NeuroToxicology 55 (2016) 65–73

Contents lists available at ScienceDirect

NeuroToxicology

Full length article

# Effect of manganese and manganese plus noise on auditory function and cochlear structures

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ARTICLE INFO

*Article history:*
Received 18 March 2016
Received in revised form 20 May 2016
Accepted 20 May 2016
Available online 24 May 2016

*Keywords:*
Manganese
Ototoxicity
Hearing
Cochlea
Noise exposure
Rats

ABSTRACT

The degenerative actions of Mn caused by persistent exposure to high atmospheric levels not only provokes irreversible damage to the CNS with symptoms comparable to that of Parkinson's disease but also may have deleterious consequences to other organs including the auditory system. The putative deleterious consequences of prolonged Mn overexposure on hearing, however, is confounded by the fact that chronically-exposed individuals often work in high noise environments where noise by itself is known to cause hearing loss. Thus, the question as to whether Mn alone is actually ototoxic and whether exposure to Mn when combined with noise increases the risk of hearing loss and cochlear pathology has never been examined. To examine whether noise effects Mn ototoxicity, we exposed rats to a moderate dose of Mn (10 mg $MnCl_2$/liter water) alone, a high level of noise (octave band noise, 8–16 kHz, presented at 90 dB SPL for 8 h/d) alone or the combination of Mn plus noise and measured the changes in auditory function and the cochlear histopathologies. Results of these studies, based on various measures of hearing including histological examination of cochlear tissue suggest that noise alone produced significant hearing deficits whereas semi-chronic exposure to moderate levels of Mn in drinking water for 90 days either in the presence or absence of noise had, at best, only a minor effect on hearing.


## 1. Introduction

Although manganese (Mn) is an essential transition metal required for normal health, high atmospheric levels in various occupational settings can lead to a syndrome called manganism (Racette, 2014; Roth, 2006). Initial neurological symptoms consist of reduced response speed, irritability, intellectual deficits, mood changes, and compulsive behaviors whereas prolonged exposures lead to more severe deficits within the extrapyramidal system that includes dystonic movements associated with Parkinson's disease, a masklike face, limb rigidity, mild tremors, gait disturbance, slurred speech, excessive salivation, sweating and a marked disturbance of balance. Other patients with chronic liver disease display increased Mn level in serum and brain, as well as behavioral deficits and neurodegenerative features resembling those seen in Mn-exposed workers because the liver is the major organ responsible for its elimination. The behavioral components, also seen upon Mn intoxication, suggest that Mn may affect a variety of neurotransmitter systems in the central nervous system (CNS) outside of its putative target, the globus pallidus (Bowler et al., 2006; Roels et al., 2012). Mn overexposure can lead to disturbances in dopaminergic (Higashi et al., 2004; Peneder et al., 2011; Roth et al., 2013), glutamatergic (Erikson and Aschner, 2002; Erikson and Aschner, 2003; Guilarte and Chen, 2007; Roth et al., 2012), GABAergic (Anderson et al., 2008; Burton et al., 2009; Erikson and Aschner, 2003) and cholinergic systems (Finkelstein et al., 2007) in the CNS. Although the neurobehavioral complications associated with chronic Mn exposure were thought to be reversible, more recent studies indicate that some symptoms are permanent (e.g., neuromuscular function, cognitive flexibility, and adverse mood states (Bowler et al., 2011; Roels et al., 1999).

The degenerative effects associated with persistent Mn exposure may have deleterious consequences to other organs including the auditory system (Antonini et al., 2003; Antonini et al., 2009; Da Silva et al., 2007; Ding et al., 2011; Josephs et al., 2005; Khalkova and Kostadinova, 1986; Korczynski, 2000; Zeidler-Erdely et al., 2011). However, the putative deleterious consequences of prolonged Mn overexposure is confounded by

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http://dx.doi.org/10.1016/j.neuro.2016.05.014
0161-813X/

the fact that chronically-exposed individuals often work in high noise environments where noise by itself is known to cause hearing loss. For example, several studies have reported that both welders and miners, who are exposed to high levels of Mn and noise, acquire irreversible hearing impairment (Da Silva et al., 2007; Gratton et al., 1990; Josephs et al., 2005, Khalkova and Kostadinova, 1986; Korczynski, 2000; Park et al., 2006). Since noise by itself can cause hearing loss the key question is whether the hearing loss reported in these complex environments is caused by Mn alone, noise alone or the combination of these two agent. This issue is critical important because previous studies have shown that noise exposure exacerbates hearing loss and cochlear pathology induced by a variety ototoxic agents such as aminoglycoside antibiotics, cisplatin, carbon monoxide, carbon disulfide, toluene and heavy metals including lead, mercury, cadmium, and arsenic (Brown et al., 1978; Counter and Buchanan, 2002; Gratton et al., 1990; Hwang et al., 2009; Prasher, 2009). Our *in vitro* studies with postnatal cochlear organotypic cultures (Ding et al., 2011) clearly indicate that μM levels of Mn can damage hair cells and spiral ganglion neurons (SGN). *In vitro*, Mn induced an atypical pattern of damage; SGN were more vulnerable to Mn than hair cells and among the two hair cell populations, inner hair cell (IHC) damage was greater than outer hair cells (OHC). A recent paper (Mirzaee et al., 2007) measured the effect of welding fumes and noise on the function of OHCs in rabbits by examining DPOAE. Rabbits exposed to noise at 110 dB SPL for 8 h/day for 12 days showed significant reductions in DPOAE amplitudes which were further reduced when combined with inhalation of welding fumes that contained high level of Mn plus other metals and gases. These findings suggest that welding fumes, containing a mixture of metals and gases, have the potential to exacerbate noise-induced hearing loss. Unfortunately, no histological measurements were performed to characterize the damage to hair cells and SGN.

