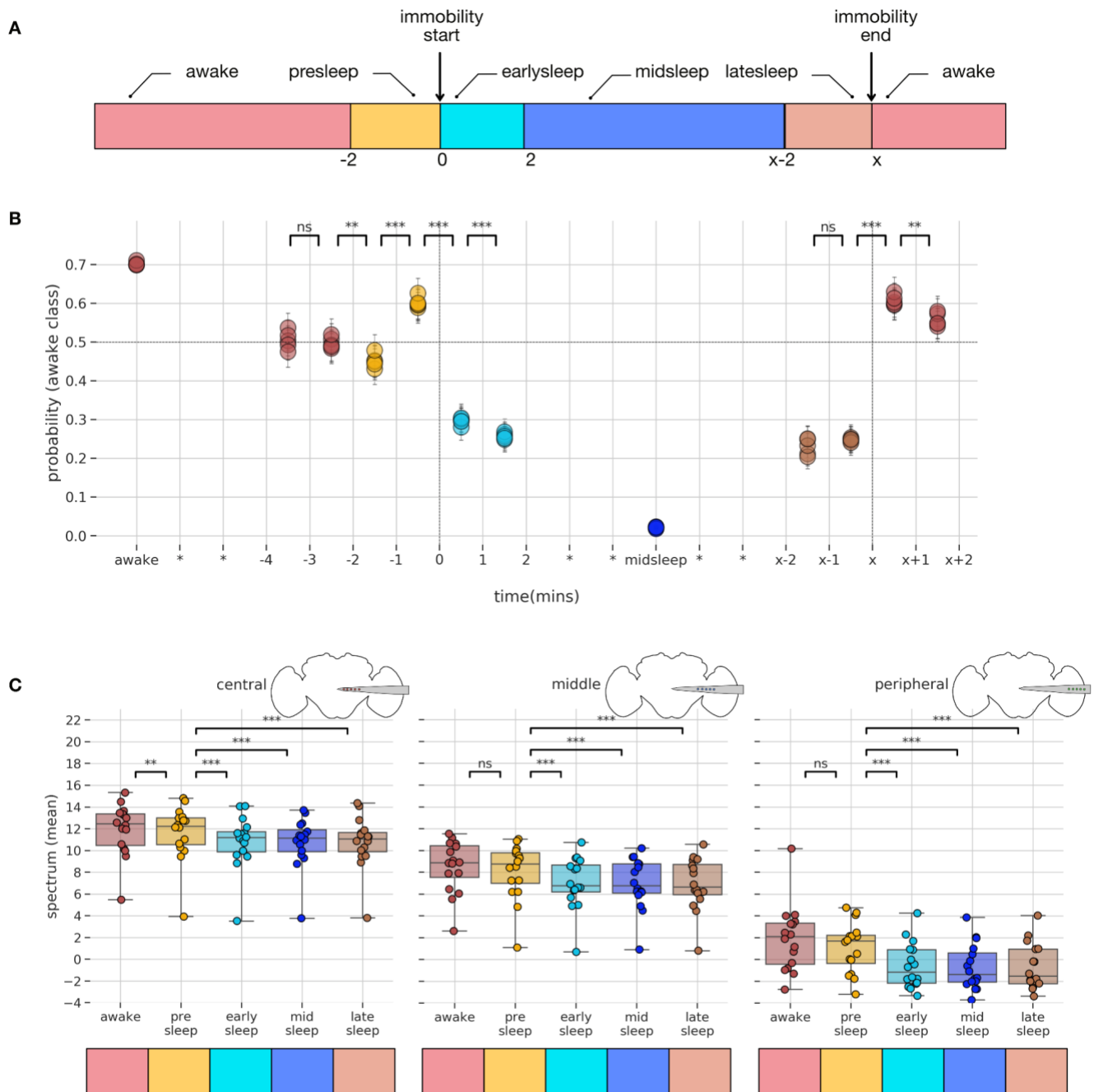
300 Our earlier analysis of micro-behaviors during sleep in this preparation (*Figure 1*) suggests that sleep is
301 not a single phenomenon, and that the requisite 5 min immobility criterion might not fully capture
302 potential LFP and behavioral changes that might occur across a sleep bout. There is evidence that sleep
303 quality (via arousal threshold probing) in wild-type *Drosophila* flies also changes across a bout of
304 quiescence (van Alphen et al. 2013; Faville et al. 2015), suggesting that flies transition from lighter to
305 deeper sleep stages. To assess whether this might also be evident in our multichannel recordings, we
306 divided our LFP data (for all channels) into five different temporal segments, analyzing only sleep
307 epochs that were 5 min or longer (*Figure 5A*): 1) ‘presleep’: the 2 mins (-2 to 0 mins) before flies stopped
308 moving; 2) ‘earllysleep’: the first 2 mins (0 to 2 mins) after the start of a sleep bout; 3) ‘latesleep’: the last
309 2 mins of sleep before mobility resumed; 4) ‘midsleep’: any time between ‘earllysleep’ and ‘latesleep’. 5)
310 ‘awake’: the rest of our LFP data. Our partitioning of the LFP data matches a similar partitioning applied
311 to whole-brain calcium imaging of flies engaged in spontaneous sleep (Tainton-Heap et al. 2021).

312

313

314

315



316

317

318

319

320 **Figure 5:** A) Sleep bouts (> 5 min) were binned into 4 segments. 2 mins before the start of immobility (presleep),
321 2 mins after the start of immobility (earlysleep), 2 mins before the end of immobility (latesleep), the period
322 between early and latesleep is midsleep, rest of the periods are categorized as awake. B) Probability estimates (of
323 awake class) plotted across different time segments. Horizontal dotted line indicates values above 0.5 are likely
324 to be classified as awake and below 0.5 as sleep. Vertical dotted line at 0 indicates start of immobility period and
325 at x indicates end of immobility period. Unpaired samples t-test was conducted across different epochs to test for
326 statistical significance. C) Comparison of mean power spectrum across different channels and different sleep
327 stages. ** p<0.01, ***p<0.001, ns indicates not significant.

328

329 To understand how LFP based signatures change within a sleep bout, we decided to perform a
330 hypothesis-agnostic analysis through machine learning techniques. To perform such machine learning
331 based classification, we first used support vector machine (SVM) based techniques. Briefly, SVM belong
332 to a class of supervised learning model, that is comprised of building a hyperplane or set of hyperplanes
333 in a high dimensional space (using the kernel trick for non-linear mapping functions) with the goal to
334 maximize the separation distance between the closest data point (in the training dataset) of any class
335 (functional margin) (Cortes and Vapnik 1995). The choice of the optimal hyperplane is made in such a
336 way that the generalization error would be lower for the new data points in the test dataset (*Figure*
337 *S7A*). For detailed steps for preprocessing of data and implementation of classifiers refer to Methods for
338 sleep staging by classifiers. The probabilistic prediction per class per iteration is shown in *Figure 5B*. It
339 is interesting to note several points. First, the probability of awake data is ~ 0.7 and of midsleep is ~ 0.0
340 indicating that the classifier performs well on classes that it has already been trained on. Second, at the
341 epoch -2 to -1min, when the fly is still moving (yellow circles), LFP data indicates that it is closer to
342 resembling sleep (<0.5), before dropping fast to ~ 0.3 (turquoise circles) in the first two minutes of sleep.

343

344 The above analysis indicates that with this approach we could predict the probability a fly will fall
345 asleep 2 mins before the start of the immobility period. Interestingly, just 1 min before flies fall asleep
346 the LFP data indicates a brief moment more closely resembling wake (yellow circles), perhaps
347 associated with grooming periods (observed in honeybees for example (Eban-Rothschild and Bloch
348 2008)). Interestingly, in the first two minutes of sleep (turquoise circles) reveal a probability metric
349 halfway between midsleep and wake, suggesting either a gradual descent into deeper sleep or a distinct
350 sleep stage. Finally, at the epoch from x-2 to x-1 min before mobility resumes (brown circles), the
351 probability metric returns to a similar level as early sleep. Immediately after mobility resumes, the LFP

352 data is classified as no different than awake, i.e, there is no post-sleep ambiguity. It is important to note
353 that only the 'awake' and 'midsleep' data has been seen by the classifier, the rest of the data -4 to +2
354 min, x-2 to x+2 min has never been seen by the classifier. Additionally, midsleep collapses a wide range
355 of different sleep durations in different flies, so could still be averaging different sleep states within.
356 Nevertheless, our results suggest that broadly dichotomizing mid sleep and wake identifies other sleep
357 stages that resemble neither.

358

359 *Model based spectral analysis differentiates wakefulness from sleep bouts across different*
360 *channels.*

361 Having revealed how multichannel LFP data can be used to differentiate across different temporal
362 stages of sleep, we next decided to identify what channels might be important for revealing this. For
363 this purpose, we employed a multilevel modeling approach. To reveal how spectral data might change
364 throughout the fly brain across a sleep bout, we calculated the mean spectral power for each of the
365 aforementioned epochs and pooled data from central, middle, and peripheral channels. Because
366 different flies had varying numbers of sleep epochs, we used multilevel models instead of traditional
367 repeated measures of analysis of variance. For details refer to the methods section (Multilevel models -
368 models for spectral analysis). The 'epoch-channel' model emerged as the winning model; here the
369 power spectrum depends on a combination of the LFP epoch type and the channel type. In the epoch-
370 channel model, we found that there was a reliable main effect of both epoch ($p < 0.001$) and channel
371 ($p < 0.001$) on power spectrum and also the interaction between epoch and channel also had a reliable
372 effect ($p < 0.001$) on power spectrum. In summary, the above model-based analysis confirms that the
373 power spectrum of the LFP data varies based on the channel location and also the epoch state of the
374 fly.

375

376 We then proceeded to examine more closely how differences in the sleep LFP might be segregated
377 across the fly brain (*Figure 5C*) using post-hoc tests (using tukey adjustment for multiple comparisons)
378 from the epoch-channel model. In the central channels, the 'awake' data was significantly different
379 compared to all sleep categories, and critically was also different to the 'presleep' data. It is important
380 to note that behaviourally the fly is still considered awake in the 'presleep' period (i.e., it is still moving).

381 Thus, the ability to predict sleep at least 2 mins before the onset of immobility, which was revealed in
382 our SVM analysis (*Figure 5B*), might be explained by these significant spectral differences only
383 observed in the central channels. In the middle channels, the ‘awake’ data was also significantly
384 different across all sleep categories, however was not different to the ‘presleep’ data. Further, the
385 ‘presleep’ period was significantly different from ‘earlysleep’, ‘midsleep’, ‘latesleep’ periods. In the
386 peripheral channels, the ‘awake’ data was significantly different across all sleep categories, however
387 was again not different to the ‘presleep’ data. Taken together, mean power spectral data across different
388 channels was thus able to differentiate between ‘awake’, ‘presleep’, and different sleep epochs of sleep.
389 However, the post-hoc analysis did not differentiate among sleep epochs (‘earlysleep’, ‘midsleep’,
390 ‘latesleep’). Since this is inconsistent with previous findings using single glass electrodes (Yap et al.
391 2017), we questioned if the pooling of channel x frequencies data (3 broad brain regions x 1 overall
392 power spectrum) could be hiding more specific effects which might become evident with the full
393 (15x145) dimension of channels x frequencies.

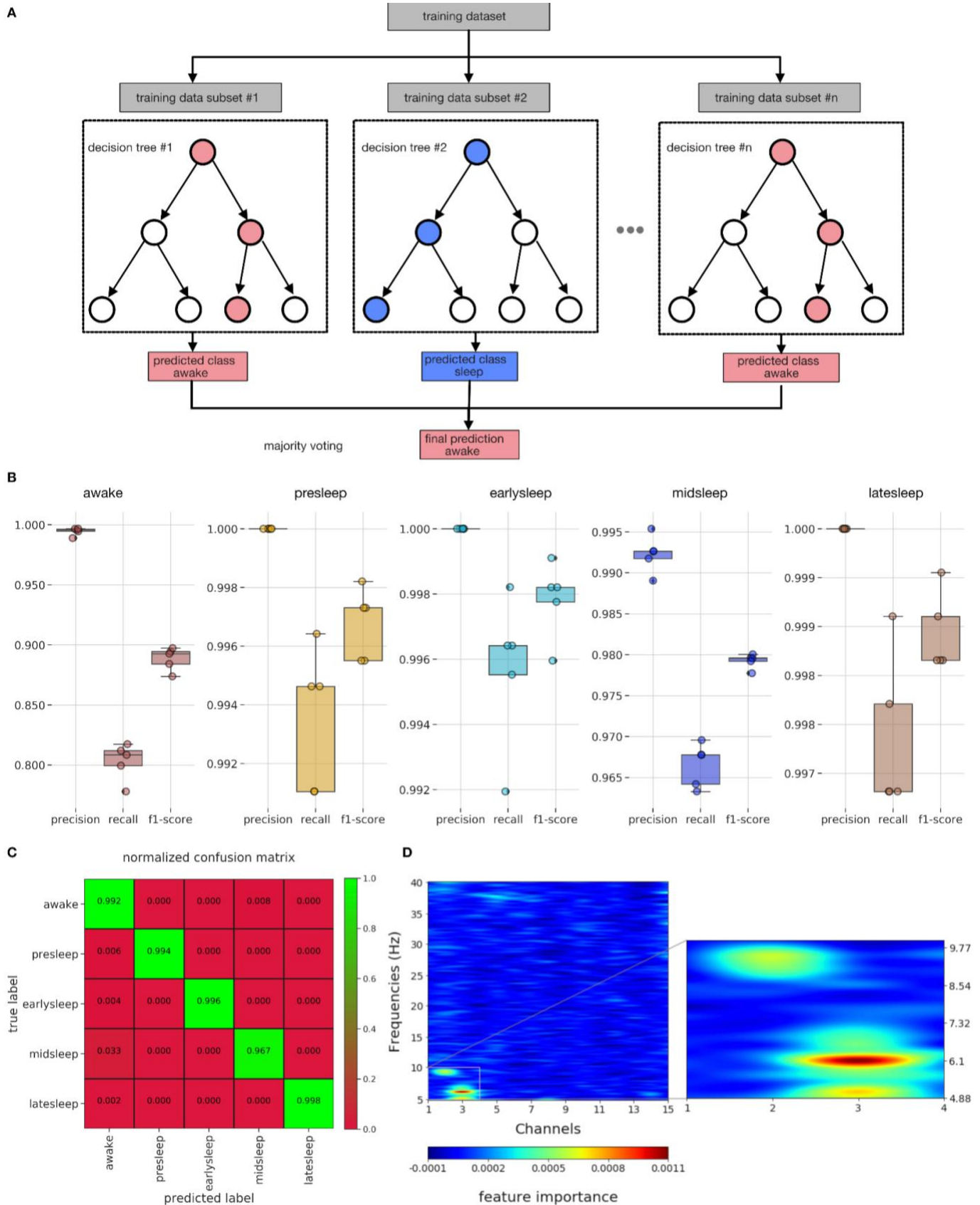
394

395 *LFP features across different temporal stages of sleep.*

396 Having established the existence of different temporal stages of sleep using a classifier based on SVM
397 and confirming the same using model-based analysis, we were next interested in the features in the
398 LFP data (which channels at what frequencies are important for distinguishing epochs within a sleep
399 bout), that helps us differentiate these stages.

400

401 For this purpose we used random forest classifiers. A random forest classifier is a class of supervised
402 learning algorithms that utilizes an ensemble of multiple decision trees for classification/regression.
403 This could be illustrated by an example (*Figure 6A*). In the first step subsets of training data (#1 to #n)
404 were created by making a random sample of size N with replacement. This allows for the ensemble of
405 decision trees (#1 to #n) to be decorrelated and the process of such random sampling is called bagging
406 (bootstrap aggregation). In the second step, each decision tree (#1 to #n) picks only a random subsample
407 of features (feature randomness) instead of all features (again allowing for the decision trees to be
408 decorrelated).



410 **Figure 6:** A) Schematic indicating the workings of a multiclass random forest classifier in identifying the predicted
411 class. B) Performance metrics like precision, recall, f1-score across different classes of the trained classifier. C)
412 Normalized confusion matrix of the trained classifier. D) Feature importance of the multiclass classifier indicates
413 an ROI across central channels and frequency bands (5-10 Hz) as critically important.

414

415 In the final step, all the decision trees create individual predictions of classes and the final outcome
416 would be resolved by simple majority voting (illustrated here with a goal of classifying ‘awake’ vs
417 ‘sleep’). Thus, bagging and feature randomness allows for the random forest to perform better than
418 individual decision trees.

419

420 We performed a multiclass classification of the following classes: ‘awake’, ‘presleep’, ‘earlysleep’,
421 ‘midsleep’, ‘latesleep’. For the detailed preprocessing and feature computation steps refer to the
422 methods section (Sleep staging by classifiers - multiclass svm analysis & feature importance). We then
423 computed classifier performance metrics (See methods for sleep staging by classifiers - classifier
424 metrics) like precision, recall, f1-score (*Figure 6B*) and further normalized confusion matrix (*Figure*
425 *6C*) which reveal excellent performance in predicting the multiple classes (green boxes). This indicates
426 that classifier features (channels x frequency) are sufficient to distinguish multiple sleep stages (classes)
427 and furthermore provide direct evidence of multiple sleep stages. Another reason for using random
428 forest classifiers is that it is possible to identify relative feature importance in the performance of
429 classifiers, thereby identifying features (channels x frequency) which are important for differentiating
430 across multiple sleep stages.

