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Up-regulation of Ca^{2+}/CaMKII/CREB signaling in salicylate-induced tinnitus in rats

Jiuhan Zhao[1] · Biao Wang[2] · Xiaohong Wang[1] · Xiuli Shang[1]

Abstract

The purpose of the study was to investigate the changes of Ca^{2+}/calmodulin-dependent protein kinases II (CaMKII)/cAMP response element-binding protein (CREB) signaling pathway in a rat tinnitus model. Eighteen Wistar rats were randomly divided into three groups: normal control (NC), normal saline (NS), and tinnitus model (TM) groups. Tinnitus model was induced by intraperitoneal injection of salicylate. The concentration of intracellular calcium level in auditory cortex cells was determined using Fura-2 acetoxymethyl ester (Fura-2 AM) method with fluorospectrophotometer. Expressions of calmodulin (CaM), N-methyl-D-aspartate receptor 2B subunit (NR2B), calcium-calmodulin kinase II (CaMKII), and cAMP response element-binding protein (CREB) were detected with Western blot. Tinnitus model was successfully established by the intraperitoneal administration of salicylate in rats. Compared with rats in NC and NS groups, salicylate administration significantly elevated CaM, NR2B, phospho-CaMKII and phospho-CREB expression in auditory cortex from tinnitus model group ($p < 0.05$), and the free intracellular Ca^{2+} concentrations ($p < 0.05$). Our data reveal that salicylate administration causes tinnitus symptoms and elevates Ca^{2+}/CaMKII/CREB signaling pathway in auditory cortex cells. Our study likely provides a new understanding of the development of tinnitus.

Keywords Tinnitus · Auditory cortex · Ca^{2+}/calmodulin-dependent kinase II · cAMP response element-binding protein

Introduction

Tinnitus is a common symptom of auditory system. Inferior colliculus and auditory cortex are the main functional areas to be responsible for tinnitus [1, 2]. Auditory center-originated mechanism has become a hotspot in the study of tinnitus [3]. Auditory system is plastic and able to reorganize own structure and function after losing part or all of its sensors [4]. Synaptic plasticity is regulated by a variety of mechanisms, including changes in the released neurotransmitters and transmission efficacy [5]. On the contrary, the change of the amount of neurotransmitter receptors can also alter synaptic plasticity [6].

Excitatory and inhibitory plasticity depend on the release of postsynaptic Ca^{2+} levels [6, 7], which is an important second messenger and plays a significant role in regulating cell metabolism. Ca^{2+} signaling pathway manipulates cell growth, differentiation, and synaptic plasticity [8]. Calmodulin (CaM) is the main Ca^{2+} binding protein, and regulates basic functions of neurons [9]. Ca^{2+}/CaM-dependent protein kinase II (CaMKII) is a key mediator of excitatory synaptic plasticity in center nerve system [10, 11]. The association between active CaMKII and NR2B is required for different forms of synaptic enhancement [25]. Meanwhile, cAMP response element-binding protein (CREB) is one of the downstream molecules of CaMKII, and plays important roles in regulating synaptic plasticity [12]. Activation of CREB can promote the expression of multiple synaptic plasticity-associated proteins [13]. In fact, Ca^{2+} binds to cAMP signal pathway, and phosphorylates serine residue 133 of CREB (CREB133), subsequently activating the transcription activity of CREB.

The plasticity involved in tinnitus is extremely complicated, which is decided by several levels in auditory pathway. Although auditory brainstem (cochlear nucleus, superior

✉ Xiuli Shang
   s459yp@163.com

[1] Department of Neurology, First Affiliated Hospital of China Medical University, No. 155 Nanjing North Street, Heping District, Shenyang 110001, China

[2] Department of Biochemistry and Molecular Biology, China Medical University, Shenyang 110001, China

olivary nucleus, inferior colliculus, etc.) and auditory cortex experience plasticity in the critical period of development, auditory pathways have lifelong plasticity towards peripheral sensory activity [14]. However, the changes of Ca^{2+} level in auditory cortex cells during tinnitus and the change of CaMKII/CREB have not been reported. In this study, we established tinnitus rat model by injecting sodium salicylate and investigated the auditory center-originated mechanism for tinnitus.

