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Manganese is Toxic to Spiral Ganglion Neurons and Hair Cells in Vitro

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Abstract

Occupational exposure to high atmospheric levels of Mn produces a severe and debilitating disorder known as manganism characterized by extrapyramidal disturbances similar to that seen in Parkinson's disease. Epidemiological and case studies suggest that persistent exposures to Mn may have deleterious effects on other organs including the auditory system and hearing. Mn accumulates in the inner ear following acute exposure raising the possibility that it can damage the sensory hair cells that convert sound into neural activity or spiral ganglion neurons (SGN) that transmit acoustic information from the hair cells to the brain via the auditory nerve. In this paper we demonstrate for first time that Mn causes significant damage to the sensory hair cells, peripheral auditory nerve fibers (ANF) and SGN in cochlear organotypic cultures isolated from postnatal day three rats. The peripheral ANF that make synaptic contact with the sensory hair cells were particularly vulnerable to Mn toxicity; damage occurred at concentrations as low 0.01 mM and increased with dose and duration of Mn exposure. Sensory hair cells, in contrast, were slightly more resistant to Mn toxicity than the ANF. Mn induced an atypical pattern of sensory cell damage; Mn was more toxic to inner hair cells (IHC) than outer hair cells (OHC) and in addition, IHC loss was relatively uniform along the length of the cochlea. Mn also caused significant loss and shrinkage of SGN soma. These findings are the first to demonstrate that Mn can produce severe lesions to both neurons and hair cells in the postnatal inner ear.

Keywords

manganese; manganism; hair cells; spiral ganglion; neurotoxicity; cochlea; hearing loss

INTRODUCTION

The biological requirement of Mn as an essential trace mineral for normal growth and development was first recognized almost 80 years ago (Kemmerer et al., 1931, Orent and McCollum, 1931). As an essential nutrient, Mn is necessary for normal homeostatic processes controlling reproduction, formation of connective tissue and bone, carbohydrate and lipid metabolism and brain function (Bourre, 2006, Keen et al., 1999). Mn deficiency

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during fetal development can result in neurological and behavioral deficits as well as abnormal growth of a variety of systems in the body (Hurley, 1981, Strause et al., 1986). Mn deficiency, however, in the adult population is essentially nonexistent because of the abundant supply of Mn in our normal diet. In contrast, Mn intoxication caused by prolonged exposures produces a severe and debilitating disorder known as manganism (Krieger et al., 1995, Pomier-Layrargues et al., 1995). The most prominent and severe disabilities associated with excess exposure to Mn include a distinct extra pyramidal syndrome which resembles the dystonic movements associated with Parkinson's disease (Huang et al., 1993, Olanow et al., 1996, Pal et al., 1999). Manganism is generally considered to be an occupational disorder being observed most often in individuals whose profession involves protracted contact with high atmospheric levels of Mn such as welders, Mn miners and individuals employed in ferroalloy processing. Patients with chronic hepatic failure also display elevated serum and brain levels of Mn and exhibit many of the behavioral deficits and neurodegenerative features observed in occupationally exposed workers primarily because the liver is the major organ responsible for its elimination from the body (Burkhard et al., 2003, Hauser and Zesiewicz, 1996, Hauser et al., 1994, Krieger et al., 1995, Pomier-Layrargues et al., 1995).

The classical symptoms of manganism were originally described almost 170 years ago by Couper (Couper, 1837, Lucchini et al., 2009, Santamaria and Sulsky, 2010) in a man using a grinding wheel composed of the black oxides of manganese. Although sporadic reports of Mn toxicity appeared in the literature within the first half of the previous century, it has only been in the last several decades that significant progress has been made in understanding the mechanisms of Mn cytotoxicity. Manganism is considered an occupational disorder largely restricted to workers in industrial environments where the Mn atmospheric levels exceed the requisite threshold limit value (TLV). Major concerns about Mn exposure in the general population, however, were recently raised with the proposed use of methylcyclopentadienyl manganese tricarbonyl (MMT) as a fuel additive to boost octane ratings in gasoline.

