Current Biology, Volume *31*

Supplemental Information

Ongoing habenular activity is driven by forebrain networks and modulated by olfactory stimuli

Ewelina Magdalena Bartoszek, Anna Maria Ostenrath, Suresh Kumar Jetti, Bram Serneels, Aytac Kadir Mutlu, Khac Thanh Phong Chau, and Emre Yaksi

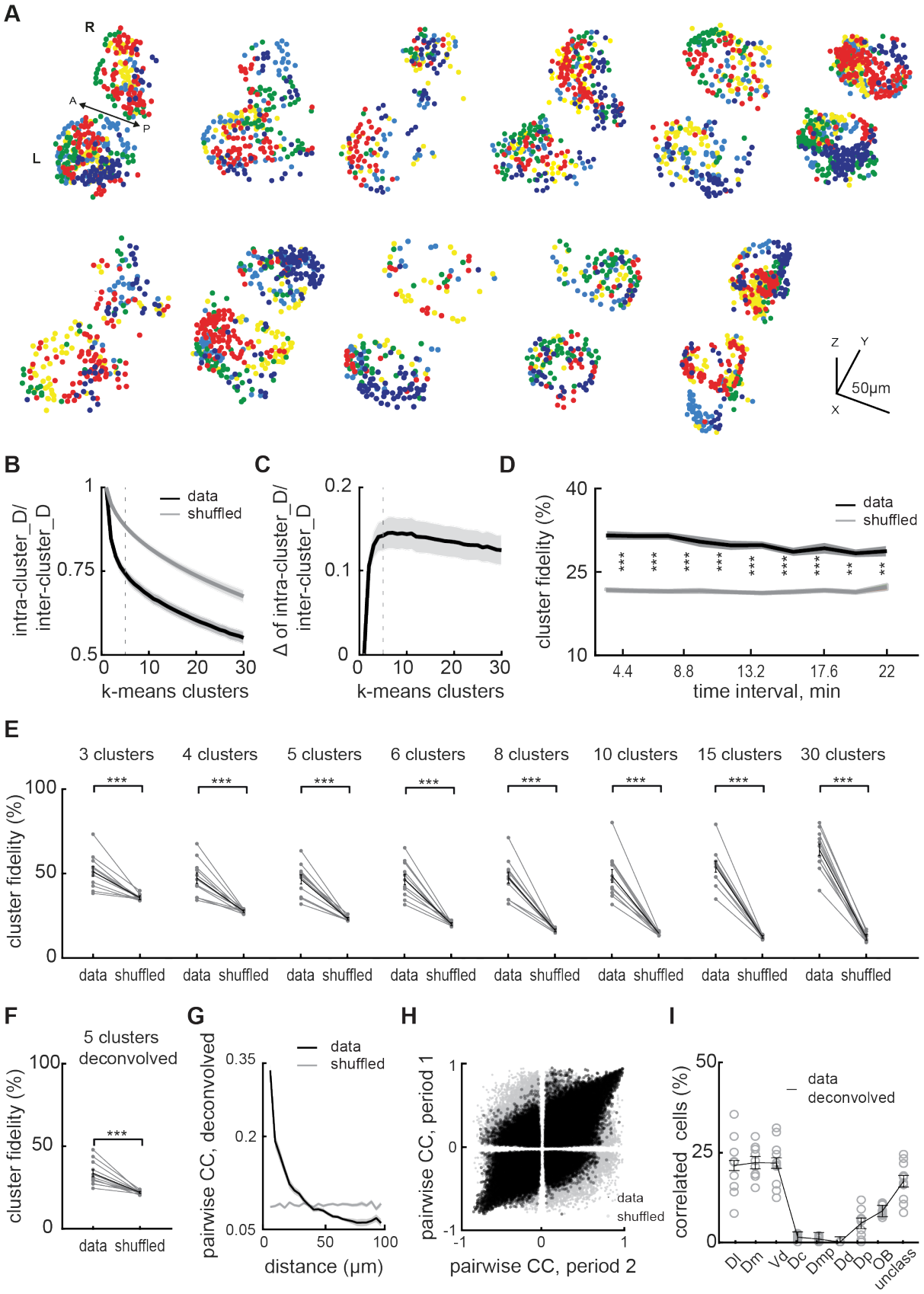

Figure S1. Functional clusters of habenular neurons are robust and stable across time. Related to Figures 1 and 2.

