Neomycin Damage and Regeneration of Hair Cells in Both Mechanoreceptor and Electroreceptor Lateral Line Organs of the Larval Siberian Sturgeon (Acipenser baerii)

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The lateral line of some aquatic amphibians and fishes can have two classes of sensory receptors: mechanoreceptors (neuromasts) and electroreceptors (ampullary organs). Lateral line mechanoreceptors (neuromasts) respond to low-frequency water movements (Bleckmann et al., 2003); electroreceptors (ampullary organs) are sensitive to low-frequency, direct-current (DC) fields (Zakon, 1988). Ampullary organs are primitive electroreceptors that are possessed by chondrichthyan and non-neopterygian fish (Gibbs and Northcutt, 2004). Like neuromasts, ampullary organs are composed of sensory hair cells and surrounding nonsensory cells (internal supporting cells and peripheral mantle cells) with distinct morphologies and distributions (Jørgensen, 2005).

The lateral line found in some amphibians and fishes has two distinctive classes of sensory organs: mechanoreceptors (neuromasts) and electroreceptors (ampullary organs). Hair cells in neuromasts can be damaged by aminoglycoside antibiotics and they will regenerate rapidly afterward. Aminoglycoside sensitivity and the capacity for regeneration have not been investigated in ampullary organs. We treated Siberian sturgeon (Acipenser baerii) larvae with neomycin and observed loss and regeneration of sensory hair cells in both organs by labeling with DASPEI and scanning electron microscopy (SEM). The numbers of sensory hair cells in both organs were reduced to the lowest levels at 6 hours posttreatment (hpt). New sensory hair cells began to appear at 12 hpt and were regenerated completely in 7 days. To reveal the possible mechanism for ampullary hair cell regeneration, we analyzed cell proliferation and the expression of neural placodal gene eya1 during regeneration. Both cell proliferation and eya1 expression were concentrated in peripheral mantle cells and both increased to the highest level at 12 hpt, which is consistent with the time course for regeneration of the ampullary hair cells. Furthermore, we used Texas Red-conjugated gentamicin in an uptake assay following pretreatment with a cation channel blocker (amiloride) and found that entry of the antibiotic was suppressed in both organs. Together, our results indicate that ampullary hair cells in Siberian sturgeon larvae can be damaged by neomycin exposure and they can regenerate rapidly. We suggest that the mechanisms for aminoglycoside uptake and hair cell regeneration are conserved for mechanoreceptors and electroreceptors.

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Most sensory hair cells in the ear and in neuromasts can be damaged by exposure to aminoglycoside antibiotics, copper, cisplatin, and noise (Song et al., 1995; Harris et al., 2003; Hernández et al., 2006; Smith et al., 2006; Owens et al., 2007; Schmitt et al., 2009; Mackenzie and Raible, 2012). However, relative sensitivity varies considerably for different hair cells. In the vertebrate inner ear, type I hair cells are generally more sensitive to gentamicin than type II hair cells (Yan et al., 1991; Pujol et al., 2014). Similarly, hair cells in superficial neuromasts are less sensitive to gentamicin than canal neuromasts (Song et al., 1995), even though a different conclusion is drawn using vital stains (Van Trump et al., 2010). Although hair cells in the mammalian cochlea cannot regenerate after damage, most non-mammalian vertebrates are able to regenerate sensory hair cells (Yals and Rubel, 1988; Groves, 2010; Burns et al., 2012).

Zakon (1991) showed that weakly electric fish can regenerate ampullary organs from existing epidermal tissue. However, the sensitivity of sensory hair cells in ampullary organs to aminoglycosides has not been determined, and the potential for regeneration after damage has not been investigated.

The regulatory mechanisms of hair cell regeneration in neuromasts and inner ear end organs have been studied widely. Cell proliferation is an important event during hair cell regeneration in neuromasts and the inner ear. In zebrafish, a subset of supporting cells proliferates during neuromast hair cell regeneration as progenitors (Ghysen and Dambly-Chaudière, 2007; Ma et al., 2008). During sensory hair cell regeneration in the chick, the new hair cells arise by direct transdifferentiation from supporting cell or proliferation of neighboring supporting cells (Brignull et al., 2009). Newborn mice can renew damaged hair cells because their non-sensory cells retain the capacity for cell proliferation (Burns et al., 2012). The proliferation of hair cell progenitors is regulated by many signaling pathways and transcription factors. Notch signaling limits progenitor proliferation in zebrafish neuromasts, and atoh1 activates cell proliferation within the mammalian cochlear epithelium (Ma et al., 2008; Kelly et al., 2012).

