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Expression and functions of ASIC1 in the zebrafish retina

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ABSTRACT

It has been demonstrated that acid sensing ionic channels (ASICs) are present in the central and peripheral nervous system of mammals, including the retina. However, it remains unclear whether the zebrafish retina also expresses ASICs. In the present study, the expression and distribution of *zasic1* were examined in the retina of zebrafish. Both *zasic1* mRNA and protein expressions were detected in the adult zebrafish retina. A wide distribution of ASIC1 in zebrafish retina was confirmed using whole mount in situ hybridization and immunohistochemistry study. Acidosis-induced currents in the isolated retinal ganglion cells (RGCs) were also recorded using whole cell patch clamping. Moreover, blockade of ASICs channel significantly reduced the locomotion of larval zebrafish in response to light exposure. In sum, our data demonstrate the presence of ASIC1 and its possible functional relevance in the retina of zebrafish.

1. Introduction

Acid sensing ionic channels (ASICs), activated by extracellular H^+, are one type of ligand-gated cation channels that widely express in the mammalian central and peripheral nerve systems [1,2]. ASICs belong to the degenerin/epithelial sodium (Na^+) channel (DEG/ENaC) superfamily, and the common feature of this superfamily is a high permeability of Na^+ that could be blocked by amiloride [1,3]. In mammals, six distinct ASICs subunits (ASIC1a, ASIC1b, 2a, ASIC2b, ASIC3 and ASIC4), encoded by four different genes, build up the functional ion channels in the form of dimers or heteropolymers [4]. In zebrafish, six ASICs subunits (*zasic1.1, zasic1.2, zasic1.3, zasic2, zasic4.1, and zasic4.2*), encoded by three different genes have been detected [5]. Strikingly, these distinct channels exhibit different ammeter types, channel nature, ion selective and distribution characteristics [6].

Several ion channels including calcium channels, potassium channel, chloride ion channel and ASICs express and regulate the visual function in the mammalian retina. [7–9]. Acid is produced and regulated by muller cells, pigment epithelium and photoreceptors in retina [10]. The activity and phototransduction in retina are influenced by ASICs [11]. The presence of ASIC1a, ASIC2 and ASIC3 in mammalian retina has been documented [12,11,13]. Among all ASICs, ASIC1 is shown to be the most abundant. It is widely expressed in the retina and involved in retinal activity, particularly in cone function [14].

Yet, the role of ASICs in retina is not fully understood. Zebrafish has a translucent embryo and larva, allowing easy analysis of gene transcription and translation profiles. Also, zebrafish develops rapidly, which means the experiment period can be greatly shorten. More importantly, the structure and functions of zebrafish retina are remarkably similar to those of humans, making the visual system of zebrafish as a powerful research tool [15]. In addition, the genome organization, the regulatory pathways controlling the signal transduction, and the retina development remain highly conserved between zebrafish and humans [16]. Thus, zebrafish provides an excellent animal model to study retina functions. However, the expression of ASICs in zebrafish retina remains unknown although its distribution was reported in mammalian retina. Herein, the main goal of this study is to examine the distribution and function of ASIC1 in zebrafish retina.

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2. Materials and methods

2.1. Reagents and antibodies

Amiloride and other chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). The sources for primary antibodies were listed as follows: anti-ASIC1 (Dallas, Santa Cruz, CA, USA) and anti-GAPDH (CMC-TAG, AT0002, San Diego, USA).

2.2. Zebrafish handling and preparation

Wild-type zebrafish (Danio rerio) were raised and maintained in a recirculating water system at 28 °C, pH 7.5 under oxygen saturation, and exposed to a photo cycle of 14 h light/10 h dark. All investigations were conducted according to the guide principles for the use and care of laboratory animals of Soochow University. The eye and brain tissues from 12 adult zebrafish (6 months old) were isolated, cleaned in cold saline solution, and processed for protein or mRNA analysis ($n = 6$ each).

