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Supplemental Information

The Zebrafish Dorsolateral Habenula Is Required for Updating Learned Behaviors

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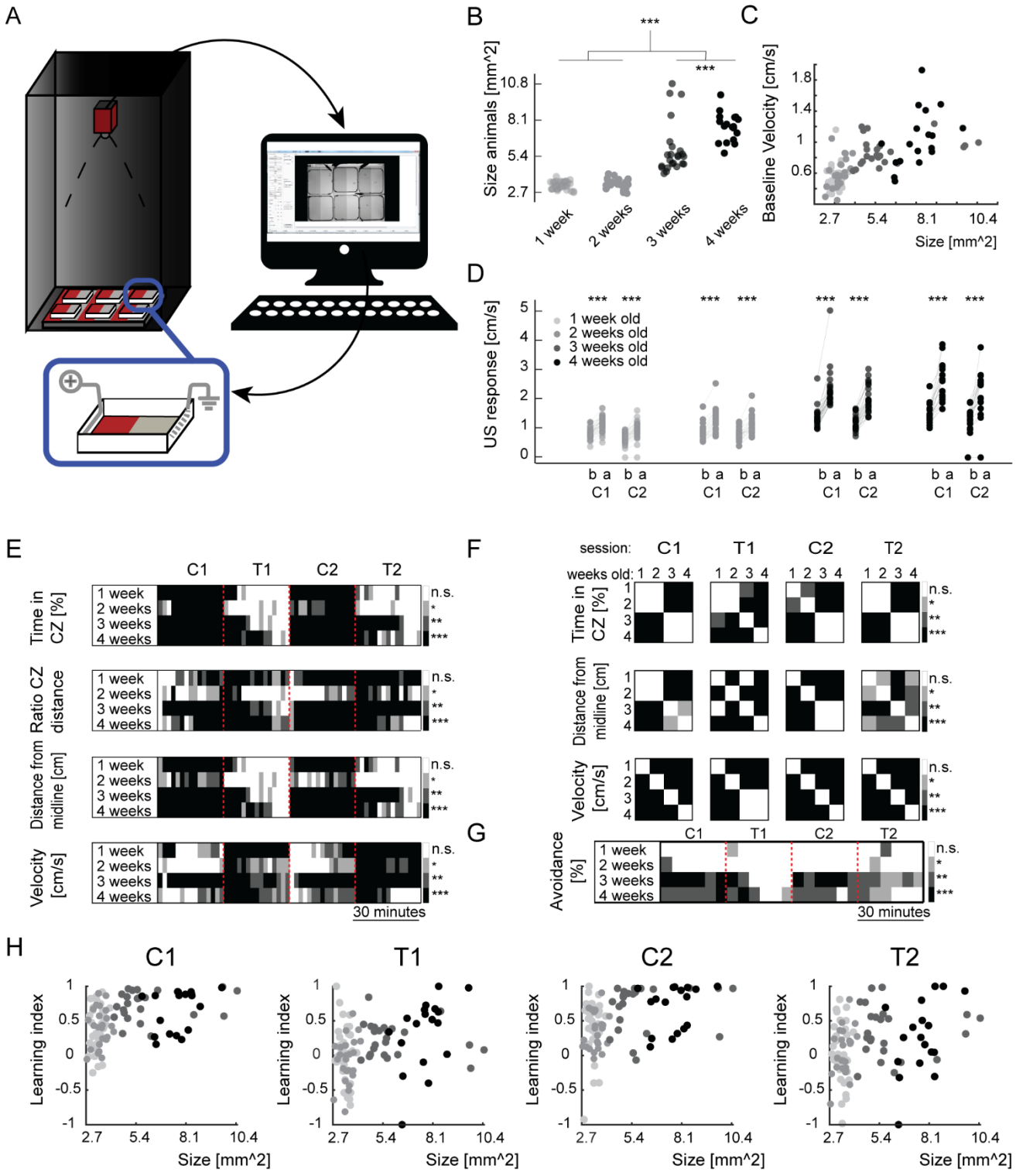