Both lateral line receptors and inner ear mechanoreceptors arise from neural placodes in early development (Northcutt et al., 1995; Schlosser, 2010). The neural placodal gene eya1 marks pan-placodal primordium and later continues to be expressed in all cranial placodes and their derivatives, including lateral line receptors and inner ear end organs (Schlosser, 2010). In eya1 mutant mice embryos, the number of proliferating cells is reduced in the otic placode (Zou et al., 2006). Coexpression of eya1/six1 can induce ectopic hair cells in cochlear epithelium (Ahmed et al., 2012). In addition, eya1 is upregulated during hair cell regeneration in zebrafish neuromasts (Hernández et al., 2007).

As eya1 expression has been found in ampullary organs in basal ray-finned fishes and cartilaginous fishes (Modrell et al., 2011; Gillis et al., 2012), its expression could facilitate identification of sensory hair cell regeneration in ampullary organs.

In this study we used neomycin to damage sensory hair cells in neuromasts and ampullary organs, and we recorded loss and regeneration of sensory hair cells by scanning electron microscopy (SEM) and 2-[4-(dimethylamino) styryl]-N-ethylpyridinium iodide (DASPEI) labeling in Siberian sturgeon (Acipenser baerii). We determined a possible pathway by which aminoglycosides enter the ampullary organ using Texas Red-conjugated gentamicin in an uptake assay. In addition, we analyzed cell proliferation and expression of eya1 during sensory hair cell regeneration in ampullary organs. Together, these results indicate that 1) ampullary hair cells are damaged after neomycin exposure, and they regenerate rapidly; 2) the ampullary organs can absorb Texas Red-conjugated gentamicin via a cation channel; and 3) the upregulation of cell proliferation and eya1-expression accompanies ampullary hair cell regeneration.

MATERIALS AND METHODS

Animals and neomycin treatment

Fertilized eggs from Siberian sturgeon (Acipenser baerii) were collected from Fangshan Shidu Fish-Breeding Base (Beijing, China) (http://www.bjfishery.com/web/base/index.asp?id=28) and reared at 18–20°C in artificial fresh water (AFW; 63 mg CaSO4, 10 mg MgSO4, 4 mg KCl, 1.1 mg Na2HPO4 per liter of dH2O) (Gibbs and Northcutt, 2004), containing 0.01 mg/L methylene blue to inhibit mold growth. The experiments were conducted at 8–9 days posthatching (dph, stage 44) when neuromasts and ampullary organs were functional, but not yet packaged in lateral line canals or ampullary sacs. All experiments were performed in accordance with Animal Care and Use Committee guidelines of Shanghai Ocean University.

The 8–9 dph Siberian sturgeon larvae were treated with a series of concentrations of neomycin (Bio Basic, Markham, Ontario, Canada, Cat. No. NB0366, Lot. No. LZ062185610Z; 50 μM, 100 μM, and 200 μM) diluted in AFW. After treatment with neomycin at 18–20°C for 1 hour, the larvae were rinsed three times quickly in AFW and returned to AFW for recovery. Thirty animals were sampled at 6 hpt, 12 hpt, 1 day posttreatment (dpt), 2 dpt, and 7 dpt. Another group of fish (n = 30) were maintained in AFW consistently and were used as the control.
Histology

Siberian sturgeon larvae were anesthetized in 0.02% 2-amino-benzoic acid ethyl ester (MS222; Sigma-Aldrich, St. Louis, MO) for 5 minutes. The heads of larvae were amputated using a fine dissecting knife and then processed for paraffin embedding. The sections (7 µm, Leica RM2235 microtome) were stained with hematoxylin/eosin (HE).

Vital staining of lateral line receptors

The fluorescent dye DASPEI (Life Technologies, Carlsbad, CA) was used as a vital dye to stain hair cells within neuromasts and ampullary organs. Five to ten Siberian sturgeon larvae were incubated in AFW containing 0.005% DASPEI for 15 minutes, and then anesthetized in 0.02% MS222 for 5 minutes. The anesthetized larvae were rinsed once in AFW and examined under a fluorescence stereomicroscope (Zeiss, Stereo Discovery V12) equipped with Filter Set 38 (excitation: 450–490 nM, emission: 500–550 nM).

Scanning electron microscopy

For SEM samples, 5–10 Siberian sturgeon larvae from each group were anesthetized in 0.02% MS222 and fixed in 4% glutaraldehyde in 0.1 M phosphate buffer (PBS with 0.1% Tween-20), and stored at 4°C overnight. Heads were dissected from the animals and dehydrated in a graded ethanol series (50%, 70%, 80%, 90%, and 100% ethanol). After critical-point drying with liquid CO₂ (Hitachi ES-2030 freeze dryer), samples were oriented ventral-side-up and mounted on aluminum stubs, sputter-coated with gold-palladium (Hitachi E-1010 ion sputter), and viewed and photographed with a scanning electron microscope (Hitachi S-3400N).

