Cognition and Behavior

Involvement of Cerebellar Neural Circuits in Active Avoidance Conditioning in Zebrafish

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Abstract

When animals repeatedly receive a combination of neutral conditional stimulus (CS) and aversive unconditional stimulus (US), they learn the relationship between CS and US, and show conditioned fear responses after CS. They show passive responses such as freezing or panic movements (classical or Pavlovian fear conditioning), or active behavioral responses to avoid aversive stimuli (active avoidance). Previous studies suggested the roles of the cerebellum in classical fear conditioning but it remains elusive whether the cerebellum is involved in active avoidance conditioning. In this study, we analyzed the roles of cerebellar neural circuits during active avoidance in adult zebrafish. When pairs of CS (light) and US (electric shock) were administered to wild-type zebrafish, about half of them displayed active avoidance. The expression of botulinum toxin, which inhibits the release of neurotransmitters, in cerebellar granule cells (GCs) or Purkinje cells (PCs) did not affect conditioning-independent swimming behaviors, but did inhibit active avoidance conditioning. Nitroreductase (NTR)-mediated ablation of PCs in adult zebrafish also impaired active avoidance. Furthermore, the inhibited transmission of GCs or PCs resulted in reduced fear-conditioned Pavlovian fear responses. Our findings suggest that the zebrafish cerebellum plays an active role in active avoidance conditioning.

Key words: cerebellum; active avoidance; operant conditioning; botulinum toxin; nitroreductase; zebrafish

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Significance Statement

An animal can associate a neutral conditioned stimulus and an aversive unconditioned stimulus, and escape to avoid an aversive stimulus. This is called active avoidance conditioning and is essential for an animal's survival. Although the amygdala and habenula nucleus are reportedly involved in active avoidance conditioning, the roles of other brain regions are largely unknown. We describe the roles of the cerebellum during active avoidance in adult zebrafish. The neurotoxin botulinum toxin-mediated inhibition of granule cells (GCs) or Purkinje cells (PCs), or the ablation of PCs, suppressed active avoidance conditioning. Our findings indicate that the cerebellum plays a positive role in active avoidance conditioning.

Introduction

An animal can associate two environmental stimuli and consequently shows certain behaviors. Broadly, there are neutral cues or conditional stimuli (CSs), such as sound or light, and aversive (noxious) cues or unconditional stimuli (USs), such as an electric shock. If an animal receives repeated pairs of CSs and USs, it associates both to show fear responses on exposure to CSs. Consequently, CS-dependent aversive effects are expected. The animal may show two types of fear responses, "passive" and "active," on exposure to a CS. Passive responses are known as "classical fear conditioning" or "Pavlovian fear

conditioning." This includes motor responses, including freezing behavior and panic movements (Sacchetti et al., 2002; Agetsuma et al., 2010; Amo et al., 2014; Lal et al., 2018), and autonomic reactions such as CS-evoked bradycardia responses (Supple and Leaton, 1990; Supple and Kapp, 1993; Supple et al., 1993; Yoshida and Hirano, 2010; Kotajima et al., 2014; Matsuda et al., 2017). It also shows active responses that result in the avoidance of noxious stimuli. Conditioning that elicits active (voluntary) responses is known as operant conditioning, and a type of operant conditioning that induces behaviors to avoid noxious stimuli is called "active avoidance" (Skinner, 1984). The amygdala in mammals is involved in both classical fear conditioning and active avoidance conditioning (Duvarci and Pare, 2014; Herry and Johansen, 2014). In zebrafish, a region in the dorsal telencephalon that is considered to be equivalent to the amygdala (Dm), and the habenula-raphe neural circuits, are reportedly involved in adaptive active avoidance conditioning (Aoki et al., 2013; Amo et al., 2014; Lal et al., 2018). However, it is still largely unknown if other neural circuits, including the cerebellum, are involved in fear conditioning.

The cerebellum plays important roles in some forms of motor coordination and motor learning, and is also involved in higher cognitive emotional functions (Ito, 2005, 2006, 2008). The functions of the cerebellum rely on neural circuits that are conserved among vertebrates (Hashimoto and Hibi, 2012; Hibi et al., 2017). Purkinje cells (PCs) and granule cells (GCs) are major GABAergic and glutamatergic neurons in the cerebellum, and receive two inputs from outside of the cerebellum. PCs receive climbing fibers (CFs), which are axons from the inferior olivary nuclei (IOs). GCs receive mossy fibers (MFs) from precerebellar nuclei located in various regions of the brain. MF information is conveyed by GC axons, called parallel fibers (PFs). PCs integrate the two inputs and send outputs to the outside of the cerebellum through efferent neurons, which are deep cerebellar nuclei in mammals and eurydendroid cells in teleosts. The cerebellum of teleosts, including zebrafish, can be divided into the rostro-medial domain [valvula cerebelli (Va) and corpus cerebelli (CCe)] and the caudo-lateral domain [lobus caudalis cerebelli (LCa) and eminentia granularis (EG)] that are composed of different neural circuit structures (for review, see Hibi and Shimizu, 2012; Hibi et al., 2017). Cerebellar neural circuits are involved in classical conditioning, such as eye-blink conditioning. In mammals, lesions of the cerebellar vermis or IOs impair the acquisition of fear-conditioned bradycardia (Supple and Leaton, 1990; Kotajima et al., 2014). Inhibition of the vermis or efferent neurons with tetrodotoxin disrupts the consolidation of conditioned freezing responses in rats (Sacchetti et al., 2002). Lesions or chemical inhibition of the cerebellum also impair fear-conditioned bradycardia responses (Yoshida et al., 2004; Yoshida and Hirano, 2010). These studies suggest that the cerebellum is involved in classical fear conditioning. However, it is uncertain whether the cerebellar neural circuits control active avoidance conditioning.

Previous studies revealed that zebrafish can acquire classical fear conditioning and active avoidance conditioning from larval stages, although the timing to acquire robust conditioned behaviors varies depending on the experimental conditions (Aizenberg and Schuman, 2011; Valente et al., 2012; Matsuda et al., 2017; Lin et al., 2020). Zebrafish reliably show classical fear-conditioned bradycardia responses from the late larval stage [~20 d postfertilization (dpf); Matsuda et al., 2017]. Cerebellar neurons in the CCe are activated during conditioned behaviors and inhibition of the GC transmission prolonged recovery from the conditioned responses (Matsuda et al., 2017), implying a role of the cerebellar neural circuits in classical fear conditioning in zebrafish. In operant conditioning, cerebellar neurons are also activated, and lesions in the cerebellum delay decision-making (Lin et al., 2020). However, specific inhibition of cerebellar neurons in active avoidance conditioning has not been reported. In this study, we transgenically expressed botulinum toxin (BoTx), a neurotoxin that inhibits neurotransmitter release, in GCs or PCs, or nitroreductase (NTR), which can convert a prodrug metronidazole (MTZ) to a cytotoxin (Pisharath et al., 2007; Tabor et al., 2014), in PCs, to study the roles of cerebellar neural circuits in active avoidance conditioning in adult zebrafish.

Materials and Methods

Ethics statement

The animal experiments in this study were approved by the Institutional Animal Experiment Committee and were conducted in accordance with the Regulations on Animal Experiments at the institute.

Zebrafish

Wild-type zebrafish with the Oregon AB genetic background and two previously reported transgenic (Tg) lines, *gSA2AzGFF152B* (Takeuchi et al., 2015), which expresses a modified version of Gal4-VP16 (GAL4FF, also referred to as GFF) in the corpus cerebelli GCs, and *Tg(UAS: BoTxBLC-GFP)^{icm21}*, which expresses the light chain of

BoTx in a GAL4-dependent manner (Sternberg et al., 2016), were used. To generate the *Tg(aldoca:BoTxBLC-GFP)* line, the 5-kbp *aldolase Ca* (*aldoca*) promoter (Tanabe et al., 2010), the BoTxBLC-GFP gene (Sternberg et al., 2016), and the polyadenylation site (pAS) of pCS2+ were subcloned to the Tol2 vector pT2KDest-RfaF (Nojima et al., 2010) by Gateway (Thermo Fisher Scientific). To generate the *Tg(cbln12:Gal4FF)* line, the 2-kbp *cerebellin12* (*cbln12*) promoter (Dohaku et al., 2019), GAL4FF (Asakawa et al., 2008), and pAS of pCS2+ were subcloned to the Tol2 vector pT2ALR-Dest (Dohaku et al., 2019) by the Gateway system. To generate the *Tg(aldoca:NTR-TagRFPT)* line, the *aldoca* promoter, the epNTR-TagRFPT gene encoding a fusion protein of zebrafish codon-optimized enhanced-potency NTR (epNTR) and TagRFPT (Tabor et al., 2014), and pAS of pCS2+ were subcloned to pT2ARL-Dest by the Gateway system. To establish the Tg lines, 25 pg of the Tol2 plasmid and 25 pg of transposase-capped RNA were injected into one-cell-stage embryos. To inhibit GC transmission, *gSA2AzGFF152B* and *Tg(cbln12:Gal4FF)* were crossed with *Tg(UAS:BoTxBLC-GFP)*. Tg adult fish that harbored both *GAL4FF* and *GFP* were identified before conditioning by genotyping with primer pairs: 5′-AAGTGCGCCAAGTGTCTGAAGAAC-3′ (F) and 5′-GACCTGGACATGCTGCCTGCTGAT-3′ (R) for *GAL4FF*, and 5′-CGAACATAGCTAGCGTGACCGTGA-3′ (F) and 5′-TGGAGCACGTGTATCAGCTCATGC-3′ (R) for *BoTxBLC*. Those that did not harbor *GAL4FF* or *GFP* were used as control fish. Similarly, adult *Tg(aldoca:BoTxBLC-GFP)* fish were identified by BoTxBLC genotyping. Adult fish that did not have the transgene were used as control fish. Fish were maintained in a 14/10 h light/dark cycle (light 9 A.M. to 11 P.M.; dark 11 P.M. to 9 A.M.) at 28.5°C. Four- to 12-month-old adult fish were used in this study. All experiments were conducted without distinction between males and females.