(A) Three-dimensional reconstructions of functional clusters of habenular neurons identified using k-means clustering in n=11 zebrafish. Functional clusters of neurons with similar ongoing activity are color-coded into 5 clusters. Colors are arbitrarily assigned by clustering algorithm, but manually matched across animals L-left; R-right hemisphere, A-anterior, P-posterior.

(B) Identification of optimal number of clusters by using elbow analysis. Elbow analysis calculate the sum of intra-cluster distances "D" of each cluster element, normalized by sum of average inter-cluster distances of each cluster element, for actual data (black), and for simulated data (100 iterations) with the same variance of actual data but with no cluster structure (grey), in n=11 zebrafish. This calculation is repeated for up to 30 clusters (x-axis). Optimal number of clusters is the elbow point, where the black curve shows a prominent bend. Dashed lines marks k-means analysis for 5 clusters. Shaded bars are SEM.

(C) Optimal number of clusters is further revealed, when actual data is compared to simulated data with similar variance but no cluster structure, by taking the difference of two curves in (B). Note that the peak point of this difference reveals 5-6 optimal number of clusters in ongoing habenular activity. 5 clusters were chosen to be used in k-means analysis of habenular activity in this paper for keeping the color scheme simple and easy to visualize.

(D) Stability of habenular clusters is investigated by calculating cluster fidelity of neurons (mean ± SEM) in the first 2.2 minute in comparison to consecutive 2.2 minute time bins, in real data (black), and in simulated data with shuffled cluster identities (100 iterations, in grey). Note that while cluster fidelity remains stable (around 40%, and with no significant change across consecutive time points), simulated data with shuffled cluster identities shows low cluster fidelity across time. Real data always remain significantly higher than shuffled control. Shaded bars are SEM. (*$P < 0.05$, **$P < 0.001$, ***$P < 0.001$, Wilcoxon signed-rank test).

(E) Cluster fidelity calculated for k-means analysis using multiple number of clusters. Note that for all numbers of k-means clusters, cluster fidelity remains significantly higher than control data with shuffled cluster identities. (***$P < 0.001$, Wilcoxon signed-rank test).

(F) Cluster fidelity is calculated for 5 k-means clusters, using temporally deconvolved calcium signals of habenular activity. (***$P < 0.001$, Wilcoxon signed-rank test).

(G) Relation between pairwise correlation of habenular neurons and the distance between each neuron pair is calculated using temporally deconvolved calcium signals of habenular activity.

(H) Pairwise correlations of habenular neurons during two consecutive time periods, calculated using temporally deconvolved calcium signals of habenular activity (black). Control pairwise comparison that are shuffled for pair identities (grey).

(I) Distribution of forebrain neurons with strong correlation (>0.1) to ongoing habenular activity into anatomically identified forebrain regions. Same as Figure 2G, but calculated using temporally deconvolved calcium signals. Note that results look similar for (G), (H), and (I) using calcium signals versus temporally deconvolved calcium signals.

