Hypoactivity, Abnormal Musculature, and Learning and Memory Defects in slc6a3 zebrafish Mutant Larvae

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Research Article

Abstract

Dopamine (DA) is one of the most common neurotransmitters in living organisms and is involved in the regulation of behavior, physiology, and disease. The dopamine transporter (DAT, encoded by the *slc6a3* gene) re-uptakes dopamine from the synaptic cleft back into neurons, which plays a critical role in regulating dopamine signaling. Clinically, mutations in *slc6a3* have been implicated in various diseases. Zebrafish are good model organisms for studying the functions of *slc6a3* and dopamine. Work using the zebrafish *slc6a3* mutant has previously been reported, but this mutant is not a complete loss-of-function mutant. In the present study, we knocked out the *slc6a3* gene using CRISRP/Cas9 technology and obtained a zebrafish mutant with complete loss of function of *slc6a3*. Behavioral assessments and quantitative reverse transcription (qRT)-PCR revealed musculature injury, decreased activity, and decreased learning and memory ability in the *slc6a3* mutant. This study provides a new theoretical basis for understanding the function of *slc6a3* and provides a new model organism for studying the molecular pathology underscoring dopamine metabolism-related diseases. It also provides a suitable model for high-throughput screening of small-molecule drugs targeting *slc6a3*.

Introduction

DA is a neurotransmitter that transmits information between neurons and regulates exercise, attention, learning, and emotional reactions[1-4]. Disorders of dopamine metabolism can lead to many physiological changes including attention deficit hyperactivity disorder (ADHD), learning defects, and depression[5, 6]. Recent studies have reported that disorders of dopamine metabolism are also associated with Parkinson's disease[7]. The main treatment for clinical Parkinson's disease is the drug L-dopa, which stimulates dopamine production[8, 9]. In addition, dopamine is associated with schizophrenia, but its role in this disorder is not fully understood[10]. Further, addictive substances such as cocaine affect dopamine secretion, which can promote the development of addiction[11].

The metabolism of dopamine includes the synthesis, release, re-absorption, and degradation of dopamine[12]. Presynaptic neurotransmitter re-uptake is mediated by the transmembrane dopamine transporter (DAT) and is the primary mechanism for terminating synaptic transmission[13]. The DAT is encoded by the *slc6a3* gene and is a sodium and chloride ion-dependent transmembrane neurotransmitter transporter[14, 15]. Since the time of dopamine activity in the synaptic cleft determines dopamine transmission, *slc6a3* is critical for the maintenance of dopamine homeostasis and controlling the duration of dopamine signaling.

Clinical research has indicated that hereditary and pharmacological changes in *slc6a3* affect human health. Polymorphisms of *slc6a3* (rs28363170, rs393795) are associated with levodopa-induced dyskinesias in Parkinson's disease during treatment of Parkinson's disease[16, 17]. Moreover, polymorphisms of *slc6a3* are associated with extreme behavioral characteristics and diseases such as anger-type personality disorder, destructive behavior disorder, overeating, attention deficit, hyperactivity,

and alcohol dependence[17-20]. In autism patients, deletion of a single amino of the *slc6a3* gene was identified to alter transmembrane structural conformation of the protein[21]. Tissues in *slc6a3* mutant mice exhibit a substantial decrease in DA content and excitatory motor activity[22]. The activity of *slc6a3* is low in patients with general anxiety disorder[23]. In addition, *slc6a3* is also a target of cocaine and alcohol[11]. Drug and alcohol abuse can reduce the expression of this gene, resulting in abnormal changes in behavior and mood.

Zebrafish are excellent model organisms that have been used successfully in the field of biomedical science for many years. The *slc6a3* zebrafish knockout mutant was successfully established in 2008 using zinc finger nucleases made by Oligomerized Pool ENgineering (OPEN) technology[24]. Subsequent studies have revealed that zebrafish *slc6a3* mutants exhibit anxiety-like behavior[25]. As *slc6a3* is mainly expressed in dopaminergic neurons, *slc6a3*:GFP has been established[26], and green fluorescence is mainly observed in ventral diencephalon (vDC) clusters, amacrine cells in the retina, olfactory bulb, pretectum, and caudal hypothalamus, similar to the results of *in situ* hybridization experiments[27]. The dopaminergic system of zebrafish is highly conserved compared to that of mice and humans; thus, dopamine-related studies are abundant in zebrafish. Zebrafish therefore serve as a good model to study DAT-related disorders and relevant mechanisms.

In this study, we first constructed an *slc6a3* mutant using CRISRP/Cas9 technology. We then used behavioral and gene expression assays to investigate the musculature, behavior, learning, and memory in the *slc6a3* mutant.

Materials And Methods

Zebrafish cultivation and egg production

Adult AB strain zebrafish were raised in a recirculating water system under a 14-/10-h light/dark cycle at 28°C and fed three times per day. To produce embryos, male and female zebrafish were paired in the evening; spawning occurred the next day within 1 h of the lights being switched on. The embryos were placed in 10-cm Petri dishes containing egg water with methylene blue (0.3 ppm) and raised in a light-controlled (14-/10-h light/dark) incubator at 28°C. At 6 days post-fertilization (dpf), samples were collected for quantitative reverse transcription (qRT)-PCR analysis.