Uptake assay with Texas Red-conjugated gentamicin

Gentamicin (Bio Basic, Cat. No. A620217, Lot No. B3198A0019) was conjugated with succinimidyl esters of Texas Red (Life Technologies, Grand Island, NY) as previously described (Wang and Steyger, 2009), and separated by SigmaSpin Sequencing Reaction Clean-Up Columns (Sigma-Aldrich). Siberian sturgeon larvae (stage 46) were treated with Texas Red-conjugated gentamicin (1 mg/ml) or unconjugated Texas-Red (2 mg/ml) in AFW. The mechanoelectrical transduction-channel blocker amiloride was used to assess uptake. The larvae were pretreated with 1 mM amiloride (Sigma-Aldrich) in AFW for 15 minutes, followed by incubation in Texas Red-conjugated gentamicin for 20 minutes. The larvae then were anesthetized and fixed with 4% PFA (4% paraformaldehyde in PBS, pH 7.4) overnight.

All treated specimens were washed with PBS 3 times, then permeabilized in cold acetone for 10 minutes. The samples were blocked by 5% bovine serum albumin (BSA) for 1 hour and incubated in mouse anti-acetylated tubulin (1:1,000; Sigma-Aldrich, Cat. No. T7451, RRID: AB_609894) at 4°C overnight. After washing with PBS, the samples were incubated with Alexa 488-conjugated goat anti-mouse IgG (H+L) (1:250, Life Technologies, Cat. No. A-11001, RRID: AB_10566289) for 1 hour. The samples were washed with PBS 3 times, and then the samples were examined and photographed with a Zeiss Axio Observer Z1.

To quantify the fluorescence intensity, 8–10 neuromasts and ampullary organs were selected respectively from each larva (n = 3) as regions of interest (ROIs). The average fluorescence intensity for each receptor was measured using FIJI-ImageJ 1.48p software (Wayne Rasband, National Institutes of Health, Bethesda, MD; RRID: nif-0000-30467). The average value for background intensity was subtracted from the value for the intensity of fluorescent pixels (Texas Red channel) observed within the ROIs, and then the resulting values for fluorescence intensity were averaged for each receptor. Student’s t-test was used to determine the significant difference between different groups.

Cell proliferation assays

A pulse-fix protocol was used to identify cell proliferation as described previously (Harris et al., 2003; Ma et al., 2008). Sturgeon larvae (stage 44) were treated with 200 µM neomycin for 1 hour and then allowed to recover for 6 hours, 12 hours, or 24 hours. The untreated larvae were used as controls. Following the recovery period, larvae were incubated in AFW containing 10 mM BrdU (5-bromo-2-deoxyuridine; Sigma-Aldrich) in 1% dimethyl sulfoxide (DMSO) for 1 hour at 18–20°C. Animals were immediately anesthetized and fixed in 4% PFA for 2 hours at room temperature (RT) or overnight at 4°C, then washed several times in PBST (PBS with 0.1% Tween-20), and stored at 4°C before being processed for immunohistochemistry. Fixed samples were washed three times in PBDT (PBS with 1% DMSO and 0.1% Tween-20) and stored at 4°C for 20 minutes before being processed for immunohistochemistry.

Damage and regeneration of ampullary hair cells

All treated specimens were washed with PBS 3 times, then permeabilized in cold acetone for 10 minutes. The samples were blocked by 5% bovine serum albumin (BSA) for 1 hour and incubated in mouse anti-acetylated tubulin (1:1,000; Sigma-Aldrich, Cat. No. T7451, RRID: AB_609894) at 4°C overnight. After washing with PBS, the samples were incubated with Alexa 488-conjugated goat anti-mouse IgG (H+L) (1:250, Life Technologies, Cat. No. A-11001, RRID: AB_10566289) for 1 hour. The samples were washed with PBS 3 times, and then the samples were examined and photographed with a Zeiss Axio Observer Z1.

