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Original Article

Changes in GABA and glutamate receptors on auditory cortical excitatory neurons in a rat model of salicylate-induced tinnitus

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Abstract: Tinnitus is associated with neural hyperactivity, which is regulated by neuronal plasticity in the auditory central system, especially the auditory cortex (AC). Excitatory neurons constitute approximately 70-85% of the total populations of neuronal cells. However, few reports have focused on the AMPA receptor (AMPAR) and the $GABA_A$ receptor ($GABA_AR$) on the excitatory neuron in animal model of tinnitus. In this study, we gave rats a single or long-term of salicylate administrations. The tinnitus-like behavior was assessed by combination of the gap prepulse inhibition of acoustic startle (GPIAS) and the pre-pulse inhibition (PPI) tests. Using immunofluorescent staining, we examined whether the AMPAR and the $GABA_AR$ on the calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) -labeled excitatory neurons in the auditory cortex underwent changes following salicylate treatment. The rats with 14 days of salicylate administration showed evidence of experiencing tinnitus, while the rats receiving a single dose of salicylate manifested no tinnitus-like behavior. Furthermore, the AMPAR and $GABA_AR$ responded in a homeostatic manner after a single dose of salicylate while those showing in a Hebbian way after long-term salicylate administration. Thus, the different patterns of plasticity change in cortical excitatory neurons might affect the generating of salicylate-induced tinnitus.

Keywords: Tinnitus, salicylate, auditory cortical excitatory neuron, GABA receptor, glutamate receptor

Introduction

Tinnitus is the phantom perception of sound in the absence of an actual external auditory stimulus [1]. With prevalence ranging from 10% to 15%, tinnitus severely affects quality of life and causes a mass of psychological disorders [2]. Salicylate, a widely applied anti-inflammatory drug in clinical scenarios, can cause reversible tinnitus and induced animal model of tinnitus reliably [3]. Amounts of studies have revealed that salicylate can exert pathological influences on almost all the constitute relays along the auditory pathway [1, 4]. Cochlear malfunction has been regarded as the trigger of tinnitus. The altered peripheral auditory stimuli continuously transmit from the cochlea to the central auditory system, which increase spontaneous neuronal activity at various auditory pathway relays [5]. The neural maladaption in the central auditory system is more likely to form and maintain tinnitus [6]. Consistent with the "tinnitus-centralization" mechanism, we previously found: augmented cubic distortion product of 2f1-f2 and increased expression of prestin in the cochlea [7, 8], increased spontaneous cochleoneural activity [9], and synapse alterations in the inferior colliculus and the AC [10]. As a crucial part of the auditory system, the AC is demonstrated to play a significant role in tinnitus generating [11, 12]. Salicylate increases the amplitude of sound-evoked local field potentials recorded from the AC in vivo [13], and attenuates the GABAergic synaptic transmission in AC slices in vitro [14]. Frequency-map reorganization appears in several sub-

fields of mouse AC after injection of salicylate [15]. In our previous study, we demonstrated that long-term salicylate administration reversibly elevated the 18F-FDG uptake [10], increased the expression of early response genes and altered the synaptic structure in AC [10, 16]. What is more, these functional and morphological changes synchronized the tinnitus-like behavior in time phase. These evidences strongly indicated that salicylate-induced tinnitus might be generated at AC level due to neuronal plasticity associated cortical heperactivity.

In the cerebral cortex, excitatory neurons constitute approximately 70-85% of the total populations of neuronal cells [17]. Excitatory neurons emanate axon and dendrites, making excitatory glutamatergic synaptic contacts along their length, while receiving inhibitory GABAergic inputs from cortical interneurons in feedforward or feedback manner. The dynamic change between excitation and inhibition of the excitatory neuron, mediates its excitatory neuronal plasticity, firing pattern and excitability, which may be involved in various of pathophysiological processes [18-20]. Salicylate can depress both the evoked and miniature inhibitory postsynaptic currents (eIPSCs and mIPSCs) recorded from pyramidal cells of the AC in a concentration-dependent manner [14]. The voltage-gated ion channel currents are also demonstrated to be reduced by salicylate in freshly dissociated rat pyramidal neurons [21]. Furthermore, salicylate is indicated to influence the firing rate in AC pyramidal neurons in vitro [22].

Although these studies seem to imply a correlation between the excitatory neuronal plasticity associated excitability and the salicylate-induced tinnitus, there are few reports about dynamic alterations of excitatory and inhibitory receptors on the excitatory neurons, which are pivotal factors in regulating neuronal plasticity and excitability. In the present study, we aimed to determine changes in GluA1R and $GABA_A$ receptors on excitatory cortical neurons after a single dose and during a long-term administration of salicylate respectively. Cortical neurons were labeled with calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) which had been proved to stain only excitatory neurons but not inhibitory neurons. We also observed the excitatory neuronal expression of immediate early genes, and Na^+-K^+-$2Cl^-$ (NKCC1) and K^+-Cl^- (KCC2) cotransporters which were known to be involved in strength and polarity of GABAergic transmission. Tinnitus-like behaviour was detected by the gap pre-pulse inhibition of acoustic startle (GPIAS) and the pre-pulse inhibition (PPI) tests. The results of the current study will help us understand the role of neural plasticity in AC involvement in salicylate-induced tinnitus.

Materials and methods

Animals

All the experimental procedures were conducted with the approval of the Animal Care and Use Committee of the Shanghai Jiaotong University School of Medicine. Male Sprague-Dawley rats, 5-6 weeks old, were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China), and housed at the animal center of Shanghai Jiaotong University School of Medicine Affiliated Ninth People's Hospital in a standard 12-hour reverse day/night cycle at an ambient temperature of 19-26°C. The rats had free access to stock laboratory diet and tap water. After a recovery period of 2 weeks, the rats were entered into subsequent study.

Salicylate administration

Sodium salicylate (Sigma-Aldrich, Shanghai, China) was dissolved in normal saline to achieve a concentration of 200 mg/ml. In an experiment of single dose injection, rats were randomly assigned to control group (Con group), 2 h group, 4 h group and 8 h group. Half of the animals underwent behavioral test of tinnitus (n=6 per group), while the other half were sacrificed for further analysis (n=6 per group) in the Con group (receiving no treatment), and at 2 (2 h group), 4 (4 h group) and 8 (8 h group) hour after a single dose of salicylate (200 mg/kg, intraperitoneally). In an experiment of long-term administration, rats were randomly assigned to NS group, SS group and RE group. Animals were given intraperitoneal injections of salicylate (200 mg/kg) twice daily at 8:00 and 16:00 for 14 consecutive days (SS group), the same volume of normal saline for 14 consecutive days (NS group) and 14 consecutive days of salicylate injection followed by 14-day recovery period (RE group). Then the animals underwent behavioral test of tinnitus (n=6 per group)

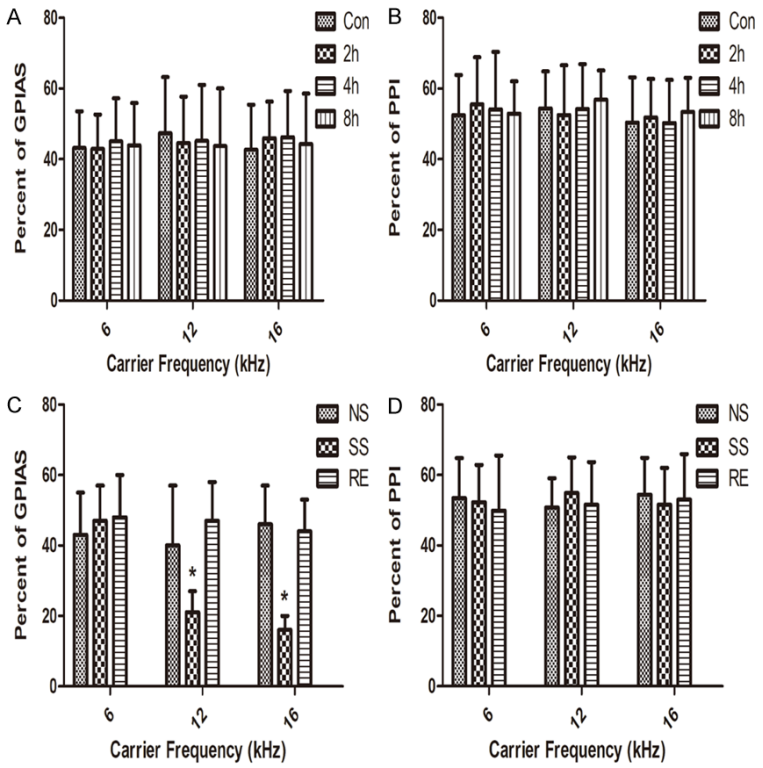

Figure 1. Gap pre-pulse inhibition of acoustic startle (GPIAS) and pre-pulse inhibition (PPI) paradigm. The data are presented as mean ± SD. A. Percent of GPIAS at 6, 12 and 16 kHz for rats after a single dose of salicylate. No significant differences were demonstrated among the four groups. B. Percent of PPI at 6, 12 and 16 kHz for rats after a single dose of salicylate. No significant differences were demonstrated among the four groups. C. Percent of GPIAS at 6, 12 and 16 kHz for rats during long-term administration of salicylate. The SS group showed a significant decrease in percent of GPIAS compared with the NS group at 12 kHz (*P<0.05) and 16 kHz (*P<0.05), but not at 6 kHz (P>0.05). There were no significant differences between NS group and RE group in percent of GPIAS. D. Percent of PPI at 6, 12 and 16 kHz for rats during long-term administration of salicylate. No significant differences were demonstrated among the three groups.

and were sacrificed for further analysis (n=6 per group) at 8:00 on day 15 after normal saline injection (NS group), day 15 (SS group) and day 29 (RE group) after salicylate injection.

