Zebrafish dorsal habenula is required for updating learned behaviors

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ABSTRACT

Operant conditioning requires multiple cognitive processes, such as learning, consolidation, prediction of potential outcomes and decision making. It is less clear how interactions of these processes led to behavioral adaptations that allows animal to cope with changing environment. We first showed that juvenile zebrafish can perform conditioned place avoidance learning, with improving performance across development. Next, we disentangled operant conditioning from contextual fear and anxiety. Our results revealed that animals' decisions and learning performance is shaped by the available information and animals' experience. Ablation of dorsal habenula (dHb), a brain region involved in learning and prediction of outcomes, led to an unexpected improvement in animals learning performance and delayed memory extinction. Interestingly, while the control animals' exhibit rapid adaptation to changing learning rules, dHb ablated animals failed to adapt. Altogether, our results showed that dHb plays a central role in switching animals' strategies while integrating new evidences with prior experience.

INTRODUCTION

The ability to predict threats or rewards, and to shape behavior in response to changing environment is essential for survival [1]. Operant conditioning has been widely used to study the process of learning, where the likelihood of a behavior is modulated by positive or negative reinforcement of the behavior. Therefore, operant conditioning requires a constant monitoring of the animal's own actions, a real time estimation of its consequences and, finally, an update of the relationship between the executed behavior and its outcome [2-4]. The constant update of this relationship between the learned behavior and its outcome is essential, since, in real-life, rules and conditions might change and therefore require a high degree of behavioral flexibility. Reversal learning has been extensively used, across species, to evaluate behavioral flexibility [5-7]. Not surprisingly, the impairment of this behavioral flexibility leads to neuropsychiatric conditions [8] such as obsessive-compulsive behaviors [9], substance abuse [10], post-traumatic stress [11] and mood disorders [9, 12]. Therefore, understanding the neural basis of operant conditioning and associated behavioral flexibility to learn new rules is essential to provide solutions to these disorders.

A brain region that play an important role in operant learning is the habenula (Hb). Lateral habenula (lHb) was shown to encode aversive experiences [13, 14] as well as prediction errors, in case of mismatch between the expectation and the outcome, in rodents and primates [15, 16]. Moreover, lesioning the lHb disrupted cost-benefit decisions in rats, without affecting the animal's ability to evaluate the magnitude of the immediate reward [17]. Complementary studies show the involvement of the medial Hb (mHb) in depression, anxiety, fear and addiction [18-20]. Lesioning mHb in a rat model of depression, reverse anhedonia-like behavior resulting in a rescued sucrose consumption after chronic stress [21]. Consistent with mammals, several studies highlight the homologous role of Hb during associative learning in zebrafish. Based on neural connectivity and molecular characteristics, zebrafish ventral habenula (vHb) and dorsal habenula (dHb) are well established homologous of mammalian lHb[22] and mHb[23] respectively. Ablation of dHb in zebrafish disrupts the execution of experience dependent fear response [23, 24] and evaluation of outcomes in social conflict [25]. Whereas vHb ablation impairs active avoidance learning, without affecting the panic behavior induced by classical fear conditioning [26]. Finally, two recent studies also highlight different roles of dHb and vHb in response to recovery after an aversive experience [27] and transition of coping behaviors in response to inescapable shock [28], respectively. All these previous studies suggest the involvement of habenula in coordinating multiple neural processes, evaluating outcomes and selecting optimal behaviors in response to experienced changes in the environment. It is however less clear, how changes in learned rules in cognitively demanding tasks are integrated in the brain and what role habenula plays in this behavioral flexibility. Investigation of such cognitively demanding behaviors in transparent juvenile zebrafish, would open new avenues in studying the neural basis of behavioral flexibility across widely dispersed brain regions.

In this study, we designed an operant conditioning assay to investigate whether transparent juvenile zebrafish can perform conditioned place avoidance

(CPA) learning, which requires coordination of multiple cognitive processes. We showed that while few of the 1-week old larval zebrafish can perform CPA successfully, animals' performance improves drastically at 4-weeks old juvenile stage with transparent brains. Moreover, we introduced a novel approach to disentangle the operant conditioning from contextual fear and the potential stress response of the animal to aversive experience. We observed that the animals' learning performance and strategy is shaped by the available visual cues. Moreover, we showed that the zebrafish learning performance and memory recall improved drastically across multiple training days. Genetic ablation of dHb led to an apparent improvement in animals learning performance likely due to delayed memory extinction. Our reversal learning experiments showed that the juvenile zebrafish exhibit prominent behavioral flexibility and can quickly learn changing CPA rules. However, dHb ablated zebrafish with delayed memory extinction lacked behavioral flexibility that is necessary for reversal learning and failed to adapt changing learning rules. Our results highlight the potential of juvenile zebrafish for performing complex behavioral tasks that requires the coordination of multiple cognitive processes and propose a new role for dHb in adaptive behaviors.

RESULTS

Juvenile zebrafish can perform conditioned place avoidance with increasing performance across development.

To investigate the ontogeny of CPA learning, we focus on the first four weeks of zebrafish development, during which animals are still transparent. We observed that the size of zebrafish increased significantly only on the third and fourth week of development, highlighting a drastic change in animals' body only at late juvenile stage (Figure S1B). Using a custom built behavioral setup, we trained zebrafish in a conditioned place avoidance protocol, where animals received mild aversive stimuli only when they entered the conditioned zone marked by red color cues presented from the bottom (Figure S1A). The training was composed of a baseline session followed by two blocks of conditioning and test sessions (Figure 1A). During baseline, we observed no apparent bias of animals to the red or grey labelled sections of the arena (Figure 1A, B). During the conditioning sessions animals received a 10 ms long mild electric stimulus every 1,3 seconds, only when they entered the conditioned zone, marked by red color. We quantified animals learning performance by using multiple measures, such as the reduction of time spent and the distance swim in conditioned zone, as well as the increase in animals' physical distance from the conditioned zone. During conditioning C1 and C2, zebrafish at all developmental stages exhibit a significant decrease of time spent in the conditioned zone (Figure 1A,B, Figure S1E, F). However, only three and four-week-old zebrafish retained a significant avoidance behavior during the entire 30 min long test sessions T1 and T2, with 4-week old animals performing significantly better then 3-weeks (Figure 1A,B, Figure S1E, F). This trend for significantly better learning performance at later developmental stages was also confirmed by other measures of learning such as distance swim in conditioned zone (Figure 1C, Figure S1E, F)) and the animals' physical distance away from the conditioned zone (Figure 1D, Figure S1E, F)). While all groups decrease their swim velocity during the conditioning protocol (Figure 1E), we did not observe substantial freezing behavior in any group (Figure S1C). All groups responded to aversive stimulus with transient speed increase (Figure S1D). Next, we investigated animals goal directed swimming trajectories during CPA learning. To achieve this, we calculated the swim direction when animals were approaching and avoiding the visual boundary marking the border between conditioned zone and safe zone, during the second conditioning and test sessions (Figure 1F). We observed that several 3-4-week-old animals take evasive action near the conditioned zone boundary (Figure 1F, gray dots on the right hemisphere, Figure 1G, top) or never approached the boundary (Figure 1F, black dots). This was also accompanied by reduced number of approaches to the border of the conditioned zone in older animals (Figure 1G, bottom). Altogether, these results showed that juvenile zebrafish can successfully perform CPA learning with increasing performance across development.

Operant learning can be disentangled from contextual fear.

To disentangle the contextual fear that is generated by the delivery of aversive stimuli in a specific environment from operant learning, we designed a "sham" training protocol for 4 weeks old juvenile zebrafish. To achieve this, we trained half of the animals with the CPA protocol, while in parallel a size-matched "sham-trained" group was exposed to same aversive stimuli, that is exactly timed and dictated by their CPA trained siblings' behavior, but in a different behavioral arena with same features (color, shape, temperature, etc.). Since the sham-trained animals' positions were independent of their aversive experience, dictated by their siblings' behavior, they were not allowed to learn the CPA task, but experienced fear in a similar arena (Figure 2A). As expected the trained zebrafish learned the CPA task and avoid the conditioned zone successfully in all measures of learning (black traces, Figure 2B-D). Whereas sham-trained animals had no opportunity to

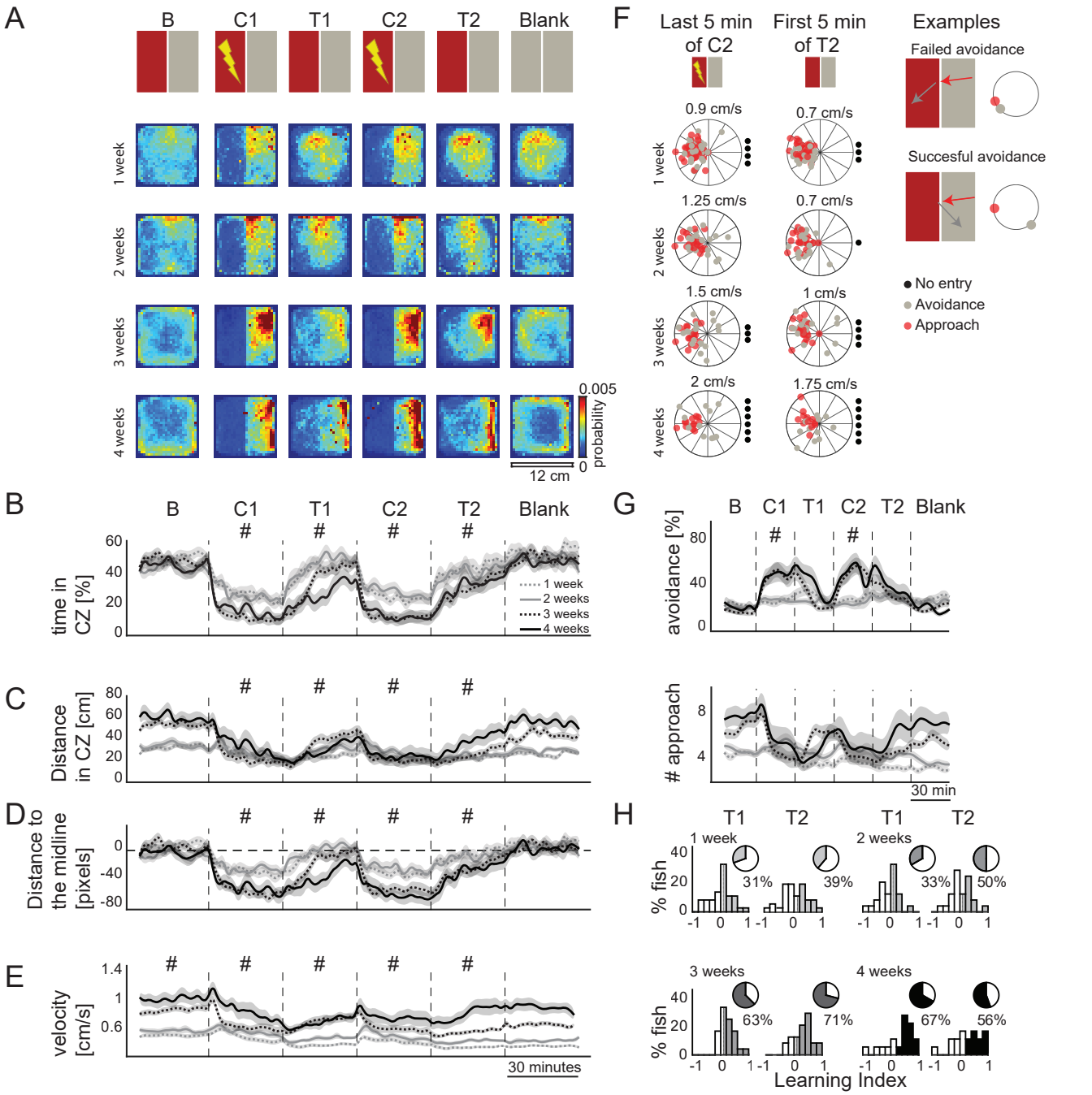

Figure 1: Juvenile zebrafish can perform conditioned place avoidance learning with increased performance across development.