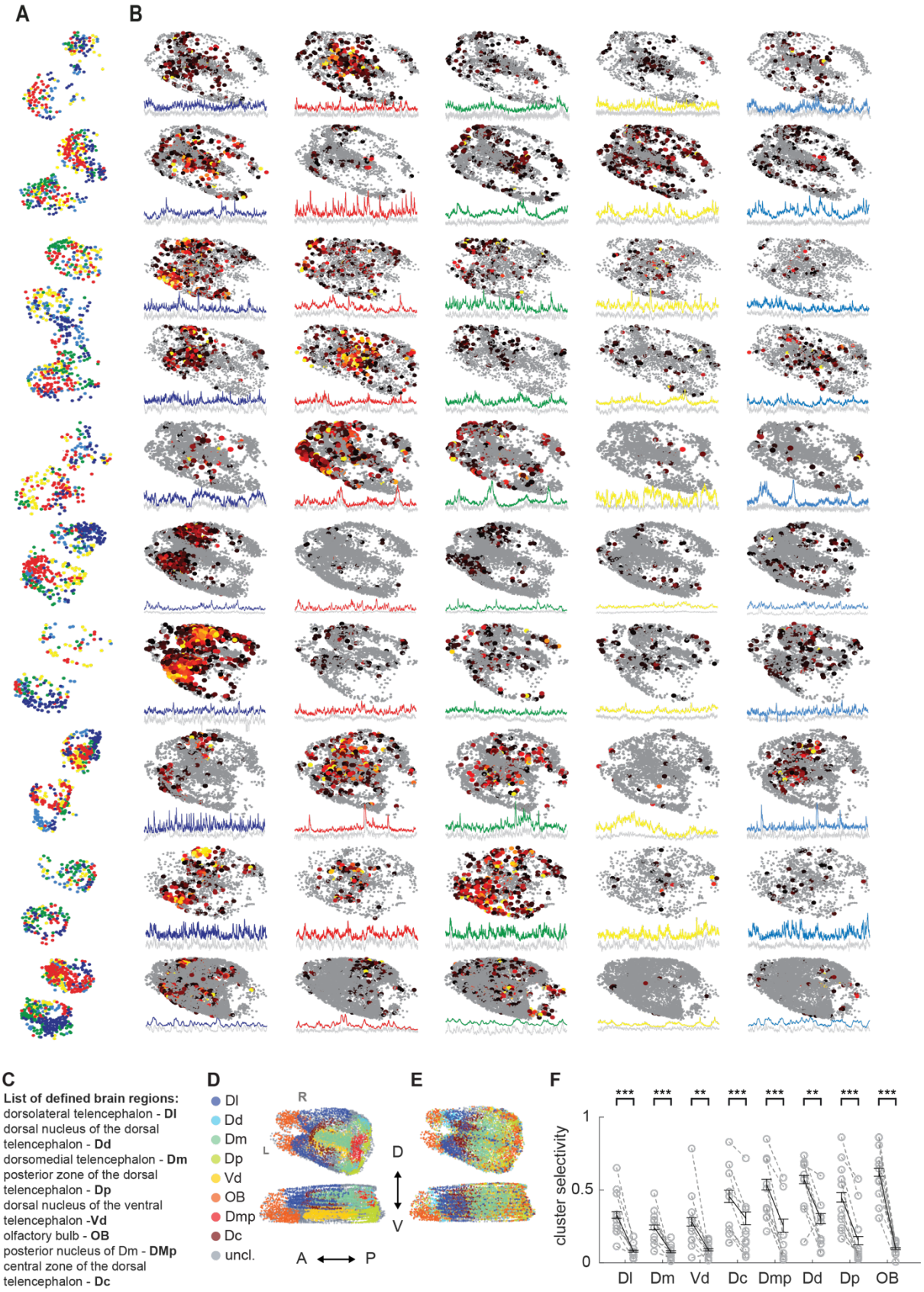

Figure S2. Functional and anatomical comparison of forebrain habenula interactions. Related to Figure 2.

(A) Three-dimensional reconstruction of habenular neurons detected in *Tg(elavl3:GCaMP6s)* zebrafish line, clustered with k-means clustering. Colors represent neural clusters with similar ongoing activity. L-left; R-right hemisphere. 315 ± 35 (mean ± SEM) habenular neurons were imaged in each fish (n=11 fish).

(B) Three-dimensional reconstruction of forebrain neurons that are strongly correlated (Pearson's correlation >0.1) to average ongoing activity of different habenular clusters in B. Warm colors represent stronger correlations. 2135 ± 345 (mean ± SEM) forebrain neurons were imaged in each fish (n=11 fish). Color-coded traces represent the average activity of forebrain neurons in each cluster. Grey traces represent the average activity of corresponding habenular clusters in A. Note that the ongoing activity of identified forebrain neurons and habenular clusters are highly similar.

(C) A list of anatomically identified zebrafish forebrain regions and their abbreviations.

(D) Three-dimensional reconstruction of forebrain neurons identified by using anatomical landmarks. Top – dorsal view, bottom – coronal view. L-left; R-right hemisphere. A –anterior, P – posterior, D- dorsal, V-ventral. Colors corresponds to individual forebrain regions in C.

(E) Three-dimensional reconstruction of forebrain neurons that are clustered by k-means functional clustering (with n=8 clusters) of their ongoing neural activity. Each arbitrary color represents functional forebrain cluster.

