

Effects of Tumor Necrosis Factor Blocker on Salicylate-Induced Tinnitus in Mice

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Abstract

Objective: Neuroinflammation is considered a novel mechanism for acute tinnitus. Here, we investigated the effects of a tumor necrosis factor (TNF) blocker on the gene expression of inflammatory-cytokine in the cochlea in a tinnitus animal model. **Methods:** Enbrel® (30 mg/kg, intraperitoneally (i.p.)) were administrated to the mice with the salicylate induced tinnitus for 3 days. Tinnitus score and mRNA expression levels of TNFR1, TNFR2, and N-methyl-d-aspartate receptor subunit 2B (NR2B) and its downstream regulatory element antagonist modulator (DREAM) in the cochlea of mice were measured and compared to the control. **Results:** The tinnitus score significantly decreased in the Enbrel® treated group. The mRNA levels of both TNFR1 and TNFR2 were significantly lower in the treatment than in the control group. The mRNA levels of NR2B and DREAM followed a similar trend. **Conclusion:** we found that treatment with 30 mg/kg Enbrel® decreased salicylate-induced behavior associated with tinnitus and reduced the mRNA expression levels of TNFR1/R2, NR2B, and DREAM in the cochlea of mice. These findings supported the hypothesis that neuroinflammation might be a novel mechanism for salicylate-induced tinnitus.

Keywords: cochlea, gene expression, salicylate, tinnitus, TNF blocker.

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INTRODUCTION

Tinnitus is the perception of sound in the absence of acoustic stimulation. Tinnitus can be perceived in one or both ears or in the head, and is usually described symptomatically as being able to hear a ringing noise. Usually, tinnitus and hearing loss occur together. To date, no effective treatment that reliably reduces the sensation of tinnitus is available, although some medications appear to be effective in certain patients¹. Currently, finding a pharmacological solution for tinnitus is a demanding and challenging task.

Salicylate-induced tinnitus in mice is a popular animal model for studying the condition². High doses of sodium salicylate (250-300 mg/kg, intraperitoneally (i.p.)) are known to elevate hearing thresholds, and reliably induce tinnitus². Based on results from a human³ and animals studies^{4,5}, neural inflammation may be a novel mechanism in tinnitus. It was found that the expression levels of the N-methyl-d-aspartate receptor subunit 2B (NR2B) gene increased moderately in the cochlea and midbrain during salicylate-induced tinnitus⁴. Further, genes for tumor necrosis factor (TNF)- α and interleukin (IL)-1 β were also up-regulated significantly in the cochlea and inferior colliculus (IC) in salicylate-induced tinnitus⁵. We hypothesized that proinflammatory cytokines might lead to tinnitus directly and/or via modulating NR gene expression⁵.

Etanercept (Enbrel[®]) induced regulatory macrophages in an Fc region-dependent manner and contributed to the resolution of inflammation^{6,7}. It was the first biological disease-modifying anti-rheumatic drug and the first TNF inhibitor to be approved for use in rheumatic diseases⁸. Enbrel[®] could prevent retinal ganglion cell loss in a rat model of glaucoma⁹, and ameliorate injury-induced neuropathic pain in sciatic nerve crush injury models¹⁰. In addition, Enbrel[®] was expected to have a beneficial effect on neurodegenerative diseases and neurogenic pain¹¹.

However, the effects of the TNF blocker on salicylate-induced tinnitus are still unclear. According to our hypothesis, these blockers can act on the inflammation pathway and potentially attenuate or stop tinnitus. Therefore, in this study, we investigated the outcome of treatment with Enbrel[®] and its interaction with NR2B and downstream regulatory element antagonist modulator DREAM in the cochlea of mice with salicylate-induced tinnitus.

ANIMALS AND METHODS

Animals

Thirty 3-month-old male C57BL/6 mice were randomly and equally divided into TNF-blocker (Enbrel[®], 30 mg/kg) treated (n = 15) and saline-treated control (n = 15) groups. All methods for animal care and experiments were carried out in accordance with relevant guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC). In addition, all experimental protocols were approved by the National Taiwan University College

of Medicine and College of Public Health IACUC (No. 20110467).

Hearing threshold testing

Auditory brain responses (ABR) to click sounds at 4 kHz, 10 kHz, and 16 kHz were performed 2 days before tinnitus training/conditioning and immediately after the end of the study.

