

Salicylate increased ascorbic acid levels and neuronal activity in the rat auditory cortex

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ABSTRACT

Importance: Clinical observations have implied a central origin for tinnitus and potential therapeutic effects of ascorbic acid (AA); however, the detailed mechanisms remain undetermined.

Objective: To investigate changes in the AA levels and neural activity in the auditory cortex (AC) during salicylate-induced tinnitus.

Methods: Rats were randomly divided into 3 groups: (1) saline group, which received an intraperitoneal saline injection; (2) SS group, which received an intraperitoneal sodium salicylate (SS) injection (350 mg/kg); and (3) SS+Lido group, which received an intraperitoneal SS injection (350 mg/kg) and lidocaine delivered to the AC by microdialysis. For each group, we firstly used an in vivo microdialysis technique to investigate the concentrations of AA in the AC; and secondly, we recorded the neural activity in the AC using a single-unit recording technique.

Results: The AA concentration in the SS group significantly increased after SS injection, whereas that of the saline group did not change. The AA concentration in the SS+Lido group also showed an increasing trend but was significantly lower than that in the SS group. In the electrophysiological study, the spontaneous firing rate of the SS group was significantly higher than that of the saline group. In addition, the proportion of short interval discharges was also higher in the SS group than in the saline group. Both differences were reversed by lidocaine treatment.

Interpretation: Our data suggest that the elevation of AA levels in the AC may be related to increased neuronal activity, which may represent the mechanism underlying salicylate-induced tinnitus.

KEYWORDS

Tinnitus, Auditory cortex, Ascorbate acid, Neural activity, Salicylate

INTRODUCTION

Tinnitus is a phantom auditory sensation, without an external stimulus, and can be debilitating for both adults and children. Epidemiological studies in different countries have shown that 10%–15% of adults are affected by tinnitus, and approximately 5% of adults

suffer significantly.¹ Although the reported frequencies of pediatric tinnitus have varied among different reports, a review by Rosing et al² showed that 4.7%–46% of the general pediatric population of children with normal hearing experienced tinnitus, and 23.5%–62.2% of children with hearing loss experienced tinnitus. In addition, studies have shown that the tinnitus prevalence

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increased with age, for both juveniles^{3,4} and adults¹. Despite great advances have been made by monitoring the neurophysiological changes associated with some forms of tinnitus and the development of animal models of tinnitus, the neural mechanisms underlying tinnitus remain unknown. Many different drugs and methods have been used to treat tinnitus, but most have had little success,⁵⁻⁸ making tinnitus one of the most difficult symptoms to treat, for both patients and otologists.

According to recent studies, tinnitus results from changes in the central nervous system and is initially triggered by peripheral insults, although some insults are not obvious to patients.⁹ Multiple recent studies have reported cortical hyperexcitability and correlated plasticity changes during tinnitus.¹⁰ Physiological studies have indicated the existence of increased synchrony of spontaneous neuronal firings, suggesting that tinnitus is associated with hyperactivity in the primary auditory cortex (AC) of animal models.¹¹ Therefore, investigators increasingly agree with the central origin theory of tinnitus, with the AC representing the most primary and important region.

Ascorbic acid (AA) is widely distributed in the central nervous system and has been found to be involved in both physiological and pathological processes in the brain.¹² AA was found to positively modulate inflamm-aging and immunosenescence, two hallmarks of biological aging.¹³ AA treatment prior to noise exposure had also been shown to play a protective role for auditory function.¹⁴ Because aging and noise exposure are both probable factors for tinnitus, AA may also play an important role during the development of tinnitus.

Since Barany¹⁵ reported that a submucosal injection of procaine hydrochloride during intranasal surgery could halt tinnitus, local anesthetic agents, such as lidocaine, have been used to treat tinnitus.¹⁶ The intravenous administration of lidocaine has been reported to relieve tinnitus in 40%–80% of patients;¹⁷ however, whether lidocaine acts on the organ of Corti or on the central auditory pathway has not yet been clarified. Considering the potential for serious adverse effects on the cardiac rhythm system caused by lidocaine administration, new treatment strategies must be developed. However, no other treatments have been found to achieve similar or better treatment effects compared with lidocaine. Therefore, understanding the mechanisms underlying the treatment effects of lidocaine on tinnitus may not only help to elucidate the pathophysiology of tinnitus, but also provide new pharmacological strategies for the treatment of tinnitus.

