

# Expression of pro-inflammatory cytokines in the auditory cortex of rats with salicylate-induced tinnitus

XIANG-HONG CHEN and LI-LI ZHENG

Department of Endocrinology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, P.R. China

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**Abstract.** Tinnitus often results in severe psychological distress. The present study hypothesized that tinnitus acts as a chronic stressor and induces dysregulation of the production of cytokines. The gap pre-pulse inhibition of acoustic startle paradigm was applied to test tinnitus-like behavior in rats. Following this, the mRNA and protein expression levels of interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6 and N-methyl D-aspartate receptor subunit 2A (NR2A) were measured in rats subjected to acute and chronic salicylate treatment, using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. The gap prepulse inhibition of acoustic startle paradigm detected the tinnitus-like behavior of rats. The expression of TNF- $\alpha$  and NR2A genes were increased in the auditory cortex (AC) following long-term administration of salicylate, whereas the expression of IFN- $\gamma$  genes decreased; however, the mRNA levels reversed back to normal baseline 14 days following the cease of salicylate administration. IL-6 gene expression, however, was not fundamentally altered by salicylate treatment. The data demonstrated that chronic salicylate administration induces tinnitus, in part, via dysregulation of cytokines and specific membrane receptors in the AC.

## Introduction

The active ingredient in the frequently used aspirin is salicylate, and previous studies and clinical examinations have demonstrated that it may result in reversible tinnitus. Salicylate-induced rats have previously been used as a model

to study the disease (1-3). The use of salicylate for long periods of time has been known to result in the modification of the average spectrum of the electrophysiological cochleoneural activity (4,5) distortion product otoacoustic emissions (6), increase outer hair cell electromotility (3,7), and reliably induce tinnitus. It has previously been demonstrated that tinnitus may be linked to alterations at one or more points along the peripheral and/or central auditory pathways (8), and even non-auditory brain structures (9-11).

Patients who have suffered for numerous years from tinnitus subsequently suffer from depression and present with symptoms of distress (12,13). The combination of tinnitus and stress creates a vicious cycle where the symptoms of each disease worsen. Anxiety and stress make the tinnitus worse which in turn, enhances the stress and anxiety (14). Further research has demonstrated that anxiety, stress and psychological distress are associated with alterations in the immune system (15-17). Uncontrollability of the stressor has been identified as a primary determinant of the immunological stress response (18), a characteristic that additionally applies to the condition of tinnitus. The present study therefore hypothesized that tinnitus, perceived as disturbing and uncontrollable, acts as a chronic stressor and thereby interferes with stress-associated cytokines and proteins. The hypothalamic-pituitary-adrenal axis and/or the sympathetic nervous system are activated by a stressor and dysregulation of the production of cytokines are induced by stressors, including interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-6 (16). A stressor may additionally modulate N-methyl D-aspartate (NMDA) receptors (NMDARs) via glutamate (19). Increased inflammatory gene expression has been demonstrated in the cochlear nucleus in a rat model of tinnitus (20). However, there are few studies assessing the inflammatory genes in the auditory cortex (AC).

The present study detected the expression levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and NR2A genes in the AC under conditions of salicylate exposure. To verify and detect the tinnitus behavior of the rats, gap prepulse inhibition of acoustic startle (GPIAS) standard was applied (21-23).

## Materials and methods

**Animals.** The Animal Care and Use Committee of Zhengzhou University School of Medicine (Zhengzhou, China) approved the procedures. A total of 36 adult male Sprague-Dawley rats

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*Correspondence to:* Professor Li-Li Zheng, Department of Endocrinology, The First Affiliated Hospital of Zhengzhou University, 1 Jianshe Eastern Road, Zhengzhou, Henan 450052, P.R. China  
E-mail: lilizheng66@126.com

**Abbreviations:** AC, auditory cortex; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6; IFN- $\gamma$ , interferon- $\gamma$ ; NMDA, N-methyl-D-aspartate; NR2A, N-methyl D-aspartate receptor subunit 2A; GPIAS, gap prepulse inhibition of acoustic startle

