SALICYLATE INDUCED NEURAL CHANGES IN THE PRIMARY AUDITORY CORTEX OF AWAKE CATS

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Abstract—Systemic administration of salicylate at high doses can induce reversible tinnitus and hyperacusis in humans and animals. For this reason, a number of studies have investigated the salicylate-induced changes of neural activity in the auditory cortex (AC); however, most previous studies of the AC were conducted on brain slices or anesthetized animals, which cannot completely represent the actual conditions. Few efforts have been made to examine the neural activity of awake animals, and only recorded the local field potential (LFP) of the AC. In this study, we recorded neural spike activities from chronically implanted electrodes in the primary AC (A1) of awake cats, and investigated the changes of neural responses to pure-tone and click-train stimuli after systemic injection of 200 mg/kg salicylate. We found that sound-evoked spike activities were significantly increased from 1 h after salicylate administration, and the increase of neural responses lasted longer than 3 days with a peak at 12 h. Salicylate not only increased the amplitude of transient responses at the onset and offset of pure-tone stimuli, but also induced a sustained response during the prolonged stimulus period and a late response at ~100 ms after stimulus offset. The significant enhancement of neural responses was observed over the entire tested frequency range (0.1–16 kHz) with a relative peak in the band of 3.2–9.6 kHz. The capability of exhibiting spikes synchronizing with successive clicks was also enhanced. All these effects were more apparent when the neurons were driven by high intensity sounds. Salicylate-administration also decreased the mean spontaneous rate in A1 units, and the decrease of spontaneous rate was larger in the units with a high initial spontaneous rate. Our data confirm that salicylate can modulate the neural activity at the cortical level and provide more information for understanding the mechanism of salicylate-induced tinnitus.

Key words: salicylate, tinnitus, primary auditory cortex, synchronization, spike activity, hyperacusis.

It is well known that the application of salicylate at high doses produces reversible tinnitus, a phantom auditory sensation without any external sound stimulus, in humans (Halla and Hardin, 1988; Day et al., 1989; Halla et al., 1991; Hicks and Bacon, 1999) and animals (Jastreboff et al., 1988; Bauer et al., 1999; Guitton et al., 2003; Ruttiger et al., 2003). For this reason, significant effort has been made to investigate how the salicylate administration affects the auditory neural system, including the auditory nerve (Evans and Borerwe, 1982; Sypulkowski, 1990; Cazals et al., 1998; Muller et al., 2003; Ruel et al., 2008), the ventral and dorsal cochlear nucleus (Basta et al., 2008; Wei et al., 2010), the inferior colliculus (Jastreboff and Sasaki, 1986; Chen and Jastreboff, 1995; Ma et al., 2006; Sun et al., 2009), the medial geniculate body (Basta et al., 2008) and the auditory cortex (AC) (Ochi and Eggermont, 1996; Eggermont and Kenmochi, 1998; Wang et al., 2006; Yang et al., 2007; Su et al., 2009). The accumulated data suggest that salicylate can increase the neural activities in both the peripheral (Evans and Borerwe, 1982) and central auditory system (Ochi and Eggermont, 1996; Sun et al., 2009). Recently, Kizawa et al. proposed that salicylate may induce an alternation from the peripheral to the central tinnitus, because the salicylate-induced high expression of a transient receptor potential cation channel subfamily V-1 (TRPV1) occurred earlier in spiral ganglia and later in dorsal cochlear nucleus (Kizawa et al., 2010). It should be noted that most previous studies were conducted on brain slices or anesthetized animals, which cannot completely represent the actual conditions. One study has shown that anesthesia affected the salicylate-induced change of AC activity: ketamine increased salicylate-induced enhancement of the AC response, whereas isoflurane suppressed such enhancement (Sun et al., 2009). For this reason, it is necessary to examine the effect of salicylate on animals under awake conditions. Recently, some studies investigated the salicylate-induced neural changes in awake animals by recording the spontaneous and sound-evoked local field potential (LFP) in AC (Yang et al., 2007; Sun et al., 2009; Norena et al., 2010). Their major finding was that salicylate could induce a reversible increase in the amplitude of LFP evoked by tone bursts over a wide range of frequencies and intensities, which may be associated with salicylate-induced hyperacusis (Myers et al., 1965); however, no such effect has been demonstrated at the level of neural spike activity. To date, only one report has presented preliminary neural spike activity at the cortical level and provide more information for understanding the mechanism of salicylate-induced tinnitus.
data recorded from 9 U (Yang et al., 2007), showing that salicylate administration caused a reversible decrease of the spontaneous spike rate in the AC of awake rats. In order to systematically investigate how the AC activities of awake animals are affected by salicylate administration, we conducted extracellular recording in the primary auditory cortex (A1) of awake cats using chronically implanted electrodes, and examined the changes of neural responses to pure-tone and click-train stimuli after systemic injection of 200 mg/kg salicylate. Our results provided more accurate information about the effect of salicylate.

