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Exploring the efficacy of (R)-PFI-2 hydrochloride in mitigating noise-induced hearing loss by targeting NLRP3 inflammasome and NF-κB pathway to reduce inner ear inflammation

Dawei Ren [a,1], Xuemin Chen [b,c,1], Hongdong Liu [c,1], Menghua Li [d], Liting Zheng [d], Pan Yong [d], Mohe Huang [e,f], Xi Shi [d,e,f], Yice Xu [g], Shujin Chen [h,**], Yan Zhang [a,***], Wei Zhu [a,*]

[a] Department of Otorhinolaryngology, the First Hospital of Jilin University, Changchun, 130021, Jilin, China
[b] Department of Otorhinolaryngology, No. 971 Hospital of People's Liberation Army Navy, Qingdao, 266000, Shandong Province, China
[c] Senior Department of Otolaryngology-Head & Neck Surgery, Chinese PLA General Hospital, National Clinical Research Center for Otolaryngologic Diseases, State Key Lab of Hearing Science, Ministry of Education, Beijing Key Lab of Hearing Impairment Prevention and Treatment, Beijing, 100083, China
[d] Artificial Auditory Laboratory of Jiangsu Province, Xuzhou Medical University, Xuzhou, 221000, Jiangsu, China
[e] Key Laboratory of Tropical Biological Resources of Ministry of Education, School of Pharmaceutical Sciences, Hainan University, Haikou, 570228, Hainan Provine, China
[f] Song Li's Academician Workstation of Hainan University, School of Pharmaceutical Sciences, Yazhou Bay, Sanya,572000, Hainan Provine, China
[g] Department of Otolaryngology-Head and Neck Surgery, The Central Hospital of Xiaogan, Xiaogan, 432000, Hubei, China
[h] ENT Department, The People's Hospital of Rongchang District, Chongqing, 402460, China

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ABSTRACT

Noise-induced hearing loss (NIHL) is primarily driven by inflammatory processes within the cochlea, where noise exposure triggers the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome, leading to an inflammatory cascade. The interaction between increased NLRP3 expression and NF-κB activity can further amplify cochlear inflammation. Our findings reveal that (R)-PFI-2 hydrochloride, a selective inhibitor of the SETD7 enzyme, effectively inhibits the activation of the cochlear NF-κB pathway, suppresses the release of pro-inflammatory factors, and prevents inflammasome assembly. This intervention disrupts the perpetuating cycle of inflammation, thereby alleviating damage to cochlear hair cells attributed to acoustic trauma. Consequently, (R)-PFI-2 hydrochloride emerges as a promising pharmacological candidate for NIHL, targeting and moderating the excessive immune and inflammatory responses implicated in the pathology of hearing loss.

1. Introduction

Noise-induced hearing loss (NIHL) manifests as sensorineural impairment primarily due to the damage to hair cells within the inner ear, a consequence of prolonged exposure to harmful noise levels. NIHL represents a significant occupational hazard across various sectors, notably in military, construction, and manufacturing environments. The condition can be transient, known as a "temporary threshold shift" (TTS), where hearing levels may temporarily decline but potentially return to baseline. Conversely, if hearing levels do not revert post-exposure, the condition is termed a "permanent threshold shift" (PTS).

Apart from the direct mechanical impact on the auditory system, noise exposure initiates a plethora of secondary metabolic disturbances, including oxidative stress, calcium overload, vascular alterations, and immune/inflammatory responses. Early intervention in managing inner ear inflammation post-exposure is crucial for hearing conservation(Li et al., 2021), aiding in the recovery from TTS and preventing the progression to PTS. This emphasizes the role of inflammation within the inner ear as a fundamental molecular mechanism underpinning NIHL (Yang et al., 2016), thereby making the regulation of

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* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: 2720451951@qq.com (S. Chen), zhangyan99@jlu.edu.cn (Y. Zhang), zhuwei@jlu.edu.cn (W. Zhu).
[1] contributed equally.

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immune/inflammatory responses within the inner ear a critical aspect of NIHL management.

The inflammasome, specifically the NOD-like receptor protein 3 (NLRP3)inflammasome, is a key multiprotein complex involved in cytoplasmic signaling. It is composed of pattern recognition receptors (PRRs), the apoptosis-associated speck-like protein (ASC), and procaspase-1. Our previous research suggests that the NLRP3 inflammasome may become activated in the cochlea following noise exposure, exacerbating inflammation and contributing to cochlear damage. This activation, potentially initiated via Toll-like receptor (TLR) signaling pathways, plays a pivotal role in amplifying immune/inflammatory responses, thus constituting a crucial mechanism in the etiology of NIHL (Vethanayagam et al., 2016; Cai et al., 2014). Meanwhile, the PRRs detect both pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), potentially leading to cell damage or apoptosis, and thus to irreversible hearing loss due to the non-regenerative nature of cochlear hair cells (Zhou et al., 2011).