MTZ treatment

Adult *Tg(aldoca:NTR-TagRFPT)* fish (8–11 months old) were treated with 10 mM MTZ solution for 18 h and returned to their tank. Eleven days after treatment, the fish were subjected to behavior tests.

Immunostaining and measurement

Cryosections 14 μm thick were prepared according to the previous publication (Bae et al., 2009). The sections were immunostained as previously described (Bae et al., 2009; Kani et al., 2010). The following antibodies were used: anti-GFP (1:1000, rat, Nacalai Tesque, catalog #04404-84, RRID: AB 10013361 or 1:1000, rabbit, MBL International, catalog #598, RRID: AB_591816) for BoTxBLC-GFP, anti-Neurod1 (1:400, mouse, ascites; Kani et al., 2010), and anti-parvalbumin 7 (1:1000, mouse monoclonal, ascites; Bae et al., 2009). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rat (H + L, Invitrogen, Thermo Fisher Scientific, catalog #A11006, RRID: AB_2534074), CF488A anti-rabbit (H + L, Biotium Inc., catalog #20019, RRID: AB_10583180), and Alexa Fluor 568 goat anti-

mouse IgG (H + L, Invitrogen, catalog #A11031, RRID: AB_144696). An LSM700 confocal laser-scanning microscope was used to obtain fluorescence images. GFP$^+$ areas in the granular layer (GL) were measured by ImageJ software (https://imagej.nih.gov/ij/) (Table 1).

Swimming performance test

Swimming performance was analyzed as previously described (Matsuda et al., 2017). Freely swimming adult fish were recorded by a CMOS camera (30 frames per second; fps). The head position of each fish was tracked using the Tracker (http://physlets.org/tracker) program. The distance and direction of head movements between two consecutive frames were calculated in Microsoft Excel. An event showing >90° in the direction change of two consecutive movements was counted as one turn. Average swimming speed and turning frequencies were calculated in Microsoft Excel.

Active avoidance conditioning, Pavlovian fear conditioning, and response to electric shocks

Active avoidance conditioning was conducted by using a previously reported apparatus (Lal et al., 2018) and protocol (Aoki et al., 2013). Fish were maintained in a tank covered by white paper on the day before conditioning to prevent them from receiving special visual cues. For the conditioning, a white opaque tank (L41 cm × W17 cm × H12 cm) with transparent walls at both ends and a trapezoidal wedge (L10–20 cm × W17 cm × H5 cm) in the center of the tank was used. For the Pavlovian fear conditioning, a side compartment of the tank for active avoidance conditioning was used (L15.5 cm × W17 cm × H12 cm). Green LEDs (3.3-V DC, 2 A) and a pair of platinum mesh electrodes (12-V AC: 0.71 V/cm, 60 Hz) were used to provide CS and US, respectively. Behaviors were monitored by a CMOS camera (30 fps). The timing of CS and US was controlled with a DAQ interface (USB-6008; National Instruments Co) and laboratory-made software written in LabVIEW (National Instruments Co). Responses to electric shocks were examined by measuring swimming speed for 2 s before and after 10-s electric shocks after habituation for 20 min.

Statistics

Data were analyzed and graphs were generated using GraphPad Prism (version 5.1) or the R software package (4.0.3; https://www.r-project.org/). Data are presented as the average ± SEM. Statistical tests were applied as indicated in the figure legends. Additional statistical details are provided in Table 2.

Results

Establishment of Tg zebrafish expressing botulinum toxin in GCs or PCs

We previously used the Tg line *gSA2AzGFF152B* that expresses a modified version of Gal4-VP16 (Gal4FF) specifically in GCs to study the roles of GCs in classical fear conditioning (Takeuchi et al., 2015; Matsuda et al., 2017;

Table 1: Tg lines and expression of BoTxBLC-GFP

Tg lines	Expression	Sample number	Lateral sections (%)	Medial sections (%)	LCa (%)
gSA2AzGFF152B;	GCs (CCe>> LCa, EG)	1	14.7	69.5	0.2
Tg(UAS:BoTxBLC-GFP)		2	13.7	63.9	0.9
		3	6.4	48.6	0.2
Tg(cbln12:Gal4FF);	GCs (CCe, LCa, EG, TL)	1	38.0	35.6	44.2
Tg(UAS:BoTxBLC-GFP)		2	37.7	42.1	55.0
		3	55.6	50.9	50.5
Tg(aldoca:BoTxBLC-GFP)	PCs	1	96.4	97.7	NA
		2	95.9	96.8	
		3	100	99.4	

Sagittal sections from three gSA2AzGFF152B;Tg(UAS:BoTxBLC-GFP) or Tg(cbln12:Gal4FF);Tg(UAS:BoTxBLC-GFP) adult fish were stained with anti-GFP and anti-Neurod1 antibodies. Sagittal sections from three Tg(aldcoa:BoTxBLC-GFP) adult fish were stained with anti-GFP and anti-Pvalb7 antibodies. Two typical lateral and medial sections from each fish were used. For the GCs, the percentage of the GFP$^+$ area in the GL area was determined by using ImageJ software. For the PCs, the number of GFP$^+$ and Pvalb7$^+$ cells was counted manually. The percentage of GFP$^+$ cells to Pvalb7$^+$ cells was determined. CCe, corpus cerebelli; EG, eminentia granularis; GC, granule cells; LCa, lobus caudalis cerebelli; NA, not applicable; TL, torus longitudinalis.

Table 1). In addition to that line, we generated a Tg line, Tg(cbln12:Gal4FF), which expresses Gal4FF in GCs by using an ~2.0-kbp promoter/enhancer of the cerebellin12 (cbln12) gene, and reportedly drives transgene expression in GCs (Dohaku et al., 2019). We crossed gSA2AzGFF152B or Tg(cbln12:Gal4FF) with Tg(UAS: BoTxBLC-GFP), which expresses a fusion protein of the botulinum toxin light chain B and green fluorescent protein (BoTxBLC-GFP), which inhibits the synaptic release of neurotransmitters, in a Gal4-dependent manner (Sternberg et al., 2016; Lal et al., 2018), and raised them to adulthood (hereafter referred as to 152B::BoTx and cbln12::BoTx lines; Fig. 1A–G,H–N). We also generated a Tg line that expresses BoTxBLC-GFP in PCs by using an ~5.0-kbp promoter/enhancer of the aldolase Ca (aldoca) gene, which drives transgene expression specifically in PCs (Tanabe et al., 2010; Takeuchi et al., 2015; Dohaku et al., 2019; Tg(aldoca:BoTxBLC-GFP), hereafter referred as to aldoca:BoTx, Fig. 1O–U). We found similar levels of BoTxBLC-GFP expression in the cerebellum of 5-dpf larvae of the same Tg lines. All of these Tg fish did not show any obvious abnormalities, including in swimming behaviors, during development. We dissected the brains from the adults of these Tg fish (more than three months old) after the behavior analyses described below and examined the expression of BoTxBLC-GFP by immunostaining with antibodies against GFP, and a GC marker (Neurod1; Kani et al., 2010) or a PC marker parvalbumin 7 (Pvalb7; Bae et al., 2009). Adult 152B::BoTx fish specifically showed BoTxBLC-GFP expression in GCs, mainly in the CCe (Fig. 1A–G), as reported previously for late-stage larvae (Matsuda et al., 2017). Adult cbln12::BoTx fish displayed BoTxBLC-GFP expression in GCs in both the CCe and LCa, as well as in GCs in the torus longitudinalis of the mesencephalon (TL; Fig. 1H–N). cbln12::BoTx fish also displayed BoTxBLC-GFP expression in some telencephalic neurons (data not shown) as the cbln12 promoter was reported to drive transgene expression in telencephalic neurons in addition to GCs (Dohaku et al., 2019). In 152B::BoTx and cbln12::BoTx fish, BoTxBLC-GFP mRNA was transcribed in the somata of GCs while BoTxBLC-GFP protein was transported to the GC axons. Consistent with this, BoTxBLC-GFP was also

detected in the molecular layer (ML) of the cerebellum and/or the stratum marginale (SM) of the optic tectum where GC axons were present (Fig. 1G,I,N). A comparison with Neurod1 expression revealed that 60.7% and 11.6% of GCs in the medial and lateral domains, respectively, of the CCe expressed BoTxBLC-GFP in 152B::BoTx fish while only 0.433% of GCs in the LCa expressed BoTxBLC-GFP. In cbln12::BoTx fish, 43.8% and 42.9% of GCs in the medial and lateral regions, respectively, of the CCe, and 49.9% of GCs in the LCa, expressed BoTxBLC-GFP (Table 1). These data suggest that the 152B::BoTx fish preferentially expressed BoTxBLC-GFP in GCs of the medial CCe whereas cbln12B::BoTx expressed it in GCs of both the CCe and LCa. A comparison with Pvalb7 expression indicated that 97.7% of Pvalb7$^+$ PCs expressed BoTxBLC-GFP in the aldoca:BoTx line (Table 1). Although we cannot exclude the possibility that BoTx was also expressed in some neurons other than PCs, they are likely to be a minor population. Our data indicate that BoTx was expressed in large numbers of GCs or PCs of the cerebellum of these Tg fish.