**EXPERIMENTAL PROCEDURES**

**Animal preparation**

All procedures were approved by the University of Yamanashi Animal Care and Use Committee. Animal preparation and recording procedures were similar to those used in our previous experiments (Qin et al., 2007, 2008a,b, 2009). The cats were premedicated with 0.1–0.2 ml atropine sulfate (0.5 mg/ml) s.c. After about 30 min they received an i.m. injection of 25 mg/kg ketamine hydrochloride (50–100 mg/ml) and an i.p. injection of 20 mg/kg sodium pentobarbital (50 mg/ml). The cat was then fixed to a stereotaxic frame (SN-3N; Narishige). The head was shaved and an incision was made in the skin overlying the skull. The skin flap was removed and the skull cleared from overlying muscle tissue. The position of A1 was marked on the bone surface according to stereotaxic coordinates. Four small holes were drilled over the occipital bone and fine jeweler’s screws were inserted to serve as an anchor for a metal block that was cemented to the skull with dental acrylic. After the cement had hardened, the head was held through the metal block and the ear bars were removed. We then drilled several small holes (0.5–1 mm diameter) in the temporal bone above the potential location of A1. A tungsten microelectrode (diameter: 250 μm; impedance: 2–5 MΩ at 1 kHz; FHC Inc.) was advanced into the cortex using a micromanipulator to examine the neural responses to tonal stimuli at each site. According to the characteristics of tonotopic gradient, we identified the location of A1.

**Electrode implant**

We implanted a microwire array following the method developed by Jackson and Fetz (Jackson and Fetz, 2007). The microwire consisted of 12 (2×6) Teflon-insulated 50 μm diameter tungsten wires (part #795500; A-M Systems, Carlsborg, WA, USA) running the location of A1.

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**Acoustic stimulus presentation**

The sound waveform was digitally generated by user-written programs in a MATLAB (Mathworks) environment. The signals were fed into a 16-bit digital-to-analog converter (PCI-6052E; National Instruments) at a sampling frequency of 100 kHz. Bilateral acoustic stimuli were played through a pair of speakers (K1000; AKG) placed 2 cm from the auricles of cats. The sound pressure level (SPL; in decibels re. 20 μPa) was measured using a Bruel and Kjaer 1/2” condenser microphone with a preamplifier 2669 situated at the position of the cat’s ear. We calibrated the sound delivery system between 0.1 and 16 kHz in frequency steps of 8 Hz, and the output varied by ±5 dB. Harmonic distortion was less than −60 dB.

We used two stimulus ensembles to evaluate sound-driven neural activity. One was the pure-tone ensemble which randomly presented 125 single tones (160 ms in duration, including 5 ms rise/fall time) in the frequency range of 128–16,000 Hz (in 128 Hz steps) and at 20, 40 and 60 dB SPLs, respectively. The other consisted of 320 ms duration click-trains. The individual clicks were 0.1 ms rectangular electric pulses. The inter-click interval (ICI) was randomly selected among 80, 60, 40, 20, 10 and 5 ms. Each ICI was repeated 12 times, constructing an ensemble of 72 stimuli. The maximum click level was calibrated to that of the pure-tone wave at 60 or 20 dB SPL. The inter-stimulus intervals were randomly set between 1 and 3 s.

**Electrophysiological recording**

After 2–3 weeks of postoperative recovery and adaptation training, the recording experiment was conducted in an electrically shielded, sound-attenuated chamber. During the recording period, the cats were immobilized on a custom-built frame, and passively listened to auditory stimuli. A video camera was placed in front of the cat to monitor its state. If a sign of drowsiness (eye closing or drifting) was detected, the cat was alerted by gently tapping the body using a remote-controlled tapping tool, or by briefly opening and closing the door.

Multi-channel neural recording was performed using hardware and software from Tucker-Davis Technologies (TDT, Alachua, FL, USA). The microwire output was connected to a multichannel preamplifier (RA16PA; TDT) using a flexible, low noise cable. The output of the preamplifier was delivered to a digital signal processing module (RX-7; TDT). Spike activities were discriminated using principal component feature space spike-sorting software (SpikePac; TDT). Using this method, 1–3 single units could be well isolated from each channel. The time stamps of these single units were stored and coded separately. As described by Jackson and Fetz (Fitzgerald et al., 2007), clean single units could often be followed for up to several weeks after positioning the microwires; however, in some cases, cells were lost during recording and new cells also occurred. Because we were not confident that all the data sets tracked the same units during the observing period, in this study we pooled the separable single-unit spike trains of each channel, again constructing multiunit data. As shown in the example (Fig. 1A), the response properties of the multiunit activities were similar between pre-administration and after recovery from salicylate administration, suggesting that our recording was stable throughout this period.