(A) The top row shows a schematic representation of the protocol. B: baseline, C1: conditioning 1, T1: test 1, C2: conditioning 2, T2: test 2, Blank. Consecutive row shows the heatmaps depicting the average density of zebrafish position for each experimental group (over the course of each entire session), 1 week (n=36), 2 weeks (n=24), 3 weeks (n=24) and 4 weeks (n=18) old zebrafish. (B) Percentage of time spent in the conditioned zone (CZ) of the arena. (C) Absolute swimming distance in the CZ of the arena. (D) Average distance of the animals away from the CZ of the arena. Dashed line from 0 indicate the animal being on the midline. (E) Average swim velocity of the zebrafish. (B-E) Line colors and style indicate different age groups. The time course of learning measures in 2 minutes time bins (mean ± SEM). # indicate significance in comparisons across several groups, which are detailed in Figure S1E,F. (F) Zebrafish swimming direction near the midline boundary of CZ and safe zone, during the last 5 min of C2 (left) and first 5 min of T2 (right), increasing age groups are ordered from top to bottom. Each dot on the polar plot depicts the average swim direction of one zebrafish during the four seconds before (red) and after (gray) encountering the midline. For each dot, the distance from the center encode average swim velocity of the animal. The numbers on top of the plots indicate the swim velocity represented by the outer-ring of the polar plots. Black dots outside the polar plots represents animals that never entered the CZ. (G) Average ratio of successful avoidance (top) and total number of approaches to the midline (bottom) over the course of the protocol, divided in five minutes time bins (mean ± SEM). # indicate significance in comparisons across several groups, which are in Figure S1G. (H) Histograms of the learning indices during the two test sessions, across development. The darker colors for each histogram represent the animal classified as learners, based on the threshold during baseline behavior. The percentage of learners are in the pie chart above each histogram.

***p= <0.001, **p= <0.01, *p= <0.05 Wilcoxon ranksum test. All the statistical comparison between groups and intra-group are shown in supplementary tables for visualization purpose (Figure S1). Duration of each session is 30min.

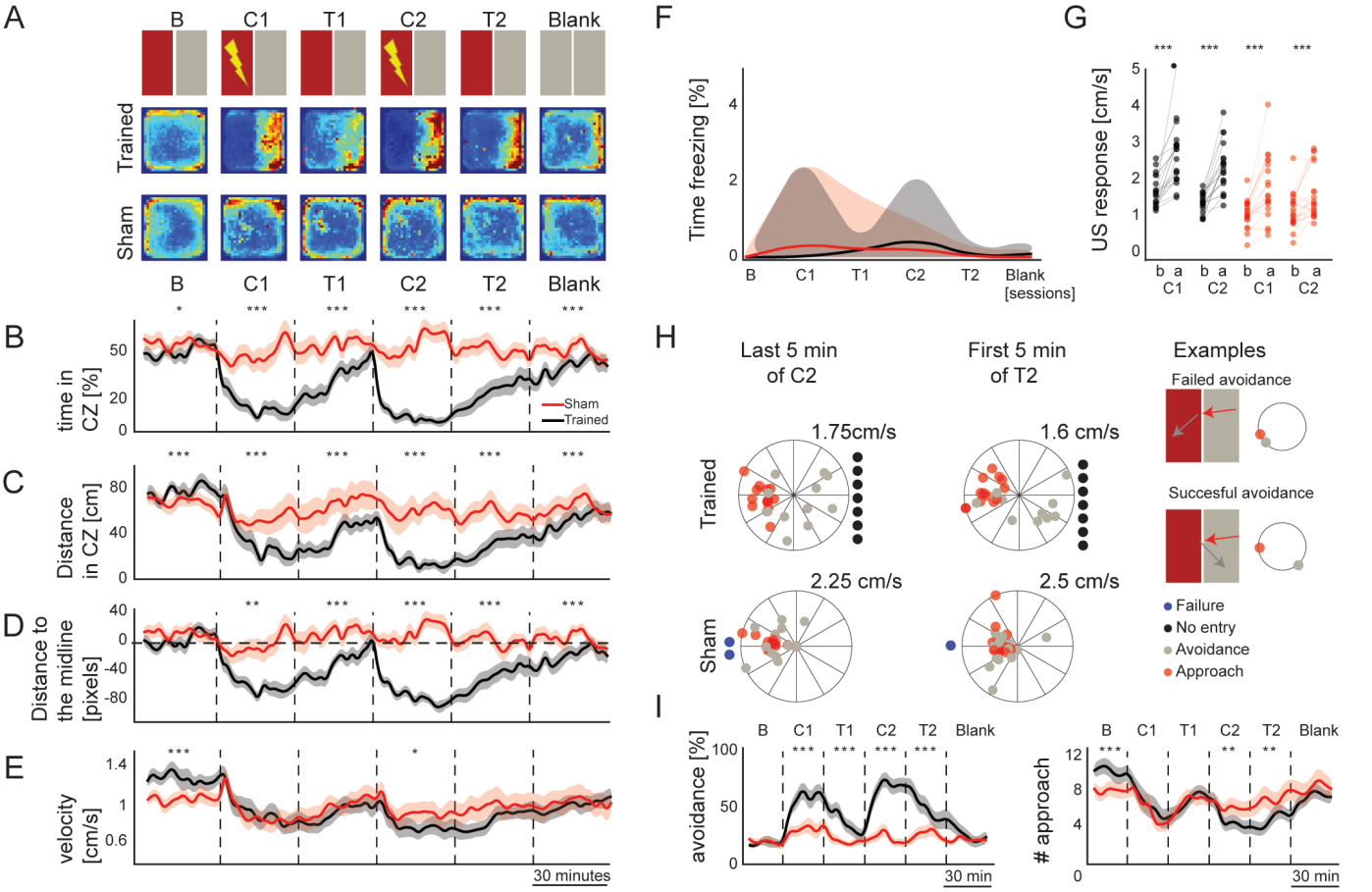

Figure 2: Disentangling contextual fear from operant learning.
(A) The top row shows a schematic representation of the protocol. Consecutive rows show the heatmaps depicting the average density of zebrafish position for trained (n=20) and sham-trained (n=20) groups. (B) Percentage of time spent in the conditioned zone (CZ) of the arena. (C) Absolute swimming distance in the CZ of the arena. (D) Average distance of the animals away from the CZ of the arena. Dashed line from 0 indicate the animal being on the midline. (E) Average swim velocity of the zebrafish. Note that the trained group shows a more prominent reduction of swim velocity when compared to the sham group. (B-E) Line colors indicate control (black) and sham (red) groups. The time course of learning measures in 2 minutes time bins (mean ± SEM). (F) Percentage of time that the zebrafish exhibit no swimming, which is defined by less then 2mm swim during 2 seconds. Note the absence of long term freezing for both contol (black) and sham (red) group. Solid lines represent median, shaded areas represent first and third quartiles. (G) Average swim velocity of the zebrafish, one second before (b) and after (a) the delivery of aversive unconditioned stimuli (US). (H) Zebrafish swimming direction near the midline boundary of CZ and safe zone, during the last 5 min of C2 (left) and first 5 min of T2 (right), in CPA trained (top) and sham (bottom). Each dot on the polar plot depicts the average swim direction of one zebrafish during the four seconds before (red) and after (gray) encountering the midline. For each dot, the distance from the center encode average swim velocity of the animal. The numbers on top of the plots indicate the swim velocity represented by the outer-ring of the polar plots. Black dots outside the polar plots represent animals that never entered the CZ, blue dots represent animals never left the CZ. (I) Average ratio of successful avoidance (left) and total number of approaches to the midline (right) over the course of the protocol, divided in five minutes time bins (mean ± SEM).
***p= <0.001, **p= <0.01, *p= <0.05 (A-E,I) Wilcoxon ranksum test, (G) Wilcoxon signedrank test. Duration of each session is 30min.

learn (red traces, Figure 2B-D), and therefore received in principle a mild level of inescapable aversive stimuli. Consequently, we observed that trained and sham animals both showed a mild reduction of their speed, but trained animals reduced their speed significantly more as a result of the CPA learning. Hence, the reduction of the speed was not merely due to aversive experience of the animals, but due to change in animals' behavioral strategy upon CPA learning. None of the trained or sham-trained animals exhibit substantial freezing (Figure 2F) and both groups responded to the aversive shock with a small transient increase in swim velocity (Figure 2G). As expected, the trained animals strategically took evasive actions along the boundaries between conditioned and safe zones, whereas sham-trained animals did not perceive these boundaries as relevant information (Figure 2H, I). These results highlight that CPA learning can be separated from contextual fear and juvenile zebrafish exhibit substantially different behaviors in response to CPA and contextual fear.

Juvenile zebrafish use different behavioral strategies for CPA learning based on the available visual information.

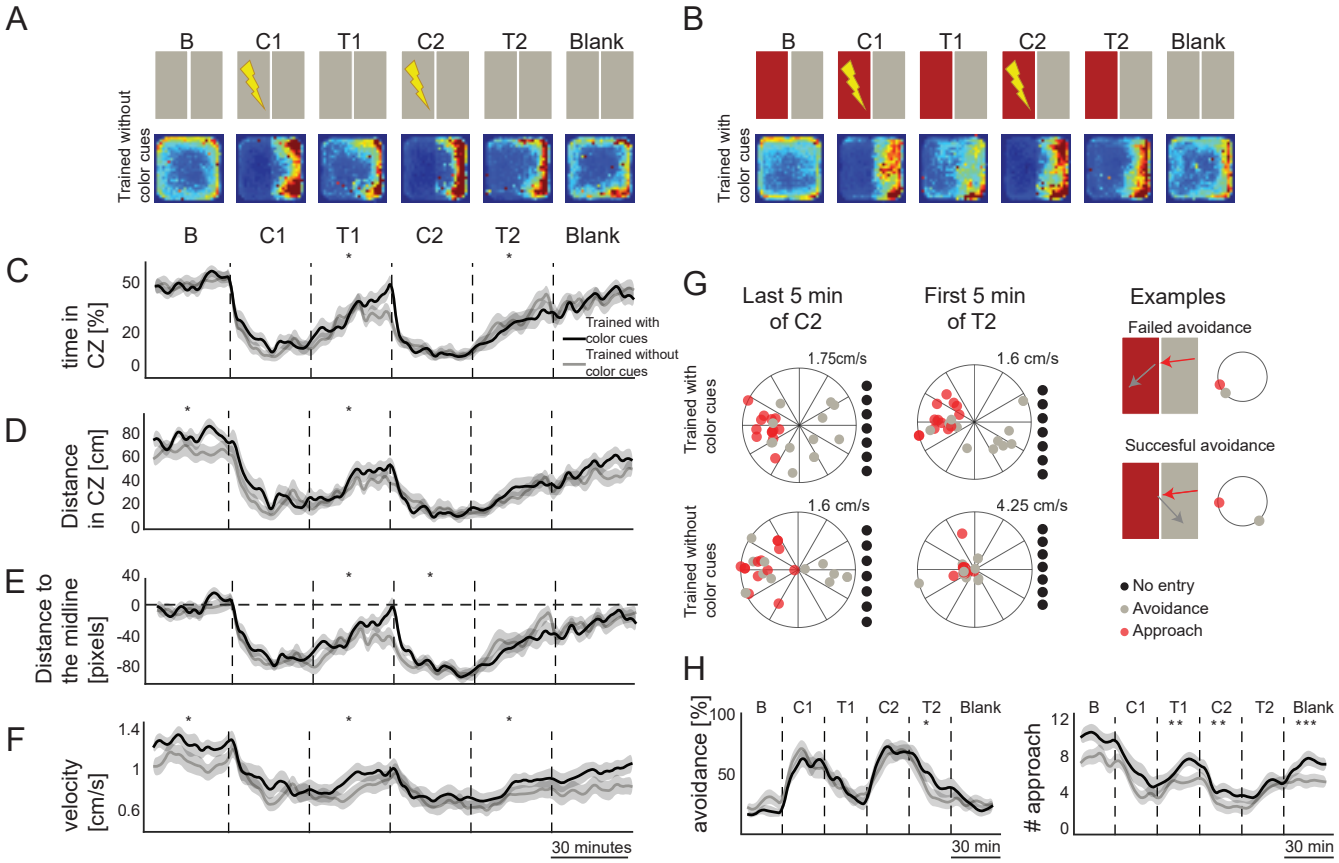

Figure 3: Zebrafish use the available vidusl information in the environment to fulfill the task.
(A-B) The top row shows a schematic representation of the protocol. Consecutive rows show the heatmaps depicting the average density of zebrafish position for the experimental groups trained without color cues (A, n=20) and with color cues (B, n=20).(C) Percentage of time spent in the conditioned zone (CZ) of the arena. (D) Absolute swimming distance in the CZ of the arena. Dashed line from 0 indicate the animal being on the midline. (E) Average distance of the animals away from the CZ of the arena. Dashed line from 0 indicate the animal being on the midline. Note that the group trained without color cues shows more prominent avoidance from the midline and slower extinction. (F) Average swim velocity of the zebrafish. (C-F) Line colors indicate zebrafish trained with (black) and without color cues (gray) groups. The time course of learning measures in 2 minutes time bins (mean ± SEM). (G) Zebrafish swimming direction near the midline boundary of CZ and safe zone, during the last 5 min of C2 (left) and first 5 min of T2 (right), in zebrafish trained with (top) and without color cues (bottom). Each dot on the polar plot depicts the average swim direction of one zebrafish during the four seconds before (red) and after (gray) encountering the midline. For each dot, the distance from the center encode average swim velocity of the animal. The numbers on top of the plots indicate the swim velocity represented by the outer-ring of the polar plots. Black dots outside the polar plots represent animals that never entered the CZ. (H) Average ratio of successful avoidance (left) and total number of approaches to the midline (right) over the course of the protocol, divided in five minutes time bins (mean ± SEM).
***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon ranksum test. Duration of each session is 30min.

