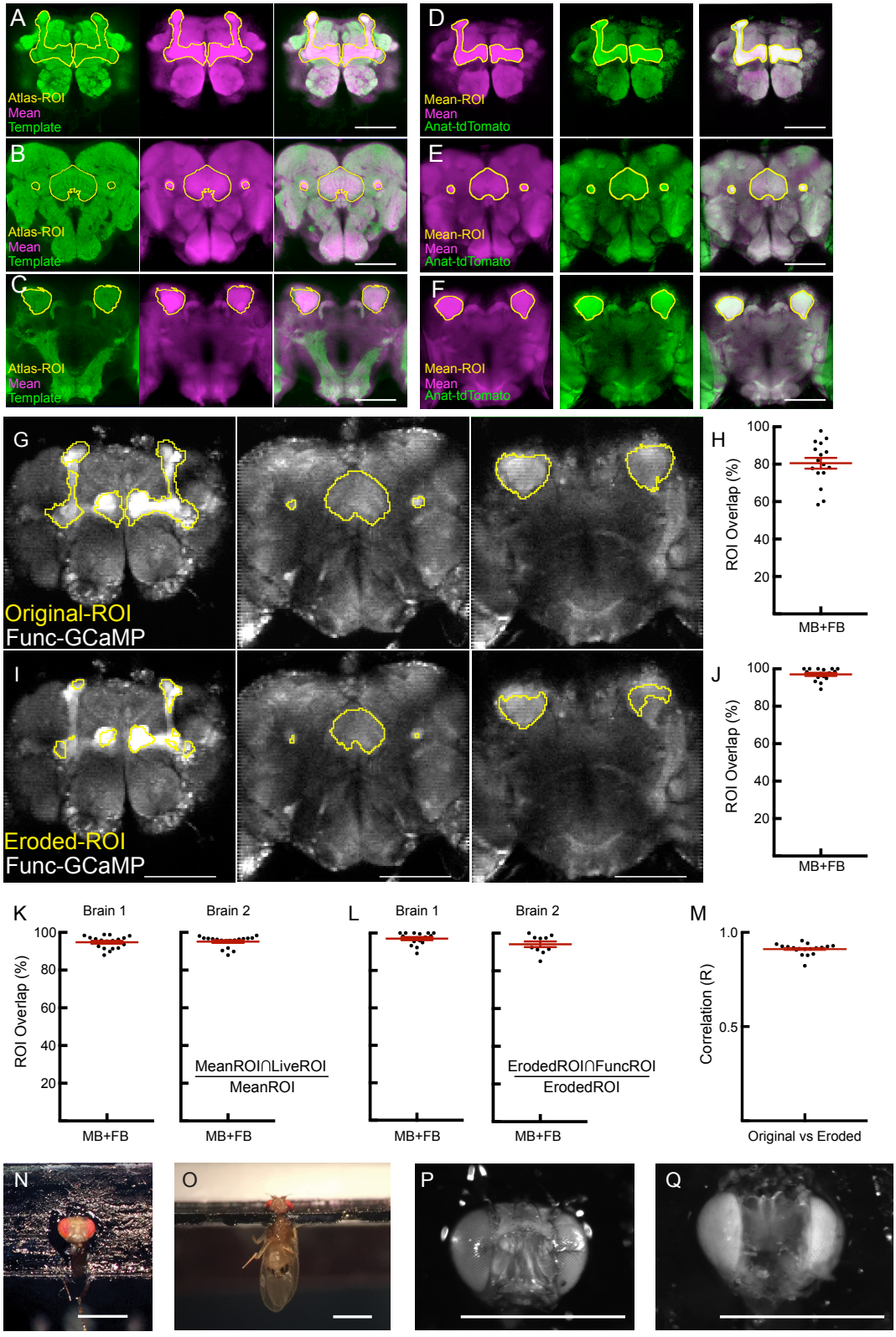
A
Atlas-ROI
Mean
Template
B
Atlas-ROI
Mean
Template
C
Atlas-ROI
Mean
Template
D
Mean-ROI
Mean
Anat-tdTomato
E
Mean-ROI
Mean
Anat-tdTomato
F
Mean-ROI
Mean
Anat-tdTomato
G
Original-ROI
Func-GCaMP
H
ROI Overlap (%)
100
80
60
40
20
MB+FB
I
Eroded-ROI
Func-GCaMP
J
ROI Overlap (%)
100
80
60
40
20
MB+FB
K
Brain 1
Brain 2
ROI Overlap (%)
100
80
60
40
20
MB+FB
MeanROI∩LiveROI
MeanROI
MB+FB
L
Brain 1
Brain 2
MB+FB
ErodedROI∩FuncROI
ErodedROI
MB+FB
M
Correlation (R)
1.0
0.5
Original vs Eroded
N
O
P
Q