Active avoidance conditioning in adult zebrafish

We employed an assay system for two-way active avoidance learning by using a tank with two compartments (Lal et al., 2018) and a previously published protocol (Aoki et al., 2013). In this system, the two compartments were separated by a fixed trapezoidal wedge allowing fish to move freely from one compartment to the other (Fig. 2A). Light exposure to green LEDs and electric shocks were considered as the CS and US, respectively. After habituation for 20 min, when a fish was located in a compartment, it was exposed to CS for 15 s during the training session. When the fish did not escape to the other side after 10 s, a 5-s US was applied (Fig. 2B). A 15- to 20-s interval was inserted between trials. When the fish escaped before US during the presentation of CS, it was considered as a successful trial (Movie 1). When the fish did not escape before US, it was considered as a failed trial (Movie 2). We found that no fish (n = 43) escaped to the other side within the first 10 s after the onset of CS during the first trial before

Table 2: Summary of statistical analyses

Figure	Measurement	Type of test	Comparison	Statistical value
2G	Number of turns during free swimming	Welch's t test	Learner WT vs non-learner WT	$p = 0.5924$ $t_{(41)} = 0.5397$
2H	Swimming speed during free swimming	Welch's t test	Learner WT vs non-learner WT	$p = 0.4405$ $t_{(41)} = 0.7791$
2J	Swimming speed before and after electric shock	Welch's t test	Before US vs after US in WT	$p = 0.002975$ $t_{(12)} = -4.539$
3A	Number of turns during free swimming	Welch's t test	Control vs 152B::BoTx	$p = 0.6224$ $t_{(86)} = -0.4942$
3B	Swimming speed during free swimming	Welch's t test	Control vs 152B::BoTx	$p = 0.7603$ $t_{(86)} = -0.3062$
3C	Number of turns during free swimming	Welch's t test	Control vs cbln12::BoTx	$p = 0.03522$ $t_{(75)} = -2.145$
3D	Swimming speed during free swimming	Welch's t test	Control vs cbln12::BoTx	$p = 0.3511$ $t_{(75)} = -0.9397$
3E	Swimming speed before and after electric shock	Welch's t test	Before US vs after US in 152B::BoTx	$p = 4.043\text{e-}05$ $t_{(12)} = -6.314$
3F	Swimming speed before and after electric shock	Welch's t test	Before US vs after US in cbln12::BoTx	$p = 0.01427$ $t_{(12)} = -3.130$
3G	Learning rate of active avoidance	Fisher's exact test	Control vs 152B::BoTx	$p = 5.574\text{e-}08$
3H	Learning rate of active avoidance	Fisher's exact test	Control vs cbln12::BoTx	$p = 0.01080$
4A	Number of turns during free swimming	Welch's t test	Control vs aldoca:BoTx	$p = 0.2164$ $t_{(78)} = 1.246$
4B	Swimming speed during free swimming	Welch's t test	Control vs aldoca:BoTx	$p = 0.6001$ $t_{(78)} = -0.5264$
4C	Swimming speed before and after electric shock	Welch's t test	before US vs after US in aldoca:BoTx	$p = 0.0001143$ $t_{(12)} = -6.248$
4D	Learning rate of active avoidance	Fisher's exact test	Control vs aldoca:BoTx	$p = 0.002116$
4E	Number of trials in training session 1	Welch's t test	Control vs aldoca:BoTx	$p = 0.3255$ $t_{(20)} = -1.031$
4E	Number of trials in training session 2	Welch's t test	Control vs aldoca:BoTx	$p = 0.7545$ $t_{(20)} = 0.3182$
4E	Number of trials in training session 3	Welch's t test	Control vs aldoca:BoTx	$p = 0.3865$ $t_{(20)} = 0.8856$
4E	Number of trials in test session	Welch's t test	Control vs aldoca:BoTx	$p = 0.2301$ $t_{(20)} = -1.360$
5M	Number of turns during free swimming	Welch's t test	Control vs aldoca:NTR	$p = 0.2728$ $t_{(54)} = -1.143$
5N	Swimming speed during free swimming	Welch's t test	Control vs aldoca:NTR	$p = 0.02462$ $t_{(54)} = 2.509$
5O	Swimming speed before and after electric shock	Welch's t test	Before US vs after US in aldoca:NTR	$p = 0.0003567$ $t_{(12)} = -6.371$
5P	Swimming speed before and after electric shock	One-way ANOVA		$F_{(18,621)} = 2.88$ $p = 0.03943$
5P	Swimming speed before and after electric shock	One-way ANOVA with Tukey's *post hoc* test	WT vs 152B::BoTx	$p = 0.6201$
5P	Swimming speed before and after electric shock	One-way ANOVA with Tukey's *post hoc* test	WT vs cbln12::BoTx	$p = 0.08734$
5P	Swimming speed before and after electric shock	One-way ANOVA with Tukey's *post hoc* test	WT vs aldoca:BoTx	$p = 0.7949$
5P	Swimming speed before and after electric shock	One-way ANOVA with Tukey's *post hoc* test	WT vs aldoca:BoTx	$p = 0.9959$
5Q	Learning rate of active avoidance	Fisher's exact test	Control vs aldoca:NTR	$p = 0.008326$
6B	Change of speed in training session	Two-way repeated measures ANOVA lines × trials interaction		$F_{(18,621)} = 3.515$ $p = 1.398\text{e-}06$
6B	Change of speed in training session	Two-way repeated measures ANOVA lines factor		$F_{(2,69)} = 19.92$ $p = 1.483\text{e-}07$
6B	Change of speed in training session	Two-way repeated measures ANOVA trials factor		$F_{(9,621)} = 5.725$ $p = 1.189\text{e-}07$
6B	Change of speed in training session	Two-way repeated measures ANOVA with Tukey's *post hoc* test	WT vs 152B::BoTx in trial 6	$p = 0.03243$
6B	Change of speed in training session		WT vs 152B::BoT in trial 7	$p = 0.04779$

(Continued)

Table 2: Continued

Figure	Measurement	Type of test	Comparison	Statistical value
		Two-way repeated measures ANOVA with Tukey's *post hoc* test		
6B	Change of speed in training session	Two-way repeated measures ANOVA with Tukey's *post hoc* test	WT vs 152B::BoT in trial 8	p = 4.1e-09
6B	Change of speed in training session	Two-way repeated measures ANOVA with Tukey's *post hoc* test	WT vs aldoca:BoTx in trial 8	p = 2.6e-05
6B	Change of speed in training session	Two-way repeated measures ANOVA with Tukey's *post hoc* test	WT vs 152B::BoTx in trial 9	p = 0.005936
6B	Change of speed in test session	Two-way repeated measures ANOVA lines × trials interaction		$F_{(18,621)} = 1.083$ $p = 0.3649$
6B	Change of speed in test session	Two-way repeated measures ANOVA lines factor		$F_{(2,69)} = 28.35$ $p = 1.398e-06$
6B	Change of speed in test session	Two-way repeated measures ANOVA trials factor		$F_{(9,621)} = 1.431$ $p = 0.171$
6B	Change of speed in training session	Two-way repeated measures ANOVA with Tukey's *post hoc* test	WT vs 152B::BoTx	$p < 1e-22$
6B	Change of speed in training session	One-way ANOVA with Tukey's *post hoc* test	WT vs aldoca:BoTx	$p < 1e-22$
6B	Change of speed in training session	One-way ANOVA with Tukey's *post hoc* test	aldoca:BoTx vs 152B::BoTx	p = 0.9425
6C	Learning rate of Pavlovian fear conditioning	Fisher's exact test with BH *post hoc* test	WT vs 152B::BoTx	p = 0.02892
6C	Learning rate of Pavlovian fear onditioning	Fisher's exact test with BH *post hoc* test	WT vs aldoca:BoTx	p = 0.02892
6C	Learning rate of Pavlovian fear conditioning	Fisher's exact test with BH *post hoc* test	152B::BoTx vs aldoca:BoTx	p = 1.000

In all figures, the data distribution was normal. WT, wild-type.

receiving any US. Fish that had eight successful trials among 10 consecutive trials were considered to have established active avoidance in the session, that session was terminated, and the fish were subjected to the next session following a 20 min interval (Fig. 2C). Each session contained up to 60 trials. The fish that did not establish active avoidance within 60 trials were not subjected to further trials, while those that established active avoidance in three consecutive sessions (sessions 1–3) were subjected to the test session in which only CSs were provided (Fig. 2C). When the fish succeeded in eight trials among the 10 consecutive trials within 60 trials, they were considered as learners. Fish that did not establish active avoidance in either training or test sessions were considered as non-learners. A small number of fish that established active avoidance in the training sessions failed in the test session (n = 3/43). It is currently unknown why these fish failed after the establishment of active avoidance. In this experimental condition, 51.2% (n = 43) of wild-type adult fish succeeded in establishing active avoidance learning (Fig. 2D). Learner fish established active avoidance in 32.5 ± 3.2, 20.4 ± 2.2, 13.6 ± 1.1, and 10.5 ± 0.5 trials (average ± SE) in training sessions 1, 2, 3, and the test session, respectively (Fig. 2E). Although we could not determine exactly when learners responded to CS to

escape, we found that learner fish escaped from the compartment where they received CS at 4.0 ± 0.63, 4.6 ± 0.44, 5.0 ± 0.60, and 4.0 ± 0.59 s (average ± SE) after receiving CS in training sessions 1, 2, 3, and the test session, respectively (Fig. 2F). We examined average swimming speed and turn frequency during 1 min of free swimming after the habituation session and before the training session. They were not significantly different between learners and non-learners (Fig. 2G,H). The data indicate that about half of the adult zebrafish could acquire active avoidance conditioning and progressively improved active avoidance. Our findings further suggest that the ability of zebrafish to acquire active avoidance conditioning was not directly related to their ability to swim. Furthermore, non-learner fish did not show a freezing response, i.e., reduction of swimming speed after CS (Fig. 2I), indicating that the freezing response was not the cause of the failure to learn.