The preponderance of clinical and basic research concerning the toxic actions of Mn has primarily focused on central nervous system (CNS) effects with almost complete indifference to other pernicious manifestations which may be equally irreversible though considerably less perceptible. What is becoming evident is that chronic exposure to Mn may also have harmful effects to other tissues in the body including the auditory system. For example, several reports in the literature have described hearing deficits both in welders and alloy worker who are normally exposed to chronic high levels of Mn and in individuals exposed simultaneously to noise and Mn (Bouchard et al., 2008, Josephs et al., 2005, Khalkova and Kostadinova, 1986, Korczynski, 2000). However, because of the confounding effects of workplace and recreational noise, it is uncertain if the hearing loss is caused by Mn exposure, noise exposure or the combined effects of manganese and noise (Nikolov, 1974). Based on the limited findings in the literature, it is unclear as to whether Mn alone is actually responsible for the hearing deficits reported or if hearing loss is due to other confounding factors such as noise exposure. Given the recent report demonstrating that Mn accumulates in the inner ear (Ma et al., 2008), it is reasonable to hypothesize that it has the potential to exert its cytotoxic effects on the sensory hair cells, neurons or supporting cells which in turn would be expected to result in significant hearing loss. To explore its potential toxic effects on the inner ear, we treated postnatal cochlear organotypic cultures with varying doses of Mn.

2.0 MATERIAL AND METHODS

2.1 Cochlear Organotypic Cultures

Cochlear organotypic cultures were prepared from postnatal day 3 SASCO Sprague–Dawley rats as described previously (Corbaccia et al., 2004, Ding et al., 2002, Wei et al., 2010). In brief, the cochlea was removed and the organ of Corti and SGN were transferred onto rat tail type I collagen gel in basal medium Eagle containing 2% sodium carbonate. A 15- μ L drop of the collagen solution was placed on the surface of a 35 mm culture dish and allowed to gel for approximately 30 min. Afterwards, 1.3 ml of culture medium (0.01 g/ml bovine serum albumin, 1% Serum-Free Supplement [Sigma I-1884], 2.4% of 20% glucose, 0.2% penicillin G, 1% 200 mM glutamine, 95.4% of 1 X BME) was added to the dish. The cultures were maintained in an incubator at 37 °C and 5% CO₂ overnight. On the following day, fresh medium was added alone or containing various concentrations of Mn.

2.2 Mn Chloride Treatments

MnCl₂ stock solution was freshly made at a stock concentration of 10 mM in serum-free medium and diluted to final concentrations varying from 0.01 to 5.0 mM. Cochlear explants (n = 6/group) were incubated in the presence or absence of Mn in 5% CO₂ and 37°C in humidified atmosphere from 24 to 96 hr.

2.3 Histological Evaluation

Cochlear explants were fixed for two hr in 4% formalin and subsequently washed with 0.1 M phosphate buffered saline (PBS). As described in our previous publications, the specimens were immunolabeled with a primary monoclonal antibody against neuronal class III β -tubulin (Covance, MMS-435P) which was detected using a secondary antibody labeled with Cy3 (goat anti-mouse IgG, Jackson ImmunoResearch; #115-165-206) (Ding et al., 2002, Lanzoni et al., 2005, McFadden et al., 2003, Qi et al., 2008). To visualize F-actin that is heavily expressed in the cuticular plate and stereocilia bundles of hair cells, specimens were labeled with phalloidin conjugated Alexa Fluor 488 (Invitrogen A12379, diluted by 1:200). After rinsing with 0.1 M PBS, specimens were mounted on glass slides in glycerin, coverslipped and examined using a confocal microscope (Zeiss LSM-510 meta, step size 0.5 μ m per slice) using appropriate filters to detect the fluorescence of Cy3 labeled product in nerve fibers and spiral ganglion neurons (SGN) (excitation 550 nm, emission 570 nm) and green fluorescence of Alexa 488-labeled phalloidin (excitation 488 nm, emission 520 nm) that labels the bundles of stereocilia and the cuticular plate of the hair cells. Confocal images were stored on disk and processed using Confocal Assistant, ImageJ and Adobe Photoshop 5.5 software.