(F) Neurons of anatomically identified forebrain regions exhibit cluster selectivity that are significantly higher than chance levels.

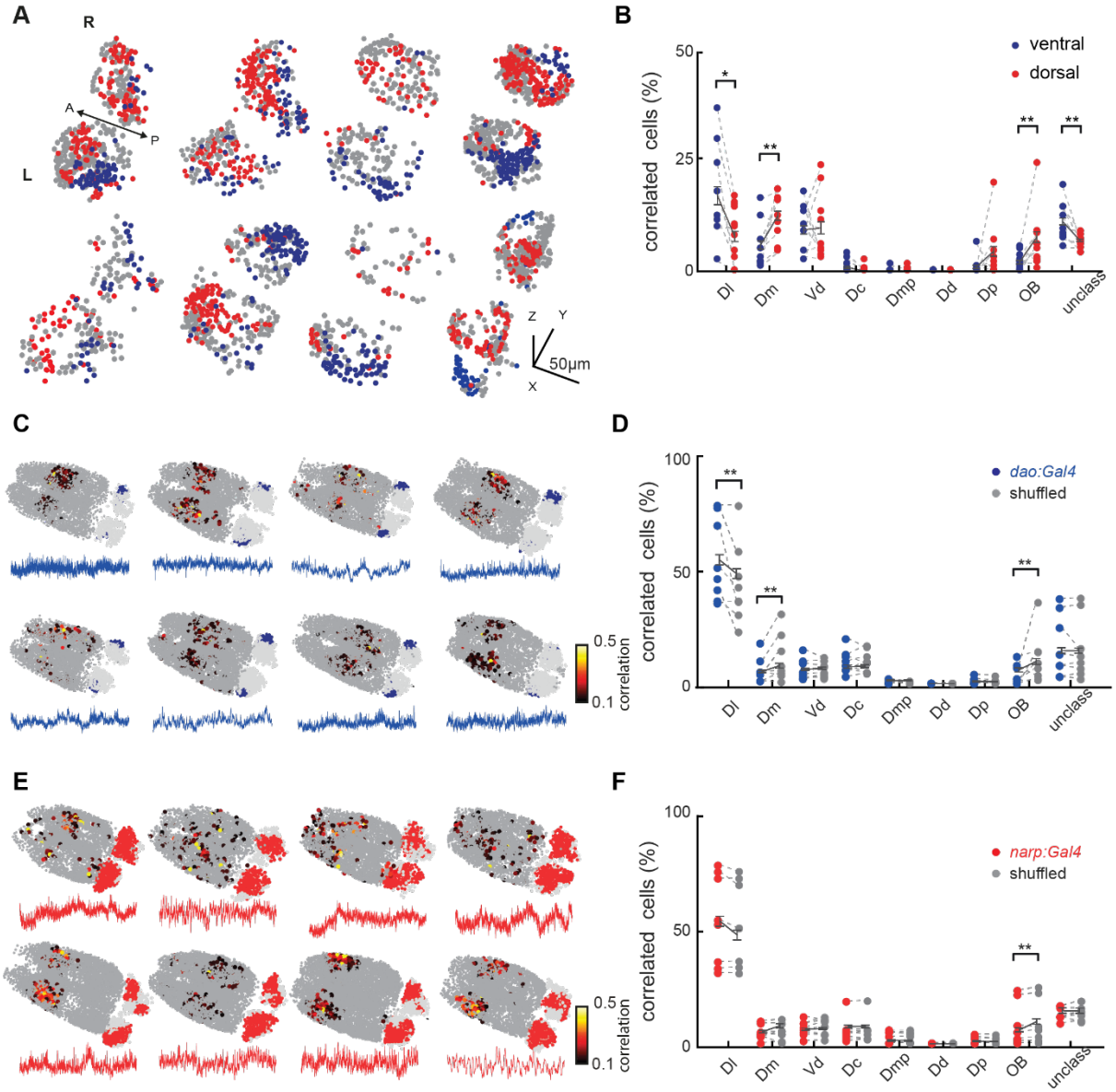

Figure S3. Ongoing activity of ventral and dorsal habenular neurons correlate preferentially with different forebrain regions. Related to Figure 2.