Behavioural measurement of tinnitus score

All mice were trained to perform an active avoidance task, which was performed in a conditioning box with an electrical floor and a climbing pole, according to the design by Guitton et al.¹².

Conditioning to the task

The conditioning paradigm consisted of 6 sessions performed daily for 5 days. Each session lasted for 15-20 minutes, and there were 10 trials per session. Inter-trial intervals lasted for at least 1 minute each. For each trial, the conditioning stimulus was a 50 dB sound pressure level (SPL) pure tone with a frequency of 10 kHz for 3 s. The unconditioned stimulus was a 3.7 mA electric foot-shock for 30 s, as described in the protocol used by Guitton et al.¹², achieved by adjusting the electric voltage in Copper wire with fixed resistance on the floor. Time between conditioned and unconditioned stimuli was 1 s. The mice would climb up through the pole into a safe area after the combined conditioned and unconditioned stimuli. Electrical shocks were stopped by the experimenter when the animal climbed correctly. The "true-positive" score was the level of performance assessed by the number of times the mice climbed correctly in response to sound. Mice were considered to be conditioned when the "true-positive" score reached at least 80% in three consecutive sessions. Only conditioned mice were selected for the tinnitus experiments.

Induction and testing of tinnitus

Once conditioned, the mice rested for 1 day. The following day, a tinnitus baseline test was measured 1 h before i.p. injection with 300 mg/kg sodium salicylate. Two treatment strategies were used as follows: i.p. injection of 30 mg/kg Enbrel[®] or saline 1 h after the sodium salicylate injection. An active avoidance task of one session with 10 trials was performed 1 h after treatment for 3 days. To avoid changes attributable to hearing loss induced by salicylate (about 10 to 20 dB during salicylate injections, as revealed by our preliminary experiment), the intensity of sound that elicited the behavioural responses was increased to 70 dB SPL in both groups. By doing so, the sound sensation levels of all mice in the study were similar. During testing, first, a sound of 3 s duration was given in each trial, and the mice were observed for another 5 s to see whether they would climb up the pole. The mice were returned to the floor for observations if they stayed on the safe area for more than 10 s (true-positive). If the mice did not climb up the pole, an electrical shock was given by the experimenter to remind the mice to

climb up. Again, the mice were returned to the floor for observations if they stayed on the safe area for more than 10 s. Finally, observed the total number (false-positive or tinnitus score) of times the mice climbed during the inter-trial silent period of 1 minute for 10 trials.

Sample isolation and RNA extraction from the cochlea

The mice were euthanized by decapitation to determine the gene expression levels of TNF receptor-1/2 (TNFR1/2) and NMDA receptor (NR2B) subunit and levels of DREAM in the cochlea.

The soft tissues of both cochleas were pooled together as one sample for each mouse and then the total RNA was isolated from the tissue homogenates of each sample using a modified Trizol protocol (Gibco BRL). Each sample was centrifuged at 12000 g for 10 minutes at 4°C and the clear supernatant was transferred to a new tube and incubated for 5 minutes at 15-30°C to permit the complete dissociation of nucleoprotein complexes. To each ml of Trizol reagent, 0.2 ml of chloroform was added and the tube was shaken vigorously by hand for 15 s, then incubated at 15-30°C for 2 minutes and centrifuged at 12000 g for 15 minutes at 4°C. The aqueous phase was transferred to a new tube, 0.5 ml of isopropyl alcohol per 1 ml Trizol reagent was added, and then it was incubated at 15-30°C for 10 minutes. This was followed by centrifuging at 12000 g for 10 minutes at 4°C. The supernatant was separated and the RNA pellet was washed once with 1 ml 75% ethanol. The sample was mixed by vortex and then centrifuged at 7500 g for 5 minutes at 4°C. The new RNA pellet was air-dried, dissolved in 10-20 µl of RNase-free water, and incubated at 42°C for 5 minutes. RNA quality was assessed by Agilent Bioanalyzer 2100, and the ratio of absorbance at 260 nm and 280 nm measured using the Nanodrop.

Real-time quantitative polymerase chain reaction (RT-qPCR)

The sequences of the primer and probe number used in this study are listed in Table 1. The primers and the probes were designed using Universal Probe Library (UPL) of Roche Applied Science (Indianapolis, IN, USA).

