Research Article

Inhibition of Histone Methyltransferase G9a Attenuates Noise-Induced Cochlear Synaptopathy and Hearing Loss

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ABSTRACT

Posttranslational modification of histones alters their interaction with DNA and nuclear proteins, influencing gene expression and cell fate. In this study, we investigated the effect of G9a (KMT1C, EHMT2), a major histone lysine methyltransferase encoded by the human *EHMT2* gene and responsible for histone H3 lysine 9 dimethylation (H3K9me2) on noise-induced permanent hearing loss (NIHL) in adult CBA/J mice. The conditions of noise exposure used in this study led to losses of cochlear synapses and outer hair cells (OHCs) and permanent auditory threshold shifts. Inhibition of G9a with its specific inhibitor BIX 01294 or with siRNA significantly attenuated these pathological features. Treatment with BIX 01294 also prevented the noise-induced decrease of KCNQ4 immunolabeling in OHCs. Additionally, G9a was increased in cochlear cells, including both outer and inner sensory hair cells, some spiral ganglion neurons (SGNs), and marginal cells, 1 h after the completion of the noise exposure. Also subsequent to noise exposure, immunoreactivity for H3K9me2 appeared in some nuclei of OHCs following a high-to-low frequency gradient with more labeled OHCs in the 45-kHz than the 32-kHz region, as well as in the marginal cells and in some SGNs of

the basal turn. These findings suggest that epigenetic modifications of H3K9me2 are involved in NIHL and that pharmacological targeting of G9a may offer a strategy for protection against cochlear synaptopathy and NIHL.

Keywords: noise-induced hair cell loss, lysine methyltransferase G9a, epigenetic modification, cochlear synapses, protection of noise-induced hearing loss

INTRODUCTION

Noise-induced hearing loss (NIHL) has become a major public health problem due to the rapid increase in the number of people exposed to the detrimental effects of noise in both industrialized and developing countries. Excessive noise damages the sensory hair cells of the inner ear and results in permanent hearing loss, as these cochlear hair cells cannot regenerate in adult mammals. Even noise-induced temporary hearing loss without apparent loss of outer hair cells (OHCs) may be associated with a slow degeneration of auditory nerve fibers leading to accelerated age-related hearing impairment (Kujawa and Liberman 2006). Although numerous molecular and biological events are involved in NIHL, no established clinical therapies for prevention or amelioration of NIHL are available (Sha and Schacht 2017). This dearth of treatment options is due to a lack of comprehensive understanding of the causal molecular mechanisms underlying noise-induced damage.

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Posttranslational modifications of histones have emerged as key regulators of genomic activity without affecting DNA sequence. All histone core proteins share long amino-terminal tails that are accessible to enzymatic modifications including methylation, acetylation, and phosphorylation (Kouzarides 2007). Recently, we have reported that histone deacetylases (HDACs) increase, while acetylation of histone H3 lysine 9 (H3K9ac) decreases in sensory hair cells after noise exposures that induce permanent hearing loss (Chen et al. 2016). In addition to acetylation, methylation is a major form of histone modification and also involved in multiple cellular processes, such as cell cycle regulation and cellular differentiation, cell survival and death, and proliferation. Dimethylation of histone 3 on lysine 9 (H3K9me2) is one of the best-studied modifications of eukaryotic chromatin and one of the most well-known markers in gene silencing and is catalyzed by lysine dimethyltransferase G9a and G9a-like protein (GLP) (Shinkai and Tachibana 2011). The major strategy for modifying H3K9me2 has been via inhibition of G9a (EHMT2). Among other actions, G9a is essential for epigenetic silencing of potassium channel genes as shown in the acute-to-chronic pain transition (Laumet et al. 2015). Interestingly, the potassium voltage-gated channel subfamily Q member 4 (*KCNQ4*) is mainly localized to auditory sensory hair cells (Kubisch et al. 1999; Kharkovets et al. 2006; Jaumann et al. 2012). Mutation of *KCNQ4* at the DFNA2 locus on the human chromosome 1p34 causes autosomal dominant nonsyndromic progressive hearing loss (Kubisch et al. 1999; Nie 2008). *KCNQ4* knockout mice exhibit hearing loss at several weeks of age and dominant-negative DFNA2 mutated mice show progressive hearing loss (Kharkovets et al. 2006). Additionally, polymorphisms in the *KCNQ4* gene are associated with susceptibility to NIHL (Pawelczyk et al. 2009). Since noise exposure decreases *KCNQ4* expression in OHCs (Jaumann et al. 2012), we hypothesize that inhibition of G9a will prevent this decrease and reduce NIHL.

A previous study reported that BIX 01294, a specific inhibitor of G9a, significantly decreases H3K9me2 levels and prevents neomycin-induced ototoxicity (Yu et al. 2013). In order to test the effect of BIX 01294 on noise trauma, we have employed our previously characterized noise conditions for CBA/J mice at the age of 12 weeks. First, we measured noise-induced losses of OHCs and inner hair cell (IHC) synaptic ribbons and NIHL in the presence of BIX 01294 or by silencing the *EHMT2* gene via small interfering RNA (siG9a). Then, we assessed immunoreactivity of G9a and H3K9me2 in the cochlea. Finally, we evaluated levels of *KCNQ4* in OHCs after inhibition of G9a with BIX 01294. Our results suggest a role

for histone methylation in NIHL and that inhibition of G9a may provide a potential target for treatment of NIHL.

MATERIALS AND METHODS

Animals

Male CBA/J mice at 10 weeks of age were purchased from The Jackson Laboratory. All mice had free access to water and a regular mouse diet (Irradiated Lab Diet no. 5V75) and were kept at 22 ± 1 °C under a standard 12:12-h light-dark cycle to acclimate for at least 1 week before conducting any the experiments. At 11 weeks of age, baseline auditory brainstem responses (ABRs) were measured. At the age of 12 weeks, mice were exposed to noise. All mice were housed in the animal facility of the Children's Research Institute at the Medical University of South Carolina (MUSC). All research protocols were approved by the Institutional Animal Care and Use Committee at MUSC. Animal care was under the supervision of the Division of Laboratory Animal Resources at MUSC.