Generation of *slc6a3* mutant zebrafish

slc6a3 mutants were generated using CRISPR-Cas9 technology. We designed the target site at the first exon and synthesized gRNA *in vitro*. gRNA (50 pg) and Cas9-capRNA (300 pg) were co-injected into zebrafish embryos at the one-cell stage. The fragment containing the mutation was PCR-amplified with primers P1 and P2 (Table 1), and confirmed by DNA sequencing. Two types of base deletion existed; both resulted in a premature stop codon leading to a truncated protein of only 39 or 73 amino acids (aa).

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was extracted from more than 30 larvae with TRIzol® reagent (Invitrogen) and reverse transcribed into cDNAs using SuperScript™ III Reverse Transcriptase (Invitrogen). qRT-PCR was performed in an ABI StepOnePlus™ instrument (7500) using the TB Green® system (TaKaRa) with thermal cycling through 40 cycles at 95°C for 10 sec and 60°C for 30 sec. Each qRT-PCR analysis was performed in triplicate on three independent biological samples. The housekeeping gene *β-actin* was amplified as a control. All results were standardized to the expression level of *β-actin*. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Calculations were performed in Microsoft Excel. qRT-PCR primers for exon boundaries were designed using Primer Express 3.0 software and checked for self-annealing, heterodimerization, and hairpin structure formation with Oligo Analyzer 3.1. Primer sequences are listed in Table 1.

Touch-evoked escape response

Wild-type (WT) and *slc6a3*-/- embryos were raised in the same standard conditions described above. At 48 and 96 hours post-fertilization (hpf), mechanical stimulation was performed using an insect pin attached to a micromanipulator. Light touches were applied to the side of the embryo at somites 14–16, at the level of the caudal part of the yolk tube. Each trial consisted of a recording of the spontaneous activity for 2 min, followed by three to five stimulations, each separated by at least 1 s. Responses of larvae to pin stimulation were video captured.

Behavioral analysis

Embryos were raised to 72 hpf in the same standard conditions described above. All analyses were performed during the light portion of the 14 h light:10 h dark cycle at the same time of day. The larvae were transferred to clear 96-well plates with lids (one individual per well) and allowed to acclimate overnight. Analyses were performed in an isolated room maintained at 28.5°C. The movement of 4 dpf larvae was video captured and quantified using an infrared camera setup and tracking extension of the software system (Noldus Information Technology). In all behavioral protocols, animal color was set to black, and background-subtracted detection threshold was set to 20 sec. This value represented a greyscale pixel intensity value; any pixels darker than this threshold in the video were detected as the animal. The integration period (bin time) for movement *slc6a3* mutant was set to 10 sec. *slc6a3* mutant were processed and analyzed using Excel, and Fast Monitor (Noldus Information Technology).

Statistical analyses

Data are presented as means ± SD. Statistical differences between groups were determined using Student's t-test. All experiments were repeated at least three times. A p-value <0.05 was considered statistically significant.

Results

Construction of *slc6a3* null mutant zebrafish

The CRISPR Cas9 system was used to construct *slc6a3* mutant zebrafish. The zebrafish *slc6a3* gene includes 17 exons encoding 629 amino acids. The transcription initiation site ATG is located in the first exon (Fig. 1A). We designed the gRNA locus behind ATG, then synthesized gRNA and Cas9 Capped mRNA *in vitro*, which were co-injected into zebrafish single-cell fertilized eggs. We then screened heritable zebrafish mutants in adulthood. The results revealed that two line *slc6a3* mutants with four and five base deletions were successfully screened (Fig. 1B). Bioinformatics analysis of the mutant sequences revealed that the predicted *slc6a3* mutant sequences encoded 39 and 73 truncated polypeptides, respectively (Fig. 1C). qRT-PCR was used to quantify *slc6a3* mRNA, revealing that the expression of the *slc6a3* gene was significantly decreased in the *slc6a3* mutant (Fig. 2A). DA content was detected using ELISA assay. DA content in the *slc6a3* mutant was significantly decreased (Fig. 2B).

Weak touch-evoked escape response in *slc6a3* mutants

Touch-evoked escape response assays are a method to assess muscle performance and function in zebrafish[28]. To explore the role of *slc6a3* in muscle function, we performed touch-evoked escape response assays in WT and *slc6a3* mutant zebrafish (Fig. 3A-H). At 48 hpf, WT zebrafish rapidly swam out of the field of view after tip stimulation. In contrast, most *slc6a3* mutant larvae remained in the field of view despite swimming after stimulation (Fig. 3A-B and E-F). Similar results was observed in 96 hpf larvae (Fig. 3C-D and G-H).