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA (1 μg) of each sample was reverse-transcribed into cDNA and amplified using PCR Master Mix kit (Fermentas, Vilnius, Lithuania) with the primers (Genscript, Nanjing, China) as follows: *zasic1* (D. rerio), 5′-TGA AGT GCT CCA GCT TTC CC-3′ (forward), 5′-TCA TGC CTG GAG TTG AGC AG-3′ (reverse); *β-actin* (D. rerio), 5′-CAC AGA TCA TGT TCG AGA CC-3′ (forward), 5′-GGT CAG GAT CTT CAT CAG GT-3′ (reverse). PCR products were separated in 2% agarose gels. The optical band densities were analyzed with Image J software (National Institute of Health, Bethesda, MD, USA).

2.4. Whole mount in situ hybridization

Probes for *zasic1* genes were cloned by PCR amplification using the primers designed according to their EST clone sequences. The *zasic1* riboprobes were generated with a DIG RNA Labeling Kit (Roche, Germany). For in situ hybridization, larvae were fixed with 4% paraformaldehyde and stored in methanol at −20 °C. Rehydrated embryos were prehybridized with 50% formamide, 5 × saline sodium citrate(SSC), 5 mM EDTA, 0.1% Chaps, 50 μg/ml heparin, and 1 mg/ml torula RNA (Sigma, St. Louis, MO) for 3 h at 65 °C followed by hybridization overnight with DIG-labeled RNA. DIG-labeled sense RNA was used as a control. After washing, embryos were incubated with 2% blocking reagent (Roche Diagnostics GmbH), 5% lamb serum, 0.1 M maleic acid, pH 7.5, 150 mM NaCl, followed by incubation with anti-DIG-Alkaline phosphatase (AP, Roche Diagnostics GmbH), 1:5000 in blocking solution. After extensive washing, samples are visualized by incubating with NBT/BCIP AP substrate solution (Roche Diagnostics GmbH) in 0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 5 mM levamisol. Photographs were taken using a fluorescent dissection microscope, a Leica MZFL III (Leica, Wien, Austria).

2.5. Western blotting

Tissue homogenates were prepared using lysis buffer (150 mM NaCl, 25 mM Tris, 5 mM EDTA, 1% Nonidet P-40, pH7.5) with protease inhibitor cocktail tablets (Roche Diagnostics, Penzberg, Germany). The lysates were sonicated for 16 s and centrifuged at 13,400*g* for 15 min at 4 °C. The protein concentration was determined by BCA assay. Next, equal amounts of protein lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes. After that, membranes were blocked in 5% (w/v) non-fat dry milk powder in 0.1% Tris buffered saline/Tween 20 (TBST) for 1 h, and incubated with goat anti-ASIC1 (1:500) at 4 °C overnight. Subsequently, membranes were washed in TBST and incubated with HRP-conjugated secondary antibodies for 1 h. Membranes were finally visualized using ECL chemiluminescence (Thermo, West Chester, PA, USA). The band densities were analyzed with Image J software (National Institute of Health, USA).

2.6. Immunofluorescence microscopy

A total of twelve days 5 post-fertilization (5 dpf) zebrafish larvae were used to perform the ASIC1 immunostaining. Larvae were fixed with 4% paraformaldehyde, placed in 30% sucrose in PBS overnight, transferred to Tissue-Tek® O.C.T.™ and then stored at −26 °C. Sections (14 μm thick) were prepared using a cryostat (Leica CM1850). Staining was performed as follows: slides were washed with 0.1% phosphate buffered saline (PBS)/Tween 20 for 10 min × 3 times. Afterward, sections were blocked in 3% bovine serum albumin (BSA) in PBS for at least 30 min and incubated with goat anti-ASIC1 (1:200) overnight at 4 °C. Next, sections were washed with PBS and incubated with Alexa Fluor® 488 rabbit anti-goat IgG (1:2000, Invitrogen A-11055) for two hours. After brief washing with PBS, slides were cover-slipped using mounting solution with DAPI (Vector Lab, H1200). Images were taken under the fluorescent microscope (Zeiss, AXIO Scope A1, Goettingen, Germany).

2.7. Dissociation of retinal ganglion cells (RGCs) and ASIC current recording

To isolate RGCs, 6-month-old zebrafish was anesthetized with 1% 3-aminobenzoic acid and sacrificed by decapitation. Isolated retinas were transferred into enzyme solutions (papain 20 U/ml, L-cysteine 1 mg/ml, EDTA 0.1 mM, NaCl 140 mM, KCl 5 mM, NaH_2PO_4 0.3 mM, Na_2HPO_4 0.3 mM, glucose 10 mM, pH 7.4) and mechanically dissociated by gently triturating with a fire-polished Pasteur pipette. The resulting single cell suspension was plated in culture dishes containing L-15 medium (Gibco, Grand Island, NY). Cells were used for experimentation after culture for 12–60 h.