Figure S1. Conditioned place avoidance (CPA) setup and additional analysis of physiological and behavioral parameters during training and multi-group comparisons, Related to Figure 1. *(A) Graphical representation of the CPA training setup. (B) Quantification of animal size reveals a significant enlargement of developing zebrafish at 3-4 weeks. Wilcoxon rank-sum test. (C)*

*Relationship between animal size and the average baseline swim velocity. Note that larger zebrafish display faster swim velocity. (D) Average swimming velocity of the zebrafish, one second before (b) and after (a) the delivery of aversive unconditioned stimuli (US). Green dots represent animals never receiving an US. Wilcoxon signed-rank test. (E) Tables showing intra-group statistical comparison of the parameters measured of animal behavior dividing each session in two-minute time-bins. (F) Tables showing statistical comparison across the 4 experimental groups. (G) Tables showing intra-group statistical comparison of the successful avoidance performed by the animal. (E, F, G) ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon rank-sum test. (H) Relationship between animal size and animal CPA performance across the protocol. Note that older/larger zebrafish perform better than smaller ones.*

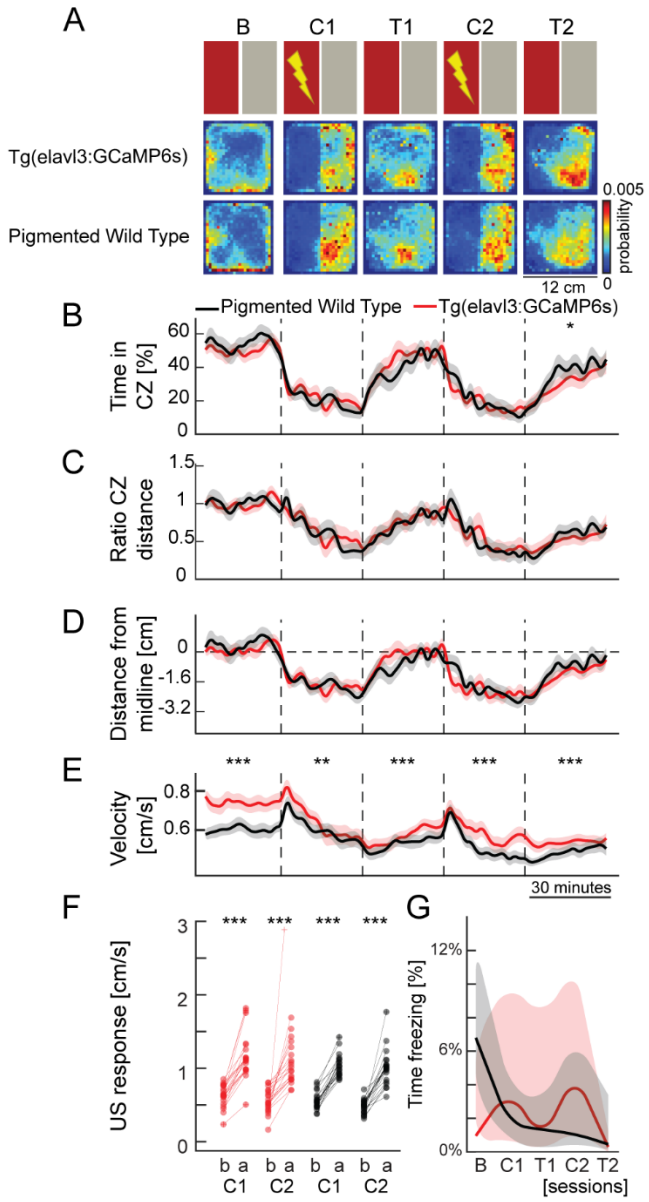

Figure S2. Pigmentated wildtype and pigmentless "nacre" juvenile zebrafish perform similarly in conditioned place avoidance learning, Related to Figure 1.