Figure S1. Detailed channel information of alignment between template and mean brain, mean brain and live anatomical brain, and comparison between original and eroded ROI overlap in functional data. Summary of a second brain alignment. Related to Figure 1.
(A-C) Individual channels from Figure 2C. Yellow lines indicate atlas ROIs. (D-F) Individual channels from Figure 2E. (G) Overlay of non-eroded fly brain atlas (Original-ROI) and functional data (Func-GCaMP). Views of ROIs drawn around the MB and FB in the anterior (left), middle (middle), and posterior (right). (H) Summary of overlap between non-eroded ROIs and func-ROIs. (I,J) Same data as presented in Figure 2F,G. (I) Overlay of eroded fly brain atlas (Eroded-ROI) and functional data (Func-GCaMP). (J) Summary of overlap between eroded-ROIs and func-ROIs. (K) Combined overlap in MB and FB between mean brain and live anatomical scan of two brains. Brain 1 is the same data as presented in Figure 2F. (L) Combined overlap in MB and FB between the eroded atlas ROIs and the functional data of two brains. Brain 1 is the same data as presented in Figure 2H. (M) Correlation of original and eroded correlation matrices from each individual fly, similar to comparison made in Figure S2B. This metric indicates that the correlation matrices before and after erosion are very similar (mean R = 0.91). (N) Top view of mounted fly prior to dissection. The fly is inserted into a slit in the mount at the cervix, separating the head from the body. The edges of the head are fixed with nail polish and the legs and wings are immobilized with nail polish. (O) Front view of mounted fly prior to dissection. (P) Detailed top view of mounted fly before dissection. (Q) Top view of mounted fly after dissection. The top compartment with the head has fly saline flowing over the brain during imaging, while the body and appendages remain dry. Data is shown with individual replicates and mean ± SEM. Scale bars in A-I are 100μm. Scale bars in N, O, P, Q are 1mm.