Decreased locomotor activity in *slc6a3* mutants

To investigate the effects of *slc6a3* mutations on autonomic activity, behavioral experiments were performed to examine the activity of *slc6a3* mutants. At 10 mins, the swimming distance of *slc6a3* mutants was significantly lower than that of WTs (Fig. 4A). Analysis of the cumulative swimming distance revealed that the difference between *slc6a3* mutants and WTs increased over time (Fig. 4B). Statistical analysis of total swimming distance revealed that the total swimming distance was significantly lower in *slc6a3* mutants than in WTs (Fig. 4C). The speed of swimming is also an important indicator of the amount of activity. Swimming speed of *slc6a3* mutants was significantly lower than that of WTs (Fig. 4D). These results indicated that the *slc6a3* mutation reduced zebrafish locomotor activity.

Deficits in learning and memory in *slc6a3* mutants

To further investigate the role of *slc6a3* in learning and memory, a single multiple light experiment was used to detect learning and memory ability in *slc6a3* mutants. When the number of repeated light-dark cycles increased, the swimming speed of fish larvae began to decrease, and the speed of *slc6a3* mutants was always lower than that of WT fish (Fig. 5A). In addition, the swimming speed of WT fish remained stable (platform) after a period of time, while the swimming speed of *slc6a3* mutants continued to decrease (Fig. 5A). Data analysis of behavior indicated that *slc6a3* mutant zebrafish took longer to reach

the platform than did WT zebrafish (Fig. 5B). These results implied that learning and memory in *slc6a3* mutants were impaired.

Alteration in expression patterns of learning and memory-related genes in *slc6a3* mutants

To further investigate the reasons underlying learning and memory abnormalities in *slc6a3* mutants, qRT-PCR was used to detect the expression of learning and memory-related genes in WT and *slc6a3* mutants. There was no significant difference in the expression of *creb1a* and *bdnf* in *slc6a3* mutants when compared with WT. *fosl2* and *hat1* were up-regulated in *slc6a3* mutants when compared with WT.

Discussion

Numerous studies have used zebrafish as a model to study DA or drug addiction[25, 29-31]. These investigations have been facilitated by the evolution of the relatively primitive DA metabolism and response system in zebrafish. In this study, zebrafish *slc6a3*-deficient mutants were successfully established using CRISP/Cas9 technology. We observed that *slc6a3* mutants exhibited weaker responses to stimulation, decreased motor activity, and impaired learning and memory.

Src6a3 belongs to the *SLC6* gene family, which comprises a variety of neurotransmitter transporters[14]. The protein encoded by *slc6a3* has a membrane structure composed of 12 transmembrane helices (TMs), an intracellular N-terminus, a C-terminus, and an extracellular glycosylation loop located between TM3 and TM4[14]. Zebrafish *slc6a3* mutants have been reported in previous studies, but the mutation site is located in the 12th exon[24, 25]. This mutant protein still contains TM1-TM10, N-terminal, and glycosyl rings. Further, the C-terminus of *slc6a3*-encoded protein has multiple response sites, such as PCK and CamKII regulatory sites[32]. Thus, this mutant may not be a complete loss-of-function mutant. Subsequent studies have found that this mutant exhibits an anxious phenotype. In this study, we knocked out the *slc6a3* gene using a specific CRISPR system to obtain two mutants. The gRNA design site in our mutant was adjacent to the transcription start site. Thus, the mutant we obtained completely lacked TMs, glycosylation loop, and C-terminus, and was a null mutant with complete loss of function. This provides a suitable model organism for studying DA metabolism, *slc6a3*-related functions, and drug screening.

Touch-evoked escape response assays enable investigation of neuromuscular disorders in zebrafish in response to tactile stimuli[33]. After tactile stimuli presentation, *slc6a3* mutants demonstrated significantly slower responses and did not exhibit escape responses. This indicated that the muscle tissue of zebrafish *slc6a3* mutants was negatively affected. Autonomous swimming experiments revealed that the activity of *slc6a3* mutants was significantly reduced. Whether the decrease in behavior was due to the negative effects of muscles remains unknown. Tactile responses of zebrafish are regulated by spinal nerve circuits, and Mauthner neurons are involved in escape responses to tactile stimuli in zebrafish[34]. The presence of DA receptors, expression of *slc6a3* in the spinal cord, and whether behavioral abnormalities are due to the absence of *slc6a3* in the spinal cord requires further investigation.

Zebrafish are excellent model organisms for studying learning and memory[35]. Many experimental techniques and methods are based on the behavior of adult fish such as the T-maze, but there are limited experiments based on fish larvae[36, 37]. Previous studies have revealed that zebrafish larvae can produce memories of multiple light and dark stimuli[38]. In this study, we presented 3 min dark/1 min light and examined learning and memory based on the speed and time taken by zebrafish to reach the platform. Time to reach the platform was longer for *slc6a3* mutants than for WT fish, indicating that the loss of *slc6a3* affected learning and memory of zebrafish larvae. In mammals, DA regulates hippocampal-dependent learning. In previous mouse *slc6a3* mutants, it was reported that the decrease in *slc6a3* expression in mice impaired learning in a running experiment[39]. In patients with Parkinson's disease, the expression level of *slc6a3* is significantly reduced and is accompanied by learning and memory impairments[40].