Noise Exposure

In this study, all CBA/J mice were exposed to 2–20 kHz at 101 dB sound pressure level (SPL) for 2 h unless otherwise stated. Unrestrained CBA/J male mice at the age of 12 weeks (one mouse per stainless steel wire cage, approximately 9 cm³) were exposed to noise to induce permanent threshold shifts with loss of ribbons and OHCs, but not IHCs, by 14 days after the noise exposure. The sound exposure chamber was fitted with a loudspeaker (model 2450H; JBL) driven by a power amplifier (model XLS 202D; Crown Audio) fed from a CD player (model CD-200; Tascam TEAC American). Audio CD sound files were created and equalized with audio editing software (Audition 3; Adobe System, Inc.). The background sound intensity of the environment surrounding the cages was 65 dB as measured with a sound level meter (model 1200; Quest Technologies). Sound levels for noise exposure are calibrated with a sound level meter at multiple locations within the sound chamber to ensure uniformity of the sound field and are measured before and after exposure to ensure stability. Control mice were kept in silence (without use of the loudspeaker) within the same chamber for 2 h.

Treatment with BIX01294 Via Intraperitoneal Route to Mice

BIX 01294 was purchased from Sigma-Aldrich (B9311) and dissolved in dimethyl sulfoxide (DMSO)

as stock solution (80 mg/mL) and stored at -20°C , as previously described (Malmquist et al. 2012). The stock solution was diluted with 0.9 % saline solution immediately before injections. Initially, we tested two doses of BIX 01294 (20 and 40 mg/kg) for prevention of NIHL. Since 40 mg/kg of BIX 01294 attenuated NIHL, we used 40 mg/kg for the rest of the experiments. For immunohistochemistry, each animal received a total of three intraperitoneal (IP) injections of BIX 01294 at a dose of 40 mg/kg per injection. The three IP injections were administered 24 h before, 2 h before, and immediately after noise exposure. The mice used for experiments to observe the progression of ABR thresholds received two additional IP injections on the following day post noise exposure (am and pm). Vehicle control mice received the same volume of DMSO (0.5 mL/kg).

Intra-Tympanic Delivery of G9a Small Interfering RNA In Vivo

The methods for siRNA delivery into the adult mouse inner ear in vivo were modified from our previous publication (Oishi et al. 2013; Hill et al. 2016). Briefly, after anesthesia, a retroauricular incision was made to approach the temporal bone. The otic bulla was identified ventral to the facial nerve and a shallow hole was made in the thin part of the otic bulla with a 30-G needle and enlarged with a forceps to a diameter of 2 mm in order to visualize the round window. A customized sterile micro medical tube was inserted into the hole just above the round window niche (RWN) to slowly deliver 10 μL (0.6 μg) of pre-designed G9a siRNA (Thermo Fisher, siRNA ID s99721) to completely fill the mouse RWN. After the siRNA was delivered, the hole was covered with surrounding muscle and glued with tissue adhesive. Finally, the skin incision was closed with tissue adhesive and the mouse was kept in the surgical position for about 1 h. Seventy-two hours after siRNA delivery, the animals were exposed to noise for 2 h.

Auditory Brainstem Response Measurement and Analysis of ABR Wave I Amplitudes

ABRs were measured in the left ears of anesthetized mice before and 2 weeks after noise exposure. Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then placed in a sound-isolated and electrically shielded booth (Acoustic Systems). Body temperature was monitored and maintained near 37°C with a heating pad. Acoustic stimuli were delivered monaurally to a Beyer earphone attached to a customized plastic speculum inserted into the ear canal. Subdermal electrodes were inserted at the

vertex of the skull (active), mastoid region under the left ear (reference), and mastoid region under the right ear (ground). ABRs were measured at 8, 16, and 32 kHz. Tucker Davis Technology (TDT) System III hardware and SigGen/Biosig software were used to present the stimuli (15 ms duration tone bursts with 1 ms rise-fall time) and record the response. Up to 1024 responses were averaged for each stimulus level. ABR wave I was used to determine ABR thresholds for each frequency. Thresholds were determined for each frequency by reducing the intensity in 10-dB increments and then in 5-dB steps near threshold until no organized responses were detected. Thresholds were estimated between the lowest stimulus level where a response was observed and the highest level without response. All ABR measurements were conducted by the same experimenter. The ABR values were assigned by an expert who was blinded to the treatment conditions. The waveforms were saved and analyzed post hoc to measure the wave I amplitudes.

Immunocytochemistry for Cochlear Surface Preparations

We have followed a procedure as previously described (Chen et al. 2012; Zheng et al. 2014; Yuan et al. 2015). The temporal bones were removed and perfused locally with a solution of 4 % paraformaldehyde in PBS, pH 7.4, and kept in this fixative overnight at 4°C . The cochleae were then rinsed in PBS and decalcified in a 4 % solution of sodium EDTA (adjusted with HCl to pH 7.4). The EDTA solution was changed daily for 3 days at 4°C . The cochleae were dissected under a microscope by removing the softened otic capsule, stria vascularis, Reissner's membrane, and tectorial membrane. The remaining tissue, including the modiolus and cochlear sensory epithelium, was permeabilized in 3 % Triton X-100 solution for 30 min at room temperature. The specimens were washed three times with PBS and blocked with 10 % normal goat serum for 30 min at room temperature, followed by incubation with primary antibodies: rabbit monoclonal anti-H3K9me2 (RevMAb Biosciences, 31-1059-00), rabbit monoclonal anti-G9a (Abcam, ab185050), and mouse monoclonal anti-KCNQ4 (Abcam, ab84820) at 4°C for 48 h. After washing three times (10 min each wash), the tissues were incubated with the Alexa Fluor-594 or 488-conjugated secondary antibody at a concentration of 1:200 at 4°C overnight in darkness. The specimens were washed three times with PBS and incubated with propidium iodide (PI) or Hoechst 33342 at 1 $\mu\text{g/mL}$ in PBS for 1 h at room temperature. After the final wash with PBS, the tissue was dissected by removing the modiolus. The epithelia were divided into three segments (apex, middle, and base). Specimens were

mounted on slides with anti-fade mounting media. Control incubations were routinely processed without primary antibody treatments.

Of note, although the anti-H3K9me2 and anti-G9a antibodies used are rabbit monoclonal antibodies, we still confirmed the specificity by Western blot using total cochlear homogenates before using them for immunohistochemistry.

