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# Too much of a good thing: Long-term treatment with salicylate strengthens outer hair cell function but impairs auditory neural activity

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## Abstract

Aspirin has been extensively used in clinical settings. Its side effects on auditory function, including hearing loss and tinnitus, are considered as temporary. A recent promising finding is that chronic treatment with high-dose salicylate (the active ingredient of aspirin) for several weeks enhances expression of the outer hair cell (OHC) motor protein (prestin), resulting in strengthened OHC electromotility and enhanced distortion product otoacoustic emissions (DPOAE). To follow up on these observations, we carried out two studies, one planned study of age-related hearing loss restoration and a second unrelated study of salicylate-induced tinnitus. Rats of different strains and ages were injected with salicylate at a dose of 200 mg/kg/day for 5 days per week for 3 weeks or at higher dose levels (250–350 mg/kg/day) for 4 days per week for 2 weeks. Unexpectedly, while an enhanced or sustained DPOAE was seen, permanent reductions in the amplitude of the cochlear compound action potential (CAP) and the auditory brainstem response (ABR) were often observed after the chronic salicylate treatment. The mechanisms underlying these unexpected, permanent salicylate-induced reductions in neural activity are discussed.

## 1. Introduction

Aspirin (acetylsalicylic acid), synthesized in 1860s, is one of the most widely used antipyretic, analgesic and anti-inflammatory drugs. Daily, low-dose aspirin therapy protects against cardiovascular injury (Danese et al., 1971; Kojda, 2004; Nunez et al., 1984) and also lowers the risk of colorectal and other forms of cancer (Bosetti *et al.*, 2002; Jacobs *et al.*, 2005). However, aspirin has several side effects such as gastrointestinal irritation, renal dysfunction, hepatic and allergic reactions (Bergman et al., 1976; Bjorklund et al., 2009; de



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Weck et al., 2006; Gibson et al., 1956; Rupp et al., 1983; Saltzman et al., 1976; Zucker et al., 1975).

High doses of aspirin have long been known to cause hearing loss and tinnitus. However, aspirin-induced hearing loss and tinnitus are considered completely reversible within a few days after discontinuing use (Bernstein et al., 1967; Falbe-Hansen, 1941; Jager, 1946; Janssen et al., 2000; Johnsen et al., 1982; McCabe et al., 1965; Myers et al., 1965a; Myers et al., 1965b; Oudot et al., 1979; Perez de Moura et al., 1968; Perlman, 1966; Ramsden et al., 1985; Waltner, 1955). Animal experiments showed losses of cochlear sensitivity of up to 40–50 dB and reductions in distortion product otoacoustic emission (DPOAE) after treatment with high doses of salicylate, the active ingredient of aspirin; these salicylate-induced changes completely recovered within a few days (Cazals et al., 1988; Guitton et al., 2003; Gunther et al., 1989; Gunther et al., 1988; Huang et al., 2005; Mitchell et al., 1973; Oliveira et al., 1976; Silverstein et al., 1967; Woodford et al., 1978; Yu et al., 2008). Based on the DPOAE and the analysis of input/output (I/O) function of cochlear compound action potentials (CAP), salicylate appears to specifically target the cochlear active process or cochlear amplifier (Gold et al., 1966; Guitton et al., 2003; Stypulkowski, 1990; Thalmann et al., 1973). Thus, the salicylate-induced temporary loss of cochlear sensitivity may result from a reversible outer hair cell (OHC) dysfunction. Salicylate purportedly competitively binds to prestin, resulting in a reversible elimination of OHC electromotility and temporary loss of DPOAE and cochlear amplification.

In contrast to the acute functional loss, DPOAE were shown to be enhanced after a chronic high-dose salicylate treatment. The improvement in DPOAE amplitude was associated with enhanced OHC electromotility and elevated prestin expression (Huang et al., 2005; Yang et al., 2009; Yu et al., 2008). Collectively, the improvements in DPOAE, OHC electromotility and prestin expression suggested that long-term treatment with high doses of salicylate might exert different effects on the auditory system than acute treatments.

To follow up on these observations, we carried out two studies, one planned study to determine if salicylate would reverse age-related hearing loss and a second unrelated study of salicylate-induced tinnitus; both studies examined the functional consequences of long-term, high-dose salicylate-treatment on cochlear function and auditory brainstem function in two different strains of rats. Since age-related hearing loss appeared to be related to a reduction of prestin in OHC (Chen *et al.*, 2009), we hypothesized that chronic salicylate treatment would increase prestin expression and enhance DPOAE thereby restoring auditory function in aged Fischer 344/NHsd (F344) rats. Although DPOAE were generally enhanced or remained at the pre-exposure levels after the chronic salicylate treatment; we unexpectedly observed a permanent reduction in CAP amplitude. In the second, unrelated study of salicylate-induced tinnitus, the auditory brainstem response (ABR) was measured in Sprague-Dawley rats that received multiple injections of salicylate for several weeks. A large reduction in ABR amplitude was often observed after long-term, high-dose salicylate treatment. These results, which were completely unexpected, show that prolonged, high-dose salicylate treatment may result in permanent auditory neural impairments.