The process of learning and memory involves many neurochemical systems and mainly occurs at the synapse. In this study, gene expression of learning and memory-related genes was examined. The expression of *creb1a* and *bdnf* was not significantly altered in *slc6a3* mutants. The expression of *fosl2* and *hat1* was significantly elevated in *slc6a3* mutants. *Creb1a* encodes an effector molecule of the cAMP signaling pathway and is a classical learning and memory-related gene[41]. *Bdnf* encodes a neurotrophic factor in the brain that regulates the differentiation and growth of neurons[42]. No changes in expression of these two genes were observed, suggesting that *slc6a3* does not affect these two genes in the context of learning and memory. *Fosl2* is an early expression gene related to learning and memory, and *hat1* encodes histone acetyltransferase 1[43, 44]. Expression of these genes in *slc6a3* mutants was elevated, suggesting that the deletion of *slc6a3* or disorders of DA signaling may affect functional changes in learning and memory via these genes, but the intrinsic mechanisms require further research.

To conclude, in this study, zebrafish *slc6a3* mutants were successfully established by CRISP/Cas9 technology. Using behavior and qRT-PCR experiments, we demonstrate musculature injury, decreased activity, and decreased learning and memory in *slc6a3* mutants. This study provides a new model organism for researching the molecular pathology underscoring DA metabolism-related diseases and for screening high-throughput small-molecule drugs targeting *slc6a3*.

Declarations

Acknowledgments

Availability of data and material

Not applicable

Compliance with ethical standards

Conflict of interest

All authors have no conflicts of interest.

Ethical approval

All procedures were approved by the Nanjing Medical University Animal Care and Use Committee and were performed in accordance with the governmental regulations of China.

Authors' contributions

Xiaojie Lu and Yu Jiang design the whole experiments, Yu Jiang, Nan Gen and Peisong Wang performed all the experiments, Xiaojie Lu and Yu Jiang prepare the manuscript.

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Tables

Table 1 The primer sequence

| Genes	Sequence	
slc6a3-P3	TAATACGACTCACTATAGGCCGCCGGGCCCCG	KO
	AGAGGGTTTTAGAGCTAGAAATAGC	
slc6a3	GCTTCTGTGACAAACTGGCG	qRT-PCR
	CAAACCACCAGCCAGTGATG	
creb1a	GAGTCAGTGGACAGTGTGAC	qRT-PCR
	GTTATGGCTGGAGTGGAGTC	
bdnf	TGCAAGTGTTGGTCTTGGAG	qRT-PCR
	CAGCTCTCATGCAACTGAAG	
fosl2	GTACCAGGATTACACCGGGA	qRT-PCR
	CAGGCATGTCTATTCGGTAC	
hat1	GATCCAGCTTTACCAGAGCG	qRT-PCR
	CCAGCAGGAATGATCTCTCG	
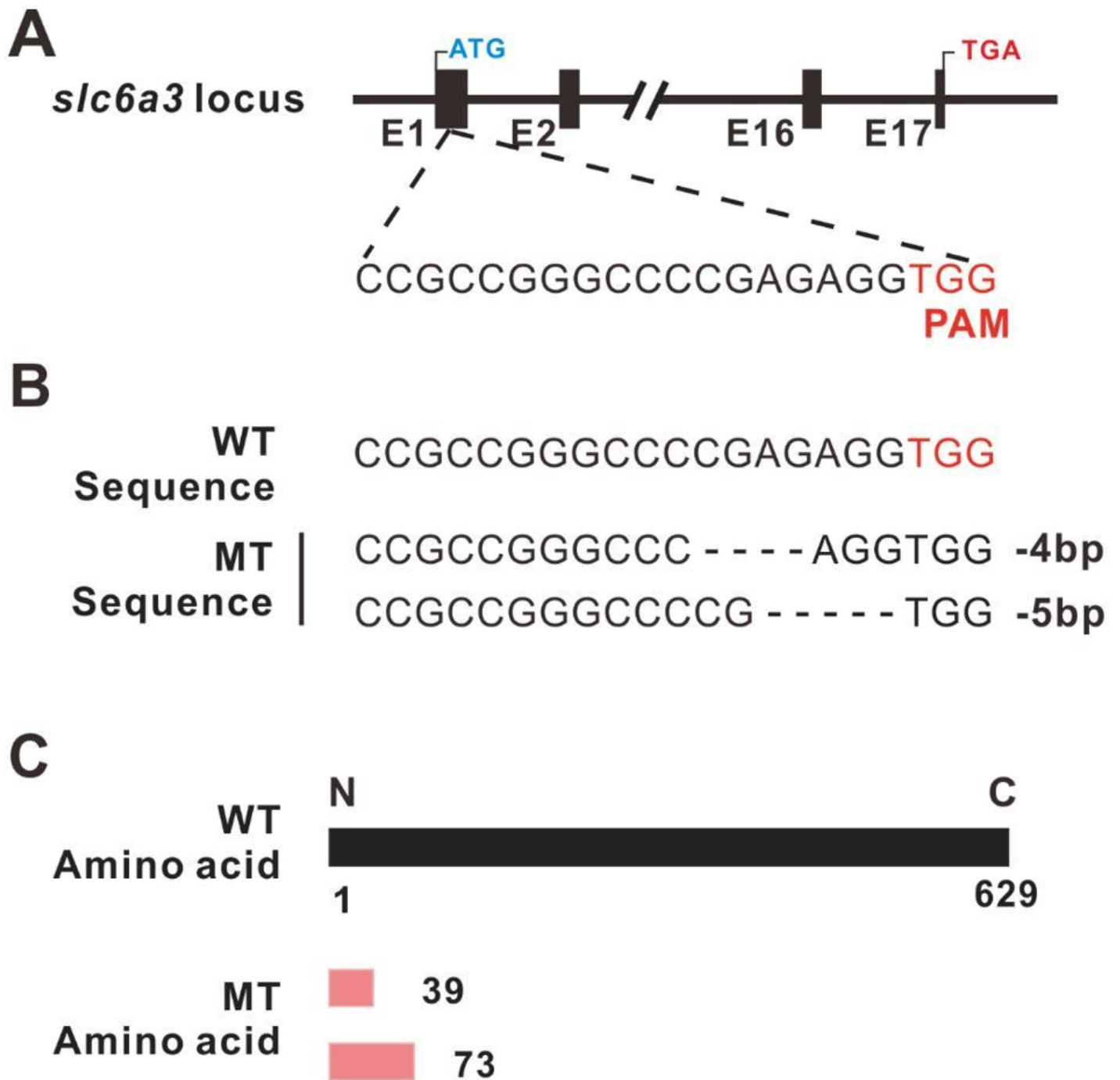
Figures