431

432 To identify the LFP features most likely discriminating among sleep stages, we utilized the multiclass
433 random forest classifier (described above), and uncovered the features that are important in this
434 classifier (*Figure 6D*) with permutation importance technique. Interestingly, the most important
435 features fall within a narrow range of channels (1-3) and frequencies (5-10 Hz). This indicates that the
436 5-10 Hz frequency range within the central channels are the most important in resolving different
437 sleep stages. Next, we decided to cross-validate the utility of this permutation-based technique in
438 resolving across different epochs. For this purpose, we created a multiclass random forest classifier,
439 with target classes as: ‘awake’, ‘sleep’, and identified the features that are important in this classifier
440 (*Figure S8A*). The most important features are actually distributed evenly among all the features

441 (channels x frequency), thus cross-validating our previous clustering results (*Figure 4D*) wherein we
442 showed that the LFP differences across ‘awake’ and ‘sleep’ are distributed across all channels and
443 frequencies.

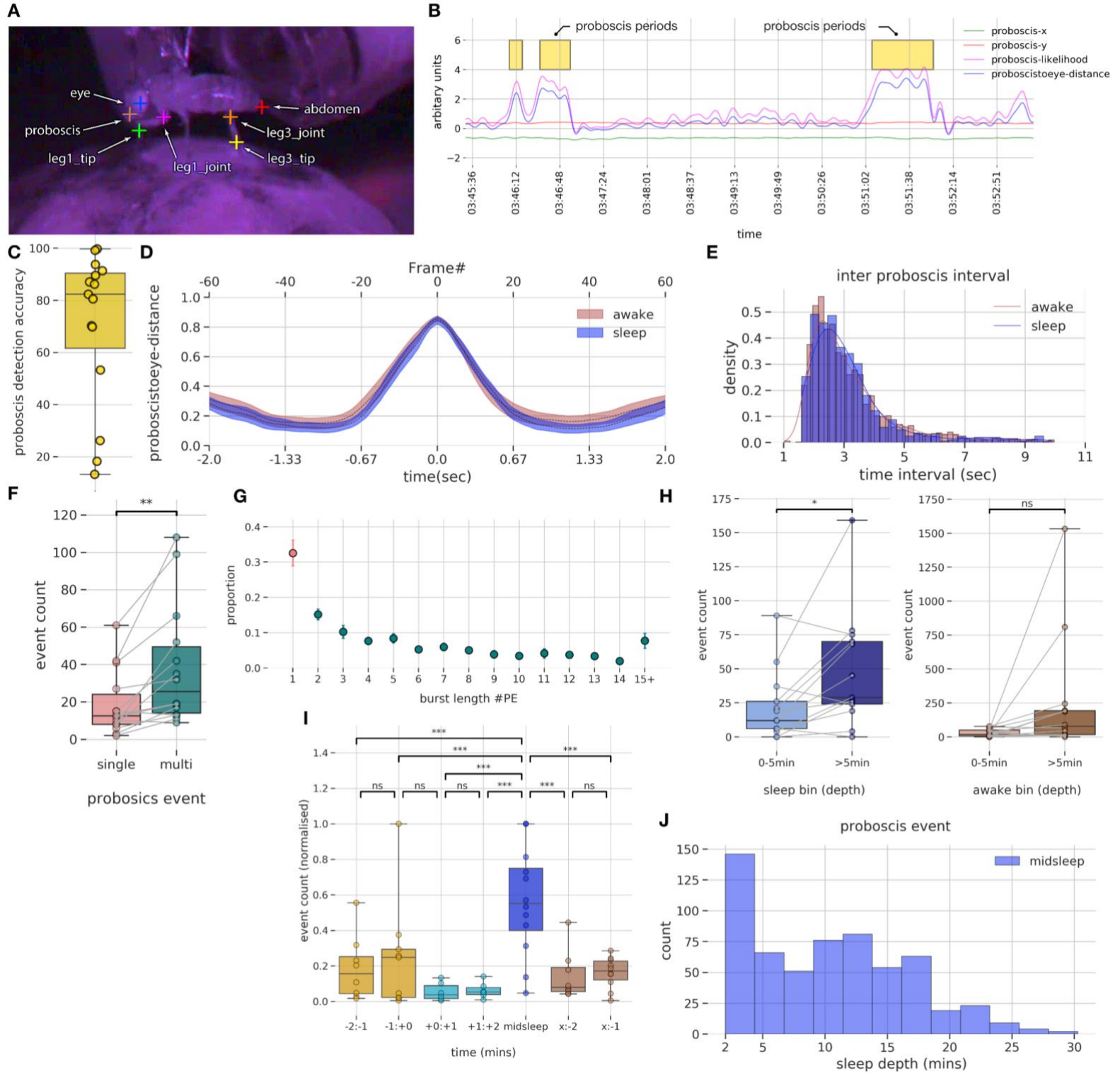
444

445 *Proboscis extension behavior during sleep in multichannel recordings.*

446 Earlier, we identified rhythmic proboscis extensions (PEs) during midsleep (*Figure 1*), which we
447 propose describe a distinct sleep stage in *Drosophila* (van Alphen et al. 2021). However, it is unclear if
448 brain activity associated with PEs are sleep-like or PE-specific. This distinction is important, as it would
449 disambiguate a unique brain state (deep sleep) from a specific behavior associated with that state (PEs).
450 In order to identify PEs in our electrophysiological dataset, we again used DeepLabCut (Mathis et al.
451 2018) to track different body parts of the fly (*Figure 7A*). We further used multiple classifiers based on
452 the tracking data, followed by manual verification to identify the PEs. Sample proboscis extension
453 periods in an example fly along with a few of the features (x,y proboscis location, likelihood of location,
454 distance of proboscis to eye) are shown in *Figure 7B*. For more details on the proboscis detection steps
455 refer to the section - Methods for proboscis tracking for flies on electrophysiology setup. Our classifier
456 accuracy was over 80% for most flies (*Figure 7C*): the ground truth was validation by a human observer
457 on classifier detected events. In *Figure 7D*, we plot the mean proboscis to eye distance for all the flies
458 averaged across awake and sleep bouts. As described earlier for flies without implanted electrodes, PEs
459 executed during wake and sleep are behaviourally similar and hence would be difficult to distinguish
460 from each other using video alone. Similar to our behavioral dataset, PE events usually occur in
461 rhythmic bouts of more than one, rather than single events. In *Figure 7E*, we plot the inter-proboscis
462 interval period, which is the interval between consecutive PE events in a single proboscis bout. It can
463 be seen that most proboscis events occur within 1.8 sec (95th percentile) of each other. As shown before
464 in our behavioral data without implanted electrodes, the inter-proboscis interval does not vary across
465 awake and sleep periods. Next in *Figure 7F*, we decided to probe the number of single (one PE event)
466 and multi (>1 PE event) across different flies. We found that occurrences of single PE events are
467 significantly lower than multi PE events using a pairwise t-test with $t(13) = 3.72$, $p < 0.01$.

468

469



470
471
472

473 **Figure 7:** A) Seven different body parts were annotated using *DeepLabCut* for pose estimation. B) Identified PE
474 periods (yellow boxes) were plotted along with filtered body parts of proboscis and other estimated metrics. C)
475 PE events detection accuracy across different flies. D) Average proboscis to eye distance (across all flies) plotted
476 across frames and time periods are similar in awake and sleep states. E) During PE burst events, inter proboscis
477 intervals are highly regular, with one PE occurring every 1.5 s at group level. F) PE events are more likely to
478 occur as multiple events (bursts) instead of a single event. G) About 33% of PE events occur as single while the
479 rest are bursts of varying length. H) Number of PE events occurring after the first 5 mins of sleep is significantly
480 higher than in the first 5 mins, indicating that more PE events occur in deeper stages of sleep. Also displayed is
481 the control analysis, with awake depth showing no increase with PE event count. I) Normalized proboscis event
482 count across different sleep segments: -2:-1 indicates 2 mins before start of sleep, -1:0 indicates 1 mins before start
483 of sleep, +0:+1 indicates 1 mins after start of sleep, +1:+2 indicates 2 mins after start of sleep, x:-2 indicates 2 mins
484 before end of sleep, x:-1 indicates 1 mins before end of sleep. The normalized count is significantly higher in the
485 midsleep segments compared to other segments. J) Proboscis events occurring in midsleep across different sleep
486 depths. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns indicates not significant.

487
488 To further illustrate this point in *Figure 7G*, we plotted the burst length of a PE event (number of
489 extension events within a PE bout) and found that only 33% of the events are single PE while the rest
490 are multiple PE events. Overall, our investigation of PEs in this multichannel recording dataset is in
491 concurrence with our first (electrode-free) dataset, suggesting that inserting probe into the fly brain
492 does not alter several measures associated with this micro-behavior.

493
494 Previous work has linked PEs with a deep sleep stage in flies (van Alphen et al. 2021). We therefore
495 next investigated whether the number of PEs varied across a sleep bout in our LFP recording dataset,
496 as suggested in our purely behavioral dataset (*Figure 1G*). We found that more PE events occur after 5
497 min of a sleep bout, compared to those occurring before the 5th min of a sleep (*Figure 7H*) (pairwise t-
498 test, $t(12) = -2.8$, $p < 0.05$), suggesting that PEs indeed predominate during deeper sleep. We also
499 compared PEs immediately after flies had awakened from sleep, which revealed no significant
500 difference (*Figure 7H*) (pairwise t-test, $t(13) = -1.92$, $p > 0.05$) between PE bouts occurring after the 5th
501 min of an awake bout compared to those occurring before the 5th min of an awake bout, confirming
502 that transitions into sleep (rather than transitions back to wake) were associated with increased PE
503 events.

504

505

506

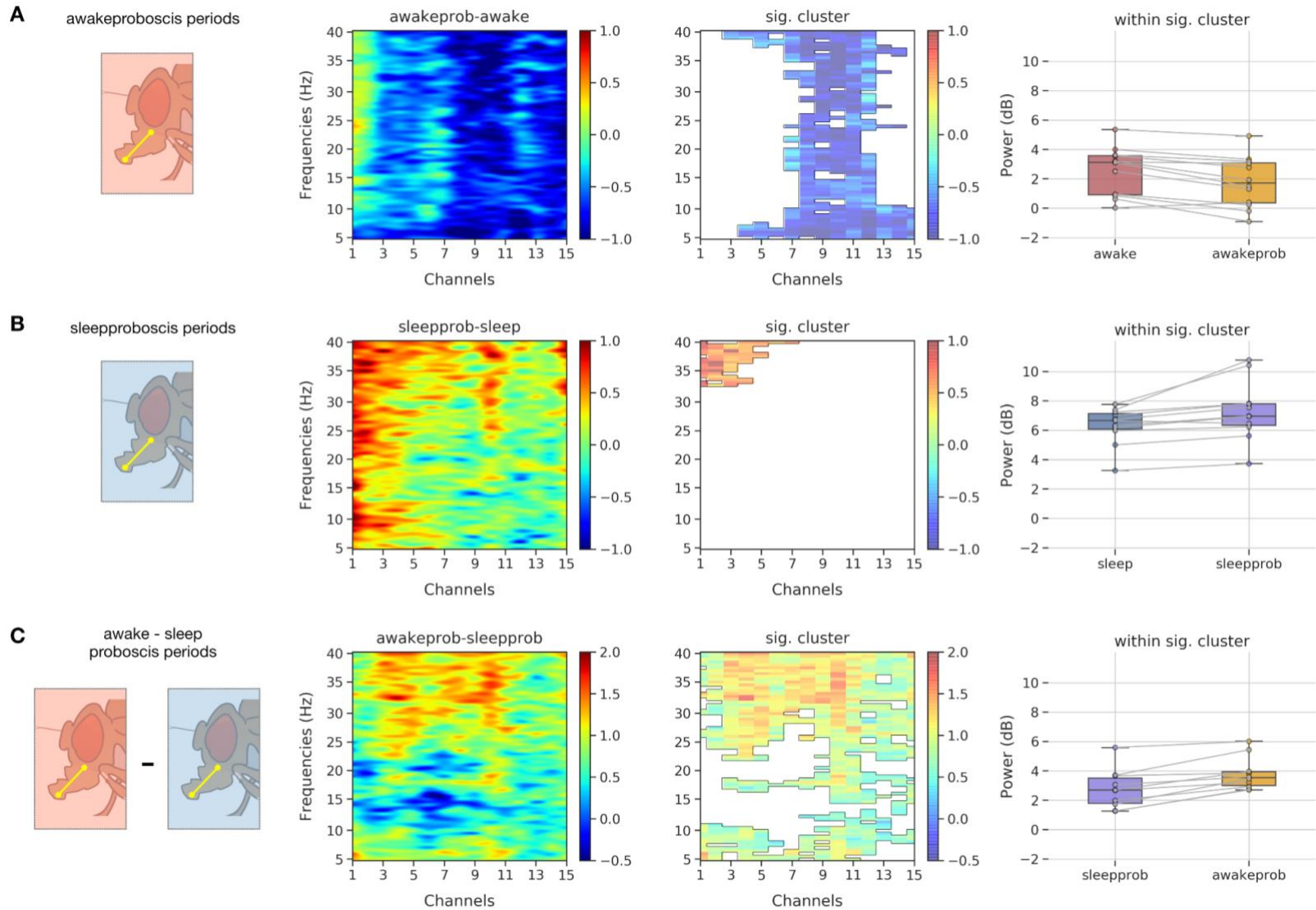
507 We next asked if the number of PE events changed across a sleep bout in our multichannel recording
508 preparation. To determine if the PE event count varies across different temporal sleep stages (*Figure*
509 *7I*) we used multilevel models. For details refer to the methods section (Multilevel models - models for
510 PE event counts). The time_label model (where the PE event count depends only on the specific
511 temporal sleep stage) emerged as the winning model. Further, we performed post-hoc tests using tukey
512 adjustment (for multiple comparisons) to identify differences between pairs that are significant. We
513 found that PE events occur more often in midsleep compared to other sleep stages. Returning to our
514 original observation that most PEs occur after 5min of sleep, we plotted the distribution of PE events
515 occur in the midsleep epoch across all flies (*Figure 7J*), and found that 95 percentile of all PE events in
516 midsleep indeed occur after 2.5 minutes of the midsleep epoch (thus, 4.5 mins from sleep onset).

517

518 *LFP features of a deep sleep stage with proboscis extension.*

519 We next questioned whether PEs occurring during sleep and wake had similar neural correlates, or if
520 the sleep-related events were indeed different and thus indicative of a unique sleep-related function.
521 We therefore focused on the multichannel data to identify any differences in the LFP activity associated
522 with PEs during wake and sleep epochs. We first identified the PE periods (Refer to Methods LFP
523 analysis - proboscis: Identification of proboscis periods) and extracted the LFP data and epoched them
524 into 1 sec bins. Second, we used spectral analysis to determine if epochs characterized by PEs differ in
525 frequencies across different channels, for wake compared to sleep. For this purpose, we computed the
526 spectral power for every 1 sec epoch per channel (See Methods for LFP analysis - proboscis: power
527 spectrum analysis), using as before a common reference system for re-referencing the LFP data. Third,
528 we employed non-parametric resampling tools to identify the precise patterns (frequency x channel
529 pairs) differing in proboscis periods within awake and sleep at the group level. For this purpose, we
530 first computed the difference in mean spectral data across non-proboscis periods (awake or sleep) and
531 proboscis periods (awake proboscis and sleep proboscis respectively) for individual flies. We then
532 performed a cluster permutation test (flies x frequencies x channels) on the difference data to reveal
533 significant clusters (frequency x channel pair).

534



535

536 **Figure 8:** A) Spectrogram showing the mean difference across 'awakeprob' (PE events in awake periods) and
 537 'awake' periods, while clustering analysis reveals a single significant cluster across middle channels in all
 538 frequencies. Activity within the significant cluster indicates activity in the 'awakeprob' is comparatively lower
 539 than 'awake' periods. B) Spectrogram showing the mean difference across 'sleepprob' (PE events in sleep periods)
 540 and 'sleep' periods, while clustering analysis reveals a single significant cluster across central channels in higher
 541 frequencies (35 - 40 Hz). Activity within the significant cluster indicates activity in the 'sleepprob' is
 542 comparatively higher than 'sleep' periods. C) Spectrogram showing the mean difference across 'awakeprob' and
 543 'sleepprob' periods, while clustering analysis reveals a single significant cluster mostly across all channels in
 544 higher frequencies (25 - 40 Hz). Activity within the significant cluster indicates activity in the 'sleepprob' is
 545 comparatively lower than 'awakeprob' periods, thereby elucidating a significant difference across proboscis
 546 events occurring in sleep and awake periods (though phenotypically they look the same - Figure 7D).
 547

548

549 In *Figure 8A*, we show the difference data (awake proboscis - awake period) and clustering analysis,
550 which reveals a significant cluster in the middle channels (6-10) across all frequencies. Further, within
551 the significant cluster we also performed a post hoc analysis revealing that spectral activity within the
552 awake proboscis periods are lower than awake periods. In *Figure 8B*, we show the difference data (sleep
553 proboscis - sleep period) and clustering analysis reveals a significant cluster in the central channels (1-
554 5) across higher frequencies (32-40 Hz). Further, within the significant cluster we also performed a
555 post hoc analysis revealing that spectral activity within the sleep proboscis periods are higher than
556 sleep periods (in contrast to the awake proboscis periods). In *Figure 8C*, we directly compared the
557 awake and sleep proboscis periods and showed the difference data (awake proboscis - sleep proboscis)
558 and clustering analysis, which reveals a significant cluster in the central, middle channels (1-9) across
559 higher frequencies (25-40 Hz). Further, within the significant cluster we also performed a post hoc
560 analysis revealing that spectral activity within the sleep proboscis periods are lower than awake
561 proboscis periods. This suggests that PEs occurring during sleep are qualitatively different from
562 identical PE events occurring during wake. This suggests that the brain activity state (e.g., quiet or deep
563 sleep (Tainton-Heap et al. 2021; Anthony et al. 2023)) overrides the neural correlates associated with
564 the same behavior occurring during wake.

