Electrophysiological characterization of acutely isolated spiral ganglion neurons in neonatal and mature sonic hedgehog knock-in mice

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1. Introduction

In the inner ear, the organ of Corti along the cochlear axis contains inner hair cells (IHCs), which are responsible for the sensory transduction and synaptic activation of the primary afferent auditory neurons, spiral ganglion neurons (SGNs) [1]. IHCs detect mechanical stimulus and transmit precise and reliable information of acoustic waves [2]. IHCs are innervated by unbranched and myelinated type I SGNs [3]. Compared with vision, the accuracy of human hearing response time is up to the level of microseconds [4]. Such precise implementation relies on rapid adaptation which is regulated via low-threshold voltage-activated (LVA) K\(^+\) channels in SGNs [5]. Thus, potassium channel plays an important role in temporal precision function of hearing.

In mice, after birth, with the development of the cochlear synaptic structures of neurons and nerve fibers, hearing functions appear only after these structures develop to a certain degree of maturity [6,7]. Therefore, a period of ~2 weeks is required for the onset of hearing in mice [8]. Moreover, during postnatal development, the spatiotemporal distribution of ion channel subtypes changes in SGNs. Hence it should
be noted that the ion currents of SGNs in neonatal and mature mice must be recorded separately while doing patch-clamp experiment [9,10], particularly before hearing onset and after hearing development. However, few electrophysiology studies have been reported of SGNs from mature animals owing to the technical difficulties in the isolation of fragile post-hearing neurons from the rigid bony labyrinth of the inner ear as well as SGNs in mature mice have myelin [11]. Here we successfully performed electrophysiological experiments on mature mice and performed aging competition of potassium channels in SGNs.

Cisplatin is a widely used chemotherapy drug with ototoxicity which is a major dose-limiting side effect and causes permanent hearing loss in 40%–80% of treated patients [12,13]. The exact mechanism of how cisplatin influences the cells in the organ of Corti and the hearing system remains poorly understood [14]. Recently, potassium channels...
have been shown to contribute to apoptosis induced by cisplatin administration in various cell lines [15]. Besides, KCa3.1 blockade was found to have protective effects against cisplatin-induced acute kidney injury (AKI) [16]. However, few articles focus on the characteristic of SGNs and deafness effects by cisplatin with electrophysiology studies. Here we provide a 0.5 mM dose cisplatin administration which corresponds to the therapy dose to fill this research gap.

Transgenic animals have been widely used in science research [17]. Sonic hedgehog (Shh) signaling plays important roles in the formation of the auditory epithelium, and shh is specifically expressed only in SGNs in mouse cochlea [18]. Here we used a shh knock-in mice whose SGNs could specifically express Tdtomato and were fluoresced which is a prominent advantage in some experiments. Nevertheless, genetic knock-in technology could have potential risks and the Cre may also be toxic [19], therefore, it is necessary to verify the functional effects on hearing performance and reliability of gene editing animals in this study.

2. Materials and methods

2.1. Generation of ShhCreEGFP/+; Rosa26-Tdtomatoloxp/+ mice

All animal experiments were approved and followed the guidelines of the Animal Care and Use Committee of the Shanghai Jiaotong University School of Medicine (Shanghai, China).

ShhCreEGFP/+ and Rosa26-Tdtomatoloxp/+ strains were purchased from The Jackson Laboratory (Bar Harbor, MN, USA). Male ShhCreEGFP/+ mice were crossed with female Rosa26-Tdtomatoloxp/+ mice to obtain ShhCreEGFP+/+; Rosa26-Tdtomatoloxp/+ mice. ShhCreEGFP/+ mice were PCR genotyped using shh-cre forward (GCCTGCATTACCGGTCGATGC) and reverse (CAGGGTGTTATAAGCAATCCCC) primers (Transgene, 481 bp), Rosa26 mice were PCR genotyped using tdTomato forward (CTGTTCCTGTACGGCATGG) and reverse (GGCATTAAAGCAGCGTATCC) primers and control forward (AAGGGAGCTGCAGTGGA GTA) and reverse (CCGAAAATCTGTGGGAAGTC) primers (wt, 297 bp; mutant, 196 bp). Further details on the generation and genotyping of Shh mice have been described by Liu [18].

2.2. ABR test

Stimuli were generated with an RZ6 workstation (Tucker-Davis Technologies, Alachua, FL, USA) and a free-field was presented via an MFI speaker (TDT). The sound level was decreased from a 90 to 0 dB SPL in 5-dB steps. Audiograms were determined according to the disappearance of all peaks at 5656 Hz, 8000 Hz, 11,314 Hz, 16,000 Hz, and 22,627 Hz.