Inhibition of GC transmission suppresses active avoidance conditioning

We next analyzed the GC-silenced 152B::BoTx and cbln12::BoTx fish, and compared them with control siblings that did not have Gal4FF and/or BoTxBLC-GFP
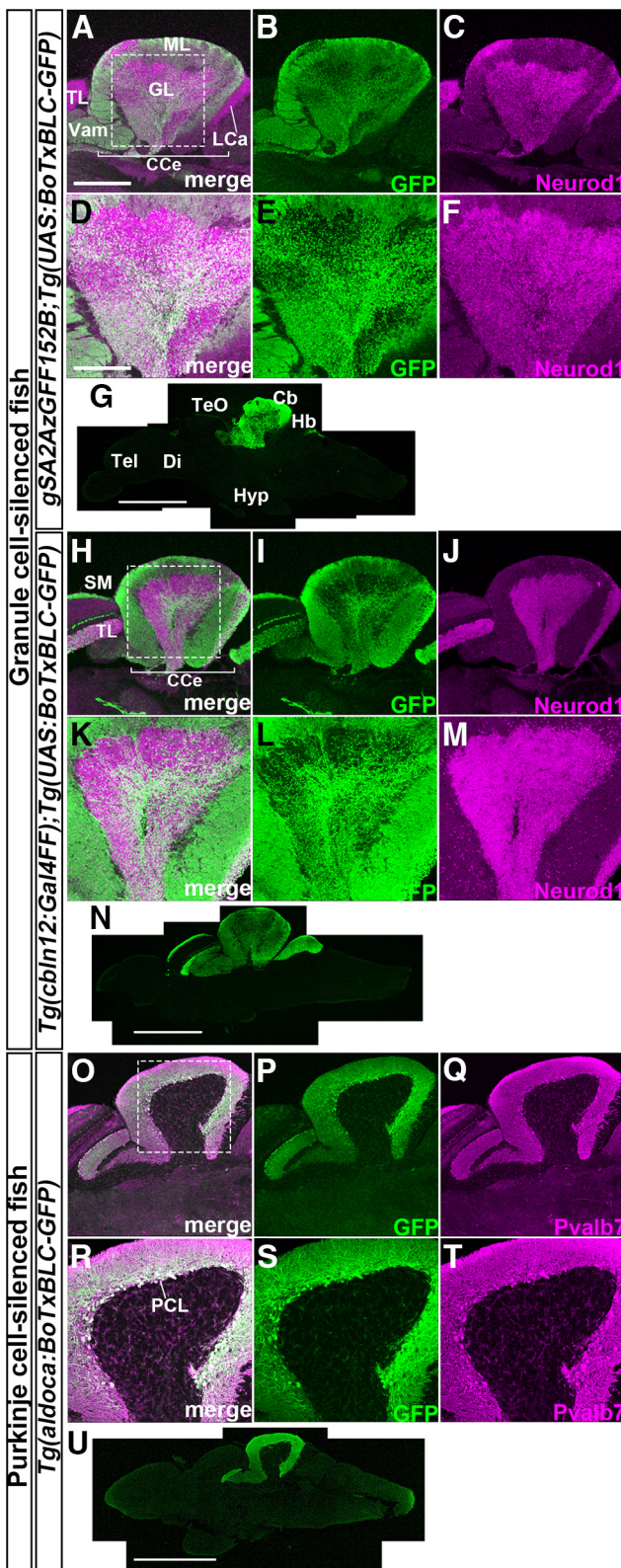
Figure 1. Establishment of Tg fish that express botulinum toxin in GCs or PCs. Sagittal sections of adult *gSA2AzGFF152B;Tg(UAS:BoTxBLC-GFP)* (*A–G*), *Tg(cbln12:Gal4FF);Tg(UAS:BoTxBLC-GFP)* (*H–N*), and *Tg(aldoca:BoTxBLC-GFP)* (*O–U*) brains were stained with anti-GFP (green), and anti-Neurod1, or anti-parvalbumin 7 (Pvalb7, magenta) antibodies. *A–C*, *H–J*, *O–Q*, Cerebellum region.

genes (Fig. 3). Although the cerebellum is involved in some forms of motor control, there was no significant difference in the average swimming speed and turn frequency between 152B::BoTx and control fish (Fig. 3*A*,*B*). Although turn frequency was slightly lower in cbln12::BoTx fish than in control fish (Fig. 3*C*), average swimming speed was not significantly different between cbln12::BoTx and control fish (Fig. 3*D*). Both 152B::BoTx and cbln12::BoTx fish responded to electric shocks as did wild-type fish (Figs. 2*J*, 3*E*,*F*), suggesting that the expression of BoTx did not strongly affect their swimming behaviors or response to US. When 152B::BoTx and control fish were subjected to active avoidance conditioning, 44.2% (*n* = 43) of control fish were learners while no (*n* = 45) 152B::BoTx fish were learners (Fig. 3*G*). When cbln12::BoTx and control fish were examined, 55.3% (*n* = 38) of control fish were learners while 25.6% (*n* = 39) of cbln12::BoTx fish were learners (Fig. 3*H*). The data indicate that the inhibition of neurotransmitter release in the GCs suppressed active avoidance conditioning. A relatively lower suppression of learner ratio observed in the cbln12::BoTx fish may be because of a relatively lower expression of BoTxBLC-GFP at the single-cell level in cbln12::BoTx fish than in 152B::BoTx fish (Table 1). The data indicate that the BoTx-mediated inhibition of GC transmission suppressed active avoidance conditioning without strongly inhibiting the swimming behaviors or aversive stimuli-dependent escape responses.

Inhibition of PC transmission suppresses active avoidance conditioning

We then analyzed the PC-silenced aldoca:BoTx fish, and compared them with control siblings that did not have the transgene. The average swimming speed and turn frequency were not significantly different between aldoca:BoTx and control fish (Fig. 4*A*,*B*). The aldoca:BoTx fish responded to electric shocks in a manner smilar to wild-type fish (Figs. 2*J*, 4*C*). When fish were subjected to active avoidance conditioning, 45.7% of control sibling fish (*n* = 35) were learners whereas 13.3% (*n* = 45) of aldoca:BoTx fish were learners (Fig. 4*D*). Furthermore, although there was no statistically significant difference, the aldoca:BoTx learner fish took more trials than control sibling fish to establish active avoidance in training session 1 and in the test session (Fig. 4*E*). These data indicate that inhibition of neurotransmitter release in PCs suppressed active avoidance conditioning but did not significantly affect conditioning-independent swimming behaviors or aversive stimuli-dependent escape responses.

continued
D–F, *K–M*, *R–T*, High-magnification views of the boxes in *A*, *H*, *O*. *G*, *N*, *U*, Low-magnification views. Cb, cerebellum; CCe, corpus cerebelli; Di, diencephalon; GL, granular layer; Hb, hindbrain; Hyp, hypothalamus; LCa, lobus caudalis cerebelli; ML, molecular layer; PCL; PC layer; SM, stratum marginale; Tel, telencephalon; TeO, tectum opticum; TL, torus longitudinalis; Vam, medial division of valvula cerebelli. Scale bars: 400 μm (*A*; applies to *A–C*, *H–J*, *O–Q*), 200 μm (*D*; applies to *D–F*, *K–M*, *R–T*), 1 mm (*G*, *N*, *U*).
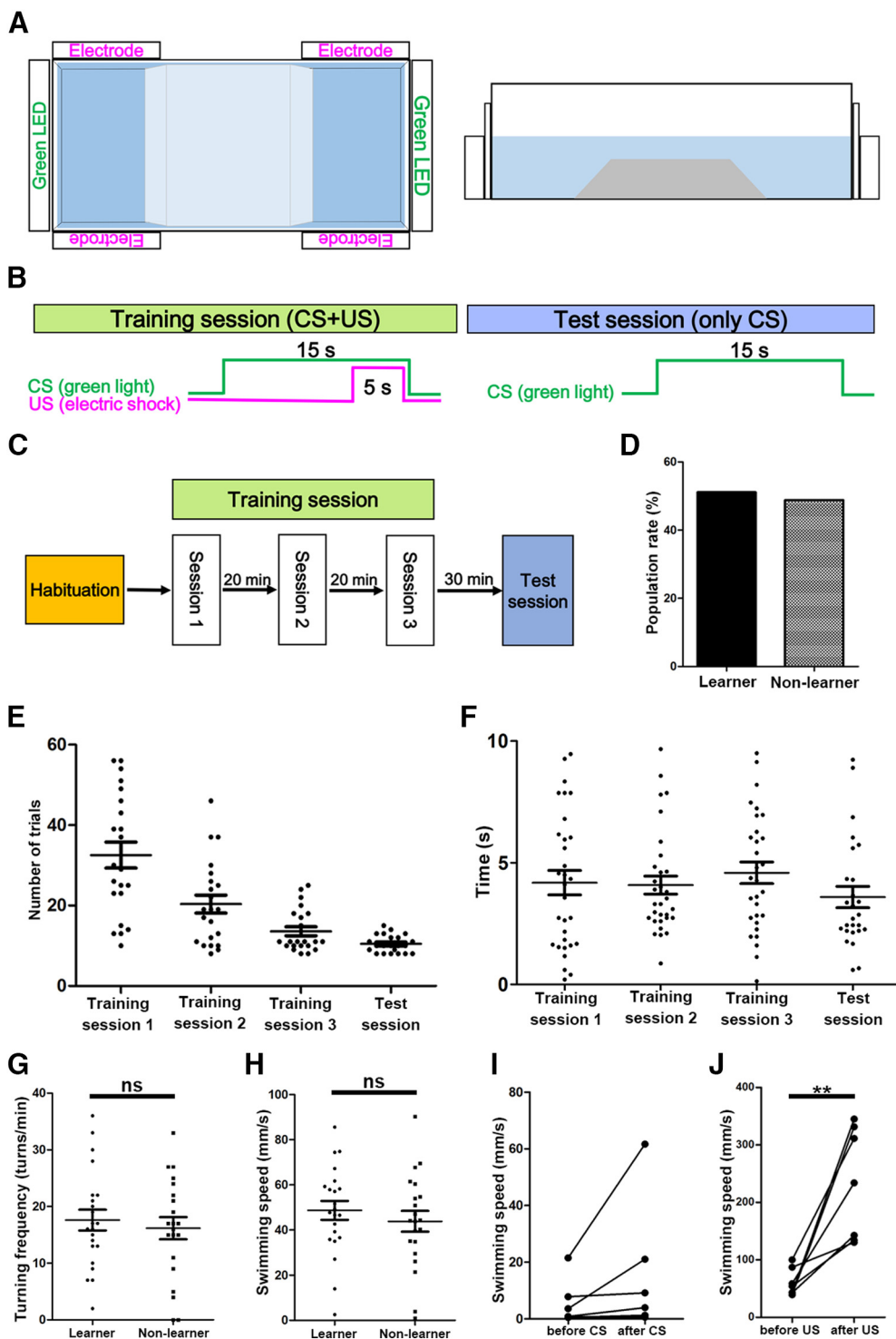
Figure 2. Active avoidance conditioning of wild-type fish. **A**, Tank used for active avoidance conditioning. A white opaque tank (L41 cm × W17 cm × H12 cm) with transparent walls at both ends, and a trapezoidal wedge (L10–20 cm × W17 cm × H5 cm) in the center, were used. Green LEDs and a pair of electrodes were placed on each side. Top view (left panel) and side view (right panel). **B, C**, Protocol for active avoidance. In the habituation session, a fish was allowed to swim freely for 20 min in the tank. In the training session, when a fish was located in a side compartment, the LED was turned on for 15 s (CS). If the fish did not escape to the other side after 10 s, an electric shock was administered for 5 s (US) in each trial. When the fish moved before the electric shocks, the trial was successful and was followed by a 30-s interval and the next trial. When fish had eight successful trials among 10 consecutive trials, they were considered to have established active avoidance in the training session, and were subjected to the next trial session. When fish did not establish active avoidance within 60 trials, the training session was terminated. When fish established active avoidance in three consecutive training sessions, they were subjected to the test session. In the test session, only light stimuli