Figure 1

Generation of zebrafish slc6a3 mutants. Schematic showing the construction of slc6a3 mutants using wangtuoyi CRISPR-Cas9 technology. (A) The gRNA site is located at the first exon in the slc6a3 gene locus. (B) The sequencing results revealed two mutations, which were missing four and five base pairs at the target site, respectively. (C) The truncated predicted protein length of slc6a3 mutants
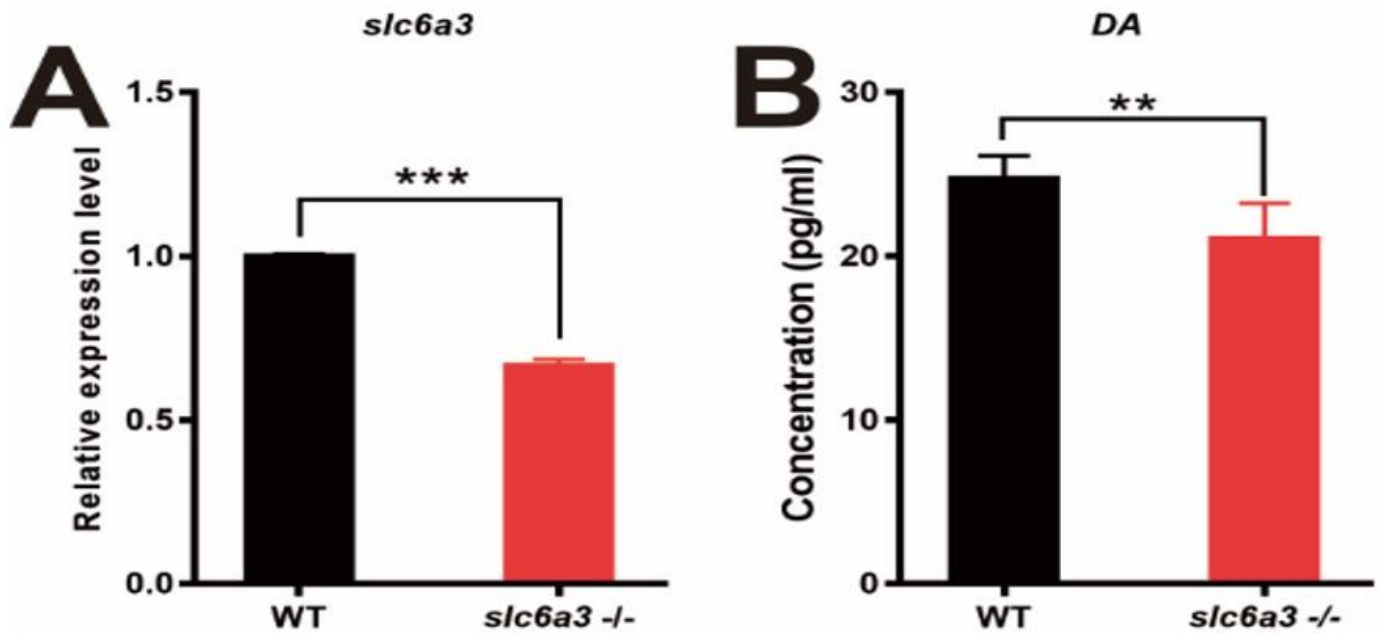
Figure 2