## 2. Methods

### 2.1. Subjects

The Sprague-Dawley (SD) rats (3 months of age) were purchased from Charles River Laboratories Inc. (Wilmington, MA). The young (3 months) and aged (18 months) Fischer 344/NHsd (F344) inbred rats were purchased from Harlan Sprague Dawley NIA (Bethesda, MD). The animals in crates were shipped by truck. The rats were housed after delivery in the State University of New York at Buffalo laboratory animal facility. Background noise in the colony room was 45 dBA. Temperature was maintained at 22°C with 12 h/12 h light dark cycle. All procedures regarding the use and handling of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo. The twenty young SD rats were originally part of a salicylate-induced tinnitus study in which ABR were measured (hereafter referred to as ABR study). The six young F344 rats and the twelve middle-aged F344 rats were used for a salicylate study of age-related hearing loss (hereafter referred to as aging study).

### 2.2. Salicylate treatments and auditory functional assessments

Sodium salicylate (cat#: S3007, Sigma) was dissolved in saline at a concentration of 50 mg/ml. The animals were injected with the salicylate solution (i.p.) at different daily doses (200, 250, 300, or 350 mg/kg) and for different periods (2 or 3 weeks). DPOAE, ABR, and CAP were measured at different times (pre-, during, and post-exposure). Details are presented in Table-1.

**2.2.1. DPOAE measurement—**The subjects were initially anesthetized by inhalation of 4% isoflurane in $O_2$ at a flow rate of 1 L/minute and subsequently maintained at 1.5% isoflurane in $O_2$. The body temperature was maintained at 37°C using a homeothermic blanket (Harvard Apparatus). DPOAE were recorded using a Smart Distortion Product Otoacoustic Emission System (version 4.53, Intelligent Hearing System, Miami, FL). Recordings were performed in a sound attenuating chamber. The earpiece, containing a microphone (Etymotic10B+) and two sound delivery tubes, was inserted into the ear canal. Two high frequency transducers (IHS-3738, Intelligent Hearing System, Miami, FL) were used to deliver the primary tones, $F_1$ and $F_2$, to the ear canal via flexible tubes connected to the earpiece. $F_2$ was set at 4, 8, 12, 16, 20, and 32 kHz. The $F_2/F_1$ ratio was set at 1.2. The intensity of $F_2$ was varied from 15 to 60 dB SPL in 5-dB steps and the intensity of $F_1$ was 10 dB higher than that of $F_2$. The output of the microphone was fed to the input of the Smart DPOAE system, digitized and evaluated using the system software. At 4, 8, 12 and 16 kHz ($F_2$), the acoustic signal was sampled at a rate of 40 kHz over a period of 204 ms and averaged 32 times. The noise floor was measured in a 24 Hz band surrounding $2F_1$-$F_2$. At 20 and 32 kHz ($F_2$), the acoustic signal was sampled at a rate of 127 kHz over a period of 64 ms. The noise floor was measured in a 46.7 Hz band surrounding $2F_1$-$F_2$.

**2.2.2. Cochlear compound action potential (CAP)—**The CAP from the salicylate-treated rats was recorded prior to cochlear histology and compared to normal controls. The subjects were anesthetized by intramuscular (i.m.) injection with ketamine (50 mg/kg) and xylazine (6 mg/kg). The body temperature was maintained at 37°C using a homeothermic

blanket (Harvard Apparatus). The cochlear round window was exposed and a silver wire electrode was placed on the round window to collect the cochlear responses. A silver chloride reference electrode was placed beneath the neck skin. Tone burst signals at 2, 6, 8, 12, 16, 20, 24, 30, and 40 kHz for eliciting cochlear responses were generated by a real time processor (RP2.1, System 3, TDT, Gainesville, FL) and presented in a fixed starting phase. The signals (duration: 10 ms; rise/fall time: 1 ms) were amplified and delivered to a high frequency earphone (ACO 1/2″ microphone, 7013) placed within a speculum that opened to the ear drum. Stimulation intensities were controlled with a TDT PA5 programmable attenuator. Cochlear responses in a time window of 20 ms were amplified with a Grass A.C. preamplifier (Model P15, 1000x, 0.1 Hz – 50 kHz). The amplified cochlear responses were averaged 50 times in the TDT RP2.1 real time processor using the custom-written data acquisition software and stored on a disk in a personal computer. The CAP component was separated from the cochlear response off-line by low-pass filtering at 3 kHz. Vertical distance between N1 and P1 was measured as CAP amplitude.