Behavioral test for tinnitus

Behavioral evidence for tinnitus was tested by the gap pre-pulse inhibition of acoustic startle (GPIAS) and pre-pulse inhibition (PPI) paradigm as described in previous studies [23-26]. The GPIAS and PPI test was performed using the Acoustic Startle Reflex Starter Package for Rat or Mouse (Med Associates, St. Albans, VT, USA). The PPI test was performed in parallel to the GPIAS test to confirm that the GPIAS deficits were not due to temporal processing issues. Animals that fail GPIAS test but pass PPI test were thought to have tinnitus. In brief, rats were put into an acrylic holder, mounted on a sensitive piezoelectric platform, in a sound-attenuating chamber. Holder movements generated a voltage proportional to the downward force exerted by animal onto the piezoelectric platform. The analogue output was digitized and collected using Med Associates hardware connecting to a PC. Startle response magnitude was measured as the peak-to-peak values of the amplitude.

For GPIAS test, different band-pass-filtered sounds (1 kHz bandwidth centered at 6, 12 or 16 kHz) was presented at 65 dB SPL. Startle responses were elicited by acoustic startle stimuli (white noise, 110 dB SPL, 20 ms, in an interval of 30-35 s randomly). In 50% of trials, a gap was introduced into the narrowband noise 100 ms before the acoustic startle stimulus and lasted for 50 ms (5 ms rise/fall time). The percentage of GPIAS was calculated in each frequency by the following formula: [(AvgTnogap-AvgTgap)/AvgTnogap]×100%, where AvgTgap is the average amplitude of the gap trials, while AvgTnogap is the average amplitude of the no-gap trials.

For PPI test, the acoustic startle stimulus (the same frequencies and levels used for GPIAS test) was preceded by a noise-burst pre-pulse (1 kHz narrowband, 1 kHz bandwidth centered at 6, 12 or 16 kHz, 50 ms, 5 ms rise/fall time, 65 dB SPL), which began 100 ms before the acoustic startle stimulus in 50% of trials. While startle stimulus was presented without pre-pulse in the other half of the trials. The percentage of PPI was calculated in each frequency by the following formula: [(AvgT without pre-pulse-AvgT pre-pulse)/AvgT without pre-pulse×100%],

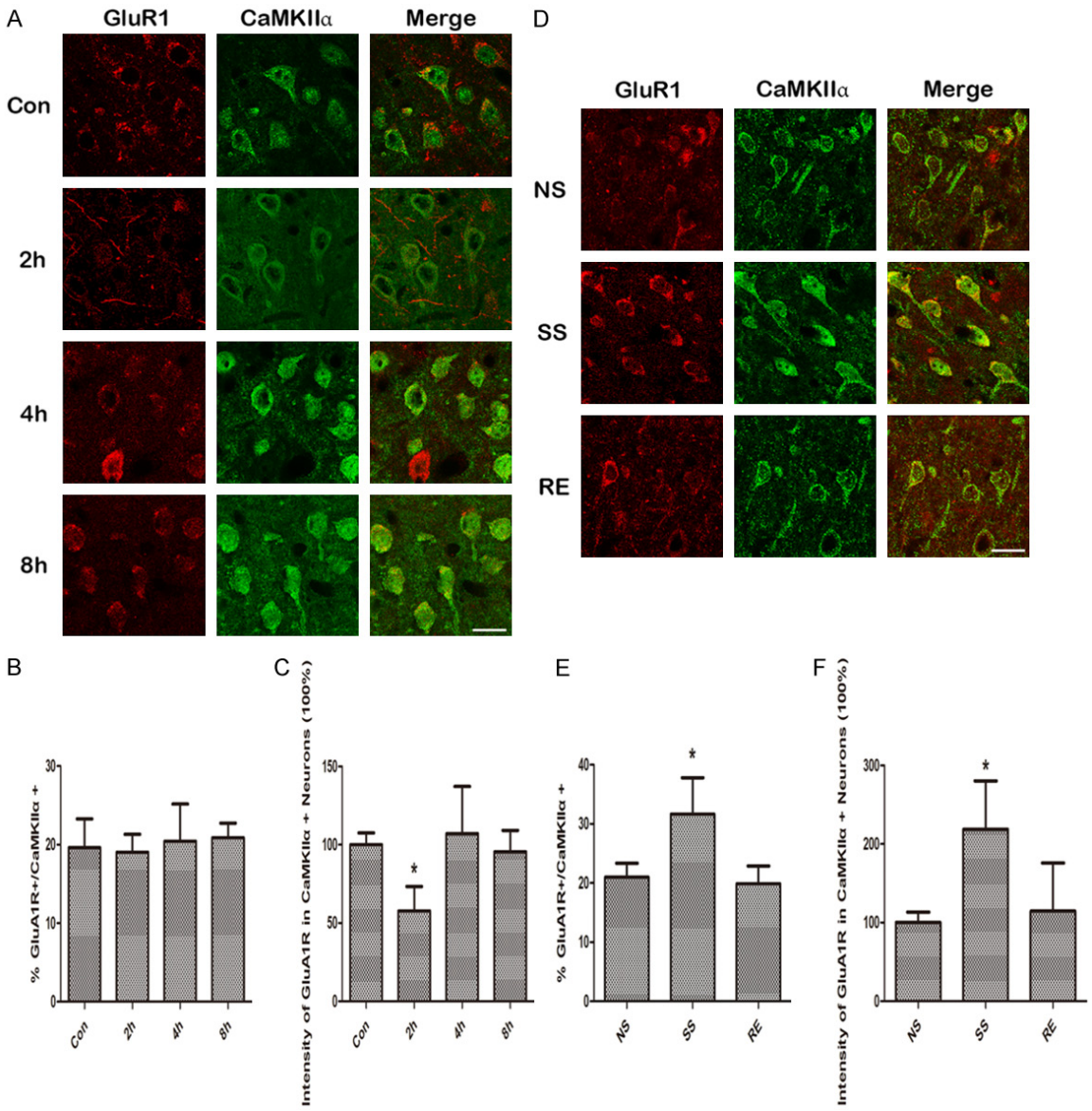

Figure 2. GluA1R expression of excitatory neurons. A. Fluorescent microscopic images of sections dual-immunostained for GluA1R (red), and CaMKII-α (green) in auditory cortex of rats after a single dose of salicylate. B. Percent of GluA1R+/CaMKII-α+ neurons for rats after a single dose of salicylate. There were no significant differences among the four groups. C. Intensity of GluA1R staining for rats after a single dose of salicylate. The 2 h group showed a significant decrease in intensity of GluA1R staining compared with the Con group (*P<0.01). No significant differences were demonstrated in the 4 h group or the 8 h group compared with the Con group. D. Fluorescent microscopic images of sections dual-immunostained for GluA1R (red), and CaMKII-α (green) in auditory cortex of rats during long-term administration of salicylate. E. Percent of GluA1R+/CaMKII-α+ neurons for rats during long-term administration of salicylate. The SS group showed a significant increase in percent of GluA1R+/CaMKII-α+ neurons compared with the NS group (*P<0.01). There were no significant differences between NS group and RE group. F. Intensity of GluA1R staining for rats during long-term administration of salicylate. The SS group showed a significant increase in intensity of GluA1R staining compared with the NS group (*P<0.05). There were no significant differences between NS group and RE group. Scale bar, 20 μm.

where AvgT pre-pulse is the average amplitude of the starle trials with pre-pulse, while AvgT without pre-pulse is the average amplitude of the startle trials without pre-pulse.