2.4 Nerve Fibers

The fascicles of auditory nerve fiber (ANF) bundles projecting out from the SGN to the organ of Corti were counted across the width (120 μ m) of the field of view of the microscope at a magnification of 630X. All the fibers were counted in the same region in the middle of the cochlear culture. Five organotypic cultures were examined for each experimental condition. Data were analyzed using a one-way ANOVA followed by Newman-Keuls post-hoc analyses (GraphPad Prism 5 software).

Cochlear hair cells were observed under a fluorescent microscope with the appropriate filter to visualize the stereocilia and cuticular plate of hair cells that are intensely labeled by Alexa 488-labeled phalloidin. A hair cell was counted as missing if the stereocilia was missing or severely damaged. The three rows of outer hair cells (OHC) and single row of inner hair cells (IHC) were counted along the entire length of cochlea from apex to base. A cochleogram was used to determine the percent of IHC and OHC as a function of percent

distance from the apex to the base. Using custom cochleogram software and laboratory norms from control animals, the average ($n = 5/\text{condition}$) percentage of hair cells missing was plotted as a function of percent distance from the apex of the cochlea for each experimental group as previously described (Wei et al., 2010).

3.0 RESULTS

3.1 Mn Damages Hair Cells and Nerve Fibers

Studies were performed to determine the effect of Mn concentration and exposure time on ANF viability. For these experiments, cochlear organotypic cultures were treated for 24, 48 or 96 h with doses of Mn ranging from 0.01 to 5 mM. Fig. 1 shows the condition of the IHC, OHC, ANF and SGN in a typical control specimen cultured for 96 h without Mn treatment (0 mM). The actin in the stereocilia bundle and cuticular plate of the OHC and IHC is heavily labeled with phalloidin-Alexa Fluor 488. The three rows of OHC and single row of IHC are arranged in orderly rows that spiral from the base to the apex of the cochlea. The SGN, ANF and nerve terminals (NT) are intensely labeled with β -tubulin. The peripheral ANF of the SGN radiate outward toward the IHC and OHC and form NT on the hair cells. The hair cells, ANF, NT and SGN in untreated (0 mM) controls appeared normal and showed no obvious signs of pathology after being cultured for 96 h or less as illustrated in Fig. 1. The normal appearance of these untreated control cultures is consistent with our previous results (Wei, Ding, 2010).

The photomicrographs in Fig. 2 illustrate the degenerative changes after 24 hr exposure to Mn. Doses of Mn ranging from 0.01 to 1 mM (Figure 2A – E) had little effect on the ANF as they radiate out towards the single row of IHC and three rows of OHC. The ANF terminate in a dense plexus of NT as they approach the IHC. The stereocilia tufts on the apical surface of the OHC and IHC are heavily labeled with Alexa 488 phalloidin (green). The stereocilia bundles form an inverted U or V shape characteristic of stereocilia bundles on healthy hair cells. Similar to the ANF, the hair cells and nerve terminals (NT) also appeared normal after 24 hr treatment with doses of Mn ranging from 0.01 to 1 mM (Figure 2A – E). In contrast, the highest dose of Mn, 5 mM, as shown in Fig. 2F, caused significant damage to both the nerve fibers and hair cells as indicated by the profuse degeneration of the formerly thick, linear fascicles which now have disintegrated into small pixels or larger clusters of debris. Most of the stereocilia on the OHC were missing, but the IHC stereocilia were present and largely intact.