**Data analysis**

Spike activities driven by pure-tone stimuli were aligned along the stimulus onset, constructing a raster plot of each tone frequency (Fig. 1A). The peri-stimulus time histogram (PSTH), counting the spikes across the 125 trials of different frequencies, was computed in 1 ms bin width and smoothed by Gaussian function with 5 ms SD (Fig. 1B). The neural responses of pure-tone stimuli were analyzed in four segments: “onset” response (during 0–50 ms after stimulus onset), “sustained” response (from 50 ms after stimulus onset to stimulus offset), “late” response (0–50 ms after stimulus offset) and “offset” response (0–50 ms after stimulus offset) and “late” response (0–50 ms after stimulus offset). The tuning curves of each kind of responses were constructed by plotting the average firing rate in the “onset”, “sustained”, “offset” or “late” segment, respectively, against tone frequency in a linear step of 128 Hz (Fig. 2A–D). The threshold to identify a significant
response was set at the level of the mean±2SD of the background firing rate obtained in the segment preceding the sound onset (dotted line in Fig. 2). The mean firing rate of each segment was obtained by averaging the firing rates at the frequencies, where the tuning curve was higher than the threshold. The characteristic frequency (CF) of each unit was estimated by the frequency at which the unit’s showed a peak response to 20 dB tones. Because the A1 units usually showed only “onset” response when driven by the lower intensity sounds, the search of CF was based on the “onset” tuning curve. If a unit had no significant response to 20 dB tones, the CF was estimated by using the tuning curve of 40 dB tones.

The click-following capacity of the neural response was evaluated from the significance of the vector strength calu-
lated by assuming a sinusoidal stimulation with a period equal to the repetition period of the clicks. This method has been well used in previous studies (Eggermont, 1991; Liu et al., 2003, 2007). In detail, we computed the mean vector strength, $R$, for stimulus-synchronized responses (excluding onset responses to the click train) according to the following formulae (Goldberg and Brown, 1969):

$$R = \frac{1}{n} \sqrt{\sum_{i=1}^{n} \cos \theta_i \cdot \sin \theta_i}$$

where $n$ is the total number of spikes, $t_i$ is the time of spike occurrence, and $T$ is the inter-click interval. Rayleigh statistics, $2nR^2$, were then calculated to indicate the significance of the stimulus-synchronized response (Shofner et al., 1996; Mardia and Jupp, 2000). A Rayleigh statistics value of 13.8 corresponds to a $P$-value of 0.001 for the Rayleigh test (Mardia and Jupp, 2000).

**Experiment procedure and salicylate administration**

Before salicylate administration, we completed a recording sequence composed of the presentation of three different intensity series (20, 40 and 60 dB) of pure-tone stimuli and two different intensity series (20 and 60 dB) of click-train stimuli. The cats were then administrated i.p. with sodium salicylate at a dose of 200 mg/kg (50 mg/ml). The recording sequences were repeated at 1, 4, 8, 12, 24, 48, 72, 96 and 120-h intervals following the injection. At the end of the experiments the animals were deeply anesthetized and perfused with 10% formalin. The brain was removed and the sites of recording electrodes were confirmed histologically. This report was based on the units collected from the sites of A1.
Statistical tests between the data obtained before and after administration were based on ANOVA with post hoc test. Changes for the same recording site before and after drug application were evaluated using paired t-tests. Changes at the P<0.05 level were considered significant. All tests were performed using the Statistics Toolbox of MATLAB.

RESULTS

Six male cats (aged from 2 to 3 years) were used to observe salicylate-induced neural changes through the implanted 12-channels microwires in the A1 of both hemispheres. A total of 144 electrodes (6 cats×12 channels×2 sides) were implanted, in which 91 multiunit data were stably collected before and 1, 4, 8, 12, 24, 48, 72, 96 and 120 h after salicylate injection (200 mg/kg i.p.). The CF of the collected units ranged from 0.5 to 15.8 kHz (lower quartile, median and upper quartile was 3.0, 6.4 and 11.5 kHz, respectively).

Effects of sodium salicylate on pure-tone evoked responses in an example unit

Fig. 1A shows the representative spike activities at one electrode driven by pure-tone stimuli before and after administration. Each plot presents a raster display of spike time in response to pure-tone stimuli at 60 dB SPL for a frequency range from 128 to 16,000 Hz. Before administration, the neurons showed phasic responses at the onset and offset of pure-tone stimuli (vertical lines in Fig. 1A), resulting in two separate peaks higher than the threshold (mean±2SD of the background firing rate) in the PSTH (Fig. 1B, dotted line shows the threshold). Fig. 1A also shows that the “onset” response could be evoked by a wide range of frequencies, while the “offset” response was evoked by a limited range of frequencies. The tuning curves of “onset” and “offset” responses are plotted in Fig. 2A, C.