Immunofluorescence

Rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine

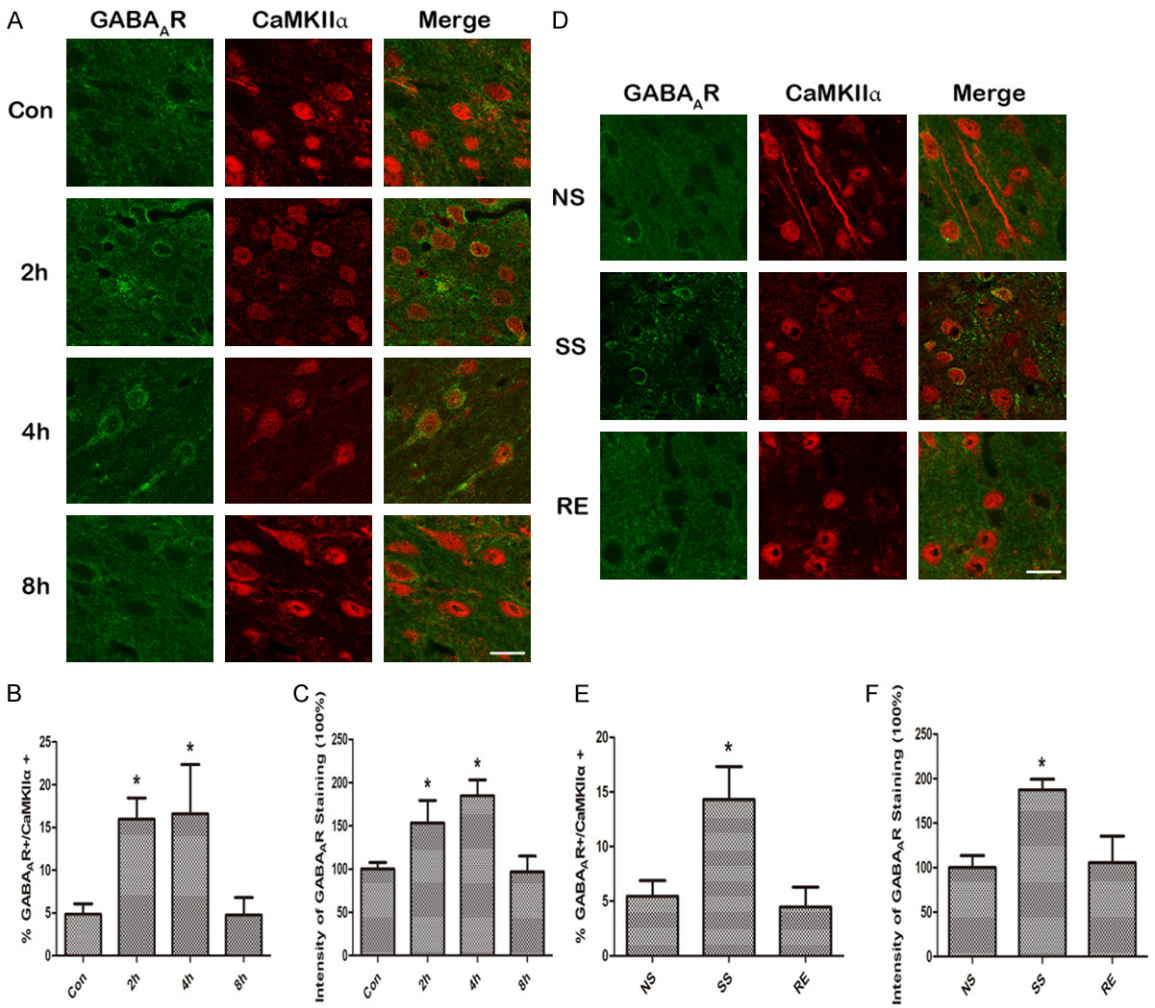

Figure 3. GABA$_A$R expression of excitatory neurons. A. Fluorescent microscopic images of sections dual-immunostained for GABA$_A$R (green), and CaMKII-α (red) in auditory cortex of rats after a single dose of salicylate. B. Percent of GABA$_A$R+/CaMKII-α+ neurons for rats after a single dose of salicylate. The 2 h group and the 4 h group showed significant increases in percent of GABA$_A$R +/CaMKII-α+ neurons compared with the Con group (Con group vs. 2 h group, *P<0.001; Con group vs. 4 h group, *P<0.001). No significant difference was demonstrated between the Con group and the 8 h group. C. Intensity of GABA$_A$R staining for rats after a single dose of salicylate. The 2 h group and the 4 h group showed significant increases in intensity of GABA$_A$R staining compared with the Con group (Con group vs. 2 h group, *P<0.05; Con group vs. 4 h group, *P<0.01). No significant difference was demonstrated between the Con group and the 8 h group. D. Fluorescent microscopic images of sections dual-immunostained for GABA$_A$R (green), and CaMKII-α (red) in auditory cortex of rats during long-term administration of salicylate. E. Percent of GABA$_A$R+/CaMKII-α+ neurons for rats during long-term administration of salicylate. The SS group showed a significant increase in percent of GABA$_A$R+/CaMKII-α+ neurons compared with the NS group (*P<0.01). There were no significant differences between NS group and RE group. F. Intensity of GABA$_A$R staining for rats during long-term administration of salicylate. The SS group showed a significant increase in intensity of GABA$_A$R staining compared with the NS group (*P<0.001). There were no significant differences between NS group and RE group. Scale bar, 20 μm.

(10 mg/kg), and perfused via the ascending aorta with normal saline followed by 4% paraformaldehyde. Brains were removed, cut into two hemispheres, post-fixed in 4% paraformaldehyde, dehydrated successively in 10%, 20% and 30% sucrose solution at 4°C, and frozen in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA, USA). Coronal sections were cut with a freezing microtome at 20 μm thickness through the auditory cortex according to the stereotaxic atlas (approximately bregma -3.96 to -6.24 mm). Every 20th section was used for immunohistochemistry in one series, such that the sections were sepa-

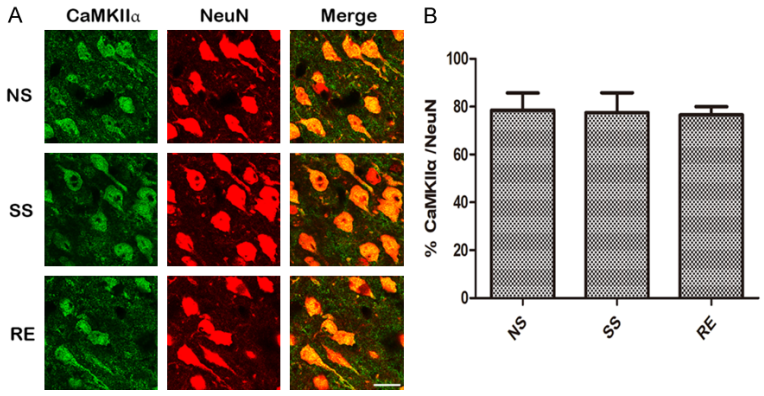

Figure 4. The proportions of excitatory neurons in total numbers of neurons. A. Fluorescent microscopic images of sections dual-immunostained for CaMKII-α (green), and NeuN (red) in auditory cortex of rats during long-term administration of salicylate. B. Percent of CaMKII-α+/NeuN+ neurons for rats during long-term administration of salicylate. There were no significant differences among the three groups. Scale bar, 20 μm.

rated by 400 μm intervals in each series. For immunolabelling, sections were rinsed three times in 0.1% TBST (TBS with 0.1% Tween 20) and blocked in 6% normal donkey serum/1% TBST at room temperature for 1 hour. Then the sections were incubated with combinations of two of the following antibodies in 1% normal donkey serum/1% TBST at 4°C overnight: mouse anti-CaMKII-α (1:200, Cell Signaling Technology, 13185S); rabbit anti-CaMKII-α (1:20, Thermo Fisher Scientific, PA5-14315); rabbit anti-NeuN (1:500, Abcam, Ab-177487); rabbit anti-GluA1R (1:500, Millipore, AB1504); mouse anti-β2-3 chain subunits $GABA_AR$ (1:100, Millipore, MAB341); rabbit anti-KCC2 (1:100, Cell Signaling Technology, 94725S); rabbit anti-NKCC1 (1:200, Cell Signaling Technology, 85403); rabbit anti-c-Fos (1:1000, Cell Signaling Technology, 2250S); rabbit anti-EGR1 (1:1000, Cell Signaling Technology, 4153S). After rinsing three times in 0.1% TBST, the sections were incubated at room temperature for 1 hour with combinations of appropriate following secondary antibodies: Alexa-Fluor 488-conjugated donkey anti-mouse (1:200, Jackson Immunoresearch laboratories); Alexa-Fluor 594-conjugated donkey anti-rabbit (1:400, Invitrogen); Alexa-Fluor 488-conjugated donkey anti-rabbit (1:200, Invitrogen); Alexa-Fluor 594-conjugated donkey anti-mouse (1:400, Invitrogen). Following rinsing in 0.1% TBST, the sections were mounted with mounting solution (Invitrogen, S36936), coverslipped and examined with confocal laser scanning microscopy (Leica, TCS SP8). We used same settings of exposure and gain adjustment for image capturing. For cell counting, 25 fields of 10 sections at a magnification of 630X were randomly selected for each subject in one series. CaMKII-α positive cells were counted for colocalization with GluA1R, $GABA_AR$, KCC2, NKCC1, c-Fos and EGR1 respectively, and reported as a percentage of the number of CaMKII-α positive cells; NeuN positive cells were counted for colocalization with CaMKII-α and reported as a percentage of the number of NeuN positive cells. To quantify the fluorescence intensity level of GluA1R, $GABA_AR$, KCC2 and NKCC1, CaMKII-α positive cells were outlined and the intensity levels were measured after background subtraction using Image J. The background immunofluorescence was measured by calculating the fluorescence intensity in a selected area devoid of neurons or any other stained structures. Where indicated these data were normalized relative to data for Con group and NS group. The investigator who performed the image acquisition and quantification, were blind to the experimental conditions.