565

566

567 **Discussion**

568 Sleep is most likely a whole-brain phenomenon, meaning that its presumed varied functions
569 (Kirszenblat and van Swinderen 2015) are understood to be of benefit to the entire brain rather than
570 to only specific sub-circuits. There is good evidence for this in the *Drosophila* model, with synaptic
571 physiology for example changing during sleep in the optic lobes of flies (Donlea, Ramanan, and Shaw
572 2009) as well as brain-wide (Gilestro, Tononi, and Cirelli 2009). Similarly, in mammals, subcortical as
573 well as cortical brain regions experience sleep-related changes that are thought to be important for
574 maintaining neuronal homeostasis (Tononi and Cirelli 2014). Accordingly, to better understand sleep
575 in an animal model such as *Drosophila melanogaster* requires sampling associated changes in neural
576 activity across the fly brain, and not only in specific sub-circuits of interest. Unlike in larger animal
577 models such as mice, recording from multiple brain regions in behaving (and sleeping) flies has been

578 challenging, so there has been limited capacity to investigate dynamic brain processes during sleep in
579 this otherwise powerful model system. While genetically encoded reporters of neural activity (e.g.,
580 GCaMPs) have been successfully used to describe spontaneous sleep in flies (Tainton-Heap et al. 2021;
581 Flores-Valle and Seelig 2022; Bushey, Tononi, and Cirelli 2015), these are typically still limited to a
582 narrow region of interest (e.g., the mushroom bodies, or the central complex), and imaging conditions
583 are rarely commensurate with the typical day-night cycles of normal sleep. In this study, we overcame
584 these drawbacks by recording electrical activity from 16 channels across the fly brain, in behaving flies
585 across long-lasting recordings that spanned a typical day and night. Our multichannel recording
586 preparation therefore approximates as closely as possible - in flies - a sleep EEG, which has been the
587 starting point for most discussions on sleep physiology in other animals. The human sleep EEG has
588 defined the sleep stages that are now being investigated in other animals (Kirszenblat and van
589 Swinderen 2015; Van De Poll and van Swinderen 2021; Raccuglia et al. 2019, 2022), although this is
590 obviously a neocortical view with potentially little relevance to animals lacking the neural architecture
591 giving rise to sleep signatures such as delta (1-4Hz) during slow-wave sleep or theta (5-8Hz) during
592 REM sleep (Jaggard, Wang, and Mourrain 2021).

593
594 Rather than focus on specific frequency bands such as delta and theta, we conducted an agnostic
595 analysis of our multichannel LFP data using machine learning techniques. These unbiased classifiers
596 identified distinct stages of sleep, in flies that were otherwise entirely quiescent (apart from certain
597 micro-behaviors, which we discuss further below). These identified sleep stages align closely with
598 similar changes in brain activity dynamics observed in calcium imaging data in spontaneously sleeping
599 flies (Tainton-Heap et al. 2021). For example, in the calcium imaging data we showed that even before
600 sleep onset, the number of 'active' neurons is already different (lower) than wake; accordingly, in the
601 current electrophysiological data the classifiers predict sleep onset 2min before flies stop moving. This
602 also aligns with an older (single channel) electrophysiological sleep study in flies showing that brain
603 LFP activity becomes uncorrelated from behavior 5min before sleep onset (B. van Swinderen, Nitz, and
604 Greenspan 2004). Together, these findings make a compelling case for dissociative states in the fly
605 brain, which is consistent with the view that such states might also be changing within a sleep bout.
606

607 Our multichannel recordings also revealed that changes in sleep physiology are likely to encompass the
608 entire fly brain, from the optic lobes to the central complex. This is also consistent with other studies,
609 although this has not been previously demonstrated using a comprehensive multichannel approach.
610 An early study in honeybees showed that visually responsive neurons in the optic lobes become
611 unresponsive during sleep (Kaiser and Steiner-Kaiser 1983), and that these cells become rapidly
612 responsive again when bees are woken up with an air puff. Immunochemical studies investigating
613 synaptic proteins found that these were downregulated in the optic lobes during sleep (Donlea,
614 Ramanan, and Shaw 2009), as well as in the whole brain (Gilestro, Tononi, and Cirelli 2009). It is
615 understood that the insect optic lobes receive significant feedback from the central brain, as well as
616 from the contralateral lobes (Scheffer et al. 2020; Mu et al. 2012), and it has been shown that oscillatory
617 neural activity extends throughout the fly brain (Paulk et al. 2013), so our finding that the optic lobes
618 also ‘sleep’ is not quite surprising. Recent work using a similar multichannel recording preparation
619 found that isoflurane anesthesia impacted feedback from the central brain to the optic lobes (Cohen,
620 van Swinderen, and Tsuchiya 2018), suggesting that such efferent communication is a feature of the
621 waking fly brain. Yet, sleep in the central fly brain is different from sleep in the periphery.
622 Interestingly, only central channels were predictive of sleep onset, and only the central channels
623 revealed the 5-10Hz frequency features that we have previously identified in single channel recordings
624 (Yap et al. 2017). This suggests a sleep-regulatory role for the central complex, which aligns well with
625 previous studies (Donlea et al. 2011; Troup et al. 2018; Tainton-Heap et al. 2021).

626
627 Sleep in *Drosophila* was originally defined by inactivity criteria, based on locomotion-based readouts
628 (P. J. Shaw et al. 2000; Hendricks et al. 2000). Subsequent studies employing video monitoring and
629 probing arousal thresholds confirmed these simple readouts to be accurate estimates of sleep in flies
630 (van Alphen et al. 2013; Faville et al. 2015; Wiggin et al. 2020), but these behavioral studies also showed
631 that flies slept in distinct stages. Only recently has closer video monitoring of fly micro-behaviors
632 revealed that these animals are not entirely immobile during sleep (van Alphen et al. 2021), although
633 some micro-behaviors were already anecdotally observed in the first reports of fly sleep, such as
634 changes in posture (P. J. Shaw et al. 2000; Hendricks et al. 2000). Other insects, such as honeybees,
635 display characteristic micro-behaviors during sleep, such as changes in posture (Eban-Rothschild and
636 Bloch 2008) and antennal movements (Sauer et al. 2003). Interestingly, in our study we also found

637 evidence of altered antennal movements during fly sleep, alongside the previously reported proboscis
638 extensions (van Alphen et al. 2021). These micro-behaviors are not necessarily correlated, although
639 they do seem to be increased during mid-sleep epochs. PEs have been associated with a deep sleep
640 function (waste clearance) in a previous study (van Alphen et al. 2021), so their occurrence in rhythmic
641 spells during mid-sleep is consistent with that interpretation.

642

643 Interestingly, PEs during wake and sleep are electrophysiologically different, even though they are
644 behaviorally identical. We found that the neural signatures of PEs occurring during wake are
645 concentrated in the middle channels, and spread across all frequencies (5-40Hz). It is interesting to
646 note that these middle channels could coincide with the location of neuropils of the antennal
647 mechanosensory and motor center (AMMC). Several studies (Kain and Dahanukar 2015; Kim, Kirkhart,
648 and Scott 2017) have implicated the AMMC as the location of axons of gustatory projection neurons
649 (GPNs) and thus an immediate higher order processing center for taste. Other studies (Flood et al. 2013)
650 have also shown that persistent depolarization of motor command activity of the Fdg (feeding) neurons
651 could also result in PEs. In this context, it is pertinent to note that LFP activity during PE events in the
652 awake periods are higher than those in the 'awake' periods without PE events, suggesting a distinct PE
653 signature. But this is not the case for the exact same behaviors during sleep. We found that LFP activity
654 for PEs occurring during sleep bouts are concentrated instead in the central channels and engage
655 primarily the higher frequencies (32-40 Hz). This suggests a distinct control mechanism for PEs
656 occurring during sleep versus wake, with central brain circuits potentially involved in regulating this
657 sleep-related function.

658

659 There are obviously several drawbacks to studying sleep physiology in a tethered animal that has been
660 skewed by a recording electrode. Sleep cannot be quite normal in such a preparation. For example, it
661 is possible that the damage caused by the electrode evokes an increased need for repair (Stanhope et al.
662 2020) and consequently waste clearance (van Alphen et al. 2021), thus increased PE behavior.
663 However, this would also be the case for windows in the brain created for calcium imaging (Tainton-
664 Heap et al. 2021) (and in the latter scenario the proboscis is typically glued in place to prevent brain
665 motion artifacts), so no fly brain recording preparation (yet) can realistically sidestep these concerns.

666 Nevertheless, it is evident that even in this somewhat contrived context, flies do still sleep and their
667 sleep displays unequivocal evidence of distinct stages.

668

669 Our study also paves the way for asking fundamental questions about fly sleep in the following fashion.
670 First, the LFP activity of mutant strains (with higher, or lower baseline sleep) could be recorded and
671 its differences across the wild type could be quantified. Second, for understanding and probing the
672 exact spatial patterns of specific sleep stages identified in this study with higher resolution, 2-photon
673 imaging at the whole brain level could be recorded for longer duration (controlled by closed loop
674 detection of events), while optimizing for signal loss with photo bleaching. Third, closed loop
675 techniques could be employed to disrupt sleep either at the PE stage or at other relevant stages to
676 identify behavioral phenotypes, thereby providing casual evidence for function of the specific stage.

677

678 Our multichannel data add to the growing realization that the entire insect brain engages in dynamical
679 patterns of activity during both sleep and wake (Tainton-Heap et al. 2021; Troup, Tainton-Heap, and
680 van Swinderen 2023), and does not simply shut off when insects become immobile or quiescent. To
681 understand these patterns of activity and how they might relate to conserved sleep functions (Van De
682 Poll and van Swinderen 2021) requires novel approaches derived from machine learning, as done in
683 this study, rather than approximations inspired from human EEG.

684

685

686

687

688

689

690

691

692

693

694

695

696 **Materials and Methods**

697 *Animals.*

698 Flies (*Drosophila melanogaster*) were reared on a standard fly medium under a 12h light/dark cycle
699 (lights on at 8 A.M). Flies were raised on a 25°C incubator (Tritech research inc) with 50-60% humidity
700 and fewer than 5 flies were maintained per vial to ensure optimal nutrition and growth. Adult female
701 flies (<3 days post eclosion) of wild-type Canton-S (CS) were used for the electrophysiological
702 recordings. The choice of age of flies was based on pilot data that suggested a higher survival rate of
703 younger flies over a 12h period on the air supported ball setup (after electrode insertion). Flies used for
704 the behavioral dataset were between 3 - 7 days post eclosion. For thermogenetic experiments refer to
705 (Yap et al. 2017) for further details.

706

707 *Fly tethering.*

708 First, flies were anesthetized on a thermoelectric cooled-block maintained at a temperature of 1-2°C.
709 Second, the thorax, dorsal surface and wings of the fly were glued to a tungsten rod using dental cement
710 (Coltene Whaledent Synergy D6 Flow A3.5/ B3) and cured using high intensity blue light (Radii Plus,
711 Henry Scheinn Dental) for about 30-40 sec. Further, dental cement was also applied to the necks to
712 stabilize them and prevent lateral movement of the head during electrode insertion (next section).
713 Third, to prepare the fly for the multichannel overnight recording, we placed a sharpened fine wire
714 made of platinum into the thorax (0.25 mm; A-M systems). The platinum rod serves as a reference
715 electrode and helps filter the noise originating from non-brain sources. The insertion of a platinum
716 electrode (while providing minimal discomfort to movement of animal) was done using a custom
717 holder with a micro-manipulator to enable targeted depth of insertion. For flies in the behavioral
718 dataset, the procedure was the same, except that no reference wire was inserted.

719

720 *Multichannel preparation.*

721 First, the tethered fly from the previous step was placed on an air supported ball (polystyrene) that
722 served as a platform for walking/rest. Humidified air was delivered to the fly using a tube below the
723 ball (also from the side) to prevent desiccation. Second, to record from half of the regions in the fly
724 brain (half-brain probe) we used a 16-electrode linear silicon probe (model no. A1x16-3 mm50-177;

725 NeuroNexus Technologies). Third, the probe was inserted into the eye of the fly laterally using a micro-
726 manipulator (Merzhauser, Wetzlar, Germany). The probe was inserted such that the electrode sites
727 faced the posterior side of the brain. The final electrode position (depth of insertion) was determined
728 using the polarity reversal procedure described below. For flies recorded in the behavioral dataset the
729 setup was similar, except that a custom chamber was lowered over the ball and fly to maintain a
730 humidified environment during recordings.

731

732 *Polarity reversal.*

733 Variability in spatial location of recording sites across different flies is a primary impediment when
734 comparing data across different flies. This occurs mainly due to the angle and depth of insertion of the
735 probe, both of which cannot be precisely controlled. To overcome this issue and to obtain comparable
736 recording sites across flies, we designed a novel paradigm using visual evoked potentials (*Figure S2*).

737 First, while the probe was being inserted from the periphery to the center of the brain, we used visual
738 stimuli (square wave of 3 sec in duration with 1Hz frequency) from a blue LED. When the visual stimuli
739 was displayed we simultaneously recorded the local field potentials from the 16 electrode sites. During
740 the initial stage of insertion, most of the electrodes are outside of the brain and only a few are inside
741 the eye, optic lobe. The recordings in the electrodes inside the eye, brain show a visual evoked potential
742 corresponding to the leading edge and the trailing edge of the square wave. Second, we move the probe
743 slowly towards the center of the brain so more of the electrode sites would now be inside the brain.
744 Third, we notice that some electrodes have a negative deflection and some have a positive deflection
745 with respect to the leading edge of the square wave. The electrodes in the eye, optic lobe regions display
746 a positive deflection and electrodes further to the central parts of the brain display a negative deflection.
747 However this polarity change usually happens in the electrodes that are coincident on the regions right
748 after the medulla. Fourth, for all flies we made sure that the polarity change coincided with the
749 electrodes 11-13 in order to establish consistency in terms of the spatial locations.

750

751

752

753

754

755 *Dye based localization.*

756 In order to identify the possible locations in the brain targeted by the electrodes, we used a three step
757 procedure. In the first stage, we used immunohistochemistry to identify the locations of electrodes
758 using a fluorescent dye and neuropils using antibodies against nc82 (presynaptic marker bruchpilot)
759 respectively. In the second stage, we used a registration procedure to map the dye locations to an EM
760 dataset (using nc82 images). In the third stage, we used principal component analysis to identify the
761 precise neuropils targeted.

762 *a) Immunohistochemistry.*

763 First, we labeled the probe with Texas red fluorescent dye conjugated to 10,000-Da mol mass dextran
764 dissolved in distilled water (Invitrogen) to identify the recording locations. Second, after removing the
765 flies from the tether, the brains were dissected in ice cold 1x phosphate buffer solution (PBS) and fixed
766 in 4% paraformaldehyde diluted in PBS-T (1× PBS, 0.2 Triton-X 100) for 20 minutes in dark to preserve
767 the fluorescence of the dye. Third, after fixation, tissues were washed 3 times with PBS-T (0.2% Triton
768 X-100 in PBS (PBST) with 0.01% sodium azide (Sigma Aldrich)) and blocked for 1 hour in 10 % Goat
769 Serum (Sigma Aldrich). Fourth, the brains were then incubated overnight in a primary antibody
770 solution (mouse anti-nc82 1:20 DSHB). Fifth, on the next day brains were washed 3 times with PBS-T
771 (10 min per wash) and incubated overnight in a secondary anti-body solution (1:250 goat anti-mouse
772 Alexa 647). Finally, the brain was washed in PBST and embedded in Vectashield and imaged using a
773 confocal microscope (Zeiss).

774 *b) Image registration.*

775 First, for each fly we used the nc82 image as source space to align to the JFRC2 template space (which
776 is a spatially calibrated version of JFRC (Jenett et al. 2012) from FlyLight). The registration process
777 involved two steps: i) rigid affine registration that roughly aligned the source image to the template
778 space with 12 degrees of freedom (translation, rotation, scaling). ii) non-rigid registration that allowed
779 different brain regions to move independently with a smoothness penalty. The entire process was
780 carried out using the CMTK plugin (Fiji toolbox) as described here (Ostrovsky, Cachero, and Jefferis
781 2013). Second, we then used the JFRC2 (light-level) registration as bridging registration to FAFB14
782 (EM dataset) using the natverse toolbox (Bates et al. 2020) and mapped both the nc82 images and the
783 dye locations to the FAFB14 space.