(A) The top row shows a schematic representation of the protocol. Consecutive rows show the heatmaps depicting the average density of pigmented wild-type (n=21) and pigmentless "nacre" Tg(elavl3:GCaMP6s) juvenile three-weeks-old zebrafish (n=21). (B) Relative time spent in the conditioned zone (CZ). (C) Relative distance of swim in CZ. (D) Distance to the midline of CZ/SZ boundary. Dashed line indicates the midline. (E) Average swimming velocity of the zebrafish. (B-E) Line colors indicate wild-type pigmented (black) and pigmentless 'nacre' Tg(elavl3:GCaMP6s) (red) zebrafish groups. The time course of learning measured in 2-minute time-bins, mean ± SEM. (F) Average swimming velocity of the zebrafish, one second before (b) and after (a) the delivery of aversive unconditioned stimuli (US). (G) Percentage of time that the zebrafish exhibit no swimming, which is defined by less then 2mm swimming during 2 seconds. Solid lines represent

*median, shaded areas represent first and third quartiles. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon rank-sum test.*

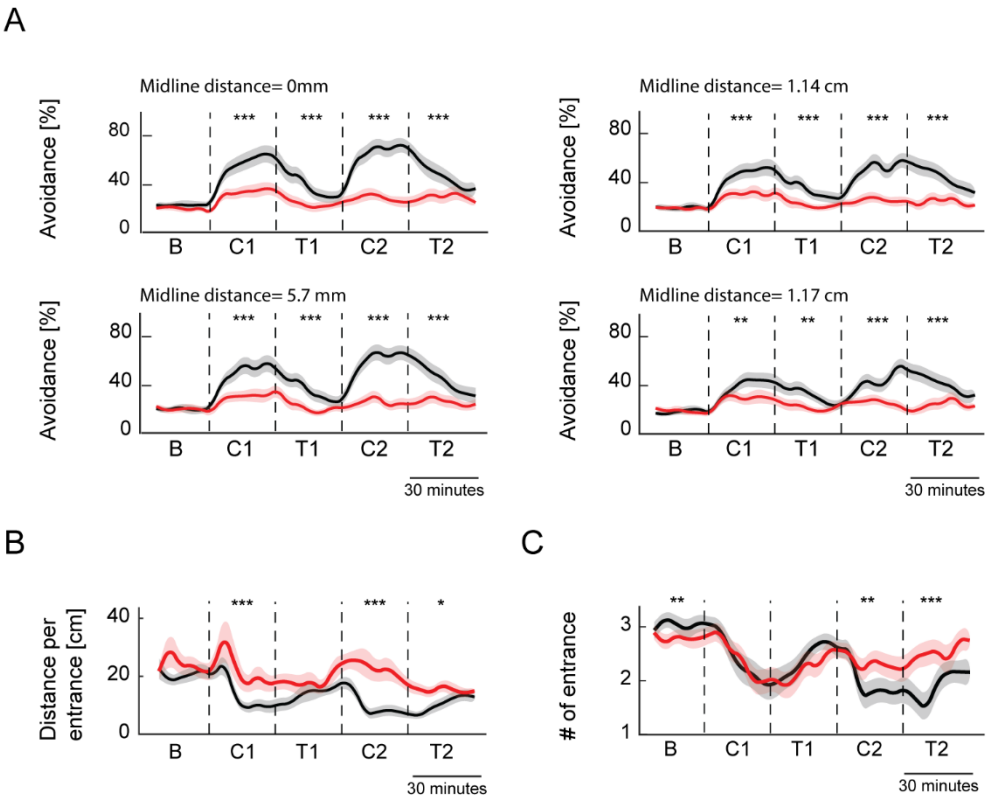

Figure S3. Trained zebrafish avoid the visual boundary between conditioned and safe zone, Related to Figure 2.