METHODS

Subjects

Forty-six adult male Wistar rats (Vital River Laboratory

Animal Technology Co. Ltd., Beijing, China) weighing 280–320 g were used in this study. Each rat was individually housed in a $22 \pm 1^\circ\text{C}$ cage, with a 12/12 h reversed light/dark schedule and full access to food and water. All experimental procedures were approved by the Laboratory Animal Care and Use Committee of Peking University Health Science Center.

Drugs and reagents

Sodium ascorbate and lidocaine was purchased from Sigma (St. Louis, MO, USA). Other drugs and reagents were purchased from local commercial sources. Artificial cerebrospinal fluid (aCSF) was composed of 126 mM NaCl, 2.4 mM KCl, 1.1 mM CaCl_2 , 0.85 mM MgCl_2 , 27.5 mM NaHCO_3 , 0.5 mM Na_2SO_4 , and 0.5 mM KH_2PO_4 .

Experiment design

Experiment 1

Twenty-eight rats were randomly assigned to three groups: (1) Saline ($n = 4$), rats received intraperitoneal (i.p.) saline injections; (2) SS ($n = 12$), rats received i.p. sodium salicylate (SS) injections (350 mg/kg); and (3) SS+Lido ($n = 12$), rats received i.p. SS injections (350 mg/kg) and were treated with lidocaine (0.2%, dissolved in aCSF) by microdialysis. AC microdialysates were collected from each rat and AA concentrations were detected.

All rats were anesthetized and placed in a stereotaxic head frame on a heating pad, to maintain the body temperature at 37°C . The left AC was exposed, using procedures described previously.¹⁸ Briefly, after shaving the dorsum of the skull, a 1-cm incision over the scalp was made, and the soft tissue covering the parietal and temporal bones was removed completely. A 1.5-mm hole above the AC was drilled, according to the Paxinos and Watson brain atlas,¹⁹ 4.8 mm posterior to bregma and 6.4 mm lateral to bregma. Then, a microdialysis guide cannula (MAB6.14.2ss, BAS) was implanted in the AC and secured in place permanently with supporting screws and dental cement. After the surgery, the animals were allowed to recover for at least 48 h before the experiment.

At the beginning of the experiment, stylets were replaced with microdialysis probes (MD-2200, BAS), with a semipermeable membrane extending 2.0 mm beyond the ventral tip of the guide cannulas. During the experiment, the rats were able to move freely. The microdialysis probes were connected to a perfusion pump (CMA100, Stockholm, Sweden), which pumped at a flow rate of $2 \mu\text{L}/\text{min}$. Microdialysates were perfused continuously through an online electrochemical system²⁰ to monitor AA concentrations.

The microdialysis continued until 6 hours after either the saline or SS injection. At the end of the experiment, methylene blue was perfused to dye the path of the probe.

The rats were injected with a lethal dose of pentobarbital sodium and cardiac perfused with PBS (0.1 M, pH 7.4), followed by paraformaldehyde solution (4%). The brains were removed and placed in paraformaldehyde solution (2 h) and then transferred to sucrose solution (30%). Coronal sections were made with methylthionine chloride staining the track of the probes to determine the placement of the dialysis probes.

Experiment 2

Eighteen rats were randomly divided into three groups, as described in Experiment 1, with six rats in each group. Neuronal activity in the AC was recorded using the single-unit discharge technique.