SET domain-containing lysine methyltransferase 7 (SETD7) has been identified for its lysine methyltransferase activity, initially recognized for its specificity in methylating H3K4 (Wang et al., 2001). Further investigations have extended the substrate range of SETD7 to include non-histone proteins such as p65 (Li et al.a) and Tau (Bichmann et al., 2021), highlighting its role in various physiological and pathological processes (Soshnikova and Liu, 2019). This includes transferring methyl groups to lysine or arginine residues, influencing gene expression, and regulating cell proliferation, differentiation, and apoptosis (Watanabe et al., 2008). The dysfunction of SETD7 has been implicated in the pathogenesis of diseases such as cancer, diabetes, and renal fibrosis. Notably, SETD7 acts as a positive regulator in the TNF-α-induced NF-κB signaling pathway activation (Wu et al., 2019), and its inhibition can diminish NF-κB pathway activation, reduce the release of inflammatory cytokines, and inhibit NLRP3 inflammasome activation (Chao et al., 2022; Shen et al., 2019; Pan et al., 2022; He et al., 2015).

In light of these insights, this study hypothesizes that SETD7 represents a potential therapeutic target for NIHL. Utilizing (R)-PFI-2 hydrochloride, identified as a potent, selective inhibitor of SETD7, this research elucidates the auditory protective effects of (R)-PFI-2 hydrochloride in a mouse model of noise-induced deafness. Our findings indicate that the inhibition of SETD7 effectively blocks the activation of NLRP3 and NF-kB signaling pathways in inner ear hair cells, thereby optimizing the cochlea's immune microenvironment. This positions SETD7 as a novel drug target for addressing NIHL.

2. Materials and methods

2.1. Experimental design and animal grouping

Specific pathogen-free (SPF) C57BL/6J male mice, aged 4–5 weeks, were procured from Speibio (Beijing) Biotechnology Co., Ltd. Following a 3-day acclimatization period to mitigate stress and the impact of transportation noise, auditory brainstem response (ABR) testing screened for auditory impairments, excluding mice with hearing deficits. Sixteen mice with confirmed normal hearing were randomly allocated into two groups for the noise exposure study: a noise exposure group (NE) and a control group (Ctrl), each comprising 8 mice. For the pharmacological intervention study, 24 mice with normal hearing were similarly selected and randomly divided into three groups: a control group (Ctrl), a noise-exposed group treated with saline (NE + saline), and a noise-exposed group treated with (R)-PFI-2 hydrochloride (NE + PFI-2), with 8 mice per group. All treatment groups received intraperitoneal injections.

2.2. Noise exposure and treatment protocol

The NE group was exposed to 120 dB SPL broadband white noise for 4 h in two batches of 4 mice each. Noise intensity was verified using a sound level meter. Mice were placed in specialized cages with overhead speakers. Day 0 (NE-0d) marked the conclusion of noise exposure, followed by ABR assessments on days 4 (NE-4d) and 7 (NE-7d). Subsequently, mice were euthanized for cochlear extraction and analysis. In the pharmacological study, the NE + saline and NE + PFI-2 groups underwent identical noise exposure. Starting from day 0 (NE-0d), these groups received daily intraperitoneal injections of saline or (R)-PFI-2 hydrochloride (10 mg/kg) sourced from Shanghai Taoshu Biotech Co., Ltd., respectively, for one week. Auditory Brainstem Response (ABR) tests were performed under anesthesia on days 4 and 7 post-exposure, including the control (Ctrl) group. After the final ABR test, the mice were euthanized for cochlear analysis.

2.3. Auditory brainstem response (ABR) assessment

ABR testing was conducted on noise-exposed mice anesthetized with 1% pentobarbital sodium (10 μL/g, IP). The testing protocol included frequency-specific threshold determinations, starting at 90 dB SPL and decreasing in 5–10 dB steps until the waveform disappeared, then increased by 5 dB to confirm threshold consistency. Wave I amplitude and latency at 90 dB SPL were recorded for each frequency. Assessments were carried out on days 4 and 7 post-exposure for all groups.

2.4. Immunofluorescence staining procedure

Cochlear sections from four mice per group were prepared for immunofluorescence staining. Following anesthesia, the mice underwent cardiac perfusion with 4% paraformaldehyde. The cochleae were then fixed and decalcified in 10% EDTA for 3 days. After dehydration, clearing, and paraffin embedding, 4 μm sections were stained. The protocol included dewaxing, antigen retrieval, blocking, overnight primary antibody incubation with NLRP3 (NBP2-12446, Novus Biologicals) and p65 (ab16502, Abcam), secondary antibody incubation, and DAPI counterstaining. Imaging was performed using an LSM980 laser confocal microscope, and image analysis was conducted with ImageJ software.

