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Long-term Administration of Salicylate-induced Changes in BDNF Expression and CREB Phosphorylation in the Auditory Cortex of Rats

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Hypothesis: We investigated whether salicylate induces tinnitus through alteration of the expression levels of brain-derived neurotrophic factor (BDNF), proBDNF, tyrosine kinase receptor B (TrkB), cAMP-responsive element-binding protein (CREB), and phosphorylated CREB (p-CREB) in the auditory cortex (AC).
Background: Salicylate medication is frequently used for long-term treatment in clinical settings, but it may cause reversible tinnitus. Salicylate-induced tinnitus is associated with changes related to central auditory neuroplasticity. Our previous studies revealed enhanced neural activity and ultrastructural synaptic changes in the central auditory system after long-term salicylate administration. However, the underlying mechanisms remained unclear.
Methods: Salicylate-induced tinnitus-like behavior in rats was confirmed using gap prepulse inhibition of acoustic startle and prepulse inhibition testing, followed by comparison of the expression levels of BDNF, proBDNF, TrkB, CREB, and p-CREB. Synaptic ultrastructure was observed under a transmission electron microscope.
Results: BDNF and p-CREB were upregulated along with ultrastructural changes at the synapses in the AC of rats treated chronically with salicylate ($p < 0.05$, compared with control group). These changes returned to normal after 14 days of recovery ($p > 0.05$).
Conclusion: Long-term administration of salicylate increased BDNF expression and CREB activation, upregulated synaptic efficacy, and changed synaptic ultrastructure in the AC. There may be a relationship between these factors and the mechanism of tinnitus. **Key Words:** Auditory cortex—Brain-derived neurotrophic factor—cAMP-responsive element-binding protein—Salicylate—Tinnitus.

Otol Neurotol **39:**e173–e180, 2018.

Salicylate is a well-known ototoxic drug that can cause reversible tinnitus, and it has been used to induce animal models for the study of tinnitus (1–3). Tinnitus is the perception of sounds in the absence of acoustic stimulation. As a frequent clinical symptom, it has a significant negative effect on a patient's quality of life. Moreover, severe tinnitus is often accompanied by affective disorders such as depression, insomnia, and anxiety (4–6). We have previously demonstrated that long-term administration of salicylate increases distortion product otoacoustic emissions (7), outer hair cell electromotility (8), the average spectrum of electrophysiological cochleoneural activity (2,9), and reliably induces tinnitus (3). Recently, changes to auditory neuroplasticity, especially abnormal neuronal excitability in the central and peripheral auditory system, have been found to be closely associated with salicylate administration (10–12).

Brain-derived neurotrophic factor (BDNF) is a key neurotrophin involved in neuronal survival, differentiation, cortical development, changes of synaptic efficacy and structure, and neuroplasticity within the central auditory system (13–16). ProBDNF, the precursor of BDNF,

has opposing effects on neuronal structure and synaptic plasticity, compared with mature BDNF (17,18). BDNF probably acts through activation of the pan-neurotrophin receptor (p75[NTR]) and tyrosine kinase receptor B (TrkB, a high affinity tyrosine kinase receptor) (13,19,20). Targeted deletion of *TrkB* has effects identical to those of *Bdnf* deletion (21,22). Full-length TrkB undergoes autophosphorylation to regulate ERK/MAPK signaling, which may increase the cAMP levels, phosphorylate cAMP-responsive element-binding protein (CREB) at Ser133, or activate CREB-regulated gene transcription. All these changes in turn promote the transcription of BDNF (23,24). This is a potential positive feedback mechanism that could promote BDNF synthesis by itself.

Tinnitus can result from damage at any level along the auditory pathway (i.e., cochlear or acoustic nerve or central auditory pathways). This damage can result in abnormal neuronal activity and maladaptive plasticity in the central auditory pathways (10,12,25) as well as in a wide array of non-auditory brain areas and networks (12). Salicylate has been used to induce a model of tinnitus in rats (1–3). Our previous studies using this model revealed ultrastructural synaptic changes in the dorsal cochlear nucleus (DCN) and inferior colliculus (IC) of rats experiencing tinnitus (26–28). However, the underlying mechanisms remained unclear. Whether the alteration of BDNF and the BDNF/TrkB/CREB signaling pathway in the AC, the end point of the auditory pathway, contributes to the progression of tinnitus symptoms is worth investigating. In the present study, salicylate-induced tinnitus-like behavior in rats was confirmed using a gap detection test. Further, we determined the expression of BDNF, proBDNF, CREB, phosphorylated CREB (p-CREB), and TrkB, and assessed ultrastructural synaptic changes in the auditory cortex (AC).