The effects of Mn on ANF and hair cells treated for 48 hr are illustrated in Fig. 3. The hair cells and ANF appeared normal at the lowest concentration of 0.01 mM. ANF density, however, started to decline around 0.05 mM; most ANF were missing at 1 mM and virtually all the fibers were absent at 5 mM. Hair cell loss first appeared at 0.05 mM Mn and increased with dose so that most were missing at 5 mM Mn. As revealed in Fig. 4, a similar though slightly greater pattern of degeneration occurred after 96 hr exposure to Mn with significant loss of ANF at a low concentration of 0.01 mM Mn. At this concentration, many ANF, but only a few hair cells, were missing implying that hair cell are less sensitive to the toxic actions of Mn. These data clearly demonstrate there was a time and dose dependent effect of Mn on ANF and hair cells.

To quantify its neurotoxic activity, the numbers of surviving nerve fiber bundles were determined for the different doses and durations of Mn exposure. Figure 5 shows the mean numbers of nerve fibers/120 μm after 24, 48, 72 and 96 hr treatment with Mn doses ranging from 0.01 to 5 mM. Approximately 45 fibers/120 μm are present in untreated control cultures (0 mM). The 24 hr Mn treatment caused a statistically significant decline in the number of nerve fibers (One-way ANOVA, $F = 56.02$, $p < 0.0001$) at only the 5 mM

treatment compared to the controls (Newman-Keuls, $p < 0.05$). In contrast, treatment for 48 hrs caused a statistically significant decline in the number of nerve fibers for Mn doses of 0.05 mM and higher (One-way ANOVA, $F = 173.4$, $p < 0.0001$; Newman-Keuls, $p < 0.05$). At the longer exposure times, the numbers of nerve fibers in all Mn treated groups were significantly less than in controls for treatments lasting 72 hrs (One-way ANOVA, $F = 163.2$, $p < 0.0001$, Newman-Keuls post-hoc analysis, $p < 0.05$) and 96 hrs (One-way ANOVA, $F = 199.7$, $p < 0.001$, Newman-Keuls, $p < 0.05$).

3.2 Mn Damage to SGN

As shown in Fig. 6, Mn not only damaged the peripheral ANF, but also injured the soma of SGN. As illustrated by Fig. 6F, 48 hr treatment with 5 mM Mn caused significant shrinkage, degeneration and loss of SGN soma whereas at the three lowest doses of Mn the SGN appeared normal (Fig. 6A–C). The cell bodies were heavily labeled with β -tubulin except for the centrally located nucleolus. As the dose of Mn increased, the soma and nucleolus decreased in size, soma shape became more irregular and SGN density decreased. The same general trends were observed in SGN after 72 hr treatment with Mn (Fig. 7); however, soma shrinkage and cell loss were more severe for a given dose of Mn. After treatment for 72 hrs, SGN degeneration occurred at concentrations as low as 0.05 mM.

3.3 Mn Dose-Response to OHC and IHC

To quantify the toxic effects of Mn on the sensory cells, mean ($n = 5/\text{group}$) cochleograms were computed for different durations and doses of Mn. Figure 8 shows the mean percentage loss of OHC and IHC as function of percent distance from the apex of the cochlea after 48 and 96 hr treatment with Mn doses ranging from 0.1 to 5 mM. Several trends were evident with respect to dose and the pattern of IHC and OHC loss. The lowest dose of Mn, 0.1 mM, caused greater OHC loss in the basal half (50–100%) of the cochlea than the apex (Figure 8A). For the 48 hr treatment, OHC loss decreased from around 40% at the extreme base to less than 10% in the apical half of the cochlea (0–50%). IHC losses were approximately 20% over most of the cochlea. As the dose of Mn increased (Figure 8C–E), OHC loss continued to show a base to apex gradient except at the highest dose, 5 mM, where all the OHC were missing. IHC losses increased with dose and maintained a fairly uniform pattern of damage along the length of the cochlea at all concentrations. When considering the overall cell loss, the magnitude of IHC loss was generally greater than that for the OHC. For the 96 hr treatment, the 0.1 mM dose again caused more OHC loss near the base than the apex whereas IHC losses (~40%) were relatively uniform along the length of the cochlea (Figure 8E). As Mn dose increased, OHC losses continued to show a base to apex gradient except at the two highest doses where all the OHC were missing. IHC loss increased with dose and the pattern of degeneration was relatively uniform along the length of the cochlea. Again, the overall magnitude of sensory cell loss was greater for IHC than OHC.