**2.2.3. Auditory brainstem response (ABR)—**The subjects were initially anesthetized by inhalation of 4% isoflurane in $O_2$ at a flow rate of 1 L/minute and subsequently maintained at 1.5% isoflurane in $O_2$. The body temperature was maintained at 37°C using a homeothermic blanket (Harvard Apparatus). Alternating phase tone bursts (5-ms duration with 1-ms rise/fall time) at 4, 8, 12, 16, 20, and 32 kHz were generated using TDT SigGen software and presented at a rate of 21/sec. The stimuli were delivered to the left and right ears through a high frequency transducer. The transducer was calibrated with a sound level meter system 824 (Larson Davis Inc,) using a microphone (½″, model 2540). Needle electrodes were placed at the vertex (active), posterior bulla (reference) and behind the shoulder blade (ground). The responses were amplified 5000 times by a TDT Headstage-4 bio-amplifier (band-pass filter: 10–3000 Hz with a notch at 60 Hz). The responses were averaged 600 times. The vertical distance between P3 and N3 was measured as ABR amplitude (Brandt *et al.*, 2006).

### 2.3. Hair cell examination

Rats were decapitated after cochlear functional assessment and the cochleae were removed immediately and fixed in 10% buffered formalin over night. The basilar membranes with the organs of Corti were dissected out, and the specimens were stained with FITC-labeled phalloidin (5 μg/ml, P5282, Sigma) for 40 min at room temperature. After incubation in the FITC-labeled phalloidin, the specimens were stained again in a solution of propidium iodide (PI, #P-3566, Molecular Probes) at a concentration of 5 μg/ml for 10 min. The stained specimens were mounted on slides with ProLong Gold antifade reagent (P36934, Molecular probes). Hair cells were counted and plotted as a function of cochlear length (cochleogram).

### 2.4. Statistical analysis

To compare frequency - or intensity-dependent differences between groups, two-way ANOVAs were performed using GraphPad Prism software (version 5). Differences between two groups were compared using an independent samples t-test. A p-value $< 0.05$ was considered to be statistically significant.

# 3. Results

## 3.1. DPOAE

Figure 1 presents data showing the effects of chronic salicylate treatments on DPOAE in rats of different strains and ages. Six young adult SD rats participating in the ABR study were exposed to a long-duration, moderate dose of salicylate (200 mg/kg/day for 5 days per week for 3 weeks). DPOAE amplitudes were measured before and 4 weeks after the 3-week treatment to determine if long-term salicylate treatment had an effect on DPOAE. Figure 1A shows the DPOAE I/O function at 12 kHz ($F_2$). The 200 mg/kg salicylate treatment did not have a significant effect on the 12 kHz DPOAE I/O function ($p>0.05$ by two-way ANOVA); similar results were obtained at the other test frequencies (data not shown). Figure 1B presents DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) at different $F_2$ frequencies. The 200 mg/kg salicylate treatment did not induce a significant change at any of the test frequencies from 4 to 32 kHz at 4 weeks post-treatment ($p>0.05$ by two-way ANOVA and $p>0.05$ at each frequency by t-test).

To determine if there were strain effects, DPOAE were also measured in 3 young adult F344 rats (3 months) and 5 aged F344 rats (18 months) using the same SA-dose as above (200 mg/kg/day for 5 days per week for 3 weeks). Three young and six aged F344 rats were used as saline controls. Figure 1C presents DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) at different $F_2$ frequencies in the young F344 rats. Compared to the saline controls (opened and filled triangles), the salicylate treatment did not cause a significant change in DPOAE amplitude (opened and filled circles, $p>0.05$ by two-way ANOVA). Thus, the 200 mg/kg salicylate dose did not have an effect on DPOAE in young F344 rats. Figure 1D presents DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) at different F2 frequencies in the aged F344 rats. Similar to the results from young rats, the salicylate treatment did not cause a significant change in DPOAE amplitude in the aged F344 rats ($p>0.05$, two-way ANOVA). However, in the saline control group, a significant reduction in DPOAE amplitude was observed ($p<0.001$ by two-way ANOVA) probably reflecting an age-related change. Thus, salicylate treatment in the aged F344 rats did prevent the age-related decline in DPOAE amplitude.