Mn can be taken up from the bloodstream and into tissues by metal transporters such as DMT1, ZIP8 and ZIP14; these metal transporters have been identified in the inner ear by quantitative RTPCR and immunohistochemistry (Ding et al., 2014; Ma et al., 2008). Mn levels in the whole otic capsules including the surrounding bone increased roughly 10 fold after intraperitoneal administration of $MnCl_2$ every other day for three days. Mn levels were still four fold greater than controls 14 d after treatment was discontinued. Mn can also be ingested from food and dietary supplements and chronic treatment could increase the levels of Mn in the brain and cochlea. By carefully dissecting out separate regions of the cochlea, we found that Mn concentrations in the basilar membrane, stria vascularis and modiolus of the cochlea increased by 73%, 62% and 27% respectively when rats were fed $MnCl_2$ in drinking water (10 mg/ml) for 30 d (Mullin et al., 2015). Similar increases in Mn were seen in these three subdivisions of the cochlea when the duration of $MnCl_2$ treatment was extended to 60 d suggesting that Mn levels had reached a plateaus after 30 days (Mullin et al., 2015). Mn levels were also measured in the inferior colliculus, located in the auditory midbrain, the striatum and globus pallidus. With the exception of the GP, there was a significant increase in Mn levels in all brain areas in animals treated with Mn for both the 30 and 60 days.

Taken together, these *in vitro* and *in vivo* studies suggest that exogenous Mn accumulates in the cochlea; that Mn alone may be ototoxic and that combined exposure to Mn plus noise increases the risk of hearing loss and cochlear pathology over that caused by noise alone. To test these hypotheses, we exposed rats to drinking water containing a high dose of Mn alone, a high level of noise alone or the combination of Mn plus noise and measured the changes in auditory function and the cochlear histopathologies.

## 2. Materials and methods

### 2.1. Animals

The twenty-four male Sprague Dawley rats (2 months of age, Charles River Laboratories Inc.) used in this study were housed in the Laboratory Animal Facility (LAF) at the University at Buffalo and given free access to food and drinking water (see details of water during experimental treatment described below). The colony room was maintained at 22 °C with a 12-h light-dark cycle. All procedures used in this project were approved by the Institutional Animal Care and Use Committee (HER05080Y) at the University at Buffalo and carried out in accordance with NIH guidelines.

### 2.2. Mn treatment and noise exposure

The rats were randomly divided into 4 groups (n = 6 per group). Each animal in the four groups was housed in a separate cage during the experiment in order to monitor the water and Mn intake. The rats in the control group (Ctrl) were provided with flavored water (1 g unsweetened Kool-Aid plus 1.5 g saccharin per liter water) ad libitum for 90 d in their home cage in a conventional room in the animal facility. Rats in the Mn group (Mn) were supplied ad libitum for 90 d with the same flavored drinking water as above that also contained 10 mg $MnCl_2$/liter water similar to the procedure of Aliva et al. (Avila et al., 2008). The rats in Mn group were housed in the same room as the Ctrl group. The daily water intake and body weight of each animal were measured every second day. From these measures, we calculated the amount of $MnCl_2$ consumed by each animal over the course of the 90-d treatment. The rats in the noise group (Noise) were exposed to an octave band noise (8–16 kHz) presented at 90 dB SPL for 8 h/d (9:00am – 5:00pm) for 90 d; these Noise group rats received the same flavored water as the Ctrl group. Each rat was noise exposed in its home cage; the noise exposure took place in a separate room in the animal facility. A calibrated loudspeaker (Fostex, FT28 1–50 kHz) was mounted above each cage. The rats in the Mn + Noise group were treated for 90 days with Mn (as above) plus Noise (as above). Water intake and body weight were measured every other day and used to compute the daily $MnCl_2$ intake (as above).

### 2.3. Noise exposure

The noise (8–16 kHz) was generated using a TDT RP2 real time signal processor (TDT, Gainesville, FL), amplified and delivered to a loudspeaker (Vifa D25AG35, Madisound Speaker Components) mounted 8.9 cm above top of each cage. The noise levels in each cage (L = 48.3, W = 25.4, H = 20.3 cm) were measured at the center of the cage directly below the speaker approximately 7.6 cm above the cage floor (*i.e.*, level of the animal's ears); sound levels near the perimeter of the cage were 1–2 dB lower. The sound level was measured with a sound level meter (Larson Davis System 824) equipped with half-inch, free-field condenser microphone (model 2540, Larson Davis).

### 2.4. Distortion product otoacoustic emissions (DPOAE)

Approximately six weeks after termination of the 90-d treatments, DPOAE ($2F_1$–$F_2$) were measured by using the Smart Distortion Product Otoacoustic Emission System (Intelligent Hearing System, version 4.53). The animals were initially anesthetized by inhalation of 4% isoflurane in oxygen at a flow rate of 0.6 l/min and subsequently maintained at 1.5% isoflurane. The earpiece containing a microphone (Etymotic10B+) and two sound delivery tubes was inserted into the ear canal. Two IHS-3738

high frequency transducers (Intelligent Hearing System, Miami, FL, USA) were used to deliver primary tones ($F_1$ and $F_2$) to the ear canal *via* flexible tubes connected to the earpiece. The $F_2/F_1$ ratio was set at 1.2 and the intensity of $F_2$ ($L_2$) was 10 dB lower than the intensity of $F_1$ ($L_1$). The output of the microphone was fed to the input of the Smart DPOAE system, digitized and evaluated using system software. At F2 frequencies of 4, 8, 12, and 16 kHz, the output of the microphone was sampled at 40 kHz over a period of 204 ms; the spectrum of each sweep was computed and averaged over 32 repetitions. The noise floor was measured in a 24 Hz band surrounding $2F_1–F_2$. At F2 frequencies of 24 and 30 kHz, the output of the microphone was sampled at 127 kHz over a period of 64 ms; the spectrum of each sweep was computed and averaged over 32 repetitions. The noise floor was measured in a 46.7 Hz band surrounding $2F_1–F_2$. DPOAE amplitudes at $F_2$ frequencies of 4, 6, 8, 12, 16, 24, 30, and 35 kHz were plotted as a function of $L_2$ intensity (DP I/O function). The $L_2$ was varied from 25 to 70 dB SPL in 5-dB steps. A DP-gram was obtained by plotting the DPOAE amplitude at an $L_2$ of 55 dB SPL as a function of frequency (DP-gram).