MATERIALS AND METHODS

Animals

Twenty-seven Sprague–Dawley male rats (weight, 200–300 g) were obtained and maintained under pathogen-free conditions. All experimental procedures involving the animals were approved by the Institutional Review Board of Xinhua Hospital and were performed in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH publication No 80–23). The animals were randomly divided into three groups: 1) a chronic treatment group (S14) with repetitive injections of salicylate for 14 days (n = 9); 2) a recovery group (S14 + R14) with 14 days of recovery after chronic salicylate administration (n = 9); and 3) a control group with repetitive injections of saline (n = 9).

Study Design and Salicylate Administration

The tinnitus model was based on our previous studies (2,7,8,28). In brief, sodium salicylate (Sigma–Aldrich, Shanghai, China) was dissolved in saline to a concentration of 200 mg/ml. The chronic treatment group was given intraperitoneal injections (200 mg/kg) twice daily at 08:00 and 16:00 for 14 consecutive days. The recovery group received intraperitoneal injections for 14 consecutive days, followed by a 14-day

recovery period. The control group received a corresponding volume of normal saline during the same period of time. The rats in the chronic treatment group and the control group were sacrificed at 08:00 on day 15. The rats in the recovery group were sacrificed at 08:00 on day 29.

Gap Prepulse Inhibition of Acoustic Startle (GPIAS) and Prepulse Inhibition (PPI)

Tinnitus was assessed using the GPIAS and PPI protocol, as described previously (27–30). This protocol uses the acoustic startle reflex test in animals treated with salicylate. In brief, GPIAS and PPI testing was performed using the Acoustic Startle Reflex Starter Package for Rat or Mouse (Med Associates, St. Albans, VT). Sound stimuli were generated digitally by a digital signal processor (RZ6; Tucker Davis Technologies, Alachua, FL). An acrylic animal holder for the startle response was mounted interchangeably on the load-cell platform. Animal holder movement resulted in the displacement of the load cell, generating a voltage proportional to the velocity of displacement. The amplitude of the startle response was recorded by a personal computer and analyzed offline. GPIAS and PPI testing began 1 hour before the rats were sacrificed. The peak-to-peak values of the amplitude of the startle response were collected.

GPIAS sessions were composed of 30 gap trials and 30 non-gap trials. Rats underwent testing with different bandpass-filtered (1000 Hz bandwidth) sounds centered at 6, 12, or 16 kHz at a sound pressure level (SPL) of 65 dB. Startle responses were elicited by a 20-ms burst of white noise at an SPL of 100 dB. The gap in the narrowband noise began 100 ms before the onset of the broadband startling noise and lasted for 50 ms (5 ms rise/fall time). The interval between each startling noise was 30 to 35 seconds and each test lasted approximately 30 minutes. PPI sessions were composed of 30 startle trials and 30 prepulse trials. The startle stimulus was either presented alone or preceded by a 65-dB SPL narrowband noise-burst (50, 5 ms rise/fall time) prepulse centered at 6, 12, or 16 kHz (the same frequencies and levels used for gap detection testing). The onset of the noise-burst prepulse preceded the onset of the startle stimulus by 100 ms, and each test took approximately 30 minutes.

The percent GPIAS was calculated by computing the average ratio of trials with a gap versus non-gap trial for each frequency using the formula: $(\mathrm{AvgT_{non\text{-}gap}} - \mathrm{AvgT_{gap}}/\mathrm{AvgT_{non\text{-}gap}} \times 100)$, where $\mathrm{AvgT_{gap}}$ is the average amplitude during gap trials and $\mathrm{AvgT_{non\text{-}gap}}$ is the average amplitude of non-gap trials. The percent PPI was calculated by computing the average ratio of the startle versus prepulse trials for each frequency using the formula: $([\mathrm{AvgT_{startle}} - \mathrm{AvgT_{prepulse}}]/\mathrm{AvgT_{startle}} \times 100)$, where $\mathrm{AvgT_{prepulse}}$ is the average amplitude during the prepulse trials, while $\mathrm{AvgT_{startle}}$ is the average amplitude of the startle trials (31).