4.0 DISCUSSION

Given that the extra pyramidal symptoms are the most conspicuous impairment seen in individuals exposed to excess Mn, it is not surprising that most research has focused on this issue. What is becoming evident is that Mn can have deleterious effects on other systems in the body; however, because the symptoms are less discernible, they have received scant attention in the medical literature. Chronic exposure to Mn, however, can lead to increased propensity to develop pulmonary infections including pneumonia and bronchitis (Antonini et al., 2009a, Antonini et al., 2009b, Bencko and Cikrt, 1984, Bowler et al., 2007, Jafari and Assari, 2004, Maigetter et al., 1976, Saric, 1992, Saric and Piasek, 2000). There is also growing evidence in the literature, although limited in scope, that Mn may cause or

contribute to hearing loss in workers exposed to chronic high levels of the metal or that noise-induced hearing loss may be exacerbated in the presence of Mn (Bouchard et al., 2008, Josephs et al., 2005, Khalkova and Kostadinova, 1986, Korczynski, 2000, Nikolov, 1974).

Current PEL for Mn fume levels established by OSHA is 5 mg/m³ and the TLV is 0.2 mg Mn/m³ for elemental Mn whereas the NIOSH recommended exposure limit (REL) is 1 mg/m³. Without proper ventilation in the workplace, welding fumes can greatly exceed these values. As already noted, hearing loss has been detected in individuals exposed to elevated levels of Mn and the data in this paper demonstrate for the first time that the ANF and sensory hair cells can be damaged in vitro by micromolar levels of Mn.

Although Mn readily accumulates in both the lung and inner ear (Kalliomaki et al., 1983, Ma et al., 2008, Park et al., 2007), we are unaware of any study that has documented Mn-induced lesions to the inner ear. Here we demonstrate for first time that Mn causes significant damage to the sensory hair cells, peripheral ANF and SGN in cochlear organotypic cultures isolated from postnatal day 3 rats (Figure 2–4) at Mn concentrations comparable to those employed previously to investigate cellular damage in other tissues (Crooks et al., 2007, Rovetta et al., 2007). While the damage seen with our highest Mn concentrations (1–5 mM) could be due in part to osmotic effects, we believe this is unlikely since this has not been reported in other in vitro studies using high concentration of Mn (Crooks et al., 2007, Rovetta et al., 2007). Moreover, osmotic effects clearly cannot account for the hair cell and neuronal damage seen with micromolar concentrations of Mn.

The peripheral ANF that synapse on the sensory hair cells were particularly vulnerable to Mn toxicity. ANF damage developed slowly between 24 and 96 hrs of Mn exposure and the damage was dose-dependent. Only the highest dose of Mn, 5 mM, caused significant ANF damage after 24 hrs of treatment. However, when the exposure was extended to 96 hrs, virtually all of the ANF were destroyed by the highest 5 mM dose and roughly two-thirds were destroyed by doses as low as 0.01 mM of Mn. Interestingly, the sensory hair cells were generally more resistant to Mn toxicity than the ANF. This is clearly seen by the observation that the 96 hr treatment with 0.1 mM Mn produced roughly 80% damage to the ANF whereas less than 40% of both the IHC and OHC were missing. The magnitude and pattern of hair cell loss and neuronal damage induced by Mn was both similar and different from that observed with classic ototoxic drugs such as cisplatin or aminoglycoside antibiotics (Corbacella et al., 2004, Ding and Salvi, 2005, Rybak and Ramkumar, 2007, Schacht, 1993). Mn, like the platinum-based ototoxic drug cisplatin, damaged both the hair cells, ANF and SGN at micromolar concentration in vitro (Zhang et al., 2003). However, Mn was more toxic to IHC than OHC whereas the reverse pattern occurs with cisplatin and aminoglycoside antibiotics (Corbacella et al., 2004, Zhang et al., 2003). Second, the IHC lesions induced by Mn were relatively uniform along the length of the cochlea while those caused by cisplatin and aminoglycoside antibiotics are more severe near the base of the cochlea than the apex. The only other drug that seems to preferentially damage the IHC is carboplatin and this unique pattern has only been observed in chinchillas (Ding et al., 1999, Takeno et al., 1994). On the other hand, the base to apex gradient of OHC loss induced by Mn follows a similar pattern to that seen with most other ototoxic drugs.