Whole cell patch-clamp techniques were performed in a voltage-clamp mode, and membrane currents were recorded with HEKA EPC-10 patch-clamp amplifier (HEKA) connected to a compatible computer via D/A and A/D converter as described previously [5]. Pulse Plus Pulsefit software 8.6 (HEKA) was used for data processing. Borosilicate glass pipettes (1.5 mm diameter) were pulled with P-97 microelectrode puller (Sutter Instruments Company, USA). The resistance of the pipettes ranged from 2 to 4 M when filled with pipette solution (140 mM KCl, 10 mM NaCl, 1 mM $MgCl_2{\cdot}6H_2O$, 5 mM EGTA, 2 mM MgATP, 10 mM HEPES, pH 7.4 with Tris-OH). After a whole cell configuration was established, the adjustment of capacitance compensation and series resistance compensation was done before recording. Cells were voltage clamped at −60 mV. A multibarrel perfusion system was used to achieve a rapid exchange of extracellular solutions (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2{\cdot}6H_2O$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4 or 6.0 with Tris-OH). All experiments were performed at room temperature (22–25 °C).

2.8. Locomotor activity measurement

5 post-fertilization (5 dpf) wild-type larvae were put into a 48-well square plate added with 300 μl system water. Larvae were exposed to a solution containing amiloride 50 μM or 100 μM at 0–5 dpf. Control groups received vehicle only. There were 8-empty

well in the middle of each group to prevent the interference between different groups. Then the plate was put into the zebrabox (ViewPoint Life Sciences, Montreal, France) to monitor the locomotor activity under the light/dark condition. The zebrabox contains a high sensitivity infrared camera (60 Frames/Sec) on the above of the plate and white light matrix in the bottom. To minimize any unintended disturbance, the larvae were first acclimated in the dark for 10 min. The light program was set as: 90 s 100% light immediately followed by 0% light for 90 s, then shifted to 100% light for 90 s and recycled. Generally, upon a switch from light to dark, locomotor activity of zebrafish larvae increased, which was substantially higher than the activity level in the initial dark period, and then declined gradually. Upon a switch from dark to light, the activity level plummeted and then increased slowly until another lighting cycle started. All the signals during the behavior test were processed using the software Zebralab3.10 (ViewPoint Life Sciences).

2.9. Data analysis

All Data were expressed as mean ± SEM. Statistical analysis was performed by SPSS Version 6.1 software (SPSS, Chicago, IL, USA) using Student's *t*-test or one-way analysis of variance followed by Tukey post hoc analysis where applicable. Differences were considered significant when P value <0.05.

3. Results

3.1. zasic1 mRNA transcription and protein expression in zebrafish eye

The mRNA transcription and protein expression of ASIC1 in zebrafish eyes were determined using RT-PCR and Western blotting. Adult zebrafish brain tissue was used as a positive control since the existence of ASIC1 in zebrafish brain was reported previously [5,17]. *zasic1* mRNA was detected in both brain and the whole eye of adult zebrafish (Fig. 1A). In Western blotting study, the specific antibody against ASIC1 recognized a band with an estimated molecular weight at about 60 kDa (Fig. 1B), consistent with the estimated molecular mass of zASIC proteins in zebrafish. Moreover, the band density in the eye was less prominent than that in the brain tissue.

3.2. ASIC1 is widely expressed in zebrafish retina

The presence and distribution of ASIC1 in zebrafish retina was verified using whole mount in situ hybridization on whole zebrafish larvae and immunostaining on retina slices. Zebrafish larvae, which are transparent, were chosen here to observe the structure of retina. In situ hybridization showed that *zasic1* transcript was detected in 5 dpf larval zebrafish (Fig. 2A). Surprisingly, the immunostaining study showed that the ASIC1 immunoreactivity was widely detected in the zebrafish retina (4–5 dpf, Fig. 2B), including the RGC layer, the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, and photoreceptor layer (Fig. 2B). The distribution pattern of ASIC1 in the zebrafish retina implies that ASIC1 may play an important role in normal retinal function.