### 2.5. Auditory brainstem response (ABR)

The ABR was assessed approximately six week after the 90-d treatment. Acoustic stimuli for the ABR were generated with TDT hardware (TDT RP2.1, System 3, TDT PA5 attenuator, TDT SA1 amplifier) as described previously (Chen et al., 2014, 2010). Tone bursts were synthesized with TDT SigGen software (5-ms duration, 1-ms rise/fall time, cosine$^2$-gated, alternating phase, 21/s) at 4, 8, 12, 16, 20, 24 and 36 kHz and presented through a high frequency transducer (FT28D, Foster) calibrated with a sound level meter (Larson Davis System 824) and half-inch microphone (model 2540, Larson Davis). Rats were anaesthetized with ketamine and xylazine (50 and 6 mg/kg respectively, i.p.) and body temperature maintained at 37 °C using a homeothermic blanket. Needle electrodes (Grass Technologies) were placed at the vertex (active), posterior bulla (reference) and behind the shoulder blade (ground) and the signals from the electrodes were recorded (25 kHz) over 10 ms from stimulation onset, amplified 5020X by a TDT Headstage-4 bio-amplifier, filtered (10–3000 Hz and 60 Hz notch filter) and averaged 200 times. At each frequency, the sound level was decreased in 10 dB steps from 100 dB SPL to 0 dB SPL. As previously reported, the ABR threshold was defined as the lowest level that produced a just detectable and noticeable ABR response (Chen, Decker, 2014).

### 2.6. Compound action potential (CAP)

After completing the DPOAE and ABR measurements, the CAP was measured. As described previously, the cochlear round window was surgically exposed under ketamine/xylazine anesthesia (as above) and a silver wire electrode was placed on the round window membrane to record the CAP (Chen et al., 2010, 2013). Tone bursts at 4, 8, 12, 16, 20, 24 and 35 kHz (10 ms of duration, 1 ms rise/fall time, fixed starting phase) were generated with TDT hardware (TDT RP2 signal processor), amplified, attenuated (TDT PA5) and delivered to a high frequency earphone (ACO 1/2″ microphone, 7013) located in a speculum in the ear canal in front of the tympanic membrane. Sound levels were calibrated in a cavity approximating the volume of the ear canal using a sound level meter (Larson Davis System 824) equipped with a half inch microphone (model 2540, Larson Davis). The cochlear responses were filtered (0.1 Hz to 3 kHz), amplified (1000X, WPI), digitized and averaged (50 kHz, 20 ms, n = 50) using custom data acquisition and analysis software. CAP $N_1$ amplitudes were measured offline at each frequency from 0 to 80 dB SPL (10 dB steps).

### 2.7. Cochleograms

After completing the CAP measurements, the anesthetized (ketamine/xylazine) rats were decapitated and the cochleae quickly removed and prepared for histological analysis as described in our previous publications (Jamesdaniel et al., 2011; Kane et al., 2012; Newman et al., 2015). The round and oval windows were opened and 10% buffered formalin carefully perfused through the cochlea. The cochlea was subsequently infused with Harris' hematoxylin staining solution for 5 min and afterwards the basilar membrane was carefully removed and mounted as a flat surface preparation in glycerin on a glass slide. The surface preparations were evaluated using a light microscope (Zeiss Standard, 400X). Missing IHC and OHC were counted over 0.24 mm intervals along the entire length of the cochlea and data were used to construct a cochleogram showing the percent missing IHC and OHC as a function of the percent distance from the apex of the cochlea. Cochlear location was related to frequency using a rat frequency-place map (Müller et al., 2005).

## 3. Results

### 3.1. Mn intake

Water and $MnCl_2$ intake was measured every other day during the course of the experimental treatment. Mean daily (n = 6/group) intake of flavored water over the entire treatment period ranged from 23.1-26.9 ml/d in the Ctl, Mn, Noise and Mn + Noise groups (Fig. 1A); there was no significant differences in water intake between groups (One-ANOVA, F = 1.22; 3, 16 DF, p > 0.05). Based on fluid intake and the concentration of $MnCl_2$ in the flavored water, the mean daily intake of $MnCl_2$ in the Mn and the Mn + Noise group ranged from approximately 260–310 mg/d; the fluid intake remained fairly stable over the 90 d treatment period (Fig. 1B). The average total intake of $MnCl_2$ over the 90 d treatment period was 26,380 mg (11,724 as Mn) in the Mn group and 24,422 mg (10,854 as Mn) in the Mn + Noise group; the total intake in the Mn group was not significantly different from the Mn + Noise group

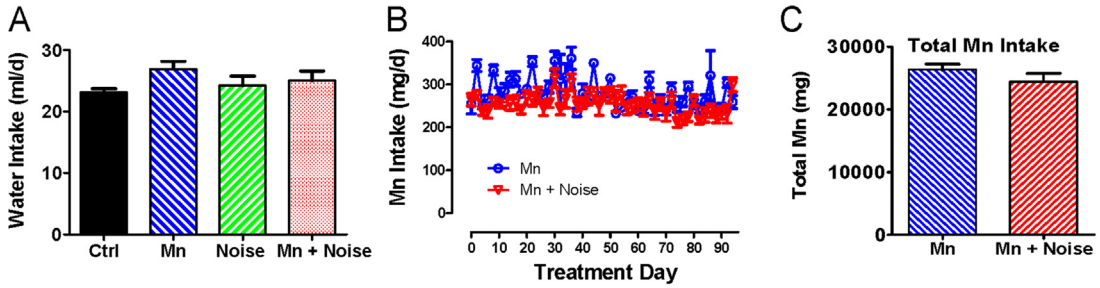


**Fig. 1.** (A) Mean (± SEM) daily water intake in the Ctrl, Mn, Noise and Mn + Noise groups. (B) Mean (± SEM) daily Mn intake (mg/d) in the Mn and Mn + Noise group over the course of the treatment period. (C) Mean (± SEM) total intake of Mn in the Mn and Mn + Noise groups over the entire treatment period.