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room temperature. Mouse anti-BrdU (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. No. sc-32323, RRID: AB_626766) was used at 1:100 dilution in the blocking solution. After washing in PBST, samples were incubated in Alexa 568-conjugated goat anti-mouse IgG (1:500, Life Technologies, Cat. No. A-11004, RRID: AB_10562368) for 1 hour at room temperature, counterstained by DAPI (Sigma-Aldrich), then examined and photographed with a Zeiss Axio Observer Z1.

cDNA sequencing and eya1 gene cloning

Total RNA was extracted from stage 26–45 Siberian sturgeon embryos using TRIzol Reagent (Life Technologies). The embryonic cDNA library was constructed with a SMART cDNA Library Construction Kit (ClonTech, Palo Alto, CA) according to the instructions and sequenced with Roche 454 GS FLX DNA Sequencer. Comparison against the GenBank nonredundant database was performed using the BLAST network server at the National Center for Biotechnology Information (Altschul et al., 1997). The specific fragment of Siberian sturgeon eya1 cDNAs was amplified by specific primers (sense: AACACCAAGCCGACAACAC; antisense: CAAATCCCAAATGAAAACGC), which were designed based on the cDNA library. The eya1 cDNA fragment was cloned into the pBSK+ vector (gift from Yingbin Zhong, Soochow University, Jiangsu, China) and sequenced to check the recombinant plasmids.

Whole-mount in situ hybridization

Siberian sturgeon larvae were treated with 200 μM neomycin as described above, then fixed at 6 hpt, 12 hpt, and 24 hpt with 4% PFA at 4°C overnight. The samples were rinsed in PBS several times, dehydrated with methanol, and stored at −20°C. The samples were rehydrated stepwise into PBST, bleached in bleaching solution (0.5× sodium chloride-sodium citrate buffer [SSC], 3% H2O2, 5% formamide) under strong white light for 1 hour, permeabilized in proteinase K (10 μg/ml in PBST) for 45 minutes at 37°C, and postfixed in 4% PFA for 30 minutes. After prehybridization in a hybridization buffer (50% deionized formamide [v/v], 5× SSC, 0.1% Tween-20, 50 μg/ml of heparin, and 500 μg/ml of RNase-free tRNA adjusted to pH 6.0 by adding citric acid) at 65°C for 3 hours, they were hybridized in hybridization buffer with 1 μg/ml antisense DIG-labeled riboprobes (using sense DIG-labeled RNA probe as negative control) at 65°C for 12–16 hours. DIG-labeled RNA probe of eya1 was transcribed in vitro using linearized recombinant plasmids as the template. The posthybridization procedure was as described previously (Fan et al., 2007). After the staining reaction, samples were incubated in methanol for 2 hours to eliminate background, rehy-
peripheral mantle cells were both labeled in the ampullary organs (Fig. 1I).

**Morphology of cilia in sensory cells of the lateral line system**

Neuromasts in lateral line canal and ampullary sacs were filled with a great deal of mucus as Siberian sturgeon larva developed to the juvenile stage (~23 dph). We chose larvae at stage 44 to survey the morphology of cilia in neuromasts and ampullary organs (Fig. 2A,B). The neuromast was oblong and sunk in a canal. The apical surface of hair cells in the neuromast had a bundle of stereocilia and a single kinocilium located on one side of each hair cell (Fig. 2C). The ampullary organ was round. The sensory hair cells of the ampullary organ had a single cilium that was located centrally (Fig. 2D). The morphology of the neuromast kinocilium and ampullary sensory cilium did not change substantially after they became mature at stage 44.

We selected the neuromasts in the infraorbital line and the ampullary organs in the ventral infraorbital field for ciliary analyses because there were more lateral line sensory organs in the ventral side than in the dorsal side, and the smooth surface was convenient for DASPEI labeling and SEM observation (Figs. 1 and 2). We measured the length and diameter of cilia attached to the skin surface in the neuromasts and ampullary organs. The length of the kinocilium in the neuromasts was longer than the ampullary sensory cilium (7.15 ± 0.58 μm versus 2.89 ± 0.28 μm; n = 12, P <
The diameter of the kinocilium was uniform from the bottom to the tip (diameter: 0.24 ± 0.02 μm). In contrast, the bottom and the tip of the ampullary sensory cilium were thinner (diameter: 0.21 ± 0.01 μm), and the middle was thicker (diameter: 0.35 ± 0.02 μm). There were also several microvilli on the apical surface of ampullary hair cells.

The components of the neuromast and the ampullary organ were similar. Both were composed of sensory hair cells, internal supporting cells, and peripheral mantle cells. Sensory hair cells were surrounded by internal supporting cells with basal nuclei and apical projections intercalated between the sensory hair cells. The internal supporting cells were surrounded by peripheral mantle cells (Fig. 2E).