3.3. Acidosis-induced current in RGCs

To further explore the functional role of ASICs in RGCs, we examined the acid-evoked currents in the isolated RGCs using whole cell patch clamp technique. A rapid drop of extracellular pH evoked a transient and rapidly inactivated inward current, and the currents induced by acid were pH-dependent (pH 7.4–5.5), as shown in Fig. 3A. Moreover, the currents could be blocked by amiloride (1, 10, 100 μM) in a concentration-dependent manner (Fig. 3B and C), indicating the expression of functional ASICs in the RGCs of adult zebrafish.

3.4. Blockage of ASICs with amiloride affects the light-dependent locomotion of larval zebrafish

Zebrafish larvae were exposed to different concentrations of amiloride (50 and 100 μM), and their locomotor activity was studied. Previous studies showed the locomotor activity of zebrafish larval was sensitive to light changes [18]. To explore the role of ASICs in retina, the locomotor activity of larval zebrafish (5 dpf) was studied in response to light/dark shifting cycles, in the presence or absence of ASICs blocker amiloride at 50 or 100 μM. Amiloride caused a significant locomotor deficit in light condition (Fig. 4A). The total activity during the light periods was reduced in the groups treated with 50 and 100 μM amiloride (Fig. 4B and C). But the amiloride failed to reduce the total activity in darkness (Fig. 4B and C), implying that the amiloride effect may not be an unspecific inhibition of zebrafish moving ability. This indicates that ASIC1 blockade may reduce the sensitivity of zebrafish to light.

4. Discussion

Retina is the light-sensing organ where visual information is transduced to the central nervous system through visual pathway. Starting from the inside layer, the zebrafish retina contains ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) [19]. In terms of the composition, zebrafish retina is made up of seven different types of cells: one is Müller cells and the other six are neurons, including retinal ganglion cell (RGC), amacrine cell (AC), horizontal cell (HC), bipolar cell (BC), as well as rod and cone cells [19]. Recent studies indicated ASICs, particularly ASIC1, affected the activity of the retina in mammals [20]. However, no data exists to support the expression of ASIC1 in the retina of fishes. In this study, we provide the evidence for the first time that ASIC1 expresses and functions in the zebrafish retina.

Subtle changes in pH may modulate the phototransduction and activity of retina [21]. RGCs are also influenced by transient interstitial acidification with rapid, constant stimulation under both darkness and light conditions [22]. ASICs, activated by protons, take part in the visual signals transduction [13]. In addition to

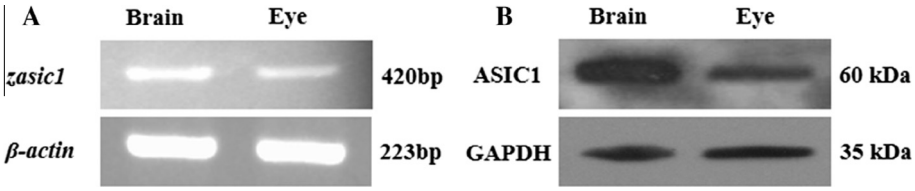

Fig. 1. mRNA and protein expression of ASIC1 gene and protein expression in zebrafish eye. (A) PCR analysis of *zasic1* and *β-actin* mRNA expression in the eye and brain of adult zebrafish. A 420 bp fragment of *zasic1* mRNA was detected. (B) Western blot detection of ASIC1 eye and brain homogenates from adult zebrafish. The anti-ASIC1 antibody used recognizes a protein band of about 60 kDa consistent with the complete ASIC1.