Quantitative Real-Time Polymerase Chain Reaction

Rats were euthanized after deep anesthesia with 2% sodium pentobarbital given intraperitoneally. The AC samples were dissected rapidly and total RNA was extracted from each sample with TRIzol reagent (TaKaRa, Otsu, Japan) according to the manufacturer's protocol. Extracted RNA was quantified spectrophotometrically at 260 and 280 nm, followed by reverse transcription into complementary DNA (cDNA) using a Prime-Script RT Master Mix (RR036A; TaKaRa). Primers for BDNF, TrkB, CREB, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Shanghai, China. The polymerase chain reaction (PCR) primer sequences were as follows:

BDNF-F, 5′-CCTGTGGAGGCTAAGTGGAG-3′;
BDNF-R, 5′-CCTGCTCTGAAGGGTGCTT-3′;
TrkB-F, 5′-CTTATGCTTGCTGGTCTTGG-3′;
TrkB-R, 5′-TGTTCTCTGGGTCAATGCTG-3′;
CREB-F, 5′-CAAACATACCAGATTCGCACA-3′; and
CREB-R, 5′-CGACATTCTCTTGCTGCTTC-3′.

PCR amplification was performed with SYBR Premix Ex Taq (RR420A; TaKaRa). The PCR cycles were as follows: 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds, 60 °C for 34 seconds, and a final dissociation stage, using the ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). We assumed the amplification efficiencies of the target and reference were approximately equal. Relative quantification and calculations were performed using the comparative threshold (Ct) cycle method ($2^{-\Delta\Delta Ct}$) (32).

Western Blot Analysis

Total proteins were extracted from the samples and their concentrations were determined using an ultraviolet spectrophotometer (DR/4000UV-VIS; Hach, Loveland, CO). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% [w/v] polyacrylamide gel) was used to electrophorese the TrkB protein and SDS-PAGE (10% [w/v] polyacrylamide gel) was used to electrophorese the BDNF, proBDNF, CREB, and p-CREB proteins. Equal quantities of protein were loaded onto each gel lane. After separation, proteins were transferred to polyvinylidene difluoride membranes, which were blocked in Tris-buffered saline (0.1% [v/v] Tween 20, 5% [w/v] skimmed milk powder) and then incubated with primary antibodies overnight and washed in Tris-buffered saline and 0.1% Tween 20. Secondary antibodies were diluted in blocking buffer and incubated with the membranes for 2 hours at room temperature. Finally, the chemiluminescence activity was captured by exposure to x-ray films. The band intensities of BDNF, proBDNF, TrkB, CREB, and p-CREB were expressed relative to GAPDH. The following antibodies were used: 1:1,000 rabbit anti-BDNF antibody (Sigma–Aldrich), 1:1,000 rabbit anti-proBDNF antibody (Abcam, Shanghai, China), 1:1,000 mouse anti-TrkB antibody (BD Biosciences, Franklin Lakes, NJ), 1:1,000 rabbit anti-CREB antibody (Cell Signaling Technology, Danvers, MA), 1:1,000 rabbit anti-phospho-CREB (pSer133) antibody (Cell Signaling Technology), 1:2,000 goat anti-rabbit IgG-HRP (Jackson, West Grove, PA), and 1:2,000 goat anti-mouse IgG-HRP (Jackson).