Damage and regeneration of sensory hair cells in the lateral line system

As reported, hair cells of neuromasts and inner ear end organs in fish are susceptible to aminoglycoside antibiotics, which can induce oxidative stress and cause hair cell death (Ton and Parng, 2005). To explore whether sensory hair cells in ampullary organs are susceptible to aminoglycosides, we analyzed lateral line sensory hair cells by SEM in Siberian sturgeon larvae (stage 43–50) after exposure to neomycin. Previous studies have shown that new ampullary organs and new ampullary hair cells are continually added to the lateral line system in shovelnose sturgeon and Adriatic sturgeon during this phase (Gibbs and Northcutt, 2004; Camacho et al., 2007). In addition, the number of
sensory hair cells in each neuromast and ampullary organ in untreated Siberian sturgeon larvae varies according to their position in the lateral line system (data not shown). To distinguish between newly developed hair cells and regenerated hair cells in neuromasts and ampullary organs, we analyzed large neuromasts (major axis of neuromast: ~30 μm) and ampullary organs (diameter of ampullary organ: ~20 μm). To consistently evaluate the changes in hair cell number, we selected neuromasts in the infraorbital canal and ampullary organs in the ventral infraorbital field for the hair cell analyses.

Figure 3 illustrates the status of sensory hair cells treated by the highest dose of neomycin (200 μM) for 1 hour. Viewed with SEM, both the neuromasts and ampullary organs had substantial ciliary bundle loss at 6 hpt compared to the controls. Some small sacs were visible on the surface of the neuromasts and ampullary organs at this stage (Fig. 3B,F), which may be expelled, dead sensory hair cells, or broken cilia (Weisleder and Rubel, 1993). Furthermore, the number and brightness of DASPEI-positive sensory hair cells in neuromasts and ampullary organs decreased remarkably compared to the control group. In addition, the ampullary organ became disorganized at this stage, as revealed by DASPEI staining (Fig. 3J).

We analyzed sensory hair cell recovery in Siberian sturgeon larvae using SEM to explore whether sensory hair cells of ampullary organs can regenerate after damage. The number of ciliary bundles declined to the lowest level at 6 hpt, and then new sensory hair cells with a shorter kinocilium formed in neuromasts and thick cilia began to form in ampullary organs at 12 hpt. Interestingly, most of the new sensory hair cells were at the edge of the ampullary organ (Fig. 3C,G). The number of hair cells and the length of cilia in neuromasts and ampullary organs recovered at 7 dpt (Fig. 3D,H).

We also used DASPEI staining to view the vital hair cells in neuromasts and ampullary organs. DASPEI-positive sensory hair cells also increased at 12 hpt, although the signal was weak (Fig. 3K). By 7 dpt, DASPEI-positive hair cell recovered to control levels (Fig. 3L).

To determine the dose–response relationship between the concentration of neomycin and hair cell survival, we treated Siberian sturgeon larvae with 50 μM, 100 μM, and 200 μM neomycin, and then analyzed hair cell numbers using SEM. Figure 4A,B show the data from all treatment groups in neuromast and ampullary organs, respectively. Both the neomycin treatment and time of recovery on the number of hair cells in neuromast and ampullary organ were highly
significant \((P < 0.0001)\). By 6 hpt, the numbers of hair cells in neuromasts and ampullary organs of all treatment groups decreased significantly compared with the control group \((P < 0.001)\). The overall decrease in neuromast and ampullary organ hair cell number as neomycin concentration increased was highly significant \((P < 0.0001)\). Following the 200 \(\mu\)M neomycin treatment, differences between treatment and controls were significant at 6 hpt, 12 hpt, 1 dpt, 2 dpt, and 7 dpt \((P < 0.001)\); however, the differences were not significant at 7 dpt. Taken together, these results indicate that neomycin treatment reduced the number of functional hair cells in both neuromasts and ampullary organs, and that sensory hair cells were nearly completely regenerated by 7 dpt.

Uptake of Texas Red-conjugated gentamicin by the ampullary organ

We have shown that hair cells in an ampullary organ can be damaged by neomycin. However, the mechanism by which neomycin kills ampullary hair cells is unknown. Previous studies showed that uptake of Texas Red-conjugated gentamicin by hair cells in neuromasts and the inner ear occurs through mechanoelectrical transduction channels (Wang and Steyger, 2009; Vu et al., 2013). To test for this mechanism in the sturgeon larvae, we used Texas Red-conjugated gentamicin to visualize the entrance of the antibiotic into the hair cells. We used unconjugated Texas Red in another group of sturgeon larvae as a control for uptake of Texas Red. Pretreatment with the mechanoelectrical transduction channel blocker amiloride was used in combination with the Texas Red-conjugated gentamicin to examine the relative entry of gentamicin in the presence of the channel blocker. We labeled the cilium with anti-acetylated tubulin antibody to identify the apical surface of the hair cells in the neuromasts and ampullary organs (Fig. 5).