Materials and methods

Experimental animals and groups

Wistar rats (2-month old) were purchased from Animal Center of China Medical University (Shenyang, China). All rats had normal auditory brainstem response (ABR) threshold and those with middle ear/internal ear diseases were excluded from the study. Finally, 18 healthy rats were randomly divided into three groups (six rats for each group): a normal control group (NC), a normal saline control group (NS), and a tinnitus model group (TM). Tinnitus model was established with administrated of 10% sodium salicylate (350 mg/kg) via intraperitoneal injection. The rats in NS group were intraperitoneally administrated with equal volume of saline. The duration of medicine injection depends on the result of conditioned training (10–14 days). We followed the Laboratory Animal Administration Rules (China, 2011) for animal care and use. The light in the animal house is in a dark/light (12/12 h) cycle. Room temperature is kept at 22–26 °C, and humidity was kept at 30–60%. All rats were raised in quiet environment with free access to food and water and did not receive any medicine and training of conditioned reflex.

Establishment of conditioned reflex and tinnitus animal models

Conditioned reflex and tinnitus animal models were established as previously described [15, 16]. After one-week adaptive feeding, the rats were deprived of water for 1–2 days. Then, the experiment was conducted in a sound-proof box in a sound-proof room. The conditioned stimulus background sound was 8 Hz, 50 dB SPL pure tone, and delivered in the 3rd, 10th, 15th, 20th, and 25th min (duration: 1 min). After the stop of background noise, an electric shock (50 V) was delivered if the rat licked the water for over 5 s, to make an association with the unconditioned stimulus of electric shock, thus forming a conditioned stimulus—"stop of background noise-rats' licking less or no water." We recorded the licking time 60 s before the background noise stops as A, and the licking time of rats 60 s after background

noise stops as B, and used the licking inhibition ratio $R = B/(A + B)$ to reflect the establishment of conditioned reflex. Before the training, R was stable. After the training, R declined to < 0.2 and was supposed to successfully establish the conditioned reflex.

Surgery and sampling auditory cortex

After modeling, the rats were anesthetized with 1.5% pentobarbital sodium solution (44 mg/kg). Then, the rats were fixed on the stereotaxic apparatus, and self-made thermostatic pad was applied to keep their body temperature at 37 °C. Coronary brain tissue was sliced to contain auditory cortex in 41 region of neocortex [17, 18]. The location of primary auditory cortex (A1) was identified as previously described [19]. The reference offers the electrophysiological mapping used to identify tonotopy in both A1 and anterior auditory field (AAF). The boundary between A1 and AAF was identified using the reversal of tonotopy and was marked with a dye. According to the fixed markers (hippocampal feet), 3–6 slices were coronally sectioned (40 μm thickness for each) in cryostat, which contained A1. Coronal sections with the identified boundary were compared with sections in Paxinos mouse brain atlas. It provided the landmarks (primarily hippocampal shape) to identify A1 sections used in the present study.

Detection of intracellular Ca^{2+} level

Cell suspension (2×10^6/mL) was prepared from the isolated tissue using D-Hank's solution as previously described [20], and the cell activity was confirmed with Trypan Blue Rejection Test (cell survival rate reached over 95%). Then, the cell suspension was heated at 37 °C for 5 min, and calcium fluorescent indicator-Fura-2/AM (final concentration: 4 μmol/L) was added. After oscillation and incubation (35 min in a constant temperature of 37 °C), Hank's solution without Mg^{2+} (Unit: mmol/L) (HEPES 10, KCl 5.0, NaCl 145, $CaCl_2$ 1.3, L-glucose, and Na_2HPO_4 10) was applied to suspense the cells into 10^6 /mL. Finally, intracellular $[Ca^{2+}]_i$ was determined by fluorospectrophotometer under following conditions: 340 and 380 nm excitation wavelength/510 nm emission wavelength. Then, 3 μL of 10% (v/v) TritonX-100 was added to determine maximum fluorescence ratio (R_{max}). After the curve became stable, 20 μL of chelating agent -5 mmol/L EDTA was applied to determine minimum fluorescence ratio (R_{min}).

The formula used to calculate $[Ca^{2+}]_i$ was as follows: $[Ca^{2+}]_i = K_d [(R - R_{min})/(R_{max} - R)](F_{min}/F_{max})$. In the formula, $K_d = 224$ nmol/L; F_{max} refers to the measured fluorescence intensity after adding TritonX-100; F_{min} refers to the measured fluorescence intensity after adding EDAT.