The 2 µg of RNA was treated with DNase and then reverse transcribed into first-strand cDNA in a 20 µl reaction volume using SuperScript III Reverse Transcriptase (Invitrogen, CA, USA). Quantitative real-time reverse transcription (RT)-PCR assays of cDNA were performed using a gene-specific double fluorescently labeled probe in an ABI Prism 7900 Sequence Detection System (Applied Biosystems) in accordance with the manufacturer's recommendations. The reactions' thermal cycle conditions were adjusted as 10 minutes initial setup at 95°C, followed by 40 cycles, each of which consisted of 15 s denaturing at 95°C and 1 minute annealing/ extending at 60°C. The relative mRNA expression level was determined by the 2-ΔCt analysis method, and calculations were performed using the software provided by the manufacturer (Applied Biosystems). The expression levels of TNF-R1/2, NR2B, and DREAM were presented as relative ratios in comparison to the expression of β-actin.

Statistical analysis

Unless otherwise stated, all data are presented as either mean ± standard deviation (SD) or boxplots including median, 75 and 25 percentile values. 'n' is the number of animals per experimental group. The statistical differences between means were compared either by Student's t-test and the median compared by Mann-Whitney U-test, depending on the distribution pattern of the variable, test by Shapiro-Wilk normality test, and sample size. P values < 0.05 were considered statistically significant.

Table 1. Primers and probes used in this study.

Genes	Sequences of primers and probes (5'-3')	Amplicon size (bp)
TNFR1	F, CCGCAACGTCCTGACAAT	121
	R, GCCTCTCGATCTCGTGCT	
	Probe number: # 12	
TNFR2	F, TCTGAGTCTGGCCTCAT	61
	R, GGCTTCCGAGATGACAGAAC	
	Probe number: # 12	
NR2B	F, TCTAAGGAAGGCCATTGAACC	69
	R, CCATGCAGCAAGAATTCAGA	
	Probe number: # 68	
DREAM	F, TGGAGCTAAGACTCGGAAGG	77
	R, GCAATTGTCACTGGAGGTTTC	
	Probe number: # 12	
β-actin	F, CTAAGGCCAACCGTGAAAAG	104
	R, ACCAGAGGCATACAGGGA CA	
	Probe number: # 64	

RESULTS

ABR hearing thresholds

Figure 1 shows the ABR hearing thresholds in both groups. In the control group, the pre-injection mean hearing thresholds were 35.7 ± 3.6 dB (4 kHz), 30.8 ± 2.1 dB (10 kHz), and 37.7 ± 3.7 dB (16 kHz), respectively. The post-injection mean hearing thresholds were 44.7 ± 3.9 dB (4 kHz), 38 ± 3.1 dB (10 kHz), and 47.5 ± 2.8 dB (16 kHz), respectively. The post-injection mean hearing thresholds were significantly elevated compared to pre-injection thresholds, being 4 kHz ($p = 6.61 \text{ E-}07$), 10 kHz ($p = 1.36 \text{ E-}07$), and 16 kHz ($p = 1.78 \text{ E-}08$). Thus, 3 consecutive days of injection with 300 mg/kg sodium salicylate induced significant shifts in hearing thresholds.

In the treatment group, the pre-injection mean hearing thresholds were 33.9 ± 3.4 dB (4 kHz), 29.1 ± 3.7 dB (10 kHz), and 35.6 ± 4.3 dB (16 kHz), respectively. The post-injection hearing thresholds were 35.4 ± 3.4 dB (4 kHz), 33.9 ± 2.8 dB (10 kHz), and 39.9 ± 4.3 dB (16 kHz), respectively. The post-injection mean hearing thresholds were slightly elevated from pre-injection thresholds for 4 kHz ($p = 0.27$), 10 kHz ($p = 0.001$), and 16 kHz ($p = 0.02$). These results revealed that treatment with Enbrel® decreased the level of salicylate-induced shifts in ABR hearing thresholds.

Figure 2 shows the tinnitus scores for both the groups. The mean tinnitus scores at baseline and 3 days after i.p. injection with 300 mg/kg sodium salicylate were 4.9 ± 2.7 , 19.5 ± 7.5 , 24.7 ± 12 , and 22.7 ± 7.4 , respectively, in the control group, and 4.5 ± 2.9 , 21.6 ± 12.8 , 15.7 ± 6.6 , and 15.1 ± 5.1 , respectively, in the treatment group. Student's t-test revealed that treatment with Enbrel® significantly decreased salicylate-induced behaviour associated with tinnitus each day after i.p.