While we did not explore the mechanisms of Mn-induced ototoxicity in detail, morphological inspection of the SGN (Figure 6–7) revealed considerable shrinkage and condensation of the soma, a morphological feature of cells dying by apoptosis in contrast to necrotic cell death that is associated with cellular swelling and rupture of the cell membrane. Previous studies with primary cultures of striatal neurons suggest that Mn toxicity is associated with mitochondrial dysfunction, ROS formation and DNA fragmentation,

features linked to apoptotic cell death (Malecki, 2001). The ROS induced by Mn exposure leads to activation of many of the classical signaling pathways associated with programmed cell death, including increased TUNEL staining, internucleosomal DNA cleavage, activation of JNK, p38 (stress activated protein kinase), caspase-3 like activity, and caspase-3 dependent cleavage of PARP (Chun et al., 2001a, Chun et al., 2001b, Desole et al., 1996, Desole et al., 1997, Hirata et al., 1998a,b, Latchoumycandane et al., 2005, Roth et al., 2000, Schrantz et al., 1999). In addition, Mn also interferes with oxidative phosphorylation by inhibiting both mitochondrial F1-ATPase (Gavin et al., 1992, 1999) and complex I (Galvani et al., 1995) leading to the depletion of ATP (Chen and Liao, 2002, Roth et al., 2000). Collectively, these findings suggest that cell death may be a combination of both apoptosis as well as necrosis.

One of the potential limiting factors in this study concerns the selective use of postnatal day 3 rats to determine the actions of Mn on the inner ear. At this point, we do not know whether a similar response to Mn will also be observed in adult animals but we are limited by the fact that adult cultures of the inner ear are difficult to maintain. Prior studies have indicated that the major transport protein for Mn, divalent metal transporter 1 (DMT1), increases with age in brain and may partially be responsible for iron accumulation seen in Parkinson's disease (Salazar et al., 2008). DMT1 has been reported to be a major divalent metal transporter present in the inner ear (Ma et al., 2008); however, the specific cellular location of DMT1 has not been determined. If a similar age-related increase in DMT1 also occurs for neurons and hair cells in the inner ear, we would anticipate increased sensitivity and hearing deficits associated with excess exposure to Mn in adult animals. Studies are currently underway to determine the effect of age and the mechanism by which Mn induces cell death in cells within the inner ear. Thus, Mn may join a list of other heavy metals such as Pb, Cd and Hg which are linked to hearing loss or cochlear pathology (Lasky et al., 2001, Murata et al., 1993, Ozcaglar et al., 2001, Rice and Gilbert, 1992, Wassick and Yonovitz, 1985, Whitworth et al., 1999, Yamamura et al., 1989).

4.1 Conclusion

Our results demonstrate for the first time that Mn is toxic to neurons and sensory cells in cochlear cultures from postnatal rats. Damage occurred with Mn concentrations as low as 10 micromolar and increased in a dose-dependent manner. Mn was more toxic to SGN than hair cells. Among OHC, those in the base of the cochlea were more vulnerable to Mn toxicity than those in the apex. Surprisingly, the toxic effects of Mn on IHC were relatively uniform across the length of the cochlea. These results suggest that Mn may be ototoxic and lead to hearing loss.