Statistical analysis

Results are expressed as the means ± SD. Differences among groups were examined by one-way analysis of variance followed by the Bonferroni post-tests for data that had homogeneity of variance, or Dunnett T3 post-tests for data that exhibited heterogeneity of variance when indicated by significance of difference. All statistical analyses were performed using SPSS Statistics for Windows (version 22.0, IBM-SPSS, Inc., Chicago, IL, USA). Curve fitting and bar charts were performed by GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

Salicylate-induced tinnitus-like behavior in rats

After a single dose of salicylate, there were no significant differences among the four groups in percent of GPIAS values or PPI values (**Figure**

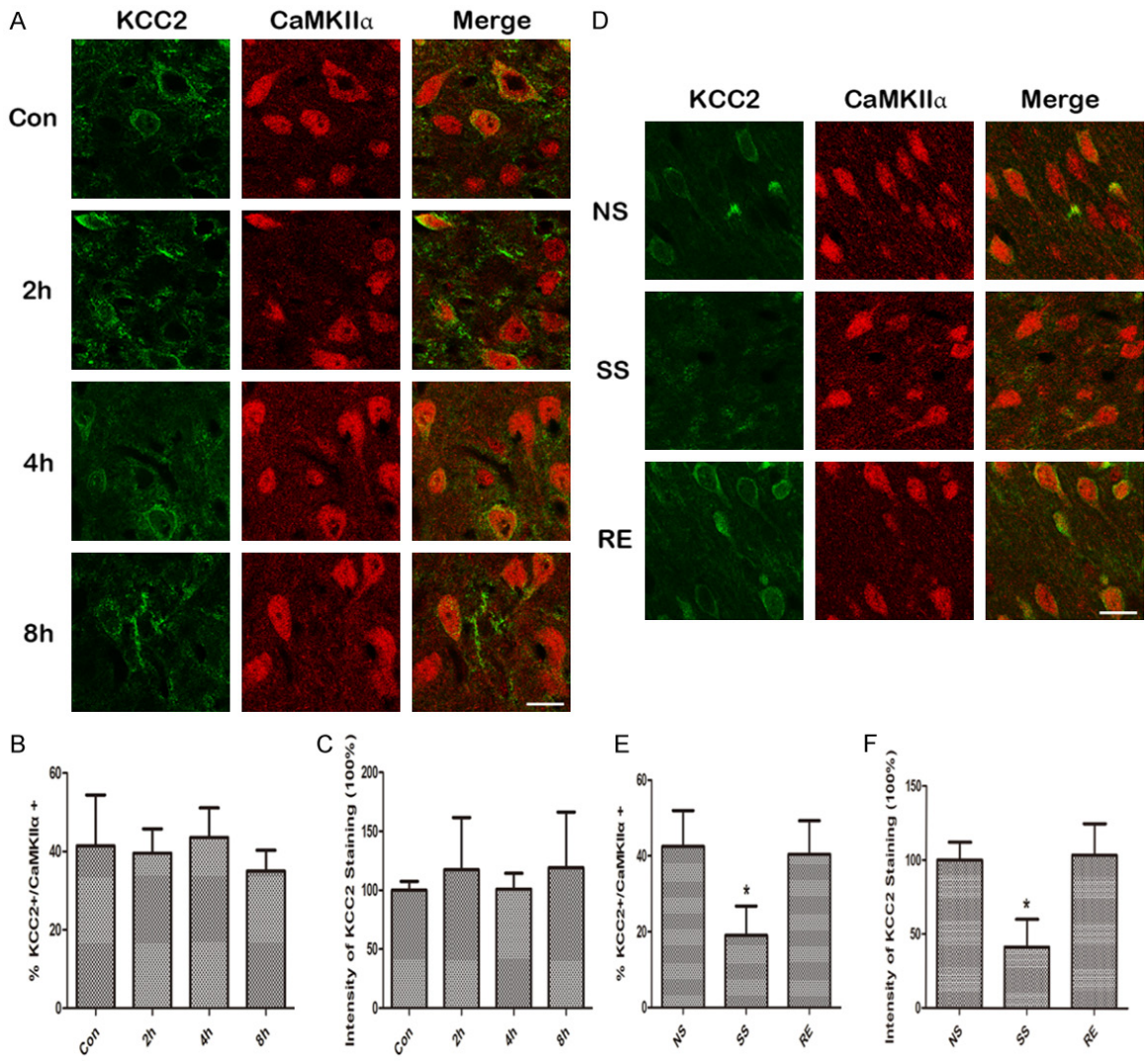

Figure 5. KCC2 expression of excitatory neurons. A. Fluorescent microscopic images of sections dual-immunostained for KCC2 (green), and CaMKII-α (red) in auditory cortex of rats after a single dose of salicylate. B and C. Percent of KCC2+/CaMKII-α+ neurons and intensity of KCC2 staining for rats after a single dose of salicylate. There were no significant differences among the four groups in percent of KCC2+/CaMKII-α+ neurons or intensity of KCC2 staining. D. Fluorescent microscopic images of sections dual-immunostained for KCC2 (green), and CaMKII-α (red) in auditory cortex of rats during long-term administration of salicylate. E and F. Percent of KCC2+/CaMKII-α+ neurons and intensity of KCC2 staining for rats during long-term administration of salicylate. The SS group showed significant decreases in percent of $GABA_AR$+/CaMKII-α+ neurons (*P<0.001) and intensity of KCC2 staining (*P<0.001) compared with the NS group. There were no significant differences between NS group and RE group. F. Intensity of $GABA_AR$ staining for rats during long-term administration of salicylate. The SS group showed a significant increase in intensity of $GABA_AR$ staining compared with the NS group (*P<0.001). There were no significant differences between NS group and RE group. Scale bar, 20 μm.

1A, **1B**). During long-term administration of salicylate, the SS group showed a statistically significant decrease in percent of GPIAS values relative to the NS group at 12 kHz (P<0.05) and 16 kHz (P<0.05), but not at 6 kHz. There was no significant difference in percent of GPIAS values between the NS group and the RE group, or in percent of PPI values among the three groups (**Figure 1C**, **1D**). This indicated that these rats in the SS group were experiencing tinnitus.

Changes in AMPAR and $GABA_AR$ after administration of salicylate

After a single dose of salicylate, no significant differences were demonstrated in the propor-