continued

with LEDs were administered. When the fish had eight successful trials among 10 consecutive trials in the training session, they were considered to be learners. When fish did not establish active avoidance in the training session or did not succeed in the test session, they were considered to be non-learners. ***D***, Acquisition of active avoidance conditioning in wild-type adult fish. Percentages of learners and non-learners are indicated ($n = 43$). ***E***, Number of trials when learner fish established active avoidance in the training and test sessions ($n = 22$). The graph shows averages and SEs of the data. ***F***, Time from CS to escape in each session of learner fish ($n = 7$). The graph shows averages and SEs of the data. ***G***, ***H***, Swimming behaviors. Turning frequency (turns/min) and swimming speed (mm/s) of learners and non-learners during free swimming (learner; $n = 22$, non-learner; $n = 21$). The graph shows averages and SEs of the data (ns indicates non-significance, Welch's *t* test). ***I***, Freezing response of non-learners. Average swimming speed (mm/s) of seven non-learners before and after the onset of CS in the 44th–53rd trials of training session 1 was calculated. ***J***, Test for responsiveness to electric shocks in wild-type adult fish ($n = 7$). Swimming speed for 2 s before and after electric shocks was calculated (**$p < 0.01$, Welch's *t* test). ns, not significant.

Ablation of PCs in adult fish suppresses active avoidance conditioning

Since BoTx was expressed in 152B::BoTx, cbln12::BoTx and aldoca:BoTx fish from an early larval stage, re-wiring of cerebellar neural circuits might occur and compensate for the deficiency of GC/PC transmission during development. We ablated PCs in adult fish with NTR. We established a Tg line that expresses a fusion protein of modified NTR and TagRFP-T (NTR-TagRFPT; Tabor et al., 2014) in PCs by using the *aldoca* promoter/enhancer [*Tg (aldoca:NTR-TagRFPT)*]. NTR-TagRFPT was expressed from early larval stages to adult stages (Fig. 5*A,B,D,E*). NTR-TagRFPT signals completely overlapped with Pvalb7 signals in the cerebellum but not in the optic tectum where Pvalb7-expressing Type I neurons are present (Fig. 5*C*), indicating that NTR-TagRFPT was specifically expressed in PCs. We found that 11 d after treatment with MTZ, most NTR-TagRFPT-positive and Pvalb7-positive cells disappeared from the cerebellum although Pvalb7-positive Type I neurons remained in the optic tectum while the ML was abrogated (Fig. 5*G–L*), indicating that most PCs were ablated. Turn frequency and swimming speed were reduced in PC-ablated fish compared with control wild-type fish (the difference in turn frequency was not statistically significant; Fig. 5*M, N*). However, the PC-ablated fish responded to electric shocks, as did wild-type, 152B::BoTx, cbln12::BoTx,

and aldoca:BoTx fish, and swam similarly to wild-type fish after electric shocks (Figs. 2*J*, 5*O,P*), suggesting that the acute ablation of PCs slightly affected free swimming but not aversive stimuli-dependent swimming. In active avoidance conditioning, 51.2% of control fish ($n = 43$) became learners whereas only 7.69% of PC-ablated fish ($n = 13$) were learners (Fig. 5*Q*), indicating that the ablation of PCs in the adult cerebellum also perturbed active avoidance conditioning.

Inhibition of GC/PC transmission also suppresses Pavlovian fear conditioning

Although the inhibition of GCs or PCs suppressed active avoidance conditioning, it is not clear whether it inhibited classical fear conditioning. We conducted Pavlovian fear conditioning in which we determined CS-evoked panic movements by using 152B::BoTx (GC-silenced) and aldoca:BoTx (PC-silenced) fish. In the Pavlovian fear conditioning, light exposure to green LEDs was provided for 2 s as a CS in the habituation session (10 trials), electric shocks were delivered as 0.2-s USs with CSs after the onset of 1.8-s CS in the training session (10 trials), and only CSs were administered in the test session (10 trials; Fig. 6*A*). We assessed the Pavlovian fear conditioned responses by measuring changes in swimming speed for a total of 3 s, i.e., 1.5 s before and after the onset of CS. Wild-type fish progressively increased CS-evoked changes in swimming speed in the training
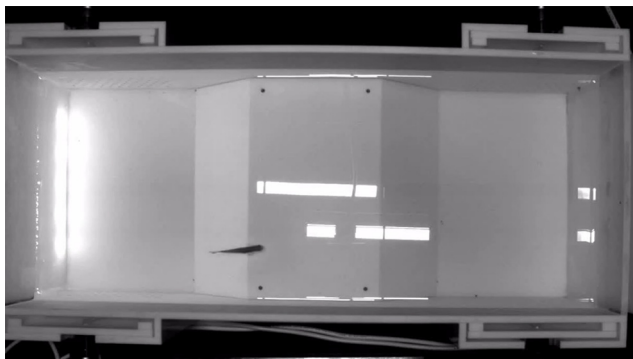
Movie 1. Active avoidance of a wild-type fish, successful trial. A successful trial of active avoidance of a wild-type learner fish in the 25th trial of training session 1 is shown. The timing of the CS (light exposure with a green LED) and US (electric shock) are indicated. Note that the fish responded to the CS and escaped into the compartment on the other side of the tank. [View online]

Movie 2. Active avoidance of a wild-type fish, failed trial. A failed trial of active avoidance of a wild-type non-learner fish in the 25th trial of training session 1 is shown. The timing of the CS and US are indicated. Note also that the fish did not escape after the presentation of CS but responded to US. [View online]
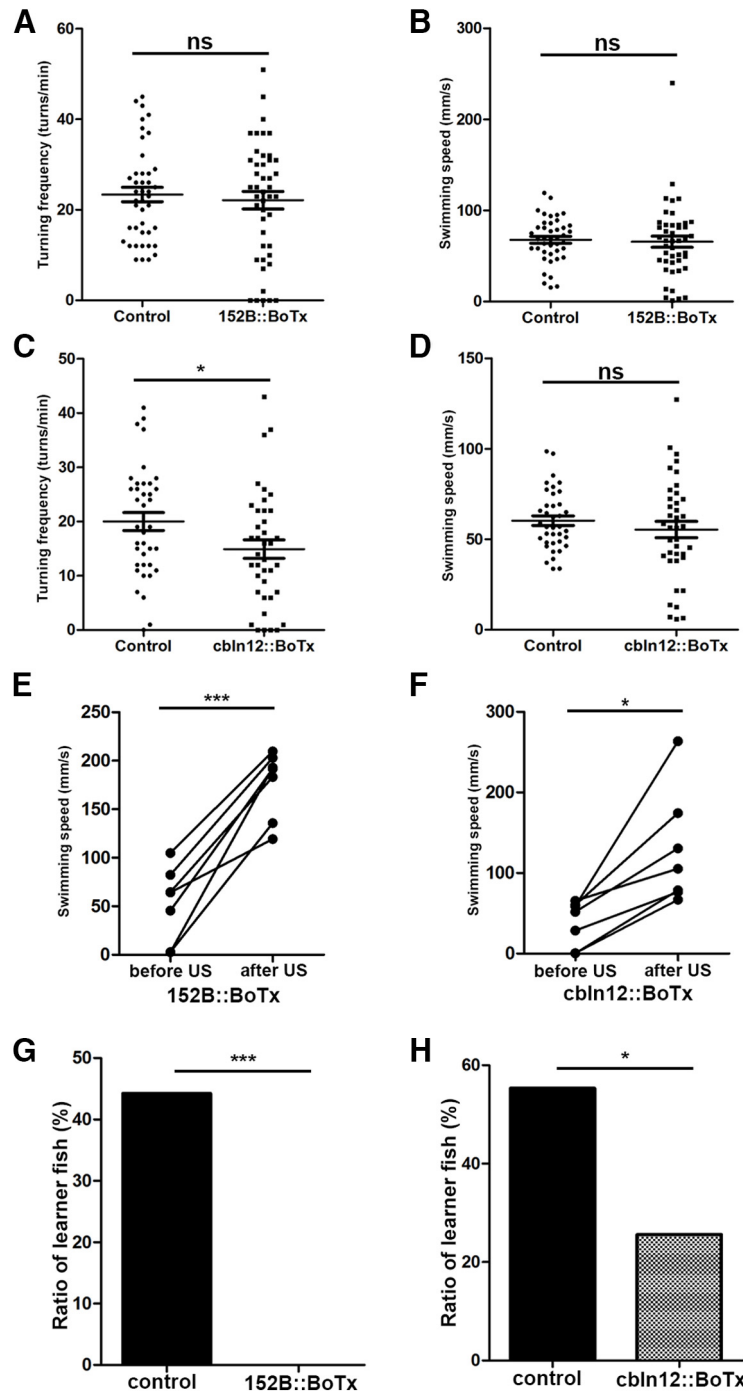
Figure 3. Expression of botulinum toxin in GCs suppresses active avoidance conditioning. *A*, *B*, Turning frequency (turns/min) and swimming speed (mm/s) of *gSA2AzGFF152B;Tg(UAS:BoTxBLC-GFP)* (152B::BoTx) and control sibling fish during free swimming (152B::BoTx; $n = 47$, control; $n = 43$). The graph shows the averages and SEs of the data (ns indicates non-significance, Welch's *t* test. *C*, *D*, Turning frequency and swimming speed of *Tg(cbln12:Gal4FF);Tg(UAS:BoTxBLC-GFP)* (cbln12::BoTx) fish during free swimming (cbln12::BoTx; $n = 39$, control; $n = 38$). The graph shows the averages and SEs of the data (ns indicates non-significance, *$p < 0.05$, Welch's *t* test). *E*, *F*, Response to electric shocks in 152B::BoTx ($n = 7$) and cbln12::BoTx ($n = 7$) fish. Swimming speed was calculated for 2 s before and after the electric shocks (***$p < 0.001$, *$p < 0.05$, Welch's *t* test). *G*, *H*, Percentages of active avoidance learners for 152B::BoTx ($n = 47$) and control sibling fish ($n = 43$; *G*), and for cbln12::BoTx ($n = 39$), and control sibling fish ($n = 38$; *H*; ***$p < 0.001$, *$p < 0.05$, Fisher's exact test). ns, not significant.