As expected, the unconjugated Texas Red did not appear in the hair cells of neuromasts and ampullary organs (Figs. 5A,D), and the Texas Red-conjugated gentamicin was seen in the hair cells of neuromasts and ampullary organs (Fig. 5B,E). We quantified the fluorescence intensity of Texas Red in neuromasts and ampullary organs using ImageJ. The Texas red fluorescence increased significantly in the Texas Red-conjugated gentamicin group compared to the unconjugated Texas Red group \((P < 0.0001); \text{Fig. 6}\). Pretreatment with the mechanoelectrical transduction channel blocker amiloride caused a significant reduction in the signal from Texas Red-conjugated gentamicin in hair cells in neuromasts and ampullary organs \((P < 0.0001); \text{Figs. 5C,F, 6}\).

Cell proliferation during ampullary organ regeneration

Hair cell regeneration in neuromasts is dependent on cell proliferation (Harris et al., 2003; Hernández et al., 2007). To further explore whether cell proliferation occurs during ampullary sensory hair cell regeneration, we assessed proliferation using BrdU. Representative ampullary organs
from untreated and neomycin-treated animals are compared in Figure 7. In the untreated sturgeon larvae, a few BrdU-positive cells were located in the periphery of the ampullary organ (Fig. 7A), in accordance with the increase of sensory hair cells documented in the control ampullary organs. Based on their location in the ampullary organ, we believe that the BrdU-positive cells were peripheral mantle cells (Fig. 2E).

By 6 hpt, BrdU-positive cells in the ampullary epithelium of treated animals had changed little (Fig. 7B); however, at 12 hpt treated animals had more BrdU-positive cells in the periphery of the ampullary organ than untreated animals (Fig. 7C). The BrdU-positive cells in the whole epithelium decreased to control level at 24 hpt (Fig. 7D). Figure 8 shows the average number of ampullary peripheral mantle cells in S-phase from control larvae and at 6 hours, 12 hours, and 24 hours following 200 μM neomycin treatment. Analyses of these data with two-way ANOVA indicated highly significant differences in the number of BrdU-positive cells between neomycin treatment and controls at 6 hpt and 12 hpt (P < 0.0001). In addition, treatment groups differed significantly between 12 hpt and the two other recovery timepoints (P < 0.001). These results indicate that cell proliferation in the peripheral region of the ampullary organ was upregulated significantly at 12 hpt during sensory hair cell regeneration.

**Eya1 expression during sensory hair cell development and regeneration**

Both neuromast and ampullary organs are derived from neural placode. The Pan-placode marker *eya1* is
expressed in the neuromast and ampullary organs in chondrichthians and chondrosteans (Modrell et al., 2011; Gillis et al., 2012). To understand the regulation of ampullary organ regeneration in the Siberian sturgeon, we cloned the sturgeon eya1 gene (GenBank: ADU05418) and characterized the expression pattern of eya1 during development and regeneration. During embryonic development, eya1 was abundant in the olfactory placode, lens placode, anterior lateral line placode, posterior lateral line placode, otic placode, epi-branchial placode, hindbrain, and forebrain at stage 32 and stage 35 (Fig. 9A,B). Expression of eya1 was found in the primordia of sensory organs and ganglia at stage 40 (Fig. 9C). At stage 45, the expression of eya1 continued in the lateral line sensory organs (neuromasts and ampullary organs), but was especially concentrated in peripheral mantle cells of the neuromast and ampullary organs (Fig. 9D). These data indicate that eya1 expression is related to the normal development of the lateral line system in Siberian sturgeon.

To explore whether eya1 was involved in lateral line sensory hair cell regeneration, we performed whole-mount in situ hybridization during regeneration. Although eya1 expression decreased after the neuromast and ampullary organs had formed, it was still detectable in the peripheral mantle cells of neuromasts and ampullary organs (Fig. 10A). We analyzed eya1 expression in the supraorbital line and the ampullary fields flanking the line. After exposure to neomycin, the expression of eya1 in the peripheral mantle cells of neuromasts and ampullary organs decreased (Fig. 10B). However, eya1 expression was highly upregulated at 12 hpt and 24 hpt at those locations (Fig. 10C,D). These results are consistent with the hypothesis that the pan-placode gene eya1 is involved in lateral line sensory hair cell regeneration.

DISCUSSION

Previous studies have shown that both neuromast mechanoreceptors and ampullary organ electroreceptors originate from lateral line placodes, have similar cell components, and express neural placodal genes (e.g., eya1 and six1), although they have distinct functions (Zakon, 1988; Northcutt et al., 1995; Jørgensen, 2005; Schlosser, 2010; Modrell et al., 2011). In this study, we found that ampullary organs and neuromasts also share aminoglycoside sensitivity and regeneration capacity.