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Fig. 1.

Photomicrograph of control organotypic culture from the middle of the cochlea after 96 h without Mn treatment (0 mM Mn). Hair cells labeled with Alexa Fluor 488-phalloidin. Nerve fibers labeled with antibody against β -tubulin and Cy3 conjugated secondary antibody. Bracket shows three rows of OHC; arrowhead marks the single row of IHC; blue arrow shows soma of a SGN; white arrow points to a bundle of ANF radiating out from the SGN towards the hair cells; yellow arrow points to NT from the ANF that terminate beneath the hair cells. Scale bar in (A): 15 μ M.

Fig. 2.

Photomicrographs show cochlear organotypic cultures from the middle of the cochlea after 24 hr treatment with Mn. Hair cells labeled with Alexa Fluor 488-phalloidin. Nerve fibers labeled with antibody against β -tubulin and Cy3 conjugated secondary antibody. Mn concentration is shown in each panel. Bracket shows three rows of OHC; arrowhead marks the single row of IHC, white arrow points to a bundle of ANF radiating out towards the hair cells, yellow arrow points to clusters of NT from the ANF that terminate beneath the hair cells. Scale bar in (A): 15 μ m.

Fig. 3.

Photomicrographs show cochlear organotypic cultures from the middle of the cochlea after 48 hr treatment with Mn; concentration is shown in each panel. OHC, bracket; arrowhead, IHC, white arrow, ANF, yellow arrow, NT; missing hair cells indicated by jagged arrowhead. Scale bar in (A): 15 μ m. Note loss of ANF as dose increases from 0.5 mM to 5 mM. OHC and IHC loss (jagged arrows) begins at 1 mM; nearly complete hair cell loss at 5 mM.

Fig. 4.

Cochlear organotypic cultures from the middle of the cochlea after 96 hr treatment; Mn concentration is shown in each panel (Bracket, OHC; arrowhead, IHC, white arrow, ANF, yellow arrow, NT; missing hair cells indicated by jagged arrowhead). Scale bar in (A): 15 μ m. Note increasing loss of ANF as dose increases from 0.5 mM to 5 mM. OHC and IHC loss begins with 1 mM and nearly complete destruction seen with 5 mM.

Fig. 5. Mean numbers of ANF/120 um versus dose in groups treated for (A) 24 hrs, (B) 48 hrs, (C) 72 hrs and (D) 96 hrs. Asterisks show Mn conditions that were significantly different ($p < 0.05$) from untreated control cultures (0 mM).

Fig. 6. Photomicrographs show SGN after 48 h treatment with Mn (0.01 to 5 mM). Specimens labeled with antibody against β -tubulin and Cy3 conjugated secondary antibody (red). (A-C) At the three lowest doses, 0.01 – 1 mM, the somas are large and round (arrowheads) and the unstained nucleolus is clearly visible. (D-F) Note shrinkage of spiral ganglion soma (jagged arrowhead), decrease in nucleolus visibility and decrease on soma number as Mn dose increases from 0.5 to 5 mM.

Fig. 7.

Photomicrographs show SGN after 72 hr treatment with Mn (0.01 to 5 mM). Specimens labeled with antibody against β -tubulin and Cy3 conjugated secondary antibody (red). (A-B) At the two lowest doses, 0.01 and 0.05 mM, the somas are large and round (arrowheads) and the unstained nucleoli are clearly visible. (C-F) Note shrinkage of spiral ganglion soma (jagged arrowhead), decrease in nucleolus visibility and decrease on soma number as Mn dose increases from 0.5 to 5 mM.

Fig. 8. Mean cochleograms showing the percentage of missing OHC (dashed line) and IHC (solid line) versus percent distance from the apex of the cochlea for 48 (left column) or 96 hr (right column) treatment with dose of Mn shown in upper left of each panel.