(Student *t*-test, t = 1.241, 10 DF, p > 0.05). Since intestinal uptake of Mn is at best 5% of that ingested, the maximum uptake of Mn which was actually transported ranged between 542 mg and 586 mg of Mn or approximately 6 mg/day over a 90-day period. For the adult human male, 2.3 mg of Mn is the recommended daily dose or 0.115 mg taken up based on the absorption rate of 5%. Thus, based on these human values, for a rat weighing 250 g, 6 mg represents approximately 52 times the recommended daily dose.

### 3.2. DPOAE

DPOAE, which reflect the functional status of the OHC together with the endolymphatic potential, were assessed approximately six-weeks post-treatment to determine if any of the treatment caused permanent changes in otoacoustic emissions. Fig. 2 shows the DPOAE I/O functions at F2 frequencies of 12, 16, 24 and 30 kHz, frequencies within and above the noise exposure and therefore most likely to show an effect. DPOAE amplitudes in the Mn group and Ctrl group were nearly the same suggesting that Mn alone had no effect on the organ of Corti. In contrast, DPOAE amplitudes in the Noise group and the Mn + Noise group were consistently lower than those in the Ctrl group and Mn group. However, DPOAE amplitudes in the Mn + Noise group were similar to (e.g., 24 kHz) or slightly lower (e.g., 16 and 30 kHz) than those in the Noise group.

A statistical analysis using a two-way, repeated measure ANOVA showed that at 12 kHz (Fig. 2A) there was a significant effect of treatment ($F = 5.52$; 3, 171 DF, $p < 0.0067$), L1 intensity ($F = 129.6$; 9, 171 DF; $p < 0.0001$) and an intensity x treatment interaction ($F = 3.415$; 27, 171 DF; $p < 0.001$). A Bonferroni *post-hoc* analysis indicated that: (1) DPOAE amplitudes in the Noise group were significantly less ($p < 0.05$) than the Ctrl group from 65 to 75 dB SPL, (2) DPOAE amplitude in the Mn + Noise group were significantly less than the Ctrl group from 50 to 75 dB SPL, (3) DPOAE amplitudes in the Noise group were less than Mn group only at 65 and 75 dB SPL and (4) DPOAE amplitudes in the Mn + Noise group were significantly less than the Mn group from 50 to 75 dB SPL. The Mn group was not significantly different from the Ctrl group (*i.e.*, no effect of Mn) and the Mn + Noise group was not significantly different from the Noise group (*i.e.*, Mn did not exacerbate the effect of noise).

At 16 kHz (Fig. 2B), the analysis indicated that there was a significant effect of treatment ($F = 5.54$; 3, 171 DF; $p < 0.0074$) and L1 intensity ($F = 129.6$; 9, 171 DF; $p < 0.0001$). A Bonferroni *post-hoc* analysis indicated that at 16 kHz: (1) DPOAE amplitudes in the Mn + Noise group were significantly less ($p < 0.05$) than the Ctrl group from 40 to 70 dB SPL, (2) DPOAE amplitude in the Mn + Noise group were significantly less than the Ctrl group from 50 to 75 dB SPL and (3) DPOAE amplitudes in the Mn + Noise group were less than Mn group from 50 to 70 dB SPL. The Mn group was not significantly different from the Ctrl group (*i.e.*, no effect of Mn) and the Mn + Noise group was not significantly different from the Noise group (*i.e.*, Mn did not exacerbate the effect of noise).

The analysis of 24 kHz (Fig. 2C) indicated that there was only a significant effect of L1 intensity ($F = 105.6$, 9, 171 DF; $p < 0.0001$); the interaction and treatment effects were not significant ($p > 0.05$). The analysis at 30 kHz (Fig. 2D) indicated that there was a