At 1 h after salicylate administration, the firing rates of “onset” and “offset” responses were increased (second row in Fig. 1) compared to those at pre-administration. From 4 h post-administration, an additional response peak appeared at about 100 ms after stimulus offset, and gradually increased in amplitude and duration with time. Such a long-latency response was named as “late” response in this paper. The tuning curves of “late” responses are presented in Fig. 2D. Until 8 h post-administration, no significant response has been observed at the time window of 50–160 ms after stimulus onset, resulted in a lower tuning curve during this period (Fig. 2B). The most significant increase of the neural response occurred at 12 h. At that time point, the peak firing rate of the “onset” response reached about four times that at pre-administration, and after the transient response peak, the unit also showed a sustained response throughout the sound stimulus. The offset of stimulation also evoked a transient response peak, followed by a dip. Another response peak appeared at 60 ms after stimulus offset and extended to 160 ms post-stimulus. As shown by the tuning curves in Fig. 2, the firing rates of the “onset”, “sustained”, “offset” and “late” response at 12 h post-administration were all largely elevated.

During the period of 24 and 72 h post-administration, the “sustained” response and “late” response decayed a little, while the transient response peaks occurring at the onset and offset of stimulus remained largely higher than those at pre-administration (Fig. 1). Consequently, the tuning curves of “onset” and “offset” responses still exceeded the threshold level at most frequencies (Fig. 2). A clear recovery started at 96 h after salicylate administration and a complete recovery was achieved at 120 h, when the amplitude of the phasic response fell back to the original level, and the “late” response disappeared (Fig. 1). The tuning curves also recovered (Fig. 2). The similarity between the response properties at pre- and 120 h post-administration reflected the stability of the recording over time.

Effects of sodium salicylate on the population responses to pure-tone

The effects of salicylate on the neural population are illustrated in Fig. 3A, in which the average PSTHs of 91 U driven by 60 dB pure-tones at different times are plotted together. For clarity, we included only the results of pre-, 12 h and 120 h post-administration in one plot (blue, red and green lines, respectively). The width of each line in Fig. 3A represents the mean±SEM of PSTH averaged across 91 individual units. Comparing the red and blue lines, it is clear that salicylate administration increased the transient response peaks at sound onset and offset, and also induced a sustained response during the stimulus period and late response at ~100 ms after sound offset. These changes were all reversed at 120 h after administration, reflected by the close match between the green and blue lines. This result was consistent with the findings in the above example unit.

We then explored the salicylate effects on the neural responses to pure tones at lower intensities. The results of 40 and 20 dB are shown in Fig. 3B, C. The increase of the neural response at sound onset remained clear at lower sound intensities, while the effect on the other responses, including the “sustained”, “offset” and “late” responses, gradually decreased with the decrease of sound intensity.

To illustrate the salicylate-induced changes more clearly, we present the time course of neural population responses according to tone frequency in a 3-dimension plot (Fig. 4). For each of the 91 U, we sampled seven points from its tuning curve, which corresponded to the frequency of the unit’s CF, and CF±0.5, ±1, ±2, ±3 octaves, respectively. Then, the firing rate averaged over the 91 re-sampled tuning curves are plotted as a column and represented by a grayscale in Fig. 4. The results obtained at different time points are arranged along the abscissa, therefore, the change of firing rate at a specific frequency is represented by a raw. The time course of “onset”, “sustained”, “offset” and “late” responses at 60 dB SPL are presented in Fig. 4A, B, C, D, respectively. All of the plots clearly show that the firing rate was elevated and the responsive frequency range was extended after the
administration of salicylate. The response enhancement concentrated on the period between 1 and 72 h post-administration and showed a peak at 12 h.

Frequency-specific effect of salicylate administration

The other question is whether the salicylate-induced change depended on the tone frequency. This issue was examined on the neural population responses to 60 dB tones. We computed the difference of mean firing rate between 12 h post- and pre-administration across five different frequency bands: 0.1–3.2, 3.2–6.4, 6.4–9.6, 9.6–12.8, and 12.8–16.0 kHz, respectively (Fig. 5). As shown by Fig. 5A, the firing rate of “onset” response was significant elevated in all the five frequency bands ($P < 0.05$, ANOVA). Though the elevation in 3.2–9.6 kHz was a little higher than in other bands, statistical analysis revealed that the variation among different bands was not significant ($P = 0.32$, ANOVA). Also, the firing rates of “sustained” and “offset” response were significantly elevated in all the frequency bands (Fig. 5B, C), while the variation of elevation among different bands was significant ($P < 0.05$, ANOVA). The higher elevation of neural response in 3.2–9.6 kHz may suggest a frequency-dependency of the salicylate-induced enhancement. This tendency was also observed in the “late” response, because the significant elevation of the “late” response only occurred in 3.2–9.6 kHz (Fig. 5D). Taken together, our results suggest that salicylate-administration generally enhanced the neural responses to all the tone frequencies, while the enhancement effect was more salient in the frequencies of 3.2–9.6 kHz.