To estimate the relevance of visual cues in CPA learning, we trained a group of 4 weeks old, size-matched juvenile zebrafish with no color cues in the behavioral arena. We observed that even in the absence of distinct color cues, separating conditioned zone from safe zone, juvenile zebrafish can perform CPA learning as good as animals trained with color cues (Figure 3A-C). In fact, our results showed that zebrafish trained with no color cues exhibit stronger aversion to conditioned zone, swimming closer to the distant wall of the safe zone (Figure 3D), and a stronger reduction of their swim velocity up on CPA training (Figure 3E). Both groups respond to unconditioned stimuli and did not exhibit prominent freezing (Figure S2). These results suggest that in the absence of color cues, zebrafish might use different strategies for preforming CPA learning. In line with this, we observed that the spatial position of the boundary between conditioned and safe zone does not carry any relevant information for the zebrafish trained with no color cues (Figure 4G, H). Hence during the test session, zebrafish with no color cues did not take any evasive action near the spatial boundaries of the conditioned and safe zones (Figure 4G, H). These results suggest that while juvenile zebrafish can perform CPA learning even in the absence of color cues using alternative strategies, zebrafish use the additional color/visual information, when it is available.

CPA performance and memory recall improve across multiple training sessions.

Next, we investigated whether juvenile zebrafish would

improve their performance across multiple training days and whether such training would also facilitate long-term learning and memory consolidation. To achieve this, we trained a set of 4 weeks old juvenile zebrafish across 4 consecutive days, where each session started with a blank session with no color cues, followed by a recall session with color cues and a conditioning session followed by a test session (Figure 4A). Finally, each session ended with a last conditioning session. In line with our hypothesis, multi-day training improved the learning performance in all measurement of learning performance during the conditioning and test sessions (Figure 4 A-D, Figure S3), especially when compared to training day 1 (Figure S3, Figure 4F). In particular, the percentage of animals classified as good learners increase from 67% in day1 up to 89% in the consecutive training days (Figure 4F). In order to test the memory recall after multi-day training, we analyzed the transient ten-minute period (boxes labelled B' and R' in Figure 4B, C), where the color pattern of the behavioral arena switch from only grey, to CPA pattern with red and gray color cues that the animals were trained with (Figure 4A, top). In fact, from the third day of training on, we observed a transient but significant recall, when CPA color cues are presented right after the blank session with no color cues, evident by reduced time spent (Figure 4G) and reduced distance swam (Figure 4G) in the conditioned zone. We also observed that during the recall period, the zebrafish perceived the boundary between the color cues and took evasive action as they approach the boundary of the conditioned red zone (Figure 4J, K, black arrow). The ratio of successful avoidance during recall significantly increased (Figure 4K) across multi-day training. We did not observe any prominent freezing behavior even after multi-day training (Figure 4I). Our results demonstrated that CPA performance of zebrafish improve across multiple training days and zebrafish can form long-term CPA memories, which are more effectively recalled after multi-day training.

Dorsal habenula ablation improves CPA learning performance.

Dorsal habenula ablation in adult zebrafish was shown to interfere with the execution of experience dependent fear response [23]. We hypothesize that dHb ablation might also interfere with the CPA learning. We achieved chemogenetic ablation of dHb by incubating 4 weeks old juvenile zebrafish Tg(narp:GAL4VP16; UAS-E1b:NTR-mCherry) with 10mM metronidazole (MTZ) (Figure 5A,B). In juvenile zebrafish, narp:Gal4 is expressed exclusively in dHb neurons, and it is comparable to adult zebrafish [23] (Figure S5 A-F). Slightly more lateral location of narp:Gal4 expressing dHb neurons in adults is due to sequential habenular neurogenesis across development [29]. Next, we compared dHb ablated juvenile zebrafish with the control group in a multi-day training protocol. To our surprise, we observed that dHb ablated animals performed better already at the first day of training session and their CPA performance stayed higher than the control group over consecutive days of training (Figure 5C-N). From the third day of multi-day training on, we also observed that dHb ablated animals exhibit improved CPA memory recall, when the CPA color pattern was first shown after an initial period of habituation with blank gray color (Figure 5O, N). We did not observe prominent freezing behavior in dHb ablated animals even after several days of CPA training (Figure S5G). Moreover, both groups increase their swim velocity in response to stimulus delivery (Figure S5H). These results suggest that dHb ablation led to an apparent improvement of CPA performance and a better memory recall, across multi-day training sessions.

Dorsal habenula is important for behavioral flexibility during reversal learning.

We hypothesized that one reason for dHb ablated zebrafish to perform better than control animals in CPA learning can be the reduced memory extinction rate of these animals during test session. To test this hypothesis, we introduced an extended extinction session test3 (T3) after the initial CPA training. In line with our hypothesis, we observed that the dHb ablated zebrafish showed significantly slower extinction of CPA behavior, even during the extended extinction session T3 (Figure 6A-C). Slower memory extinction in dHb animals can in principle be due to inefficiency of integrating new information about the lack of aversive cue during the test session. Since dHb animals performed better during the initial CPA protocol (Figure 5 and 6), we inferred that the dHb animals might not be integrating new information about the lack of aversive experience. Inspired with these results, we next reversed the learning rule after the initial CPA training and paired the aversive stimuli with the part of the CPA arena, marked with grey visual cue (Figure 6, conditioning periods C3 and C4). In this reversal learning protocol, zebrafish were required to update their learned CPA behavior, and relearn to associate the initially safe zone with the aversive experience. Excitingly, dHb ablated zebrafish did significantly worse than control animals in reversal learning, when the learning rules were switched, quantified by all indicators of reversal learning performance (Figure 6A-D). We observed no significant differences in the velocity of dHb ablated versus control group as well as no prominent freezing behavior (Figure S5G). Altogether our results revealed that while dHb ablation led to better performance in CPA learning, dHb ablated zebrafish exhibit reduced ability to integrate new evidence over

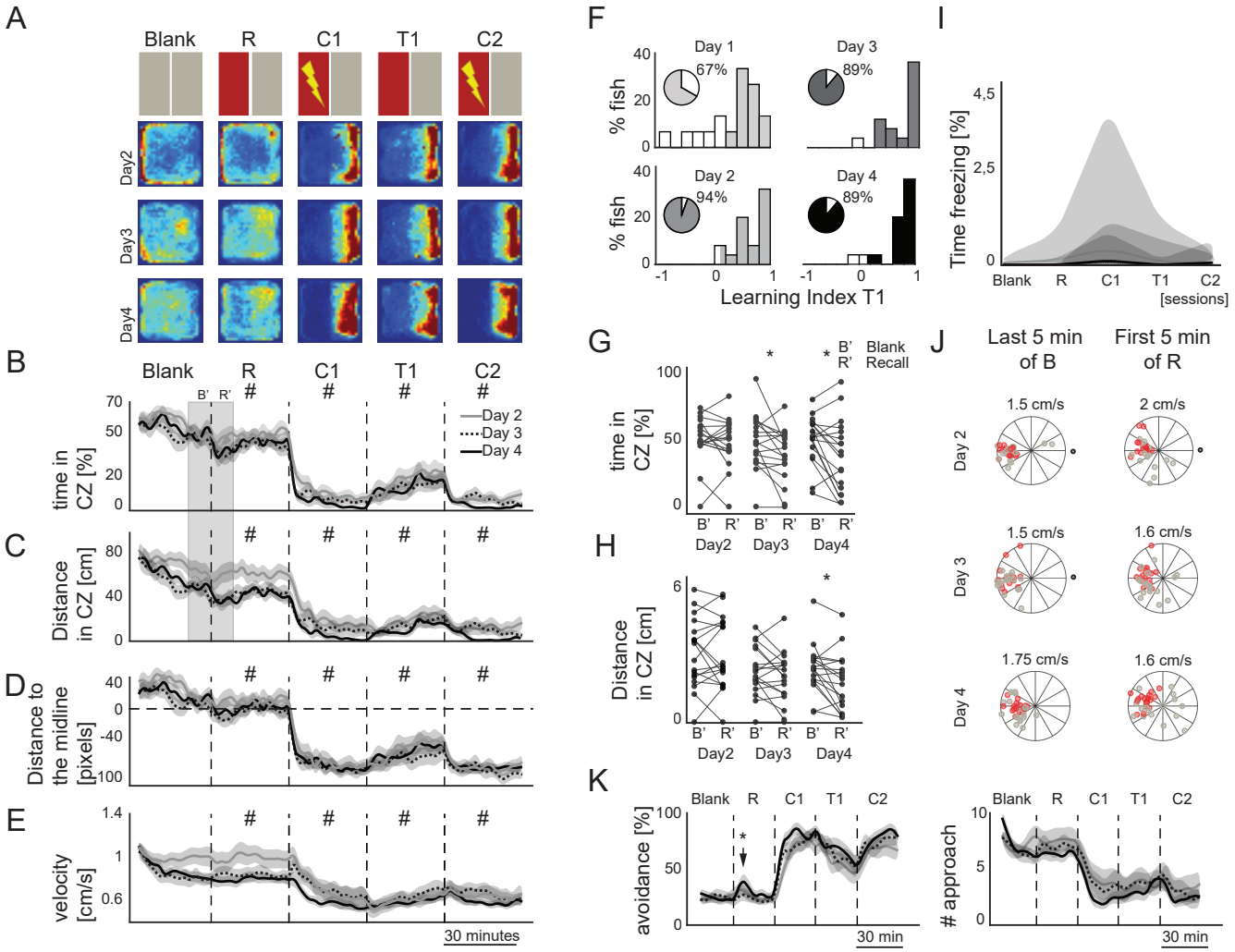

Figure 4: Learning performance and memory recall improves with multi-day training.
(A) The top row shows a schematic representation of the protocol. Blank: baseline with no color cues, R: recall, C1: conditioning 1, T1: test 1, C2: conditioning 2. Consecutive row shows the heatmaps depicting the average density of zebrafish position during multiple training days (n=18). (B) Percentage of time spent in the conditioned zone (CZ) of the arena.
(C) Absolute swimming distance in the CZ of the arena. (D) Average distance of the animals away from the CZ of the arena. Dashed line from 0 indicate the animal being on the midline. (E) Average swim velocity of the zebrafish. (B-E) Line colors and style indicate zebrafish performance during multiple training days. The time course of learning measures in 2 minutes time bins (mean ± SEM). # indicate significance in comparisons across several groups, which are detailed in Figure S4A. Note the improved performance over consecutive training days. (F) Histograms of the learning indices during the test sessions, across multiple days of training. The darker colors for each histogram represent the animal classified as learners, based on the threshold during baseline behavior. The percentage of learners are in the pie chart above each histogram. (G) Average time spent by the animal in the CZ during the last ten minutes of the blank session (B') and in the first ten minutes of the recall session (R') over multiple training days. B' and R' periods are marked with the grey box in panel B and C. Note that from third training day on zebrafish exhibit aversion to CZ when the color cues are presented during recall ression. (H) Average swimming distance in the CZ during the last ten minutes of the blank session (B') and in the first ten minutes of the recall session (R') over multiple training days. B' and R' periods are marked with the grey box in panel B and C. Note that on the fourth day of training zebrafish exhibit aversion to CZ when the color cues are presented during recall ression. (I) Percentage of time that the zebrafish exhibit no swimming, which is defined by less then 2mm swim during 2 seconds. Note the absence of long term freezing for any of the training day. Solid lines represent median, shaded areas represent first and third quartiles. (J) Zebrafish swimming direction near the midline boundary of CZ and safe zone, during the last ten minutes of Blank (B', left) and first ten minutes of Recall (R', right), consecutive training days are ordered from top to bottom. Each dot on the polar plot depicts the average swim direction of one zebrafish during the four seconds before (red) and after (gray) encountering the midline. For each dot, the distance from the center encode average swim velocity of the animal. The numbers on top of the plots indicate the swim velocity represented by the outer-ring of the polar plots. Black dots outside the polar plots represent animals that never entered the CZ. (K) Average ratio of successful avoidance (left) and total number of approaches to the midline (right) over the course of the protocol, divided in five minutes time bins (mean ± SEM). # indicate significance in comparisons across several groups, which are detailed in Figure S4. Note the improved performance over consecutive training days.
***p= <0.001, **p= <0.01, *p= <0.05 Wilcoxon rank sum test. All the statistical comparison between groups and intra-group are shown in supplementary tables for visualization purpose. Duration of each session is 30min.

learned memories during memory extinction and reversal learning.