session, and moved quickly after CS in the test session (Fig. 6B; Movie 3). The CS-evoked responses were gradually reduced in the test session (Fig. 6B). In contrast, the conditioned responses of 152B::BoTx and aldoca:

BoTx fish barely improved in the training session (Fig. 6B). The conditioned responses in the test session were significantly weaker in 152B::BoTx and aldoca:BoTx fish than in wild-type fish (Fig. 6B; Movies 4, 5). We defined
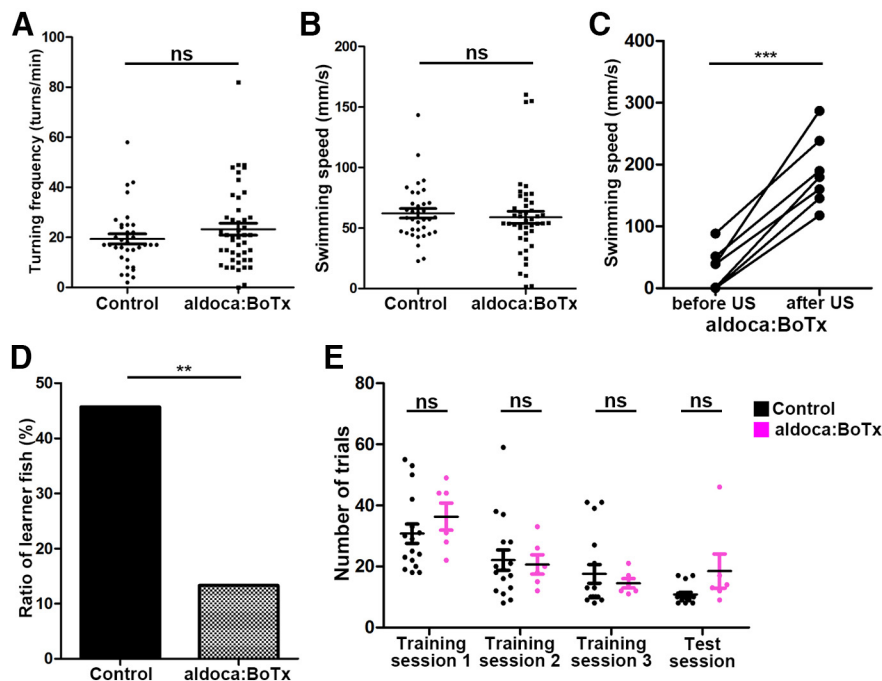
Figure 4. Expression of botulinum toxin in PCs suppresses active avoidance conditioning. *A*, *B*, Turning frequency (turns/min) and swimming speed (mm/s) of *Tg(aldoca:BoTxBCL-GFP)* (aldoca:BoTx) and control sibling fish during free swimming (aldoca:BoTx; $n = 45$, control; $n = 35$). The graph shows the averages and SEs of the data (ns indicates non-significance, Welch's t test). *C*, Response to electric shocks in aldoca:BoTx fish ($n = 7$). Swimming speed was calculated for 2 s before and after the electric shocks (***$p < 0.001$, Welch's t test). *D*, Percentages of active avoidance learners of aldoca:BoTx ($n = 45$) and control ($n = 35$) fish (**$p < 0.01$, Fisher's exact test). *E*, Number of trials required to establish active avoidance conditioning (aldoca:BoTx: $n = 6$; control: $n = 16$). The graph shows the averages and SEs of the data (ns indicates non-significance, Welch's t test).

"learners" as fish whose conditioned responses were significantly higher in the test session than in the habituation session; 57.1% ($n = 14$) of wild-type fish were learners whereas a significantly lower number of 152B::BoTx (8.33%, $n = 12$) and aldoca:BoTx (7.69%, $n = 13$) fish were leaners (Fig. 6C). These data indicate that the inhibition of GC or PC transmission also perturbed the Pavlovian fear conditioning. We further examined Pavlovian fear responses by measuring changes in speed for 1.5 s before and after the onset of CS in active avoidance conditioning; 70% ($n = 10$) of wild-type fish showed CS-evoked quick movements (Fig. 6D), indicating that Pavlovian fear conditioning was also established during active avoidance conditioning.

Discussion

Genetic inhibition of cerebellar neurons in zebrafish

The roles of cerebellar neural circuits in fear conditioning have traditionally been studied by inhibiting the cerebellum with physical or laser-induced lesions, or by local administration of an anesthetic drug (Yoshida et al., 2004; Yoshida and Hirano, 2010; Ahrens et al., 2012; Lin et al., 2020). In this study, we inhibited neurotransmitter release from GCs or PCs by expressing BoTx using the promoter/enhancer of GC-expressed or PC-expressed genes (i.e., *cbln12* and *aldoca*) or a Gal4 trap line that drives transgene expression in GCs (Fig. 1). These genetic inhibitions are thought to be

reliable, reproducible, and cell-type-specific as the neurotoxin was stably expressed in the cells of interest. The Gal4-UAS system induces transgene expression more strongly than when driven directly by cell-type-specific enhancer/promoters. However, methylation-mediated silencing of UAS-regulated transgenes occurs more as generations progress, resulting in variegated transgene expression (Akitake et al., 2011). Although the *gSA2AzGFF152B* line expresses *Gal4FF* in most GCs of the medial CCe (Takeuchi et al., 2015) and the *cbln12* promoter/enhancer drives transgene expression in all differentiated GCs (Dohaku et al., 2019), the Gal4-dependent expression of BoTxBLC-GFP was mosaic (Fig. 1; Table 1). In contrast, the expression of BoTxBLC-GFP, which was driven directly by the *aldoca* promoter/enhancer (Tanabe et al., 2010), was detected in most, if not all, PCs (Fig. 1; Table 1). Therefore, careful examination of BoTx expression is required to validate results from neuronal inhibition with Gal4-UAS-mediated toxin expression. In this study, even when BoTx was expressed in about half of the GCs of the medial CCe in 152B::BoTx and cbln12::BoTx fish, this suppressed active avoidance conditioning (Fig. 3), suggesting that the establishment of active avoidance conditioning is sensitive to the number of functional GCs in the cerebellum. cbln12::BoTx expressed BoTxBLC-GFP in neurons in the telencephalon and GCs in the TL, in addition to GCs in the cerebellum (Fig. 1). Although the roles of these neurons outside the cerebellum are not necessarily excluded, it is likely that
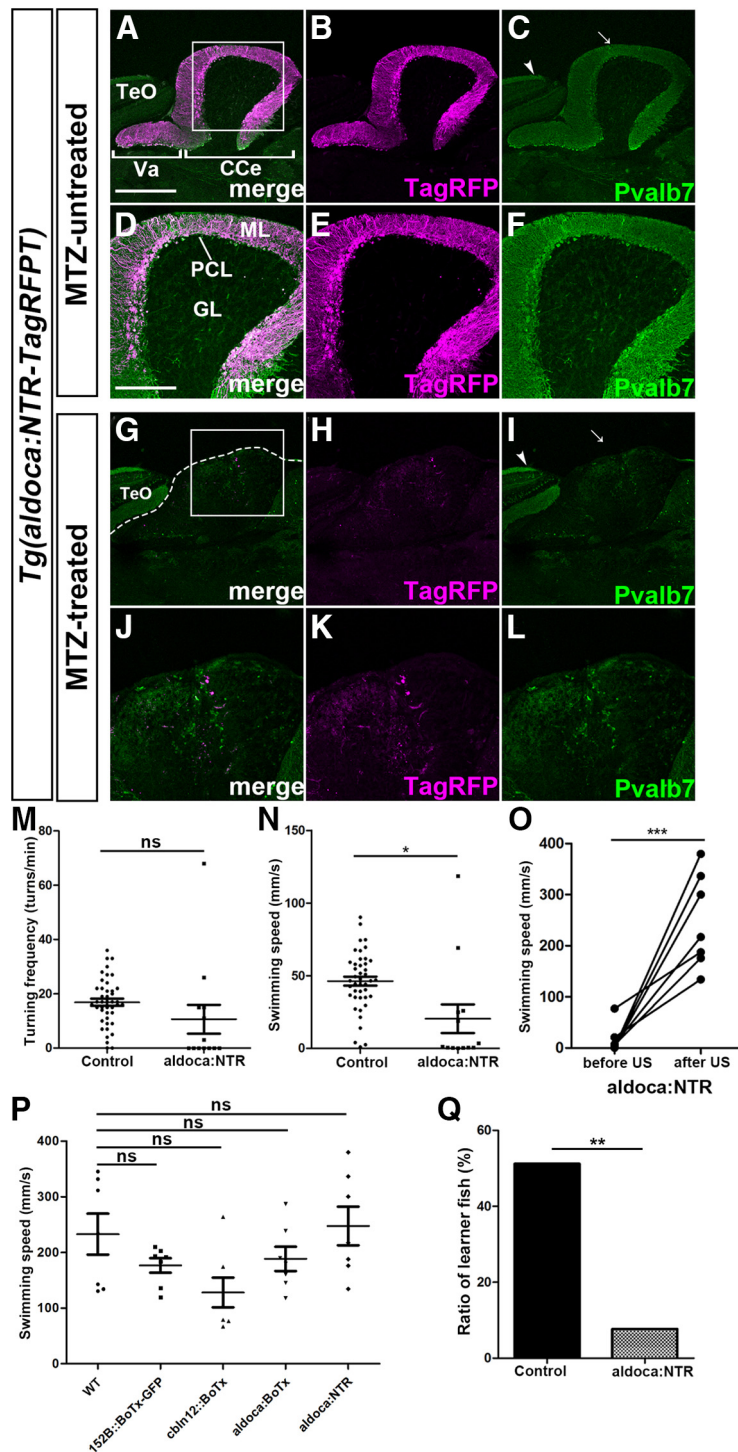
Figure 5. NTR-mediated ablation of PCs in adult fish suppresses active avoidance conditioning. *A–L*, Ablation of PCs. Adult *Tg(aldoca:NTR-TagRFPT)* fish were treated with MTZ for 18 h (*A–F*) or left untreated (*G–L*). The fish were subjected to behavior assays and subsequent histologic analysis 11 d after MTZ treatment. Sagittal sections were stained with anti-Pvalb7 antibody (green). Expression of NTR-TagRFPT (TagRFP, magenta) is also shown. *D–F, J–L*, High-magnification views of the boxes in *A, G*. Arrows and arrow heads indicate Pvalb7-positive dendrites of PCs (in the cerebellum) and Type I neurons (in the optic tectum), respectively. The dotted line in *G* indicates the limit of the cerebellum. Note that the Pvalb7 signal in PCs but not in Type I neurons disappeared and no ML was observed in the MTZ-treated fish. *M, N*, Turning frequency (turns/min) and swimming speed (mm/s) of *Tg(aldoca: NTR-TagRFPT)* (aldoca:NTR) and control fish during free swimming (aldoca:NTR; $n = 13$, control; $n = 43$). The graph shows the averages and SEs of the data (ns indicates non-significance, *$p < 0.05$, Welch's *t* test). *O*, Response to electric shocks in adult aldoca: NTR fish treated with MTZ ($n = 7$). Swimming speed was calculated for 2 s before and after the electric shocks (***$p < 0.001$, Welch's *t* test). *P*, Swimming speed for 2 s after US in each strain. The graph shows the averages and SEs of the data (ns indicates