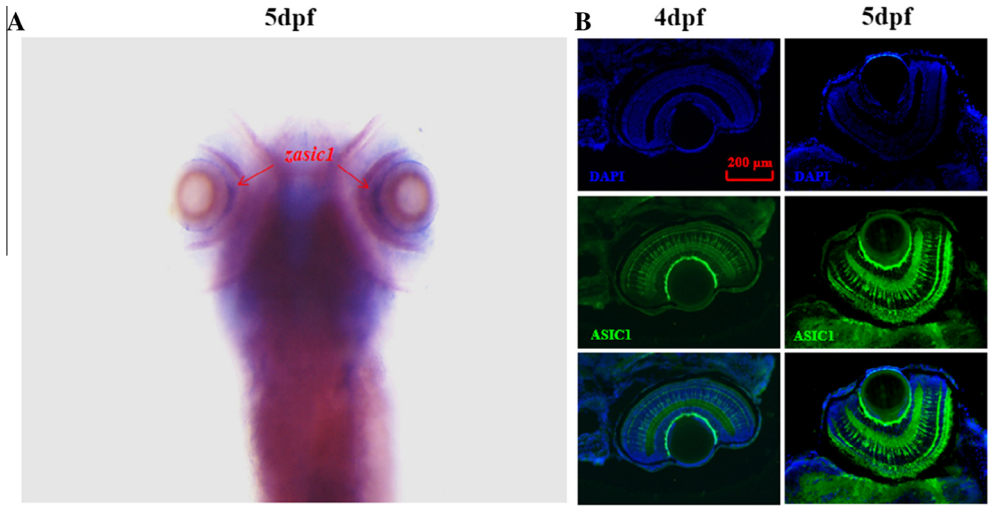

Fig. 2. Expression of ASIC1 in the zebrafish retinal. (A) Whole mount in situ hybridization of *zasic1* was done using larval zebrafish at 5 dpf. (B) Immunohistochemical analyses with the same antibodies showed positive ASIC1 immunoreactivity in the retinal at 4 dpf and 5 dpf. Immunoreactivity for ASIC1 was detected distributed in the RGC layer, the inner plexiform layer, the inner nuclear layer, the outer plexiform layer, and photosensory cell layer.

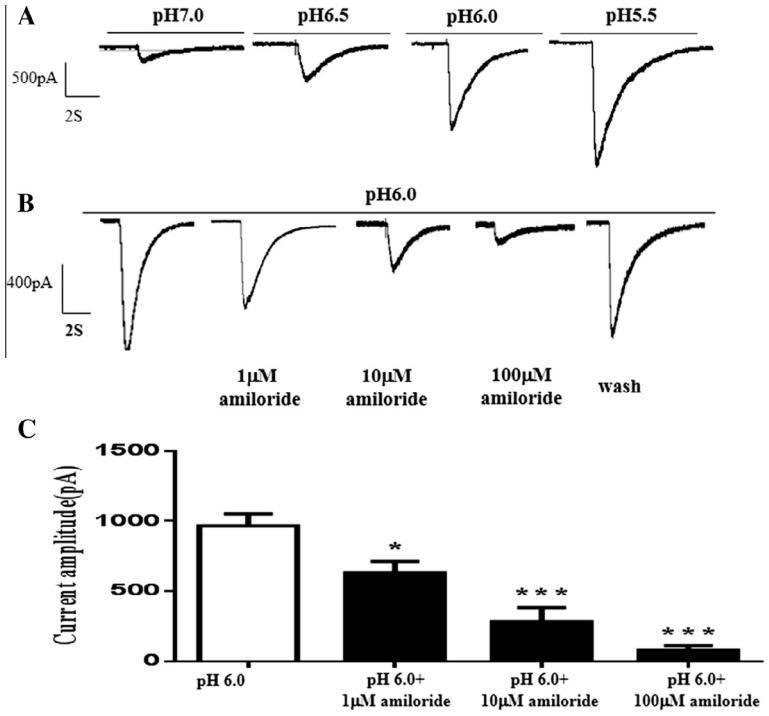

Fig. 3. Electrophysiological properties of acid-sensing ion channels (ASICs) in RGCs. (A) Currents were recorded in whole cell configuration at −60 mV. Representative traces showed the pH-dependent (7.0–5.5) activation of ASIC currents in RGCs (n = 20). (B and C) Amiloride blocked the ASIC currents in RGCs. Representative recordings showed the dose-dependent blockade of ASIC currents activated by pH drop from 7.4 to 6.0 (n = 6 for each group). Holding potential is −60 mV. Data were presented as mean (n = 6 for each group) ± SEM ($^{*}P < 0.05$, $^{***}P < 0.001$ compared with control).

the mRNA transcription and protein expression, we show the ASIC1 currents in the isolated RGCs from adult zebrafish retina. Moreover, the acidosis-induced current was blocked by amiloride [3]. In addition, immunohistochemistry and in situ hybridization technique revealed that ASIC1 channels were widely expressed in the retina of larval zebrafish.