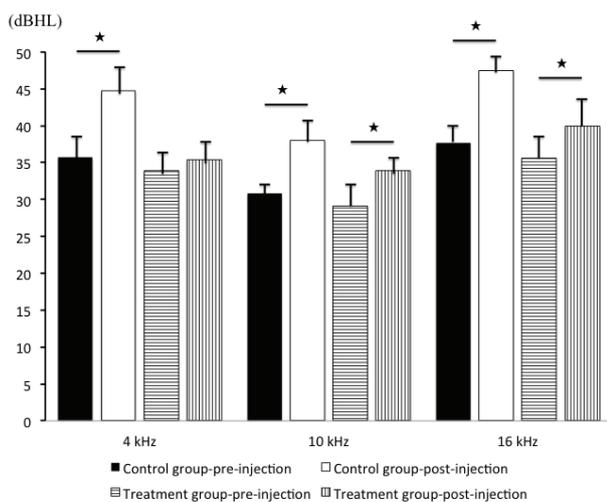


Figure 1: ABR hearing thresholds in both the groups. Consecutive 3 days of injection with 300 mg/kg sodium salicylate induced significant shift in the hearing thresholds (P-values for frequency were 6.61 E-07 at 4 kHz, 1.36 E-07 at 10 kHz, and 1.78 E-08 at 16 kHz, respectively). Treatment with Enbrel® decreased salicylate-induced shift in ABR hearing thresholds (P-values for frequency were 0.27 at 4 kHz, 0.001 at 10 kHz, and 0.02 at 16 kHz, respectively).

injection with 300 mg/kg sodium salicylate ($p = 0.72$ for baseline, $p = 0.61$ for day 1, $p = 0.02$ for day 2, and $p = 0.005$ for day 3). These findings indicate that treatment with Enbrel® significantly decreased salicylate-induced behaviour associated with tinnitus each day, especially on day 1.

TNFR1/2 gene expression

Figure 3 shows the expression levels of the TNFR1/R2 genes relative to that of β -actin in the cochlea. The mRNA level of TNFR1 was significantly lower in the treatment than in the control group (0.22 vs. 1.03; Mann-Whitney U-test, $p = 0.0009$). The mRNA expression level of TNFR2 also decreased significantly in the treatment group compared with that in the control group (18.61 vs. 66.86; Mann-Whitney U-test, $p = 0.01468$).

These findings indicate that treatment with Enbrel® significantly reduced the mRNA expression levels of TNFR1 and TNFR2 in the cochlea after i.p. injection with 300 mg/kg sodium salicylate. Furthermore, according to the findings in the control groups (Figures 4 and 5), i.p. injection with 300 mg/kg sodium salicylate could alter the mRNA expression level of TNFR1 more than that of TNFR2 (66.86 vs. 1.03; Mann-Whitney U-test, $p = 0.0001$).

NR2B gene expression

Figure 5 shows the expression level of NR2B relative to that of β -actin. The mRNA level of NR2B was significantly lower in the treatment than in the control group (37.9 vs. 224.25; Mann-Whitney U-test, $p = 0.01878$). These findings indicate that treatment with Enbrel® decreased the mRNA expression level of NR2B in the cochlea after i.p. injection with 300 mg/kg sodium salicylate.

DREAM gene expression

Figure 6 shows the expression level of DREAM relative to that of β -actin. The mRNA level of DREAM was significantly lower in the treatment than in the

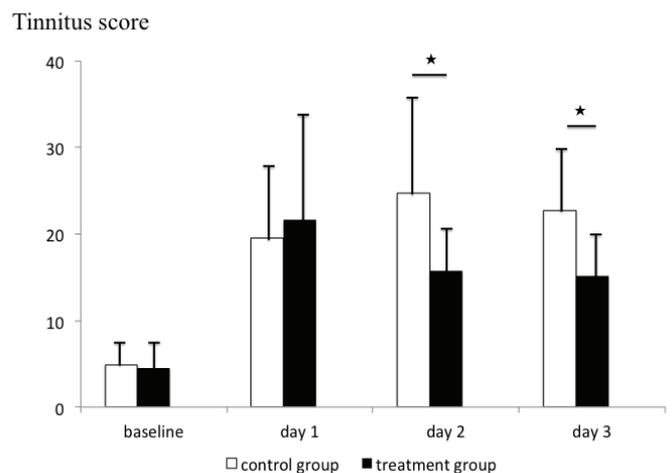


Figure 2: The tinnitus scores for both the groups. Enbrel® significantly decreased salicylate-induced tinnitus at day 2 and day 3 after i.p. injection with 300 mg/kg sodium salicylate (P = 0.72 for baseline, P = 0.61 for day 1, P = 0.02 for day 2, and P = 0.005 for day 3).