slc6a3 expression and dopamine (DA) content was decreased in mutants. qRT-PCR and ELISH analysis to investigate the slc6a3 mRNA and DA levels in wild-type (WT) and slc6a3 mutants. (A) slc6a3 expression was significantly reduced in slc6a3 mutants. (B) DA content was decreased in slc6a3 mutants. Results are presented as mean ± SD for independently performed triplicate experiments. Statistical significance was assessed via Student's t-test (***$P<0.001$)
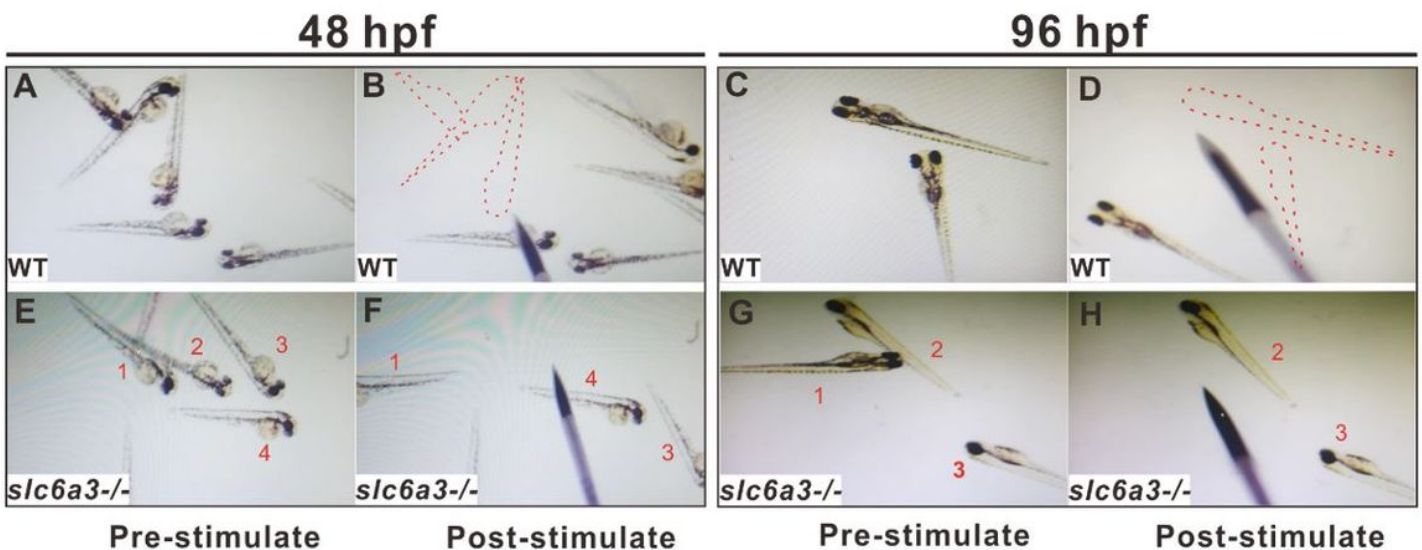

Figure 3

The ability to respond to stimuli was decreased in slc6a3 mutants. Embryos obtained from wild-type (WT) and slc6a3 mutant fish were studied using touch-evoked escape response assays. (A, B) At 48 hpf, WT fish were able to rapidly escape from view following disturbance (a'). (E, F) Most slc6a3 mutant fish

remained within view of the camera after their tails were touched (b'). (C, D) At 96 hpf, (c,c') WT fish swam rapidly but (G, H) mutants remained stationary (d,d'). Similar results were observed in three independent experiments. More information is provided in the supplementary video
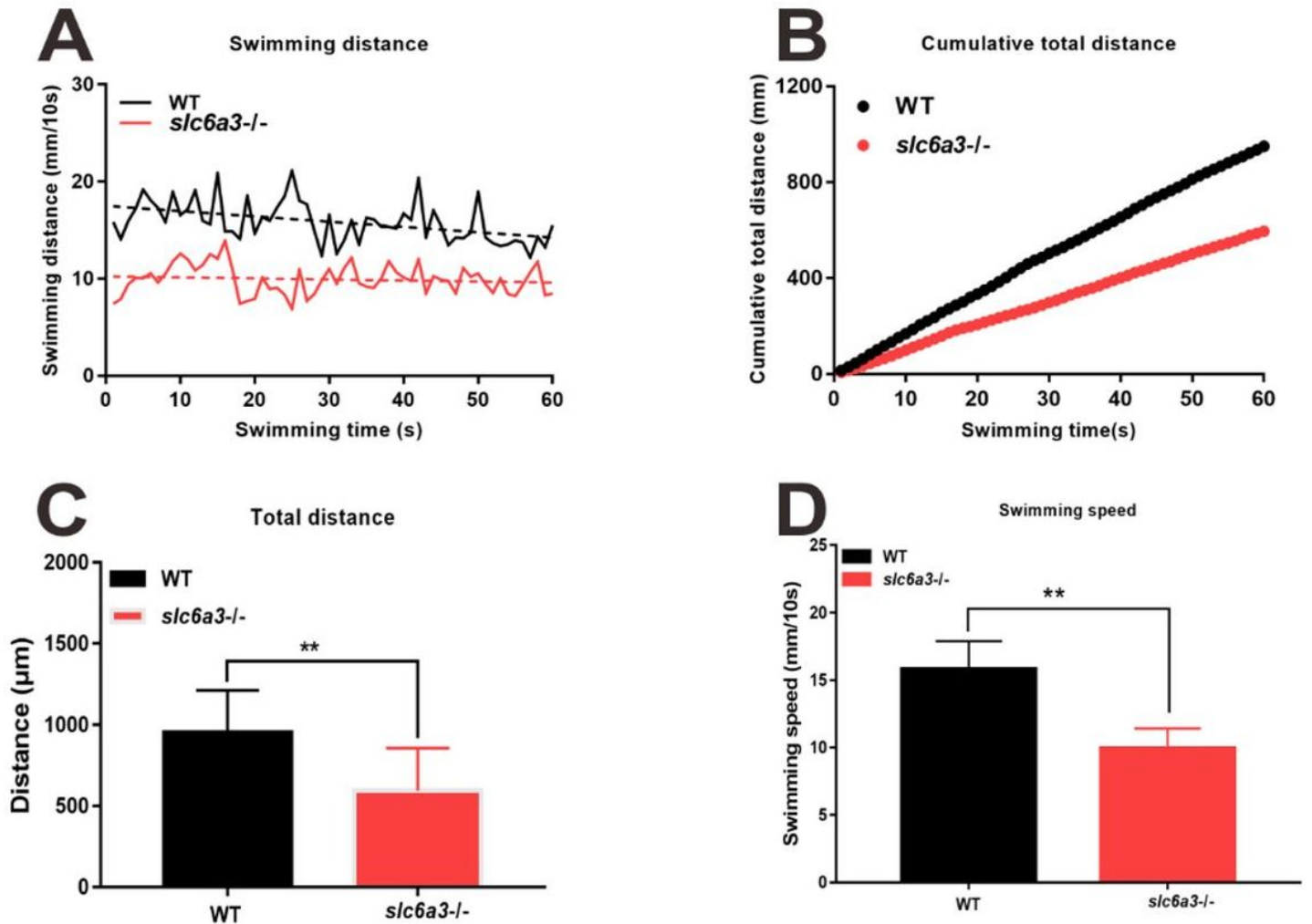