Transmission Electron Microscopy (TEM)

Twelve rats (three from each group) were used for the TEM study. The rats were anesthetized with 2% sodium pentobarbital given intraperitoneally, then perfused through the ascending aorta with 2% glutaraldehyde. The AC was located on the basis of stereotactic coordinates as provided in the Mouse Brain Atlas, removed bilaterally and dissected into cubes of $1 \times 1 \times 1$ mm with the aid of a dissecting microscope, and washed in 0.1 M phosphate buffer (pH 7.2). The tissue samples were then immersed in 2% glutaraldehyde and 1% osmium tetroxide for 2 hours at 4 °C. Subsequently, the tissue blocks were routinely dehydrated using a graded ethanol series. After displacing ethanol by propylene oxide, the tissues were embedded with epon. A series of consecutive ultrathin sections were prepared with a thickness of 70 μm, using a diamond knife in the coronal plane. They were then stained with lead citrate and observed under a CM-120 TEM (Philips, Eindhoven, North Brabant, The Netherlands). ImageJ software (National Institutes of Health, Bethesda, MD) was used for quantitative analysis on six sections of each tissue block, separately for the two hemispheres. Asymmetric synapses in layers 2 or 3 of the primary AC were examined. The number of synaptic vesicles, the thickness of postsynaptic densities (PSDs), the width of synaptic clefts, the curvature of synaptic interfaces, and the length of the synaptic active zones were measured in each photograph taken at 33,000× magnification (33).

Statistical Analysis

Results are presented as the mean ± standard errors of the mean. According to the distribution of the data and the homogeneity of variance, unpaired, two-tailed Student's t test and one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) post-hoc tests were used for comparisons among groups, except that GPIAS and PPI testing were tested for significance with a two-way analysis of variance (frequency and treatment groups) followed by Tukey's post-hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Salicylate-induced Tinnitus-like Behavior in Rats

GPIAS values were significantly lower in the chronic treatment group and the recovery group at 14 days than in the control group at 12 kHz (29.24 ± 7.51) and 16 kHz (30.81 ± 4.08), but not at 6 kHz (48.09 ± 8.97), indicating that these animals were experiencing tinnitus ($p < 0.05$). There was no difference in GPIAS values between the control group and the recovery group at 28 days ($p > 0.05$), indicating that the tinnitus-like behavior was not present in the control group, and it was not observed 14 days after salicylate discontinuation. There was no significant difference in PPI values among the three groups at 6, 12, and 16 kHz (Fig. 1).
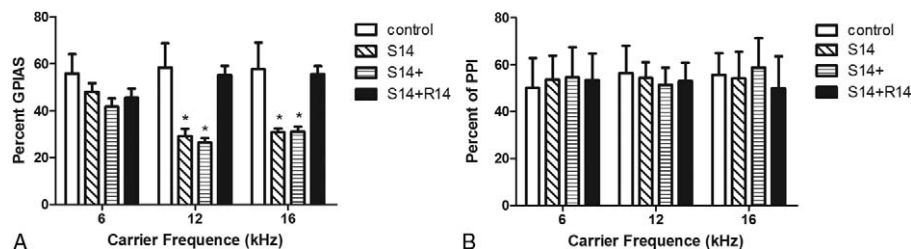
FIG. 1. Effects of salicylate on gap prepulse inhibition of acoustic startle (GPIAS) values and prepulse inhibition (PPI). *A*, The chronic treatment group (S14) and the recovery group at 14 days (S14+) showed a significant decrease in GPIAS values compared with the control group at 12 kHz (29.24 ± 7.51, *$p < 0.05$) and 16 kHz (30.81 ± 4.08, *$p < 0.05$), but not at 6 kHz (48.09 ± 8.97, $p > 0.05$). There were no differences in GPIAS values between the recovery group at 28 days (S14 + R14) and the control group ($p > 0.05$). *B*, There was no significant difference in PPI values among the three groups at 6, 12, and 16 kHz.