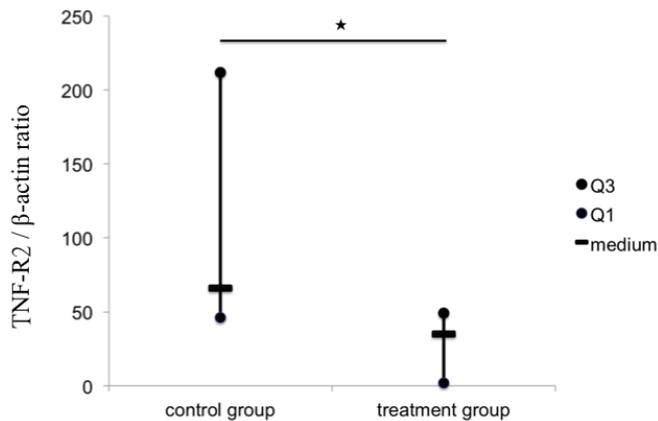


Figure 3: The expression levels of the TNFR1 gene relative to that of β -actin in the cochlea. The mRNA level of the TNFR1 gene was significantly lower in the treatment than in the control group (0.22 vs. 1.03; Mann-Whitney U-test, $P = 0.0009$).

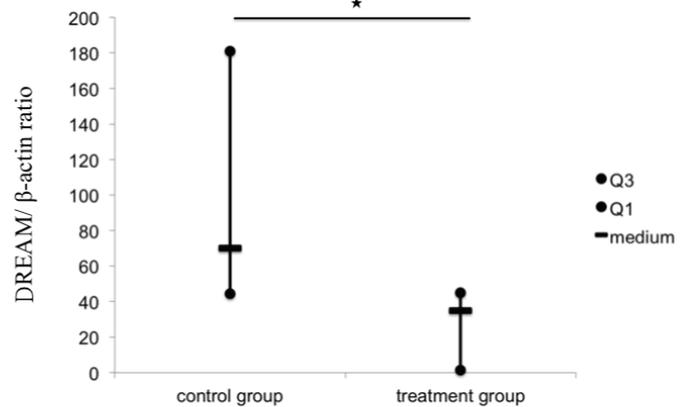


Figure 6: The expression level of DREAM relative to that of β -actin. The mRNA level of the DREAM gene was significantly lower in the treatment than in the control group (10.18 vs. 70.04; Mann-Whitney U-test, $P = 0.00988$).

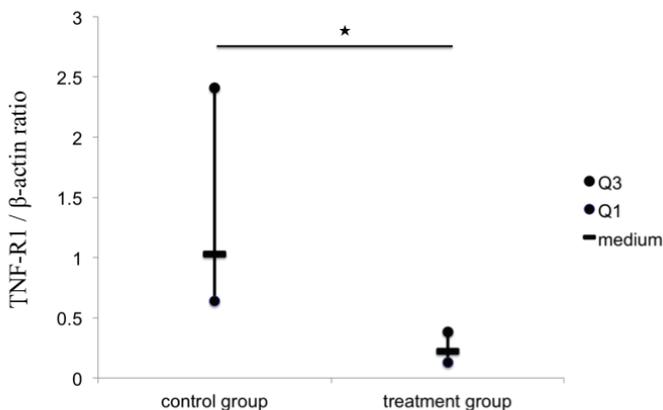


Figure 4: The expression levels of the TNFR2 genes relative to that of β -actin in the cochlea. The mRNA expression level of the TNFR2 gene decreased significantly in the treatment group compared with the control group (18.61 vs. 66.86; Mann-Whitney U-test, $P = 0.01468$).