(A) Three-dimensional reconstruction of functional clusters in habenula corresponding to ventral (blue) and dorsal (red) zones of habenula in 11 *Tg(elavl3:GCaMP6s)* zebrafish.

(B) Anatomical distribution of forebrain neurons with strong correlation (>0.1) to ongoing activity of ventral (blue) versus dorsal (red) functional clusters of the habenula. Note that the functional clusters of neurons in ventral zones of the habenula shows significantly higher fraction of correlated neurons in Dl. Also note that the functional clusters of neurons in dorsal zones of the habenula shows significantly higher fraction of correlated neurons in Dm, Dp, OB and unclassified neurons across the forebrain. (*P < 0.05, **P < 0.01, Wilcoxon signed-rank test).

(C) Three-dimensional reconstruction of forebrain neurons in *Tg(elavl3:GCaMP6s); Tg(dao:GAL4VP16; UAS-E1b:NTR-mCherry)* zebrafish, which genetically labels vHb neurons (blue), in 8 zebrafish. Individual forebrain neurons with strong correlation (>0.1) to vHb are highlighted with warm colors. Non-correlated

forebrain neurons are in grey. Average ongoing activity of vHb neurons are plotted under each brain.

(D) Anatomical distribution of forebrain neurons with strong correlation (>0.1) to the average ongoing activity of genetically labelled vHb neurons (blue) versus similar number of randomly selected other habenular neurons (grey). Note that the vHb shows significantly higher fraction of correlated neurons in Dl, and significantly smaller number of correlated neurons in Dm and OB. (**P < 0.01, Wilcoxon signed-rank test).

(E) Three-dimensional reconstruction of forebrain neurons in *Tg(elavl3:GCaMP6s); Tg(narp:GAL4VP16; UAS-E1b:NTR-mCherry)* zebrafish, which genetically labels neurons in 68% of habenula, in 8 zebrafish. Individual forebrain neurons with strong correlation (>0.1) to *narp:GAL4VP16* labelled neurons are highlighted with warm colors. Non-correlated forebrain neurons are in grey. Average ongoing activity of vHb neurons are plotted under each brain.

(F) Anatomical distribution of forebrain neurons with strong correlation (>0.1) to the average ongoing activity of *narp:GAL4VP16* labelled neurons (blue) versus similar number of randomly selected other habenular neurons (grey). Note that the *narp:GAL4VP16* labelled dorsal habenular neurons do not show any significant preference of correlations in the forebrain, except the OB. (**P < 0.01, Wilcoxon signed-rank test).