Fig. 3. SGN counting and diameter measurement by Tuj1 immunohistochemistry staining. A and B, Cochleae from wild type mice (A) and shh mice (B) paraflin sections with magnified apical, middle, and basal turns in three frames below. C, Average optical density of SGN. D, Average cell diameter of SGN from apical, middle, and basal turns. There were no statistically significant differences between the Shh group and wild type group in the apical, middle and basal turns (p > 0.05).
analyzed between groups in BioSigRZ software (TDT). Latency referred to the time from the onset of the signal to the peak, and amplitude was determined by averaging the ΔV of both sides of the peak.

2.3. Paraffin section and tuJ immunohistochemistry

Cochleae were fixed in 4% PFA overnight and decalcified in PBS with 10% EDTA for 7 days at 4 °C, and dehydrated in a graded series of xylene and absolute ethanol; Ten paraffin sections (1μm thick) approximately parallel to the modiolus at intervals of approximately 30μm were taken from each cochlea. The samples were washed with distilled water for 10 min, blocked in 0.1% Triton X-100 with 3% BSA for 30 min and incubated with the primary antibody mouse anti-TUJ1 (Covance, catalog # MMS-435 P, 1:500 dilution) overnight at 4 °C. After washing with 10% EDTA for 7 days at 4 °C, and dehydrated in a graded series of xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with distilled water for 10 min, blocked in 0.1% Triton X-100 with 3% BSA for 30 min and incubated with the primary antibody mouse anti-TUJ1 (Covance, catalog # MMS-435 P, 1:500 dilution) overnight at 4 °C. After washing with 10% EDTA for 7 days at 4 °C, and dehydrated in a graded series of xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with distilled water for 10 min, blocked in 0.1% Triton X-100 with 3% BSA for 30 min and incubated with the primary antibody mouse anti-TUJ1 (Covance, catalog # MMS-435 P, 1:500 dilution) overnight at 4 °C. After washing with 10% EDTA for 7 days at 4 °C, and dehydrated in a graded series of xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with distilled water for 10 min, blocked in 0.1% Triton X-100 with 3% BSA for 30 min and incubated with the primary antibody mouse anti-TUJ1 (Covance, catalog # MMS-435 P, 1:500 dilution) overnight at 4 °C. After washing with 10% EDTA for 7 days at 4 °C, and dehydrated in a graded series of xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with xylene and absolute ethanol; Ten paraﬃn sections were rehydrated with distilled water for 10 min, blocked in 0.1% Triton X-100 with 3% BSA for 30 min and incubated with the primary antibody mouse anti-TUJ1 (Covance, catalog # MMS-435 P, 1:500 dilution) overnight at 4 °C.

2.4. Isolation of neonatal and mature SGNs

C57BL/6 J mice with ages ranging from p3 to p30 were sacrificed, and the temporal bones were removed in Hank’s balanced salt solution (Gibco). The spiral ganglion tissue was digested in a 700 μl mixture containing Protease type XIV (20 mg/ml, 35 μl, Sigma) and ACSF (665 μl) at 25 °C for 40 min. Subsequently, we changed the solution to ACSF/FBS to stop the digestion reaction. A series of gentle trituration and centrifugation (100 RCF, 10 min) was necessary. We coated the glass slides with poly-γ-lysine (0.1 mg/ml, Sigma P-6282) to allow SGN adherence. The attachment process required 2 h.