**Key words:** tinnitus, cytokines, auditory cortex

(age, 2-3 months; weight, 200-250 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Rats with normal pinna reflexes that did not have a middle ear infection were used in the present study. The animals were divided into 4 groups: i) Control group (n=10); ii) salicylate injected once (n=6) acute treatment group; iii) continuous injections of salicylate for 7 days (Sal-7d/S7 n=10; ) sustained treatment group and iv) rehabilitation group with 14 days rest post-chronic salicylate administration for 7 days (n=10; S7+R14). Rats were housed in a temperature-controlled room at 21±2°C, with 55±3% relative humidity, under a 12-h light/dark cycle with free access to water and food.

**Experimental design and salicylate administration.** Sodium salicylate (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was melted to a density of 200 mg/ml in regular saline [9% (w/v) NaCl]. Rats in the acute group were administered an intraperitoneal (i.p.) injection of salicylate (400 mg/kg) and were sacrificed following 2 h. i.p. injections of salicylate were administered to animals in the sustained treatment groups every day at 8:00 a.m. for 7 continuous days. The rehabilitating group received intraperitoneal injections for 7 continuous days and then underwent 14 days of recovery following completion of treatment (S7+R14). At 8:00 a.m. on day 7, the rats in S7 group were sacrificed, whereas the rats in the S7+R14 group were sacrificed at 8:00 a.m. on day 21 (6,7). Rats were anesthetized with pentobarbital sodium (60 mg/kg) intraperitoneally (i.p.) prior to sacrifice.

**Gap detection testing.** The GPIAS paradigm was used to assess tinnitus as previously described (20,21). The procedure utilizes acoustic starlet reflex tests within animals which have been given salicylate. GPIAS testing began two h prior to sacrifice. The rats were in an acoustically transparent cage placed over a sensitive piezoelectric transducer. The transducer produced voltage in proportion to the response of the rats, generated by a digital signal processor. The result of the reaction from the rats was recorded with a computer and analyzed offline.

The gap detection tests were conducted using different band-pass-filtered sounds. The reactions of starlet had been obtained with a 20 msec spurt with white noise. The narrow-band gap sound started 100 msec prior to the broadband astonishing noise starting. Furthermore, the time between each sound was 30-35 sec (22).

GPIAS percentages were calculated as the average ratio of trials with a gap vs. trials without gaps, for each frequency, according to the following formula: [(AvgTnogap-AvgTgap)/AvgTnogap x100%], where AvgTgap was the average amplitude in the gap trials, and AvgTnogap was the average amplitude of trials without gaps (23,24).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The AC was dissected following sacrifice of rats. TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total RNA, following the manufacturer's protocol. The pureness and amount of isolated RNA had been measured at optical density (OD) 280 and OD260. Primers obtained from Sangon Biotech Co., Ltd. (Shanghai, China) were utilized in the amplification of IL-6, TNF- $\alpha$ , NR2A, IFN- $\gamma$  and GAPDH as listed in Table I. Isolated

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Genes	Primer sequence (5'-3')	
TNF- $\alpha$	Forward	TCAGTTCCATGGCCCAGAC
	Reverse	GTTGTCCTTTGAGATCCATGCCATT
IL-6	Forward	ATTGTATGAACAGCGATGATGCAC
	Reverse	CCAGGTAGAAACGGAACTCCAGA
IFN- $\gamma$	Forward	TGGATGCTATGGAAGGAAAGA
	Reverse	GATTCTGGTGACAGCTGGTG
NR2A	Forward	TGGCTATCCTGCAGCTGTTTG
	Reverse	TGGCTGCTCATCACCTCATTC
GAPDH	Forward	GGCACAGTCAAGGCTGAGAATG
	Reverse	ATGGTGGTGAAGACGCCAGTA

TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6; IFN- $\gamma$ , interferon- $\gamma$ ; NR2A, N-methyl D-aspartate receptor subunit 2A.

RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent kit (DRR037A; Takara Bio, Inc., Otsu, Japan) according to the manufacturer's instructions. The reverse transcription reaction was performed at 37°C for 15 min, and at 85°C for 5 sec. SYBR Premix Ex Taq was used to perform the PCR amplification (DRR420A; Takara Bio, Inc.), which was conducted using ABI 7500 real time PCR systems (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mixture was amplified for 2 min at 50°C and for 3 sec at 95°C, followed by 40 cycles of 5 sec at 95°C, then 34 sec at 60°C. The optimal concentration of the templates and primers utilized within each reaction was established in accordance with the standard curve that was produced earlier to the response when it was equal to almost 100% of the reaction efficiency. In addition, relative calculations and quantifications were performed using comparative threshold cycle methods ( $2^{-\Delta\Delta C_q}$ ) (25).

**Western blotting.** Total protein was extracted using radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA) for 30 min on ice and the concentration was measured using a bicinchoninic acid protein assay. Equal amounts of extracted protein samples (20  $\mu$ g) were then loaded onto 8% (w/v) SDS-PAGE for electrophoresis of NR2A protein, with 12% (w/v) SDS-PAGE utilized for electrophoresis of IL-6, TNF- $\alpha$ , and IFN- $\gamma$  proteins. The proteins were then transferred to polyvinylidene difluoride membranes. The membranes had been blocked within TBS containing 0.1% Tween-20 (TBST) and 5% skimmed milk powder at room temperature for 4 h. The membranes were then incubated overnight with primary antibodies at 4°C and washed with TBST. Secondary antibodies had been diluted with blocking buffer and incubated for 2 h with the membranes at room temperature. Finally, immunoreactive bands were imaged using the

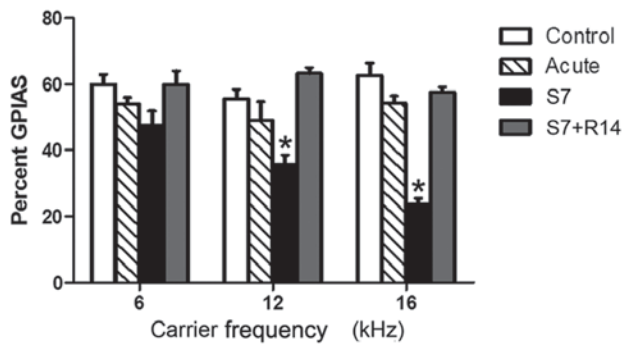


Figure 1. Effects of salicylate on GPIAS values. Rats in the S7 group demonstrated a significant decrease in GPIAS values compared with control group at 12 and 16 kHz, however not at 6 kHz. There was no difference in GPIAS values between rats in the acute treatment group, the 14 days following S7 group and the control group. \* $P < 0.05$  vs. control. GPIAS, gap prepulse inhibition of acoustic startle; acute, salicylate injected once ( $n=6$ ) intense treatment group; S7, continuous injections of salicylate for 7 days ( $n=10$ ) sustained treatment group; S7+R14, rehabilitation group with 14-day rest post-chronic salicylate administration for 7 days ( $n=10$ ).

Super Signal Chemiluminescent Substrate system (Pierce; Thermo Fisher Scientific, Inc.). Blots were semi-quantified by densitometry using Image Lab software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and normalized to GAPDH.