continued

non-significance, one-way repeated measures ANOVA with Tukey's *post hoc* test). **Q**, Percentage of active avoidance learners of al-doca:NTR ($n = 13$) and control wild-type ($n = 43$) fish (**$p < 0.01$, Fisher's exact test). Va, valvula cerebelli. The other abbreviations are described in Figure 1. Scale bars: 400 μm (**A**; applies to **A–C**, **G–I**) and 200 μm (**D**; applies to **D–F**, **J–L**). ns, not significant.
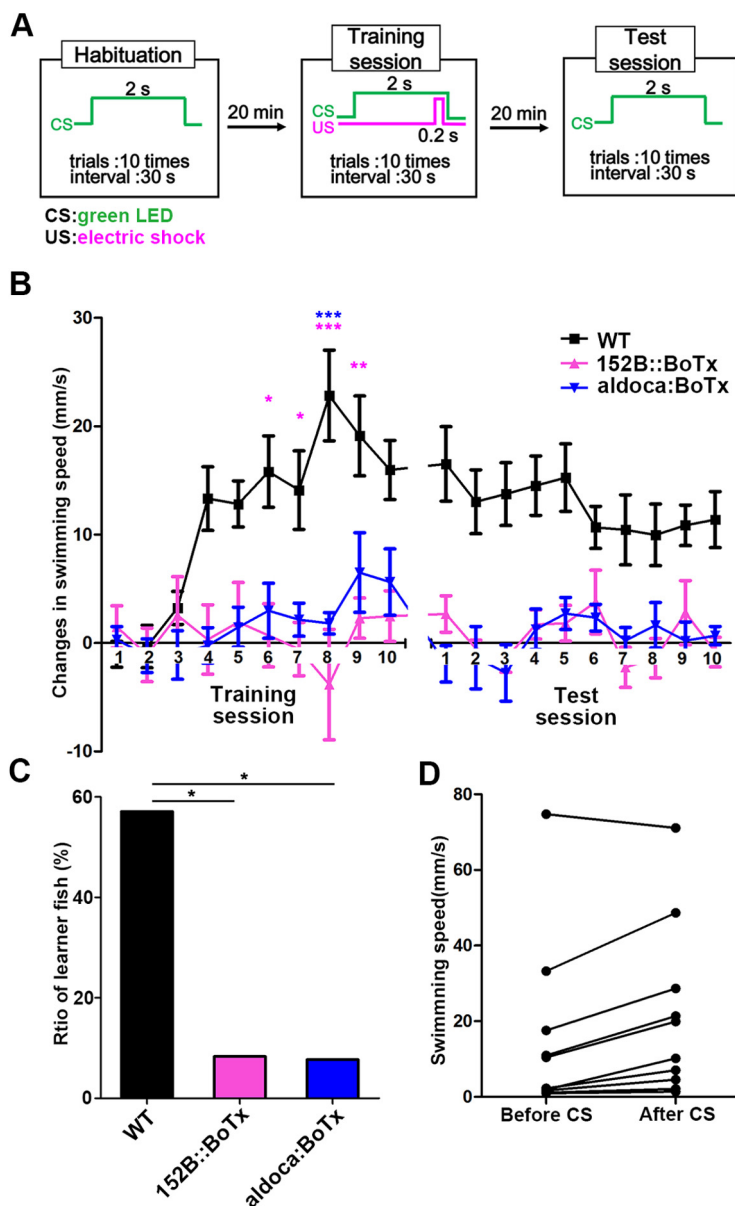

Figure 6. Expression of botulinum toxin in GCs or PCs also perturbs classical conditioning responses. **A**, Protocol for classical fear condition-ing. A compartment on one side of the tank in Figure 2A was used. In the habituation session, a light stimulus (CS) was provided for 2 s per trial (10 trials). In the training session, a paired CS and US (0.2-s electric shock given 1.8 s after the onset of CS) was administered in each trial (10 trials). In the test session, CS alone was administered (10 trials). The interval between trials was 30 s, and the interval between sessions was 20 min. **B**, Changes in swimming speed before and after the CS of wild-type (WT, $n = 25$), 152B::BoTx ($n = 23$), and aldoca:BoTx ($n = 24$) fish. Swimming speed was measured for 1.5 s before and after the CS in each trial, and average changes in swimming speed in the training and test sessions were calculated. The graph shows the averages and SEs of the data (Training session; lines factor: $p = 1.483e-07$, trials fac-tor: $p = 1.189e-07$, lines $\times$ trials interaction: $p = 1.398e-06$, two-way repeated measures ANOVA; ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, two-way repeated measures ANOVA with Tukey's *post hoc* test. Test session; lines factor: $p = 1.398e-06$, trials factor: $p = 0.171$, lines $\times$ trials interac-tion: $p = 0.3649$, two-way repeated measures ANOVA; WT vs 152B::BoTx in test session: $p < 1e-22$, WT vs aldoca:BoTx in test session: $p < 1e-22$, 152B::BoTx vs aldoca:BoTx in test session: $p = 0.9425$, one-way ANOVA with Tukey's *post hoc* test). **C**, Percentages of Pavlovian conditioning learners of WT ($n = 14$), 152B::BoTx ($n = 12$), and aldoca:BoTx ($n = 13$) fish (*$p < 0.05$, Fisher's exact test with BH *post hoc* test). **D**, Pavlovian panic responses during active avoidance conditioning. Data from 10 wild-type fish that were subjected to active avoidance con-ditioning were used. Since wild-type fish established active avoidance in the 13th trial at the earliest, swimming speed for 1.5 s before and after the CS was measured in each trial from the fourth to the 13th trial. Average speed is plotted in the graph.
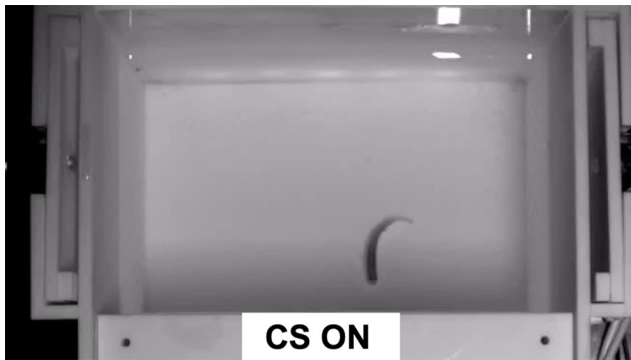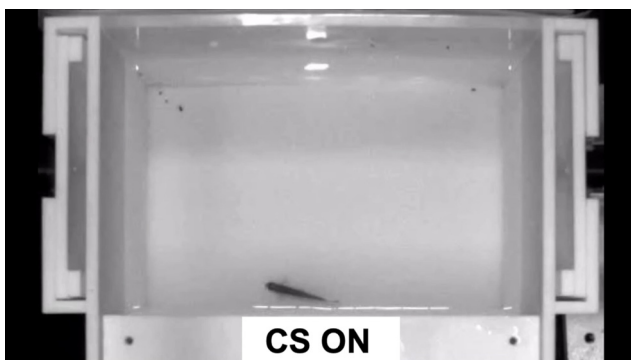
Movie 3. Pavlovian fear conditioning of a wild-type fish. Behavior of a wild-type learner fish in trial 1 of the test session is shown. The timing of the CS is indicated. Note that the fish moved quickly after the presentation of CS. [View online]

the inhibition of GCs in the cerebellum had a major impact on active avoidance conditioning in both 152B::BoTx and cbln12::BoTx fish. The BoTx-mediated inhibition of PCs also perturbed active avoidance conditioning (Fig. 4). Collectively, BoTx expression driven by both Gal4-UAS or a cell-specific promoter/enhancer successfully inhibited the transmission of GCs and PCs.