DISCUSSION

Series of recent studies highlight the increasing popularity of zebrafish for studying diverse aspects of animal behavior [30-39], including the more complex behaviors such as learning [25, 26, 40-46] and social behaviors[47, 48]. While studying complex behaviors in adult zebrafish allow investigation of brain function in a fully mature brain, larval and juvenile zebrafish has the tremendous advantage of a small and transparent brain, when it comes to studying brain

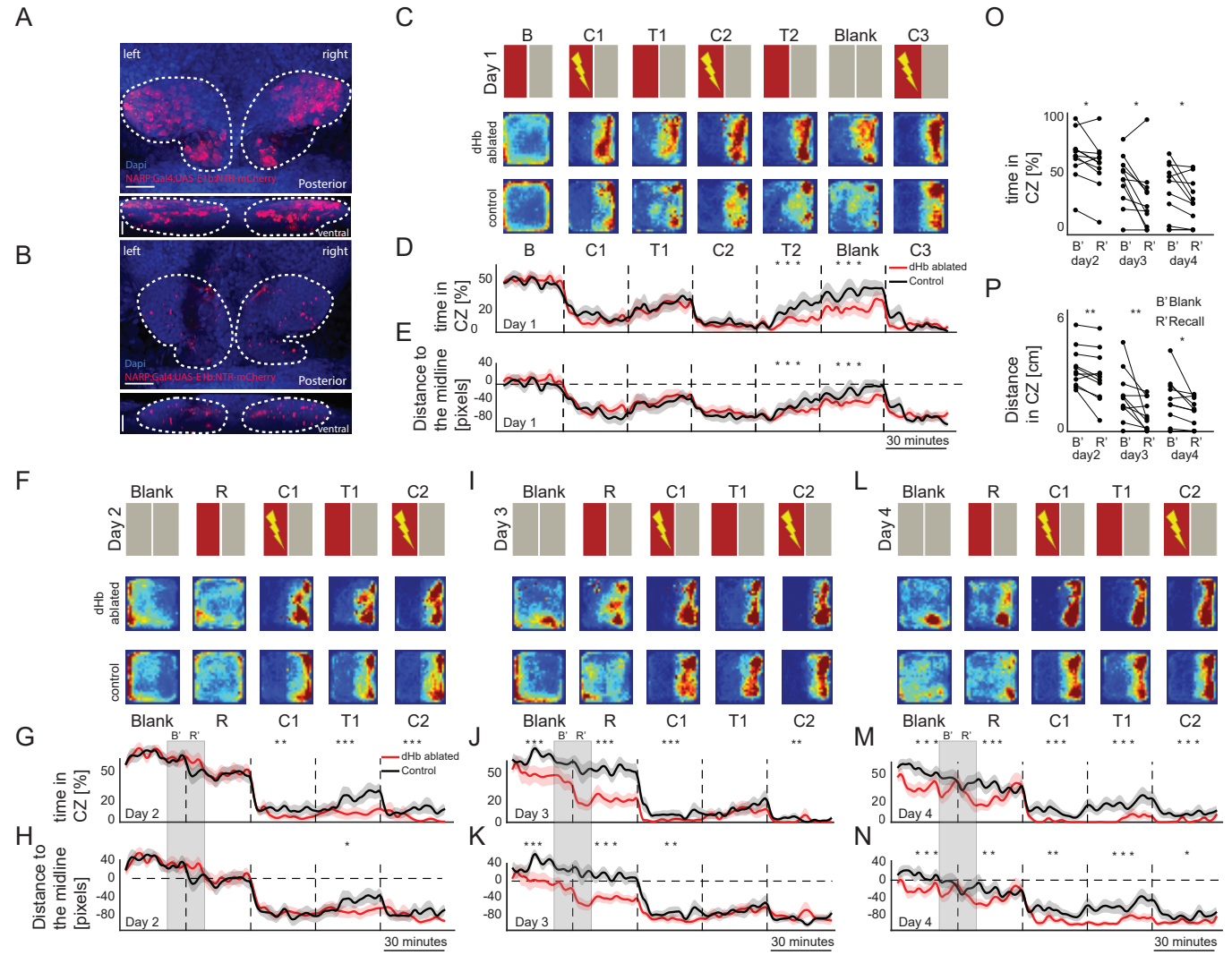

Figure 5: Dorsal habenula ablation improve CPA learning performance and delay memory extinction.
(A,B) Confocal microscopy images of Tg(narp:Gal4;UAS-E1b:NTR-mCherry) zebrafsh before (A) and after (B) Metrodiniazole (MTZ) treatment. Scale bar is 25 µm. (C,F,I,N) The top row shows a schematic representation of the protocol. Blank: baseline with no color cues, R: recall, C1: conditioning 1, T1: test 1, C2: conditioning 2. Consecutive rows show the heatmaps depicting the average density of zebrafish position for dHb ablated (n=11) and control (n=12) groups, across multiple training days. (C) First day of training. (F) Second day of training. (I) Third day of training. (L) Fourth day of training. (D, G, J, M) Percentage of time spent in the conditioned zone (CZ) of the arena, during multiple training days for dHb ablated (red) and control (black) zebrafish. Note the improved performance for dHb ablated zebrafish during the recall and test sessions. (E, H, K, N) Average distance of the animals away from the CZ of the arena, during multiple training days for dHb ablated (red) and control (black) zebrafish. Dashed line from 0 indicate the animal being on the midline. Note the improved performance for dHb ablated zebrafish during the recall and test sessions. (O) Average time spent by the dHb ablated zebrafish in the CZ during the last ten minutes of the blank session (B') and in the first ten minutes of the recall session (R') over multiple training days.. Note that already from the second training day on zebrafish exhibit aversion to CZ when the color cues are presented during recall ression. (P) Average swimming distance of the dHb ablated zebrafish in the CZ during the last ten minutes of the blank session (B') and in the first ten minutes of the recall session (R') over multiple training days. Note that already from the second day of training zebrafish exhibit aversion to CZ, when the color cues are presented during recall ression.
***p= <0.001, **p= <0.01, *p= <0.05 (D, E, G, H, J, K, M, N) Wilcoxon ranksum test (O, P) Wilcoxon signedrank test. Duration of each session is 30min.

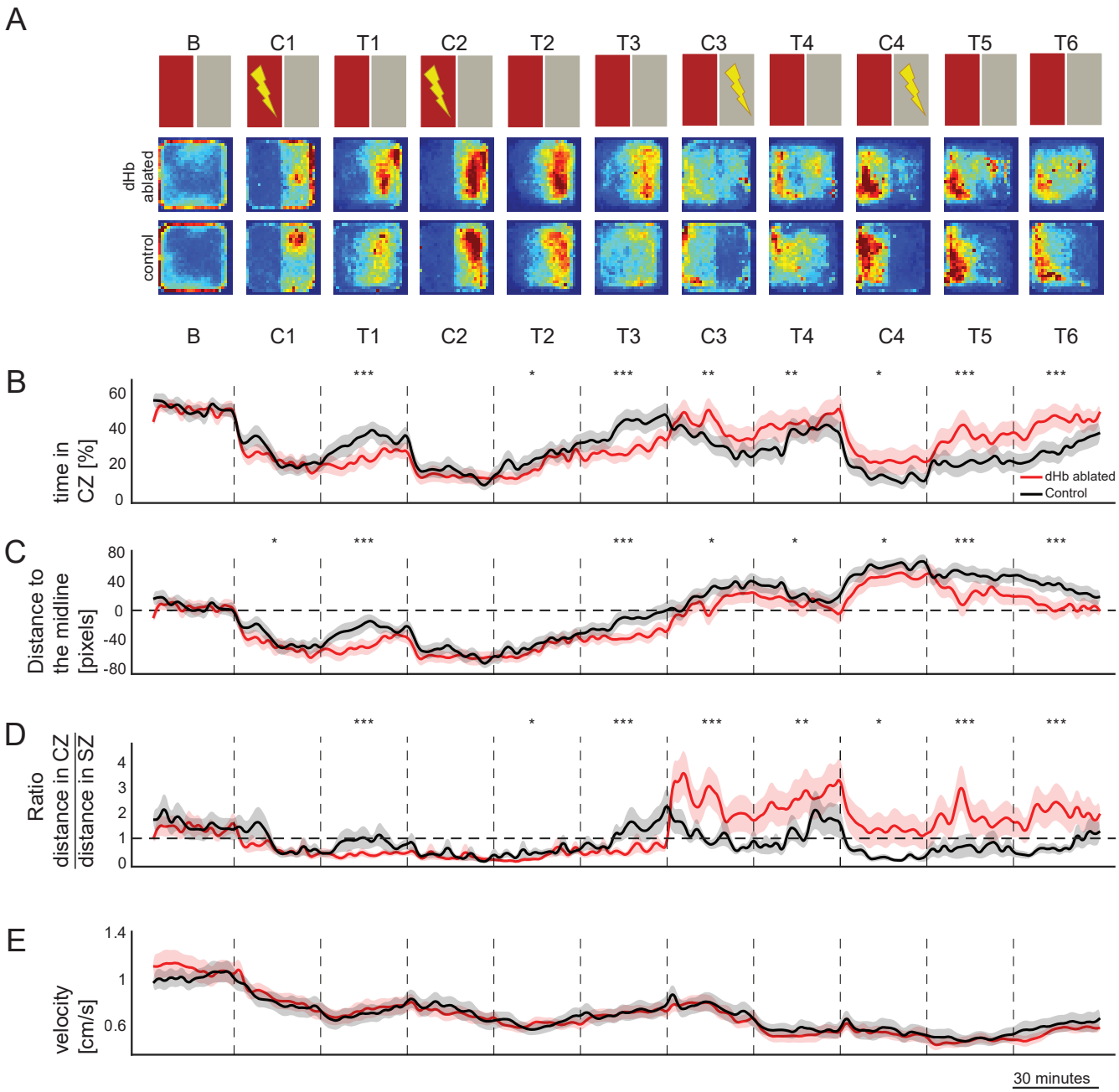

Figure 6: Dorsal habenula provides the animal with the behavioral flexibility required to complete cognitive demanding task under stressful situations.
(A)The top row shows a schematic representation of the protocol. B: baseline with color cues, C1: conditioning 1, T1: test 1, C2: conditioning 2, T2: 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. Consecutive rows show the heatmaps depicting the average density of zebrafish position for control (n=21) and dHb ablated (n=22) groups. (B) Percentage of time spent in the conditioned zone (CZ) of the arena.
(C) Average distance of the animals away from the CZ of the arena. Dashed line from 0 indicate the animal being on the midline. (D) The ratio of swimming distance in conditioned zone (CZ) versus safe zone (SZ). (E) Average swim velocity of the zebrafish. (B-E) Line colors indicate control (black) and dHb ablated (red) groups. The time course of learning measures in 2 minutes time bins (mean ± SEM).
***p= <0.001, **p= <0.01, *p= <0.05, no mark=non significant, Wilcoxon ranksum test. Duration of each session is 30min.

activity [33, 38, 49-58]. Hence, we investigated the ontogeny of a complex operant behavior, namely CPA learning, and showed that the learning performance of young zebrafish improves across development. Our findings are in line with a previous study [45], but also shows that even at younger ages some zebrafish can perform such CPA learning. At four weeks the learning performance of juvenile zebrafish reaches to very high levels and become more robust across animals. Interestingly, we observed that the CPA learning strategy of younger (one-two weeks) animals are substantially different than older (three-four weeks) animals. While young animals swim away from the aversive stimuli towards to the safe

zone during conditioning, during the test session the visual boundary between conditioned zone and safe zone has no impact on their behavior. Whereas older animals perceive the visual boundary and adopt their swimming strategy to take evasive action, in order to avoid conditioned zone during the test session. Moreover, after multi-day training, 4 weeks old juvenile zebrafish can recall these memories even after 24hr. Adult zebrafish were shown to recall memories encoded by telencephalic neural activity [43] homologous to mammalian cortex [59], resembling the memory engrams of the mammalian brain [60-62]. Our results showed for the first time such long-term memory recall in juvenile zebrafish with small and transparent brains that are amenable for brain-wide optical imaging, at single neuron resolution. Future studies will be necessary to further investigate the neural basis of memory at different time scales, and to understand how brain regions involved in short-term and long-term learning interacts with each other to generate memory engrams.