2.5. Electrophysiology recording

Digested SGNs from mature mice (after p21) or neonatal mice (before p7) were placed into a custom-made recording chamber and viewed under an OLYMPUS LUMPlanFL upright microscope (Olympus, Japan) with a U-CMAD3 CCD camera (Olympus, Japan). Cells were bathed in standard external solution that contained (in mM): 135 NaCl, 5.8 KCl, 1.3 CaCl2, 0.9 MgCl2, 5.6 d-glucose, 10 HEPES; pH 7.4 (adjusted with NaOH), 303 mOsmol/kg. For Potassium currents recording in aging competition and cisplatin administration: External NaCl and CaCl2 is replaced by NMDG and MgCl2 to suppress sodium and calcium current. And the potassium currents were divided by the cell capacitance (picofarads) to generate the current density-voltage relationship for aging competition. Recording pipettes were filled with standard internal solution that contained (in mM): 135 KCl, 5.0 HEPES, 5.0 EGTA, 0.1 CaCl2; pH 7.4 (adjusted with KOH), 283 mOsmol/kg. Data from SGNs were recorded with an EPC10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Cells were held at -80 mV (unless otherwise indicated). Traces were recorded immediately after the cell membrane was broken through at a giga-ohm (GΩ) seal, and the series resistance (Rs) and membrane capacitance (Cm) were corrected. Whole-cell membrane capacitance (Cm) measurements were performed with the lock-in feature and “Sine + DC” method in Patchmaster (HEKA). Cells were charged and discharged by the sine-wave and the membrane capacitance was calculated in Patchmaster automatically. Offline analysis of electrophysiological data was performed mainly with Igor Pro 6.22 software (Wavemetrics, Lake Oswego, OR, USA) and Graphpad Prism 8.0.2 (GraphPad, La Jolla, CA).

2.6. Cisplatin administration

Four groups were included in this study: control, 0.5 mM cisplatin, 5 mM cisplatin, and washout groups. Cisplatin (P4394, Sigma) was used at two concentrations (0.5 mM correspond to clinical therapeutic doses and 5 mM correspond to lethal doses) in external solution. For the washout group, digested SGNs were treated with cisplatin concentrations at 0.5 mM for 5 min., then the bath solution was replaced with normal external solution and the I-V curves were re-measured.

Conductance-voltage relationships were calculated from K+ currents (bottom) recorded from digested SGNs from control and shh mice at P3. K+ currents were evoked by voltage steps (200 ms) from −64 mV to 14 mV in 10 mV increments from a holding potential of −80 mV. Na+ currents were evoked by 100 ms pre-pulse to −140 mV from a holding potential of −80 mV, followed by voltage steps (200 ms) from −64 mV to 14 mV in 10 mV increments. Stimulus protocol was shown on the right. B and C, Mean (± SEM) sodium current-voltage (I-V) (B) and mean (± SEM) potassium current-voltage (I-V) (C) relations. D, Representative current-clamp traces recorded from P3 wild type and shh mice in response to 500 ms current injections in 10pA increments from rest. Depolarizing current elicit action potential in wild type and shh mice. E-I, Comparison of AP amplitude (E), AP threshold (F), AP latency (G), Resting potential (H) and Input Resistance (I) between shh and wt mice. Input resistance was calculated by dividing the −140 mV stimulus to the recording currents (currents not shown in figure). AP threshold was measured as the minimum membrane potential required to evoke an AP. AP latency was quantified as the time from the current step to the AP peak for a 40-pA current step.
currents responses to steps stimulation from \(-110\) mV to \(+70\) mV and fitted to the Boltzmann equation:

\[
f(v) = \frac{G_{\text{max}}}{1 + e^{(v - V_{\text{half}})/k_{\text{slope}}}}
\]

where \(V\) is the command membrane potential, \(G_{\text{max}}\) is the maximum conductance, \(V_{\text{half}}\) is the half-activation voltage, \(k_{\text{slope}}\) is the slope factor that defines steepness of voltage dependence in current activation and \(V_{\text{rev}}\) is the reversal potential.

### 2.7. Data analysis

Depending on the nature of data set, statistical significance was assessed with unpaired Student’s t-test, ordinary one-way ANOVA or two-way ANOVA followed by Bonferroni post-hoc test, and the level of significance was set to \(p < 0.05\).

All the data are reported as mean data ± standard deviation (SD) in the text and mean data ± standard error of the mean (SEM) in the figure.

### 3. Results

#### 3.1. SGNs from shh mice are fluorescent

Shh is specifically expressed in the spiral ganglion area at E13.5. In the absence of Cre, Tdtomato expression is blocked by the floxed stop sequences preceding the Tdtomato coding frame. When the floxed stop sequences are deleted by Cre, Tdtomato is expressed permanently as long as the Rosa26 promoter is continually active (Fig. 1E). Almost 100% of the cochlear spiral ganglion neurons from shh mice specifically expressed Tdtomato, whereas the surrounding glial cells did not (Fig. 1A-D).