2.5. Western Blot analysis

Cochlear proteins from four mice per group were analyzed via Western blot. After extraction with RIPA lysis buffer and concentration measurement using a BCA protein assay, proteins (20–30 μg per lane) were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked and incubated overnight at 4 °C with primary antibodies against NLRP3 (NBP2-12446, Novus Biologicals) , phosphorylated p65 (ab86299 , Abcam) , and β-actin (ab8227 , Abcam) , followed by HRP-conjugated secondary antibody incubation. Development was achieved with ECL, and band densitometry was analyzed using Image J software.

2.6. Statistical analysis

Statistical evaluations were performed using GraphPrism 9.0 software. Differences between the two groups were analyzed using the *t*-test, while one-way ANOVA was employed for comparisons among three groups. Significance levels were denoted as ****P $<$ 0.0001, ***P $<$ 0.001, **P $<$ 0.01, *P $<$ 0.05. Image processing was conducted using Image J (National Institutes of Health) and Adobe Illustrator (Adobe Company, California, USA).

3. Results

3.1. Impact of noise exposure on the auditory system in mice

In this study, male C57BL/6J mice, aged 4–5 weeks, were subjected to high-intensity broadband noise at 120 dB SPL for a duration of 4 h.

Auditory Brainstem Response (ABR) assessments were performed on days 4 (NE-4d) and 7 (NE-7d) post-exposure, alongside a control group evaluated within the same timeframe to determine auditory conditions after noise exposure. Measurements included thresholds for click stimuli and short pure tones across frequencies of 4k/8k/16k/24k/32K, alongside latency and peak-to-trough amplitude of Wave I. By day 4 post-exposure (Fig. 1A), ABR thresholds across all tested frequencies in the noise-exposed group (NE-4d) were significantly elevated compared to the control group (Ctrl), indicating statistical significance ($P < 0.05$). Wave I latency exhibited prolongation, with statistically significant differences observed at Click and 32k frequencies ($P < 0.05$). A decline in Wave I peak-to-trough amplitude was noted, particularly at the 4k frequency, demonstrating statistical significance ($P < 0.05$). By day 7 post-exposure (Fig. 1B), the noise-exposed group (NE-7d) exhibited significantly heightened ABR thresholds across all frequencies in comparison to the control group (Ctrl), affirming statistical significance ($P < 0.05$). Again, Wave I latency was extended, with significant differences at Click and 32k frequencies ($P < 0.05$), and a reduction in Wave I peak-to-trough amplitude was specifically significant at the Click frequency ($P < 0.05$) (Fig. 1).

On day 4 (NE-4d) (A) and day 7 (NE-7d) (B) post-noise exposure, the auditory threshold, latency, and wave I peak-to-trough amplitude were assessed at various frequencies in the mice.

3.2. (R)-PFI-2 hydrochloride mitigates noise-induced hearing loss

SETD7, implicated in gene expression modulation and cellular processes including proliferation, differentiation, and apoptosis, emerges as a pivotal factor in disease pathogenesis. The primary pathological mechanism of noise-induced hearing loss encompasses immune/inflammatory responses. Literature indicates SETD7 as an enhancer of the TNF-α-induced NF-κB inflammatory signaling pathway; its inhibition could diminish NF-κB pathway activation, curtail inflammatory cytokine release, and attenuate NLRP3 inflammasome activation, positioning SETD7 as a viable target for therapeutic intervention in noise-induced hearing loss. Utilizing (R)-PFI-2 hydrochloride, a selective inhibitor of SETD7, in vitro assays demonstrated its protective efficacy against hydrogen peroxide-induced cytotoxicity in HEI-OC1 cells. Post-noise exposure, mice were administered (R)-PFI-2 hydrochloride intravenously on a daily regimen, in contrast to the control group, which received saline injections. ABR evaluations conducted on days 4 and 7 post-treatment revealed that (R)-PFI-2 hydrochloride administration significantly preserved hearing thresholds across various frequencies in noise-exposed mice, notably at Click, 4k, and 8k frequencies ($P < 0.05$) on day 4 (Fig. 2A). Most frequencies showed reduced Wave I latency, with a notable statistical difference at the Click frequency ($P < 0.05$), and an elevation in Wave I peak-to-trough amplitude, particularly significant at the Click frequency ($P < 0.05$). By day 7 (Fig. 2B), significant improvements in hearing thresholds were observed only at Click and 8k frequencies ($P < 0.05$) within the (R)-PFI-2 hydrochloride treatment group (NE + PFI-2), suggesting a potential for spontaneous recovery in the saline-treated group's hearing thresholds. Reductions in Wave I latency were recorded, with a significant difference at the Click frequency ($P < 0.05$), and an increase in Wave I peak-to-trough amplitude, notably at the 8k frequency ($P < 0.05$), underscoring the therapeutic efficacy of (R)-PFI-2 hydrochloride in mitigating early stages of noise-induced hearing loss by attenuating cochlear inflammation and facilitating hearing recovery (Fig. 2).