*(A) Iterative analysis of animals' avoidance behavior upon approach to the visual boundary between CZ and SZ. The analysis is performed at four different distance away from the CZ-SZ boundary, ranging from 0 to 1.17 cm. (B) Average swim distance up on each entry to CZ. (C) Average number of entries into CZ. (A-B) Line colors indicate control (black) and sham (red) groups. The time course of learning measured in 5-minute time-bins, mean ± SEM. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon rank-sum test*

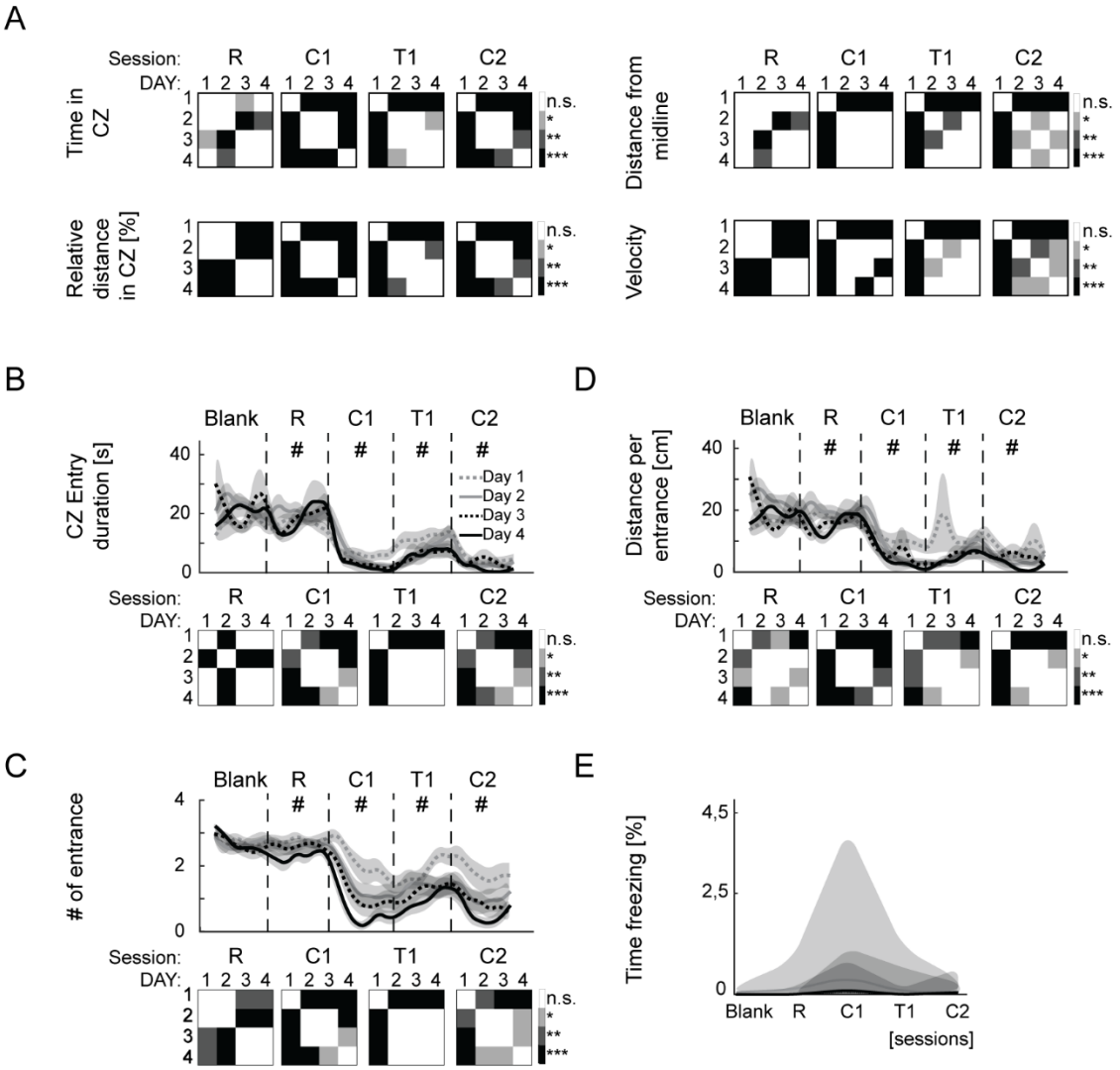

Figure S4. Multi-day training significantly improves CPA performance of the animal, Related to Figure 3.

*(A) Statistical comparison across the different days of training. R: recall, C1: conditioning 1, T1: test 1, C2: conditioning 2. Note the significant improvement of learning performance (in T1) and recall (R) over consecutive training days, using different learning measures. White color marks non-significant comparison. (B) Average swim duration up on each entry to CZ. (C) Average number of entries into CZ. (D) Average swim distance up on each entry to CZ. (B-D) Time course measured in 5-minutes time bins, mean ± SEM. Bottom raw represent statistical comparison across the different days of training. R: recall, C1: conditioning 1, T1: test 1, C2: conditioning 2. (A, B, C, D) # indicate significance comparisons, detailed underneath the graphs. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon rank-sum test. White color marks non-significant comparison. (E) Percentage of time that the zebrafish exhibit no swimming, which is defined by less then 2mm swimming during 2 seconds. Solid lines represent median, shaded areas represent first and third quartiles.*