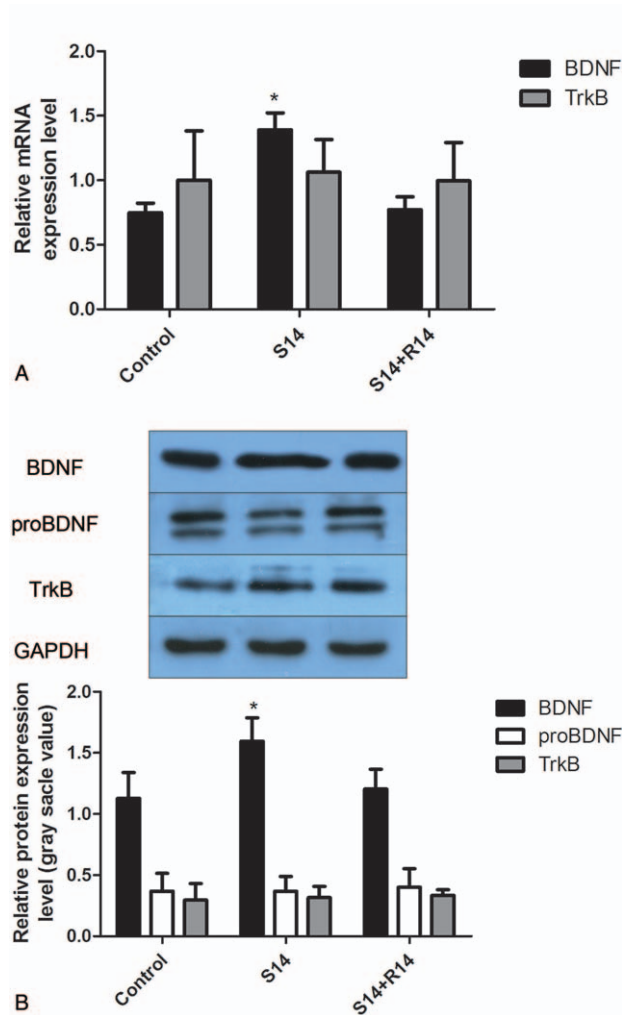**FIG. 2.** The expression of brain-derived neurotrophic factor (BDNF), proBDNF, and tyrosine kinase receptor B (TrkB) in the auditory cortex (AC). *A*, Real-time PCR showed a significantly higher level of BDNF mRNA in the chronic treatment group (S14; 1.39 ± 0.13) than in the control and recovery (S14 + R14) groups (*$p < 0.05$). There was no significant difference in TrkB mRNA expression among the three groups ($p > 0.05$). *B*, The protein expression levels of BDNF, indicated by a Western blot assay, were markedly increased in the chronic treatment group (S14; 1.60 ± 0.19) compared with any other group (*$p < 0.05$). There was no significant difference in proBDNF and TrkB protein expression among the three groups ($p > 0.05$).

Expression of BDNF, proBDNF, and TrkB in the AC

Compared with the control group, *Bdnf* gene (1.39 ± 0.13) and protein (1.60 ± 0.19) expressions were significantly upregulated in the chronic treatment group ($p < 0.05$), but not in the recovery group. There was no difference in proBDNF protein expression or *TrkB* gene or protein expression among the three groups (Fig. 2).

Expression of CREB and p-CREB in the AC

Compared with the control group, p-CREB protein expression (0.20 ± 0.03) was upregulated in the chronic

treatment group ($p < 0.05$), but not in the recovery group. There was no difference in *Creb* gene and protein expression among the three groups (Fig. 3).