The primary antibodies used were: mouse anti-IL-6 antibody (cat. no. ab9324; 1:1,000; Abcam, Cambridge, UK), mouse anti-TNF- $\alpha$  antibody (cat. no. ab1793; 1:1,000; Abcam), goat anti-IFN- $\gamma$  antibody (cat. no. AF-585-NA; 1:5,000; R&D Systems Inc., Minneapolis, MN, USA), rabbit anti-NMDAR2A antibody (cat. no. D15B3; 1:1,000; Cell Signaling Technology Inc.) and rabbit anti-GAPDH antibody (cat. no. 5174S; 1:1,000; Cell Signaling Technology, Inc.). The secondary antibodies used were as follows: horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G (cat. no. 111-005-003; 1:5,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and HRP-conjugated goat anti-mouse IgG (cat. no. 115-005-062; 1:5,000; Jackson ImmunoResearch Laboratories, Inc.).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation of 6 independent experiments. Statistical analysis was performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). Based on the similarity of the variations and the distribution of the information, either a Kruskal-Wallis H test or one-way analysis of variance with a Bonferroni post hoc test were used to compare the significance of the differences between the groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Tinnitus-like behaviour in rats is induced by salicylate.** There had been an essential decline in GPIAS values in the S7 group when compared with control group at 12 kHz ( $P < 0.05$ ) and 16 kHz ( $P < 0.05$ ), however not at 6 kHz ( $P > 0.05$ ).

It was observed that the rats that appeared to be experiencing tinnitus were those that were accepting a repetitive injection of salicylate for 7 days. Conversely, it appeared that

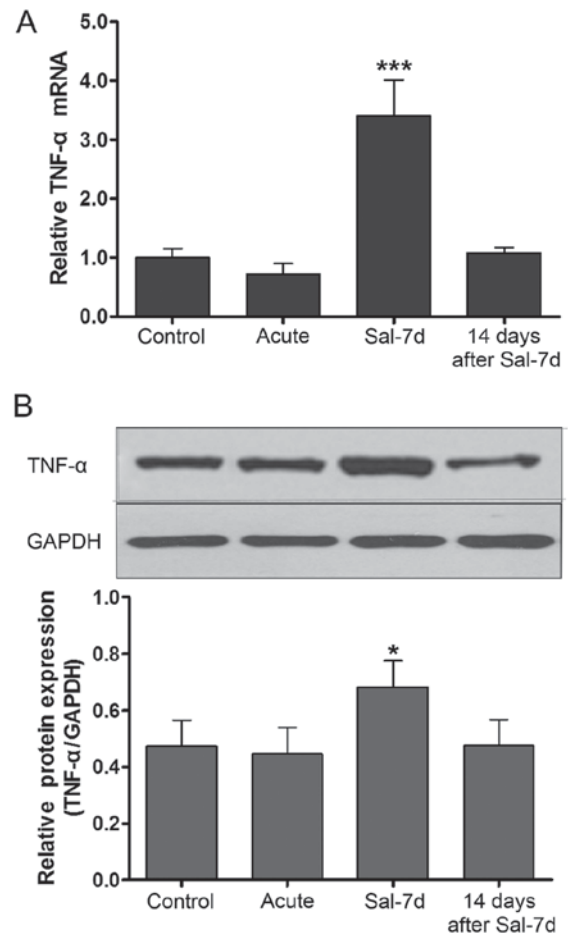


Figure 2. Expression of TNF- $\alpha$  in the auditory cortex. (A) Quantitative polymerase chain reaction demonstrated a significantly increased level of TNF- $\alpha$  mRNA in the S7 group compared with the control group, acute treatment group and 14 days following the S7 group. (B) Protein expression levels of TNF- $\alpha$ , indicated by western blot analysis, were markedly increased in the S7 group compared with other groups. \* $P < 0.05$ ; \*\*\* $P < 0.001$  vs. all groups. TNF- $\alpha$ , tumor necrosis factor; acute, salicylate injected once ( $n=6$ ) intense treatment group; S7, continuous injections of salicylate for 7 days ( $n=10$ ) sustained treatment group; S7+R14, rehabilitation group with 14-day rest post-chronic salicylate administration for 7 days ( $n=10$ ).

there were no differences between the acute treatment group, S7+R14 group and control group values ( $P > 0.05$ ), indicating that tinnitus-like behavior was not induced with salicylate injected only once, and that the tinnitus-like behavior induced in the S7 group vanished 14 days following the end of the salicylate treatment period (Fig. 1).