Major brain structures required in operant learning such as the amygdala and the hippocampus, are evolutionary conserved across vertebrates [44, 59, 63-68]. However, all these brain regions maturate during vertebrate development by the addition of new cell types, new layers or even entire brain structures [69-71]. For instance, most neurons in the zebrafish homolog of amygdala [44] are born later at juvenile stage but not during larval stage [65]. Similarly, mammalian entorhinal-hippocampal circuitry maturates gradually as animals develop [69, 72]. A recent study in zebrafish telencephalon revealed a sequential addition of distinct neural layers across development [73], which likely represent developmentally expanding functionality of this ancestral cortico-limbic circuitry in zebrafish brain. Our findings can therefore reflect an overall maturation of the zebrafish forebrain allowing animals to integrate new types of information about their environment with their experiences, and evaluate them in a context specific framework to take critical decisions.

Increasing number of studies use fear learning for studying the molecular and neural basis of learning [60-62, 74-76]. However, it is not always easy to disentangle the role of fear (or anxiety) from learning, for modifying molecular and neuronal processes in the brain. Here, we introduced a novel approach, which can address some of these challenges for disentangling fear from learning. We propose that one way to address this challenge is to simultaneously train control animals in CPA protocol while comparing the results to a sham group, which experienced the same environment and exact same aversive stimuli at the same time as the control group but with no CPA rule to learn. We argue that comparing the control group to the sham group, allow to disentangle the learning related processes in the brain from all other processes related to the subjective experiences of the animals. For example, our data suggest that the sham animals, did not develop a preference for a particular corner of the arena or its visual features. We also observed that the swim patterns or speed of the sham animals were not modulated as prominently as the animals that were allowed to learn. Finally, we also observed no freezing behaviors neither in trained or sham animals, suggesting that our CPA protocol induce very mild aversive experience. Likely due to very low aversive stimulus intensity, we observed no helplessness behaviors and freezing [23, 27, 28] even after several days of CPA training. Altogether, we propose that using very mild aversive stimulus and comparing CPA trained animals with sham animals is a promising approach to be used in future studies for disentangling the neural processes underlying learning from fear, anxiety and helplessness.

Our results suggest that the juvenile zebrafish can adopt different strategies during learning, depending on the available information in their environment. When we trained the animals in the presence of visual color cues, the visual boundary between the conditioning zone and the safe zone was used as a salient information by the animals to take evasive action near those boundaries and to avoid the conditioned zone. In our multi-day training experiments, we also observed that zebrafish can store such visual pattern information for several days and avoid the conditioned zone, as soon as the color cues are presented. However, we also observed that the zebrafish doesn't necessarily need such color cues to perform CPA learning. Animals trained without color cues, solved the challenge by using other landmarks such as walls and by moving as far as possible from the conditioned zone, accompanied by slower swimming. These results may also partially reflect a contextual fear component, when animals are not provided with color cues to help them discriminate different compartments of the arena. Our results suggest that CPA learning in juvenile zebrafish is a complex behavior that is not only driven by visual cues, but integrate visual and spatial information with animals' emotional response to short- and long-term aversive experiences to make appropriate decisions, similar to mammals[77, 78].

Previous studies in mammals established a direct role for habenula for prediction of learned outcomes [13, 15, 16, 26, 79, 80], and negative motivational values[14, 81, 82]. Similarly, zebrafish dorsal and ventral habenula were implicated in classical and operant learning [23, 24, 26]. At first glance our results for improved CPA performance in dHb ablated zebrafish were surprising. In fact, dHb ablated zebrafish also outperformed the control animals in memory recall session during early period of multi-day training. After several days of training, the control animals caught up with dHb ablated animals in CPA performance and memory recall, highlighting the amount of training as

an important factor while interpreting the results. It is also important to note that due to the low intensity of the aversive stimuli, neither control nor dHb ablated animals showed any significant freezing or helpless behavior [23, 24], which might have explained the apparent improvement of CPA performance. Instead, we observed that the dHb ablated animals showed significantly slower memory extinction rate. In fact, extinction of a memory is an essential component of learning new rules [83]. Extinction of fear memories was proposed as a therapeutic target for PTSD treatment [84]. Hence, comparing predicted outcomes with the actual consequences is important for updating the previous rules and memories with the new ones [5, 85]. Our reversal learning experiments showed that the juvenile zebrafish exhibit such behavioral flexibility and can quickly adapt to changing rules. However, we also showed that the dHb ablated zebrafish with slower memory extinction have difficulty in adapting to changing CPA rules during reversal learning. In line with our findings, knockout mice lacking narp gene that is expressed both in mouse mHb and zebrafish dHb, were shown to succeed in instrumental conditioning but failed in devaluation protocol, which required adjustment of choices in the absence of rewards[86]. Primate studies highlights further roles for habenula in adaptive behaviors [16, 85] and in human subjects, impaired behavioral flexibility can led to various mood disorders [87-90]. In line with all these studies, our findings highlight an important role for the habenula in optimizing animal behavior to provide the necessary behavioral flexibility, when there is a mismatch between the predicted and the actual outcomes. In the future, it will be crucial to investigate the neural computations across widely dispersed brain regions of transparent juvenile zebrafish, that could underlie the role of habenula and its input/output structures in behavioral flexibility and learning.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.P., E.Y.; Methodology and data, F.P., B.S.; Recording software F.P., R.P.; Data Analysis, F.P.; Investigation, all authors; Writing, F.P., E.Y.; Review & Editing, all authors; Funding Acquisition and Supervision, E.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Skinner, B.F. (1984). The evolution of behavior. Journal of the experimental analysis of behavior 41, 217-221.
2. Skinner, B.F. (1981). Selection by consequences. Science 213, 501-504.
3. Catania, A.C. (1984). The operant behaviorism of B. F. Skinner. Behavioral and Brain Sciences 7, 473-475.
4. Staddon, J.E., and Cerutti, D.T. (2003). Operant conditioning. Annual review of psychology 54, 115-144.
5. Izquierdo, A., Brigman, J.L., Radke, A.K., Rudebeck, P.H., and Holmes, A. (2017). The neural basis of reversal learning: An updated perspective. Neuroscience 345, 12-26.
6. Brown, V.J., and Tait, D.S. (2016). Attentional Set-Shifting Across Species. Current topics in behavioral neurosciences 28, 363-395.
7. Hadar, R., and Menzel, R. (2010). Memory formation in reversal learning of the honeybee. Front Behav Neurosci 4, 186.
8. Baker, P.M., Oh, S.E., Kidder, K.S., and Mizumori, S.J. (2015). Ongoing behavioral state information signaled in the lateral habenula guides choice flexibility in freely moving rats. Front Behav Neurosci 9, 295.
9. Remijnse, P.L., Nielen, M.M., van Balkom, A.J., Hendriks, G.J., Hoogendijk, W.J., Uylings, H.B., and Veltman, D.J. (2009). Differential frontal-striatal and paralimbic activity during reversal learning in major depressive disorder and obsessive-compulsive disorder. Psychol Med 39, 1503-1518.
10. Bechard, A.R., LaCrosse, A., Namba, M.D., Jackson, B., and Knackstedt, L.A. (2018). Impairments in reversal learning following short access to cocaine self-administration. Drug and alcohol dependence 192, 239-244.
11. George, S.A., Rodriguez-Santiago, M., Riley, J., Abelson, J.L., Floresco, S.B., and Liberzon, I. (2015). Alterations in cognitive flexibility in a rat model of post-traumatic stress disorder. Behav Brain Res 286, 256-264.
12. Dickstein, D.P., Finger, E.C., Brotman, M.A., Rich, B.A., Pine, D.S., Blair, J.R., and Leibenluft, E. (2010). Impaired probabilistic reversal learning in youths with mood and anxiety disorders. Psychol Med 40, 1089-1100.
13. Matsumoto, M., and Hikosaka, O. (2007). Lateral habenula as a source of negative reward signals in dopamine neurons. Nature 447, 1111-1115.
14. Lazaridis, I., Tzortzi, O., Weglage, M., Martin, A., Xuan, Y., Parent, M., Johansson, Y., Fuzik, J., Furth, D., Fenno, L.E., et al. (2019). A hypothalamus-habenula circuit controls aversion. Mol Psychiatry.
15. Trusel, M., Nuno-Perez, A., Lecca, S., Harada, H., Lalive,

A.L., Congiu, M., Takemoto, K., Takahashi, T., Ferraguti, F., and Mameli, M. (2019). Punishment-Predictive Cues Guide Avoidance through Potentiation of Hypothalamus-to-Habenula Synapses. Neuron 102, 120-127 e124.
16. Kawai, T., Yamada, H., Sato, N., Takada, M., and Matsumoto, M. (2015). Roles of the Lateral Habenula and Anterior Cingulate Cortex in Negative Outcome Monitoring and Behavioral Adjustment in Nonhuman Primates. Neuron 88, 792-804.
17. Stopper, C.M., and Floresco, S.B. (2014). What's better for me? Fundamental role for lateral habenula in promoting subjective decision biases. Nat Neurosci 17, 33-35.
18. Yamaguchi, T., Danjo, T., Pastan, I., Hikida, T., and Nakanishi, S. (2013). Distinct roles of segregated transmission of the septo-habenular pathway in anxiety and fear. Neuron 78, 537-544.
19. Shih, P.Y., McIntosh, J.M., and Drenan, R.M. (2015). Nicotine Dependence Reveals Distinct Responses from Neurons and Their Resident Nicotinic Receptors in Medial Habenula. Mol Pharmacol 88, 1035-1044.
20. Frahm, S., Slimak, M.A., Ferrarese, L., Santos-Torres, J., Antolin-Fontes, B., Auer, S., Filkin, S., Pons, S., Fontaine, J.F., Tsetlin, V., et al. (2011). Aversion to nicotine is regulated by the balanced activity of beta4 and alpha5 nicotinic receptor subunits in the medial habenula. Neuron 70, 522-535.
21. Xu, C., Sun, Y., Cai, X., You, T., Zhao, H., Li, Y., and Zhao, H. (2018). Medial Habenula-Interpeduncular Nucleus Circuit Contributes to Anhedonia-Like Behavior in a Rat Model of Depression. Front Behav Neurosci 12, 238.
22. Amo, R., Aizawa, H., Takahoko, M., Kobayashi, M., Takahashi, R., Aoki, T., and Okamoto, H. (2010). Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. J Neurosci 30, 1566-1574.
23. Agetsuma, M., Aizawa, H., Aoki, T., Nakayama, R., Takahoko, M., Goto, M., Sassa, T., Amo, R., Shiraki, T., Kawakami, K., et al. (2010). The habenula is crucial for experience-dependent modification of fear responses in zebrafish. Nat Neurosci 13, 1354-1356.
24. Lee, A., Mathuru, A.S., Teh, C., Kibat, C., Korzh, V., Penney, T.B., and Jesuthasan, S. (2010). The habenula prevents helpless behavior in larval zebrafish. Curr Biol 20, 2211-2216.
25. Chou, M.Y., Amo, R., Kinoshita, M., Cherng, B.W., Shimazaki, H., Agetsuma, M., Shiraki, T., Aoki, T., Takahoko, M., Yamazaki, M., et al. (2016). Social conflict resolution regulated by two dorsal habenular subregions in zebrafish. Science 352, 87-90.
26. Amo, R., Fredes, F., Kinoshita, M., Aoki, R., Aizawa, H., Agetsuma, M., Aoki, T., Shiraki, T., Kakinuma, H., Matsuda, M., et al. (2014). The habenulo-raphe serotonergic circuit encodes an aversive expectation value essential for adaptive active avoidance of danger. Neuron 84, 1034-1048.
27. Duboue, E.R., Hong, E., Eldred, K.C., and Halpern, M.E. (2017). Left Habenular Activity Attenuates Fear Responses in Larval Zebrafish. Curr Biol 27, 2154-2162.e2153.
28. Andalman, A.S., Burns, V.M., Lovett-Barron, M., Broxton, M., Poole, B., Yang, S.J., Grosenick, L., Lerner, T.N., Chen, R., Benster, T., et al. (2019). Neuronal Dynamics Regulating Brain and Behavioral State Transitions. Cell 177, 970-985. e920.
29. Fore, S., Cosacak, M.I., Verdugo, C.D., Kizil, C., and Yaksi, E. (2019). Functional properties of habenular neurons are determined by developmental stage and sequential neurogenesis. bioRxiv, 722462.
30. Orger, M.B., Smear, M.C., Anstis, S.M., and Baier, H. (2000). Perception of Fourier and non-Fourier motion by larval zebrafish. Nat Neurosci 3, 1128-1133.
31. Huang, Y.Y., and Neuhauss, S.C. (2008). The optokinetic response in zebrafish and its applications. Front Biosci 13, 1899-1916.
32. Kubo, F., Hablitzel, B., Dal Maschio, M., Driever, W., Baier, H., and Arrenberg, A.B. (2014). Functional architecture of an optic flow-responsive area that drives horizontal eye movements in zebrafish. Neuron 81, 1344-1359.
33. Portugues, R., Haesemeyer, M., Blum, M.L., and Engert, F. (2015). Whole-field visual motion drives swimming in larval zebrafish via a stochastic process. J Exp Biol 218, 1433-1443.
34. Braubach, O.R., Wood, H.D., Gadbois, S., Fine, A., and Croll, R.P. (2009). Olfactory conditioning in the zebrafish (Danio rerio). Behav Brain Res 198, 190-198.
35. Koide, T., Miyasaka, N., Morimoto, K., Asakawa, K., Urasaki, A., Kawakami, K., and Yoshihara, Y. (2009). Olfactory neural circuitry for attraction to amino acids revealed by transposon-mediated gene trap approach in zebrafish. Proc Natl Acad Sci U S A 106, 9884-9889.
36. Burgess, H.A., and Granato, M. (2007). Sensorimotor gating in larval zebrafish. J Neurosci 27, 4984-4994.
37. Lacoste, A.M., Schoppik, D., Robson, D.N., Haesemeyer, M., Portugues, R., Li, J.M., Randlett, O., Wee, C.L., Engert, F., and Schier, A.F. (2015). A convergent and essential interneuron pathway for Mauthner-cell-mediated escapes. Curr Biol 25, 1526-1534.
38. Vendrell-Llopis, N., and Yaksi, E. (2015). Evolutionary conserved brainstem circuits encode category, concentration and mixtures of taste. Sci Rep 5, 17825.
39. Kermen, F., Franco, L.M., Wyatt, C., Yaksi, E. (2013). Neuralcircuits mediating olfactory-driven behavior in fish. Frontiers in Neural Circuits 7.
40. Sison, M., and Gerlai, R. (2010). Associative learning in zebrafish (Danio rerio) in the plus maze. Behav Brain Res 207, 99-104.
41. Aoki, R., Tsuboi, T., and Okamoto, H. (2015). Y-maze avoidance: an automated and rapid associative learning paradigm in zebrafish. Neurosci Res 91, 69-72.
42. Xu, X., Scott-Scheiern, T., Kempker, L., and Simons, K. (2007). Active avoidance conditioning in zebrafish (Danio rerio). Neurobiology of learning and memory 87, 72-77.
43. Aoki, T., Kinoshita, M., Aoki, R., Agetsuma, M., Aizawa, H., Yamazaki, M., Takahoko, M., Amo, R., Arata, A., Higashijima, S., et al. (2013). Imaging of neural ensemble for the retrieval of a learned behavioral program. Neuron 78, 881-894.
44. Lal, P., Tanabe, H., Suster, M.L., Ailani, D., Kotani, Y., Muto, A., Itoh, M., Iwasaki, M., Wada, H., Yaksi, E., et al. (2018). Identification of a neuronal population in the