Changes in auditory threshold, latency, and wave I peak-to-trough amplitude at various frequencies were evaluated on day 4 (NE-4d) (A) and day 7 (NE-7d) (B) post-noise exposure in the (NE + PFI-2) treatment group compared to the (NE + saline) treatment group.

3.3. Suppression of NLRP3 expression in noise-induced hearing loss by (R)-PFI-2 hydrochloride

Prior research has demonstrated that noise exposure elevates NLRP3 inflammasome complex expression within the murine cochlea, activating caspase-1 and triggering the release of inflammatory mediators IL-1β and IL-6. Consequently, the NLRP3 inflammasome is identified as a critical component in cochlear inflammatory responses and a potential mechanism underlying noise-induced hearing loss. Our investigations affirm the significance of the NLRP3 inflammasome in the pathogenesis of noise-induced hearing loss. Given its high selectivity as a SETD7

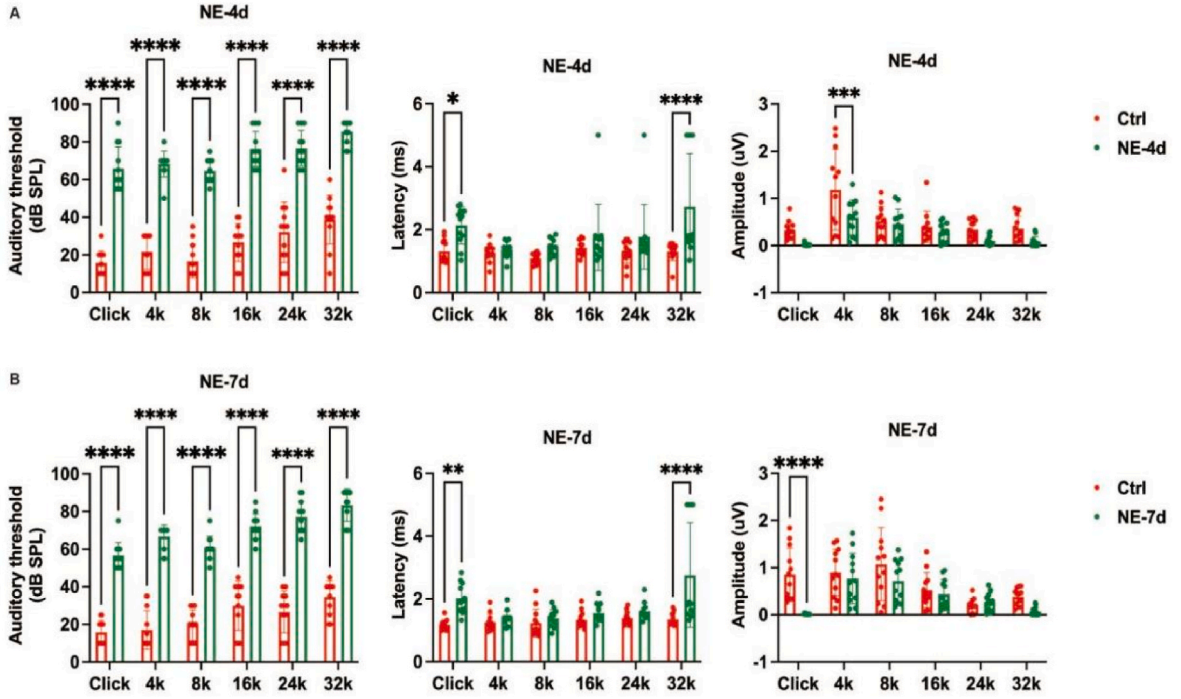

Fig. 1. Changes in ABR Measurements following noise exposure.