**TNF- $\alpha$  expression levels increase in the AC.** TNF- $\alpha$  gene expression was upregulated in the AC of the rats which had been chronically treated with salicylate for 7 days ( $P < 0.05$ ). There were no alterations in the precise treatment or S7+R14 groups (Fig. 2).

**IL-6 expression levels remain unaltered in the AC.** IL-6 mRNA and protein expression was not significantly altered in any of the treatment groups compared with control (Fig. 3).

**IFN- $\gamma$  expression decreases in the AC.** Compared with control group, IFN- $\gamma$  gene expression was downregulated in the AC of rats which had been chronically treated with salicylate for

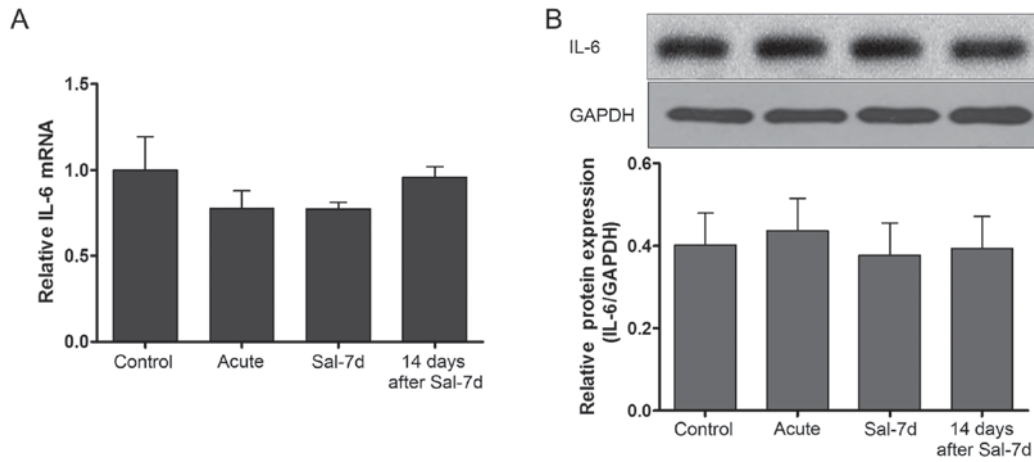


Figure 3. Expression of IL-6 in the cochlear nucleus. Differences in the expression levels of IL-6 (A) mRNA and (B) protein in the AC. Compared with control group, IL-6 gene expression exhibited no significant alterations in any of the groups. IL, interleukin; acute, salicylate injected once (n=6) intense treatment group; S7, continuous injections of salicylate for 7 days (n=10) sustained treatment group; S7+R14, rehabilitation group with 14-day rest post-chronic salicylate administration for 7 days (n=10).