Quantification of the Immunofluorescence Signals from Outer Hair Cells of Surface Preparations

Immunohistochemistry is well accepted as a semi-quantitative methodology when used with careful consideration of the utility and semiquantitative nature of these assays (Taylor and Levenson 2006; Walker 2006). The specificity of antibodies must be first detected by Western blot analysis. An antibody showing only a single band with the correct molecular weight was then used for immunolabeling on surface preparations and quantification of signaling in OHCs. The regions of interest were outlined within individual OHCs based on the counterstaining. The grayscale value was determined in only the hair cells to quantify the change in fluorescence intensity. This procedure provided quantitative measurements that are not confounded by protein expression in other cell types of the cochlea.

Immunolabeling for G9a was quantified from original confocal images with 8-bit grayscale values, each taken with a $\times 63$ magnification lens under identical conditions and equal parameter settings for laser gains and photomultiplier tube (PMT) gains within linear ranges of the fluorescence, using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The cochleae from the different groups were fixed and stained simultaneously with identical solutions and processed in parallel. All surface preparations were counterstained with PI (red) for staining OHC nuclei in order to identify the comparable parts of the OHCs in confocal images. The regions of interest of individual OHC nuclei were outlined with the circle tool based on the PI staining. The immunolabeling for G9a in OHCs was measured in the upper-basal region, corresponding to sensitivity to 22–32 kHz, of surface preparations in 0.12-mm segments, each containing about 60 OHCs. The intensity of the background fluorescence was subtracted and the average fluorescence per cell was then calculated. For each repetition, the relative grayscale value was determined by normalizing the ratio to control.

Immunocytochemistry for Synaptic Ribbons on Cochlear Surface Preparations

Depending on the time points, mice were euthanized either 1 h or 14 days after the end of exposure. The temporal bones were removed immediately following

euthanasia, perfused locally with a fresh solution of 4 % paraformaldehyde in PBS, pH 7.4, and fixed for 1 h at room temperature. After the decalcification with 4 % EDTA for 3 days, each cochlea was dissected by removing the softened otic capsule, stria vascularis, Reissner's membrane, and tectorial membrane. The remaining tissue, including the modiolus and cochlear sensory epithelium, was permeabilized in fresh 3 % Triton X-100 solution for 30 min at room temperature. The specimens were washed three times (10 min each) with PBS and blocked with 10 % normal goat serum for 30 min at room temperature and then incubated in darkness at 37 °C overnight with primary monoclonal mouse anti-CtBP2 IgG1 at 1:200 (BD Biosciences, no. 612044). After washing three times, the tissues were incubated with the Alexa Fluor 594-conjugated secondary antibody at a concentration of 1:1000 at 37 °C for 1 h in darkness. After washing three times, the tissues were re-incubated with Alexa Fluor-conjugated secondary antibodies for an additional 1 h at 37 °C to increase the immunolabeling for CtBP2 (Wan et al. 2014; Hill et al. 2016). Following washing three times, the tissues were incubated in darkness at 4 °C overnight with polyclonal rabbit anti-myosin VIIa at 1:200 (Proteus Biosciences, 25-6790). Following the washing steps, the tissues were incubated with Alexa Fluor 350-conjugated secondary antibody at a concentration of 1:200 at 4 °C overnight in darkness. For all immunolabeling samples, after at least three final washes with PBS, the specimens were microdissected in PBS by removing the modiolus and divided into three segments (apex, middle, and base). Each segment was mounted on slides with Fluoro-gel with Tris buffer (Electron Microscopy Sciences, no. 17985-10). Immunolabeled images were taken with a $\times 63$ magnification lens under identical Z-stack conditions using Zeiss LSM 880 or Leica SP5 confocal microscope.

Quantification of the Immunolabeled Ribbons from Z Projections on Surface Preparations

We have followed a procedure as previously described (Hill et al. 2016). Immunofluorescence of CtBP2 on surface preparations was quantified from original confocal images, each taken with a $\times 63$ magnification lens under identical Z-stack conditions in 0.25-mm intervals and equal parameter settings for laser gains and PMT gains. The z-stack images in each 0.12-mm segment (containing about 16 IHCs) were captured from cochlear surface preparations. The number of synaptic ribbons was counted using ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, the background of the images was subtracted, the noise was despeckled once, and the threshold was set to isolate the immunolabeling of ribbon signals. The

image was then converted to a binary file and the number of ribbon particles was counted using the 3D Object Counter and divided by the total number of IHC nuclei within the image.

Surface Preparations and Diaminobenzidine Staining of Myosin VII-Labeled Hair Cells for Hair Cell Counts

Following decalcification, the cochleae were dissected as described in the section of immunocytochemistry for cochlear surface preparations. The specimens were permeabilized in 3 % Triton X-100 solution, blocked with 10 % normal goat serum for 30 min at room temperature, incubated with a primary antibody (rabbit polyclonal anti-myosin VII at a 1:100 dilution) overnight at 4 °C, and then incubated overnight at 4 °C with biotinylated goat anti-rabbit antibody at a 1:100 dilution. The specimens were washed with PBS three times between each step and then incubated in ABC solution (Vector Laboratories no. PK-4001) overnight. Following another washing, the cochleae were incubated in diaminobenzidine (DAB) for 3 h, as necessary for sufficient staining intensity, followed by washing to stop the DAB reaction. Finally, the cochleae were microdissected under a microscope into apical, middle, and basal segments and mounted on slides with Fluoromount-G mounting media. Images were taken with a Zeiss AxioCam MRc5 camera with Axioplan 2 imaging software under a Zeiss microscope.

Images from the apex through the base of the DAB-stained preparations were captured using a $\times 40$ lens on the Zeiss microscope. The lengths of the cochlear epithelia were measured and recorded in millimeters. OHCs were counted from the apex to the base along the entire length of the mouse cochlear epithelium. The percentage of hair cell loss in each 0.5-mm length of epithelium was plotted as a function of the cochlear length as a cytocochleogram.

Mapping of frequencies as a function of distance along the length of the cochlear spiral was done using the ImageJ plugin. In addition, we also calculated frequency mapping with the equation [d (%) = $156.5 - 82.5 \times \log(f)$] from Müller's paper (Müller et al. 2005). The results were in agreement with the literature (Viberg and Canlon 2004).