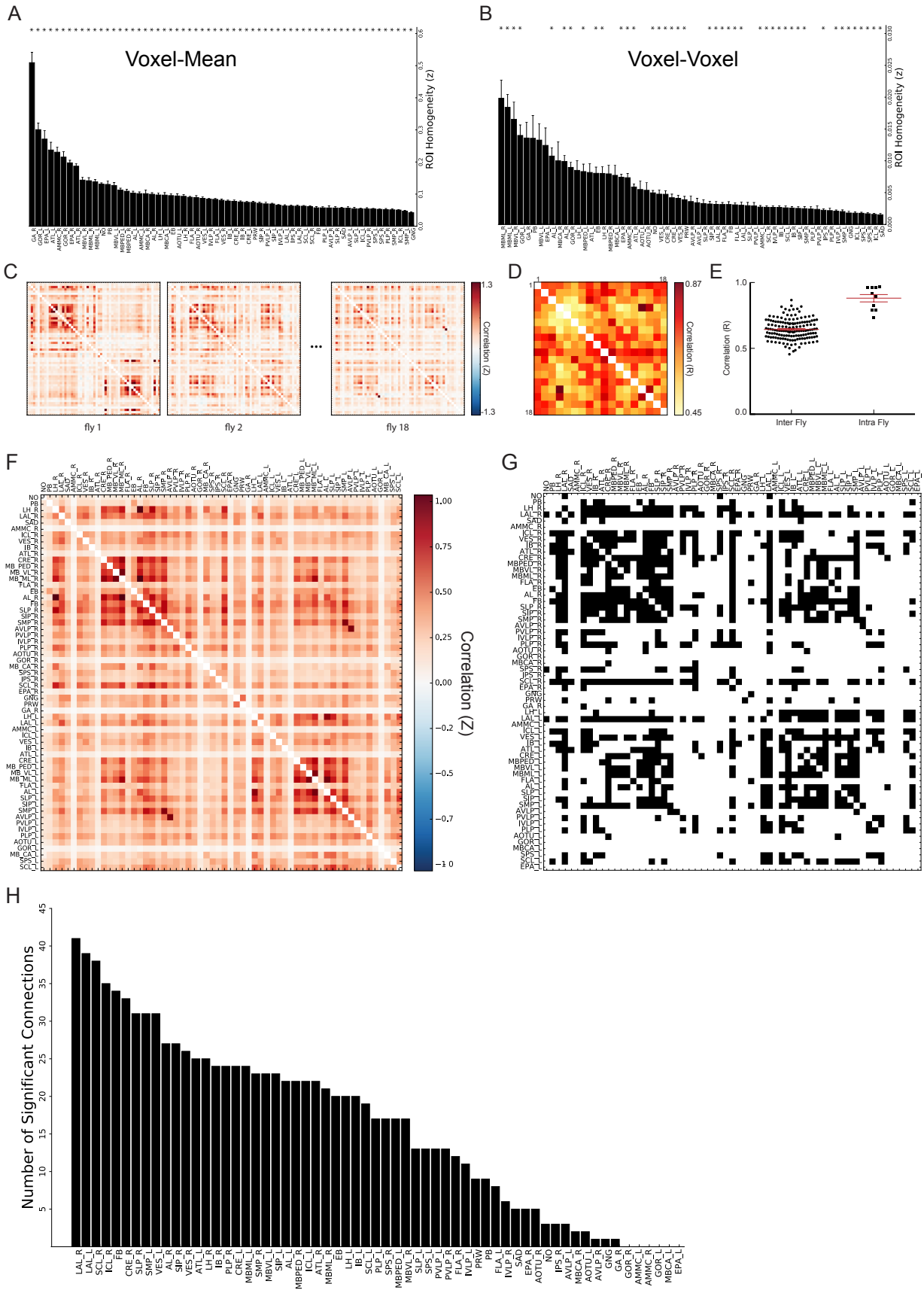
A
Voxel-Mean
ROI Homogeneity (z)
B
Voxel-Voxel
ROI Homogeneity (z)
C
fly 1
fly 2
fly 18
Correlation (Z)
D
Correlation (R)
E
Correlation (R)
Inter Fly
Intra Fly
F
Correlation (Z)
G
H
Number of Significant Connections

Figure S2. ROI homogeneity, reliability of correlations between and within animals, and significance of functional connections across flies. Related to Figures 2 and 3.

(A) Homogeneity between average ROI time series and each ROI's voxels. Bars are plotted as mean ± SEM across flies. Significance of homogeneity was calculated using a one-sample t-test against zero across all flies, with $p < 0.05$ Bonferroni-corrected for all regions tested (number of regions = 61, $p < 8.20e\text{-}4$). (B) Homogeneity between the voxels in each ROI. Bars are plotted as mean ± SEM across flies. Significance of homogeneity was calculated using a one-sample t-test against zero across all flies, with $p < 0.05$ Bonferroni-corrected for all regions tested (number of regions = 61, $p < 8.20e\text{-}4$). (C) Example correlation matrices from three individual flies. Correlation coefficients are Fisher z-transformed. Order of ROIs is the same as in Figure 4A. (D) Correlation matrix of all 18 flies' correlation matrices. Each individual correlation matrix is treated as a linear set of values and is correlated with all other individual correlation matrices. This is a metric of how similar each correlation matrix is with others. (E) Left, summary values from (D) showing the correlation values between individual fly correlation matrices. Right, the correlation values within individual flies between two imaging sessions for 10 flies that were stably imaged for two sessions. Intra-fly correlations were generated in a manner similar to (D) except that comparisons were only made between each individual fly's two sessions (n=10). *$p < 0.05$. (F) Average correlation matrix across all flies (N = 18). Cell values represent the average Fisher z-transformed correlation value across animals for that ROI-ROI connection. (G) Significant functional connections (black cells) calculated using a one-sample t-test against zero across all flies at $\alpha = 0.001$ Bonferroni-corrected for all connections tested (number of connections = 1830, $p < 5.46e\text{-}7$). (H) Total number of functional connections for each atlas ROI from (G).