To determine the effects of higher dose of salicylate on DPOAE in young rats, 8 SD rats were treated with a salicylate dose of 250 mg/kg/day (n=3), 300 mg/kg/day (n=3), or 350 mg/kg/day (n=2) for 4 days during the first week of treatment followed by 300 mg/kg/day in the rats for 4 days in the second week. DPOAE amplitudes were measured 2 hours after each salicylate injection and 24 hours after the last treatment in each week. DPOAE amplitudes were also measured 8 weeks after the 2-week treatment. Figure 1E presents DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) at 8, 12, and 16 kHz. For simplicity, the DPOAE data obtained with the 250, 300 and 350 mg/kg doses were averaged together during the first week of the study. During the first week of treatment, DPOAE amplitudes measured 2 h post-salicylate dropped below the pre-treatment baseline. However, they recovered or rebounded above pre-treatment baseline values by 1–2 dB 24 h after the last treatment in the first week (Figure 1E, Rest). During the second week of treatment with 300 mg/kg for 4 days, DPOAE measured 2 h post-salicylate dropped below pre-treatment

baseline. Twenty-four hours after the second week of treatment, DPOAE amplitudes were enhanced by approximately 4–5 dB compared to the pre-treatment. Importantly, the DPOAE amplitudes remained enhanced even 8 weeks after the last salicylate treatment. The differences were statistically significant ($p<0.05$ by t-test).

### 3.2. ABR

As noted above, salicylate injections at 200 mg/kg/day for 5 days per week for 3 weeks did not cause a permanent change in DPOAE in the 6 young SD rats (see Fig. 1A and B); however, this treatment caused an unexpected reduction in the amplitude of ABR. Figure 2A presents ABR amplitudes at 12 kHz as a function of stimulus intensity. Baseline ABR amplitude increased from less than 1 μV near 30 dB SPL to approximately 6.8 μV at 100 dB SPL. ABR amplitudes measured 3 days, 2 weeks and 4 weeks post-salicylate were smaller than the normal values. The ABR amplitude reduction was greatest at high intensities and decreased with level. ABR amplitude showed no recovery between 3 days and 4 weeks post-treatment suggesting that the effects were permanent. Figure 2B shows the ABR amplitudes as a function of frequency at 100 dB SPL at baseline and 3 days, 2 weeks and 4 weeks post-salicylate. ABR amplitudes post-salicylate were significantly smaller than baseline ($p<0.0001$ by two-way ANOVA); the amplitude reduction was greatest at 4 and 8 kHz and declined at higher frequencies.

### 3.3. CAP

Figure 3A and B present CAP amplitudes measured in the 6 SD rats treated with salicylate at a dose of 200 mg/kg/days for 5 days per week for 3 weeks (filled circles) (ABR data from these animals presented in Figure 2). Figure 3A shows the CAP I/O function measured at 16 kHz in the 6 salicylate-treated SD rats versus 6 control SD rats (age-matched); CAP amplitudes in the salicylate-treated group (filled circles) were significantly smaller than those in the control group (opened circles, $p<0.001$ by two-way ANOVA). Figure 3B shows the CAP amplitudes at 70 dB SPL as a function of frequency. CAP amplitudes in the salicylate-treated animals were lower than those in age-matched SD control rats at most frequencies; ($p<0.05$ by two-way ANOVA).

To identify potential strain or age effects, CAP measurements were obtained from 3 young (3 months) and 6 aged (18 months) F344 rats that received the same treatment as the SD rats (200 mg/kg/day for 5 days per week for 3 weeks). Figure 3C shows the CAP amplitudes measured at 70 dB SPL from 2 kHz to 40 kHz; measurements were made 4 weeks post-treatment. CAP amplitudes in the young F344 rats treated with salicylate were smaller than those in the saline control group ($n=3$) at every frequency; CAP amplitudes in the young, salicylate-treated F344 rats (Fig. 3C) were statistically smaller than those in the saline control group ($p<0.0001$ by two-way ANOVA). Figure 3D shows the CAP amplitudes in aged rats measured at 70 dB SPL from 2 kHz to 40 kHz at 4 weeks post-treatment. CAP amplitudes in the aged, salicylate-treated F344 rats were generally smaller than those in the saline control group except at 40 kHz; however, the differences did not reach statistical significance in the aged F344 rats ($p=0.07$ by two-way ANOVA, Fig. 3D).

To examine the effects of higher doses of salicylate, CAP were also measured in the 8 SD rats used in Figure 1E. Figure 3E presents CAP amplitudes at 8, 20, and 40 kHz measured at 90 dB SPL; the data are plotted as a function of the total amount of salicylate that each animal received. CAP amplitude showed a noticeable decline when the total dose of salicylate exceeded a critical level around 2000 mg/kg.

### 3.4. Hair Cells

To determine if chronic salicylate treatment had an effect on hair cell survival, the cochleae were removed and OHC and IHC evaluated. Occasional OHC loss was observed, but there was no IHC loss in any of the animals studied. Figure 4 presents mean OHC losses in both young F344 rats (n=3) and SD rats (n=5) exposed to salicylate at a dose of 200 mg/kg/day for 5 days per week for 3 weeks. The average loss of OHCs was less than 1%. Hair cells in the aged rats were not counted.