All rats were anesthetized and placed in a stereotaxic frame, and a craniotomy was performed above the AC, in accordance with the Paxinos and Watson brain atlas,¹⁹ 3–7 mm posterior to bregma, and 3.5–5.5 mm lateral to bregma. Another small hole near bregma was drilled, and a fine jeweler's screw was inserted to connect to the reference electrode. Under a surgical microscope, the dura was removed, and the AC was exposed. The microdialysis probe was then lowered into position with the stereotaxic carrier so that the end of the microelectrode rested in place. A self-made microelectrode (drawn from borosilicate glass, filled with 3 M KCl solution, with a tip diameter of 0.5–5 µm and impedance of 5–10 MW) was inserted into the AC region near the probe and fixed with a carrier to the stereotaxic frame. The craniotomy was then covered with agar. The microelectrode output and the reference electrode were connected to a multichannel preamplifier (SWF-2W, Chengdu instrument factory, China) with a flexible wire. The output from the preamplifier was delivered to a digital signal processing module (RM6240B, Chengdu instrument factory, China), with a 2 000× gain and a band-pass filter set between 500 Hz and 10 KHz. Single-unit spike activity could be well-isolated.

During the experiment, rats were kept anesthetized and received the same treatments as described in Experiment 1. Spontaneous activity was recorded approximately every 5 min until 2 hours after injections.

At the end of the session, the electrode track was marked by iontophoretically ejecting Fast Green from the electrode at 2–3 points. Then, the animals were sacrificed and brain sections were made, as described in Experiment 1, to determine the placement of the microdialysis probe and the microelectrode.

Data analysis

The online electrochemical system reported the AA concentrations of the microdialysates, with a current

response. Chart 5 from AD Instruments (USA) was used as waveform analysis software to analyze the single-unit discharges. The discriminator module was used to distinguish neuronal discharge waveforms into single waveforms. For each unit, the spontaneous firing rate (SFR, spikes/s) was calculated, and a discharge interval histogram was created to determine the firing pattern of the neuron (resolution 2 ms; the proportion of discharge intervals shorter than 50 ms was calculated).

The data were statistically analyzed using SPSS 19 software. The data were presented as the mean ± standard deviation (SD). Differences in microdialysate AA concentrations between groups were assessed with a two-way analysis of variance (ANOVA), followed by the least significant difference test of multiple comparisons. A one-way ANOVA was used to compare the differences in SFRs and short interval discharge proportions between groups. The accepted two-tailed level of significance was 0.05.

RESULTS

Effects of SS and lidocaine on AA levels in the AC

Given that AA is thought to be involved in auditory protection, we investigated the extracellular AA concentrations in the AC in animal models with SS-induced tinnitus, both with and without lidocaine treatment. A typical microdialysis probe path is shown in Figure 1A. The extracellular AA levels of the three groups are shown in Figure 1B. After the injection, the AA concentration of the saline group did not change significantly. The AA concentration of the SS group began to increase significantly 30 min after the injection and gradually increased to as high as 517% ± 162% of baseline after 4 hours. In the SS+Lido group, the AA level also showed an increasing trend, but it increased much slower than the SS group. At 4 hours after injection, the AA level of SS+Lido group was 222% ± 66% of the baseline. The two-way ANOVA indicated significant effects for treatment ($F_{(2, 600)} = 64.65$, $P < 0.0001$) and time ($F_{(24, 600)} = 33.17$, $P < 0.0001$). Bonferroni post hoc tests showed that the differences between the SS group and the other two groups were significant, starting 90 min after treatment. In addition, the differences between the saline group and the SS+Lido group were only significant at 200 min and 240 min after treatment; this implied that lidocaine inhibited the SS-induced increases of the AA concentrations in the AC.

Effects of lidocaine on SS-induced single-unit AC neuron discharges

Because AA modulates neural activity, we next detected the neuronal activity of AC neurons in rat models of SS-induced tinnitus. Three or more contiguous spikes with

interspike intervals shorter than 50 ms constituted a short interval discharge. Examples of simple discharges and short interval discharges are shown in Figure 2A and 2B, respectively. The SFR and the interspike interval histogram (ISIH) of spontaneous activities were used as indices. A total of 30 neurons were recorded in each group. A typical microelectrode path is shown in Figure 3A. The tip of the microelectrode was placed in the AC, and 2 hours after treatment, the SFRs were 2.71 ± 1.55 spikes/s, 10.54 ± 5.92 spikes/s, and 3.32 ± 1.45 spikes/s in the saline, SS, and SS+Lido groups, respectively. A significant difference was detected using a one-way ANOVA among the three groups ($F = 38.47$, $P < 0.0001$). Bonferroni's Multiple Comparison Test showed significant differences between the Saline group and the SS group ($P < 0.001$) and between the SS group and the SS+Lido group ($P < 0.001$). However, no significant difference was found between the saline group and the SS+Lido group (Figure 3B). Typical examples of ISIHs for the three groups are shown in Figure 4. The percentages of short interval discharges were 9.3% in the saline group, 56% in the SS group, and 16.7% in the SS+Lido group. These results indicated that neural activity was increased by SS and that the increase could be effectively reversed by lidocaine.

