Glutamate/Aspartate Transporter (GLAST), Taurine Transporter and Metallothionein mRNA Levels are Differentially Altered in Astrocytes Exposed to Manganese Chloride, Manganese Phosphate or Manganese Sulfate

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Abstract:

Manganese (Mn)-induced neurotoxicity can occur due to environmental exposure (air pollution, soil, water) and/or metabolic aberrations (decreased biliary excretion). High brain manganese levels lead to oxidative stress, as well as alterations in neurotransmitter metabolism with concurrent neurobehavioral deficits. Based on the few existing studies that have examined brain regional Mn concentration, it is likely that in pathological conditions, Mn concentration can reach between 100 and 500 μM. Environmental Mn exposure as a result of methylcyclopentadienyl manganese tricarbonyl (MMT) combustion is in the form of phosphate or sulfate ($MnPO_4$, $MnSO_4$, respectively). Pharmacokinetic studies have shown that the Mn salt will determine the rate of transport into the brain: $MnCl_2 > MnSO_4 > MnPO_4$. The salt-specific neurotoxicity of these species is unknown. The primary goal of this study was to examine gene expression of glutamatelaspartate transporter (GLAST), taurine transporter (tau-T), and metallothionein-I (MT-I) in astrocytes exposed to manganese chloride ($MnCl_2$), manganese sulfate ($MnSO_4$), and manganese phosphate ($MnPO_4$). We hypothesized that the effects of $MnPO_4$ and $MnSO_4$ exposure on GLAST expression in astrocytes would be similar to those induced by $MnCl_2$, since irrespective of salt species exposure, once internalized by astrocytes, the Mn ion would be identically complexed. At the same time, we hypothesized that the magnitude of the effect would be salt-dependent, since the chemical speciation would determine the rate of intracellular uptake of Mn. $MnCl_2$ caused a significant overall decrease ($P < 0.0001$) in astrocytic GLAST mRNA levels with $MnSO_4$ causing a moderate decrease. $MnPO_4$ exposure did not alter GLAST mRNA in astrocytes. We also sought to examine astrocytic metallothionein and taurine transporter gene expression as markers of manganese exposure. Our findings suggest that manganese chloride significantly decreased ($P < 0.0001$) astrocytic metallothionein mRNA compared to both the sulfate and phosphate species. However, astrocytic taurine transporter mRNA was not affected by Mn exposure, irrespective of the salt species. These data are consistent with the hypothesis that astrocytic neurotoxicity due to Mn exposure is dependent upon its species, with solubility, and by inference, intracellular concentration, representing a major determinant of its neurotoxicity.

Keywords: Astrocytes; Manganese; Taurine transporter; Glutamate; Metallothionein; mRNA; Rat

Article:

INTRODUCTION

Manganese (Mn) is an essential nutrient and is important for cellular functioning. However, excess Mn is known to cause neurotoxicity (Cotzias et al., 1968). Manganese has been implicated in oxidative stress (Stokes et al., 2000; Desole et al., 1997), as well as disturbance of neurotransmitter metabolism (Miele et al., 2000; Montes et al., 2001). Few reports exist on brain Mn concentration levels upon manganese intoxication. A study performed almost three decades ago, examined Mn concentration in striatum and globus pallidus (two regions known to accumulate Mn) of monkeys dosed for 3 months with manganese dioxide (Suzuki et al., 1975). Striatal Mn concentration reached 264 μM, while globus pallidus Mn concentration peaked at 334 μM. In rats, after Mn dosing, Mn content can reach up to 200 μM depending upon brain region examined and dosing regimen (Ingersoll et al., 1999; Lai et al., 1999; Roels et al., 1997). Thus, during manganese toxicity, it is possible for brain levels to exceed 350 μM.

It has also been shown that Mn neurotoxicity may be due to an indirect excitotoxic event caused by altered glutamate metabolism (Brouillet et al., 1993). In the brain, both Mn uptake (Aschner et al., 1992) and glutamate uptake predominantly occur in astrocytes (see Danbolt, 2001 for review). In fact, it has been suggested that glutamate uptake by astrocytes, its conversion to glutamine via glutamine synthetase and deamination to glutamate is the primary glutamate recycling pathway (Van den Berg and Garfinkel, 197 1; Westergaard et al., 1995; Ottersen et al., 1992). There-fore, an attenuation of this pathway could be detrimental to neurons dependent on this function.