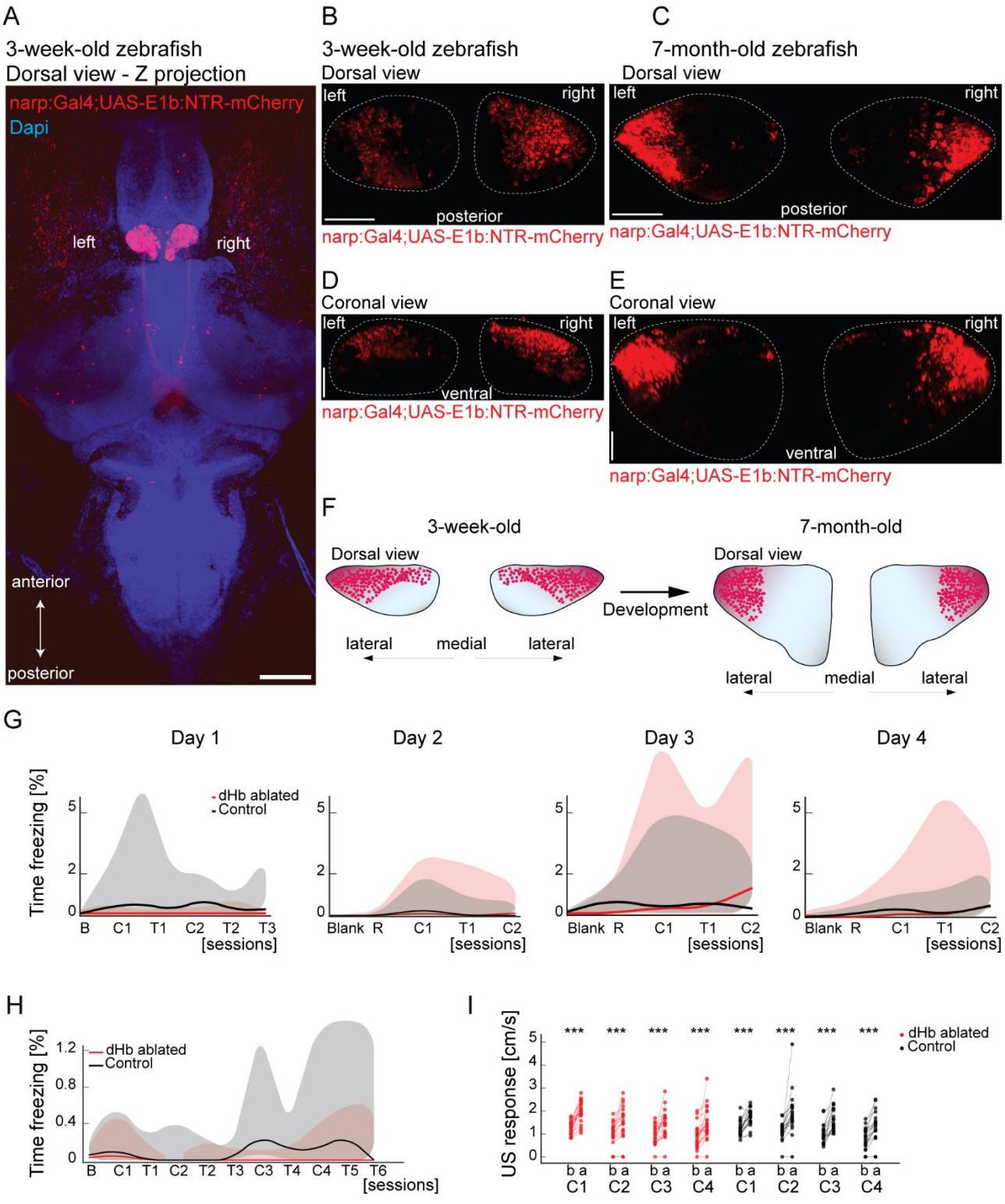

Figure S5 Dorsolateral habenula (dlHb) ablation does not lead to freezing behavior, Related to Figure 4 and Figure 5.

(A) Confocal microscopy image of the entire brain in three-week-old Tg(narp:Gal4;UAS-E1b:NTR-mCherry) zebrafish, dorsal view, scale bar is 100µm. Please note that the narp:Gal4

*expression is exclusively in the dorsolateral habenula and its projections to midbrain. (B-E) Closeup confocal microscopy images of the habenula in three-week- (B, D) and seven-month-old (C, E) Tg(narp:Gal4;UAS-E1b:NTR-mCherry) zebrafish. Dorsal views (B, C) and coronal views (D, E). Scale bars are 50μm. (F) Schematic representation of narp:Gal4-expressing dorsal habenular neurons in juvenile and adult zebrafish, coronal view. Please note that the relative position of dorsal habenular neurons labelled with narp:Gal4 expression moves towards the lateral end of dorsal habenula in adult zebrafish, in line with sequential neurogenesis of habenular neurons across development, which was described by Fore et al. 2019. (G) Percentage of time that the zebrafish exhibit no swimming, which is defined by less than 2mm swimming during 2 seconds. Note the absence of long-term freezing for both control (black) and dlHb-ablated (red) group. B: baseline, Blank: baseline with no color cues, R: recall, C1: conditioning 1, T1: test 1, C2: conditioning 2, T2: test 2, T3: test 3. Solid lines represent median, shaded areas represent first and third quartiles. (H) Percentage of time that the zebrafish exhibit no swimming, which is defined by less than 2mm swimming for 2 seconds. Note the absence of long-term freezing for both control (black) and dlHb-ablated (red) group. B: baseline with color cues, C1: conditioning 1, T1: test 1, C2: conditioning 2, T2: test 2, T3: test 3 (or extinction), C3: conditioning 3 (reversal learning), T4: test 4 (reversal learning test), C4: conditioning 4, T5: test 5, T6: test 6. Solid lines represent median, shaded areas represent first and third quartiles. (I) Average swimming velocity of the zebrafish, one second before (b) and after (a) the delivery of aversive unconditioned stimuli (US). Green dots represent animals never receiving an US. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon signed-rank test. dlHb-ablated animals are displayed in red, control in black.*