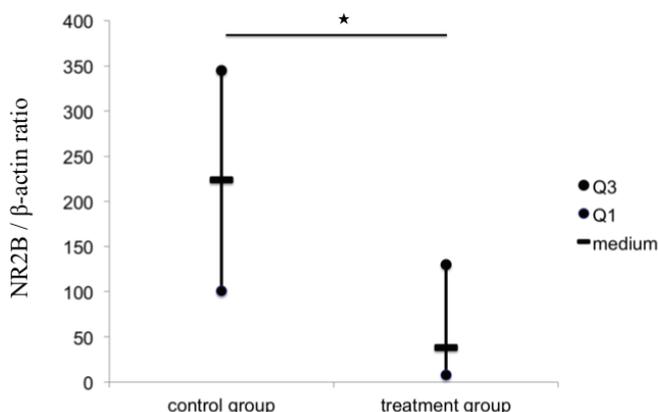


Figure 5: The expression level of the NR2B gene relative to that of β -actin. The mRNA level of the NR2B gene was significantly lower in the treatment than in the control group (37.9 vs. 224.25; Mann-Whitney U-test, $P = 0.01878$).

control group (10.18 vs. 70.04; Mann-Whitney U-test, $p = 0.00988$). These findings indicate that Enbrel[®] reduced the mRNA expression level of DREAM in the cochlea after i.p. injection with 300 mg/kg sodium salicylate.

DISCUSSION

It is well known that a large dose of aspirin causes reversible hearing loss and tinnitus¹³. The underlying mechanisms for salicylate-induced hearing impairment and tinnitus are numerous. For example, high doses of salicylate might reduce cochlear blood flow and electromotility of outer hair cells¹⁴. In addition, neuroinflammation and oxidative stress are associated with age-related hearing impairment. Our results showed that Enbrel[®] significantly decreased salicylate-induced behaviour associated with tinnitus. Further, Enbrel[®] could reduce the mRNA expression levels of TNFR1/R2, NR2B, and DREAM in the mice cochlea after i.p. injection with 300 mg/kg sodium salicylate. These findings support the hypothesis that neuroinflammation is a novel mechanism for salicylate-induced tinnitus³⁻⁵.

TNF- α exerts its biological functions via interactions with two cognate membrane receptors, TNF-R1 and -R2^{15,16}. TNF-R1 contains a conserved protein-protein interaction motif of 80 amino acids, termed the “death domain,” which interacts with the adapter molecule TNF receptor-associated protein with death domain (TRADD)¹⁷. TNF-R2 interacts with TRAF2 and exclusively activates proinflammatory pathways. Although TNF-R2 does not induce apoptosis directly, it can modulate TNF-R1-induced effects, including apoptosis, via cross-talk between TNF-R2 and -R1 signal pathways¹⁸. In this study, it was observed that i.p. injection with 300 mg/kg sodium salicylate could increase the mRNA expression level of TNFR1 more than that of TNFR2 in the mice cochlea. However, the salicylate-induced TNFR1 mRNA elevation did not result in cell apoptosis in the cochlea because aspirin only causes reversible hearing loss and tinnitus¹³. Thus, Enbrel[®] was expected to have a beneficial effect on neurodegenerative diseases and neurogenic pain via inducing regulatory macrophages in an Fc region-dependent manner and blocking downstream inflammation-related signaling pathways^{6,7,11}.

Our previous studies indicated that changes in COX and/or NR play a minor role in salicylate-induced tinnitus, although tinnitus was reported to be highly associated with NR activity¹³ and/or its mRNA expression⁴. In fact, interaction between TNF- α and/or IL-1 α and the functions of NR were present^{5,18,19}. With respect to NR-related signal pathways, DREAM had a neuroprotective effect against excitotoxic neuronal injury by directly binding to the C0 domain of the NR1 subunit and inhibiting surface expression of NRs and NR-mediated current²⁰. Knockdown of DREAM could enhance NMDA-induced toxicity²⁰. The results of this study showed that Enbrel[®] could inhibit both NR2B and DREAM gene expression, which had a reciprocal effect on neural cell function. Here, we conclude that the net effect of this inhibition of DREAM and NR2B by Enbrel[®] is beneficial for tinnitus reduction.

CONCLUSION

This study showed that the TNF α blocker Enbrel[®] decreased salicylate-induced behavior associated with tinnitus. This TNF- α blocker might exert its effect via TNF-R directly, in which TNF-R2 plays a more dominant role than TNF-R1 in the cochlea. Further, this TNF- α blocker might reduce salicylate-induced tinnitus indirectly via interaction between TNF-R and the NMDA receptor. Therefore, future research in this field should be directed at conducting clinical trials for treatment with Enbrel[®] in patients with tinnitus.

CONFLICT OF INTEREST

None declare

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