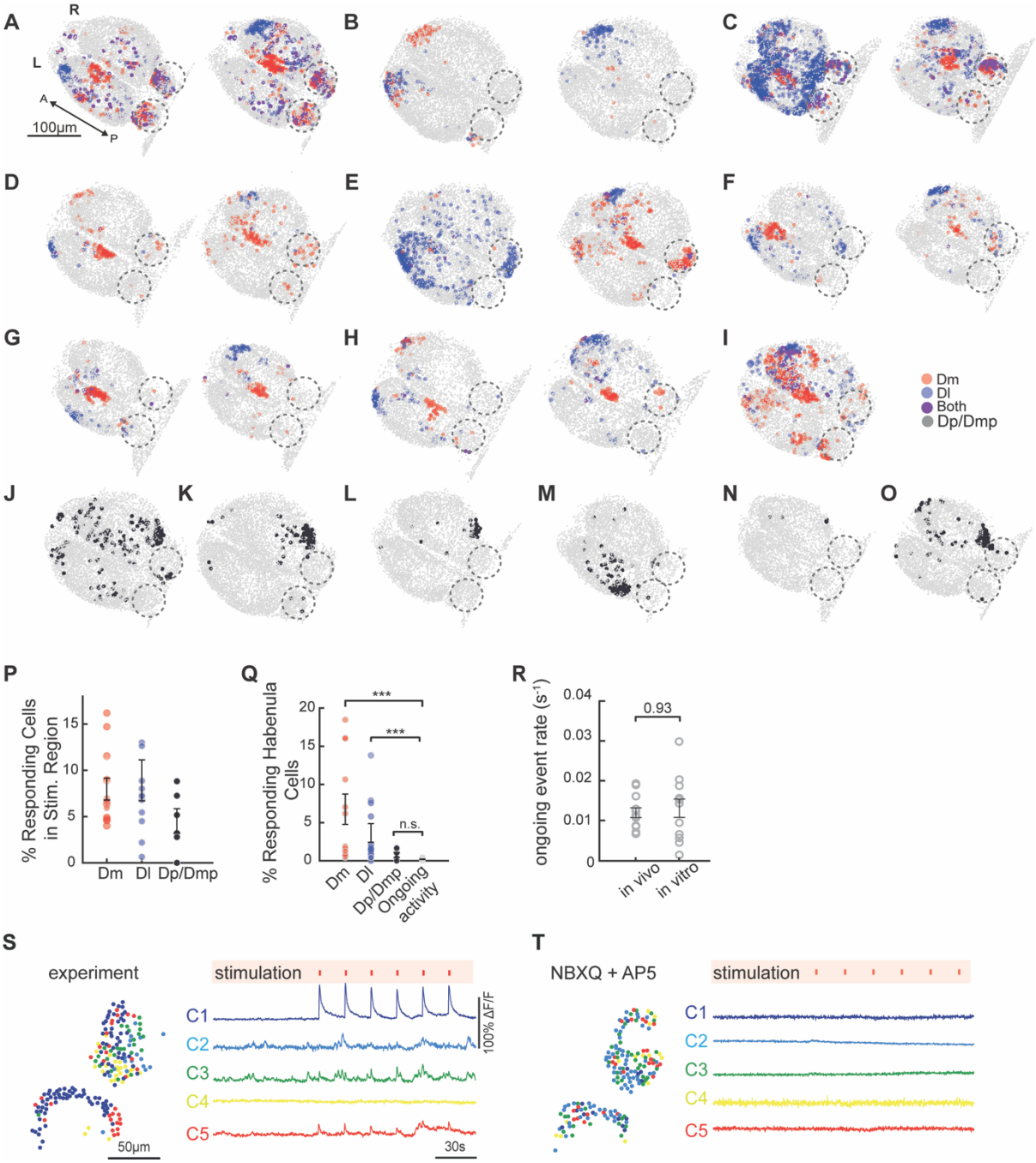

Figure S4. Three dimensional reconstructions of all micro-electrode stimulations in zebrafish forebrain. Related to Figures 3 and 4.

(A-I) All three-dimensional forebrain reconstructions in *Tg(eval3:GCaMP6-nuclear)* juvenile zebrafish brain explant, upon sequential Dm and Dl stimulations. Each panel represents an individual fish. Neurons responding to only Dm (red), only Dl (blue), both (magenta) stimulations, and non-responsive (grey). Scale bar 100µm, L-left; R-right; A-anterior; P-posterior.

(J-O) All control stimulations upon inserting micro-electrode to regions near Dp/Dmp. Each panel represents an individual fish. Neurons responding to Dp/Dmp stimulation (black), non-responsive (grey). Habenulae are delineated by dashed black circles.

(P) Fraction of forebrain neurons in Dm, Dl and Dp/Dmp regions activated by (above 2SDs) to the micro-electrode stimulation of these specific brain regions. No significant differences were observed in the fraction of neurons activated in these brain regions.

(Q) Fraction of habenular neurons responding to Dm, Dl and Dp/Dmp stimulation (black), when compared to randomly selected periods of ongoing habenula activity (grey). Habenular ongoing activity periods was selected from 100 random time points. Note that while Dm and Dl activation recruits habenular neurons significantly more than random periods of ongoing habenular activity different (***$P < 0.001$, Wilcoxon ranksum test), this is not the case for control stimulations of Dp/Dmp region.

(R) Frequency of ongoing calcium events detected in vivo versus brain-explant preparation. Note that no significant difference was observed between these two preparations (Wilcoxon ranksum test).

(S) Left, representative example of habenular neurons detected in Tg(eval3:GCaMP5) zebrafish line during forebrain micro-stimulation, clustered with k-means clustering. Neurons are color-coded based on their cluster identity (C1-5). Right, average activity of each habenular functional cluster (colors corresponds to panel A) during 6 consecutive, 50ms forebrain micro-stimulations. Clusters are defined by k-means clustering. Forebrain micro-stimulations are marked in red.