Figure 4

Locomotor activity was decreased in slc6a3 mutants. Autonomous swimming behavior in slc6a3 mutants and wild-type (WT) zebrafish larvae. (A) The swimming distance curve; solid lines represent the real-time distance, and dotted lines represent the mean distance. (B) Cumulative locomotor distance of WT and slc6a3 mutants. (C) Total locomotor distance of WT and slc6a3 mutants. (D) Swimming speed of WT and slc6a3 mutants. Results are presented as mean ± SD for independently performed triplicate experiments (n=24/group). Statistical significance was assessed via Student's t-test (**P < 0.01)
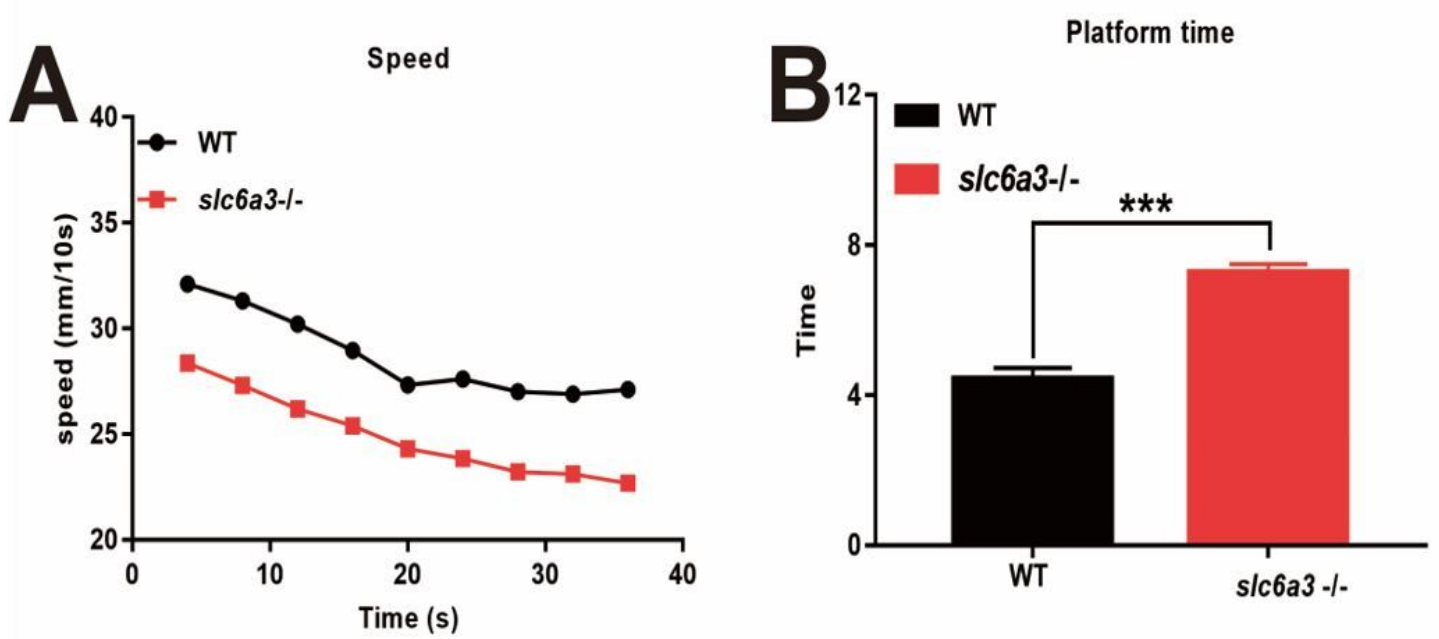
Figure 5