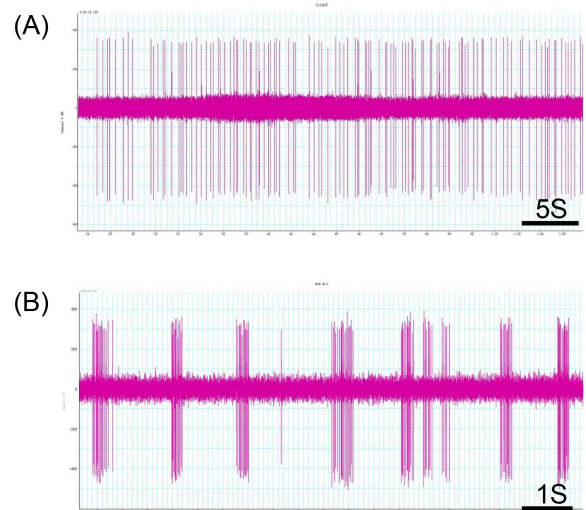


FIGURE 2 Example of spike discharges, recorded from the microelectrode in the auditory cortex. (A) Simple discharge. (B) Short interval discharge (interspike interval shorter than 50 ms).

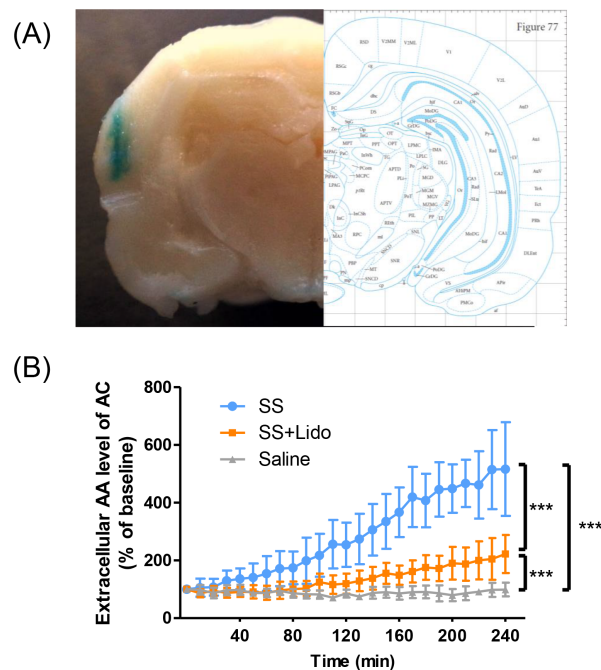


FIGURE 1 Effect of SS and lidocaine on the extracellular concentrations of AA in the AC. (A) Representative coronal brain section, showing the placement of a probe track in the AC. (B) The AA concentration in the AC of SS-treated rats was significantly higher than that of Saline-treated rats from 1.5 h to 4 h post-injection. Lidocaine significantly inhibited the increase of AA (Saline: $n = 4$; SS: $n = 12$; SS+Lido: $n = 12$. Two-way ANOVA with Bonferroni post hoc tests). SS, sodium salicylate; AA, ascorbic acid; AC, auditory cortex; Lido, lidocaine; ***, $P < 0.001$.