## 4. Discussion

Our results show that DPOAE amplitudes are initially depressed after treatment with a high dose of salicylate (Figure 1E) consistent with previous studies (Guitton *et al.*, 2003; Huang *et al.*, 2005). The temporary reduction of DPOAE amplitude presumably occurs because salicylate competitively inhibits the binding of chloride at its anion-binding site on prestin thereby suppressing OHC electromotility (Oliver *et al.*, 2001). The reversible loss of OHC electromotility presumably accounts for the temporary elevation of threshold that occurs during the first 8–12 hours following salicylate treatment.

Twenty-four hours after discontinuing our high-dose (300 mg/kg/d), long-term salicylate treatment, DPOAE amplitudes were enhanced 3–5 dB for up to 8 weeks post-treatment consistent with previous studies (Huang *et al.*, 2005) (Figure 1E). This post-treatment increase in DPOAE amplitude has been associated with increased expression of the motor protein, prestin, in outer hair cells (Yang et al., 2009; Yu et al., 2008). Although the long-term high-dose salicylate treatment increased DPOAE amplitudes, it had the opposite effect on neural activity reducing CAP or ABR amplitudes; this amplitude reduction results in a slight increase in neural thresholds (Figure 2A, 3A).

The current study confirmed the enhancement of DPOAE after a chronic salicylate exposure; however, auditory sensitivity was not improved despite the fact that DPOAE amplitude was increased 3–5 dB. In fact, the chronic salicylate treatment caused a permanent reduction in CAP and ABR amplitude during the time when OHC motility was enhanced. Interestingly, our preliminary study showed that when a high-dose salicylate (250 mg/kg) was injected with an increased interval between injections (twice or once per week), the DPOAE enhancement was observed without a salicylate-related reduction of ABR threshold (data not shown). Thus, an optimized salicylate-treatment could conceivably be found, which enhances both DPOAE and auditory sensitivity.

Two major new findings emerged unexpectedly from this study. First, salicylate treatment for 15 days with 200 mg/kg/d significantly reduced CAP amplitudes in young F344 and SD rats (Figure 3A–C); CAP amplitudes were also reduced in aged F344 rats, but to a lesser

degree (Figure 3D). Second, young SD rats treated for 15 days with 200 mg/kg/d showed a significant reduction in ABR amplitude. Both the CAP and ABR amplitude reductions were observed up to 4 weeks post-treatment suggesting that these neurophysiological deficits were permanent. The CAP and ABR amplitude reductions are most likely neural in origin, including the spiral ganglion neuron (SGN) and the synapse at the IHC, because (1) there was no evidence of hair cell loss and (2) there was no long-term reduction in DPOAE amplitude. The persistent reductions in CAP and ABR amplitude run counter to the long held view that salicylate ototoxicity is temporary and that the auditory function recovers completely after salicylate treatment is discontinued. This raises questions as to why these neurophysiological deficits have not been reported previously. First, most laboratory studies of salicylate ototoxicity involve acute treatments that produce changes that are most likely completely reversible (Bernstein et al., 1967; Didier et al., 1993; Jastreboff et al., 1988a; McFadden et al., 1984a; McFadden et al., 1984b; Myers et al., 1965a). Second, most behavioral and neural measures of salicylate ototoxicity rely on threshold as the primary indicator of impairment (Bancroft et al., 1991; Cazals, 2000; Day et al., 1989; Wecker et al., 2004). Inspection of the pre- and post-treatment ABR (Figure 2A) and CAP (Figure 3A) input/output functions show that the salicylate-induced threshold shifts to be rather small, on the order of 5–10 dB, and therefore insignificant. Third, the reductions in CAP amplitude (Figure 3) are suggestive of auditory nerve fiber or spiral ganglion dysfunction. Auditory nerve fiber dysfunctions do not have a significant effect on behavioral thresholds; the gold standard in clinical assessment (Butinar et al., 2000; Schuknecht, 1994; Schuknecht et al., 1971; Starr et al., 1996). Therefore, prolonged high-dose treatment with salicylate would likely have little effect on audiometric thresholds.

The reductions in CAP amplitude suggest that high-dose long-term treatment with salicylate may damage the neurites, soma or axons of spiral ganglion neurons (SGN). Previous in vitro studies with cochlear organotypic cultures indicate that high doses of salicylate damage the spiral ganglion neurites, but not hair cells (Gao, 1999). In more recent work, we have shown that 1.4 mM of sodium salicylate causes significant loss of SGN in cochlear cultures; cell loss is preceded by soma shrinkage, a morphological feature of apoptosis, and caspase activation (Wei, 2009). The 1.4 mM dose used in our culture is clinically relevant since it is similar to the concentration seen in the cerebrospinal fluid and perilymph of animals that develop salicylate-induced tinnitus and hearing loss (Jastreboff *et al.*, 1986; Jastreboff *et al.*, 1988b). Our results plus previous in vitro studies suggest that further studies are needed to determine if high-dose, long term salicylate treatment damages SGN. Damage is likely to develop slowly over weeks or months of treatment and may be difficult to detect during the early stage of the pathology, as it is in noise-induced auditory nerve damage (Kujawa et al., 2009).