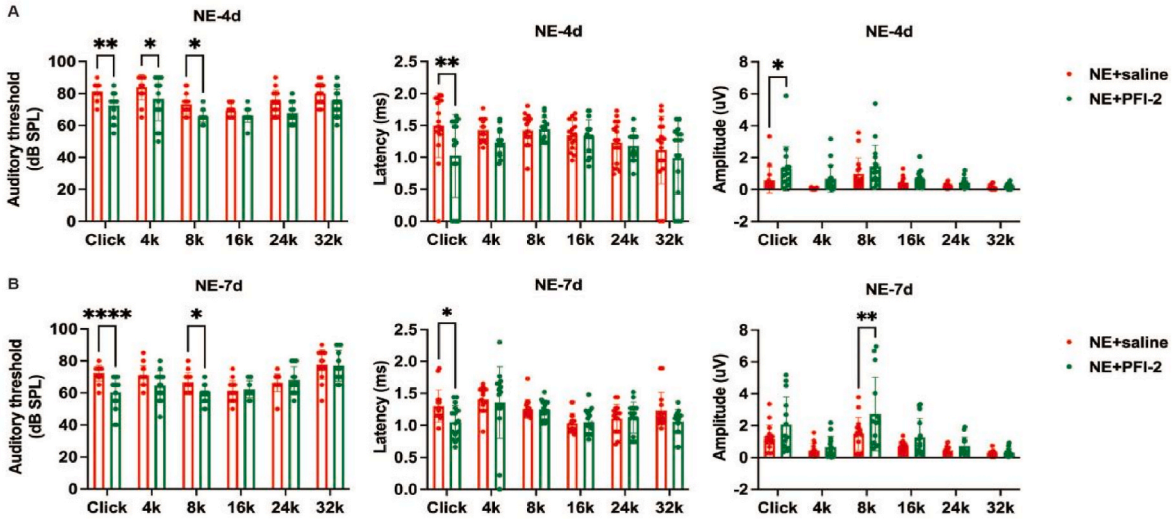

Fig. 2. Therapeutic effects of (R)-PFI-2 hydrochloride on noise-induced hearing loss.

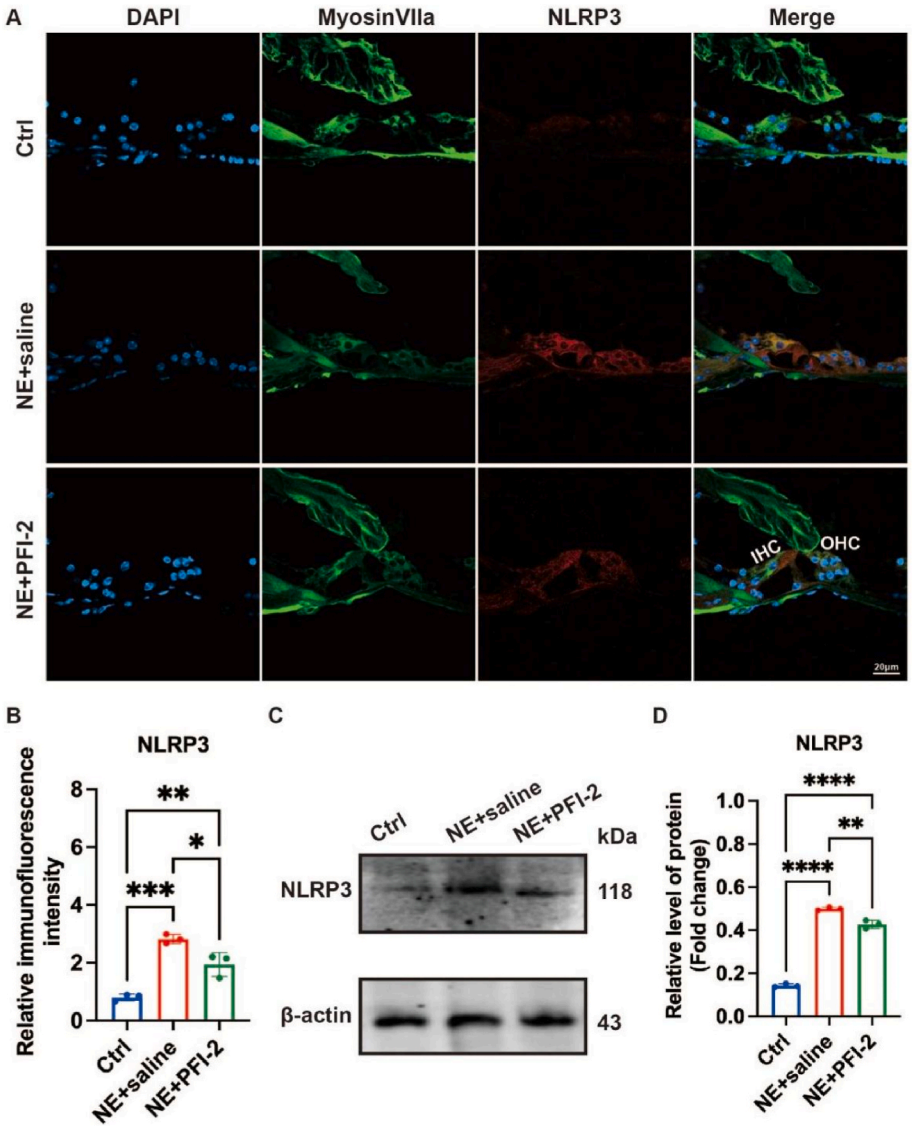

Fig. 3. Impact of (R)-PFI-2 hydrochloride on cochlear NLRP3 expression in mice post-noise exposure.