Ultrastructural Alterations of Synaptic Endings

The synaptic endings were observed under TEM. Quantitatively, the ultrastructure of the AC neurons showed a significant increase in the number of synaptic vesicles ($p < 0.01$), PSDs ($p < 0.01$), synaptic interface curvature ($p < 0.01$), and synaptic active zone length ($p < 0.05$) in the chronic treatment group, as compared with the control group. There were no significant differences in the ultrastructure between the control group and the recovery group (Fig. 4). These results are summarized in Table 1. There were no significant differences in synaptic ultrastructure between the two hemispheres.
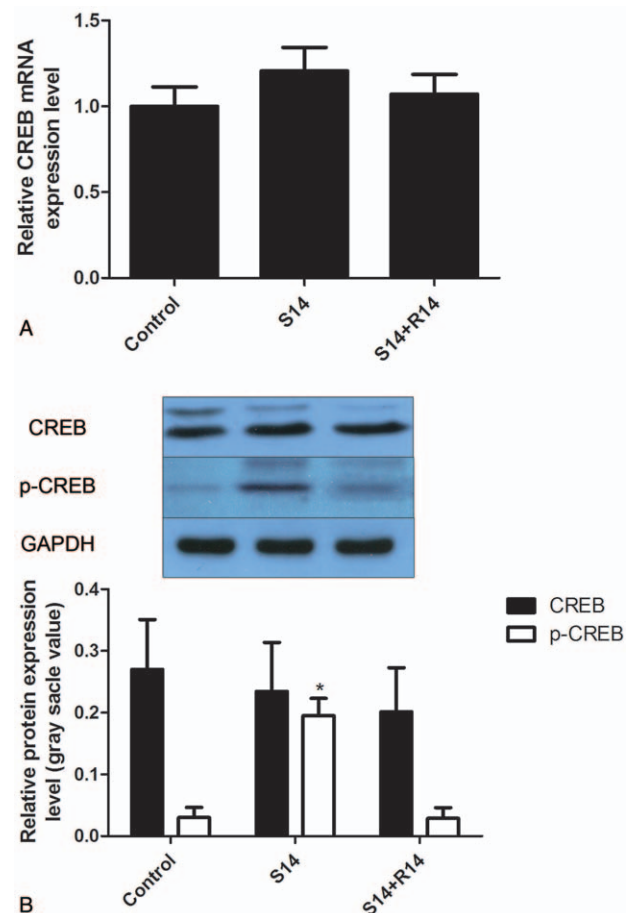
FIG. 3. The expression of cAMP-responsive element-binding protein (CREB) and phosphorylated CREB (p-CREB) in the auditory cortex (AC). *A*, Real-time PCR showed no difference in CREB mRNA expression among the three groups ($p > 0.05$). *B*, The protein expression levels of p-CREB, indicated by a Western blot assay, were markedly increased in the chronic treatment group (S14; 0.20 ± 0.03) compared with the other two groups (*$p < 0.05$). There was no difference among all three groups for CREB protein expression in the AC ($p > 0.05$).

FIG. 4. Ultrastructural alterations of synapses in the auditory cortex (AC). Compared with the control group, synapses of rats in the chronic treatment (S14) group contained a greater number of presynaptic vesicles (*white arrows*), a greater number of postsynaptic densities (PSDs, *black arrows*), increased synaptic interface curvatures, and longer synaptic active zones. There were no significant differences between the control group and the recovery group (S14 + R14). Scale bar, 0.2 μm.
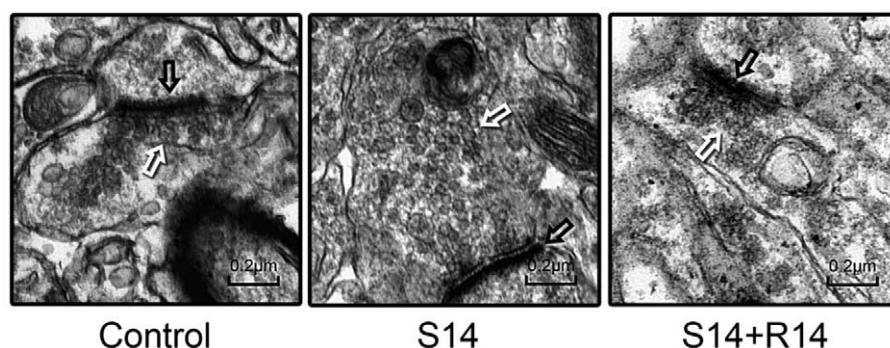
DISCUSSION

The progression of tinnitus is associated with altered neuronal excitability in peripheral and central auditory neurons. Changes in the central auditory system are more likely to induce tinnitus perception and auditory plasticity (10–12). As one of the key neurotrophins for neuroplasticity (i.e., synaptic plasticity), *BDNF* is known as an immediate early gene with mRNA transcription occurring rapidly without the need of new protein synthesis (34). *BDNF* can regulate self-synthesis through the BDNF/TrkB/CREB signaling pathway (23,24) and its transcription is regulated by neuronal activity (35). It is well known from previous studies that salicylate administration induces tinnitus in humans and in animals (36,37). These features were earlier observed in response to aspirin and sodium salicylate, because salicylate was soon thought to be the active compound. Sodium salicylate-induced tinnitus is the most widely used animal model to study tinnitus in rats (1–3,38). In previous studies, BDNF was found to play a key role in the onset and the persistence of salicylate-induced tinnitus (39). Upregulated BDNF expression in spiral ganglion neurons combined with increased spontaneous activity in the cochlear nerve and the DCN have been reported in a salicylate-induced tinnitus model (40). How BDNF participates in the process of tinnitus is unknown. We, therefore, determined whether there is an increase in the BDNF/TrkB/CREB signaling pathway in the AC in a salicylate-induced tinnitus model.