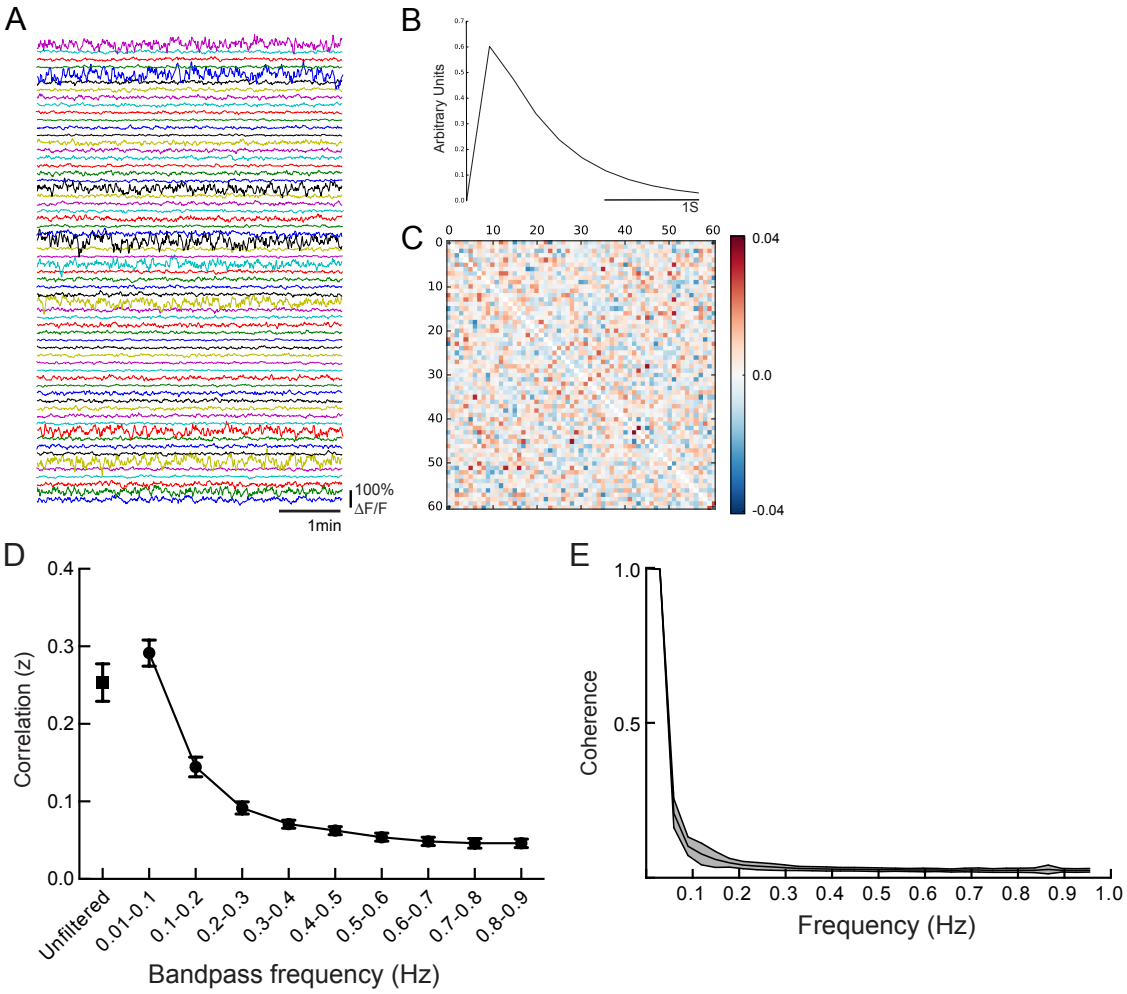

Figure S3. Simulated data and functional connectivity after temporal filtering. Related to Figure 3.

(A) Example simulated data. Simulated data was generated such that it would recapitulate the mean and standard deviation from each of the 61 ROIs from each of the 18 animals in our data set. This data was also convolved with GCaMP6m kinetics (right) to accurately simulate GCaMP6m-generated data. (B) GCaMP6m curve used for the convolution in (A). This accurately reflects the kinetics of GCaMP6m as previously reported (rise time = 280ms, $t_{½}$ = 1162ms) at a 1.9Hz sampling rate. (C) Example correlation matrix generated from a single simulation. Correlation values are quite low and only one significant connection was found across the 1000 simulations. Significance criteria were the same as those used on our actual data (Figure 4). (D) Average correlation matrix values (Fisher z-transformed) for raw, unfiltered data (Unfiltered) and for data at various frequency bands ranging from 0.01 - 0.9 Hz, in 0.1 Hz steps. Data are presented mean ± SEM of average connectivity values across flies (N = 18). Lower frequency bands have higher correlation values. (E) Average coherence (the spectral analog of cross-correlation) of all ROI-ROI pairs. Data are presented as mean ± SD of average coherence values across flies (N = 18).