inhibitor, (R)-PFI-2 hydrochloride exhibits therapeutic potential against noise-induced hearing loss. To elucidate the mechanism of action of (R)-PFI-2 hydrochloride and its capacity to inhibit NLRP3 activation through SETD7 inhibition, immunofluorescence, and Western blot analyses were conducted (Fig. 3), utilizing cochlear samples from the Ctrl group, NE + saline group, and NE + PFI-2 group on day 7 post-exposure (NE-7d). Immunofluorescence findings indicated a marked increase in NLRP3 expression within the NE + saline group post-noise exposure, with localization in inner and outer hair cells, as well as spiral ganglion cells. NLRP3 expression in the NE + PFI-2 group was significantly reduced compared to the NE + saline group but remained elevated above the Ctrl group levels (Fig. 3A). Immunohistochemical analysis corroborated an upsurge in NLRP3 protein expression post-noise exposure, which was substantially mitigated in the NE + PFI-2 group relative to the NE + saline group, yet exceeded Ctrl group levels (Fig. 3C). Statistical analysis of fluorescence and grayscale values (Fig. 3B and D)

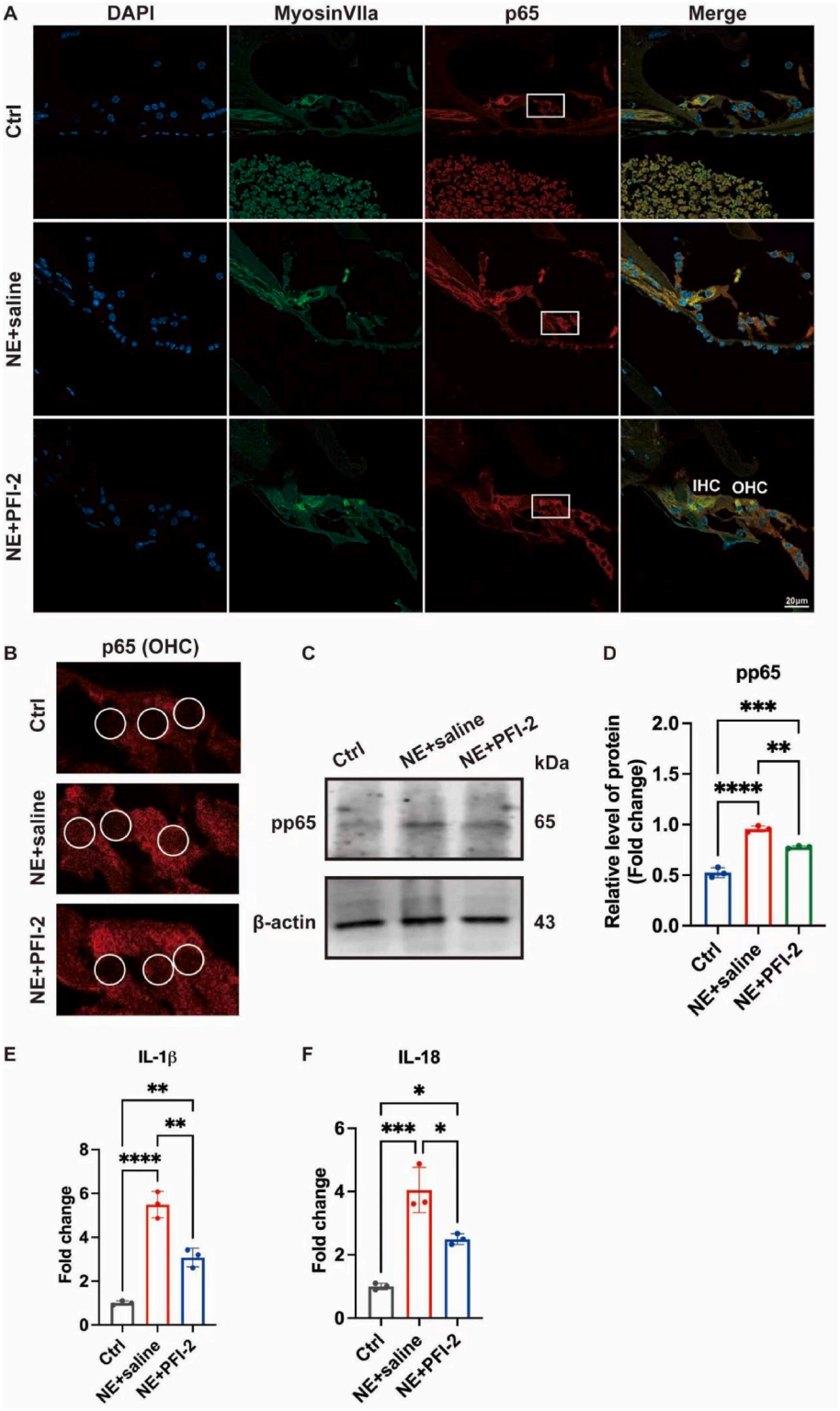

Fig. 4. Effects of (R)-PFI-2 Hydrochloride on Cochlear p65 and Cytokine Expression in Mice Following Noise Exposure.