Mechanoreceptive hair cells in neuromasts and in the inner ear are sensitive to aminoglycosides and can be killed by these drugs (Song et al., 1995; Harris et al., 2003; Owen et al., 2007). In the present study, the cilia of electroreceptive hair cells in Siberian sturgeon larva were damaged after treatment with 200 μM neomycin

![Figure 7](image7.png)  
**Figure 7.** Cell proliferation during ampullary hair cell regeneration.  
A: Cell proliferation in representative ampullary organs from an untreated, control larva. B–D: Cell proliferation in representative ampullary organs from larvae treated with 200 μM neomycin. The boundary of each ampullary organ is encircled by a dotted line. Ampullary organs were immunostained with BrdU to label dividing cells (red) and counterstained with DAPI (blue). Scale bar = 20 μm.

![Figure 8](image8.png)  
**Figure 8.** The time course of proliferating ampullary cells in control and 200 μM neomycin exposure Siberian sturgeon larvae. The numbers of BrdU-positive cell in each ampullary organ were plotted at 6 hpt, 12 hpt, and 24 hpt. The number of proliferating cells in an ampullary organ is upregulated at 6 hpt and 12 hpt. n = 4 fish per condition, 3–4 ampullary organs per fish. The time of recovery and neomycin treatment had significant effects on the number of proliferating cells (two-way ANOVA, P < 0.0001). Bars are mean ± SEM.
for 1 hour (Fig. 3F), and the ampullary organs appeared to be disorganized by 6 hpt, with evidence that cells were ejected (Fig. 3F,J). Furthermore, the DASPEI signal was reduced. Combined, these results indicate that ampullary sensory hair cells are also sensitive to neomycin.

Aminoglycosides are thought to enter the mechanoreceptive hair cells via endocytosis and apical cation channels, including the epithelial sodium channel (ENaC) and transient receptor potential (TRP) channel (Meyers et al., 2003; Wang and Steyger 2009; Huth et al., 2011). Ampullary organs also contain many cation channels for sensory signal transduction (Struik, 2001; Peters and Denizot, 2005). Brown (2010) speculated that some TRP channels may lie in the electroreceptor membrane; however, the type of channel has not been determined.

Amiloride is a blocker for epithelial sodium channels, which can also block electroreception in catfish (Struik, 2001). Therefore, we used amiloride in experiments with Texas Red-conjugated gentamicin to evaluate uptake of the antibiotic when cation channels were blocked (Fig. 5D,F). The results suggested that aminoglycoside can enter the ampullary hair cell through a cation channel; however, the precise mechanism for aminoglycoside entrance is yet to be determined.

The susceptibility of mechanoreceptive hair cells to aminoglycosides varies among different receptors, for

Figure 9. Expression of eya1 gene during lateral line development in Siberian sturgeon. A: At stage 32, eya1 is expressed in hindbrain and multiple placodes including olfactory, otic, epibranchial, and lateral line. B: At stage 35, eya1 is expressed in developing neuromast canal lines and presumptive ampullary organ fields flanking those lines. C: At stage 40, expression of eya1 continues in elongating lateral line primordium and neuromast canals. D: At stage 45, expression of eya1 continues in developing lateral line organs as well as in the migrating posterior lateral line primordium. E: A negative control at stage 40. adp, anterodorsal lateral line placode; ao, ampullary organ; aop, ampullary organ primordium; avp, anteroventral lateral line placode; e, eye; epi, epibranchial placode; hb, hindbrain; mlp, middle lateral line placode; nm, neuromast; nmp, neuromast primordium, olf, olfactory; otp, otic lateral line placode; plp, posterior lateral line placode; stp, supratemporal lateral line placode. Scale bar = 500 μm.
example, canal neuromast hair cells versus superficial neuromast hair cells in the lateral line system; outer hair cells versus inner hair cells, and basal hair cells versus apical hair cells in the cochlea (Yan et al., 1991; Song et al., 1995; Vu et al., 2013). The lethal effect of neomycin (200 μM) affected fewer hair cells in ampullary organs than in neuromasts at 6 hpt (measured as the proportion of hair cell loss compared to the control, paired t-test *P* < 0.01). One explanation is that the channel responsible for aminoglycoside uptake in ampullary organs is different from the mechanism for entry into neuromasts. In support of this hypothesis, the Texas Red-conjugated gentamicin uptake in neuromasts is stronger than in the ampullary organ (Fig. 5). Furthermore, ampullary organs develop later than neuromasts at 6 hpt (measured as the proportion of hair cell loss compared to the control, paired t-test *P* < 0.01). One explanation is that the channel responsible for aminoglycoside uptake in ampullary organs is different from the mechanism for entry into neuromasts. In support of this hypothesis, the Texas Red-conjugated gentamicin uptake in neuromasts is stronger than in the ampullary organ (Fig. 5). Furthermore, ampullary organs develop later than neuromasts in primitive electrosensory animals (Northcutt et al., 1994; Gibbs and Northcutt, 2004). There may be more naive hair cells, which are not susceptible to aminoglycosides, in ampullary organs than in the neuromasts at stage 44 (Song et al., 1995; Santos et al., 2006).