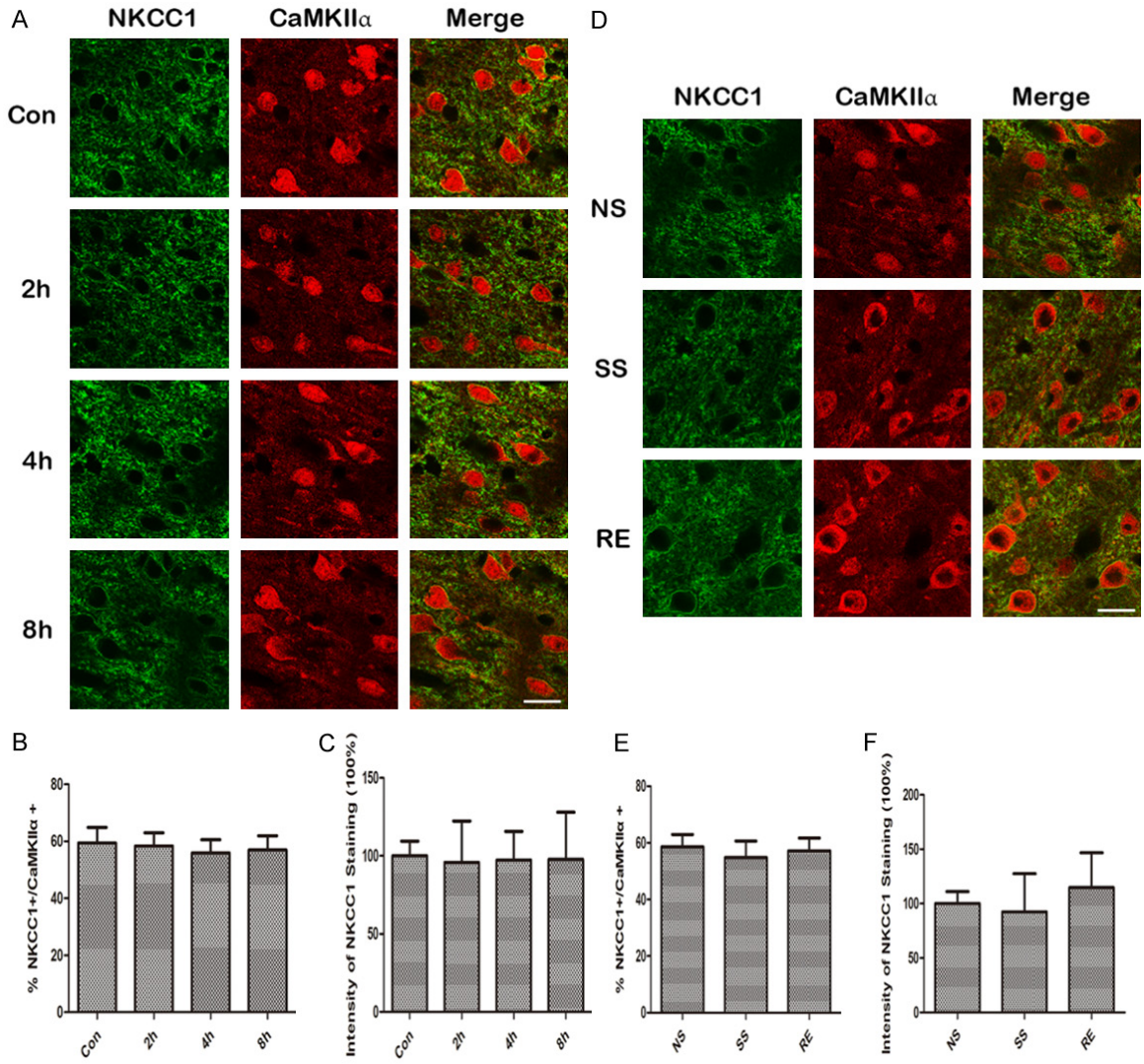

Figure 6. NKCC1 expression of excitatory neurons. A. Fluorescent microscopic images of sections dual-immunostained for NKCC1 (green), and CaMKII-α (red) in auditory cortex of rats after a single dose of salicylate. B and C. Percent of NKCC1+/CaMKII-α+ neurons and intensity of NKCC1 staining for rats after a single dose of salicylate. There were no significant differences among the four groups in percent of NKCC1+/CaMKII-α+ neurons or intensity of NKCC1 staining. D. Fluorescent microscopic images of sections dual-immunostained for NKCC1 (green), and CaMKII-α (red) in auditory cortex of rats during long-term administration of salicylate. E and F. Percent of NKCC1+/CaMKII-α+ neurons and intensity of NKCC1 staining for rats during long-term administration of salicylate. There were no significant differences among the three groups in percent of NKCC1+/CaMKII-α+ neurons or intensity of NKCC1 staining. Scale bar, 20 μm.

tion of GluA1R/CaMKII-α+ neurons among the four groups (**Figure 2B**). The intensity of GluA1R staining in 2 h group was significantly decreased ($P<0.01$) while those in 4 h and 8 h group were not significantly different compared with the Con group (**Figure 2C**). The proportions of $GABA_AR$+/CaMKII-α+ neurons and the intensity of $GABA_AR$ staining in 2 h and 4 h group were significantly increased (Con group vs. 2 h group, $P<0.001$; Con group vs. 4 h group, $P<0.001$) while that in 8 h group was not significantly different compared with the Con group (**Figure 3B**, **3C**). During long-term administration of salicylate, the proportion of GluA1R/CaMKII-α+ neurons ($P<0.001$) and the intensity of GluA1R staining ($P<0.05$) in SS group were significantly increased while those in RE group was not significantly different compared with the NS group (**Figure 2E**, **2F**). The proportion of $GABA_AR$+/CaMKII-α+ neurons ($P<0.001$) and the intensity of $GABA_AR$ staining ($P<0.001$) in SS group was significantly elevated while that in RE group

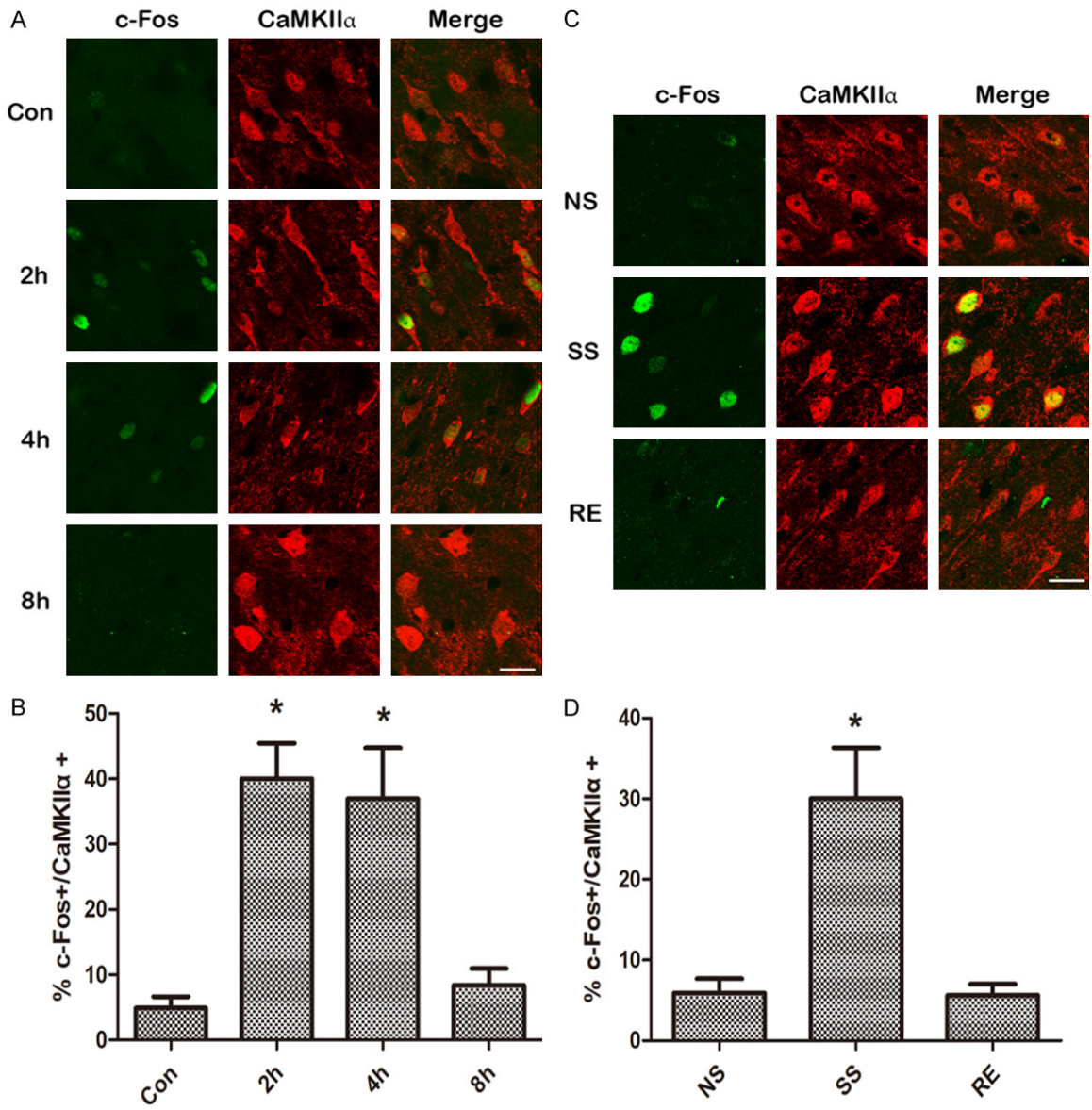

Figure 7. c-Fos expression of excitatory neurons. A. Fluorescent microscopic images of sections dual-immunostained for c-Fos (green), and CaMKII-α (red) in auditory cortex of rats after a single dose of salicylate. B. Percent of c-Fos+/CaMKII-α+ neurons for rats after a single dose of salicylate. The 2 h group and the 4 h group showed significant increases in percent of c-Fos+/CaMKII-α+ neurons compared with the Con group (Con group vs. 2 h group, *P<0.001; Con group vs. 4 h group, *P<0.001). No significant differences were demonstrated between the Con group and the 8 h group. C. Fluorescent microscopic images of sections dual-immunostained for c-Fos (green), and CaMKII-α (red) in auditory cortex of rats during long-term administration of salicylate. D. Percent of c-Fos+/CaMKII-α+ neurons for rats during long-term administration of salicylate. The SS group showed a significant increase in percent of c-Fos+/CaMKII-α+ neurons compared with the NS group (*P<0.001). There were no significant differences between NS group and RE group. Scale bar, 20 μm.

was not significantly different compared with the NS group (**Figure 3E**, **3F**). There was no significantly difference in the proportion of CaMKII-α+/NeuN cells among the three groups during long-term of salicylate administration (**Figure 4**).