784 *c) Electrode localisation.*

785 The electrode dye locations inside the brain are usually visible as fragments (points) instead of a single
786 continuous (line) segment, mainly because the insertion of the probe causes the smearing of the dye on
787 the neuropils in the brain. In order to identify the precise locations of the recording electrodes in the
788 brain, we first used the points and performed principal component analysis to find the eigenvector or
789 line (1st principal component) that would have minimize the distance between the different points to
790 the line itself. This line could be thought of as the main path of the probe as it entered into the brain.
791 Next, we choose the innermost electrode as the projection of the innermost point (dye location)
792 projected onto the eigenvector. The rest of the recording electrode sites were obtained by sampling the
793 same eigenvector at intervals of 25 μm (which is the interelectrode distance on the probe) from the
794 innermost point.

795

796 *LFP recording.*

797 The LFP data from the 16-electrode probe was acquired using Tucker–Davis Technologies (Tucker-
798 Davis Technologies, US) multichannel data acquisition system at 25 kHz coupled with a RZ5 Bioamp
799 processor and RP2.1 enhanced real-time processor. Data was acquired and amplified using a pre-
800 amplifier (RA16PA/RA4PA Medusa PreAmp). The pre-amplifier used can only record data of up to 20
801 hours on a single charge cycle, hence we limited the recording of the LFP signals to 20 hour duration.
802 Further, as file sizes tend to be larger over longer recording periods, we recorded data in chunks of 1
803 hour which was automatically controlled via a MATLAB script.

804

805 *Video recording for flies on electrophysiology setup.*

806 The ball setup was illuminated with visible light, switched ON at 8 AM and switched OFF at 8 PM
807 (mimicking the light/dark cycle conditions in the incubator). Further, we used Infrared LEDs for
808 monitoring the movement of the fly on the ball (which allowed us to quantify movements under both
809 the light and the dark cycle. We recorded the fly in profile view with a digital camera from Scopetek
810 (DCM 130E) and to achieve optical magnification, we used a zoom lens (from Navitar). As done
811 previously (Yap et al. 2017), we removed the IR filter in front of the camera sensor, to allow for filming
812 under IR light, thereby achieving constant illumination under both day and night. We made a custom

813 script with Python (2.7.15), OpenCV (3.4.2.17), that allowed for recording videos automatically and
814 saving them in hourly intervals. The video was recorded with a resolution of 640 x 480 pixels at 30
815 frames per second using Xvid codec and further with additional metadata (time stamps in a csv file)
816 that allowed a later matching up of the LFP data with the video data.

817

818 *Video recording for flies on behavioral dataset setup.*

819 The camera in this setup was a Point Grey/Teledyne FLIR Firefly perpendicular to the fly, in addition
820 to an extra camera (ProMicroScan) placed on the trinocular output of a Nikon SZ7 stereomicroscope.
821 This second camera was used to record a close-up view of the head of the fly for the purposes of tracking
822 movements of the antennae. Illumination was as above with infrared LEDs and recordings were
823 obtained with the same Python scripts.

824

825 *Movement analysis.*

826 The fly movement was quantified with the video files using Python (3.6.1), OpenCV (3.4.9) in the
827 following manner. First, every video file (1 per hour of recording) was read frame by frame. Second,
828 for each frame, we clipped the image such that the main focus was on the fly while ignoring items in
829 the background. Third, we converted the color space for each frame from BGR to grayscale. Fourth,
830 we computed the 'deltaframe' as the absolute difference of the current frame with the previous frame.
831 Fifth, we thresholded the deltaframe using a custom defined threshold per fly and converted them into
832 binary. Sixth, we dilated the thresholded image and identified contours in the dilated image and looped
833 over the different contours selecting those above a specific threshold (area). Finally, we drew rectangles
834 around the contours above the threshold on the original (color) image to manually verify the
835 movement location. Only those frames that had contours above threshold were regarded as 'moved'
836 frames, other frames would be classified as 'still'. Thus, each frame would be either 0 (still) or 1 (moved).
837 In the next stage, we used the frame by frame movement data to identify segments of LFP data as 'sleep'
838 or 'awake' etc in the following fashion. First, we synced the LFP data with the video data by using the
839 time stamps in both the LFP data and video metadata (csv files). Second, we clipped both the LFP and
840 video data to the first 8 hours of recording. Though 23 flies survived for more than 24 hours, we only
841 used the first 8 hours to ensure that the fly's health was completely optimal (considering the

842 circumstances) in both the behavior and brain recordings. Further only 16 flies were used for the
843 analysis, as 7 of them had issues with calibration (noisy or no calibration) or abnormal activity (either
844 no sleep trials or very active). Third, we pruned movement data to ensure brief noise in movements
845 are avoided. Fourth, we identified the segments of data wherein the fly was immobile for more than 5
846 mins as ‘sleep’ and the segment immediately preceding 2 mins before the sleep data as ‘presleep’ and
847 the rest of the data as ‘awake’.

848

849

850 *LFP analysis.*

851 *a) Preprocessing.*

852 LFP data was analyzed with custom-made scripts in MATLAB (The MathWorks) using EEGLAB
853 toolbox (Delorme and Makeig 2004). The preprocessing steps were as follows: First, the binary data was
854 extracted for every hour from Tucker-Davis technology ‘tank’ file format to MATLAB ‘mat’ file format.
855 Second, the data were resampled to 250 Hz and bandpass filtered with zero phase shift between 0.5 and
856 40 Hz using hamming windowed-sinc FIR filter, further line noise at 50 Hz was removed using a notch
857 filter. Third, the hourly LFP data was saved to EEGLAB ‘.set’ file format. Fourth, the hourly LFP data
858 were interpolated in a linear way to avoid any discontinuities between the hourly segments of data.
859 Fifth, the movement data (see Movement analysis) was added to the EEGLAB file along with the start
860 and end time based on video data. Sixth, the multi-hour LFP data (along with the movement data) is
861 collated for the first 8 hours of the recording. Seventh, we created separate epochs based on movement
862 data into ‘sleep’, ‘presleep’, ‘awake’ (where preceding 2 mins of immobility (-2 to 0 mins) is ‘presleep’
863 and immobility is ‘sleep’ and the rest of the data is ‘awake’, here 0 mins is the start of the immobility).
864 Eighth, the epochs were now re-referenced based on the channel where the polarity reversal occurred.
865 For this we identified the channel wherein the polarity reversal occurred (see Polarity reversal section)
866 and subtracted all the channels from this channel, thus resulting in 15 channels after the re-referencing.
867 This brain based referencing technique (similar to the Cz based reference in human EEG recordings)
868 allows for filtering of non-brain based physiological noise (like heartbeat etc). Previous multichannel
869 recordings used only the thorax based referencing (followed by bipolar referencing) along with
870 Independent Component Analysis (ICA) to remove physiological noises. However, the identification

871 of noise components like heartbeat etc from ICA is subjective and further depends on the expertise of
872 the human curator. Our technique overcomes these issues while simultaneously providing a method to
873 remove physiological noises not originating from the brain.

874 *b) Power spectrum analysis (sleep vs awake).*

875 The power spectra of the LFP data was computed for each fly in the following fashion. First, each
876 condition ('awake', 'sleep' etc) of varying duration was re-epoched into trials of 60 sec duration. Second,
877 each trial was bandpass filtered with zero phase shift between 5 and 40 Hz using hamming windowed-
878 sinc FIR filter. Third, for each trial, power spectra (in decibels) was computed using the 'spectopo'
879 function in the EEGLAB toolbox in MATLAB. Fourth, the mean power spectra for all the trials per
880 condition per fly was computed. The goal of the power spectra analysis was to identify the cluster of
881 frequency bands and channels that differ across the sleep, awake periods at the group level. To perform
882 these group level comparisons (sleep vs awake periods) we only used flies that had at least 10 trials
883 under each condition. We performed a cluster permutation test (flies x frequencies x channels) using
884 MNE (0.22.0) in python (permutation_cluster_1samp_test) (Gramfort et al. 2013) with all possible
885 permutations to identify clusters that differ across awake and sleep periods. We also computed the
886 effect sizes for every channel x frequency combination using cohen's d measure (difference of means/
887 standard deviation).

888

889 *Thermogenetic sleep induction.*

890 The thermogenetic sleep induction data was collected using 104y-Gal4 lines as part of the study (Yap
891 et al. 2017). This multichannel recording consisted of a 16-electrode full-brain probe (model no. A1x16-
892 3mm50-177; NeuroNexus Technologies) covering the whole of the brain (*Figure S6B*) (in contrast to
893 the half-brain probe mentioned before) with interelectrode distance of 50 μ m. The rest of the recording
894 parameters were the same as mentioned in the previous section. Sleep induction was achieved by
895 transient circuit activation of the sleep promoting circuit innervating the dorsal fan shaped body (dFB).
896 For example, this was done by using the 104y gal4 lines (offering cell type specificity in the dFB regions)
897 to control the expression of UAS driven TrpA1 (temperature sensitive cation channel), thereby
898 allowing for the activation of the specific neurons in dFB with temperature changes. As described in
899 (Yap et al. 2017), before the induction of sleep, the baseline activity was recorded in the 'baseline'

900 condition for 3 secs, followed by stimulation in the ‘sleep induction’ condition for 3 secs before
901 returning to recovery for 3 secs.

902 *a) Preprocessing.*

903 LFP data was analyzed with custom-made scripts in MATLAB (The MathWorks) using EEGLAB as
904 mentioned before. The preprocessing steps were as follows: First, the LFP data per condition (‘baseline’,
905 ‘sleep induction’, ‘recovery’) was converted to EEGLAB ‘.set’ file format with a sampling rate of 1 KHz.
906 Second, the LFP data was re-referenced using a differential approach, wherein nearby channels are
907 subtracted with each other resulting in 15 channels.

908 *b) Power spectrum analysis (baseline vs sleep induction).*

909 The power spectra of the LFP data was computed for each fly in the following fashion. First, each
910 condition (‘baseline’, ‘sleep induction’ etc) was reepoched into trials of 1 sec duration. Second, each
911 trial was bandpass filtered with zero phase shift between 5 and 40 Hz using hamming windowed-sinc
912 FIR filter. Third, for each trial, power spectra (in decibels) was computed using the ‘spectopo’ function
913 in the EEGLAB toolbox in MATLAB. Fourth, the mean power spectra for all the trials per condition
914 per fly was computed. The group level comparison was performed using cluster permutation test
915 methods (as described in previous sections) to identify differences in frequency x channels across
916 ‘preheat’ and ‘heaton’ conditions.

917

918

919

920 *Sleep staging by classifiers.*

921 The main goal of this analysis was to use classifiers to identify the existence of sleep stages using LFP
922 data.

923 *a) Labeling of sleep states.*

924 Here, we relabelled the segments of data (already identified as ‘sleep’, ‘awake’ based on movement data)
925 in the following fashion. First, we labeled the segments of data in the first 2 mins (0 to 2 mins) after
926 the start of immobility as ‘earlysleep’ and the segments of the data in the preceding 2 mins (-2 to 0
927 mins) as ‘presleep’. Second, we labeled the segments of data in the last 2 mins of sleep as ‘latesleep’ and

928 the segments of data in between the ‘earllysleep’ and ‘latesleep’ as ‘midsleep’. The rest of the data is
929 considered as ‘awake’.

930 *b) Preprocessing & power spectrum computation.*

931 The preprocessing steps were the same as mentioned in the previous section (LFP preprocessing). For
932 the computation of the power spectrum, we followed similar procedures as mentioned before, however
933 we saved the individual power spectrum per trial (channels x frequency) per fly in a csv file along with
934 the corresponding label of the sleep state.

935 *c) Classifier probability analysis.*

936 We implemented a support vector machine (svm) based classifier using scikit-learn (0.24.2) to classify
937 the LFP data using the following steps. First, we collated the features based on power spectrum
938 (channels x frequency) from all the flies across different sleep states. Second, we filtered the features to
939 only ‘awake’ (5106 epochs) and ‘midsleep’ (1165 epochs) states. Here, we also did not feed (for training)
940 the preceding 2 mins of ‘presleep’ and succeeding 2 mins of ‘earllysleep’ and the last 2 mins of sleep
941 ‘latesleep’ into the classifier (we used those minutes for sanity check purposes - Refer to Figure 5A).
942 Third, we encoded the target labels (‘awake’, ‘midsleep’) into binary states using ‘LabelEncoder’ from
943 scikit-learn. Fourth, we balanced the composition of labels (or classes) to prevent bias due to unequal
944 distribution of classes in the training dataset. Fifth, we divided the dataset into train and test sets (80%
945 train, 20% test) using ‘train_test_split’ from scikit-learn in a stratified fashion. Sixth, we subjected both
946 the train and test data to a standard scaler using ‘StandardScaler’ from scikit-learn, which removes the
947 mean of the data and scales it by the variance. Seventh, we implemented a svm based classifier using a
948 ‘linear’ kernel along with probability estimates per class and fit the classifier to the train dataset. Eighth,
949 we used the trained classifier on the test dataset and computed different metrics of classifier
950 performance like accuracy, roc_auc, recall, precision, f1-score etc using ‘metrics’ from scikit-learn
951 (*Figure S7B*). Ninth, we used the trained classifier on all class labels (‘awake’, ‘presleep’, ‘earllysleep’,
952 ‘midsleep’, ‘latesleep’, preceding 2 mins of ‘presleep’ and succeeding 2 mins of ‘latesleep’) from the
953 original dataset and computed the probability estimates per class. It is pertinent to note that none of
954 the ‘presleep’, ‘earllysleep’, ‘latesleep’, preceding 2 mins of ‘presleep’ and succeeding 2 mins of ‘latesleep’
955 the data have not been seen by the classifier beforehand. The above process from Step 5 onwards is

956 repeated a further 4 times with different test, train splits to create five different iterations of classifiers
957 and performance metrics.

958

959 *d) Multiclass svm analysis & Feature importance.*

960 To identify differences across multiple classes ('awake', 'presleep', 'earlysleep', 'midsleep', 'latesleep')
961 we implemented a random forest classifier using scikit-learn (0.24.2) to classify the LFP data using the
962 following steps. First, we collated the features based on power spectrum (channels x frequency) from
963 all the flies across different sleep states. Second, as the different labels (or classes) were unbalanced viz:
964 'awake'(5585 epochs), 'presleep'(258 epochs), 'earlysleep'(262 epochs), 'midsleep' (1165 epochs),
965 'latesleep' (262 epochs), we used SMOTE (Synthetic Minority Over-sampling Technique) from
966 imblearn (0.8.1) to balance the distribution of classes in the dataset. Third, we divided the dataset into
967 train and test sets (80% train, 20% test) using 'train_test_split' from scikit-learn in a stratified fashion.
968 Fourth, we subjected both the train and test data to a standard scaler using 'StandardScaler' from scikit-
969 learn, as mentioned in the previous section. Fifth, we encoded the target labels into binary states using
970 'LabelBinarizer' from scikit-learn. Sixth, we implemented a random forest classifier for this multiclass
971 classification problem. As the random forest classifier has multiple hyperparameters that need to be
972 tuned, we first used a random grid (using 'RandomizedSearchCV' from scikit-learn) to search for the
973 hyperparameters and then further used these parameters in a grid search model (using 'GridSearchCV'
974 from scikit-learn) to identify the best hyperparameters. Seventh, we used the trained classifier on the
975 test dataset and computed different metrics of classifier performance like recall, precision, f1-score etc
976 using 'metrics' from scikit-learn separately for all the 5 classes. Furthermore, we also computed a
977 normalized confusion matrix using 'confusion_matrix' from scikit-learn. The above process from Step
978 5 onwards is repeated a further 4 times with different test, train splits to create five different iterations
979 of classifiers and performance metrics. Finally to identify and rank the importance of different features
980 we utilized the permutation importance metric (using 'permutation_importance' from scikit-learn).
981 The permutation feature importance works by randomly shuffling a single feature value and further
982 identifying the decrease in the model score (Breiman 2001). The process breaks the relationship
983 between the shuffled feature and the target, thus if the feature is very important, it would be indicated
984 by a high drop in model score, on the other hand if it is relatively unimportant, then the model score
985 would not be affected so much. We used the permutation importance with a repeat of 5, and for each

986 train/test split we computed a permutation importance score. Finally, the mean permutation
987 importance score was computed using all the splits.

988 *e) Classifier metrics.*

989 The performance of the above-mentioned classifiers (both SVM based, random forest based) was
990 evaluated using metrics like accuracy, recall, precision, roc_auc, f1-score. The definition of these
991 metrics are as follows:

992 *Recall:* This refers to the ability of a classifier to correctly detect the true class of the epoch among the
993 classifications made. It is obtained by the $(TP/TP + FN)$. It is also known as sensitivity. TP: True
994 Positives, FN: False Negatives.

995 *Precision:* This refers to the exactness of the classifier. It is obtained by the $(TP/TP + FP)$. TP: True
996 Positives, FP: False Positives.

997 *F1-score:* This refers to the harmonic mean between precision and recall.

998 *roc_auc:* This refers to the area under the receiver operating curve. In general, it refers to how efficient
999 the classifier is in identifying different epochs. Scores closer to 1 indicate a highly efficient classifier
1000 whereas those closer to 0 indicate otherwise.

1001 *Accuracy:* This is defined as the number of correctly classified epochs divided by the overall number
1002 of epochs classified.

1003 *Confusion matrix:* This enables visualization of the classifier performance, by tabulating the predicted
1004 classes against actual classes. For multiclass problems (random forest classifiers here), the values in the
1005 diagonal indicate where the predicted and actual classes converge, whereas those on the off-diagonal
1006 indicate misclassifications.