In this study, we found that BDNF and p-CREB were upregulated in the AC of rats with salicylate-induced tinnitus. BDNF transcripts have been suggested to play a key role during plasticity-associated changes of synaptic efficacy (13–15). It is reported that variant BDNF might act as a common susceptibility factor in chronic tinnitus (41,42). Several clinical studies also reported that plasma BDNF levels are increased in patients with tinnitus compared with healthy controls, and it may serve as a useful biomarker for evaluating the therapeutic effects in patients with tinnitus (43,44). BDNF induces the expression of genes coding for the regulators of synaptic activity (45), PSD scaffold protein in rat forebrain synaptoneurosomes (46) and cultured cerebrocortical neurons (47), several synaptic vesicle proteins, and in proteins related to their traffic, such as vesicular glutamate transporters (48,49). Using cultured neurons and muscle cells, it has been reported that BDNF can potentiate synaptic transmission at developing neuromuscular junctions on a fast timescale (minutes) by enhancing neurotransmitter release from presynaptic terminals, such as glutamate and acetylcholine (16,50). In particular, BDNF appears to be critical for the ability of synapses to undergo changes in synaptic efficacy (16). Keifer et al. (51) demonstrated that application of BDNF significantly increases the size of presynaptic boutons.

TABLE 1. *Comparisons of synaptic parameters in the auditory cortex among three groups*

n = 18	Control	S14	S14 + R14
Synaptic vesicles (number/μm^2)	16 ± 6	52 ± 24[b]	15 ± 6
Cleft width (nm)	15.98 ± 1.47	19.09 ± 4.17	20.87 ± 3.46
postsynaptic density (PSD) thickness (nm)	32.03 ± 6.83	42.21 ± 11.20[b]	26.96 ± 4.61
Synaptic curvature	1.05 ± 0.05	1.13 ± 0.06[b]	1.04 ± 0.02
Length of synaptic active zone (nm)	287.09 ± 77.34	411.80 ± 96.10[a]	324.99 ± 59.68

[a]$p < 0.05$, compared with controls.
[b]$p < 0.01$, compared with controls.
N indicates the total number of photographs in each group, bilaterally from each rat. Control group (n = 3 rats), chronic treatment group (S14, n = 3 rats), recovery group (S14 + R14, n = 3 rats).

Therefore, the extracellular proteolytic regulation of proneurotrophins such as BDNF in the synaptic cleft has a key role in coordinating activity-dependent pre- and postsynaptic modifications during synaptic plasticity and learning. Synaptic plasticity is one of the most important aspects of neuroplasticity, which include both short-term changes in the strength or efficacy of neurotransmission and long-term changes in the structure of the synapses (52). In this study, rats with long-term administration of salicylate showed more presynaptic vesicles, thicker and longer PSDs, and increased synaptic interface curvatures. These changes demonstrated that long-term administration of salicylate caused changes in neural plasticity at the AC level. We hypothesize that repeated salicylate administration upregulates the expression of BDNF, which could correlate with long-term changes in synaptic ultrastructure. With enhanced neurotransmitter release, PSD scaffold proteins and synaptic vesicle proteins will be increased. These effects will constantly increase, with over activity in the AC or other auditory centers leading to continuous tinnitus. Moreover, plasma BDNF levels are decreased in patients with severe tinnitus after effective tinnitus retraining therapy (53). BDNF may therefore play an important role in these processes.