highlighted profound differences among the groups ($P < 0.05$), indicating that (R)-PFI-2 hydrochloride effectively diminishes NLRP3 protein expression and impairs the assembly and activation of the NLRP3 inflammasome, thereby interrupting the cochlear inflammatory cascade.

On day 7 post-noise exposure (NE-7d), changes in NLRP3 expression were analyzed in cochlear sections from the Ctrl, NE + saline, and NE + PFI-2 groups. (A) Immunofluorescence staining of paraffin-embedded cochlear sections was used to assess NLRP3 expression. (C) NLRP3 protein levels were evaluated through immunohistochemistry. (B, D) Fluorescence intensity and gray values were recorded and subjected to statistical analysis for each group.

3.4. Modulation of P65 expression in cochlear hair cells by (R)-PFI-2 hydrochloride in noise-induced deafness

Considering that noise-induced cochlear damage invokes immune/inflammatory responses through the NF-κB pathway, SETD7, which is identified as a facilitator of TNF-α-induced NF-κB pathway activation, plays a role in mediating inflammatory responses. Cochlear samples from the Ctrl group, NE + saline group, and NE + PFI-2 group were collected on day 7 post-exposure (NE-7d) for further analysis. Immunofluorescence and Western blot assays assessed the nuclear localization of NF-κB and the expression of phosphorylated p65 (PP65), a pivotal component of the NF-κB signaling cascade, in the cochlear tissues across the three groups (Fig. 4). Immunofluorescence observations revealed no significant upregulation of cytoplasmic p65 in outer hair cells post-noise exposure; however, a notable augmentation in nuclear p65 fluorescence was detected, indicating an active state. This nuclear fluorescence was reduced in the NE + PFI-2 group compared to the NE + saline group. Western Blot analysis (Fig. 4C) confirmed an increase in pp65 expression post-noise exposure, with a discernible reduction in the NE + PFI-2 group relative to the NE + saline group. Statistical evaluation of gray-scale values (Fig. 4D) evidenced significant intergroup disparities ($P < 0.05$), suggesting that (R)-PFI-2 hydrochloride can effectively curtail the cochlear inflammatory response by inhibiting nuclear translocation of p65, thereby offering a therapeutic advantage in the management of noise-induced deafness.

To corroborate these findings, Western Blot was employed to assess the expression levels of downstream inflammatory cytokines IL-1β and IL-18 in cochlear tissues across the three groups. Western Blot results demonstrated increased expression of IL-1β and IL-18 following noise exposure, with IL-1β showing a particularly notable increase. However, the NE + PFI-2 group exhibited reduced levels of these cytokines compared to the NE + saline group. Gray values were recorded and subjected to statistical analysis (Fig. 4E,F), revealing highly significant differences between groups ($P < 0.05$). These results suggest that (R)-PFI-2 hydrochloride mitigates inner ear inflammation by reducing the release of inflammatory cytokines.

On day 7 post-noise exposure (NE-7d), cochlear tissues were collected from the Ctrl, NE + saline, and NE + PFI-2 groups. (A, B) Immunofluorescence analysis of p65. (C) Western Blot analysis of pp65 expression. (D) Gray values were recorded and statistically analyzed. (E, F) Western Blot analysis of IL-1β and IL-18 expression.

4. Discussion

Cochlear hair cells are integral to the transduction of sound energy into electrical signals. Unfortunately, once damaged, human hair cells lack the capacity for regeneration, with broad-spectrum noise exposure predominantly causing metabolic, rather than mechanical, damage. Identifying the molecular underpinnings of noise-induced hearing loss (NIHL) and uncovering novel therapeutic targets for early intervention post-noise exposure are crucial. Such strategies may attenuate hair cell necrosis and apoptosis, potentially thwarting the development of NIHL. Our findings corroborate the hypothesis that inhibition of SETD7 significantly curtails the overactivation of noise-induced inner ear inflammatory responses, thereby conferring auditory protection. This underscores the relevance of SETD7 as a promising pharmacological target for the innovation of treatments against noise-induced deafness and auditory preservation.