Changes in chloride co-transporters after administration of salicylate

There was a decreased proportion of KCC2+/CaMKII-α+ neurons and intensity of KCC2 staining after 14 days of salicylate administra-

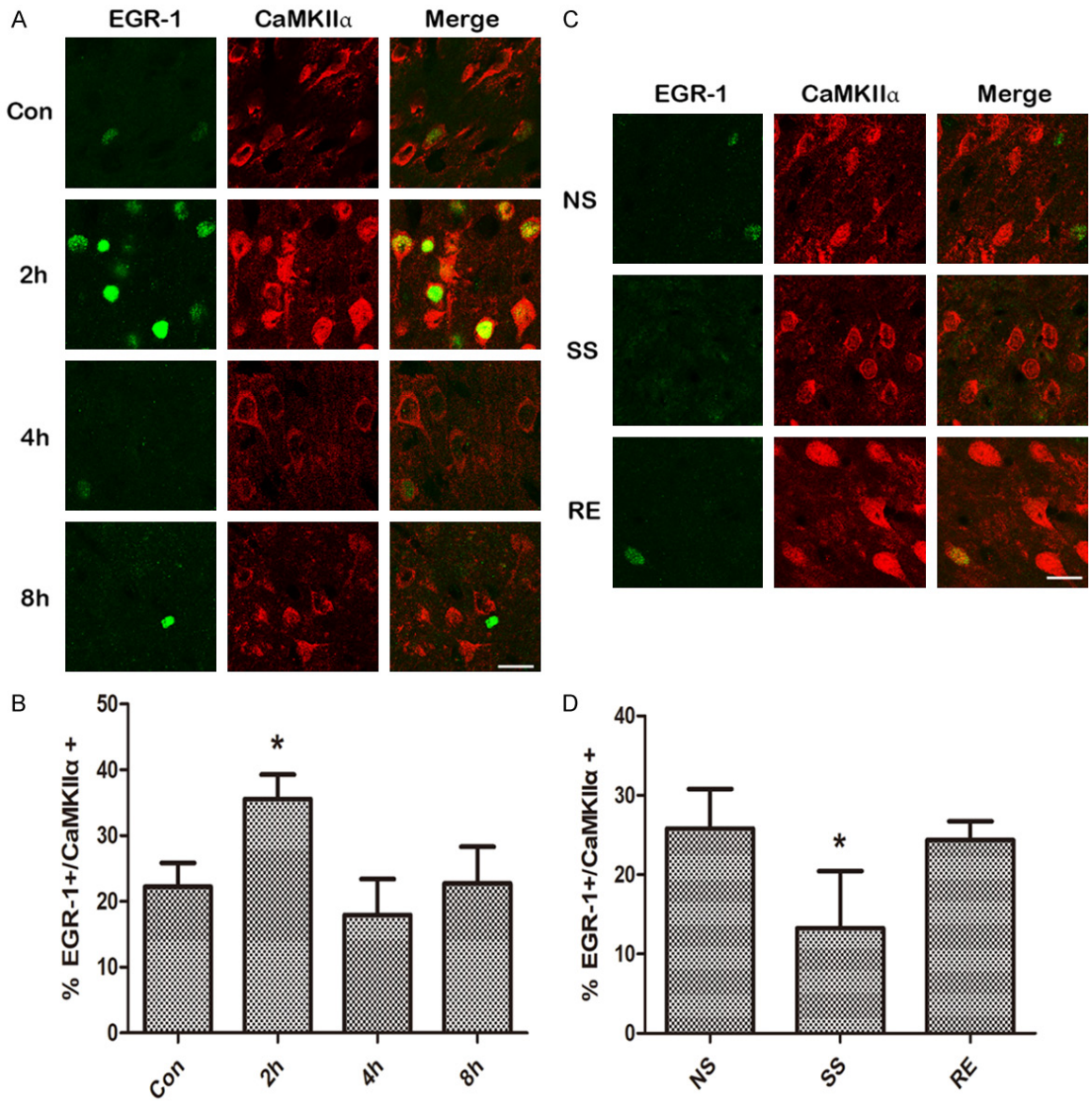

Figure 8. EGR-1 expression of excitatory neurons. A. Fluorescent microscopic images of sections dual-immunostained for EGR-1 (green), and CaMKII-α (red) in auditory cortex of rats after a single dose of salicylate. B. Percent of EGR-1+/CaMKII-α+ neurons for rats after a single dose of salicylate. The 2 h group showed a significant increase in percent of EGR-1+/CaMKII-α+ neurons compared with the Con group (*P<0.001). No significant differences were demonstrated between the Con group and the 4 h group or the Con group and the 8 h group in percent of EGR-1+/CaMKII-α+ neurons. C. Fluorescent microscopic images of sections dual-immunostained for EGR-1 (green), and CaMKII-α (red) in auditory cortex of rats during long-term administration of salicylate. D. Percent of EGR-1+/CaMKII-α+ neurons for rats during long-term administration of salicylate. The SS group showed a significant decrease in percent of EGR-1+/CaMKII-α+ neurons compared with the NS group (*P<0.01). There were no significant differences between NS group and RE group. Scale bar, 20 μm.

tion (SS group) compared with the NS group (P<0.001), there was no change in proportion of KCC2+/CaMKII-α+ neurons intensity of KCC2 staining in the RE group compared with the NS group (**Figure 5E**, **5F**) or among the four groups after a single administration of salicylate (**Figure 5B**, **5C**). There were no significant differences in the proportion of NKCC1+/CaMKII-α+ neurons or intensity of NKCC1 staining among the four groups within 8 hours after salicylate injection or during long-term administration (**Figure 6**).

Changes in immediate early genes after administration of salicylate

After a single dose of salicylate, the proportions of c-Fos+/CaMKII-α+ neurons in 2 h and 4 h group were significantly increased (Con group vs. 2 h group, P<0.001; Con group vs. 4 h group, P<0.001) while that in 8 h group was not significantly different compared with the Con group (**Figure 7B**). The proportion of EGR1+/CaMKII-α+ neurons in 2 h group was significantly elevated (P<0.001) while those in 4 h and 8 h group were not significantly different compared with the Con group (**Figure 8B**). During long-term administration of salicylate, the proportion of c-Fos+/CaMKII-α+ neurons in SS group was significantly increased (P<0.001) while those in RE group was not significantly different compared with the NS group (**Figure 7D**). The proportions of EGR1+/CaMKII-α+ neurons in SS group was significantly downregulated (P<0.01) while that in RE group was not significantly different compared with the NS group (**Figure 8D**).

Discussion

The exact mechanisms that give rise to tinnitus are not fully elucidated. An increasing numbers of researches have focused on changes regarding to neuroplasticity in central auditory system [3, 12, 16]. In the present study, we studied the plasticity changes on membrane receptors of excitatory neurons in the AC. We found that $GABA_A$Rs were increased with decreased AMPARs on the membrane of the excitatory cortical neurons within 8 hours after a single dose of 200 mg/kg salicylate. However, both $GABA_A$Rs and AMPARs were increased with decreased expression of KCC2 on excitatory cortical neurons after a long-term of salicylate which induced tinnitus-like behavior. The transition from homeostatic to Hebbian manner of receptor changes might partially underline the machenism of salicylate-induced tinnitus.

AMPAR and $GABA_A$R changes after a single dose of salicylate

We previous found that AC shown an augment of F18-FDG uptake, which indicated increased metabolic activity 2 h after a single dose of salicylate administration [10]. The hyperactive state of the AC was thought to be a stress response to acute administration of salicylate, as we had not detected any synaptic structural alterations or tinnitus-like behavioral manifestation. Consistent with the evidences indicating increased excitability of AC, we found increased proportion of c-Fos+/CaMKIIα+ cells 2 h after salicylate administration. The expression of IEGs (c-Fos and EGR-1) is associated with neural activity, synaptic efficacy and plasticity changes in neurons [26], and also they have been validated as indirect markers of cellular activity [27]. However the increased $GABA_A$Rs expression and decreased AMPARs expression of the excitatory neurons observed after a single dose of salicylate seemed to contradict the long-standing recognition of salicylate-induced neural hyperactivity. This opposite response of receptors to neural activity might reflect a feedback in a homeostatic process [28]. The dynamic changes in $GABA_A$Rs seen here can be an analogy to the increased receptor clusters on the membrane responding to increased activity induced by pharmacological or electrophysiological stimulus in neuronal cultures [29]. Moreover, increases in $GABA_A$Rs have been seen in pyramidal neurons and motor trigeminal neurons following sleep deprivation induced hyperactivity in vivo [30, 31]. The β2-3 chain subunit of the $GABA_A$Rs as labeled here, exists in both synaptic and extrasynaptic sites [32]. Thus the augmentation of $GABA_A$Rs would result in enhancement of inhibitory postsynaptic currents which derives synaptically; and increased phasic and tonic GABAergic inhibition which origins from extrasynaptic $GABA_A$Rs. AMPARs changed in a different manner, but in a homeostatic way. The homeostatic changes of the excitatory receptors have also been reported in vivo and in vitro before [33, 34]. In addition, increased proportion of EGR-1+/CaMKIIα+ neurons were observed 2 h after salicylate administration. Besides being a molecular marker of cellular activity, EGR-1 plays an important role in mediating the homeostatic scaling of AMPA receptor by enhancing endocytosis [35]. The increased ERG-1 expression in the excitatory neurons, as shown here, might be responsible for down scaling of AMPA receptor. By such compensatory regulation of GABAergic and glutamatergic receptors, the excitatory neurons are allowed to dampening its excitability and activity in a homeostatic manner when faced with the stimulus of salicylate. For this reason, the animals receiving a

single dose of salicylate showed no sign of tinnitus-like behavior.