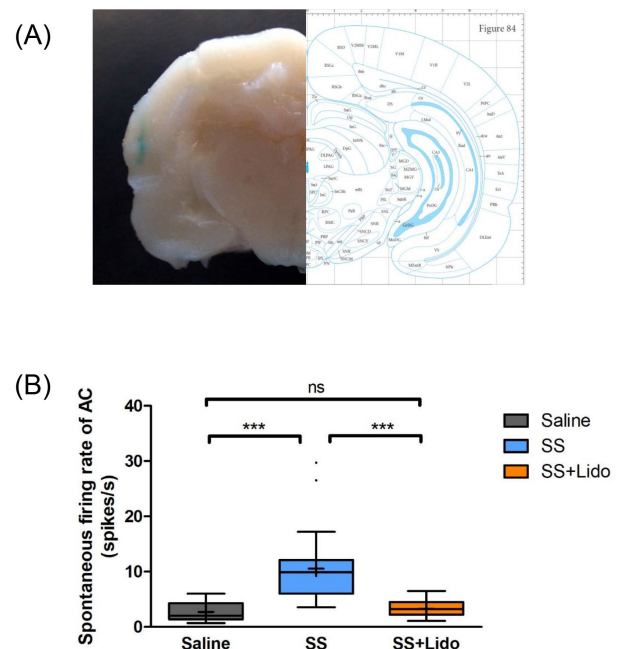


FIGURE 3 Effects of SS and lidocaine on neural activity in the AC. (A) Representative coronal brain section showing the placement of a microelectrode track in the AC. (B) The SFR of SS-treated rats was significantly higher than that of saline-treated rats. Lidocaine significantly weakened this difference. (Saline: $n = 30$; SS: $n = 30$; SS+Lido: $n = 30$. One-way ANOVA with Bonferroni post hoc tests). SS, sodium salicylate; AC, auditory cortex; SFR, spontaneous firing rate; Lido, lidocaine; ***, $P < 0.001$; ns, $P > 0.05$.

DISCUSSION

The clinical observation that tinnitus continues to exist after systemic or local treatments indicates that tinnitus may have a central origin. Along the auditory pathway, the

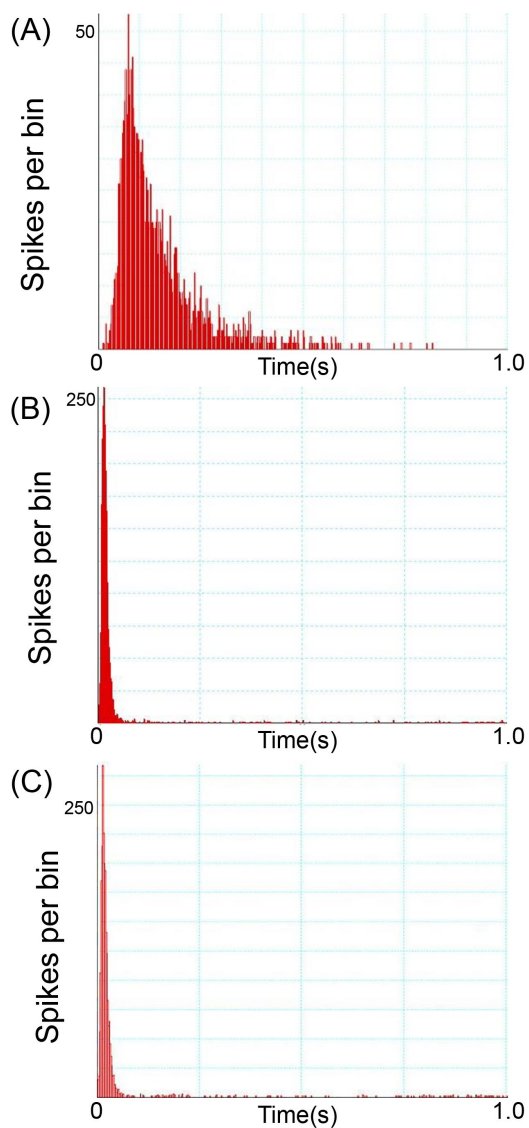


FIGURE 4 Examples of interspike interval histograms (ISIHs) for the ascorbic acid in the Saline group (A), SS group (B) and SS+Lido group (C). SS, sodium salicylate; Lido, lidocaine.