ProBDNF interacts preferentially with $p75^{NTR}$, whereas BDNF selectively binds and activates TrkB (20,54). ProBDNF and BDNF elicit opposite biological effects by activating two distinct receptor systems (55). While the involvement of BDNF, in activity-dependent synaptic plasticity has been demonstrated in various systems, it is not at all clear which intracellular signaling pathways originating at the TrkB receptors are involved and how they exert their action (16). If the TrkB-Shc site-activated pathway is not involved in changes of synaptic function, a prime candidate is the TrkB-PLCγ1-dependent pathway. It can release Ca^{2+} from intracellular stores via IP_3 and thereby activate CREB, which is an important mediator of neurotrophin function (16). However, there were no changes in the proBDNF protein and TrkB gene and protein expression in our study. CREB is downstream of the ERK pathway and is also a major downstream signaling pathway of the *N*-methyl-D-aspartate receptors (NMDARs). CREB signaling regulates expression of several genes, including BDNF (56,57). The phosphorylation of CREB subsequently results in the transcriptional regulation of c-fos, c-jun, and bcl-2 (58–60). CREB–BDNF signaling has been suggested to be critical in numerous neuronal biological processes, including cell survival, synaptic structure, and synaptic plasticity (15,61). In a previous study, there were no changes in *Creb* gene expression and an increase in the phosphorylation of CREB protein after injection of concentrated salicylate into the spiral ganglion neurons of the rat cochlea (62). A possible explanation could be that they are prebound in an inactive state to the BDNF promoter. This supports the possibility that the phosphorylation of CREB may be involved in the signaling pathway that is initiated in the AC after salicylate treatment. The work from Jourdi et al. (47) showed that

expression of an extracellularly secreted tolloid-like metalloproteinase is regulated in the early stages of classical conditioning and functions in the conversion of proBDNF to mature BDNF, which may explain our data. Their data suggest that within minutes after the beginning of paired conditional stimulus (CS)–unconditional stimulus (US) stimulation, PKA and CaMKIV are phosphorylated and activate the transcription factor CREB. Since mature BDNF protein expression is initiated shortly after this, one obvious target for CREB was postulated to be *Bdnf*. Both tTLL mRNA and BDNF protein are expressed shortly after activation of CREB, and one possibility is that CREB, or possibly another transcription factor, directly induces transcription of the tTLL gene. Once tTLLs protein is manufactured and secreted into the synaptic cleft, it cleaves proBDNF into mature BDNF. Therefore, the activity-dependent expression of the mature form of BDNF may be regulated not at the level of *Bdnf* transcription but rather at the point of the proteolytic cleavage of the precursor proBDNF. There are still many problems to be researched in the future. Genetic studies to disrupt the PLCγ1 signaling downstream the TrkB receptor should be analyzed in detail to elucidate the mechanisms by which BDNF might regulate synaptic plasticity in the auditory pathway.

In our previous studies, expression of the NMDAR subunit, NR2A, and NR2B in the DCN was increased (26,27), and increased expression of NR2B was also found in the inferior colliculus and AC (3). NMDARs are members of the glutamate receptor channel superfamily. It is suggested that tinnitus arises from an increase in excitatory neurotransmission, and is strongly associated with NMDAR activity (63). Activated NMDARs enhance Ca^{2+} levels in the axon terminal, facilitating exocytosis of synaptic vesicles (64). It is well known that neuronal stimulation by NMDA activates a signaling cascade resulting in CREB phosphorylation (35,65). Thus, a chronic increase in the activation of NMDAR enhances the phosphorylation level of CREB and induces BDNF expression, which might be the molecular and cellular mechanism underlying NMDAR-mediated physiological and pathological processes (23). It has been reported that BDNF modulates glutamatergic synaptic transmission and plasticity (66). Alterations of BDNF and NMDARs during tinnitus may come from their complicated interactions, and the phosphorylation level of CREB may also be involved in the process. Subsequently, synaptic ultrastructural and functional changes and alterations in the plasticity of the auditory center may result in persistent tinnitus. Lopez Hill et al. (53) found a significant decrease of mice hippocampal BDNF levels induced by MK-801, the most potent non-competitive NMDARs antagonist. Our investigation of BDNF may enlighten the detailed biological ability and complicated mechanisms of action of BDNF in the context of tinnitus, which would provide a future BDNF-related remedy for tinnitus.

In conclusion, long-term salicylate administration increased the expression of BDNF and p-CREB in the

AC, accompanied with ultrastructural synaptic changes and upregulated synaptic efficacy, enhanced neurotransmitter release, and increased synthesis of PSD scaffold and synaptic vesicle proteins. All these alterations imply that long-term administration of salicylate markedly but reversibly causes neuroplasticity changes at the AC level, which may play a crucial role in persistent tinnitus. Further study is required to find the BDNF-based therapeutic strategies for tinnitus.

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