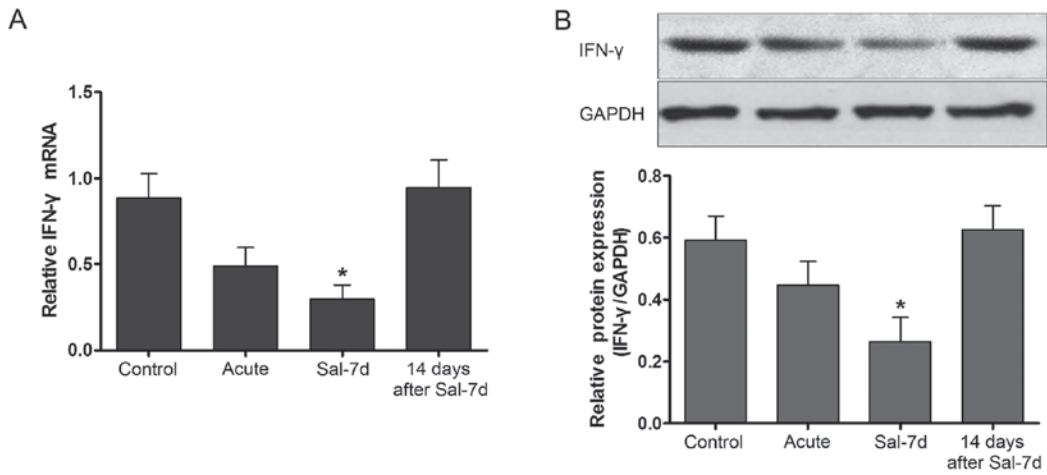


Figure 4. Expression of IFN-γ in the auditory cortex. (A) Quantitative polymerase chain reaction demonstrated a significantly decreased level of IFN-γ mRNA in the S7 group compared with control, acute treatment and 14 days following S7 groups. (B) IFN-γ protein expression level, as indicated by western blot analysis, was significantly decreased in the S7 group compared with other groups. \*P<0.05 vs. all groups. IFN-γ, interferon-γ; acute, salicylate injected once (n=6) intense treatment group; S7, continuous injections of salicylate for 7 days (n=10) sustained treatment group; S7+R14, rehabilitation group with 14-day rest post-chronic salicylate administration for 7 days (n=10).

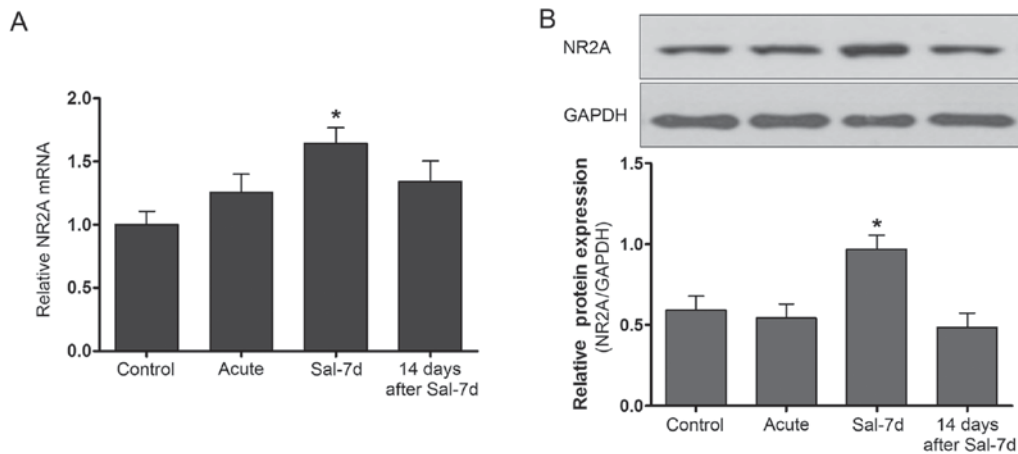


Figure 5. Expression of NR2A in the auditory cortex. The expression levels of NR2A (A) mRNA and (B) protein were significantly increased in the S7 group compared with control, acute treatment and 14 days following S7 groups, as determined by quantitative polymerase chain reaction and western blot analysis. \*P<0.05 vs. all groups. NR2A, N-methyl D-aspartate receptor subunit 2A; acute, salicylate injected once (n=6) intense treatment group; S7, continuous injections of salicylate for 7 days (n=10) sustained treatment group; S7+R14, rehabilitation group with 14-day rest post-chronic salicylate administration for 7 days (n=10).



7 days ( $P < 0.05$ ) There were no significant IFN- $\gamma$  gene expression alterations in the precise treatment and S7+R14 groups (Fig. 4).

*NR2A expression is upregulated in the AC.* qPCR and western blot analysis were conducted in order to detect the expression of NR2A in the AC. As presented in Fig. 5, levels of NR2A mRNA and protein expression were upregulated in rats which had been chronically treated with salicylate for 7 days ( $P < 0.05$ ), compared with control group. Furthermore, no increase was observed in the precise treatment or the S7+R14 groups.

## Discussion

The 'theory of cochlear origin' suggest that tinnitus originates from the central nervous system. A previous study using positron emission tomography revealed an increase in metabolic activity and continuous neuronal activation in the AC of rats with salicylate-induced tinnitus (2).