telencephalon essential for fear conditioning in zebrafish. BMC biology 16, 45.
45. Valente, A., Huang, K.H., Portugues, R., and Engert, F. (2012). Ontogeny of classical and operant learning behaviors in zebrafish. Learn Mem 19, 170-177.
46. Yashina, K., Tejero-Cantero, Á., Herz, A., and Baier, H. (2019). Zebrafish exploit visual cues and geometric relationships to form a spatial memory. iScience.
47. Hinz, R.C., and de Polavieja, G.G. (2017). Ontogeny of collective behavior reveals a simple attraction rule. Proc Natl Acad Sci U S A 114, 2295-2300.
48. Dreosti, E., Lopes, G., Kampff, A.R., and Wilson, S.W. (2015). Development of social behavior in young zebrafish. Front Neural Circuits 9, 39.
49. Barker, A.J., and Baier, H. (2015). Sensorimotor decision making in the zebrafish tectum. Curr Biol 25, 2804-2814.
50. Thiele, T.R., Donovan, J.C., and Baier, H. (2014). Descending control of swim posture by a midbrain nucleus in zebrafish. Neuron 83, 679-691.
51. Miri, A., Daie, K., Arrenberg, A.B., Baier, H., Aksay, E., and Tank, D.W. (2011). Spatial gradients and multidimensional dynamics in a neural integrator circuit. Nat Neurosci 14, 1150-1159.
52. Del Bene, F., Wyart, C., Robles, E., Tran, A., Looger, L., Scott, E.K., Isacoff, E.Y., and Baier, H. (2010). Filtering of visual information in the tectum by an identified neural circuit. Science 330, 669-673.
53. Jetti, S.K., Vendrell-Llopis, N., and Yaksi, E. (2014). Spontaneous activity governs olfactory representations in spatially organized habenular microcircuits. Curr Biol 24, 434-439.
54. Dreosti, E., Vendrell Llopis, N., Carl, M., Yaksi, E., and Wilson, S.W. (2014). Left-right asymmetry is required for the habenulae to respond to both visual and olfactory stimuli. Curr Biol 24, 440-445.
55. Randlett, O., Haesemeyer, M., Forkin, G., Shoenhard, H., Schier, A.F., Engert, F., and Granato, M. (2019). Distributed Plasticity Drives Visual Habituation Learning in Larval Zebrafish. Curr Biol 29, 1337-1345.e1334.
56. Naumann, E.A., Fitzgerald, J.E., Dunn, T.W., Rihel, J., Sompolinsky, H., and Engert, F. (2016). From Whole-Brain Data to Functional Circuit Models: The Zebrafish Optomotor Response. Cell 167, 947-960.e920.
57. Mu, Y., Bennett, D.V., Rubinov, M., Narayan, S., Yang, C.T., Tanimoto, M., Mensh, B.D., Looger, L.L., and Ahrens, M.B. (2019). Glia Accumulate Evidence that Actions Are Futile and Suppress Unsuccessful Behavior. Cell 178, 27-43.e19.
58. Kawashima, T., Zwart, M.F., Yang, C.T., Mensh, B.D., and Ahrens, M.B. (2016). The Serotonergic System Tracks the Outcomes of Actions to Mediate Short-Term Motor Learning. Cell 167, 933-946 e920.
59. Mueller, T., and Wullimann, M.F. (2009). An evolutionary interpretation of teleostean forebrain anatomy. Brain, behavior and evolution 74, 30-42.
60. Liu, X., Ramirez, S., Pang, P.T., Puryear, C.B., Govindarajan, A., Deisseroth, K., and Tonegawa, S. (2012). Optogenetic stimulation of a hippocampal engram activates fear memory recall. Nature 484, 381-385.
61. Ramirez, S., Tonegawa, S., and Liu, X. (2013). Identification and optogenetic manipulation of memory engrams in the hippocampus. Front Behav Neurosci 7, 226.
62. Kitamura, T., Ogawa, S.K., Roy, D.S., Okuyama, T., Morrissey, M.D., Smith, L.M., Redondo, R.L., and Tonegawa, S. (2017). Engrams and circuits crucial for systems consolidation of a memory. Science 356, 73-78.
63. Portavella, M., Torres, B., and Salas, C. (2004). Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. J Neurosci 24, 2335-2342.
64. Portavella, M., Vargas, J.P., Torres, B., and Salas, C. (2002). The effects of telencephalic pallial lesions on spatial, temporal, and emotional learning in goldfish. Brain Res Bull 57, 397-399.
65. von Trotha, J.W., Vernier, P., and Bally-Cuif, L. (2014). Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. Eur J Neurosci 40, 3302-3315.
66. Rodriguez, F., Lopez, J.C., Vargas, J.P., Broglio, C., Gomez, Y., and Salas, C. (2002). Spatial memory and hippocampal pallium through vertebrate evolution: insights from reptiles and teleost fish. Brain Res Bull 57, 499-503.
67. Elliott, S.B., Harvey-Girard, E., Giassi, A.C., and Maler, L. (2017). Hippocampal-like circuitry in the pallium of an electric fish: Possible substrates for recursive pattern separation and completion. J Comp Neurol 525, 8-46.
68. Tosches, M.A., Yamawaki, T.M., Naumann, R.K., Jacobi, A.A., Tushev, G., and Laurent, G. (2018). Evolution of pallium, hippocampus, and cortical cell types revealed by single-cell transcriptomics in reptiles. Science 360, 881-888.
69. Donato, F., Jacobsen, R.I., Moser, M.B., and Moser, E.I. (2017). Stellate cells drive maturation of the entorhinal-hippocampal circuit. Science 355.
70. Frangeul, L., Kehayas, V., Sanchez-Mut, J.V., Fievre, S., Krishna, K.K., Pouchelon, G., Telley, L., Bellone, C., Holtmaat, A., Graff, J., et al. (2017). Input-dependent regulation of excitability controls dendritic maturation in somatosensory thalamocortical neurons. Nat Commun 8, 2015.
71. Sur, M., and Leamey, C.A. (2001). Development and plasticity of cortical areas and networks. Nat Rev Neurosci 2, 251-262.
72. Bjerknes, T.L., Moser, E.I., and Moser, M.B. (2014). Representation of geometric borders in the developing rat. Neuron 82, 71-78.
73. Furlan, G., Cuccioli, V., Vuillemin, N., Dirian, L., Muntasell, A.J., Coolen, M., Dray, N., Bedu, S., Houart, C., Beaurepaire, E., et al. (2017). Life-Long Neurogenic Activity of Individual Neural Stem Cells and Continuous Growth Establish an Outside-In Architecture in the Teleost Pallium. Curr Biol 27, 3288-3301.e3283.
74. Redondo, R.L., Kim, J., Arons, A.L., Ramirez, S., Liu, X., and Tonegawa, S. (2014). Bidirectional switch of the valence associated with a hippocampal contextual memory engram. Nature 513, 426-430.

75. Silva, B.A., Gross, C.T., and Graff, J. (2016). The neural circuits of innate fear: detection, integration, action, and memorization. Learn Mem 23, 544-555.
76. Sananbenesi, F., Fischer, A., Wang, X., Schrick, C., Neve, R., Radulovic, J., and Tsai, L.H. (2007). A hippocampal Cdk5 pathway regulates extinction of contextual fear. Nat Neurosci 10, 1012-1019.
77. Wolff, S.B., Grundemann, J., Tovote, P., Krabbe, S., Jacobson, G.A., Muller, C., Herry, C., Ehrlich, I., Friedrich, R.W., Letzkus, J.J., et al. (2014). Amygdala interneuron subtypes control fear learning through disinhibition. Nature 509, 453-458.
78. Letzkus, J.J., Wolff, S.B., and Luthi, A. (2015). Disinhibition, a Circuit Mechanism for Associative Learning and Memory. Neuron 88, 264-276.
79. Tian, J., and Uchida, N. (2015). Habenula Lesions Reveal that Multiple Mechanisms Underlie Dopamine Prediction Errors. Neuron 87, 1304-1316.
80. Matsumoto, M., and Hikosaka, O. (2009). Two types of dopamine neuron distinctly convey positive and negative motivational signals. Nature 459, 837-841.
81. Wang, D., Li, Y., Feng, Q., Guo, Q., Zhou, J., and Luo, M. (2017). Learning shapes the aversion and reward responses of lateral habenula neurons. Elife 6.
82. Hong, S., Jhou, T.C., Smith, M., Saleem, K.S., and Hikosaka, O. (2011). Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. J Neurosci 31, 11457-11471.
83. Singewald, N., and Holmes, A. (2019). Rodent models of impaired fear extinction. Psychopharmacology 236, 21-32.
84. Kida, S. (2019). Reconsolidation/destabilization, extinction and forgetting of fear memory as therapeutic targets for PTSD. Psychopharmacology (Berl) 236, 49-57.
85. Baker, P.M., Raynor, S.A., Francis, N.T., and Mizumori, S.J. (2017). Lateral habenula integration of proactive and retroactive information mediates behavioral flexibility. Neuroscience 345, 89-98.
86. Johnson, A.W., Crombag, H.S., Takamiya, K., Baraban, J.M., Holland, P.C., Huganir, R.L., and Reti, I.M. (2007). A selective role for neuronal activity regulated pentraxin in the processing of sensory-specific incentive value. J Neurosci 27, 13430-13435.
87. Lawson, R.P., Nord, C.L., Seymour, B., Thomas, D.L., Dayan, P., Pilling, S., and Roiser, J.P. (2017). Disrupted habenula function in major depression. Mol Psychiatry 22, 202-208.
88. Ranft, K., Dobrowolny, H., Krell, D., Bielau, H., Bogerts, B., and Bernstein, H.G. (2010). Evidence for structural abnormalities of the human habenular complex in affective disorders but not in schizophrenia. Psychol Med 40, 557-567.
89. Sartorius, A., Kiening, K.L., Kirsch, P., von Gall, C.C., Haberkorn, U., Unterberg, A.W., Henn, F.A., and Meyer-Lindenberg, A. (2010). Remission of major depression under deep brain stimulation of the lateral habenula in a therapy-refractory patient. Biol Psychiatry 67, e9-e11.
90. Morris, J.S., Smith, K.A., Cowen, P.J., Friston, K.J., and Dolan, R.J. (1999). Covariation of activity in habenula and dorsal raphe nuclei following tryptophan depletion. Neuroimage 10, 163-172.
91. Vladimirov, N., Mu, Y., Kawashima, T., Bennett, D.V., Yang, C.T., Looger, L.L., Keller, P.J., Freeman, J., and Ahrens, M.B. (2014). Light-sheet functional imaging in fictively behaving zebrafish. Nat Methods 11, 883-884.