1007

1008 *Proboscis tracking for flies on electrophysiology setup.*

1009 *a) Pose detection.*

1010 We used DeepLabCut (Mathis et al. 2018) to track the different body parts of the fly using an artificial
1011 neural network trained in the following fashion. First, we extracted frames from: sample videos
1012 wherein the fly performs the following: normal walking movement on the ball ('all_body'), proboscis
1013 extension periods ('proboscis') both while asleep and awake. For each fly we extracted videos of the
1014 above mentioned categories for the purpose of creating annotation labels. Second, we extracted frames

1015 from these videos and further labeled the different body parts: eye, proboscis, leg1_tip, leg1_joint,
1016 leg3_tip, leg3_joint, abdomen (Figure 7). Third, we trained the neural network per fly using this dataset
1017 with ‘resnet_50’ weights until the loss parameter during training stabilizes. The performance of the
1018 network per fly (train, test error in pixels) was in general similar in both the train and test datasets.
1019 Fourth, we evaluated the annotation performance manually by labeling a test video and verifying the
1020 same. Finally, this trained network (per fly) was used for annotating the video for the first 9 hours of
1021 the recording.

1022 *b) Pose analysis.*

1023 In the next step, we use the pose detection output to design a classifier capable of identifying proboscis
1024 extension periods. First, we manually detected several sample time points (to be used as ground truth
1025 for training/testing the classifier) in the video of each fly and identified proboscis time periods and
1026 saved them in a ‘csv’ file. Second, we used the pose tracking data (x,y,likelihood) for the body parts of
1027 the proboscis, leg1_tip, leg1_joint, eye, abdomen and further computed low pass filtered data (0.1 Hz
1028 butterworth filter) of each body part. Further we also computed the moving average (window length
1029 of 5 samples) of the filtered data. Third, we computed ‘dist_eyeprob’ as the euclidean distance between
1030 the proboscis and eye body part and finally multiplied the same with the likelihood of the proboscis
1031 body part. Fourth, we used the above-mentioned body parts (and its derivatives) as features and used
1032 the ‘StandardScaler’ from scikit-learn for normalizing the data. Fifth, we divided the dataset into train
1033 and test sets (70% train, 30% test) using ‘train_test_split’ from scikit-learn. Sixth, we implemented a
1034 svm based classifier using a ‘rbf’ kernel and fit the classifier to the train dataset. Seventh, we used the
1035 trained classifier on the test dataset and computed different metrics of classifier performance like
1036 accuracy, recall, precision etc using ‘metrics’ from scikit-learn. The data segments (frames) identified
1037 here will be used to construct the candidate proboscis periods, which then will be further refined in
1038 the next steps.

1039 *c) Proboscis detection.*

1040 First, we use the frames identified by the classifier from the previous section and construct continuous
1041 segments to identify time periods of probable proboscis periods. Further, we add additional time periods
1042 by using the likelihood of the proboscis part with a threshold based method. Second, we identify the
1043 peak frame (where the maximum displacement of the proboscis occurs) in each proboscis extension

1044 event (each proboscis bout consists of multiple proboscis extension events) and save the identified
1045 proboscis events (frame number, time, behavior state) to a 'csv' file. Third, each event in the csv file is
1046 manually verified and only true events are further taken forward. This process is repeated for all the
1047 flies and the proboscis detection accuracy per fly is plotted in Figure 7C.

1048

1049 *Micro-behaviour tracking for flies on behavioral dataset setup.*

1050 Here, the same method for tracking micro-behaviors via DeepLabCut was used, focusing on the
1051 proboscis and abdomen for the lateral camera view (See above), and the base and tip of the left and
1052 right antennae for the dorsal view of the fly head. The data from these two streams was imported into
1053 a custom MATLAB (2020a) script, which performed synchronization based on the integrated
1054 timestamps. After preprocessing, antennal tracking with DeepLabCut was converted into an angle for
1055 both respective antennae by calculation of the respective positions of the bases and tips, with the angle
1056 of the fly's head with respect to the camera automatically derived from this data and used to correct
1057 the angle of the antennae. For the proboscis a median position was calculated for each recording -
1058 assumed to be the resting position - and the distance and angle between the proboscis at any given time
1059 point and this median position was calculated. Extensions of the proboscis were derived from this
1060 distance data with the 'findpeaks' function in MATLAB, with a number of exclusion criteria applied to
1061 remove tracking artifacts. For example, detected peaks that exceeded a biologically plausible distance
1062 threshold, lasted only for a single frame, or had an implausible instantaneous rise time were excluded.
1063 Since this method could potentially be biased towards identifying proboscis activity that follows a
1064 prototypical shape, we also employed an alternative proboscis event detection based purely on the
1065 current distance of the proboscis from resting. In this we used a manually set threshold for each fly to
1066 detect portions in the recording when the proboscis was extended versus not, and for these 'events' we
1067 calculated the duration and median angle of the proboscis during the span of the event. Periods of
1068 antennal periodicity in recordings were calculated based on a Fast Fourier Transform (FFT), applied to
1069 time segments of recordings. Since proboscis activity was not sinusoidal in nature (and thus would
1070 behave poorly if subjected to an FFT), periodicity for this organ was calculated manually as a factor of
1071 timing between individual PEs in that proboscis extensions were periodic if they occurred less than 6s
1072 after a preceding proboscis extension. This value was selected from observation of typical inter-PE
1073 intervals.

1074

1075 *LFP analysis - proboscis.*

1076 The main goal of this analysis was to identify the spectral signatures associated with the proboscis
1077 extension periods across ‘awake’ and ‘sleep’ states in the LFP data.

1078 a) *Identification of proboscis periods.*

1079 First, we used the csv file containing frame by frame detection of manually verified proboscis events
1080 (from the section above). Second, we identify periods of proboscis extensions which are close together
1081 (within 10 sec of each other) and label them as continuous periods. Third, we add activity labels like
1082 ‘awake’ (awake periods without any proboscis activity), ‘awakeprob’ (awake periods with proboscis
1083 activity), ‘sleep’ (sleep periods without any proboscis activity), ‘sleepprob’ (sleep periods with proboscis
1084 activity), ‘presleep’ (presleep periods without any proboscis activity), ‘presleepprob’ (presleep periods
1085 with proboscis activity) based on annotated behaviors. Fourth, we extract the LFP data corresponding
1086 to the different time periods across each fly.

1087 b) *Power spectrum analysis.*

1088 The preprocessing steps for the extracted LFP data were the same as mentioned in the previous section
1089 (LFP preprocessing). For the computation of the power spectrum, we followed similar procedures as
1090 mentioned before, however we computed the individual power spectrum per trial (channels x
1091 frequency) per fly by re-epoching them into trials of 1 sec in duration (instead of the 60 sec periods for
1092 sleep analysis, as the proboscis periods are usually shorter). Then the mean power spectrum for all the
1093 trials per condition per fly was computed. Next, we performed cluster permutation tests (flies x
1094 frequencies x channels) for identifying the differences across frequencies and channels across different
1095 conditions. For this analysis we only used flies that had at least 50 trials under each condition.

1096

1097 *Multilevel models.*

1098 a) *Models for antennal, proboscis periodicity.*

1099 We defined 2 different multilevel models (*Supplementary Table 1,3,5 - left, right antenna, proboscis*)
1100 to understand how the likelihood of periodicity varies by sleep epoch. In the null model, the periodicity
1101 depends only on the mean per fly (fixed effect) and the fly ID (random effect). In the second model
1102 (epoch model), the periodicity depends only on the epoch (fixed effect) and the fly ID (random effect).

1103 These models were fit using the ‘lmer’ function (‘lmerTest’ package) in R (Kuznetsova, Brockhoff, and
1104 Christensen 2017) and the winning model is identified as the one with the highest log-likelihood by
1105 comparing it with the null model, and performing a likelihood ratio chi-square test (χ^2). Finally the
1106 winning model was analyzed using the ‘anova’ function (*Supplementary Table 2,4,6 - left, right*
1107 *antenna, proboscis*) in R (Fox and Weisberg 2018).

1108 *b) Models for spectral analysis.*

1109 We defined 4 different multilevel models (*Supplementary Table 7*) to understand the modulation of
1110 the power spectrum by sleep epoch and channel type. In the null model, the power spectrum depends
1111 only on the mean per fly (fixed effect) and the fly ID (random effect). In the second model (epoch
1112 model), the power spectrum depends only on the LFP epoch type (fixed effect) and the fly ID (random
1113 effect). In the third model (channel model), the power spectrum depends only on the channel type
1114 (fixed effect) and the fly ID (random effect). In the fourth model (epoch-channel model), the power
1115 spectrum depends on a combination of the LFP epoch type and the channel type, both used as fixed
1116 effects, and the fly ID (random effect). These four models were fit using the ‘lmer’ function (‘lmerTest’
1117 package) in R (Kuznetsova, Brockhoff, and Christensen 2017) and the winning model is identified as
1118 the one with the highest log-likelihood by comparing it with the null model, and performing a
1119 likelihood ratio chi-square test (χ^2). Finally the top two winning models were compared against each
1120 other using ‘anova’ function in R (Fox and Weisberg 2018), to validate whether the winning model (if
1121 it is more complex) is actually better than the losing model (if it is simpler). The epoch-channel model
1122 emerged as the winning model, indicating an important contribution from different channels. The
1123 epoch-channel was further analyzed with the ‘anova’ function (*Supplementary Table 8*) in R (Fox and
1124 Weisberg 2018)

1125 *c) Models for PE event counts.*

1126 We defined 2 different multilevel models (*Supplementary Table 9*) to understand the modulation of PE
1127 event count by sleep epochs. In the null model, the PE event count depends only on the mean per fly
1128 (fixed effect) and the fly ID (random effect). In the second model (time_label model), the PE event
1129 count depends only on the specific temporal sleep stage (fixed effect) and the fly ID (random effect).
1130 These 2 models were fit using the ‘lmer’ function (‘lmerTest’ package) in R (Kuznetsova, Brockhoff,
1131 and Christensen 2017) and the winning model is identified as the one with the highest log-likelihood

1132 by comparing it with the null model, and performing a likelihood ratio chi-square test (χ^2). Thus, the
1133 time_label model emerged as the winning model. The time_label model was further analyzed with the
1134 ‘anova’ function (*Supplementary Table 10*) in R (Fox and Weisberg 2018)

1135

1136

1137 **AUTHOR CONTRIBUTIONS**

1138 Conceptualization: S.R.J., R.J., B.v.S.;

1139 Data Curation: S.R.J., R.J., and M.V.D.P.;

1140 Formal Analysis: S.R.J., and M.V.D.P.;

1141 Investigation: R.J., and M.V.D.P.;

1142 Visualization: S.R.J., and M.V.D.P.;

1143 Software: S.R.J.;

1144 Writing – original draft: S.R.J., and B.v.S.;

1145 Writing – review & editing: S.R.J., R.J., M.V.D.P., B.v.S.;

1146 Methodology: S.R.J., R.J., M.V.D.P., B.v.S.;

1147 Resources: R.J., M.V.D.P., B.v.S.;

1148 Project Administration: S.R.J., R.J., B.v.S.;

1149 Supervision: B.v.S.;

1150 Funding acquisition: S.R.J., and B.v.S.;

1151

1152 **Acknowledgements**

1153 The authors would like to thank Dr. Deniz Ertekin for performing immunostaining to identify
1154 electrode locations. This work was supported by the Gates Cambridge Scholarship, fieldwork funding
1155 from the Department of Psychology to S.R.J.; NIH RO1 NS076980-01 to BvS and Paul Shaw
1156 (Washington University); NHMRC GNT1164499 to BvS.

1157 We thank members of the van Swinderen lab for critical discussions and Dr. Melvyn Yap for initial
1158 code/dataset sharing. S.R.J is grateful to Dr. Tristan Bekinschtein for his continuous encouragement
1159 and support which he received while shifting to the field of fly neurobiology.

1160

1161

1162 References

1163

- 1164 Alphen, Bart van, Evan R. Semenza, Melvyn Yap, Bruno van Swinderen, and Ravi Allada. 2021. “A Deep
1165 Sleep Stage in *Drosophila* with a Functional Role in Waste Clearance.” *Science Advances*.
1166 <https://doi.org/10.1126/sciadv.abc2999>.
- 1167 Alphen, Bart van, Melvyn H. W. Yap, Leonie Kirszenblat, Benjamin Kottler, and Bruno van Swinderen.
1168 2013. “A Dynamic Deep Sleep Stage in *Drosophila*.” *The Journal of Neuroscience: The Official Journal of*
1169 *the Society for Neuroscience* 33 (16): 6917–27.
- 1170 Anthoney, Niki, Lucy A. L. Tainton-Heap, Hang Luong, Eleni Notaras, Qiongzi Zhao, Trent Perry, Philip
1171 Batterham, Paul J. Shaw, and Bruno van Swinderen. 2023. “Experimentally Induced Active and Quiet
1172 Sleep Engage Non-Overlapping Transcriptomes in.” *bioRxiv : The Preprint Server for Biology*, April.
1173 <https://doi.org/10.1101/2023.04.03.535331>.
- 1174 Bates, Alexander Shakeel, James D. Manton, Sridhar R. Jagannathan, Marta Costa, Philipp Schlegel,
1175 Torsten Rohlfing, and Gregory Sxe Jefferis. 2020. “The Natverse, a Versatile Toolbox for Combining and
1176 Analysing Neuroanatomical Data.” *eLife* 9 (April). <https://doi.org/10.7554/eLife.53350>.
- 1177 Besedovsky, Luciana, Tanja Lange, and Jan Born. 2012. “Sleep and Immune Function.” *Pflügers Archiv -*
1178 *European Journal of Physiology*. <https://doi.org/10.1007/s00424-011-1044-0>.
- 1179 Breiman, Leo. 2001. “Random Forests.” *Machine Learning* 45 (1): 5–32.
- 1180 Bushey, Daniel, Giulio Tononi, and Chiara Cirelli. 2015. “Sleep- and Wake-Dependent Changes in
1181 Neuronal Activity and Reactivity Demonstrated in Fly Neurons Using in Vivo Calcium Imaging.”
1182 *Proceedings of the National Academy of Sciences of the United States of America* 112 (15): 4785–90.
- 1183 Cirelli, Chiara, and Giulio Tononi. 2008. “Is Sleep Essential?” *PLoS Biology*.
1184 <https://doi.org/10.1371/journal.pbio.0060216>.
- 1185 Cohen, Dror, Bruno van Swinderen, and Naotsugu Tsuchiya. 2018. “Isoflurane Impairs Low-Frequency
1186 Feedback but Leaves High-Frequency Feedforward Connectivity Intact in the Fly Brain.” *eNeuro* 5 (1).
1187 <https://doi.org/10.1523/ENEURO.0329-17.2018>.
- 1188 Cohen, Dror, and Naotsugu Tsuchiya. 2018. “The Effect of Common Signals on Power, Coherence and
1189 Granger Causality: Theoretical Review, Simulations, and Empirical Analysis of Fruit Fly LFPs Data.”
1190 *Frontiers in Systems Neuroscience* 12 (July): 30.
- 1191 Cohen, Dror, Oressia H. Zalucki, Bruno van Swinderen, and Naotsugu Tsuchiya. 2016. “Local Versus
1192 Global Effects of Isoflurane Anesthesia on Visual Processing in the Fly Brain.” *eNeuro* 3 (4).
1193 <https://doi.org/10.1523/ENEURO.0116-16.2016>.
- 1194 Cortes, Corinna, and Vladimir Vapnik. 1995. “Support-Vector Networks.” *Machine Learning* 20 (3): 273–
1195 97.
- 1196 Dag, Ugur, Zhengchang Lei, Jasmine Q. Le, Allan Wong, Daniel Bushey, and Krystyna Keleman. 2019.
1197 “Neuronal Reactivation during Post-Learning Sleep Consolidates Long-Term Memory in.” *eLife* 8
1198 (February). <https://doi.org/10.7554/eLife.42786>.
- 1199 Delorme, Arnaud, and Scott Makeig. 2004. “EEGLAB: An Open Source Toolbox for Analysis of Single-Trial
1200 EEG Dynamics Including Independent Component Analysis.” *Journal of Neuroscience Methods* 134 (1): 9–
1201 21.
- 1202 Dement, W., and N. Kleitman. 1957. “Cyclic Variations in EEG during Sleep and Their Relation to Eye
1203 Movements, Body Motility, and Dreaming.” *Electroencephalography and Clinical Neurophysiology* 9 (4):
1204 673–90.
- 1205 Donlea, Jeffrey M., Narendrakumar Ramanan, and Paul J. Shaw. 2009. “Use-Dependent Plasticity in Clock