Effects of sodium salicylate on click-train evoked responses

To assess the neural capacity to follow successive sounds, we stimulated the auditory neurons using click-trains with six different ICIs (80, 60, 40, 20, 10 and 5 ms). Fig. 6A–C present an example of A1 units responding to click-trains at 60 dB SPL. As shown by the raster plot (Fig. 6A) and PSTH (Fig. 6B), the unit exhibited transient discharges to the first click for all stimuli. What differentiated the responses to click trains with different ICIs were the discharges evoked by later clicks. The discharges synchronizing each click were clear when the ICI was 40–80 ms, and unclear when the ICI was 5–20 ms. The capacity of neural response following the clicks was quantified by Rayleigh statistics (see Experimental procedures). Fig. 6C shows the Rayleigh statistics of this unit as a function of ICI, wherein the Rayleigh statistics was higher than the significant level (13.8, $P < 0.001$) at 40–80 ms ICI, and lower at 5–20 ms ICI. Such a response pattern has been well reported by previous studies on normal animals (Lu and Wang, 2000).

After treatment with salicylate, the neural capacity to follow the clicks was markedly increased. For brevity, we have presented only the results of 12 h and 120 h after administration in Fig. 6D–F, G–I, respectively. It is clear that the click-synchronized discharges were more salient at 12 h (Fig. 6D, E). Consequently, the Rayleigh statistics of 40–80 ms ICI were largely increased and even that of 20 ms ICI was also elevated above the significant level (Fig. 6F). At 120 h post-administration, the neural responses returned to a level similar to pre-administration (comparing Fig. 6G–I with A–C).

Effects of sodium salicylate on the population responses to click-train

The population responses to the click-trains at 60 dB SPL are presented in Fig. 7, in which the PSTHs of different time points are plotted together. The blue line shows the mean±SEM of PSTH averaged across 91 individual units before salicylate administration, while the red line shows that after 12 h administration. In Fig. 7A, both the blue and
red lines exhibited five transient peaks synchronizing with the five clicks in 80 ms ICI; however, each peak at 12 h post-administration was markedly higher than at pre-administration. The green line shows the PSTH at 120 h post-administration, which had completely returned to the range at pre-administration. With the decrease of ICI (Fig. 7B–F), the neural responses synchronizing to the later clicks gradually decreased, and completely disappeared when ICI was 10 and 5 ms (blue lines). After 12 h salicylate administration, the click-synchronized responses were all enhanced under the tested conditions. Especially at 20 ms ICI (Fig. 7D), the original unclear synchronized response became salient, suggesting that the administration of salicylate could increase the neural capacity to synchronize with click trains. At 5–10 ms ICI, the administration of salicylate could induce sustained responses during the stimulus period of click trains, and also a slight response at stimulus offset (Fig. 7E, F). All these changes recovered at 120 h post-administration.

The time course of changes in click-following capability is shown in Fig. 8, in which the average Rayleigh statistics of the 91 U is plotted against time. Fig. 8A shows the result of click trains at 80 ms ICI and 60 dB SPL, when the synchronizing response was most obvious. Consistent with the finding of pure-tone stimuli, the Rayleigh statistics was significantly increased above the initial level between 1 and 72 h post-administration (P<0.05, ANOVA with post hoc test) and peaked at 12 h. A similar tendency was also observed in the results of 20–60 ms ICIs (not illustrated), but not in 5–10 ms ICIs because A1 neurons could not synchronize with such fast click trains under any conditions.

Fig. 8B shows the result of click trains at 80 ms ICI and 20 dB SPL. Although salicylate administration could increase the Rayleigh statistics, it gently fluctuated at the significant level suggesting that the click-following capability could not be stably increased at low sound intensity.