Further validation of our results comes from a series of analogous investigations highlighting the inner ear's intricate immune milieu. Previous studies have elucidated a marked augmentation in the NLRP3 -caspase-1 -IL-1β signaling cascade within the cochleae of noise-exposed mice, implicating the NLRP3 inflammasome in the post-exposure accumulation of pro-inflammatory mediators. The recruitment of ASC by NLRP3 facilitated through PYD-PYD domain interactions, enables the activation of caspase-1 (Broz and Dixit, 2016; Kesavardhana and Kanneganti, 2017), fostering the maturation and dissemination of the cytokines IL-1β and IL-18. This initiates an inflammatory cascade, culminating in the extensive activation of the immune system (Frank and Vince, 2019; Zhao et al., 2020), which in turn precipitates hair cell demise through necrosis and pyroptosis (Zhou et al., 2011). These observations suggest that NLRP3 inflammasome activation serves as a proximal event in noise-induced cochlear inflammation and consequent hearing loss, positioning the blockade of NLRP3 as a viable therapeutic avenue (Li et al., 2021).

SETD7's capability to methylate a broad array of substrates, including histones and non-histone proteins, bestows upon it multifaceted roles in cellular regulation and disease pathogenesis. Notably, SETD7 is instrumental in modulating the inflammatory response, regulating cytokine expression, and participating in the pathophysiology of chronic constriction injury-induced neuropathic pain (Shen et al., 2019). In models of hypoxia and reoxygenation-induced cardiomyocyte stress in rats, SETD7 overexpression has been linked to the exacerbation of oxidative stress and apoptosis. In contrast, its inhibition mitigates these responses, suggesting a protective role against myocardial ischemia/reperfusion injury (Dang et al., 2018). Recent findings also demonstrate that silencing SETD7 attenuates the activation of the NLRP3 inflammasome induced by isoflurane in aged mice, reducing inflammatory mediator release, alleviating pyroptosis, and improving cognitive function post-anesthesia (Chao et al., 2022).

(R)-PFI-2 hydrochloride, characterized by its selective inhibition of SETD7, exhibited pronounced inhibitory effects. Our research observed a significant upregulation of NLRP3 protein within the cochlear hair cells and spiral ganglion neurons post-noise exposure, which was mitigated following (R)-PFI-2 hydrochloride treatment. The expression of IL-1β and IL-18 was elevated in the cochlea of mice after noise exposure. However, following treatment with (R)-PFI-2 hydrochloride, the levels of IL-1β and IL-18 were significantly reduced, further substantiating the inhibition of downstream inflammatory factors. We deduce that (R)-PFI-2 hydrochloride mediates its therapeutic effects by hindering NLRP3 activation via SETD7 inhibition, subsequently dampening the release of inflammatory mediators, mitigating hair cell damage, and fostering recovery from NIHL.

Moreover, intense noise levels facilitate interactions with the NF-κB signaling pathway, augmenting NLRP3 expression and promoting the release of cytokines such as TNF-α and IL-1β, thereby inducing inflammatory responses (Sangiuliano et al., 2014; Kaisho and Akira, 2006). p65, a principal component of the NF-κB family, predominantly resides in the cytoplasm under normal conditions but exhibits significant nuclear translocation in outer hair cells following noise exposure, indicative of an activated state. (R)-PFI-2 hydrochloride administration resulted in a reduction of nuclear p65 fluorescence. This, coupled with Western Blot analyses confirming a decrease in phosphorylated p65 (pp65) levels in the treated group compared to controls, suggests noise-induced NF-κB pathway activation. Literature indicates that the NF-κB pathway modulates the transcription of genes encoding NLRP3 and PRO-IL-1β, playing a pivotal role in the initiation of gene expression (Capece et al., 2022). The NLRP3 inflammasome, alongside IL-18 and IL-1β, exacerbates the secretion of TNF-α and IL-6, which, via TLR4

binding, further activates the NF-κB pathway, instigating cellular damage and necrosis. This establishes a feedback loop within the cochlea, intensifying inflammatory responses (Se et al., 2018). SETD7's role in directly activating the NF-κB pathway through methylation of p65 at specific sites (Li et al.) and its potential to enhance the affinity of methylated p65 for inflammatory gene promoters (Yang et al., 2009) further delineates its regulatory influence on inflammation. However, the precise catalytic sites within the inner ear warrant additional exploration.

In conclusion, this investigation affirms the therapeutic potential of (R)-PFI-2 hydrochloride in ameliorating NIHL by inhibiting SETD7, thereby curtailing NF-κB signaling and NLRP3 inflammasome activation within the cochlea. This highlights the utility of modulating immune and inflammatory responses as a novel strategy for addressing noise-induced auditory damage, offering a promising direction for future drug development and therapeutic interventions in NIHL.

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