#### 3.2. The ABR threshold and wave I parameter scarcely changed in shh mice

Threshold comparisons revealed similar baseline thresholds in shh and wild type mice (Fig. 2B). The average thresholds in shh mice were 

| Frequency (kHz) | shh (n = 5) | wild type (n = 5) |
|----------------|-------------|------------------|
| 5.6           | -51.70 ± 2.06 | -52.70 ± 2.40 |
| 8             | -57.60 ± 2.60 | -58.70 ± 2.74   |
| 11            | -59.50 ± 2.60 | -60.70 ± 2.74   |
| 16            | -62.60 ± 2.60 | -64.00 ± 2.74   |
| 22.6          | -64.00 ± 2.60 | -65.00 ± 2.74   |
| 32            | -65.00 ± 2.60 | -66.00 ± 2.74   |

There was no significant difference between the Shh and wild type groups, according to two-way ANOVA (\(P > 0.05\), Bonferroni’s multiple comparisons test).

The amplitude and latency of wave I were analyzed at low, middle, and high frequencies (here only 16kHz is shown) by measuring the peaks at different intensities (Fig. 2C and D), and no difference was observed between amplitude and latency at all frequencies at all intensities (\(P > 0.05\), two-way ANOVA).

#### 3.3. SGN density and diameter are almost identical between Shh and wild type mice

The SGN density values in the apical, middle, and basal turns in the Shh group were 2321 ± 361, 2268 ± 333, and 1811 ± 263, respectively. For the wild type group, the results were 2673 ± 695, 2415 ± 550, and 1921 ± 477, respectively (Fig. 3C).

The SGN diameter in the apical, middle, and basal turns in the Shh group were 7.96 ± 0.84, 8.20 ± 0.56, and 7.66 ± 0.56, respectively. For the wild type group, the results were 7.69 ± 0.79, 7.95 ± 0.96, and 8.16 ± 0.91, respectively (Fig. 3D).

No statistically significant differences were observed in SGN density (\(P > 0.05\), unpaired t-test).

### Table 1: Summary of firing and membrane properties in SGNs.

| Trait                  | shh          | wild type    |
|------------------------|--------------|--------------|
| AP Amplitude (mV)      | 9.20 ± 5.62  | 7.20 ± 3.90  |
| AP Threshold (mV)      | 67.20 ± 5.82 | 69.20 ± 5.62 |
| AP Latency (ms)        | 67.20 ± 5.62 | 69.20 ± 5.62 |
| Resting Potential (mV) | 59.50 ± 4.65 | 60.00 ± 4.65 |
| Input Resistance (MΩ)  | 968.31 ± 297.64 | 798.31 ± 271.73 |

Summary of firing and membrane properties from patch-clamp recordings in SGNs. Data are presented mean ± SD; n = number of SGNs; statistical tests and p-values are presented for each dataset.
3.4. Voltage-dependent currents and action potentials of SGNs from wild type and shh mice have no difference

Prominent inward and outward currents, indicative of voltage-dependent sodium and potassium currents was recorded (Fig. 4A). Maximum peak outward currents amplitudes were not significantly different between shh mutants and wild type controls (shh: 1.98 ± 0.37 nA, n = 5; control: 1.87 ± 0.17 nA, n = 5, p > 0.05, two-way ANOVA) and there was no significant difference in the current–voltage relationships (Fig. 4C). We also analyzed fast activating and fast inactivating inward currents, characteristic of voltage-dependent sodium currents (INa+) (Fig. 4B). Again, no significant difference was found in INa+ (shh: −1.70 ± 0.58 nA, n = 7; controls: −1.45 ± 0.68 nA, n = 7, p > 0.05, two-way ANOVA).

To assess SGN firing properties, we injected depolarizing currents to evoke action potentials (APs) in SGNs (Fig. 4D). Quantitative analyses of AP parameters were shown in Fig. 4E-I and Table 1 and all the parameters have no difference as well.

3.5. Different densities of potassium currents between neonatal and mature mice

We examined the whole-cell K+ currents from SGNs and compared the currents magnitudes between neonatal and mature neurons (Fig. 5A). The total K+ currents magnitude was comparable between shh and control SGNs in mature mice (for example at −70 mV, shh: 5.26 ± 1.96 nA, n = 7; controls: 4.98 ± 1.38 nA, n = 6) (Fig. 5B). However, the expression of the currents differed during aging while the
amplitude divided by the cell capacitance (Fig. 5C). For example, at a 40 mV step potential, the magnitude of the whole-cell K⁺ currents were (shh in pA/pF) as follows: neo, 659.15 ± 101.30; mature, 388.07 ± 183.76. Of note, a significant difference became apparent from 10 mV as potassium channels opened (**, p < 0.005).