For pain relief in clinic, aspirin daily dosage up to a few grams is usually used (Laska et al., 1982; Skjelbred, 1984; Thomas et al., 2002; Tigerstedt et al., 1981). In this study in animals, permanent auditory functional loss was observed in rats receiving salicylate injection at a daily dose of 200 mg/kg and higher doses. The functional loss at lower salicylate doses was not measured in this study.

Salicylate has antioxidant properties and has been used to protect cells from oxidative stress (Althaus et al., 1993; Kojda, 2004; Speir et al., 1998; Yiannakopoulou et al., 2009). Several studies have found that salicylate protects against ototoxicity and noise-induced hearing loss (Chen et al., 2007; Kopke et al., 2000; Li et al., 2002; Sha et al., 1999; Sha et al., 2006; Yamashita et al., 2005). In contrast, others have reported that salicylate fails to protect against noise-induced hearing loss (Lambert *et al.*, 1986; Spongr *et al.*, 1992) or that salicylate exacerbates temporary noise-induced hearing loss in humans (McFadden et al., 1984b).

High doses of salicylate can act as a pro-oxidant that promotes cell death. Salicylate enhances the production of reactive oxygen species (ROS) in C6 glioma cells (Seo *et al.*, 2005) and induces apoptosis in a variety of human cancers (Bellosillo *et al.*, 1998; Oh *et al.*, 2005). Salicylate-induced apoptosis in cancer cells is mediated by the activation of p38 mitogen-activated protein kinases leading to the activation of caspase 3 and lowering the threshold for mitochondrial pore transition both of which promote programmed cell death (Lee et al., 2003; Oh et al., 2003; Schwenger et al., 1997). Lastly, salicylate enhances NMDA receptor currents on SGN that innervate IHC; sustained activation of these receptors from prolonged salicylate treatment could result in glutamate excitotoxicity of SGN (Ruel *et al.*, 2008).

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## Abbreviations

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| **ABR** | auditory brainstem response |
| **CAP** | compound action potential |
| **DPOAE** | distortion product otoacoustic emission |
| **F344** | Fischer/HNsd 344 rat |
| **IHC** | inner hair cell |
| **I/O** | input/output |
| **OHC** | outer hair cell |
| **ROS** | reactive oxygen species |
| **SD** | Sprague–Dawley |
| **SGN** | spiral ganglion neuron |

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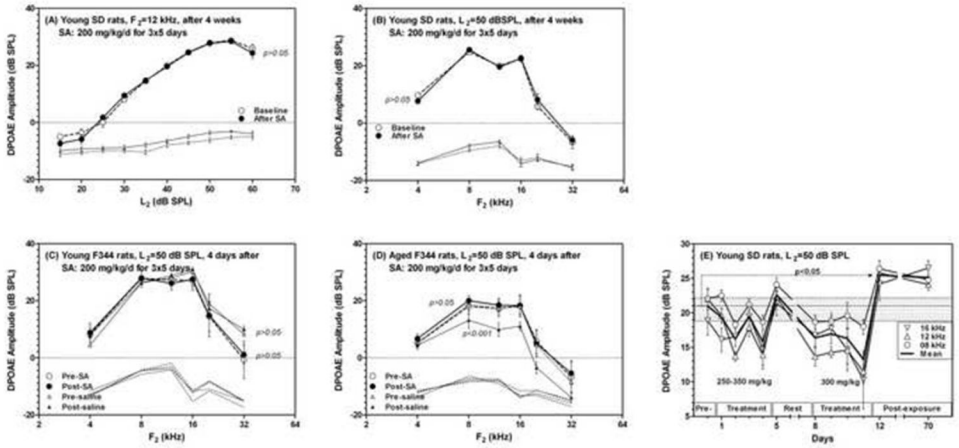


**Fig. 1. The effects of a chronic salicylate treatment on DPOAE in rats of different strain and age**

(A): DPOAE amplitudes as a function of stimulation intensity ($L_2$) at 12 kHz ($F_2$) in a group of young adult Sprague–Dawley (SD) rats (3 months, n=6). Salicylate treatment: 200 mg/kg/day for 5 days per week for 3 weeks; DPOAE recording: prior to and 4 weeks after the 3-week salicylate treatment;

(B) DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) as a function of frequency ($F_2$) in the same animals as in (A);

(C): DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) as a function of frequency ($F_2$) in the young Fischer 344 (F344) rats (3 months) receiving salicylate treatment (n=3) or saline injections (n=3). Salicylate treatment was the same as in (A); DPOAE recording: prior to and 4 days after the 3-week treatment;