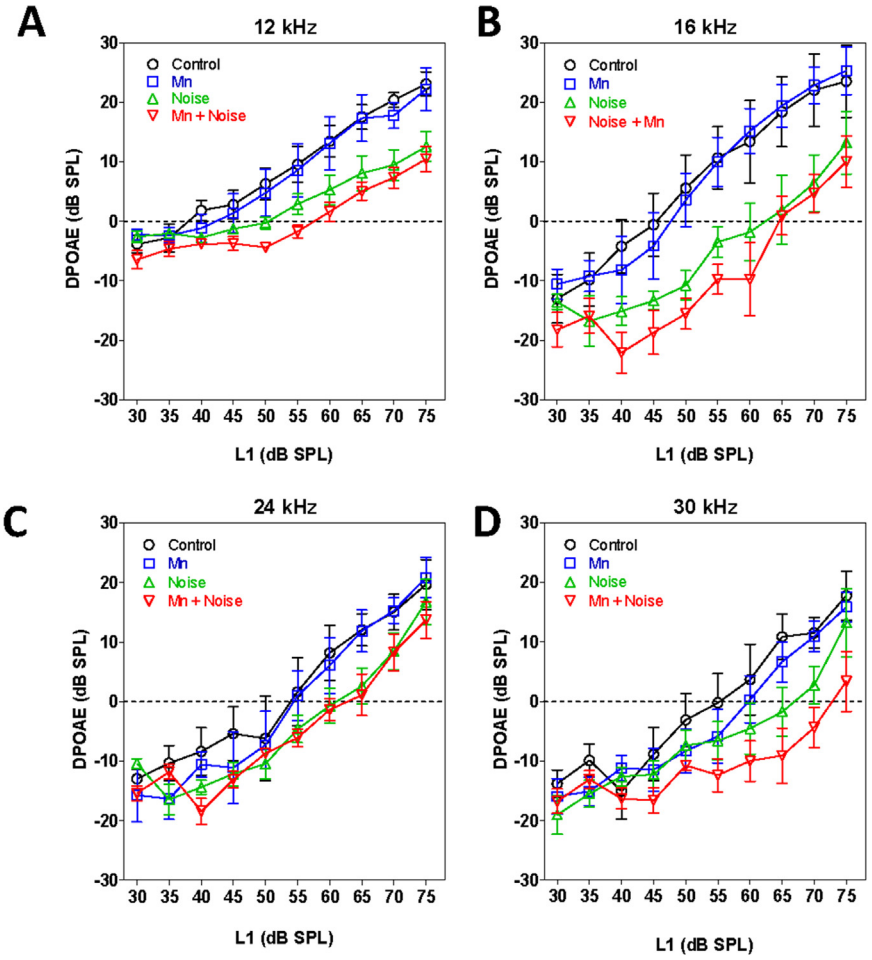


**Fig. 2.** Mean (± SEM) DPOAE input/output functions in the Ctrl, Mn, Noise and Mn + Noise groups at F2 frequencies of (A) 12 kHz, (B) 16 kHz, (C) 24 kHz and (D) 30 kHz.

significant effect of L1 intensity ($F = 105.6$, 9, 171 DF; $p < 0.0001$) and a significant intensity x treatment interaction effect ($F = 1.93$; 27, 144 DF; $p < 0.001$); however, the treatment effect was not statistically significant ($p > 0.05$). A Bonferroni *post-hoc* analysis indicated that at 30 kHz: (1) DPOAE amplitudes in the Mn + Noise group were significantly less ($p < 0.05$) than the Ctrl group from 65 to 75 dB SPL, (2) DPOAE amplitudes in the Mn + Noise group were significantly less than the Mn group ($p < 0.05$) at 65–70 dB SPL

### 3.3. CAP

The CAP, which reflects the synchronized response of auditory nerve fibers to the onset of the acoustic stimulus, reflect the gross neural output of the IHC and type I spiral ganglion neurons. The CAP I/O functions were measured approximately six-weeks post-exposure to determine in any of the treatments induced permanent changes in the neural output of the cochlea. Fig. 3 shows the CAP I/O at 12, 16, 24 and 35 kHz, frequencies within and above the noise exposure. CAP amplitudes in the Mn group were nearly the same as the Ctrl group except at 80 dB SPL at 12 and 24 kHz. CAP amplitudes in the Noise group and the Mn + Noise group were nearly the same and the amplitudes in both groups were consistently lower than those in the Ctrl group and Mn group.

At 12 kHz (Fig. 3A), a two-way, repeated measure ANOVA indicated that there was a significant effect of treatment ($F = 15.67$, 3, 144 DF, $p < 0.001$), intensity ($F = 135.0$; 8, 144 DF; $p < 0.0001$) and intensity x treatment interaction ($F = 8.08$, 24, 144, 171 DF; $p < 0.0.001$). A Bonferroni *post-hoc* analysis ($p < 0.05$) indicated that: (1) CAP amplitudes in the Noise group were significantly less than the Ctrl group from 50 to 80 dB SPL, (2) CAP amplitudes in the Noise group and the Mn + Noise group were significantly less than in the Ctrl group from 50 to 80 dB SPL and (3) CAP amplitudes in the Noise group and Mn + Noise group were significantly less than Mn group from 50 to 80 dB SPL. At 12 kHz, the Mn group was not significantly different from the Ctrl group except at 80 dB SPL where the amplitude for the Mn group was significantly less than the control group. The Mn + Noise group was not significantly different from the Noise group (*i.e.*, Mn did not exacerbate the effect of noise).

At 16 kHz (Fig. 3B), the analysis revealed a significant effect of treatment ($F = 7.41$, 3, 144 DF; $p < 0.002$), sound intensity ($F = 83.12$; 8, 144 DF; $p < 0.0001$) and treatment x intensity interaction ($F = 4.03$; 24, 144 DF; $p < 0.0001$). A Bonferroni *post-hoc* analysis ($p < 0.05$) indicated that: (1) CAP amplitudes in the Noise group and Mn + Noise group were significantly less than the Ctrl group from 60 to 80 dB SPL and (2) CAP amplitudes in the Noise group and Mn + Noise group were significantly less than in the Mn group from 60 to 80 dB SPL.

At 24 kHz (Fig. 3C), the analysis showed a significant effect of treatment ($F = 5.59$; 3, 136 DF; $p < 0.008$), sound intensity ($F = 57.73$; 8, 136 DF; $p < 0.0001$) and treatment x intensity interaction ($F = 5.13$; 24, 136 DF; $p < 0.0001$). A Bonferroni *post-hoc* analysis ($p < 0.05$) indicated that: (1) CAP amplitudes in the Noise group and Mn + Noise group were significantly less than in the Ctrl group from 50 to 80 and 60–80 dB SPL respectively and (2) CAP amplitudes in the Noise group and the Mn + Noise group were significantly less than in the Mn group from 60 to 80 dB SPL.

At 35 kHz (Fig. 3D), the analysis showed a significant effect of treatment ($F = 5.87$; 3, 144 DF; $p < 0.016$), sound intensity ($F = 40.88$; 8, 144 DF; $p < 0.0001$) and treatment x intensity interaction ($F = 4.7$; 24, 144 DF; $p < 0.0001$). A Bonferroni *post-hoc* analysis ($p < 0.05$) indicated that: (1) CAP amplitudes in