3.6. Electrophysiological characterization of potassium channel changes after cisplatin administration

Before the application of cisplatin, the amplitude of IK was stable (at 70 mV for example, control: 5.60 ± 1.02 nA, n = 5) (Fig. 6B). After changed solution to cisplatin, the amplitude of IK gradually decreased (at 70 mV for example, 0.5 mM: 2.88 ± 0.53 nA, n = 7; 5 mM: 0.72 ± 0.52 nA, n = 7). The amplitude of IK recovered after cisplatin was washed out (at 70 mV for example, washout: 4.89 ± 0.67 nA, n = 6). Statistically significant differences were observed between the control group and 0.5 mM group, and the difference was more significant between the control and 5 mM groups. However, no statistically significant difference was found between the control and washout groups at any voltage, thus indicating that the effect caused by low dose cisplatin was reversible.

To examine the voltage dependence, conductance was calculated from the currents and plotted against membrane potential for the four groups (Fig. 6C). Half-maximal activation (Vhalf) and slope (k) values were calculated by fitting Boltzmann to the data. Although there was no significant difference in either Vhalf or k values for the effect of the cisplatin, there was significant effect in the Imax values as a function of currents activation states.

The firing and membrane properties of SGNs after cisplatin administration were described in Supplementary 1 and Table 1.

4. Discussion

Transgenic mice are increasingly used in scientific research [20,21]. Here, we generated a knock-in mice exhibited markedly higher fluorescence levels, thus allowing us to easily visualize SGNs under a microscope. To determine whether the gene-modification was properly completed, we sought to address whether the hearing system might be affected by performing morphological and functional experiments. Through our verification experiments, including testing the amplitude and latency of ABR wave I which reflect the summed activity of auditory nerve fibers [22] and Tuj-1 staining which enables easy detection of SGNs for calculating the density and diameter [23,24], both experiments indicated the hearing conditions appeared to be normal. Thus, we verified that shh transgenic mice are a reliable mouse model exhibiting properties [25] because SGNs express several voltage-dependent potassium currents (IK⁺) and rapid inward sodium currents (INa⁺) [25] have also been recorded from our study which both of them have the similar amplitude in shh mice comparing to wild type mice. Quantitative analyses of AP parameters and membrane properties further proved that the firing machinery was not compromised, indicating no significant difference in the characteristics of ion channels on the cell membrane between shh and control littermates.

Potassium channel plays an important role in temporal precision of hearing function due to its regulation on rapid adaptation [5]. Moreover, channel function changes at different ages [9,10], particularly before hearing onset and after hearing development. However, few studies have performed electrophysiology experiments from mature animals probably due to the technical difficulties in isolating fragile post-hearing neurons from the rigid bony labyrinth of the inner ear. To fill this research gap, here we performed aging competition of voltage-gated outward rectifier potassium currents (IK,1) in SGNs on neonatal and mature C57 mice. We plotted the actual IK,1 density to correct the data by cell capacitance, this is the same method previously used by Ebenezer N. Yamoa who demonstrated a Kv7 current from cultured SGNs changes at different ages in 2010 [29]. Since we obtained same amplitudes but different density of IK,1, the channel characteristics or channel number should be considered. Unfortunately, we did not verify the potassium channel subtype by using Kv1.1 or Kv1.3 channel antagonists. This line of investigation may be further studied in our next project.

Potassium channels have been shown to contribute to apoptosis induced by cisplatin administration in various cell lines [15]. Besides, Kᵥ₃.₅.₁ blockade was found with having protective effect against cisplatin-induced acute kidney injury (AKI) [16]. However, the exact mechanism through which cisplatin influences the potassium channel from cells in the organ of Corti had been unknown. In this study, a low dose of cisplatin (0.5 mM, corresponding to the therapy dose) was found to cause a decrease in current, which was reversible for a short time after cisplatin washout. In addition, the decline of amplitudes become more pronounced from external solution changed to high doses of cisplatin; However, in the conductance-voltage relationship, parameters such as Vhalf and Kslope of cisplatin-administration groups did not changed comparing with the control group. Significant difference was found in the results of Imax which indicates that the cisplatin administration may inactivate the potassium channels, but not alter the number and the characteristic of ion channels on the membrane. We suppose as some of the potassium ion channel inactivated, the excess potassium ions cannot get out of the cell completely and accumulate in the cell body, which had affected the membrane properties and induce cytotoxicity and apoptosis (supplementary 2). Therefore, using Drugs or signaling molecules that alter the activity of potassium channels may reduce cytotoxicity and provide protection against ototoxicity.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neulet.2019.134536.

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