AC acts as the most important region for the perception of sounds. We used microdialysis and a single-unit recording technique to investigate the changes in AA concentrations and neuronal activity in the AC in an SS-induced tinnitus animal model. Our results showed the following: (1) SS injection significantly increased the extracellular AA level in the AC; (2) the SFR of AC neurons was significantly increased by SS treatment, and the firing pattern changed, with an increased percentage of short interval discharges; and (3) both the increased AA level and the increased SFR could be inhibited by lidocaine treatment.

SS is the active component in a number of widely used antipyretic analgesic and non-steroid anti-inflammatory

drugs. When used at high doses, however, SS can temporarily induce moderate hearing loss and high-pitched tinnitus in humans and animals. During the 1980s, Jastreboff and colleagues^{21,22} applied this characteristic to tinnitus research and identified the presence of SS-induced tinnitus in rats, based on behavioral evidence. This approach had been widely validated and used by researchers. In our study, we treated rats with a high dose of SS (350 mg/kg) to investigate changes in the AC during tinnitus. This dose was the same as that used by Jastreboff and has been used in many behavioral experiments, with a reliable behavioral manifestation of tinnitus.^{21,23-25}

AA reaches the highest concentration in the brain.¹² As an important endogenous antioxidant, SS is considered to be neuroprotective. Based on these findings, researchers have attempted to investigate the therapeutic effects of AA during tinnitus, which might result from the abnormal generation of reactive oxygen species (ROS) after noise exposure and other etiologies;^{26,27} however, the results have been controversial and the mechanism has not been revealed. We found that the extracellular AA level in the AC significantly increased starting 1.5 hours after SS injection, which was in accordance with the results reported by other studies.²⁵ In addition, this increase could be inhibited by lidocaine treatment, which currently represents the most effective treatment for tinnitus. This result implied that AA might play some role in the generation of SS-induced tinnitus.

SS has been found to induce abnormal excitability at the levels of the brainstem, the subcortex and the cortex, which may be related to tinnitus. In our study, we found that SS increased the SFR and short interval discharge rate in AC neurons, which could be reversed by lidocaine. Our results were similar to those reported by previous research.^{18,28} Because the firing pattern is related to the precise balance of transmitters, neurotransmitters in the AC may also be affected by SS administration. Considering that AA also plays an important role in the transmission and clearance of glutamate,¹³ a common excitatory neurotransmitter in the brain, we suggested that SS injections may induce a neurotransmitter imbalance in the AC, which may cause tinnitus. An overload of glutamate has been found in the AC of tinnitus animal models²⁵ and in the AC of humans²⁹, and the increased glutamate concentration could be weakened by glutamate antagonist MK-801. Changes in gamma-aminobutyric acid (GABA), another neurotransmitter associated with glutamate metabolism, have also been identified in the AC and other regions of the central auditory system.^{29,30} Taken together, our results suggest that AA might be involved in the generation of SS-induced tinnitus through the modulation of neurotransmitter levels, such as glutamate.

Our study also provides evidence for the origin of tinnitus, which remains under debate. The AC is the most important

center for sound perception and makes comprehensive connections with other centers, such as the limbic system and the autonomic nervous system, which have been found to act synergistically to promote the maintenance of tinnitus symptoms.³¹ Thus, the AC is an important site when studying tinnitus. Previous research revealed the hypermetabolism²³ and hyperactivity³² of cortical neurons, and our monitoring of changes in neuronal activity and AA levels provides further evidence of these changes.

Although we used the same dose of SS (350 mg/kg) as what has been used by Jastreboff and many others, the side effects of SS, including hearing loss and systemic toxicity, should be taken into consideration. The tinnitus-like behavior of animals could be affected by these side effects, making the result of experiments based on the behavior of tinnitus model animals difficult to interpret. For a better understanding of the electrophysiological and molecular mechanisms of tinnitus, other sites of classical auditory pathways, such as the medial geniculate body, inferior colliculus, and cochlear nucleus, as well as non-classical auditory pathways, should be examined in the future. Further examination of the behavioral expression of tinnitus will provide more significant evidence for the clinical pharmacotherapy of this intractable disease.

CONFLICT OF INTEREST

All authors have approved the final article. The authors declare no conflicts of interest.

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