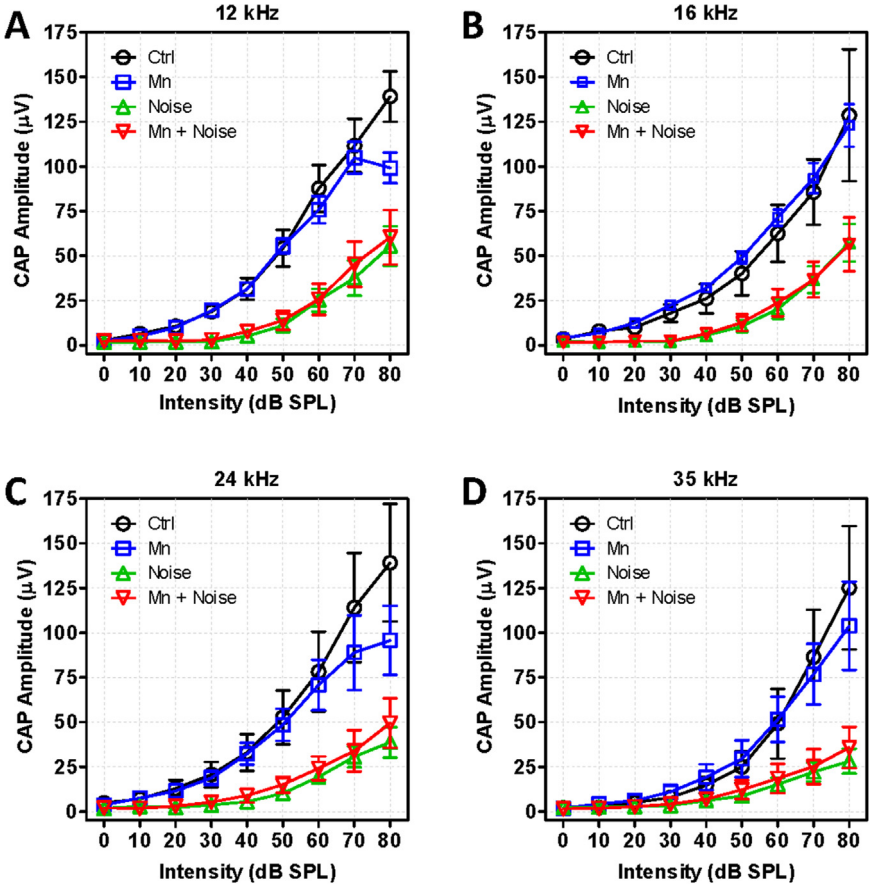


**Fig. 3.** Mean (± SEM) CAP input/output functions in the Ctrl, Mn, Noise and Mn + Noise groups at (A) 12 kHz, (B) 16 kHz, (C) 24 kHz and (D) 30 kHz.

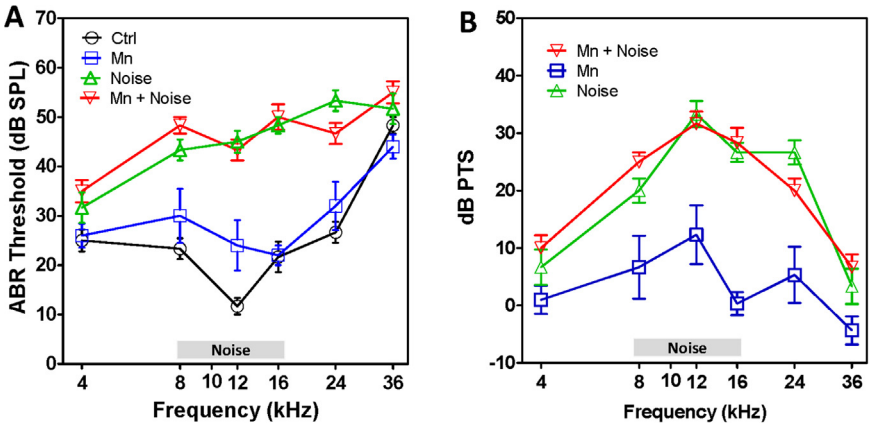


**Fig. 4.** (A) Mean (±) ABR threshold *versus* frequency. (B) dB PTS *versus* frequency; PTS calculated using thresholds of the Ctrl group. Gray area beneath both figures shows frequency of the noise exposure.

the Noise group and Mn + Noise group were significantly less than in the Ctrl group from 70 to 80 dB SPL and (2) CAP amplitudes in the Noise group and the Mn + Noise group were significantly less than in the Mn group from 70 to 80 dB SPL.

### 3.4. ABR

The threshold of the ABR, which reflects the neural response of the auditory brainstem, was evaluated approximately 6 weeks post-treatment to determine if any of the treatments produced permanent changes in the ABR thresholds. Fig. 4A plots the ABR thresholds as a function of frequency for the four experimental groups; the location of the 8–16 kHz noise is shown on the abscissa. Thresholds were lowest in the Ctrl group and highest in the Noise group and the Mn + Noise group. Thresholds in the Ctrl group were lowest at 12 kHz, ~12 dB and increased to 48 dB SPL at 36 kHz and 25 dB SPL at 4 kHz. Threshold for the Mn group were similar to or slightly higher than the Ctrl group. ABR thresholds in the Noise group and Mn + Noise were similar to one another, but substantially higher than those in the Ctrl group and Mn group. For ease of comparison, ABR permanent threshold shifts (PTS) were computed by subtracting the ABR thresholds in the Ctrl group from those in the Mn, Mn + Noise and Noise groups. As shown in Fig. 4B, the ABR PTS in the Noise group and Mn + Noise group were similar to one another and substantially higher than those in the Mn group. The maximum PTS in the Noise group was ~33 dB at 12 kHz and in the Mn + Noise group the maximum PTS was ~32 dB at 12 kHz. The maximum PTS occurred approximately one-half octave above the center frequency of the noise-exposure consistent with previous reports (Davis et al., 1950; Salvi et al., 1983). The PTS values in the Mn group ranged from −4 to 12 dB with the maximum shift at 12 kHz.