Immunohistochemistry for Cryosections

Following decalcification with 4 % EDTA for 72 h at 4 °C, cochleae were transferred to 30 % sucrose and incubated overnight and then embedded with OCT for sectioning. Sections of 8- μ m thickness were incubated in 0.3 % Triton X-100 in PBS for 30 min at room temperature. The specimens were washed

three times with PBS and blocked with 10 % normal goat serum for 30 min at room temperature, followed by incubation with primary antibody: anti-H3K9me2 (RevMAb Biosciences, no. 31-1059-00, 1:50) at 4 °C for 48 h. After washing three times (10 min each), the tissues were incubated with the Alexa Fluor 488-conjugated secondary antibody at a concentration of 1:200 at 4 °C overnight in darkness. The specimens were washed three times with PBS and incubated with PI at 1 μ g/mL in PBS for 1 h at room temperature. After the final wash with PBS, specimens were mounted on slides with anti-fade mounting media. Secondary antibody controls were routinely processed without primary antibody treatments. Immunolabeled cryosections were imaged with a Zeiss laser confocal microscope (Zeiss LSM 510).

Immunocytochemistry for Cochlear Paraffin Sections

Following decalcification with 4 % EDTA, each cochlea was transferred to 70 % ethanol and embedded in paraffin for sectioning. Five-micrometer sections were routinely deparaffinized in xylene and rehydrated in alcohol. The sections were incubated with target retrieval solution (Dako, S2367) in a steamer (Oster, no. CKSTSTMD5-W) for 10 min and then 3 % hydrogen peroxide solution for 10 min and protein block (Dako, no. 0909) for 20 min at room temperature. All primary antibodies were first optimized by titration with five different concentrations at two pH values (pH 6 and 9). Then, both G9a rabbit monoclonal primary antibodies (G9a at 1:500, pH 6, Abcam, ab185050 or G9a at 1:50, pH 6, MA5-14880, Invitrogen) were incubated overnight in a humid chamber at 4 °C, followed by biotinylated secondary antibody (Vector Laboratories,) for 30 min and ABC reagent (Vector Laboratories) for 30 min. Immunocomplexes of horseradish peroxidase were visualized by DAB reaction, and sections were counterstained with hematoxylin before mounting. Immunohistochemistry on paraffin sections of G9a reveals immunoreactivity in both nuclei and the cytoplasm in sensory hair cells and spiral ganglion cells (SGNs). We have tried two monoclonal antibodies from different companies, one a rabbit monoclonal antibody (MA5-14880) from Invitrogen, and the other also a rabbit monoclonal antibody (ab185050) from Abcam. Both rabbit monoclonal antibodies show identical immunoreactivity for G9a.

Extraction of Total Cochlear Protein

Cochleae were rapidly removed and dissected in ice-cold PBS containing complete™ mini EDTA-free

protease inhibitor cocktail tablets (Roche Diagnostic) at pH 7.4. To extract total protein, tissues from the cochleae of one mouse were homogenized in ice-cold radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich, R0278) plus Phosphatase Inhibitor Cocktails II and III (Sigma-Aldrich) and Roche Protease Inhibitor by using a glass/glass micro Tissue Grind pestle and vessel for 30 s. After 30 min on ice, tissue debris was removed by centrifugation at $15,000\times g$ at 4 °C for 10 min and the supernatants were retained as the total protein fractions. Protein concentrations were determined using the Bio-Rad Protein Assay dye reagent with bovine serum albumin as a protein standard. Two cochleae from the same mouse were pooled for each sample.

Cell Culture, Silencing EHMT2 Gene, and Extraction of Nuclear Protein from 3T3 Cells

3T3 cells were kindly provided by Gavin Wang at MUSC, originally generated at ATCC: The Global Bioresource Center. 3T3 cells were seeded in six-well dishes to about 2×10^5 cells/well and cultivated in Dulbecco's modified eagle's medium (DMEM) containing 4.5 g/L glucose, 10 % fetal bovine serum, and 1 % penicillin/streptomycin in a humidified incubator (37 °C, 5 % CO₂, 95 % humidity). After 48 h, cells were grown to 70 % confluency, 3T3 cells were silenced with G9a interfering RNA, which was the same siG9a as we used in *in vivo* experiments. According to the manufacturer's protocol, Lipofectamine RNAiMAX Reagent (Invitrogen, 13778075) was used for the transfection. Forty-eight hours after the transfection, 3T3 cells were treated with trypsin-EDTA (Thermo Fisher Scientific, 25200056) for 5 min. The trypsin was diluted with 10 mL of DMEM. In order to obtain sufficient amounts of protein, cells from two wells were collected as one sample. The collected cells were transferred to a 15-mL conical tube (Corning, 430052) and centrifuged at $500\times g$ for 5 min. After the medium was removed, the pellets of cells with 500 μ L medium were transferred to 1.5-mL Eppendorf tubes (Thermo Fisher Scientific, 05408133). The cells were washed with 1 mL of PBS (Invitrogen, 20012) and centrifuged again at $500\times g$ for 5 min. After removing the PBS, nuclear proteins were extracted using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents following the provided instructions (Thermo Fisher Scientific, 78833). Finally, the nuclear proteins were collected in a clean, labeled tube and kept at -80 °C after protein was quantified. In this study, the 3T3 cells used were between 20 and 40 culture passages.

Western Blot Analysis

Protein samples (30 μ g) were separated by SDS-PAGE. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Pierce) and blocked with 5 % solution of nonfat dry milk in PBS-0.1 % Tween 20 (PBS-T). The membranes were incubated with anti-H3K9me2 (1:1000), anti-G9a (1:1000) at 4 °C overnight, and then washed three times (10 min each) with PBS-T buffer. Membranes were then incubated with an appropriate secondary antibody at a concentration of 1:2500 for 1 h at room temperature. Following extensive washing of the membrane, the immunoreactive bands were visualized by SuperSignal® West Dura Extended Duration Substrate or Pierce® ECL Western Blotting Substrate (Thermo Scientific). The membranes were then stripped and re-probed for GAPDH at a concentration of 1:20,000 as a control for sample loading.

X-ray films of Western blots were scanned and analyzed using ImageJ software. First, the background staining density for each band was subtracted from the band density. Next, the probing protein/GAPDH ratio was calculated from the band densities run on the same gel to normalize for differences in protein loading. Finally, the difference in the ratio of the control and experimental bands was tested for statistical significance. Four samples were used for each group in all Western blotting experiments.