AMPAR and $GABA_AR$ changes during long-term administration of salicylate

Long-term administration of salicylate didn't change the proportion of excitatory neurons in the AC. Showing a discrepancy from the changes occurred during the 'single dose' experiment, both the expressions of AMPARs and $GABA_ARs$ in the excitatory neurons were increased after 14 days of salicylate administration, and returned to baseline following 14 days' cease of administration. In parallel with this, the proportion of ERG-1+/CaMKIIα+ neurons, which were supposed to mediate AMPA receptor endocytosis, was downregulated. While the augment of AMPARs on auditory cortical excitatory neurons reflecting the previous recognition of salicylate-induced neural hyperactivity, the increased $GABA_AR$ seemed to be counter to the hypothesis of central hyperactivity. Long-term of salicylate administration was demonstrated to reduce the expression of $GABA_A$ and $GABA_B$ receptors in auditory center in rat showing tinnitus-like behavior [36, 37]. However they measured the total expression of GABA receptors and could not distinguish excitatory versus inhibitory neurons.

Interestingly, we found an elevated proportion of KCC2+/CaMKIIα+ neurons with no change of NKCC1+/CaMKIIα+ neurons after 14 days of salicylate administration. KCC2 (a chloride importer) and NKCC1 (a chloride exporter) are two major chloride co-transporters which are crucial for maintaining the concentration gradient of chloride anions between the outside and the inside part of the cellular membrane [38]. The combination of GABA and $GABA_A$ receptors activates the chloride channel, allowing Cl^- entering the cell to induce a hyperpolarizing inward chloride current. Importantly, the influx of Cl^- through chloride channel is supported by the low intracellular concentration of Cl^-. Owing to less expression of KCC2 during development, $GABA_A$ receptor shows a depolarizing response to GABA as a consequence of relatively high concentration of intracellular Cl^- [39]. After maturation, the increasing expression of KCC2 leads to the switch of $GABA_A$ receptor's function from excitation to inhibition. Several studies have confirmed that under pathological state, a depolarizing response to GABA induced by reduction in KCC2 expression contributes to local neuronal excitability [40, 41]. Thus the increased $GABA_A$ receptors and decreased KCC2 we observed here would enhance the neuronal excitability, instead of dampening it with normal expression of KCC2. In addition, we previously reported activation of BDNF-TrkB signaling in the AC after the same long-term salicylate regimen applied in this study [42]. What is more, the upregulation of BDNF-TrkB signaling correlated with the synaptic structural changes and behavioral manifestation. As a neurotrophic factor, upregulation of BDNF can act on TrkB receptor to cause a depolarization effect of $GABA_AR$ activation through downregulation of KCC2, turning the GABAergic activity to be excitatory at this circumstance [43]. Both the increased excitatory receptor AMPAR and the increased inhibitory $GABA_A$ receptor which showing an excitatory property enhanced activity of the excitatory neurons, contributing to the hyperactivity of the AC. In parallel with this, the proportion of c-Fos+/CaMKIIα+ elevated after 14 days of salicylate injection, and returned to baseline after recovery from salicylate administration. And the hyperactivity of the excitatory neurons here resulted in reversible tinnitus-like behavior indicated in the GPIAS and PPI test.

Neural plasticity in salicylate-induced tinnitus

Neural plasticity regulates neuronal excitability, synaptic strength and synapse formation. It is composed of two complementary but opposite forces: one that regulates neuronal properties to stabilize the neural activity, and another that modifies the neuronal elements to progressively destabilize the neural network [28]. A battery of experimental efforts aimed at exploring the biological basis of tinnitus, especially salicylate associated tinnitus, have focused on Hebbian plasticity, which was supposed to increase synaptic strength and lead to unrestricted hyperactivity in the auditory center [44]. We previously had verified the synaptic plasticity associated hyperactivity in the AC underlining the tinnitus generation [10]. However we did not distinguish the excitatory neurons from the inhibitory neurons, nor the excitatory synapse from the inhibitory one in those studies.

Cortical excitatory neurons locate in complex neural network with extensive excitatory and inhibitory feedbacks [17]. Activity of the excitatory neurons reflects the combined action of excitatory inputs from other excitatory neurons and inhibitory inputs from GABAergic neurons.

Salicylate was reported to attenuate inhibitory postsynaptic currents recorded from pyramidal neurons of the AC in brain slices [22]. What is more, it was demonstrated that salicylate significantly increased the action potential firing rates in the cortical excitatory neurons through affecting the voltage-gated ion channels [14]. In the present study, we showed augmentation of both AMPAR and $GABA_AR$ which showing an excitatory property on excitatory neurons after long-term salicylate administration. The receptor changes synchronized with the reversible tinnitus-like behavioral manifestation. The increase in excitatory components of receptors on the excitatory neurons, which are liken to Hebbian plasticity, may thus play a partial role in the generation of tinnitus development. On the contrary, the AMPAR and $GABA_AR$ (with on change of KCC2) responded in a homeostatic manner to excitatory stimulation after a single dose of salicylate. The homeostatic plasticity changes of AMPAR and $GABA_AR$ here share some similarity with the results in the study of sleep deprivation and recovery [30]. They found an increased ratio of $GABA_AR$/AMPAR during sleep deprivation which was supposed to excite excitatory neurons in the barrel cortex and an opposite ratio during sleep recovery which helped to recover the state of the neurons. Similar homeostatic plasticity can be revealed in the hippocampus during sensory deprivation [45], the rostral cingulate cortex during esophageal acid exposure [46] and other pathological conditions [47].

Like other systems in the organism, the auditory system functions from compensatory way to decompensatory way when confronted with damage factors. We speculate that, after the single dose of salicylate, the excitatory neurons in the AC are able to make homeostatic changes to maintain their own steady state and the normal function of the auditory center. Whereas the repeated doses of salicylate gradually overwhelms the excitatory neuron, makes them be prone to undergo Hebbian plasticity and disturbs the balance between excitation and inhibition. It was the different pattern of neuronal plasticity the excitatory neurons express that affected the auditory reactions to salicylate. Actually we had demonstrated different electromotility of outer hair cell in response to a single dose or long-term administration of salicylate (the same regimen of salicylate used in the present study) before [7]. These phenomenons we observed may coincide with the data from clinical researches that patients with occurrent or early stage of tinnitus had a favourable prognosis while those with chronic tinnitus showed resistance to various interventions [4]; and the data from tinnitus animal studies that benzodiazepines, a GABA receptor agonist, showed some efficacy [48]. However, these correlations need firm evidence from further study.

In summary, we studied the effects of salicylate on the AMPAR and the $GABA_AR$ of the excitatory neurons in the AC. We found that a single dose and long-term administration of salicylate had different impacts on the the expression and feature of the membranous receptors. This study suggests that plasticity of the cortical excitatory neurons might be involved in the generation of salicylate-induced tinnitus. These finding will help to further reveal the mechanism of tinnitus, and to develop precise interventions for tinnitus.

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Disclosure of conflict of interest

None.

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References

[1] Elgoyhen AB, Langguth B, De RD and Vanneste S. Tinnitus: perspectives from human neuroimaging. Nat Rev Neurosci 2015; 16: 632-642.