A two-way repeated measure ANOVA of ABR thresholds showed a significant effect of treatment ($F = 35.71$, 3, 95 DF; $p < 0.0001$), frequency ($F = 43.9$, 5, 95 DF; $p < 0.0001$) and a frequency × treatment interaction ($F = 7.32$, 15, 95 DF; $p < 0.0001$). A Bonferroni *post-hoc* analysis indicated that ABR thresholds in the Mn group were significantly greater than those in the Ctrl group only at 12 kHz ($p < 0.05$). ABR thresholds in the Noise group were significantly greater than the Ctrl group from 8 to 24 kHz ($p < 0.05$); thresholds in the Mn + Noise group were significantly greater than the Ctrl group from 4 to 24 kHz ($p < 0.05$); thresholds in the Noise group were significantly greater than the Mn group from 8 to 24 kHz ($p < 0.05$) and thresholds in the Mn + Noise group were significantly greater than the Mn group from 8 to 36 kHz. Thus, the noise exposure was the major source of the ABR threshold shift; Mn alone or together with the noise exposure had little or no effect.

### 3.5. Hair cell loss

Cochleograms were prepared to determine the amount of hair cell loss in the three treatment groups compared to the Ctrl group. Fig. 5 shows the mean loss of OHC and IHC plotted as a function of percent-distance from the apex of the cochlea. The cochlear frequency-position map is shown below the x-axis (Muller, 1991) and the gray rectangle below shows the location of the 8–16 kHz noise exposure. There was essentially no IHC loss in any of the four groups (Ctrl, Mn, Noise and Mn + Noise) (Fig. 5B). In addition, there was little or no OHC loss in the Ctrl and Mn groups. However, in the

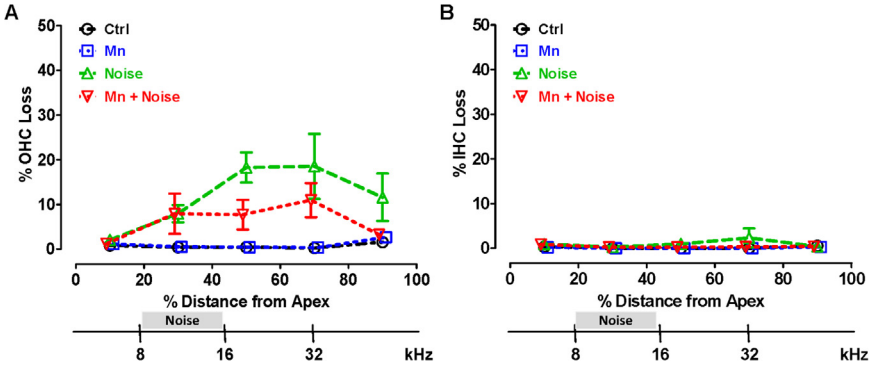


**Fig. 5.** Mean (± SEM; n = 6/group) cochleograms (20% intervals) showing the percent loss of (A) outer hair cells (OHC) and (B) inner hair cells (IHC) as a function of percent distance from the apex of the cochlea. Rat cochlear frequency-place map shown below the x-axis. Gray rectangle below shows the location of the 8–16 kHz noise exposure.

Noise group, the mean OHC loss was ~20% at the 50% and 70% cochlear locations and ~10% at the 30% and 90% locations. Thus, the maximum OHC occurred one-half to one octave above the frequency of the noise exposure consistent with previous reports (Salvi, Henderson, 1983). In the Mn + Noise group, the mean OHC lesion was ~10% at the 30, 50 and 70% cochlear locations slightly less than in the Noise alone group. A two-way, repeated measure ANOVA showed a significant effect of treatment ($F = 12.66$; 3, 76 DF; $p < 0.0001$), cochlear location ($F = 3.272$; 4, 76 DF; $p < 0.02$) and a treatment x location interaction ($F = 1.98$; 12, 76 DF; $p < 0.05$). A Bonferroni *post-hoc* analysis indicated that: (1) the OHC lesions in the Noise group were significantly greater ($p < 0.05$) than the Mn group and the Ctrl group at the 50% and 70% location and significantly greater than the Mn + Noise group at the 50% location and (2) the OHC lesions in the Mn + Noise group were significantly greater than the Ctrl group at the 70% location.

## 4. Discussion

The permissible exposure limit (PEL) for Mn set by the Occupational Health and Safety Administration is 0.2 mg/m$^3$ (8 h time weighted average) (https://www.osha.gov/dsg/annotated-pels/tablez-1.html). However, Mn exposures in many work environments often exceed these limits in industries such as ferroalloy smelting (Bast-Pettersen et al., 2004), welding (Bowler et al., 2007; Mergler et al., 1994), mining (Montes et al., 2008), battery assembly (Bader et al., 1999) and glass and ceramic manufacturing (Srivastava et al., 1991) particularly in industries or countries where regulations are lax. Besides excessive Mn, the noise intensities in many occupations are high enough to cause hearing loss either alone or in combination with other ototraumatic agents which have the potential to exacerbate the hearing loss (Morata and Campo, 2002).

### 4.1. Mn intake

The intake of water in the Mn-treated and in the Mn + Noise groups was similar to the intake of non-Mn-treated water in the Ctrl and Noise groups indicating that $MnCl_2$ did not disrupt fluid intake (Fig. 1). Mn intake remained fairly stable over the 90 treatment and total Mn intake in the Mn group was similar to that in the Mn + Noise group suggesting that the noise exposure did not disrupt water or Mn intake. The three metal transporters, DMT1, ZIP8 and ZIP14, which have a high affinity for Mn are present in the cochlea and are thought to play a major role in moving Mn from the bloodstream into the sensory epithelium (Ding et al. 2014; Ma et al., 2008). Not surprisingly, after acute intraperitoneal treatment with $MnCl_2$ (Ma et al., 2008) or long-term oral administration, Mn levels in the cochlea increased significantly and remained elevated for several weeks (Mullin et al., 2015). In our recent paper that employed the same concentration of $MnCl_2$ in drinking water as used in this manuscript, Mn levels increased significantly in the basilar membrane, stria vascularis and modiolar regions of the cochlea after 30 d or 60 d of treatment; the largest increase of 72% occurred in the basilar membrane fraction which contains the IHC and OHC (Mullin et al., 2015). Interestingly, Mn concentrations in the cochlea were substantially higher than in the globus pallidus and striatum, brain regions thought to be especially vulnerable to Mn neurotoxicity. Thus, our 90-d oral treatment with $MnCl_2$ would be expected to elevate the Mn concentrations throughout the three key subdivision of the cochlea.