Change of the single-unit spike activity

Above we showed that the multiunit activities were significant increased by the application of salicylate. These results may be just caused by recruitment of neurons that did not respond to sound stimuli before the salicylate treatment. In order to examine whether the existing neuronal responses was enhanced by salicylate, we sorted the spike waveforms using the offline spike-sorting software (OpenSorter, TDT) and identified 46 well isolated single-units which were maintained over 12 h. All the spike waveforms of an example unit are present in Fig. 9A. The similarity between the spike waveforms before and 12 h after salicylate-administration was originated from the same neuron. We compared the data of 46 single-units before and 12 h after salicylate-administration. As shown by the representative results of 60 dB pure-tone and click-train (Fig. 9B, C), the single-unit activities were obviously increased at 12 h after salicylate-administration. The patterns of response en-
hancement were quite similar to those of the multiunit data (compares to Figs. 3A and 7A), suggesting that salicylate did increase the responses of existing units.

Effects of sodium salicylate on spontaneous spike activity

We measured spontaneous activity from 91 U in six cats before and after salicylate treatment. Spontaneous spike counts were sampled over 0.5 s window every 2 s over a period of 5 min (total sample time 62.5 s). With the use of the multiunit data, we failed to find a significant change of spontaneous firing rates after salicylate-administration. Thereby, we focused on the 46 single-units sorted by the software. The effect of salicylate-administration was illustrated by the scatter plot of the spontaneous firing rate at 12 h post-administration against that at pre-administration (Fig. 10A). It is clear that the spontaneous activity in most of units was decreased after the administration of salicylate. The mean and SD of spontaneous firing rates of the 46 U were 8.4±5.0 spikes/s before salicylate injection, and decreased to 5.4±4.4 spikes/s at 12 h later. The difference between the mean spontaneous firing rates was statistically significant (paired t-test, P<0.001).

We then investigated a potential differential effect on units with different spontaneous firing rates by plotting the ratio of firing rates (post/pre) against the firing rate at pre-administration (Fig. 10B). This scatter plot shows a weak tendency that the firing rate was less decreased in the units with lower spontaneous firing rate. That means, the ratio of firing rate negatively correlated to the initial spontaneous firing rate (r=−0.3, P<0.05). The ratio of spontaneous firing rate was also plotted against the unit’s CF (Fig. 10C), in order to investigate the frequency dependency of salicylate effect. No significant correlation between the change of spontaneous firing rate and CF was found.

DISCUSSION

This study is the first to report the effect of salicylate administration on the neural spike activity recorded in A1 of awake cats. Our major findings are (1) a single administration of salicylate increased the neural responses to pure-tone and click-train stimuli from 1 to 72 h after administration, while the peak response enhancement occurred at 12 h (Figs. 1, 4 and 8); (2) salicylate not only markedly increased the amplitude of transient responses at the onset and offset of pure tones, but also induced sustained firing during the prolonged stimulus period and late firing at 100 ms after stimulus offset (Figs. 1 and 3); (3) salicylate administration could extend the frequency range of the neural responses (Fig. 4). The enhancement of neural responses was more salient at the 3.2–9.6 kHz frequency band (Fig. 5); (4) salicylate also obviously increased the neural capability to exhibit spikes synchronizing with successive clicks (Figs. 6–8); (5) effects of salicylate admin-
istration were more obvious when the neurons were driven by high intensity sounds (Figs. 3 and 8); (6) the mean spontaneous spike rate recorded from the same single-unit decreased from 8.4 spikes/s at pre-treatment to 5.4 spikes/s at 12 h post-treatment. And, the ratio of post/pre spontaneous rate was negatively correlated with the unit’s spontaneous rate before treatment. Overall, the results reported herein are in broad agreement with those from earlier studies showing that salicylate can increase neural activity in AC (Yang et al., 2007; Sun et al., 2009; Norena et al., 2010). The present work further provides more accurate information on spike activity, which is helpful to understand the mechanism of salicylate effects.

Merits of our experimental method

Using the method well developed by Jackson et al. (Jackson et al., 2006; Jackson and Fetz, 2007), we could stably record extracellular potentials for about 1-week. Although we were not confident that all the recordings were from the same single unit during the experimental period, the similarity between the neural properties pre- and 120 h post-administration indicated that the recordings were at least from a cluster of cells with identical properties (Figs. 3 and 7). Also, our results from the off-line sorted single-units were similar to those of multiunits (Fig. 9).

We found that the systemic administration of salicylate increased the neural activities of AC at 1 h. Such a rapid effect of salicylate was also observed in AC of awake rodents (Yang et al., 2007; Sun et al., 2009; Norena et al., 2010). The long-term recording in this study found that salicylate-induced enhancement continued over 3 days in cats, during which the properties of neural responses changed little (Figs. 1, 2, 4 and 8). The effective duration of salicylate in cats was obviously longer than in rodents (about 1-day), consistent with the fact that cats take more time to metabolize salicylate because they are deficient in glucuronyl transferase (Jung et al., 1993). Despite the potential confusion with non-specific toxic effects in the cat, the prolonged duration of the drug effect allowed extensive data to be collected under similar conditions.