- 1206 Neurons Regulates Sleep Need in Drosophila.” *Science* 324 (5923): 105–8.
- 1207 Donlea, Jeffrey M., Matthew S. Thimgan, Yasuko Suzuki, Laura Gottschalk, and Paul J. Shaw. 2011.
- 1208 “Inducing Sleep by Remote Control Facilitates Memory Consolidation in Drosophila.” *Science* 332 (6037):
- 1209 1571–76.
- 1210 Eban-Rothschild, Ada D., and Guy Bloch. 2008. “Differences in the Sleep Architecture of Forager and
- 1211 Young honeybees(*Apis Mellifera*.)” *Journal of Experimental Biology*. <https://doi.org/10.1242/jeb.016915>.
- 1212 Faville, R., B. Kottler, G. J. Goodhill, P. J. Shaw, and B. van Swinderen. 2015. “How Deeply Does Your
- 1213 Mutant Sleep? Probing Arousal to Better Understand Sleep Defects in Drosophila.” *Scientific Reports* 5
- 1214 (February): 8454.
- 1215 Flood, Thomas F., Shinya Iguchi, Michael Gorczyca, Benjamin White, Kei Ito, and Motojiro Yoshihara.
- 1216 2013. “A Single Pair of Interneurons Commands the Drosophila Feeding Motor Program.” *Nature*.
- 1217 <https://doi.org/10.1038/nature12208>.
- 1218 Flores-Valle, Andres, and Johannes D. Seelig. 2022. “Dynamics of a Sleep Homeostat Observed in Glia
- 1219 during Behavior.” *bioRxiv*. <https://doi.org/10.1101/2022.07.07.499175>.
- 1220 Fox, John, and Sanford Weisberg. 2018. *An R Companion to Applied Regression*. SAGE Publications.
- 1221 Fulda, Stephany, Christoph P. N. Romanowski, Andreas Becker, Thomas C. Wetter, Mayumi Kimura, and
- 1222 Thomas Fenzel. 2011. “Rapid Eye Movements during Sleep in Mice: High Trait-like Stability Qualifies
- 1223 Rapid Eye Movement Density for Characterization of Phenotypic Variation in Sleep Patterns of Rodents.”
- 1224 *BMC Neuroscience* 12 (November): 110.
- 1225 Gilestro, Giorgio F., Giulio Tononi, and Chiara Cirelli. 2009. “Widespread Changes in Synaptic Markers as
- 1226 a Function of Sleep and Wakefulness in Drosophila.” *Science* 324 (5923): 109–12.
- 1227 Grabowska, Martyna J., Rhiannon Jeans, James Steeves, and Bruno van Swinderen. 2020. “Oscillations in
- 1228 the Central Brain of Are Phase Locked to Attended Visual Features.” *Proceedings of the National Academy*
- 1229 *of Sciences of the United States of America* 117 (47): 29925–36.
- 1230 Gramfort, Alexandre, Martin Luessi, Eric Larson, Denis A. Engemann, Daniel Strohmeier, Christian
- 1231 Brodbeck, Roman Goj, et al. 2013. “MEG and EEG Data Analysis with MNE-Python.” *Frontiers in*
- 1232 *Neuroscience* 7 (December): 267.
- 1233 Hendricks, J. C., S. M. Finn, K. A. Panckeri, J. Chavkin, J. A. Williams, A. Sehgal, and A. I. Pack. 2000.
- 1234 “Rest in Drosophila Is a Sleep-like State.” *Neuron* 25 (1): 129–38.
- 1235 Iglesias, Teresa L., Jean G. Boal, Marcos G. Frank, Jochen Zeil, and Roger T. Hanlon. 2019. “Cyclic Nature
- 1236 of the REM Sleep-like State in the Cuttlefish *Sepia Officinalis*.” *The Journal of Experimental Biology* 222
- 1237 (1): jeb174862.
- 1238 Jaggard, James B., Gordon X. Wang, and Philippe Mourrain. 2021. “Non-REM and REM/paradoxical Sleep
- 1239 Dynamics across Phylogeny.” *Current Opinion in Neurobiology* 71 (December): 44–51.
- 1240 Jenett, Arnim, Gerald M. Rubin, Teri-T B. Ngo, David Shepherd, Christine Murphy, Heather Dionne,
- 1241 Barret D. Pfeiffer, et al. 2012. “A GAL4-Driver Line Resource for Drosophila Neurobiology.” *Cell Reports* 2
- 1242 (4): 991–1001.
- 1243 Kain, Pinky, and Anupama Dahanukar. 2015. “Secondary Taste Neurons That Convey Sweet Taste and
- 1244 Starvation in the Drosophila Brain.” *Neuron* 85 (4): 819–32.
- 1245 Kaiser, Walter, and Jana Steiner-Kaiser. 1983. “Neuronal Correlates of Sleep, Wakefulness and Arousal in a
- 1246 Diurnal Insect.” *Nature* 301 (5902): 707–9.
- 1247 Kim, Heesoo, Colleen Kirkhart, and Kristin Scott. 2017. “Long-Range Projection Neurons in the Taste
- 1248 Circuit of Drosophila.” *eLife* 6 (February). <https://doi.org/10.7554/eLife.23386>.
- 1249 Kirszenblat, Leonie, and Bruno van Swinderen. 2015. “The Yin and Yang of Sleep and Attention.” *Trends*
- 1250 *in Neurosciences* 38 (12): 776–86.
- 1251 Kuznetsova, Alexandra, Per B. Brockhoff, and Rune H. B. Christensen. 2017. “lmerTest Package: Tests in
- 1252 Linear Mixed Effects Models.” *Journal of Statistical Software* 82 (December): 1–26.
- 1253 Leung, Angus, Dror Cohen, Bruno van Swinderen, and Naotsugu Tsuchiya. 2021. “Integrated Information
- 1254 Structure Collapses with Anesthetic Loss of Conscious Arousal in Drosophila *Melanogaster*.” *PLoS*

- 1255 *Computational Biology* 17 (2): e1008722.
- 1256 Lin, Jian-Sheng, Perti Panula, and Maria Beatrice Passani. 2015. *Histamine in the Brain*. Frontiers Media
- 1257 SA.
- 1258 Mathis, Alexander, Pranav Mamidanna, Kevin M. Cury, Taiga Abe, Venkatesh N. Murthy, Mackenzie
- 1259 Weygandt Mathis, and Matthias Bethge. 2018. “DeepLabCut: Markerless Pose Estimation of User-Defined
- 1260 Body Parts with Deep Learning.” *Nature Neuroscience* 21 (9): 1281–89.
- 1261 Miller, Patrick J. O., Kagari Aoki, Luke E. Rendell, and Masao Amano. 2008. “Stereotypical Resting
- 1262 Behavior of the Sperm Whale.” *Current Biology: CB* 18 (1): R21–23.
- 1263 Mu, Laiyong, Kei Ito, Jonathan P. Bacon, and Nicholas J. Strausfeld. 2012. “Optic Glomeruli and Their
- 1264 Inputs in *Drosophila* Share an Organizational Ground Pattern with the Antennal Lobes.” *The Journal of*
- 1265 *Neuroscience: The Official Journal of the Society for Neuroscience* 32 (18): 6061–71.
- 1266 Muñoz, Roberto N., Angus Leung, Aidan Zecevik, Felix A. Pollock, Dror Cohen, Bruno van Swinderen,
- 1267 Naotsugu Tsuchiya, and Kavan Modi. 2020. “General Anesthesia Reduces Complexity and Temporal
- 1268 Asymmetry of the Informational Structures Derived from Neural Recordings in *Drosophila*.” *Physical*
- 1269 *Review Research* 2 (2): 023219.
- 1270 Nitz, Douglas A., Bruno van Swinderen, Giulio Tononi, and Ralph J. Greenspan. 2002.
- 1271 “Electrophysiological Correlates of Rest and Activity in *Drosophila Melanogaster*.” *Current Biology: CB* 12
- 1272 (22): 1934–40.
- 1273 Ostrovsky, Aaron, Sebastian Cachero, and Gregory Jefferis. 2013. “Clonal Analysis of Olfaction in
- 1274 *Drosophila*: Image Registration.” *Cold Spring Harbor Protocols* 2013 (4): 347–49.
- 1275 Paulk, Angelique C., Leonie Kirszenblat, Yanqiong Zhou, and Bruno van Swinderen. 2015. “Closed-Loop
- 1276 Behavioral Control Increases Coherence in the Fly Brain.” *The Journal of Neuroscience: The Official*
- 1277 *Journal of the Society for Neuroscience* 35 (28): 10304–15.
- 1278 Paulk, Angelique C., Yanqiong Zhou, Peter Stratton, Li Liu, and Bruno van Swinderen. 2013.
- 1279 “Multichannel Brain Recordings in Behaving *Drosophila* Reveal Oscillatory Activity and Local Coherence
- 1280 in Response to Sensory Stimulation and Circuit Activation.” *Journal of Neurophysiology* 110 (7): 1703–21.
- 1281 Raccuglia, Davide, Sheng Huang, Anatoli Ender, M-Marcel Heim, Desiree Laber, Raquel Suárez-Grimalt,
- 1282 Agustin Liotta, Stephan J. Sigrist, Jörg R. P. Geiger, and David Oswald. 2019. “Network-Specific
- 1283 Synchronization of Electrical Slow-Wave Oscillations Regulates Sleep Drive in *Drosophila*.” *Current*
- 1284 *Biology: CB* 29 (21): 3611–21.e3.
- 1285 Raccuglia, Davide, Raquel Suárez-Grimalt, Laura Krumm, Cedric B. Brodersen, Anatoli Ender, Sridhar R.
- 1286 Jagannathan, York Winter, et al. 2022. “Coherent Multi-Level Network Oscillations Create Neural Filters
- 1287 to Favor Quiescence over Navigation in *Drosophila*.” *bioRxiv*. <https://doi.org/10.1101/2022.03.11.483976>.
- 1288 Rasch, Björn, and Jan Born. 2013. “About Sleep’s Role in Memory.” *Physiological Reviews*.
- 1289 <https://doi.org/10.1152/physrev.00032.2012>.
- 1290 Rößler, Daniela C., Kris Kim, Massimo De Agrò, Alex L. Jordan, Cosmas Giovanni Galizia, and Paul S.
- 1291 Shamble. 2022. *Regularly Occurring Bouts of Retinal Movements Suggest an REM Sleep-like State in*
- 1292 *Jumping Spiders*.
- 1293 Sauer, S., M. Kinkelin, E. Herrmann, and W. Kaiser. 2003. “The Dynamics of Sleep-like Behaviour in
- 1294 Honey Bees.” *Journal of Comparative Physiology. A, Neuroethology, Sensory, Neural, and Behavioral*
- 1295 *Physiology* 189 (8): 599–607.
- 1296 Scheffer, Louis K., C. Shan Xu, Michal Januszewski, Zhiyuan Lu, Shin-Ya Takemura, Kenneth J. Hayworth,
- 1297 Gary B. Huang, et al. 2020. “A Connectome and Analysis of the Adult *Drosophila* Central Brain,”
- 1298 September. <https://doi.org/10.7554/eLife.57443>.
- 1299 Shafer, O. T., and A. C. Keene. 2021. “The Regulation of *Drosophila* Sleep.” *Current Biology: CB* 31 (1).
- 1300 <https://doi.org/10.1016/j.cub.2020.10.082>.
- 1301 Shaw, Paul J., Giulio Tononi, Ralph J. Greenspan, and Donald F. Robinson. 2002. “Stress Response Genes
- 1302 Protect against Lethal Effects of Sleep Deprivation in *Drosophila*.” *Nature* 417 (6886): 287–91.
- 1303 Shaw, P. J., C. Cirelli, R. J. Greenspan, and G. Tononi. 2000. “Correlates of Sleep and Waking in *Drosophila*

1304 Melanogaster.” *Science* 287 (5459): 1834–37.
1305 Siegel, J. M. 2001. “The REM Sleep-Memory Consolidation Hypothesis.” *Science* 294 (5544): 1058–63.
1306 Stanhope, Bethany A., James B. Jaggard, Melanie Gratton, Elizabeth B. Brown, and Alex C. Keene. 2020.
1307 “Sleep Regulates Glial Plasticity and Expression of the Engulfment Receptor Draper Following Neural
1308 Injury.” *Current Biology: CB* 30 (6): 1092–1101.e3.
1309 Swinderen, Bruno van, and Ralph J. Greenspan. 2003. “Salience Modulates 20–30 Hz Brain Activity in
1310 Drosophila.” *Nature Neuroscience* 6 (6): 579–86.
1311 Swinderen, B. van, D. A. Nitz, and R. J. Greenspan. 2004. “Uncoupling of Brain Activity from Movement
1312 Defines Arousal States in Drosophila.” *Current Biology: CB* 14 (2): 81–87.
1313 Tainton-Heap, Lucy A. L., Leonie C. Kirszenblat, Eleni T. Notaras, Martyna J. Grabowska, Rhiannon Jeans,
1314 Kai Feng, Paul J. Shaw, and Bruno van Swinderen. 2021. “A Paradoxical Kind of Sleep in Drosophila
1315 Melanogaster.” *Current Biology: CB* 31 (3): 578–90.e6.
1316 Tononi, Giulio, and Chiara Cirelli. 2014. “Sleep and the Price of Plasticity: From Synaptic and Cellular
1317 Homeostasis to Memory Consolidation and Integration.” *Neuron* 81 (1): 12–34.
1318 Troup, Michael, Lucy A. L. Tainton-Heap, and Bruno van Swinderen. 2023. “Neural Ensemble
1319 Fragmentation in the Anesthetized Brain.” *The Journal of Neuroscience: The Official Journal of the Society
1320 for Neuroscience* 43 (14): 2537–51.
1321 Troup, Michael, Melvyn Hw Yap, Chelsie Rohrscheib, Martyna J. Grabowska, Deniz Ertekin, Roshini
1322 Randeniya, Benjamin Kottler, et al. 2018. “Acute Control of the Sleep Switch in Reveals a Role for Gap
1323 Junctions in Regulating Behavioral Responsiveness.” *eLife* 7 (August). <https://doi.org/10.7554/eLife.37105>.
1324 Van De Poll, Matthew N., and Bruno van Swinderen. 2021. “Balancing Prediction and Surprise: A Role for
1325 Active Sleep at the Dawn of Consciousness?” *Frontiers in Systems Neuroscience* 15 (November): 768762.
1326 Walker, Matthew P., and Robert Stickgold. 2004. “Sleep-Dependent Learning and Memory Consolidation.”
1327 *Neuron* 44 (1): 121–33.
1328 Wiggin, Timothy D., Patricia R. Goodwin, Nathan C. Donelson, Chang Liu, Kien Trinh, Subhabrata
1329 Sanyal, and Leslie C. Griffith. 2020. “Covert Sleep-Related Biological Processes Are Revealed by
1330 Probabilistic Analysis in.” *Proceedings of the National Academy of Sciences of the United States of
1331 America* 117 (18): 10024–34.
1332 Xie, Lulu, Hongyi Kang, Qiwu Xu, Michael J. Chen, Yonghong Liao, Meenakshisundaram Thiyagarajan,
1333 John O’Donnell, et al. 2013. “Sleep Drives Metabolite Clearance from the Adult Brain.” *Science* 342 (6156):
1334 373–77.
1335 Yap, Melvyn H. W., Martyna J. Grabowska, Chelsie Rohrscheib, Rhiannon Jeans, Michael Troup,
1336 Angeliqne C. Paulk, Bart van Alphen, Paul J. Shaw, and Bruno van Swinderen. 2017. “Oscillatory Brain
1337 Activity in Spontaneous and Induced Sleep Stages in Flies.” *Nature Communications* 8 (1): 1815.