Western blotting

Cell lysis solution (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 μg/mL Leupeptin, 1 μg/mL Pepstatin) was applied to isolate the proteins from auditory cortex. After centrifugation (12,000 rpm) for 10 min, the supernatant was collected and the protein concentration was determined using phenol reagent method. 40 μg of total protein from each group was loaded in the 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was transferred onto nitrocellulose (NC) membranes (50 V, 2 h). After rinsing in TTBS, the NC membrane was blocked by 5% skim milk at 4 °C overnight. The primary antibodies, including rabbit anti-rat CREB (Cell Signaling Technology), rabbit anti-rat-p-CREB (Ser133) (Cell Signaling Technology), rabbit anti-rat NR2B (Santa Cruz, USA), rabbit anti-rat CaMKII (Santa Cruz, USA), rabbit anti-rat p-CaMKII (Santa Cruz, USA), rabbit anti-rat CaM (Santa Cruz), and rabbit anti-rat GAPDH (Santa Cruz, USA) were diluted into 1:800 and incubated with NC membrane for 2 hat room temperature. After rinsing in TTBS, secondary antibody labeled with HRP (1:10000) (Santa Cruz, USA) was incubated with NC membrane for 2 h at room temperature. After ECL luminescence imaging, we analyzed the gray level of blotting bands with Image J image software v17.

Statistical analysis

The data were expressed in mean ± standard deviation (SD) and analyzed by SPSS13.0 software. One-way analysis of variance (ANOVA) was applied to compare the difference. $p < 0.05$ was considered as significant difference.

Results

Establishment of tinnitus animal model

A conditioned reflex—"stop of background noise-rats' licking less or no water" model was established in rats and tinnitus was confirmed according to the subsidize period of conditioned reflex. After the conditioned reflex was established, the administration of sodium salicylate induced the rats to develop tinnitus, and when the conditioned stimulus emerged, the rats could not recognize whether the background noise was stopped due to tinnitus, and might even present with substitution effect. At this moment, it was easy for the rats to ignore conditioned stimulus, and the subsidize period was shortened (Fig. 1; Table 1). The rats showed a significantly shorter subsidize period in TM than in NC and NS groups (p < 0.05). The subsidize period in NC and NS group has no significant difference (p > 0.05). These
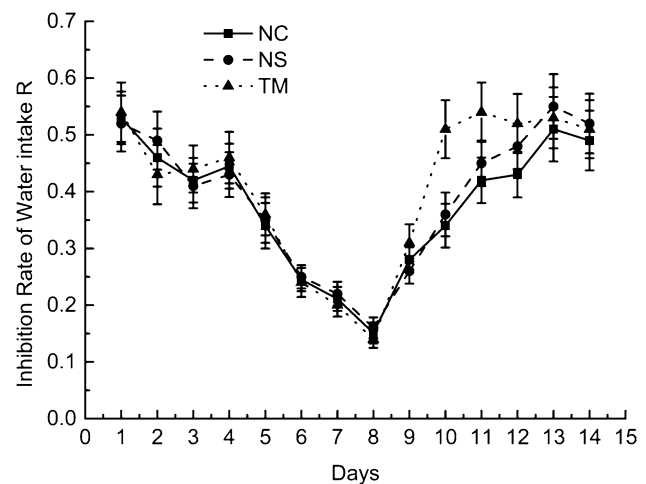

Fig. 1 The change of R value as the training time changes. Reflex training period is the 1st–7th day, subsidize period is the 8th–15th day

Table 1 Comparison of subsidize period in each group ($x \pm$ sd)

Groups	Numbers	Subsidize period (days)
Normal control	6	5.27 ± 0.42
Normal saline	6	4.32 ± 0.78
Tinnitus model	6	1.66 ± 0.53*

*Compared with NC and NS groups, $p < 0.05$

data implicate that tinnitus model has been successfully established.

Ca²⁺ concentration was increased in auditory cortex neurocytes in tinnitus model

We detected the change of intracellular free Ca^{2+} level in auditory cortex neurocytes in each group. Compared with rats in NC or NS group, $[Ca^{2+}]_i$ in the auditory cortex neurocytes was significantly elevated in TM group (226.33 ± 25.67 nmol/L) ($p < 0.05$) (Fig. 2). By contrast, the values in NC and NS groups were comparable (NC vs. NS, 168.16 ± 17.45 vs. 172.22 ± 18.35 nmol/L, $p > 0.05$).