Salicylate-induced response enhancement suggesting a reduction of central inhibition

It has been suggested that salicylate-induced enhancement of AC responses may be attributable to the decrease in central inhibition (Jastreboff, 1990; Sun et al., 2009; Norena et al., 2010). Recently, evidence has been accumulating that salicylate decreases inhibitory (GABAergic) neurotransmission in AC (Wang et al., 2006; Su et al., 2009). For example, whole cell patch clamp recordings in AC slices showed that high doses of salicylate greatly reduced inhibitory postsynaptic currents (Wang et al., 2006). On the other side, salicylate-induced enhancement of the AC response was suppressed under isoflurane an-
esthesia (Sun et al., 2009) because isoflurane can increase GABA-mediated neurotransmission while suppressing glutamatergic synaptic activity (Ranft et al., 2004).

Our results of extracellular recording in the in vivo preparation provide evidence supporting the proposal that salicylate works on the AC by reducing cortical inhibition. (1) Under normal conditions, A1 neurons generally exhibited a transient response at sound onset, which rapidly decayed during the prolonged stimulus period (blue and green lines in Fig. 3A). This has been related to stronger intracortical inhibition (Creutzfeldt et al., 1980). Some neurons also exhibited a brief response at sound offset, which was explained as a rebound from the preceding inhibition (Phillips et al., 2002). After salicylate treatment, the amplitude of the transient response at stimulus onset was largely increased, but its width was not obviously changed (red line in Fig. 3A). After the firing rate fell close to the normal level, it soon rebounded and followed a sustained response to the stimulus offset. An additional increase of neural discharge also appeared at ~100 ms after stimulus offset. This phenomenon suggests that salicylate may reduce the inhibitory input to A1 neurons, especially in the late post-stimulus period. (2) We found that salicylate administration obviously extended the frequency range of neural responses (Figs. 2 and 4). This is similar to previous results of pharmacological experiments in AC, which showed that blocking cortical GABAergic inhibition resulted in an expansion of the frequency tuning on both sides of the CF (Chen and Jen, 2000; Wang et al., 2000, 2002). Recent in vivo whole-cell voltage-clamp recordings in AC neurons have found that the tuning curve of spike responses is shaped by stronger tuning inhibitory synaptic

Fig. 7. Mean PSTHs of 91 U driven by 60 dB click-trains at 80, 60, 40, 20, 10 and 5 ms ICI, respectively (A–F). Refer to Fig. 3 for other captions. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 8. Mean Rayleigh statistics of the neural population against time relative to salicylate administration. (A) Rayleigh statistics of the neural response to click-trains at 80 ms inter-click interval (ICI) and 60 dB SPL. Circles represent the mean; dots represent the SEM. Dotted line shows the significant level to identify the stimulus-synchronized response. Asterisk marks the time point when the mean Rayleigh statistics was significantly higher than that at the pre-treatment (P<0.05, ANOVA with post hoc test). (B) Rayleigh statistics at 80 ms ICI and 20 dB SPL.
Fig. 9. Change of single-unit spike activities as a result of salicylate-administration. (A) Waveforms of all spikes of an example single-unit recorded at pre- (left) and 12 h (right) post-administration. Note that the waveforms show a similar shape. (B) Mean PSTHs of 46 single-units driven by tones at 60 dB tones. PSTHs of pre- and 12 h post-administration are shown in blue, red respectively. Refer to Fig. 3 for other captions. (C) Mean PSTHs driven by click-trains at 60 dB and 80 ms ICI. Refer to Fig. 7 for other captions. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Fig. 10. Change of spontaneous firing rate as a result of salicylate-administration. (A) Scattergram of 46 single-unit spontaneous firing rates at 12 h post-administration against those at pre-administration. (B) Ratio of post/pre spontaneous firing rates as a function of the spontaneous firing rate at pre-administration. (C) Ratio of post/pre spontaneous firing rates as a function of the characteristic frequency (CF) of the unit.
input following excitatory input (Wehr and Zador, 2003; Tan et al., 2004; Wu et al., 2006, 2008). Wehr and Zador (2003) reported that the tuning properties of inhibitory and excitatory inputs were completely overlapped, while Wu et al. (2006) later found that cortical inhibition is broader than excitation. Therefore, the salicylate-induced extension of frequency tuning in A1 neurons may be attributable to the reduction of inhibitory synaptic input. (3) AC neurons usually cannot follow the trains of clicks with ICI shorter than about 20–40 ms (Lu and Wang, 2000; Lu et al., 2001). This is widely considered a result of forward suppression, that is, the responsiveness of the succeeding sound is decreased by that of the preceding sound. After salicylate administration, the capability of AC neurons to follow clicks suppressed by that of the preceding sound. After salicylate administration, the capability of AC neurons to follow clicks was increased (Figs. 6–8); hence,forward suppression administration, the capability of AC neurons to follow clicks suppressed by that of the preceding sound. After salicylate administration, the capability of AC neurons to follow clicks was increased (Figs. 6–8); hence, forward suppression may be decreased. Because most thalamic neurons can follow click trains at ICI shorter than 10 ms, forward suppression may originate from the cortical circuit (Creutzfeldt et al., 1980; Miller et al., 2002). Postsynaptic GABAergic inhibition has been widely considered the most likely mechanism for cortical forward suppression (Calford and Semple, 1995; Brosch and Schreiner, 1997; Tan et al., 2004). However, a recent study using whole-cell recordings in vivo found that GABA-mediated inhibition does not play a major role in forward suppression beyond about 100 ms, and that synaptic depression was suggested to be the most likely candidate mechanism of long-lasting suppression (Wehr and Zador, 2005). Our results suggest that the major function of salicylate might be the reduction of long-lasting suppression.