METHODS

Experimental animals

The animal facilities and maintenance of the zebrafish, Danio rerio, were approved by the NFSA (Norwegian Food Safety Authority). Fishes were kept in 3,5 liter tanks in a Techniplast Zebtech Multilinking system at constant conditions: 28°C, pH 7 and 600µSiemens, at a 14:10 hour light/dark cycle to simulate optimal natural breeding conditions. Fishes received a normal diet of dry food (SDS 100-400, dependent of age) two times/day and Artemia nauplii once a day (Grade0, platinum Label, Argent Laboratories, Redmond, USA). For the experiments investigating the ontogeny of CPA learning, the age range in each group have a variability of plus or minus two days. For all the experiment showed in figure 1, 2, 3 and 4 we used Tg(elavl3:GCaMP6s) [91] zebrafish. In the experiment showed in figure 5 and 6 we used Tg(narp:GAL4VP16; UAS-E1b:NTR-mCherry)[23] and siblings not expressing NTR-mCherry protein as controls. All experimental procedures performed were in accordance with the directive 2010/63/EU of the European Parliament and the Council of the European Union and the Norwegian Food Safety Authorities.

Behavioral setup

A custom-made real-time tracking software was implemented to track six zebrafish simultaneously, using the OpenCV 3.0 library and the QtCreator5.2 developing platform. A microcontroller controls six integrated circuits to deliver electrical stimulation to each individual arena. Each current pulse has a duration of 10 ms and an amplitude of approximately 1.2 mA. This result in a current density of approximately 0.1 mA/cm using tungsten electrodes. A pulse of current is delivered every 740 ms, resulting in a delivery frequency of 1.33 Hz. The aversive stimulus is administrated to the fish only when it is located inside the conditioned zone. The visual stimulus is presented from the bottom of the arena using a horizontally positioned LCD monitor. Each of the six arenas was divided into 2 equal parts with red and grey colors with matched luminosity. A Manta 235B camera was used to monitor animal behavior at 15 fps. Experiments were conducted in Gosselin square Petri dishes 120mm x 120mm x 15.8 mm with opaque side walls. The entire behavioral setup was enclosed in a temperature controlled black isolation box to prevent any external inference. The water temperature during training was kept at 26°C. A schematic of the behavioral setup is shown in Figure S1A.

Experimental procedures

One fish per arena was placed in the behavioral setup between 09:00 and 11:00 am, after the morning feeding, and the experiment was started immediately. Every animal is used only in one specific protocol once. All the behavioral protocols performed in this work are composed of the following sessions that were used in various combinations:

Baseline session: animal position is monitored and recorded for 60 minutes, while the color pattern is displayed to the animal.

Blank session: animal position is monitored and recorded for 30 minutes, but no color pattern is presented to the animal; the arena is entirely covered by a uniform gray color.

Conditioning session: animal position is monitored and recorded for 30 minutes. Only when the zebrafish entered the conditioned zone a mildly aversive weak (10 ms, 1,2 mA) electric stimuli is delivered at 1,33 Hz.

Test session: animal position is monitored and recorded for 30 minutes, while the color pattern is displayed to the animal.

In Figure 1, 2, 4 and 5, the “conditioned zone” was always associated with the left half of the arena. In Figure 1,4 and 5 the conditioned side of the arena was always associated with the red color. In the experiment presented in Figure 2 half of the animals were trained against the red color pattern while the other half against the gray color pattern. We observed no substantial differences in this two groups and combined both groups for data analysis. Each of these conditions had the corresponding sham control animals. In Figure 3, the “conditioned zone” was the left side of the arena, but no color pattern was associated with it. The reversal protocol in Figure 6 was achieved by first conditioning the animals against the left/red side of the arena. From third conditioning session on the aversive stimuli was experienced only on the right/gray side of the arena.

Metronidazole treatment

To ablate the neurons of dHb, Tg(narp:GAL4VP16; UAS-E1b:NTR-mCherry) zebrafish was treated with 10mM metronidazole (MTZ, Sigma-Aldrich) for 24h followed by a washout period of at least 12h, protocol adapted from Agetsuma et al. (2010) [23]. The treatment is started two days prior the behavioral protocol. The fish were placed, in groups of three, in a Petri dish containing 50ml of 10mM MTZ +250 microliters of DMSO. Control group was subjected to the same treatment. The fish were kept in the 28°C incubator, covered in aluminum foil, for 24h. Next day the fish were transferred to a new Petri dish containing fresh artificial fish

water and they were feed with dry food and placed back in the incubator. The water was changed at least one additional time before the experiment.

Behavioral analysis

To perform all the behavioral analysis showed in this study, we used custom-made scripts written in MATLAB. The heat-maps are an average of zebrafish swim position over the entire length of each session. For all further analysis, of the baseline session only the last 30 minutes are taken into consideration. We divided each behavioral session into 2 minutes time bins to be able to better detect changes over time.
The learning index is defined as follow:
((% time in “conditioning zone” during baseline - % time in “”conditioning zone\” “ in session alanysed))/((% time in “conditioning zone” during baseline))
To discriminate between learners and non-learners fish, a lowest boundary for the learning index was calculated, by using the relatively random distribution of zebrafish swim position during the baseline session. All fish that exceed this lowest boundary was considered to exhibit significant learning up on CPA training.
For the quantifying swimming direction and avoidance behaviors, the animal position was detected when they are near the midline boundary between conditioned zone and safe zone. Next, the linearize trajectory of the animal in the four seconds preceding and following the encounter with the midline boundary were used as approach and avoidance, respectively We then quantify, in 5 minutes time bins, successful avoidance rate of the animals. If the position of the animals was in the “conditioned zone” after they encountered the midline the avoidance was considered unsuccessful, otherwise it was considered successful.
In reversal learning experiments, we only continued the reversal protocol with those animals that exhibit prominent CPA learning in the first training session and classified as learners using our learning index criteria. If animals did not learn the initial CPA task, they were not subjected to reversal learning.

SUPPLEMENTARY MATERIALS

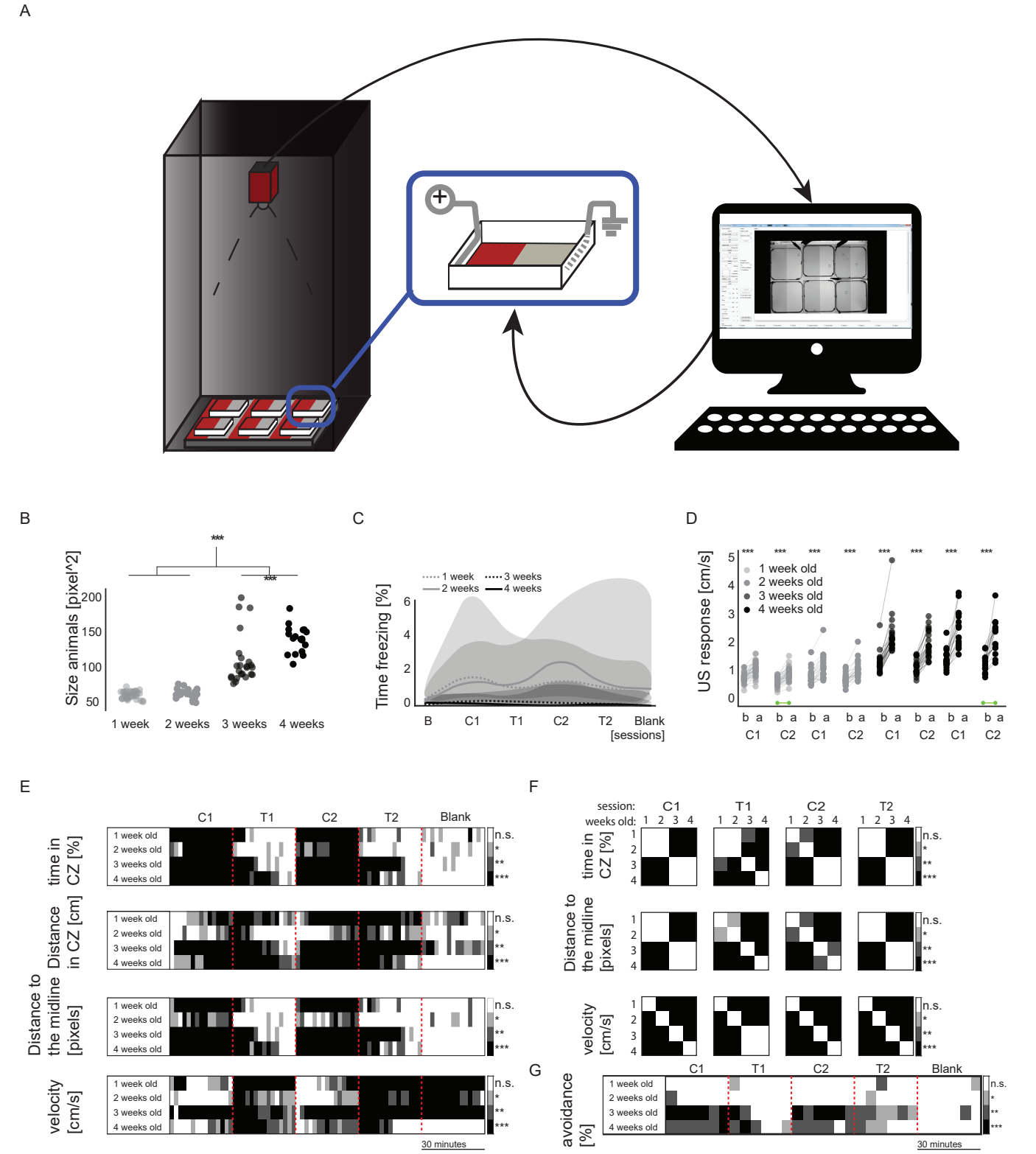

Figure S1: Conditioned place avoidance (CPA) setup and additional analysis of physiological and behavioral parameters during training and multi-group comparisons.

(A) Graphical representation of the CPA training setup. (B) Quantification of animal size reveals a significant enlargement of developing zebrafish at 3-4 weeks. ***p= <0.001, Wilcoxon ranksum test. (C) Percentage of time that the zebrafish exhibit no swimming, which is defined by less than 2mm swim during 2 seconds. Note the absence of long term freezing for all the ages. Solid lines represent median, shaded areas represent first and third quartiles. B: baseline, C1: conditioning 1, T1: test 1, C2: conditioning 2, T2: test 2. (D) Average swim 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 , Wilcoxon signrank test. (E) Tables showing intra-group statistical comparison of the parameters measured of animal behaviour dividing each session in two minutes time bin. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon ranksum test. (F) Tables showing statistical comparison across the 4 experimental group. ***p= <0.0005, **p= <0.005, *p= <0.05, Wilcoxon ranksum test. (G) Tables showing intra-group statistical comparison of the successful avoidance performed by the animal. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon ranksum test.