Statistical Analyses

Data were analyzed using IBM SPSS Statistics Premium V21 and GraphPad software for Windows. Biological sample sizes were determined based on the variability of measurements and the magnitude of the differences between groups, as well as experience from our previous studies, with stringent assessments of difference. Data of OHC loss along the length of the cochlear spiral were analyzed with repeated measures one-way analysis of variance (ANOVA) with Tukey's multiple comparisons using IBM SPSS Statistics. The remaining analysis was done using GraphPad. Differences with multiple comparisons were evaluated by one-way ANOVA with multiple comparisons. Differences for single-pair comparisons were analyzed using two-tailed unpaired Student's *t* tests. Data for relative ratios of single-pair comparisons were analyzed with one-sample *t* tests. A *p* value <0.05 was considered statistically significant. Data are presented as means $\pm$ SD or SEM based on the sample size and variability within groups. Sample sizes are indicated for each figure.

RESULTS

Treatment with BIX 01294 Prevents Noise-Induced Loss of Outer Hair Cells and Inner Hair Cell Ribbons and Attenuates Auditory Threshold Shifts

The mice tolerated treatments of BIX 01294 well. Body weights and appearance of fur were unaffected by the treatment and there were no changes in auditory thresholds after BIX 01294 or control injections with the vehicle DMSO (0.5 mL/kg DMSO). Noise exposure led to loss of OHCs and auditory threshold shifts in agreement with our previous studies (Yuan et al. 2015; Hill et al. 2016). Concomitant treatment with BIX 01294 significantly reduced OHC loss along the cochlear epithelium by about 50 % when assessed 14 days after the exposure ($F_{1, 16} = 381.33$, $p < 0.001$, Fig. 1a, b). The loss of OHCs was completely blocked in the 3.5-mm region of the cochlear spiral and significantly reduced at 4–5.5 mm from the apex (Fig. 1b). Treatment with BIX 01294

also significantly attenuated noise-induced auditory threshold shifts at both 16 ($t_{26} = 8.164$, $p < 0.0001$) and 32 kHz ($t_{26} = 7.122$, $p < 0.0001$) with an average reduction of 23 and 28 dB, respectively, 14 days after the exposure (Fig. 1c).

Furthermore, we assessed the numbers of IHC presynaptic ribbons at 1 and 14 days after the completion of noise exposure. First, we counted ribbons 14 days after the noise exposure in five frequency regions (5, 8, 16, 22, and 32 kHz) for three groups (no noise + DMSO, 101 dB + DMSO, and 101 dB + BIX 01294). Synaptic ribbon counts were significantly different in the 32-kHz region ($F_{2, 21} = 9.787$, $p = 0.001$) and a potential difference was seen at 22 kHz ($F_{2, 25} = 3.297$, $p = 0.053$) but not in 5-, 8-, and 16-kHz regions by one-way ANOVA analysis (Fig. 2a, b). Further analysis confirmed that noise exposure induced significant ribbon loss in both 32- ($p = 0.0003$) and 22-kHz ($p = 0.02$) regions. Co-treatment with BIX 01294 nearly completely prevented noise-induced loss

FIG. 1. Treatment with BIX 01294 prevents noise-induced loss of outer hair cells and reduces auditory threshold shifts. **a** Representative images of DAB staining in myosin VII-labeled OHCs at 4 mm from the apex, which is prevented by treatment with BIX 01294. Scale bar = 10 μ m. **b** Quantification of noise-induced loss of OHCs along the cochlear spiral confirms significant reduction by BIX

treatment. The distance along the cochlear duct correlating with the frequencies of 8, 16, and 32 kHz is indicated. Data are presented as means $\pm$ SD, $n = 6$. **** $p < 0.0001$. **c** Treatment with BIX significantly attenuated noise-induced threshold shifts at both 16 and 32 kHz 14 days after the noise exposure. Data are presented as individual points and means $\pm$ SD. **** $p < 0.0001$

of ribbons, bringing the ribbon number per IHC close to that of non-noise-exposed controls in both 32- and 22-kHz regions (Fig. 2b). Additionally, the non-exposed groups (naïve, DMSO alone, and BIX 01294 alone) showed similar ribbon numbers at all five analyzed frequencies (5, 8, 16, 22, and 32 kHz). In order to determine if treatment with BIX 01294 could attenuate noise-induced loss of ribbons at an earlier time point, we also counted the ribbons 1 day following the noise exposure. CtBP2-labeled presynaptic ribbons on IHCs in the 22–32-kHz region

decreased from 16 to 9 per IHC ($t_6 = 3.749$, $p = 0.009$), whereas co-treatment with BIX 01294 preserved synaptic ribbon counts at 14 per IHC ($t_8 = 2.935$, $p = 0.019$, Fig. 2c).

We then measured the ABR peak I amplitudes at 16 kHz, which is one of the most sensitive frequencies of the auditory spectrum in mice, and no loss of OHCs was seen under the three treatment conditions (no noise + DMSO, 101 dB + DMSO, and 101 dB + BIX 01294). These amplitudes reflect the summed activity of auditory nerve fibers (inferring function of

FIG. 2. Treatment with BIX 01294 attenuates noise-induced loss of synaptic ribbons and inhibition of wave I amplitudes. **a** Representative images of immunolabeling for CtBP2 as examined 14 days after the completion of noise exposure. Images were compressed from 40 Z-stack projections taken from the basal turn corresponding to the area of 32 kHz. Blue: myosin VIIa-labeled IHCs; red: CtBP2-labeled synaptic ribbons and nuclei of IHCs; scale bar = 10 μ m. **b** Quantification of CtBP2-immunolabeled ribbon particles in IHCs in locations corresponding to 5, 8, 16, 22, and 32 kHz showed noise-reduced ribbon counts at 22 and 32 kHz, while treatment with BIX 01294 prevented noise-induced loss of ribbons. The distance along the cochlear duct correlating with the frequency regions is indicated. Data from the DMSO group were combined with that of the naïve and BIX 01294 alone groups, as these three groups were not exposed to noise. Data are presented as means $\pm$ SEM, n = number of mice

per group with one ear used per mouse. * $p < 0.05$, *** $p < 0.001$ for DMSO only vs 101 dB + DMSO, # $p < 0.05$ for 101 dB + DMSO vs 101 dB + BIX. **c** Noise-induced reduction of synaptic ribbon density per IHC at 22–32 kHz was prevented by BIX treatment when evaluated 1 day after the completion of the exposure. Data are presented as means $\pm$ SD. The number in each black bar is the n of mice with one ear used per mouse, * $p < 0.05$, ** $p < 0.01$. **d** Treatment with BIX prevented reduction of wave I amplitudes induced by noise at 16 kHz when tested 14 days after the noise exposure. Data from the DMSO group were combined with that of naïve and BIX-alone groups. Data are presented as means $\pm$ SEM; n = number of mice with one ear used per mouse, **** $p < 0.0001$ for DMSO vs 101 dB + DMSO, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.0001$ for 101 dB + DMSO vs 101 dB + BIX 01294