It should be noted, however, that our results from awake cats only indicated some cues of the reduction of central inhibition. More direct and solid evidence are needed to draw a conclusion that the salicylate-induced effects are caused by the reduction of central inhibition.

Enhanced AC response and hyperacusis

In this study, we revealed that salicylate-administration significantly enhanced the neural responses to a wide range of pure-tones and click-trains. Our results also showed that the salicylate-induced enhancement was more salient in the neural responses to 3.2–9.6 kHz frequencies, which are in the low absolute-threshold band of cat's hearing (Elliott et al., 1960). The enhancement of sound-evoked response may relate to hyperacusis. Recently, Sun et al. found that systemic injection of 250 mg/kg salicylate enhanced the neural response from the AC of awake rats and also caused an increase in the amplitude of the startle response (Sun et al., 2009). Because an increase in startle amplitude has been suggested as a behavioral correlate of hyperacusis (Ison et al., 2007), both of these measures was interpreted as evidence of hyperacusis. It was also shown that high doses of salicylate may induce hyperacusis in humans (Myers et al., 1965); however, additional studies are needed to collect direct evidence of salicylate-induced hyperacusis in humans and/or animals. Moreover, it is not yet clear whether the origin of salicylate-induced change is peripheral or central. Previously, local administration of NMDA antagonists has been shown to block behavioral evidence of salicylate-induced tinnitus (Guitton et al., 2003), whereas systemic administration of NMDA antagonists did not prevent salicylate-induced tinnitus (Lobarinas et al., 2006). In the future, similar methods combined with the long-term unit recording technique might reveal more evidence about the origin of salicylate effects.

Spontaneous firing rate after salicylate administration

In this study of awake cats, we collected 46 well isolated single-units which was maintained over 12 h. We found the mean spontaneous rate averaged over the 46 U was significantly depressed by the application of salicylate (Fig. 10A). Our data are compatible with more recent data showing that salicylate selectively suppressed spontaneous rate in fusiform cells, but not cartwheel cells in dorsal cochlear nucleus (Wei et al., 2010) and AC (Yang et al., 2007). It has been shown that salicylate selectively suppressed spontaneous rate in fusiform cells, but not cartwheel cells in dorsal cochlear nucleus (Wei et al., 2010). The cell-specific effect of salicylate is also implied by the results from the A1 of anesthetized cats (Ochi and Eggermont, 1996), showing that the spontaneous rate was increased in low-spontaneous rate units and decreased in high spontaneous rate units. Similarly, our results also showed that the suppression of spontaneous rate was larger in the units with a high initial spontaneous rate. Thereby, salicylate may selectively suppress the spontaneous rate of inhibitory neurons in A1. And the phantom auditory sensation may directly relate to the increase of spontaneous rate in the higher auditory cortexes (Eggermont and Kenmochi, 1998), due to the decreased cortical inhibition from A1. Because the neuron population cannot be well categorized just using our extracellular recording method on awake animals, this possibility needs to be examined in the future study which combining both the morphological and pharmacological methods.

CONCLUSION

In conclusion, we developed a method to observe the long-term effect of salicylate on spike activity in the AC of awake cats, and found that salicylate can increase sound-evoked responses for 3 days. Salicylate administration not only increased the amplitude of transient responses at the onset and offset of pure tones, but also induced sustained firing during the prolonged stimulus period and late firing at ~100 ms after stimulus offset. The responsive frequency range of A1 neurons was largely expanded, and the capability to exhibit spikes synchronizing with successive clicks was also enhanced. Salicylate could decrease the mean spontaneous rate in A1 units, and the decrease of spontaneous rate was larger in the units with a high initial spontaneous rate. These enhancements were more obvious when the neurons were driven by high-intensity sounds. These findings provide more information to understand the effects of salicylate.
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