Sodium salicylate influenced the expression of synaptic plasticity-related proteins in auditory cortex neurocytes

To analyze the influence of sodium salicylate on synaptic plasticity-related protein expression, CaM/CaMKII/CREB signaling transduction pathway was selected. As shown in Fig. 3a, compared with NC and NS groups, CaM expression in tinnitus model group was significantly enhanced ($n = 6$, $p < 0.05$). The relative expression amount of CaM protein
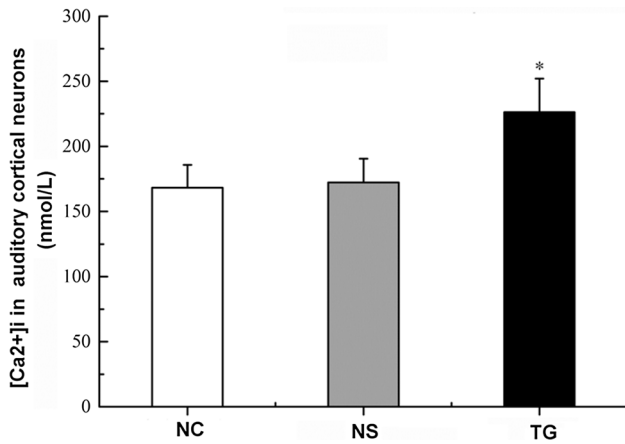
Fig. 2 $[Ca^{2+}]_i$ in auditory cortex neurocytes in each group ($x \pm sd$, $n = 6$). *Compared with NC group, $p < 0.05$

was 0.79 ± 0.073, 0.78 ± 0.075, and 1.17 ± 0.112 in NC, NS ,and TM groups, respectively. NR2B expression in tinnitus model group was significantly enhanced ($n = 6$, $p < 0.05$). The expression of NR2B was 0.41 ± 0.068, 0.42 ± 0.072, and 0.72 ± 0.108 in NC, NS, and TM groups, respectively.

As shown in Fig. 3b, compared with NC and NS groups, phosphorylated CaMKII (p-CaMKII) protein of TM group was also enhanced ($n = 6$, $p < 0.05$). The relative expressions of p-CaMKII were 0.73 ± 0.071, 0.78 ± 0.082, and 0.96 ± 0.091 in NC, NS, and TM groups, respectively. By contrast, the expression of CaMKII in each group was comparable ($n = 6$, $p > 0.05$).

Compared with NC and NS rats, the expression of phosphorylated p-CREB133 protein in TM group was significantly increased (Fig. 3c). The relative expression levels of p-CREB133 protein were 0.71 ± 0.072, 0.72 ± 0.071, and 0.98 ± 0.099 in NC, NS, and TM groups, respectively. The expression of total CREB protein in each group was comparable.

Discussion

The mechanisms for tinnitus were not entirely known, though some speculation was proposed. Recent study demonstrated that plasticity of auditory neurons was related to tinnitus [21, 22]. Due to the lifelong plasticity auditory pathways towards peripheral sensory activity, the auditory cortex from tinnitus is likely to be functionally reorganized [21, 22]. In this study, salicylate was administrated to establish tinnitus model and we also evidenced that Ca^{2+}/CaMKII/ CREB signaling pathway was elevated in the salicylate-induced tinnitus model.

We confirmed that the animals developed tinnitus according to the length of conditioned reflex subsidize period. Our results demonstrated that the animals with salicylate administration developed tinnitus after the conditioned reflex was established. When conditioned stimulus emerged, rats could not recognize whether background noise was stopped, thus leading to a shortened subsidize period. The animals in tinnitus model group had a significantly shorter subsidize period than those in normal saline control group.
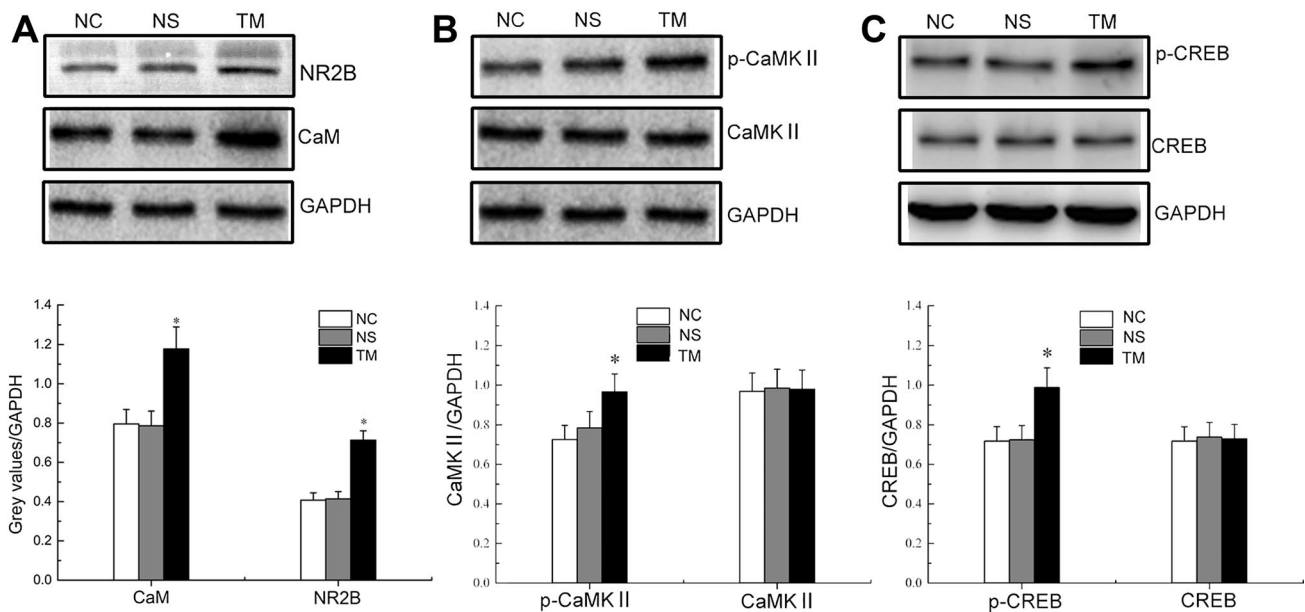