Salicylate affects neural activity in the brain, particularly in the AC (26). In addition, a strong association is present between stress and perception of tinnitus. The present study demonstrated that the expression of TNF- $\alpha$  and NR2A genes increased in the AC following long-term administration of salicylate, whereas the expression of IFN- $\gamma$  genes decreased, however returned to normal levels when salicylate treatment ceased. Additionally, the results demonstrated that IL-6 gene expression was not fundamentally altered by salicylate.

Tinnitus has previously been demonstrated to be connected with the activity of NMDA receptors (27-29). It was demonstrated that NR2A mRNA and protein expression increased significantly under the conditions of long-term administration of salicylate in the AC, and recovered back to normal levels 14 days following termination of salicylate treatment.

The results mimic those of a previous study that demonstrated NR2A is upregulated in the AC of rats that experience tinnitus (30). The etiology of tinnitus is associated with glutamate-induced excitotoxicity. NMDARs are important in excitotoxic injury (31). As modulatory subunits, the overall neurotransmission excitability in the AC is enhanced by the upregulation of NR2A receptors.

NMDAR activation, accompanied by ascending Ca<sup>2+</sup> influx, stimulation of Ca<sup>2+</sup>-dependent enzymes, ATP depletion and an increase in lipid peroxidation products, contributes to the excitotoxic damage in the auditory system under conditions of salicylate administration.

A biphasic model proposed by Dhabhar and McEwen (32) evaluates type of stress (acute or chronic) and how it affects the immune response. TNF- $\alpha$  reacts to acute stress in mice models (33), in addition to psychological stress in humans (34). Salicylate has been demonstrated to reduce the expression of TNF- $\alpha$  by inhibition of NFAT-mediated transcription in lymphocytic and monocytic cell lines (35). However, the results of the present study indicated that TNF- $\alpha$  gene expression increased reversibly in the AC of rats with tinnitus induced by long-term salicylate administration. These findings suggested that TNF- $\alpha$  may serve as a stress marker in tinnitus patients (36). TNF- $\alpha$  shifts neurons towards increased excitation and decreased inhibition (37). TNF- $\alpha$  enhances spontaneous excitatory synaptic transmission in retinotectal neurons (38) and in chronic pain;

TNF- $\alpha$  may therefore participate in the hyperactivity of the AC in tinnitus. A previous study reported an interaction between TNF- $\alpha$  and NMDARs (39).

The results of the present study demonstrated that NR2A and TNF- $\alpha$  were upregulated in the AC following chronic administration of salicylate. NR2A and TNF- $\alpha$  levels exhibited the same alterations in expression levels. Therefore, TNF- $\alpha$  may potentially contribute to tinnitus via augmentation of NMDAR expression and/or function.

Transient increases of IL-6 may be provoked psychological stressors. In plasma, stress and administration of adrenaline may increase levels of IL-6. It has previously been suggested that increased IL-6 levels are connected with psychological stress and primary depressive disorders (40). However, no alterations in IL-6 expression levels are detected in patients with tinnitus prior to, during, or following relaxation training (35), which is in accordance with the absence of alterations demonstrated in the present study. The role of IL-6 in the pathogenesis of tinnitus remains to be fully elucidated.

IFN- $\gamma$  gene expression was downregulated in the AC of rats that were chronically administered salicylate for 7 days. Psychological stressors decrease IFN- $\gamma$  levels during exam stress (41). The decreased IFN- $\gamma$  has also been attributed to the pharmacological actions of salicylate (36), and an increase in NMDARs (42). It is revealed in these data that chronic salicylate administration induces dysregulation of cytokines and NR2A, which may be involved in tinnitus.

In conclusion, the present results demonstrate that transient alterations are produced by chronically administered salicylate, in the expression of cytokines in the AC. These data suggest that alterations in neural plasticity in the AC may result from long-term administration of salicylate.

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