(T) Same example fish in (S), but after the bath application of AMPA+NMDA receptor blockers, NBXQ(5μm) + AP5(25μm). Note that in the presence of AMPA+NMDA receptors blockers, no habenular responses are detected

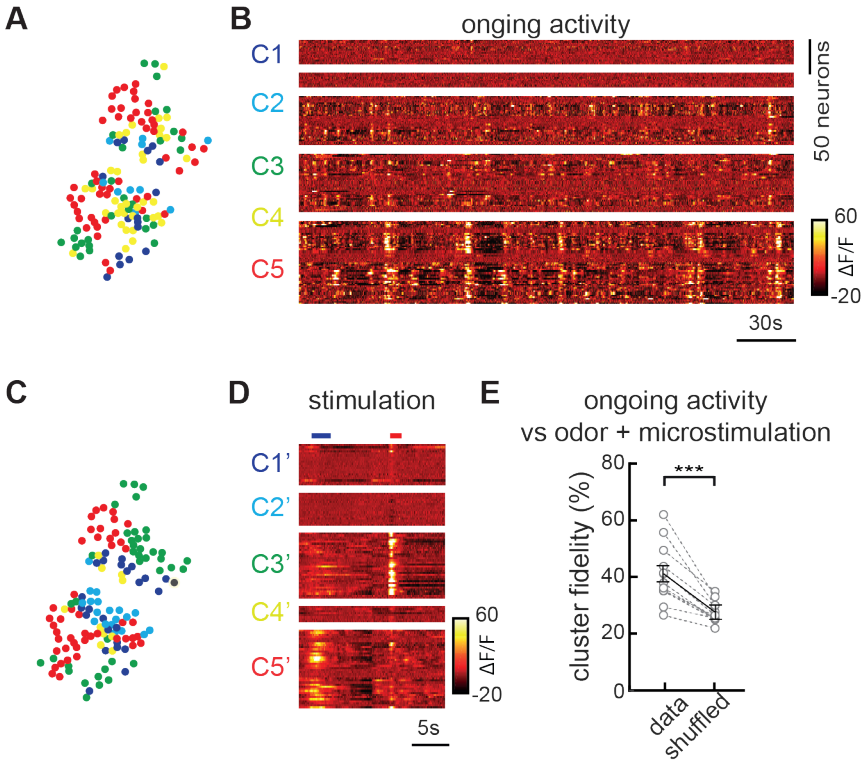

Figure S5: Cluster identities of habenular neurons remains stable during forebrain and odor stimulation. Related to Figure 5.

(A) Representative example of habenular neurons clustered with k-means functional clustering of their ongoing activity in juvenile zebrafish brain explant. Colors represent habenular clusters with similar ongoing activity.

(B) Ongoing activity of the habenular neurons corresponding to clusters in A.

(C) Representative example of habenular neurons clustered during forebrain and odor stimulation using k-means clustering. Colors represent habenular clusters with similar responses to forebrain micro-stimulation and odor stimulation. Note the similarity of clusters in A and C.

(D) Forebrain micro-stimulation and odor responses of the habenular neurons corresponding to clusters in C. Blue line indicates odor stimulation, red – micro-stimulation.

(E) The ratio of habenular neuron pairs remaining in the same functional clusters (cluster fidelity) is significantly higher than chance levels, during ongoing activity and forebrain and odor stimulation. ***$p<0.001$, Wilcoxon signed rank test.