Fig. 3 The expression of CaM, NR2B, CaMKII, and CREB. **a** The expression of CaM and NR2B in each group; **b** the expression of total CaM-KII and p-CaMKII in each group; **c** the expression of total CREB and p-CREB133 in each group; *compared with NC group, $p < 0.05$

Ca^{2+} is crucial to eukaryotic cells, and the extracellular Ca^{2+} concentration is about 1000-fold of intracellular one. Ca^{2+} is an important second messenger and is able to trigger the release of neurotransmitter. Moreover, Ca^{2+} is the key factor regulating synaptic plasticity and gene expression [23]. However, the influence of sodium salicylate on the concentration of free Ca^{2+} in auditory cortex neurocytes has not been reported. In our study, we demonstrated that the $[Ca^{2+}]_i$ in auditory cortex neurocytes in tinnitus model group was obviously increased.

The increase of intracellular Ca^{2+} can promote the binding of Ca^{2+} to its regulatory protein, thus generating a series of biological effect. There are a lot of regulatory proteins that can bind to Ca^{2+}. CaM protein is regarded as the main downstream molecule of calcium signaling pathway. After entering into cells, Ca^{2+} binds to CaM, forming Ca^{2+}/CaM complex [24], which then activates CaMKII by phosphorylating serine in 286 residue of CaMKII [25]. CaMKII is a multifunctional enzyme with ten subunits and performs critical roles in spatial learning and memory, and induction of long-term potentiation (LTP) [25]. LTP is a form of synaptic plasticity, and requires Ca2+/CaM activation of CaMKII [26]. Furthermore, calcium entries through postsynaptic NMDARs and subsequent activates CaMKII-dependent synaptic plasticity. Biochemical studies have demonstrated a high affinity binding between the catalytic domain of CaMKII and the C tail of NR2B subunit of the NMDA-Rs [27, 28]. CaMKII phosphorylates NR2B in vitro and in vivo, and both proteins coimmunoprecipitate in a Ca2+/CaM-dependent manner [27, 29]. Expression of NR2B that increases association to active CaMKII recovers LTP [25]. Our experiment demonstrated that, after the administration of sodium salicylate, the expression of CaM and NR2B was obviously up-regulated in auditory cortex neurocytes in tinnitus model group. Compared to non-treatment control group and normal saline group, the activity of CaMKII in auditory cortex neurocytes in tinnitus model group was also up-regulated.

CREB is a 43 kDa nucleoprotein and is the key molecule of the long-term change of synaptic plasticity in CREB signal pathway [30]. Serine 133 is the target of many protein kinases, including protein kinase C (PKC) and CaMKII. Once serine 133 is phosphorylated, CREB-induced transcription will initiate, thus increasing the transcription of synaptic plasticity-related protein, and stimulating the generation of LTP [31]. In a previous study, CREB phosphorylation was also revealed to be enhanced in salicylate-induced tinnitus rats [32]. In our study, we also confirmed that CREB activity was elevated in tinnitus rats. The prolonged activation of the auditory cortex neurocytes not only lowers the frequency of its spontaneous discharge, but also increases synchronic discharge among different neurons. These changes are regarded to be associated with the mechanism of tinnitus [33].

In summary, we successfully established tinnitus model in rats, in which calcium signals increased to enhance CaM and NR2B, causing activation of CaMKII and CREB. Our data might implicate that Ca^{2+}/CaM/CaMKII/CREB pathway is involved in the generation of tinnitus induced by sodium salicylate.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

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