### 4.2. Hearing impairment from Mn, Noise or Mn + Noise

Concern over the potential ototoxic effect of Mn were derived in part from our in vitro study showing that μM concentrations of $MnCl_2$ damaged the sensory hair cells and SGN in cochlear organotypic cultures. These results appeared consistent with other results showing that welding fumes, which contain high levels of Mn impaired cochlear function and that welding fumes in combination with noise caused greater cochlear impairment than noise alone. Based on these earlier findings, we predicted that 90-d oral dosing with $MnCl_2$ would lead to cochlear damage and hearing impairments and that the combination of $MnCl_2$ plus noise would cause greater cochlear damage and hearing impairment than noise alone.

A global assessment of our functional and anatomical data failed to support the hypothesis that chronic, high-dose oral intake of Mn (~260 mg/d) alone induced hearing impairments or hair cells loss. The lack of Mn-induced ototoxicity in the current study may be due to the fact that the dose employed was too low to damage the peripheral auditory system even though our prior study showed that 30 or 60 d oral treatment with this same dose used in this study elevated Mn levels in the cochlea by 50–75% (Mullin et al., 2015). Taken together, these results suggest that higher levels or longer treatments with Mn are needed to damage the cochlea.

DPOAE amplitudes, CAP amplitudes and ABR thresholds in the Mn + Noise group were generally the same or very similar to the Noise alone group. Thus, our functional data failed to support the hypothesis that Mn potentiated noise-induced hearing impairment. Unexpectedly, the OHC lesions in the Mn + Noise group were significantly less than in the Noise alone group most noticeably in the 50% region of the cochlea. One interpretation of these results suggests that Mn may protect against noise-induced OHC damage. While these results suggest a potential protective effect, the anatomical data contradict our DPOAE result that show smaller DPOAE amplitudes at 12, 16 and 30 kHz in the Mn + Noise group *versus* Noise alone. Since our CAP data show little difference between Mn + Noise *versus* Noise alone, we believe that the most parsimonious conclusion is that Mn has little or no effect on noise-induced hearing loss.

The most consistent findings was that our 90-d exposure (8 h/d; 90 dB SPL), approximating the OSHA maximum permissible level, significantly reduced DPOAE and CAP amplitudes and increased ABR thresholds. These functional deficits were most pronounced at frequencies within or slightly above the noise exposure consistent with previous reports (Chen, Decker, 2014, Davis, Morgan, 1950, Salvi, Henderson, 1983). These functional deficits were associated with mild OHC lesions (10–20%) at cochlear locations within or above octave band noise consistent with prior studies (Chen et al., 2014; Salvi et al., 1983).

Our results differ from an earlier report in which inhalation of welding fumes with a high concentration of Mn caused a large reduction in DPOAE amplitudes (Mirzaee et al., 2007). This discrepancy could be caused by many different factors. One obvious one is the route of administration, oral ingestion in our case *versus* inhalation. While uptake of Mn through the stomach is limited (Davidsson et al., 1989; Johnson et al., 1991), prior studies (Mullin et al., 2015) from our laboratory indicated a persistent and significant increase of Mn in the cochlea after chronic oral treatment. Since we are unaware of any study that has measured Mn concentrations in the sensory epithelium of the cochlea following inhalation of welding fumes, it is unclear if the route of administration is a key factor. Another critical variable is the complex mixture of noxious metals (Fe, Zn, Cu and Mn) and gases ($O_3$, CO and $CO_2$) in welding fumes *versus* just elevated Mn in our study. While the level Mn in welding fumes was roughly 9 times greater than the threshold limit value (TLV), the level of iron (Fe) was even higher, 21 times above the TLV. Thus, the neurotoxic effects of each individual component in combination provides a plausible explanation for why welding fumes caused a reduction in

DPOAE in rabbits. Welding fumes also exacerbated the DPOAE reduction induced by an 8 h/d exposure at 110 dB SPL whereas we found that Mn failed to enhance the hearing impairments caused by noise alone. Several factors could account for this difference. The most likely is the combined effects of the neurotoxic gases and metals in welding fumes as opposed to straight Mn. However, an additional factor is the 20 dB higher exposure intensity used to study the combined effects of welding fumes and noise. The 110 dB exposure intensity during welding fume exposure would likely be more effective in disrupting the blood-labyrinth barriers thereby increasing the likelihood that toxic substances would the cochlea and interact with the trauma effects induced by noise (Ding et al., 2012; Juhn et al., 2001; Suzuki et al., 2002).

Failure of finding a major effect with Mn in our studies whether in the presence or absence of noise is important especially when one considers the fact that Mn can accumulated in the inner ear. It is feasible that the lack of an ototoxic effect observed is related to the dose used. In comparison to many acute dosing regimens described in the literature which utilize very high levels of Mn, we employed a relatively modest dose of Mn; however, the exposure duration was considerably greater. Since uptake of Mn is reliant on intestinal transport which at best is 5% of the total dose administered, the actual amount of Mn that accumulates in the rats is considerably less than that consumed. Nevertheless, with respect to dosing, our study probably reflects a more semi-chronic exposure regimen than the acute exposure treatments employed in many laboratory studies in the literature. In addition, one cannot rule out the possibility that exposure of the rats to greater noise intensities may actually induce a synergistic response with Mn even at the modest oral dose employed in the current study. In summary, our studies suggest that semi-chronic exposure to moderate levels of Mn in either the presence or absence of noise has, at best, only a minor effect on hearing though we cannot exclude the possibility that higher exposure levels may induce significant hearing loss.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Disclosure

Dr. Roth reports grants from NIOSH, during the conduct of the study.

## Acknowledgement

This work was supported by the grant from the National Institute of Occupational Safety & Health (R01OH010235).

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