Same as mammals, ASIC1 is highly expressed in zebrafish RGCs, which suggests that the acidosis-induced current may participate in visual signals transduction. Indeed, the acid-sensing ion channel currents were recorded in the isolated RGCs using whole cell patch clamp technique. Amiloride, the ASICs blocker, reduced the acidosis-induced current on RGCs. To explore whether ASIC1 was involved in regulating normal visual function of zebrafish, we also studied and demonstrated that blockade of ASIC1 in larval zebrafishs (5 dpf) decreased their response to light, indicating that ASIC1 may play a role in zebrafish vision. Our present results were consistent with those from mouse and rat retina [9]. However, research in ASIC1 knockout mice provided contradictory results. Ettaiche et al. demonstrated that ASIC1 knockdown decreased oscillatory potentials and photopic a-and b-waves [21]. Render et al. did not provide evidence of obvious morphological changes in cone photoreceptors in ASIC1 knockout mice [23]. Therefore,

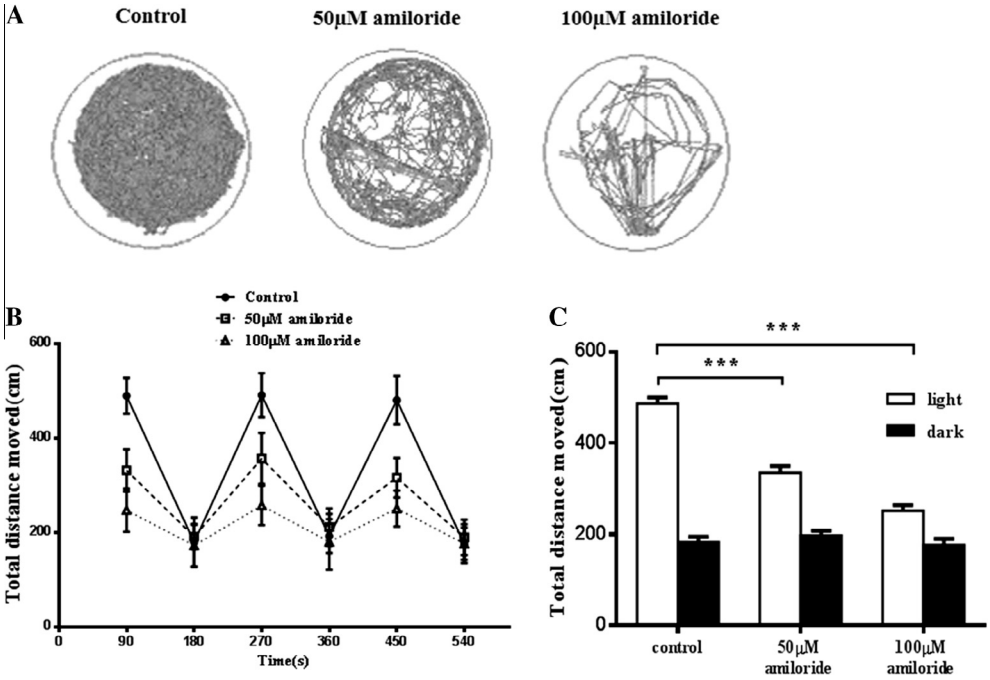

Fig. 4. Effects of amiloride on locomotion of larval zebrafish under the continuous exposure mode. (A) Characteristic swimming patterns of 5 dpf larvae (total activity in each 90 s light and dark periods). (B) Locomotion (distance moved, cm) was recorded in alternating periods of darkness and light for a total duration of 540 s. (C) Amiloride significantly reduced the locomotion of larval zebrafish during the light periods. Amiloride had no significant effect on the total activity in darkness. Data were presented as mean (n = 9–12) ± SEM ($^{***}P < 0.001$ compared with control in light periods).

future studies are needed to further elucidate the functions and mechanisms of ASIC1 in retina.

In summary, our data demonstrate for the first time that ASIC1 expresses and functions, in terms of regulating locomotor activity in response to light changes, in zebrafish retina.

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