Learning and memory were deficient in slc6a3 mutants. The light-dark response assay was used to examine learning and memory in zebrafish. (A) The average speed in wild-type (WT) and slc6a3 mutants. (B) Time to reach the platform stage. Results are presented as mean ± SD for independently performed triplicate experiments (n=24/group). Statistical significance was assessed via Student's t-test (***P < 0.001)
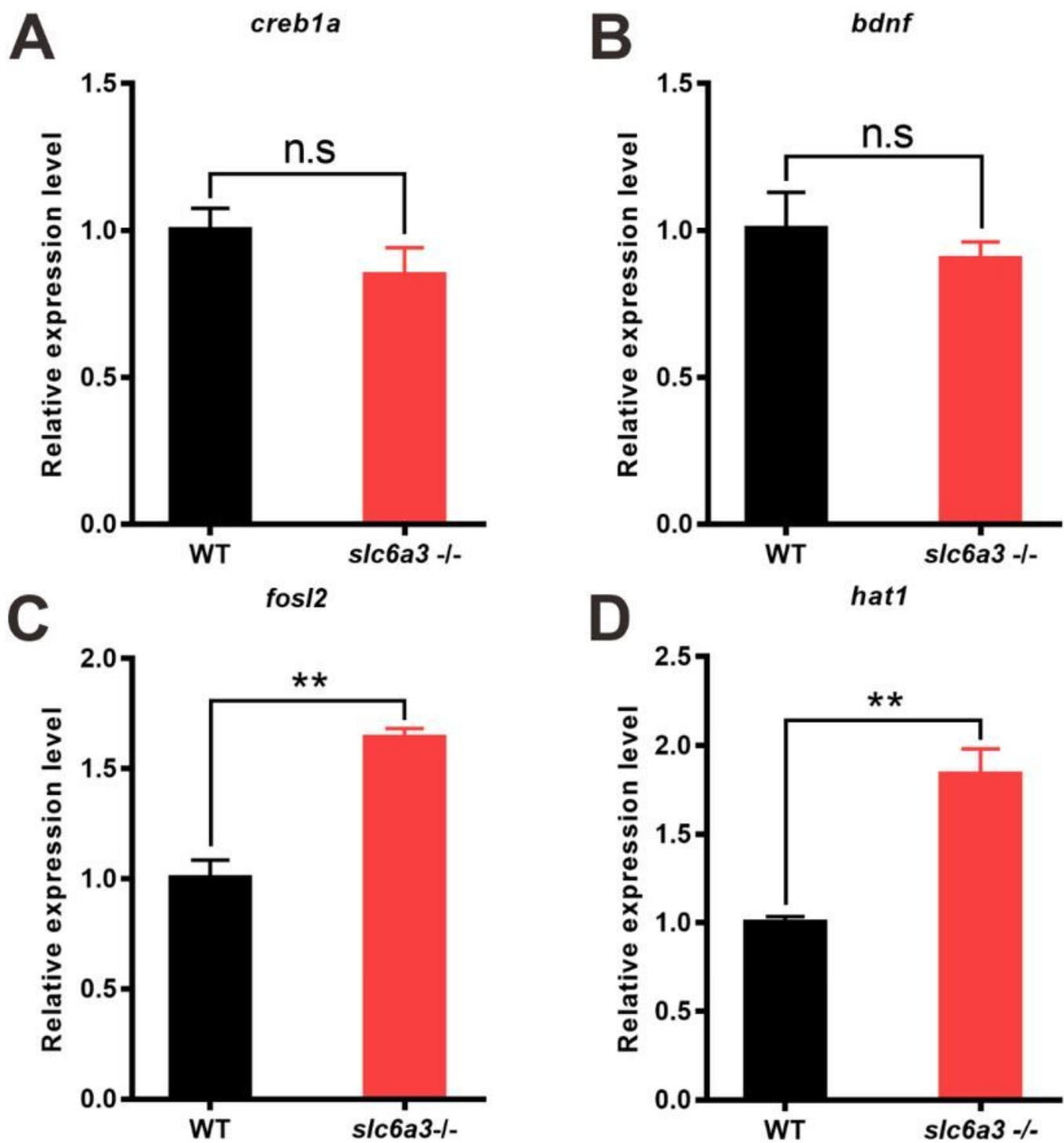
Figure 6

Alteration in expression patterns of learning and memory-related genes in slc6a3 mutants. qRT-PCR analysis was used to investigate mRNA levels of learning/memory-related genes in wild-type (WT) and slc6a3 mutants. The expression patterns of creb1a (A), bdnf (B), fosl2 (C), and hat1 (D) in WT and slc6a3 mutants. Results are presented as mean ± SD for independently performed triplicate experiments. Statistical significance was assessed via Student's t-test (n.s, not significant; **P < 0.01; ***P<0.001)