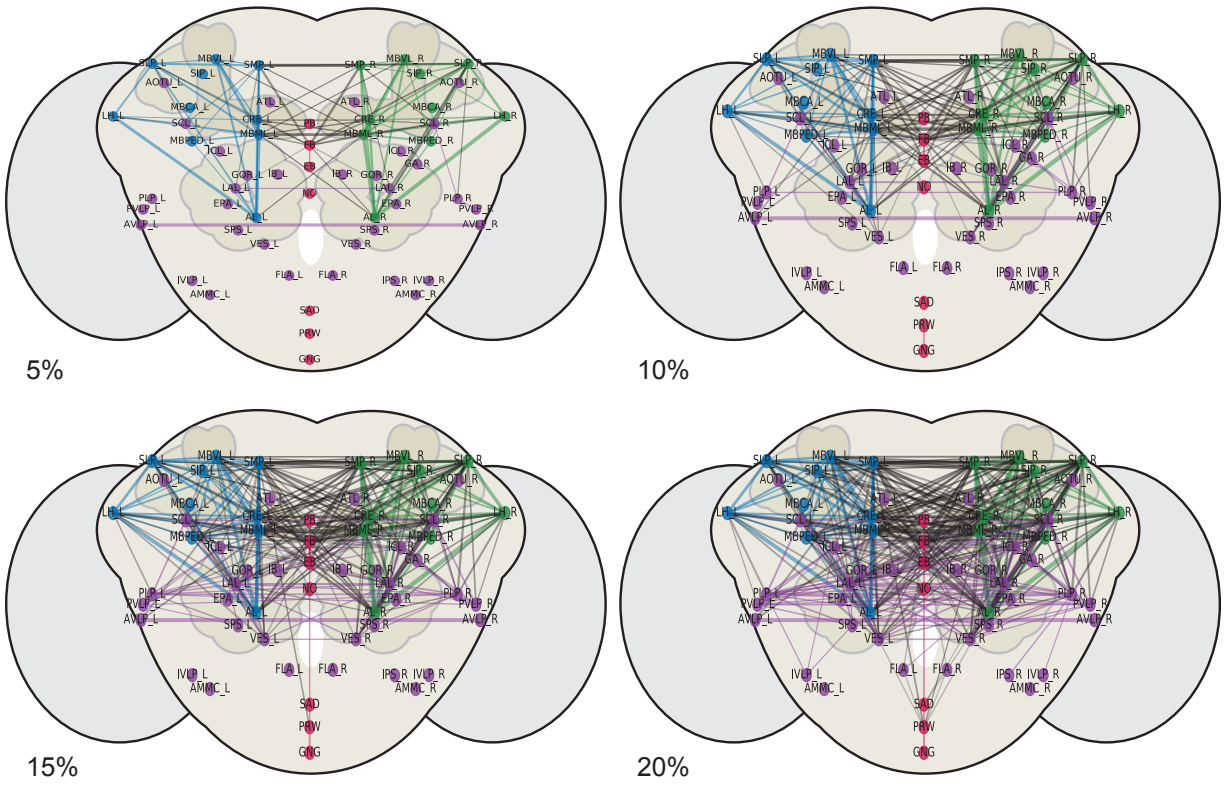

Figure S4. Fly brain networks at different thresholds. Related to Figure 4.

Fly brain network with labeled atlas regions represented as circles and connections between them represented with lines. (A) Top 5% of significant connections in the central fly brain are shown. (B) Top 10% of significant connections. (C) Top 15% of significant connections. (D) Top 20% of significant connections. ROIs are grouped and colored as in Figure 5A. Connections between brain regions of the same group are colored the same as the regions themselves, while connections between regions of different groups are colored grey. Connections are weighted according to their average connectivity strength.