TABLE 1

Post hoc testing for data of Fig. 2d (ABR wave I amplitudes)

Frequency (kHz)	Groups	Sound intensity (dB)	<i>p</i> value	<i>p</i> value key
16	DMSO vs 101 dB + DMSO	30	$p < 0.0001$	****
		40	$p < 0.0001$	****
		50	$p < 0.0001$	****
		60	$p < 0.0001$	****
		70	$p < 0.0001$	****
		80	$p < 0.0001$	****
	101 dB + Saline vs 101 dB + BIX 01294	30	$p = 0.003$	**
		40	$p = 0.005$	**
		50	$p < 0.0001$	****
		60	$p < 0.024$	*
		70	$p = 0.0005$	***
		80	$p = 0.01$	**

synapses) if no losses of OHCs are present. Fourteen days after the noise exposure, the wave I amplitudes were significantly different between the three groups by one-way ANOVA analysis (60 dB: $F_{2, 30} = 27.18$, $p < 0.0001$; 70 dB: $F_{2, 30} = 38.40$, $p < 0.0001$; 80 dB: $F_{2, 30} = 13.23$, $p < 0.0001$, Fig. 2d). Further analysis revealed that noise exposure significantly decreased wave I amplitudes for sound stimuli from 30 to 80 dB SPL compared to non-exposed samples, whereas treatment with BIX 01294 significantly attenuated this noise-induced decrease by 50 % overall (for detailed statistical values, see Table 1). These results

demonstrate that treatment with BIX 01294 prevents noise-induced losses of ribbons and OHCs and NIHL.

Pretreatment with G9a siRNA Attenuates Noise-Induced Hearing Loss

To assess whether the action of BIX 01294 was mediated by G9a, we silenced the *EHMT2* gene with G9a small interfering RNA (siG9a) in CBA/J mice and 3T3 cells. Based on our previous experiments with siRNA, a 0.6- μ g dose of siG9a or siControl (scrambled siRNA) was locally delivered to the mouse middle ear intra-tympanically

FIG. 3. Treatment with siG9a reduces G9a expression in OHCs and 3T3 cells. **a** Immunolabeling for G9a (green) in OHC nuclei decreases with siG9a treatment. Images were taken from the basal turn. PI: propidium iodide. Scale bar = 10 μ M. **b** Quantification of immunolabeling for G9a revealed significant reduction. Data are

presented as means + SD, $n = 4$ mice (left ears), $**p < 0.01$. **c** Western blots using nuclear protein revealed a single band for G9a at 170 kDa and reduced G9a band density with G9a siRNA in 3T3 cells. Data are presented as means + SD, $n = 5$, $**p < 0.01$

Fig. 4. Pretreatment with siG9a attenuates noise-induced loss of outer hair cells and auditory threshold shifts at 16 kHz, but not at 32 kHz, when measured 14 days after the exposure. **a** Noise-induced OHC loss was reduced by siG9a treatment. Data are presented as means + SD; $n=5$ in each group with one ear used per mouse, ** $p<0.01$, *** $p<0.001$. **b** Noise-induced auditory threshold shifts at 16 kHz were significantly reduced by treatment with siG9a but not at 32 kHz. Data are presented as individual points and means $\pm$ SD, ** $p<0.01$.

(Zheng et al. 2014; Han et al. 2015; Hill et al. 2016). Expression of G9a in OHCs on surface preparations was assessed 72 h after the local delivery. Treatment with siG9a showed a 25 % reduction of immunolabeling for G9a in

OHCs compared to the siControl group ($t_3=6.9136$, $p=0.006$, Fig. 3a, b). Additionally, we also checked if siG9a effectively inhibits G9a protein production using transfection of siG9a into 3T3 cells. Western blot analysis of nuclear proteins from 3T3 cell homogenates showed a 33 % ($t_4=4.7816$, $p=0.0088$) reduction of G9a band density with a single band at 170 kDa in siRNA-transfected cells (Fig. 3c). Local intra-tympanic delivery of siRNA results in temporary elevation of thresholds that completely recovers by 72 h (Oishi et al. 2013). Therefore, noise exposure was performed 72 h after siRNA delivery. Treatment with siG9a significantly attenuated noise-induced loss of OHCs compared to treatment with the siControl ($F_{1,8}=20.773$, $p=0.002$, Fig. 4a) when examined 14 days after the exposure. The reduction of OHC loss was seen from 3.5 to 5.5 mm from the apex ($p<0.001$ from 3.5 to 5 mm, $p=0.002$ at 5.5 mm). Additionally, noise-induced auditory threshold shifts were significantly attenuated at 16 kHz ($t_{18}=3.292$, $p=0.004$, Fig. 4b), but there was no difference at 32 kHz ($t_{14}=1.695$, $p=0.11$). These results imply that pretreatment with siG9a partially attenuates noise-induced permanent hearing loss via the inhibition of G9a.