(D): DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) as a function of frequency ($F_2$) in the aged F344 rats (18 months) receiving salicylate treatment (n=6) or saline injections (n=6). Salicylate treatment was the same as in (A); DPOAE recording was the same as in (C);

(E): DPOAE amplitudes at a stimulation level of 50 dB SPL ($L_2$) at 8, 12, and 16 kHz ($F_2$) as a function of time in a group of young adult SD rats (3 months, n=8). In the first week, three of them were treated with salicylate at 250 mg/kg/day, three at 300 mg/kg/day, and two at 350 mg/kg/day for 4 days and in the second week, all the animals received salicylate injection at 300 mg/kg/day for 4 days.

The vertical bars represent standard errors (SE).

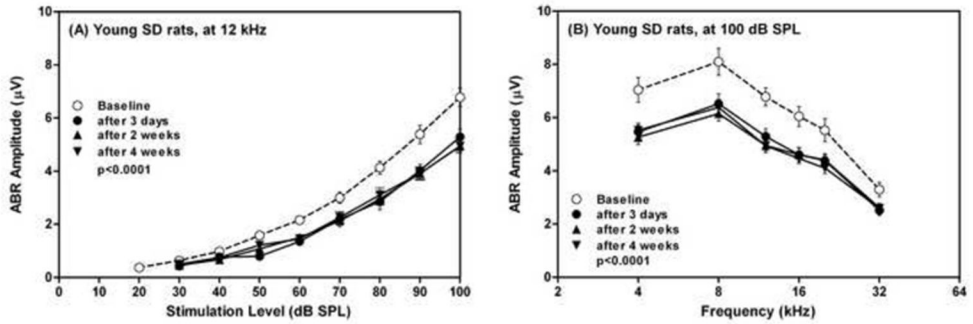


**Fig. 2.**

The permanent effects of the chronic salicylate treatment on ABRs in a group of young adult SD rats (n=6). (A): ABR amplitudes as a function of stimulation level at 12 kHz; (B): ABR amplitudes at a stimulation level of 100 dB SPL as a function of frequency. Salicylate treatment: 200 mg/kg/day for 5 days per week for 3 weeks; ABR amplitude: N3-P3; ABR was measured prior to, 3 days, 2 weeks, and 4 weeks after the 3-week salicylate treatment. The vertical bars represent SE.

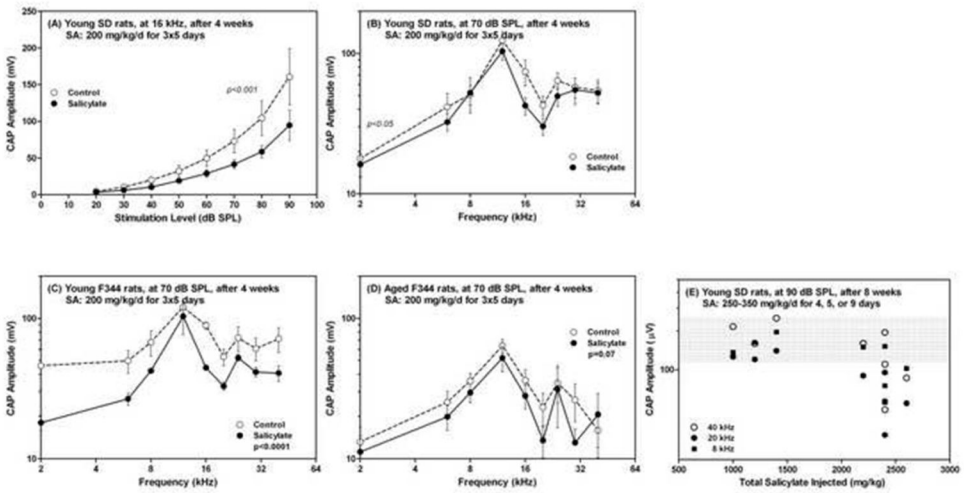


**Fig. 3. The effects of the chronic salicylate treatment on CAP in rats of different strain and age**

(A): CAP amplitudes as a function of stimulation level at 16 kHz in the young adult SD rats (3 months, n=6 in each group). Salicylate treatment: 200 mg/kg/day for 5 days per week for 3 weeks; CAP recording: 4 weeks after the last salicylate treatment;

(B) CAP amplitudes at a stimulation level of 70 dB SPL as a function of frequency in the same animals as in (A);

(C): CAP amplitudes at a stimulation level of 70 dB SPL as a function of frequency in the young F344 rats (3 months, n=3 in each group). Salicylate treatment and CAP recording were the same as in (A);

(D): CAP amplitudes at a stimulation level of 70 dB SPL as a function of frequency in the aged F344 rats (18 months, n=6 in each group). Salicylate treatment and CAP recording were the same as in (A);

(E): CAP amplitudes at a stimulation level of 90 dB SPL at 8, 20, and 40 kHz as a function of total salicylate injection (mg/kg) in a group of young adult SD rats (3 months, n=8) during 2 weeks. The vertical bars represent SE.