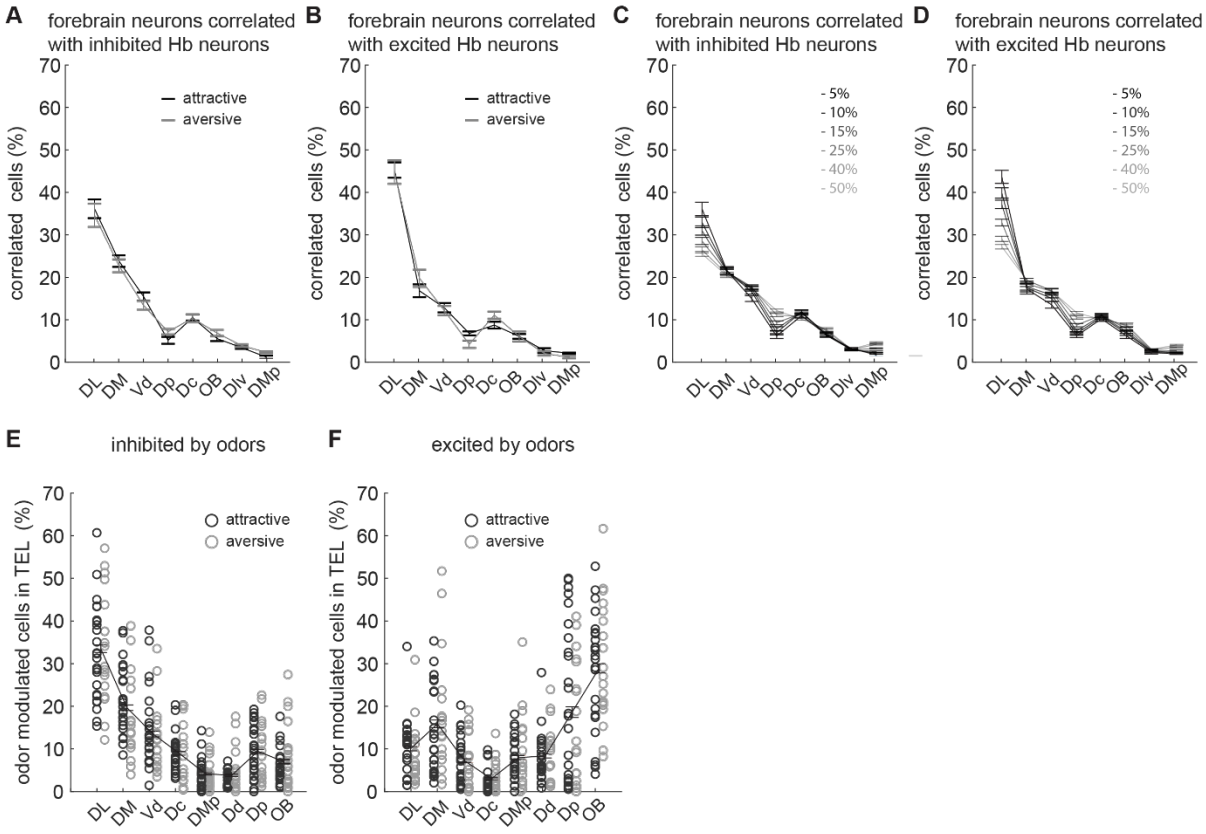

Figure S6. Distribution of forebrain neurons into anatomically identified forebrain regions, based on their odor responses, or their synchrony with odor modulated habenular neurons. Related to Figure 6.

(A) Anatomical distribution of forebrain neurons that are correlated with habenular neurons (top 5%) that are inhibited by odors in 10 fish.

(B) Anatomical distribution of forebrain neurons that are correlated with habenular neurons (top 5%) that are excited by odors in 10 fish. In A and B, attractive odors are in black, aversive odors are in grey. Lines represent mean ± SEM.

(C) Anatomical distribution of forebrain neurons with varying threshold for strong correlations (between top 5 and 50%) to ongoing activity of habenular neurons that are inhibited by odors in n=10 fish. Related to Figure 6E.

(D) Anatomical distribution of forebrain neurons with varying threshold for strong correlations (between top 5 and 50%) to ongoing activity of habenular neurons that are excited by odors in n=10 fish. Related to Figure 6E.

(E) Anatomical distribution of forebrain neurons that are most (top 5%) inhibited by odors in 10 fish. Black and gray circles represent attractive and aversive odors, respectively. Black lines represent mean ± SEM.

(F) Anatomical distribution of forebrain neurons that are most (top 5%) excited by odors in 10 fish. Black and gray circles represent attractive and aversive odors, respectively. Black lines represent mean ± SEM. Dl – dorsolateral telencephalon, Dd – dorsal nucleus of the dorsal telencephalon, Dm – dorsomedial telencephalon, Dp – posterior zone of the dorsal telencephalon, Vd – dorsal nucleus of the ventral telencephalon, OB – olfactory bulb, Dmp – posterior nucleus of dorsomedial telencephalon, Dc – central zone of the dorsal telencephalon.