Regeneration of organs or complex structures occurs in certain fish and urodeles, for example, the neuromast hair cells in zebrafish (Ma et al., 2008), the lower jaw in zebrafish (Wang et al., 2012), and limbs in salamanders (Kumar et al., 2011). As expected, the sensory hair cells in Siberian sturgeon ampullary organs can regenerate after damage by neomycin, and new ampullary hair cells are produced very quickly (at 12 hpt). Because hair cell loss occurs following neomycin exposure, we speculate that hair cell replacement by other cells is the main mechanism for ampullary sensory hair cell recovery.

Previous studies have indicated that regenerated hair cells originate from progenitor proliferation or nonsensory cell transdifferentiation in vertebrates from fishes to mammals (Stone and Cotanche, 2007; Ma et al., 2008; Burns et al., 2012). Indeed, we observed cell proliferation in peripheral mantle cells of the ampullary organ, which peaked at 12 hpt (Fig. 7), at the same time that new hair bundles were visible (Fig. 3). Mantle cells in neuromasts have been regarded as progenitors during hair cell regeneration (Ghysen and Dambly-Chaudière, 2007). We propose that during sensory hair cell regeneration in ampullary organs the progenitor cells are peripheral mantle cells that resume cell division and produce new sensory hair cells. This mechanism is supported by the observation that the new ampullary sensory hair cells occur at the edge of the ampullary organ, where the mantle cells are located.

Regeneration can be regarded as the reactivation of developmental processes to restore missing tissues.

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**Figure 10.** Expression of *eya1* gene in neuromast and ampullary organ during regeneration in Siberian sturgeon larvae. The expression of *eya1* in control larva (A) and neomycin-treated larvae at 6 hpt (B), 12 hpt (C), and 24 hpt (D). Scale bar = 500 μm.
Many important developmental genes are reactivated during regeneration (Ma et al., 2008; Wang et al., 2012). The neural placodal gene eya1 plays a key role in inner ear development and regulates cell proliferation (Zou et al., 2006; Modrell et al., 2011; Gillis et al., 2012). A previous study showed that eya1 is reactivated during neuromast hair cell regeneration (Hernández et al., 2007). During ampullary hair cell regeneration in this study, eya1 was upregulated at 12 hpt in the peripheral mantle cells (Fig. 10). The spatiotemporal pattern of eya1 expression agrees with cell proliferation, which suggests eya1 may have a role in progenitor proliferation during ampullary organ regeneration. Along with SIX1, EYA1 also can induce hair cell differentiation through activating the preneural gene atoh1 (Ahmed et al., 2012), a key gene for hair cell development and regeneration in the mammalian inner ear (Izumikawa et al., 2005; Atkinson et al., 2014). We have tried to clone atoh1 in Siberian sturgeon using a homology cloning strategy and embryonic transcriptome sequencing, but failed. We speculate Siberian sturgeon atoh1 may have low homology with mammalian atoh1. We will increase the sampling density and the sequencing depth in transcriptome sequencing to clone the gene. Because the developmental genes are expressed dynamically during ampullary hair cell regeneration, we expect to target the downstream gene of EYA1 in Siberian sturgeon lateral line receptors through transcriptome and chromatin immunoprecipitation (ChIP) analyses of regenerative lateral line receptors. Additional study of the expression of these developmental genes may help us to understand the evolution of sensory hair cells in vertebrates.

In summary, we found that primitive electoreceptors in the sturgeon were susceptible to neomycin, and they can regenerate quickly after damage. Aminoglycoside can enter and kill the ampullary hair cell via a cation channel. During sensory hair cell regeneration in ampullary organs, eya1 expression and cell proliferation in peripheral mantle cells were upregulated. Taken together, the results suggest that, to some extent, aminoglycoside sensitivity and the capacity for regeneration are conserved between electoreceptors and mechanoreceptors.

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CONFLICT OF INTEREST

The authors declare the absence of any conflict of interest.

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