Noise Exposure Increases G9a in Cochlear Cells of the Basal Turn

Since inhibition of G9a by either BIX 01294 or siG9a attenuates NIHL, we next assessed G9a protein levels with immunohistochemistry on paraffin sections 1 h after noise exposure. Immunoreactivity of G9a increased in the nuclei of OHCs (rectangular box, insert of enlarged images from OHCs), IHCs (arrow-head), and supporting cells of the organ of Corti (OC, Fig. 5), the nuclei of SGN (Fig. 5), and the nuclei of marginal cells in lateral wall tissues (LW, Fig. 5). Incidentally, some labeled G9a also appeared in the cytosol of OHCs (OC, Fig. 5). To further assess the expression of G9a in the nuclei of OHCs, we performed immunolabeling for G9a on cochlear surface preparations 1 and 24 h after completion of the 101-dB noise exposure. Immunoreactivity for G9a was stronger in the nuclei of OHCs in the basal turn at 1 h and remained stable at 24 h after the exposure (Fig. 6a). There were no changes in the nuclei of OHCs in the apical and middle turns. Quantification of G9a immunolabeling in OHC nuclei around the 32-kHz region confirmed a significant increase by about 50 % at both 1 h ($t_2=4.496$, $p=0.046$) and 24 h ($t_2=15.62$, $p=0.004$) (Fig. 6b).

Noise Exposure Modifies H3K9me2 in Cochlear Cells of the Basal Turn

Since a primary function of G9a is to dimethylate lysine 9 of histone H3 (H3K9me2), we evaluated levels

FIG. 5. Noise exposure increases immunolabeling for G9a in the basal turn of the cochlea. **a** Paraffin sections of the adult CBA/J mouse inner ear revealed an increase in immunolabeling for G9a (brown) in OHCs (rectangular box) and IHCs (arrowhead) in the organ of Corti (OC), spiral ganglion neurons (SGN), and marginal

cells (arrow) of the stria vascularis in the lateral wall (LW) of the basal turn when examined 1 h after completion of the noise exposure. These images were taken with $\times 40$ magnification lens and are representative of four individual mice in each group; scale bar = 10 μm

of H3K9me2 in the cochlea 1 h after completion of the noise exposure. Immunolabeling for H3K9me2 on cryosections showed clear labeling in the nuclei of marginal cells of the stria vascularis (Stria, arrow, Fig. 7a) and some of the SGNs (Fig. 7a). However, immunolabeling for H3K9me2 in OHCs was inconsistent between cryosections. We therefore analyzed immunolabeling for H3K9me2 in hair cells on cochlear surface preparations. In control mice without noise exposure, there was immunoreactivity for H3K9me2 in the nuclei of IHCs and Deiters cells but not in OHCs (Fig. 7b). At 1 h after the completion of noise exposure, immunolabeling for H3K9me2 was seen in some OHC nuclei in the basal turn, more prevalently in the lower base (45 kHz) than the upper base (32 kHz) (Fig. 7c). In contrast, there was no immunolabeling for H3K9me2 in the nuclei of OHCs in the apical and middle turns. Additionally, there

were no obvious changes in H3K9me2 in IHCs and Deiters cells. These results suggest that the noise exposure modifies H3K9me2 in OHCs, SGNs, and stria marginal cells in the basal turn of the cochlea. Additionally, Western blots for H3K9me2 from whole cochlear homogenates revealed a single band at 17 kDa without a difference in the band-densitometry between control and noise-exposed mice when assessed at 1 h after the completion of the noise exposure (Fig. 7d).

The Noise Exposure-Induced Decrease in KCNQ4 in OHCs Is Prevented by Treatment with BIX 01294

Since G9a is essential for epigenetic silencing of potassium channel genes (Laumet et al. 2015), we tested whether treatment with BIX 01294 affects

FIG. 6. Noise exposure increases immunolabeling for G9a in outer hair cells of the basal turn. **a** Immunolabeling for G9a (green) in OHCs of surface preparations co-stained with propidium iodide (PI; red) for nuclei was stronger at 1 h and remained stable at 24 h after the noise exposure. The inset boxes in merged images show three OHCs enlarged to provide a better view of G9a immunolabeling. Images were taken from the area of the basal turn corresponding to

22–32 kHz; $n=3-6$ mice, scale bar = 10 μm . **b** Quantification of immunolabeling for G9a in OHCs in the 22–32-kHz region showed a significant increase at 1 h after and was stable at 24 h after completion of the exposure. Data are presented as means + SD. The number in each black bar is the n of mice used with one ear used per mouse. * $p<0.05$, ** $p<0.01$

KCNQ4 levels in OHCs after noise exposure. One hour after completion of noise exposure, the expression of KCNQ4 in OHCs of the basal turn was significantly reduced (Fig. 8a, b). Our data showed that 40 mg/kg of BIX 01294 had significantly reduced noise-induced loss of OHCs and NIHL. We used this dose for three IP injections (24 h, 30 min before and immediately after noise exposure) and evaluated KCNQ4 immunolabeling in OHCs 1 h following the exposure. Co-treatment with BIX 01294 prevented the decrease in KCNQ4 expression in OHCs (Fig. 8a). Quantification of KCNQ4 immunolabeling in OHCs in the 22–32-kHz region

confirmed a 75 % decrease in KCNQ4 following the exposure ($t_4=20.9631$, $p<0.0001$), and a significant prevention of this decrease was afforded by treatment with BIX 01294 ($t_9=6.3066$, $p=0.0001$, Fig. 8b). This result implies that activation of G9a silences KCNQ4 genes.

DISCUSSION

A salient finding of this study is that noise exposure induces epigenetic modification in the cochlea. As a consequence, modification of histones alters gene

FIG. 7. Noise exposure increases immunolabeling for H3K9me2 in nuclei of outer hair cells, stria marginal cells, and spiral ganglion neurons. **a** All examined cryosections from the basal turn showed that immunolabeling for H3K9me2 increased in nuclei of stria marginal cells (Stria, arrow) and some SGNs 1 h after completion of the noise exposure. Representative images were merged from both green (H3K9me2) and red (PI for staining nuclei) images; Stria: stria vascularis, SGN: spiral ganglion neurons, $n=4$ mice. Scale bar = 10 μm . **b** Surface preparations of control mice without noise exposure showed no H3K9me2 immunolabeling in OHCs, but

H3K9me2 appeared in IHCs and Deiters cells (DCs) along the entire cochlear spiral. Representative images were taken from the basal turn. Scale bar = 10 μm . **c** Noise exposure induces immunolabeling for H3K9me2 in some OHC nuclei in the basal turn, more prevalently in the lower base (45 kHz) than the upper base (32 kHz). Scale bar = 10 μm , $n=4$ mice with one ear used per mouse. **d** Western blots using total cochlear homogenates showed a single band for H3K9me2 at 17 kDa with no difference between control and noise-exposed samples when examined 1 h after the end of the noise exposure. $n=5$ mice per group

expression. In general, histone acetylation tends to be a permissive modification, resulting in loose chromatin more easily accessible to other proteins such as transcription factors. Conversely, methylation of histone tends to be a suppressive modification (Tachibana et al. 2002; Yokochi et al. 2009). The result that noise exposure activates G9a and increases immunoreactivity of H3K9me2 points to a suppression of gene expression by noise. Our previous results showing noise-induced increases in HDACs and

concomitant decreases of H3K9ac (Chen et al. 2016) support the notion that convergent histone modifications are involved in NIHL. The fact that blockade of G9a by treatment with BIX 01294 or G9a siRNA reduces noise-induced losses of OHCs and synaptic ribbons and attenuates NIHL suggests that epigenetic modifications are causal mechanisms in NIHL.