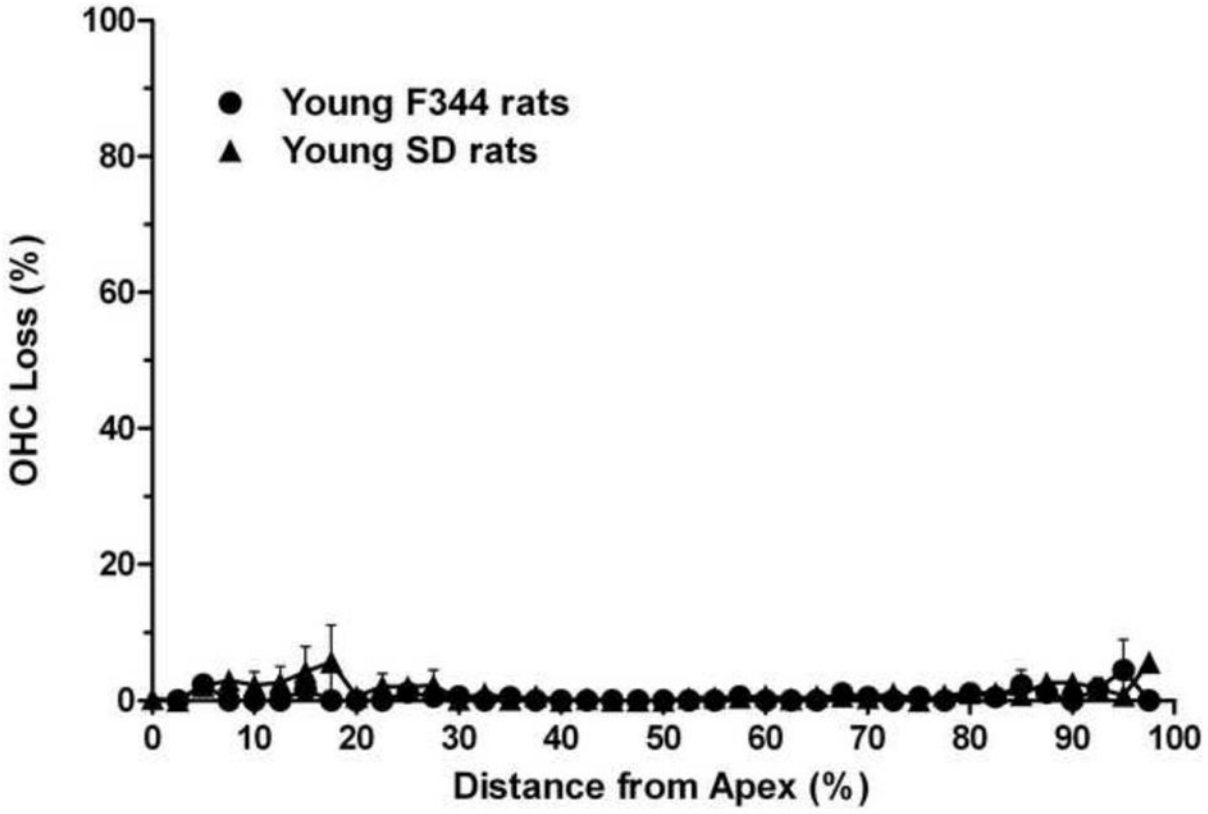


**Fig. 4.**

Cochleogram in rats chronically exposed to salicylate (200 mg/kg/day for 5 days per week for 3 weeks). The vertical bars represent SE.

**Table 1**

Salicylate (SA) treatments and functional measurements

| Treatment | DPOAE | ABR | CAP | Rats | Saline Ctrl |
|---|---|---|---|---|---|
| T1 | pre- & 4w-post | pre-, 3d-, 2w-and 4w-post | 4w-post | 6 young SD | 6 young SD |
| T1 | pre- & 4d-post | | 4w-post | 3 young F344 | 3 young F344 |
| T1 | pre- & 4d-post | | 4w-post | 6 aged F344 | 6 aged F344 |
| T2 | R | | 8w-post | 8 young SD | |

T1: 200 mg SA per kg per day (or saline) for 5 days per week for 3 weeks

T2: 250 or 300 or 350 mg SA per kg per day for 4 days in the first week and 300 mg salicylate per kg per day for 4 days in the second week

R: pre-, 2h after each SA-injection, 24h after each week of treatment and 8 weeks after the last SA-injection