PC-silenced fish did not show abnormal locomotion but generated an erratic form of body displacement in some conditions (Chang et al., 2020). However, both GC-silenced and PC-silenced fish did not show conditioning-independent swimming and responded to US in a manner similar to wild-type fish (Figs. 3, 4). Thus, although the BoTx-mediated inhibition of GCs or PCs might affect smooth and/or well-coordinated movements to some extent, its main impact on conditioning is likely to be independent of abnormal swimming behavior.

In this study, BoTx was expressed from early larval stages when GCs and PCs had differentiated. Therefore, physical or functional rewiring of neural circuits might occur and compensate for the deficiency of GC or PC transmission. It might alleviate the effects of inhibition of GCs or PCs. To address this issue, we specifically ablated PCs in the adult cerebellum using the NTR-MTZ system

Movie 4. Pavlovian fear conditioning of a 152B::BoTx fish. Behavior of a 152B::BoTx non-learner fish in trial 1 of the test session is shown. The timing of the CS is indicated. Note that the fish did not move after the presentation of CS. [View online]
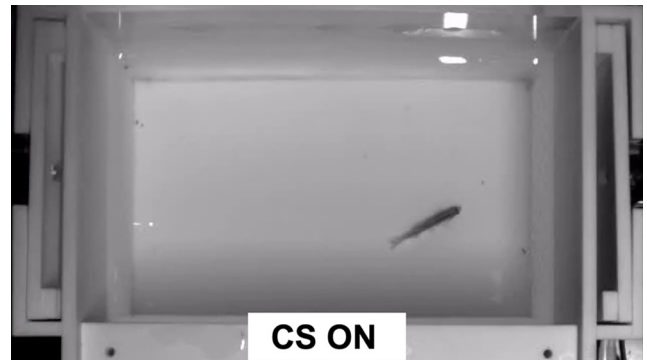
Movie 5. Pavlovian fear conditioning of an aldoca:BoTx fish. Behavior of an aldoca:BoTx non-learner fish in trial 1 of the test session is shown. The timing of the CS is indicated. Note that the fish did not move after the presentation of CS. [View online]

(Fig. 5). Although PC-ablated fish showed some abnormal swimming behaviors, they could respond to US in the same manner as wild-type fish (Figs. 2, 5). Similar to BoTx-expressing fish, PC-ablated fish showed reduced active avoidance conditioning. In contrast, NTR-MTZ-mediated ablation might induce an inflammatory response that exacerbated the cerebellar function, but it did not induce abnormal swimming, such as ataxia or rolling movements. Therefore, the BoTx-mediated inhibition of GCs/PCs and NTR-MTZ-mediated PC ablation were both able to induce defects in cerebellar neural circuits.

The cerebellum is involved in active avoidance conditioning in zebrafish

Early stage zebrafish larvae acquired operant conditioning in which they learned to turn their tail in the correct direction to obtain relief from an aversive heat stimulus (Lin et al., 2020). In that operant conditioning, cerebellar lesions affected the decision of which direction they would turn their tail (Lin et al., 2020). It is not clear whether the cerebellum is involved in other types of operant conditioning. In this study, we employed two-way active avoidance conditioning with adult zebrafish and genetically inhibited cerebellar neurons. Although the conditioning paradigm and fish ages are different, our findings clearly indicate that the zebrafish cerebellum is involved in active avoidance conditioning.

152B::BoTx fish expressed BoTx mainly in GCs of the medial CCe but only rarely in GCs in the LCa (Fig. 1C). This is consistent with previous data in which Gal4FF in *gSA2AzGFF152B* fish drove transgene expression mainly in GCs of the medial CCe in larvae and adult fish (Takeuchi et al., 2015; Matsuda et al., 2017). Since active avoidance conditioning, but not free swimming, was strongly suppressed in 152B::BoTx fish (Fig. 3), GCs in CCe play an important role in active avoidance conditioning but not in free swimming. Previously, it was reported that the inhibition of GC transmission in 152B::BoTx late-stage larvae prolonged fear-conditioned bradycardia responses in which a set of neurons, most likely GCs, in the CCe became activated by the CS and were associated with conditioned bradycardia responses (Matsuda et al., 2017). The same or similar types of neurons might be

involved in active avoidance conditioning. As Ca^{2+} and voltage imaging of neurons in freely moving adult zebrafish are still difficult, we were unable to identify conditioning-associated neurons in active avoidance conditioning. Future studies with optical fibers or body-mounted fluorescence sensors that allow for the detection of immediate early gene expression, or virtual-reality conditioning assays, may reveal GCs and PCs that are associated with active avoidance conditioning.

The cerebellar vermis in mammals was reported to be involved in classical fear conditioning, such as autonomic bradycardia and freezing responses (Supple and Leaton, 1990; Supple and Kapp, 1993; Supple et al., 1993; Sacchetti et al., 2002). A previous study (Matsuda et al., 2017), as well as the current study, revealed that GCs in the CCe of the zebrafish cerebellum are involved in conditioned fear responses, including bradycardia and active avoidance. These findings suggest that the CCe of the zebrafish cerebellum has a function similar to that of the mammalian cerebellar vermis.

Cerebellar efferent neurons, which are eurydendroid cells, receive inputs directly from GCs and possibly integrate information of GCs (Harmon et al., 2020). Since BoTx-mediated inhibition of PC transmission or PC ablation in the adult cerebellum perturbed active avoidance conditioning (Figs. 4, 5), the transmission from PCs to eurydendroid cells is required for this conditioning. In goldfish, the activity of major PCs was suppressed whereas that of minor PCs was activated during classical fear conditioning (Yoshida and Kondo, 2012). Long-term depression (LTD) and long-term potentiation (LTP) in GC-PC synapses are involved in cerebellar learning (Ito and Kano, 1982; Salin et al., 1996; Lev-Ram et al., 2002; Sacchetti et al., 2004), although the contribution of LTD or LTP may depend on the type of learning. The inhibition or ablation of PCs might induce an imbalance of PC outputs, resulting in defective active avoidance behaviors. Future analysis of PC activity during conditioning will clarify this issue.

The habenula-raphe circuit and the medial zone of the dorsal telencephalon (Dm), which are thought to correspond to the amygdala in mammals, were involved in active avoidance conditioning in zebrafish (Aoki et al., 2013; Amo et al., 2014; Lal et al., 2018). In mammals, the amygdala plays essential roles in both classical fear conditioning and active avoidance conditioning (Duvarci and Pare, 2014; Herry and Johansen, 2014). Although a functional connection between the habenula and cerebellum was suggested (Lin et al., 2020), future studies on neural circuit connections between the cerebellum and the habenula/Dm are needed and will reveal what kind of information is transmitted to, and integrated in, the cerebellum.

Roles of the cerebellum in Pavlovian fear and active avoidance conditioning

Previously, BoTx-mediated inhibition of GCs did not perturb but rather prolonged CS-evoked bradycardia responses in zebrafish larvae, suggesting that cerebellar neural circuits control recovery from the fear-conditioned response (Matsuda et al., 2017). In this study, the inhibition of GCs or PCs suppressed CS-evoked Pavlovian panic responses, indicating a positive role of cerebellar neural circuits in the conditioned fear response. Why do the results of cerebellum inhibition differ between the two different classical conditioning paradigms? Although both are forms of classical fear conditioning in which CS-induced passive responses occur after conditioning, the bradycardia response is an autonomic response while the swimming response is a motor response. One possibility is that the two different conditioned responses are controlled by different neural circuits in the cerebellum although they share the same or similar sensory or integration systems, including the habenula nuclei and Dm. A different subpopulation of cerebellar neurons may control autonomic and motor responses. Total ablation of the CCe or anesthetic inhibition of the cerebellum impaired the acquisition of the conditioned bradycardia response in goldfish (Yoshida et al., 2004; Yoshida and Hirano, 2010), supporting the positive role of the entire cerebellum in classical fear conditioning. In 152B::BoTx fish, a specific GC subpopulation that functions in recovery from the conditioned fear response might be suppressed. Alternatively, the same cerebellar neurons may control both autonomic and motor responses in different manners: they regulate the timing of recovery from the freeze response while controlling the decision of the motor response. The elucidation of neurons whose activity was associated with each type of conditioned responses and/ or specific inhibition of GC or PC subpopulations in each conditioning paradigm will clarify neural circuits that control the conditioned autonomic and motor responses.

Both active avoidance and Pavlovian fear conditioning involve associative learning of the CS and US, and behavioral decisions based on them. We found that both of them were suppressed by PC or GC inhibition. Pavlovian fear conditioning was also established during active avoidance conditioning (Fig. 6). Although we could not determine the relationship between Pavlovian and active conditioning, it is possible that zebrafish first establish Pavlovian fear conditioning then, simultaneously or subsequently, move directionally to avoid US. In this scenario, the cerebellar neural circuits may control the initial process and possibly the decision-making of active avoidance conditioning. In summary, our findings indicate that the cerebellum plays active roles in active avoidance conditioning and provide a platform for understanding the mechanisms of conditioned fear responses.

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