Noise exposure induces OHC loss in mice following a base-to-apex gradient with initial losses in the basal cochlear spiral (Wang et al. 2002; Chen et al.

FIG. 8. Noise-diminished immunolabeling for KCNQ4 in OHCs is prevented by treatment with BIX 01294. **a** Representative images showed immunolabeling for KCNQ4 (red) in OHCs when examined 1 h after 101-dB noise exposure. The counterstained Hoechst 33342 (original blue) in OHC nuclei were difficult to visualize. Therefore, the blue channel information was transferred to the green channel for

better visual identification of the focal plane. Scale bar = 10 μ m, n = 4–6 mice per group with one ear used per mouse. **b** Quantification of immunolabeled KCNQ4 in OHCs showed that noise-induced reduction of KCNQ4 is prevented by treatment with BIX. Data are presented as means + SD. The number in each black bar is the n of mice used. *** p < 0.001, **** p < 0.0001

2012; Zheng et al. 2014). In line with the notion that the activation of G9a results in demethylation of lysine 9 of histone H3, we see strong immunoreactivity for H3K9me2 in OHCs in the basal turn where cells are dying. The appearance of H3K9me2 in dying OHC nuclei more prevalently in the lower base than the upper base when examined 1 h after completion of noise exposure suggests a link between H3K9me2 and cell death. H3K9me2 is one of the prominent marks of transcriptional repression and is mediated by histone methyltransferase G9a, which is associated with silencing of a multitude of genes (Salzberg et al. 2017). The diminished immunoreactivity of KCNQ4 suggests that the *KCNQ4* gene might be one of the genes suppressed by noise exposure, a notion in agreement with a previous observation (Jaumann et al. 2012). The fact that inhibition of G9a by BIX 01294 prevents noise-

diminished KCNQ4 in OHCs places suppression of potassium channel genes into the pathological sequence of NIHL. Such a concept is in agreement with the literature showing that nerve injury upregulates G9a and HDACs and diminishes potassium channel expression in the dorsal root ganglion (Laumet et al. 2015). However, since damaged OHCs have reduced KCNQ4 expression, protective agents that reduce NIHL may preserve KCNQ4 expression. Of note, inhibition of G9a by BIX 01294 may also regulate other voltage-gated potassium channels after noise exposure. The detailed mechanisms need further investigation. Additionally, the treatment with the specific G9a inhibitor BIX 01294 reduces losses of not only OHCs but also IHC ribbon synapses and thus a potential latent hearing loss. Such synaptic preservation may also reflect preservation of overall IHC functional status.

Interestingly, in addition to the changes in G9a and H3K9me2 in OHCs, marginal cells appear to show the same response to noise. Marginal cells are important in maintaining the endocochlear potential (EP) (Fettiplace 2017), generating a positive transepithelial potential by secreting potassium ions. The changes seen in marginal cells may depend on the strain examined. A recent report showed that noise-exposed CBA/J mice results in temporary EP reduction, while the same is not true for C57B6 mice (Ohlemiller et al. 2018). Therefore, the strial effects of noise in the present findings may be characteristic for CBA/J mice but not of some other strains. Furthermore, SGNs exhibit a comparable response of an increase in G9a and H3K9me2. Thus, noise exposure broadly elicits posttranslational modification of genes in the key regions in the cochlea.

It should be noted that the actions of G9a are not restricted to catalyzing H3K9me2 for gene silencing. G9a also interacts with a large number of proteins, including NF- κ B family member RelB and growth factor independent 1 (Gfi1) protein for epigenetic silencing of transcription (Chen et al. 2009; Scheer and Zaph 2017). NF- κ B signaling is involved in inner ear trauma, including aminoglycoside- and noise-induced hearing loss (Jiang et al. 2005; Lang et al. 2006). Furthermore, Gfi1 is required for IHC development and survival (Wallis et al. 2003) and loss of *Gfi1* results in OHC degeneration (Hertzano et al. 2004). While these are potential scenarios, details of such interactions need to be further investigated. Worth noting is that BIX 01294 is a non-hazardous substance that also prevents aminoglycoside-induced ototoxicity (Yu et al. 2013). This specific inhibitor of G9a may be a promising protectant against inner ear insults and cochlear synaptopathy. Additionally, translocation of G9a out of the OHC nuclei to the cytoplasm might be associated with activation of G9a. Of note, it is a common phenomenon to find no changes in band density by Western blot analysis using whole cochlear homogenates. Since the noise-induced changes in molecular signals only occurred in the basal turn, such changes in specific regions are muted when assessed by Western blot and are diluted by other cochlear cell types (Han et al. 2015; Yuan et al. 2015; Hill et al. 2016).

In summary, our results reveal novel epigenetic mechanisms involved in the pathogenesis of noise-induced losses of OHCs and synaptic ribbons and NIHL. Pharmacological inhibition of G9a might provide a strategy for prevention or attenuation of noise-induced hair cell loss and hearing loss.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest There are no conflicts of interest for any of the authors.

The data contained in the manuscript have not been previously published, have not been submitted elsewhere, and will not be submitted elsewhere while under review.

Ethics Approval

All research protocols were approved by the Institutional Animal Care & Use Committee at the Medical University of South Carolina. Animal care was under the supervision of Division of Laboratory Animal Resources at MUSC.

All authors have reviewed the contents of the manuscript, approve of its contents, and validate the accuracy of the data.

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