[2] Eggermont JJ and Roberts LE. The neuroscience of tinnitus. Trends Neurosci 2004; 27: 676-682.
[3] Yang G, Lobarinas E, Zhang L, Turner J, Stolzberg D, Salvi R and Sun W. Salicylate induced tinnitus: behavioral measures and neural activity in auditory cortex of awake rats. Hear Res 2007; 226: 244-253.
[4] Langguth B, Kreuzer PM, Kleinjung T and De RD. Tinnitus: causes and clinical management. Lancet Neurol 2013; 12: 920-930.
[5] Lockwood AH. Tinnitus. Neurol Clin 2005; 23: 893-900.
[6] Chen GD, Stolzberg D, Lobarinas E, Sun W, Ding D and Salvi R. Review: salicylate-induced cochlear impairments, cortical hyperactivity and re-tuning, and tinnitus. Hear Res 2013; 295: 100-113.
[7] Huang ZW, Luo Y, Wu Z, Tao Z, Jones RO and Zhao HB. Paradoxical enhancement of active cochlear mechanics in long-term administration of salicylate. J Neurophysiol 2005; 93: 2053-2061.
[8] Yang K, Huang ZW, Liu ZQ, Xiao BK and Peng JH. Long-term administration of salicylate enhances prestin expression in rat cochlea. Int J Audiol 2009; 48: 18-23.
[9] Cazals Y, Horner KC and Huang ZW. Alterations in average spectrum of cochleoneural activity by long-term salicylate treatment in the guinea pig: a plausible index of tinnitus. J Neurophysiol 1998; 80: 2113.
[10] Yi B, Hu S, Zuo C, Jiao F, Lv J, Chen D, Ma Y, Chen J, Ling M and Wang X. Effects of long-term salicylate administration on synaptic ultrastructure and metabolic activity in the rat CNS. Sci Rep 2016; 6: 24428.
[11] Stolzberg D, Chen GD, Allman BL and Salvi RJ. Salicylate-induced peripheral auditory changes and tonotopic reorganization of auditory cortex. Neuroscience 2011; 180: 157-164.
[12] Ochi K and Eggermont JJ. Effects of salicylate on neural activity in cat primary auditory cortex. Hear Res 1996; 95: 63-76.
[13] Sun W, Lu J, Stolzberg D, Gray L, Deng A, Lobarinas E and Salvi RJ. Salicylate increases the gain of the central auditory system. Neuroscience 2009; 159: 325-334.
[14] Wang HT, Luo B, Zhou KQ, Xu TL and Chen L. Sodium salicylate reduces inhibitory postsynaptic currents in neurons of rat auditory cortex. Hear Res 2006; 215: 77-83.
[15] Yanagawa Y, Takasu K, Osanai H and Tateno T. Salicylate-induced frequency-map reorganization in four subfields of the mouse auditory cortex. Hear Res 2017; 351: 98-115.
[16] Hu SS, Mei L, Chen JY, Huang ZW and Wu H. Expression of immediate-early genes in the inferior colliculus and auditory cortex in salicylate-induced tinnitus in rat. Eur J Histochem 2014; 58: 2294.
[17] Radnikow G and Feldmeyer D. Layer- and cell type-specific modulation of excitatory neuronal activity in the neocortex. Front Neuroanat 2018; 12: 1.
[18] Taranenko VD, Lopantsev VÉ, Syomik LI, Gladkii TV and Topol'Nik EV. Mechanisms of epileptogenic effects of some convulsants on the neuronal activity in the neocortex. Neurophysiology 1999; 31: 85-88.
[19] Hoppenrath K and Funke K. Time-course of changes in neuronal activity markers following iTBS-TMS of the rat neocortex. Neurosci Lett 2013; 536: 19-23.
[20] Palop JJ, Chin J, Roberson ED, Wang J, Thwin MT, Bien-Ly N, Yoo J, Ho KO, Yu GQ and Kreitzer A. Aberrant excitatory neuronal activity and compensatory remodeling of inhibitory hippocampal circuits in mouse models of Alzheimer's disease. Neuron 2007; 55: 697-711.
[21] Liu Y, Zhang H, Li X, Wang Y, Lu H, Qi X, Ma C and Liu J. Inhibition of voltage-gated channel currents in rat auditory cortex neurons by salicylate. Neuropharmacology 2007; 53: 870-880.
[22] Su YY, Luo B, Wang HT and Chen L. Differential effects of sodium salicylate on current-evoked firing of pyramidal neurons and fast-spiking interneurons in slices of rat auditory cortex. Hear Res 2009; 253: 60-66.
[23] Hu SS, Mei L, Chen JY, Huang ZW and Wu H. Effects of salicylate on the inflammatory genes expression and synaptic ultrastructure in the cochlear nucleus of rats. Inflammation 2014; 37: 365-373.
[24] Mulders WH, Barry KM and Robertson D. Effects of furosemide on cochlear neural activity, central hyperactivity and behavioural tinnitus after cochlear trauma in guinea pig. PLoS One 2014; 9: e97948.
[25] Engineer ND, Riley JR, Seale JD, Vrana WA, Shetake JA, Sudanagunta SP, Borland MS and Kilgard MP. Reversing pathological neural activity using targeted plasticity. Nature 2011; 470: 101-104.
[26] Lobarinas E, Hayes SH and Allman BL. The gap-startle paradigm for tinnitus screening in animal models: limitations and optimization. Hear Res 2013; 295: 150-160.
[27] Hoffman GE, Smith MS and Verbalis JG. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Front Neuroendocrinol 1993; 14: 173-213.
[28] Turrigiano GG. Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. Trends Neurosci 1999; 22: 221-227.
[29] Marty S, Wehrlé R, Fritschy JM and Sotelo C. Quantitative effects produced by modifications of neuronal activity on the size of GABAA receptor clusters in hippocampal slice cultures. Eur J Neurosci 2015; 20: 427-440.

[30] Del Cidpellitero E, Plavski A, Mainville L and Jones BE. Homeostatic changes in GABA and glutamate receptors on excitatory cortical neurons during sleep deprivation and recovery. Front Syst Neurosci 2017; 11: 17.
[31] Toossi H, Del CE and Jones BE. Homeostatic regulation through GABA and acetylcholine muscarinic receptors of motor trigeminal neurons following sleep deprivation. Brain Struct Funct 2017; 222: 3163-3178.
[32] Kasugai Y, Swinny JD, Roberts JD, Dalezios Y, Fukazawa Y, Sieghart W, Shigemoto R and Somogyi P. Quantitative localisation of synaptic and extrasynaptic GABAA receptor subunits on hippocampal pyramidal cells by freeze-fracture replica immunolabelling. Eur J Neurosci 2010; 32: 1868-1888.
[33] Ullrich O, Reinsch S, Urbé S, Zerial M and Parton RG. Rab11 regulates recycling through the pericentriolar recycling endosome. J Cell Biol 1996; 135: 913-924.
[34] Goel A and Lee HK. Persistence of experience-induced homeostatic synaptic plasticity through adulthood in superficial layers of mouse visual cortex. J Neurosci 2007; 27: 6692-6700.
[35] Qin X, Jiang Y, Tse YC, Wang Y, Wong TP and Paudel HK. Early growth response 1 (Egr-1) regulates NMDA receptor-dependent transcription of PSD-95 and AMPA receptor trafficking in hippocampal primary neurons. Journal of Biological Chemistry 2015; 290: 29603-29616.
[36] Butt S, Ashraf F, Porter L and Zhang H. Sodium salicylate reduces the level of GABAB receptors in the rat's inferior colliculus. Neuroscience 2016; 316: 41-52.
[37] Bauer CA, Brozoski TJ, Holder TM and Caspary DM. Effects of chronic salicylate on GABAergic activity in rat inferior colliculus. Hear Res 2000; 147: 175-182.
[38] Silvestre de Ferron B, Vilpoux C, Kervern M, Robert A, Antol J, Naassila M and Pierrefiche O. Increase of KCC2 in hippocampal synaptic plasticity disturbances after perinatal ethanol exposure. Addict Biol 2016; 22: 1870.
[39] Takayama C and Inoue Y. Developmental localization of potassium chloride co-transporter 2 (KCC2), GABA and vesicular GABA transporter (VGAT) in the postnatal mouse somatosensory cortex. Neurosci Res 2010; 67: 137-148.
[40] Coull JA, Boudreau D, Bachand K, Prescott SA, Nault F, Sík A, Koninck PD and Koninck YD. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. Nature 2003; 424: 938-942.
[41] Coull JA, Beggs S, Boudreau D, Boivin D, Tsuda M, Inoue K, Gravel C, Salter MW and De KY. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. Nature 2005; 438: 1017-1021.
[42] Yi B, Wu C, Shi R, Han K, Sheng H, Li B, Mei L, Wang X, Huang Z and Wu H. Long-term administration of salicylate-induced changes in BDNF expression and CREB phosphorylation in the auditory cortex of rats. Otol Neurotol 2018; 39: 1.
[43] Dutheil S, Watabe I, Sadlaoud K, Tonetto A and Tighilet B. BDNF signaling promotes vestibular compensation by increasing neurogenesis and remodeling the expression of potassium-chloride cotransporter KCC2 and GABAA receptor in the vestibular nuclei. J Neurosci 2016; 36: 6199.
[44] Stefanescu RA, Koehler SD and Shore SE. Stimulus-timing-dependent modifications of rate-level functions in animals with and without tinnitus. J Neurophysiol 2015; 113: 956.
[45] Milshteinparush H, Frere S, Regev L, Lahav C, Benbenishty A, Beneliyahu S, Goshen I and Slutsky I. Sensory deprivation triggers synaptic and intrinsic plasticity in the hippocampus. Cerebral Cortex 2017; 27: 1.
[46] Banerjee B, Medda BK, Tuchscherer VJ, Babygirija R, Kannampalli P, Sengupta JN and Shaker R. Tu1883 homeostatic downscaling of AMPA receptor GluA1 subunits in cingulate cortex following chronic esophageal acid exposure in adult rats. Gastroenterology 2015; 148: S-927.
[47] Kapur J, Trotter S. Homeostatic plasticity hypothesis and mechanisms of neocortical epilepsies. Epilepsy Curr 2010; 5: 133-135.
[48] Bahmad FM Jr, Venosa AR and Oliveira CA. Benzodiazepines and GABAergics in treating severe disabling tinnitus of predominantly cochlear origin. Int Tinnitus J 2006; 12: 140-144.