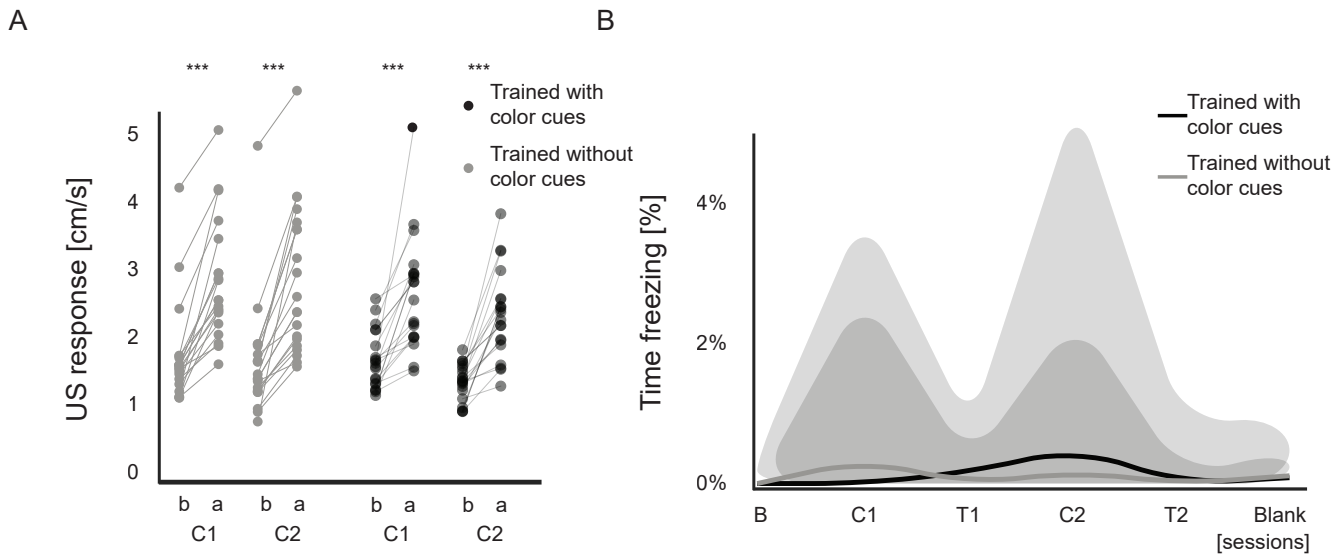

Figure S2: Training without color cues does not affect animal response to unconditioned stimulus and does not elicit prominent freezing behavior.
(A) Average swim velocity of the zebrafish, one second before (b) and after (a) the delivery of aversive unconditioned stimuli (US). ***p= <0.001, Wilcoxon signrank test. (B) Percentage of time that the zebrafish exhibit no swimming, which is defined by less than 2mm swim during 2 seconds. Note the absence of prominent freezing for both control (black) and no color cues (gray) group during B: baseline, C1: conditioning 1, T1: test 1, C2: conditioning 2, T2: test 2. Solid lines represent median, shaded areas represent first and third quartiles. Animals trained without visual cues are displayed in gray, the one trained with visual cues in black.

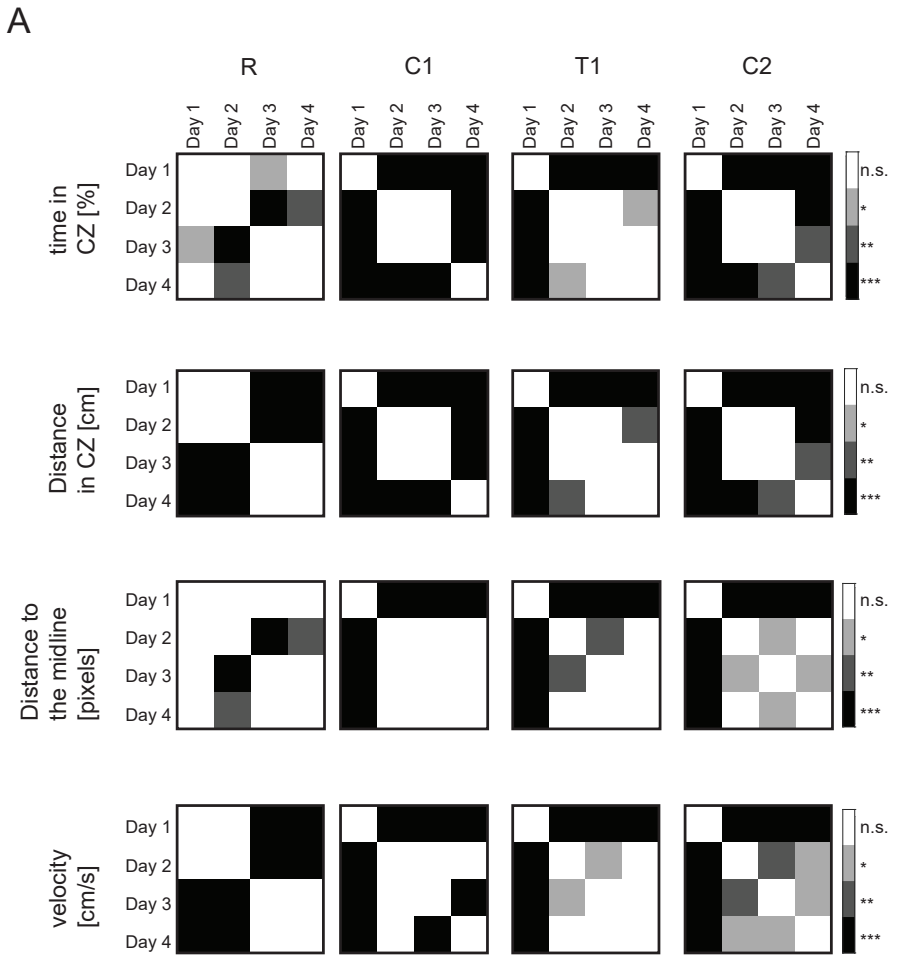

Figure S3: Multi-day training significantly improves learning performance of the animal.
(A) Statistical comparison across the different days of training. R: recall, C1: conditioning 1, T1: test 1, C2: conditioning 2. Note the significant improvement of learning performance (in T1) and recall (R) over consecutive training days, using different learning measures. White color mark non significant comparison. ***p= <0.001, **p= <0.01, *p= <0.05, Wilcoxon ranksum test.

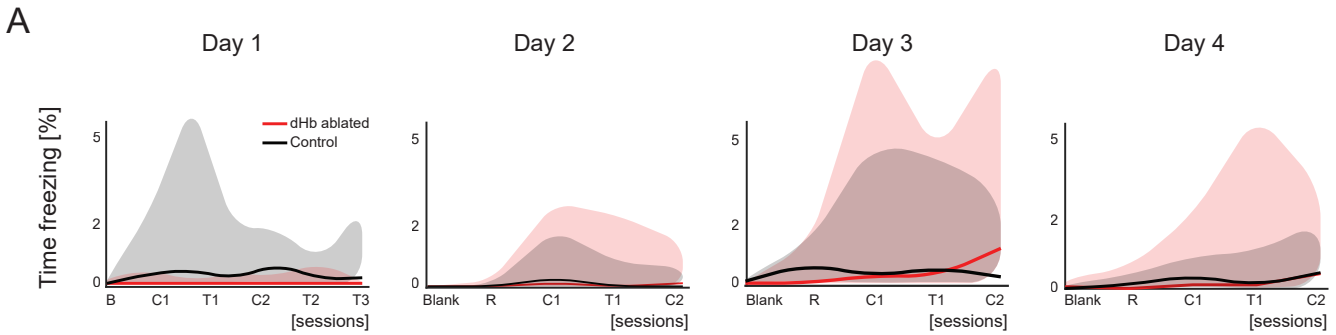

Figure S4: Dorsal habenula (dHb) ablation does not led to freezing behavior over multi-day training.
(A) Percentage of time that the zebrafish exhibit no swimming, which is defined by less then 2mm swim during 2 seconds. Note the absence of long term freezing for both control (black) and dHb ablated (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. dHb ablated animals are displayed in red, while control in black.

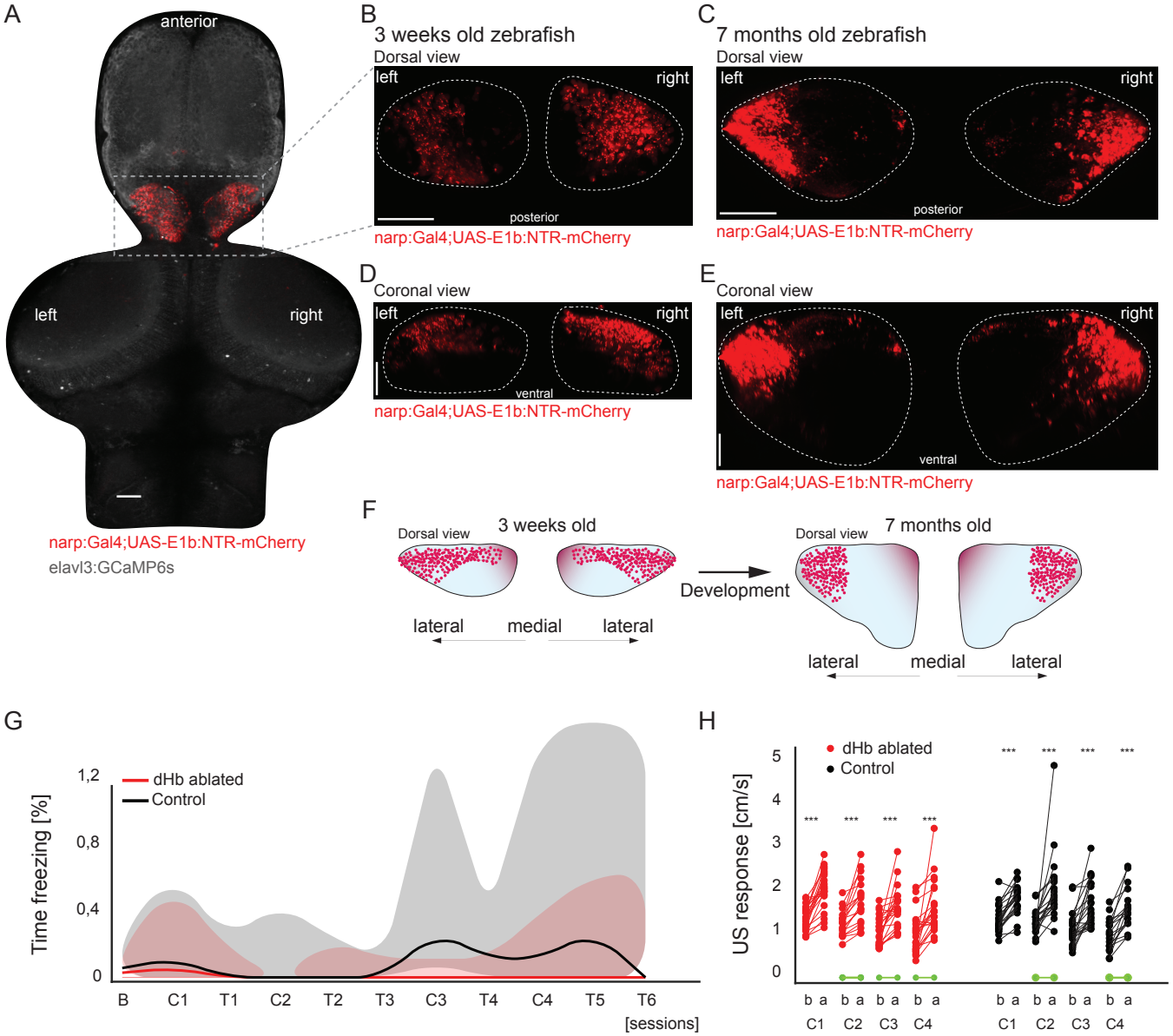

Figure S5: Dorsal habenula (dHb) ablation does not elicit freezing behavior and does not affects animal response to unconditioned stimulus during reversal learning.

(A) Confocal microscopy image of the entire brain in three weeks old Tg(narp:Gal4;UAS-E1b:NTR-mCherry;elavl3:GCaMP6s) zebrafish, dorsal view, scale bar is 50µm. Please note that the narp:Gal4 expression is exclusively in dorsal habenula. (B-E) Confocal microscopy images of the habenula in three weeks (B,D) and seven months (C,E) old Tg(narp:Gal4;UAS-E1b:NTR-mCherry) zebrafish. Dorsal views (B,C) and coronal views (D,E). Scale bars is 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 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 then 2mm swim during 2 seconds. Note the absence of long term freezing for both control (black) and dHb 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.

(H) Average swim 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 signrank test.

dHb ablated animals are displayed in red, while control in black.