1338

1339

1340

1341

1342

1343

1344

1345

1346 Supplementary material

1347

1348 Suppl Table 1: Model comparison - Left antenna

Model	Parameters	Log-likelihood	Pr(> χ^2)
Null	Fixed: mean, Random: fly ID	-106.53	-
epoch	Fixed: time_label, Random: fly ID	-73.58	<0.001

1349

1350 Suppl Table 2: Type III analysis of variance with Satterthwaite's method of the winning model (Epoch)

1351 - Left antenna

Model elements	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Epoch	4.5527	1.1382	4	1090	16.985	<0.001

1352

1353 Suppl Table 3: Model comparison - Right antenna

Model	Parameters	Log-likelihood	Pr(> χ^2)
Null	Fixed: mean, Random: fly ID	-68.415	-
epoch	Fixed: time_label, Random: fly ID	-44.468	<0.001

1354

1355 Suppl Table 4: Type III analysis of variance with Satterthwaite's method of the winning model (Epoch)

1356 - Right antenna

Model elements	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Epoch	3.1004	0.7751	4	1125	12.232	<0.001

1357

1358

1359

1360

1361 Suppl Table 5: Model comparison - PEs

Model	Parameters	Log-likelihood	Pr(>χ^2)
Null	Fixed: mean, Random: fly ID	-301.09	-
epoch	Fixed: time_label, Random: fly ID	-207.25	<0.001

1362

1363 Suppl Table 6: Type III analysis of variance with Satterthwaite's method of the winning model (Epoch)

1364 - PEs

Model elements	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Epoch	20.877	5.2192	4	795	52.923	<0.001

1365

1366 Suppl Table 7: Model comparison - LFP power spectrum

Model	Parameters	Log-likelihood	Pr(>χ^2)
Null	Fixed: mean, Random: fly ID	-68117	-
Epoch	Fixed: epoch, Random: fly ID	-67941	<0.001
Channel	Fixed: channel, Random: fly ID	-52391	<0.001
Epoch-Channel	Fixed: epoch*channel, Random: fly ID	-51593	<0.001

1367

1368

1369

1370

1371

1372

1373

1374

1375

1376 Suppl Table 8: Type III analysis of variance with Satterthwaite's method of the winning model (Epoch-

1377 Channel) - LFP power spectrum

Model elements	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Epoch	8476	2119	4	22582	378.025	<0.001
Channel	112004	56002	2	22580	9990.441	<0.001
Epoch:Channel	796	100	8	22580	17.756	<0.001

1378

1379 Suppl Table 9: Model comparison - PEs LFP dataset

Model	Parameters	Log-likelihood	Pr(>χ^2)
Null	Fixed: mean, Random: fly ID	-4.5588	-
time_label	Fixed: time_label, Random: fly ID	15.0632	<0.001

1380

1381 Suppl Table 10: Type III analysis of variance with Satterthwaite's method of the winning model

1382 (time_label) - PEs LFP dataset

Model elements	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
time_label	1.2145	0.20241	6	41	9.6039	<0.001

1383

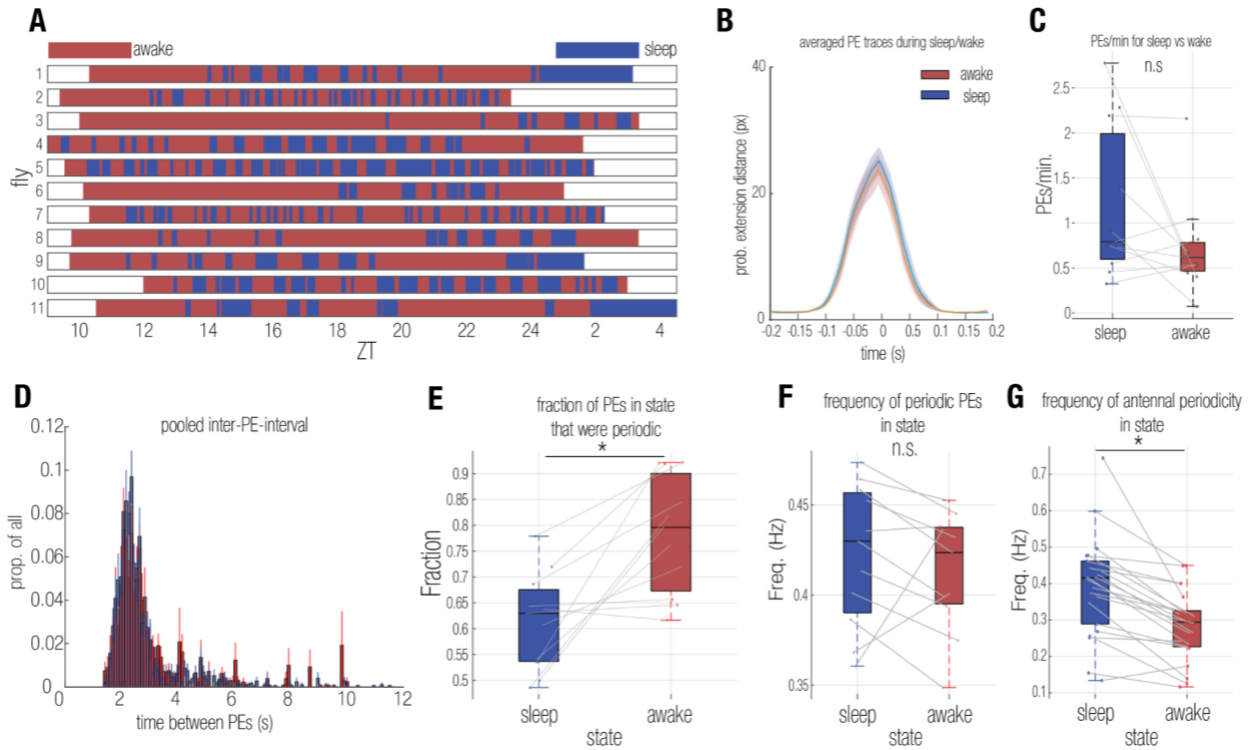
1384

1385

1386

1387

1388



1389

1390 **Supplementary Figure 1: Additional metrics of proboscis activity during sleep and wake.** A) Representation of

1391 the distribution of sleep (Blue) and wake (Red) across N=11 recorded individuals over the course of time. B)

1392 Averaged timecourse of proboscis extension distance from resting during a single event for sleep (Blue) and wake

1393 (Red). C) Comparison of proboscis extension rates during sleep and wake (n.s. ; Student's T-test). D) Histogram

1394 of the distribution of times between PEs during sleep (Blue) and wake (Red). E) Comparison of the fraction of

1395 PEs that were periodic versus isolated for sleep and wake ($p < 0.05$; Student's T-test). F) Comparison of the average

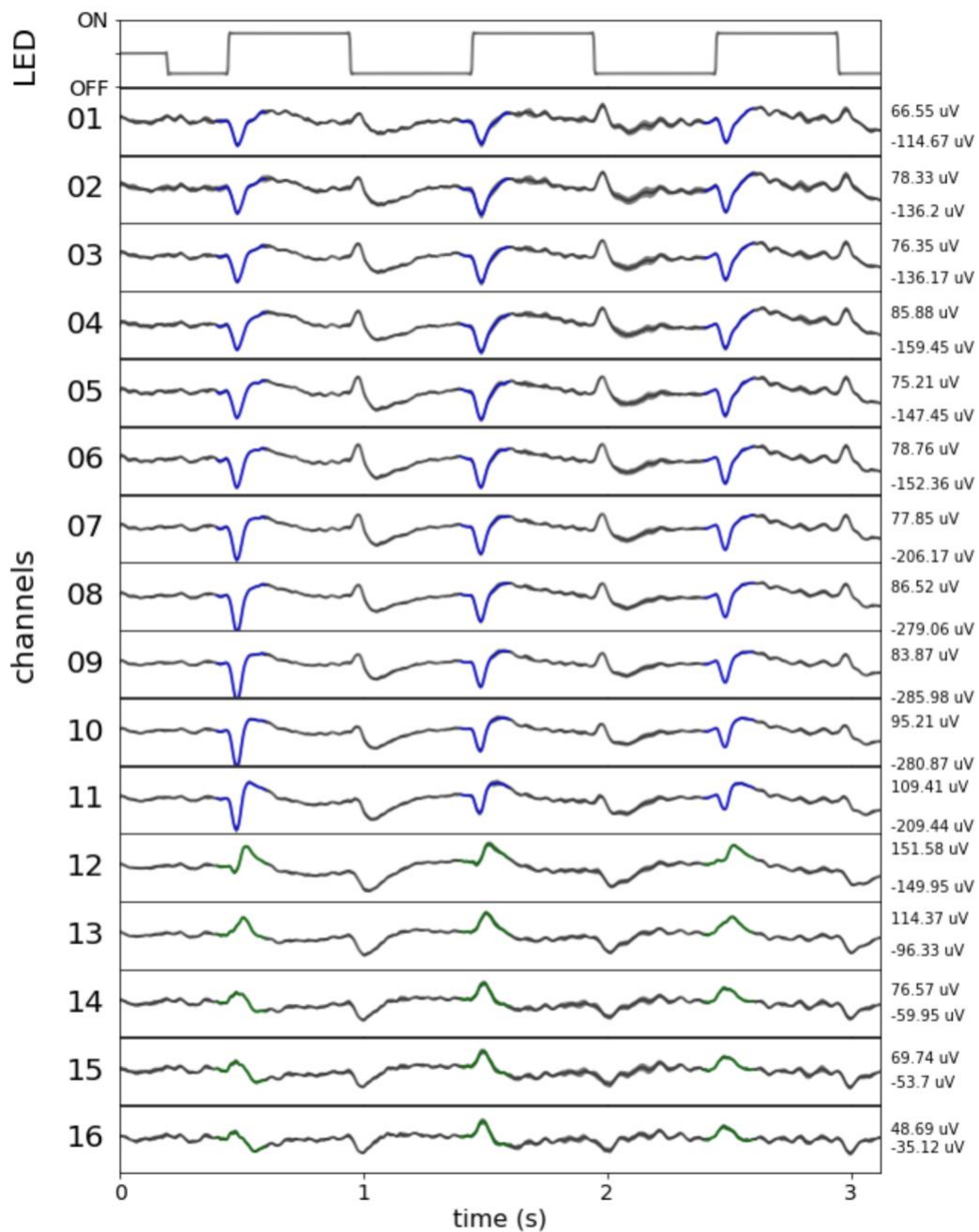
1396 frequency of PE periodicity across sleep and wake (n.s.; Student's T-test). G) As with F, for antennal periodicity

1397 ($p < 0.05$; Student's T-test).

1398

1399

1400



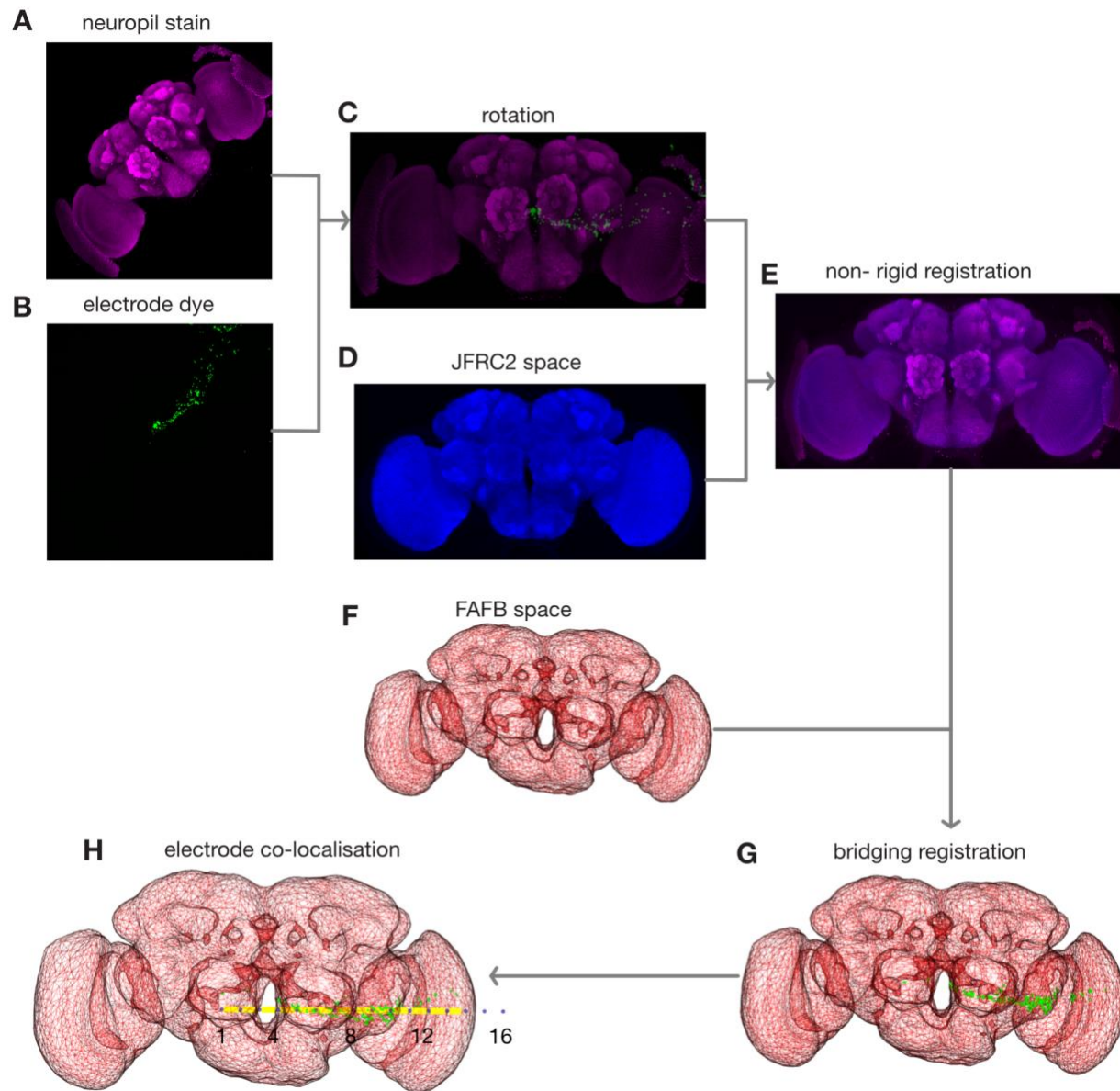
1401

1402

1403 **Supplementary Figure 2:** Electrode insertion depth was controlled by using a polarity reversal method. In this
1404 example fly, the change in LED stimulation (OFF to ON) stage, coincides with a LFP deflection. The LFP
1405 deflection changes from positive (12th channel) to negative (11th channel). The LFP amplitude depicted here is
1406 based on an average of 5 trials, with the shaded region representing the standard error.

1407

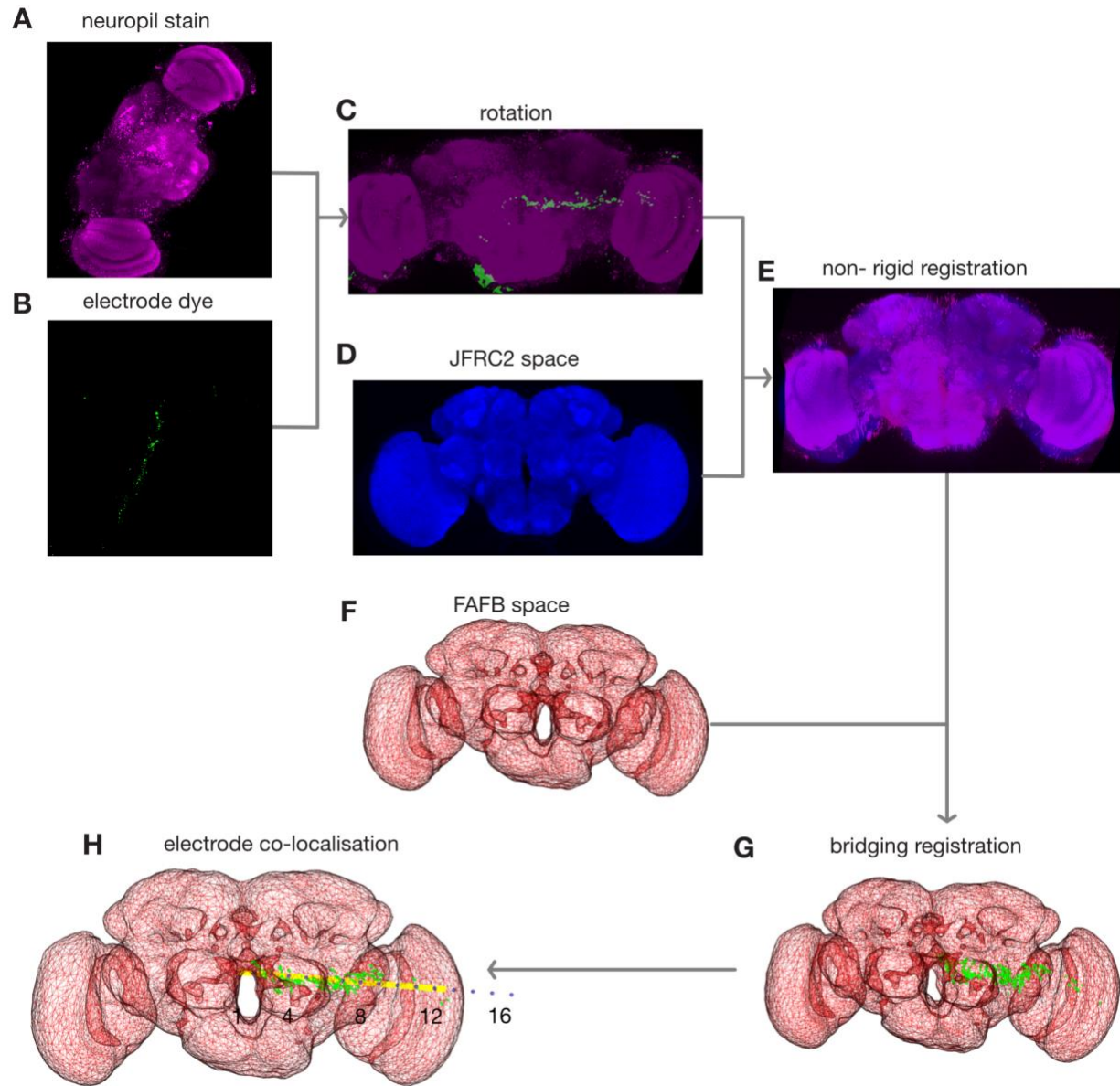
1408



1409

1410

1411 **Supplementary Figure 3:** Electrode locations were determined using a dye based localisation method. Neuropil
1412 stain (A) and electrode dye locations (B) were registered to JFRC2 space (D) via non-rigid registration. Further
1413 Bridging registration was used to register to FAFB space (F), the registration templates were applied on electrode
1414 dye locations (B) to produce co-localisation (H).



1415

1416 **Supplementary Figure 4:** Electrode locations were determined using a dye based localisation method. Neuropil

1417 stain (A) and electrode dye locations (B) were registered to JFRC2 space (D) via non-rigid registration. Further

1418 Bridging registration was used to register to FAFB space (F), the registration templates were applied on electrode

1419 dye locations (B) to produce co-localisation (H).

1420

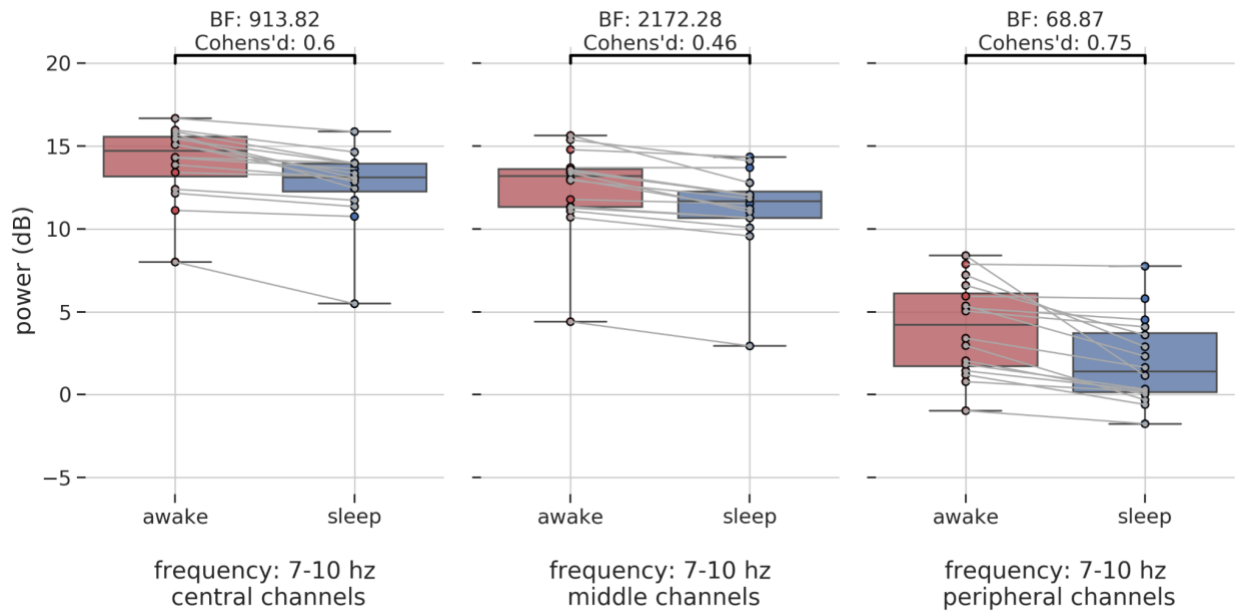
1421

1422

1423

1424

1425



1426

1427 **Supplementary Figure 5:** Power differences across central, middle, peripheral channels in the frequency bands of

1428 7-10 Hz.

1429

1430

1431

1432

1433

1434

1435

1436

1437

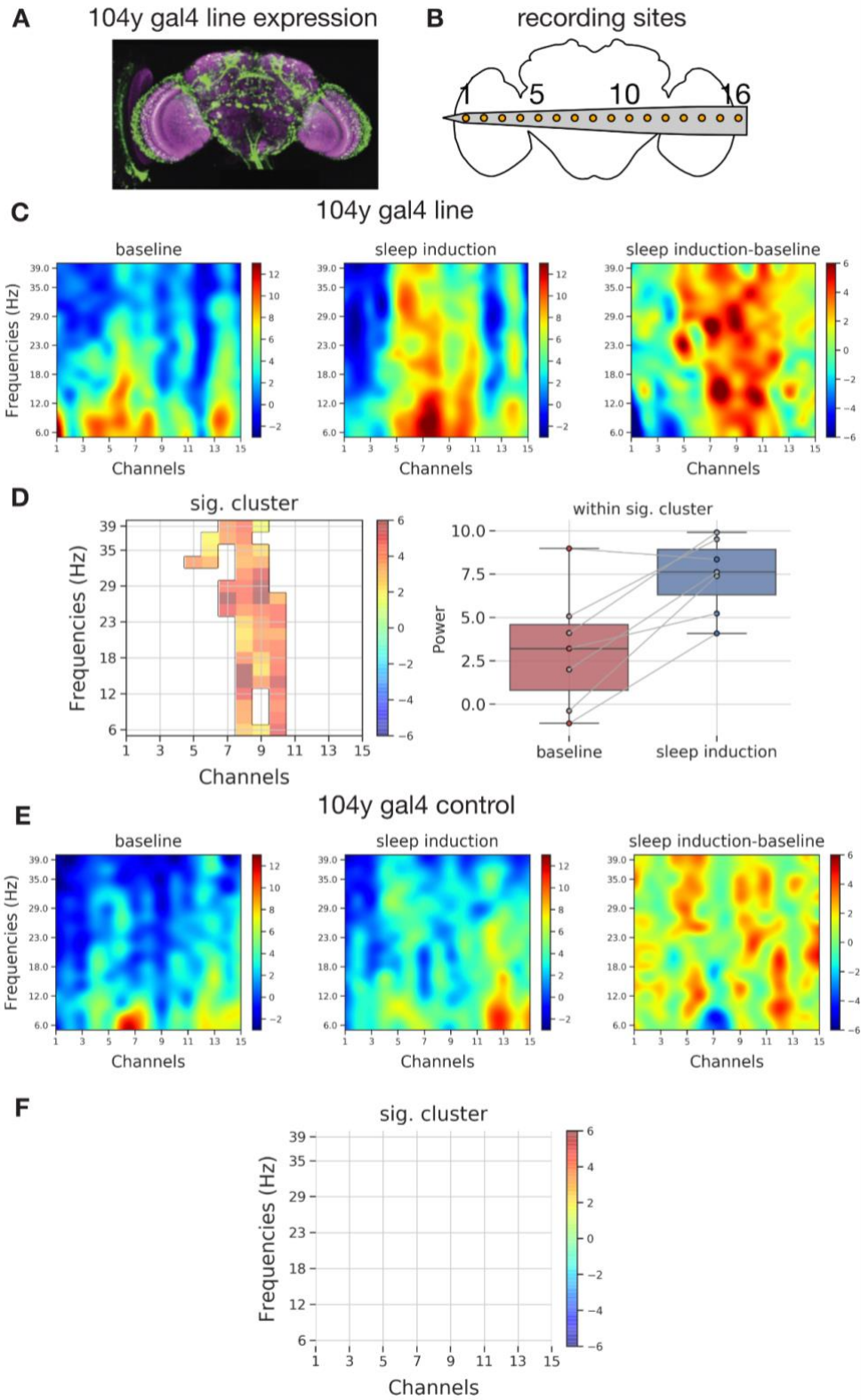
1438

1439

1440

1441

1442

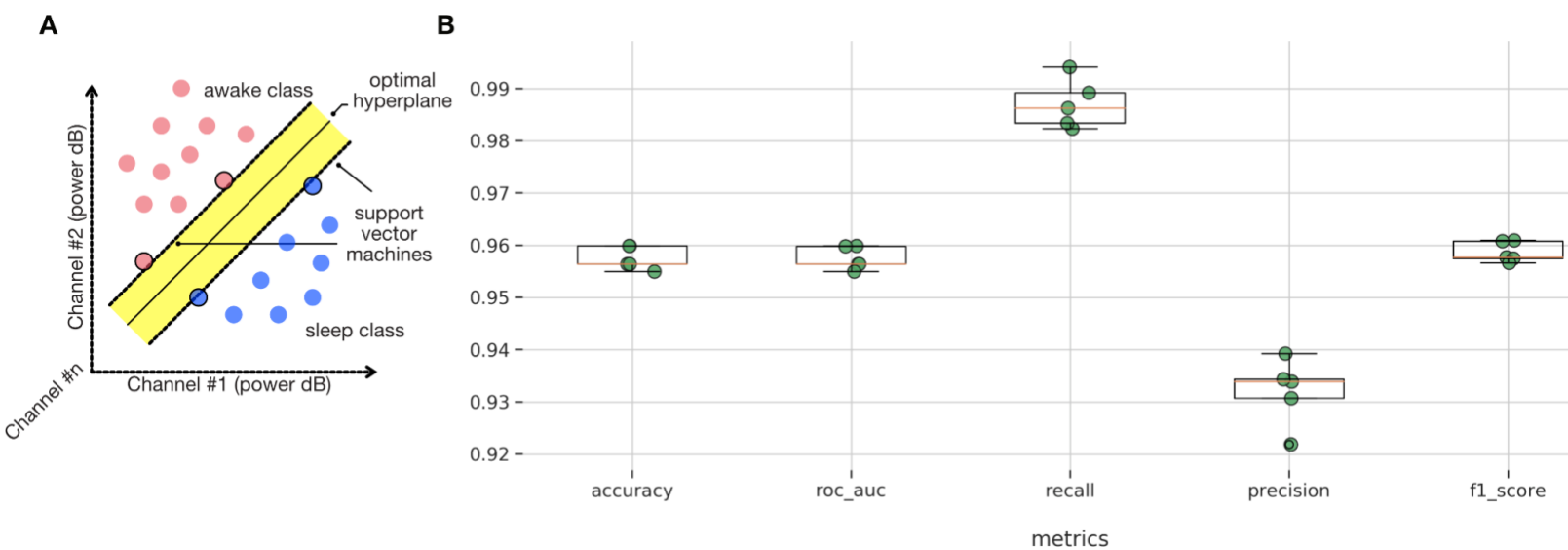


1443 **Supplementary Figure 6:** Spectral differences in thermogenetically induced sleep recorded using full brain probe.

1444

1445

1446



1447

1448 **Supplementary Figure 7:** A) Schematic indicating the optimal separation of awake and sleep classes using
1449 classifiers based on support vector machines. B) SVM based classifier performance across different metrics based
1450 on 5 different train/test data splits.

1451

1452

1453

1454

1455

1456

1457

1458

1459

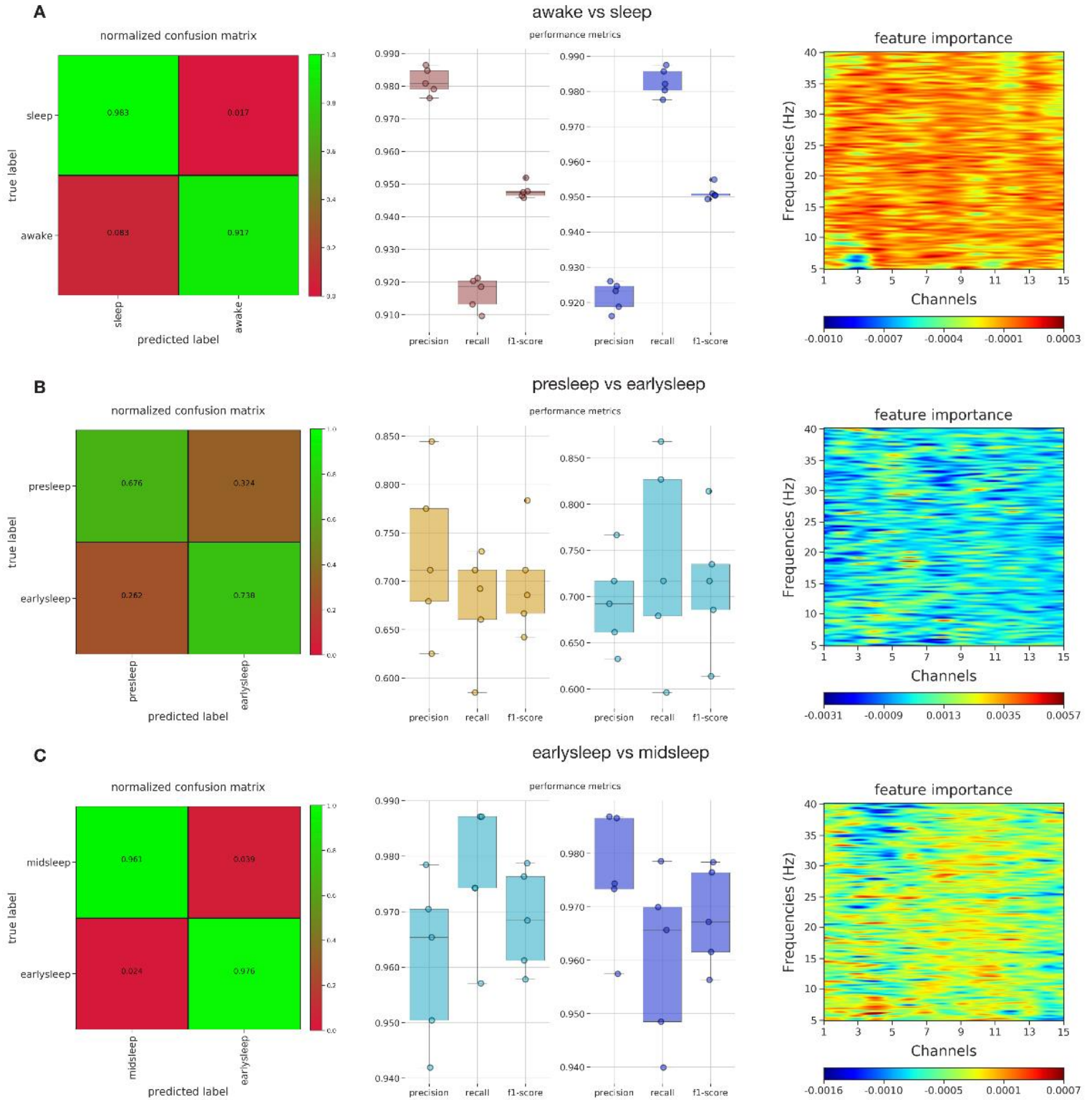
1460

1461

1462

1463

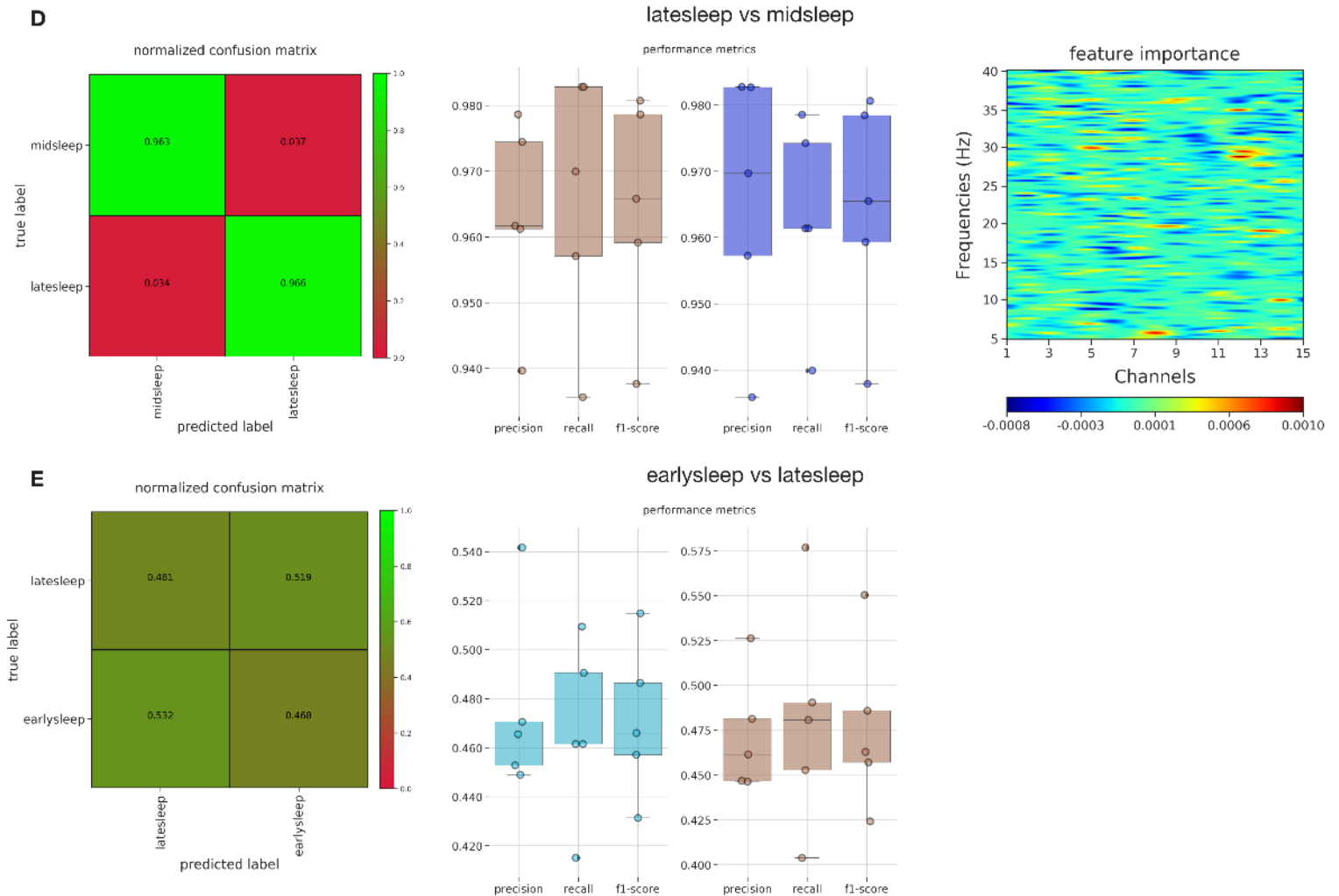
1464



1465

1466

1467



1468

1469 **Supplementary Figure 8:** A) Feature importance of the multiclass classifier (reduced to awake vs sleep) indicates
1470 an ROI across all channels and almost all frequency bands as critically important. This cross validates the
1471 differences in the power spectrum across awake and sleep as shown in Figure 4D. B,C,D,E) Feature importance
1472 of multiclass classifier for the other categories.

1473

1474

1475