It has been estimated that 80% of brain glutamate is in astrocytes (Aschner et al., 2001), and that abnormally increased extracellular glutamate levels are excitotoxic to neurons (Choi, 1988). Therefore, it is critical that astrocytes are equipped with mechanism(s) for the rapid removal of glutamate from synaptic clefts. This uptake process occurs via sodium/potassium-dependent membrane proteins known as glutamate transporters. While there are several glutamate transporters known to be important for neuronal functioning, glutamate transporter (GLT-1) and glutamate/aspartate transporter (GLAST) are the prominent astrocytic transporters (see Danbolt, 2001 for review).

In cultured rat astrocytes, GLAST is the most prevalent glutamate transporter (Kondo et al., 1995), intracellularly transporting both glutamate and aspartate. A previous study showed that overnight Mn exposure to cultured rat astrocytes caused a 30% decrease in glutamate uptake (Hazell and Norenberg, 1997). It can be inferred from this study that Mn increases the excitotoxic potential as a result of increased extracellular glutamate. While it is speculated that decreased glutamate uptake is due to altered transporter functioning, levels of glutamate transporter protein and/or mRNA have not been examined in astrocytes exposed to Mn.

Excess Mn is associated with neurotoxicity, thought to be due to oxidative stress (Stokes et al., 2000; Desole et al., 1997). Like other eukaryotic cells, astrocytes possess defense mechanisms against increased intracellular oxidant levels. Amongst these is the sequestration of pro-oxidants by metallothionein (MT, Maret, 1994). In CNS, MT exists in three isoforms (MT-I, MT-II and MT-III). MT-I and MT-II are expressed predominately in astrocytes, whereas MT-III is expressed primarily in hippocampal neurons (Aschner, 1996). MT is important for cellular metabolism of metals, particularly in the brain. Normally, zinc and copper levels will determine MT expression (i.e. increased intracellular zinc levels leads to increased MT expression). While Mn intoxication in rats has been shown to have no effect on MT-I gene expression in striatal tissue (Zheng et al., 1999), it has yet to be determined whether astrocytic MT-I expression changes upon exposure to Mn.

Taurine is an amino acid that has been implicated as a neuroprotectant. Its osmoregulatory release from astrocytes exemplifies its neuroprotective property (Vitarella et al., 1994). Taurine uptake is mediated by a sodium-dependent uptake mechanism via the taurine transporter (tau-T, Martin, 1992). Both gene expression of tau-T (Bitoun and Tappaz, 2000) and taurine uptake (Aschner et al., 2001) are increased in astrocytes exposed to a hyperosmotic condition. Currently, the effect of Mn exposure on both taurine uptake and tau-T gene expression in astrocytes are unknown.

The Mn fuel additive, methylcyclopentadienyl manganese tricarbonyl (MMT) is an anti-knock agent that replaced lead and is being utilized in the US MMT has been reported to cause increased health problems in heavily air-polluted areas (Hakkinen et al., 1983; Logroscino et al., 1997;Sierra et al., 1995), but this remains controversial. When combusted, the Mn from MMT is in the form of several aerosolized salts, the most abundant being phosphate and sulfate (Lynam et al., 1999; Zayed et al., 1999). Pharmacokinetic studies have shown that the Mn salt will determine the rate of transport into the brain: $MnCl_2$ > $MnSO_4$ > $MnPO_4$ (Drown et al., 1986;Dorman et al., 2001). It is important to note that while the toxicity associated with these Mn species is unknown, regardless of exposure, Mn would be bound to the same ligands within biological media (e.g. blood, cerebrospinal fluid). Therefore, the primary goal of this study was to examine gene expression of GLAST, tau-T, and MT-I in astrocytes exposed to $MnCl_2$, $MnSO_4$, and $MnPO_4$ under the working hypothesis that Mn exposure, regardless of species, will affect astrocytic gene expression in the same fashion. The magnitude of the effect, however, will be determined by salt solubility, and by inference, intracellular Mn concentrations.

MATERIALS AND METHODS

Cell Cultures

Primary astrocyte cultures were prepared as described by Frangakis and Kimelberg (1984). Briefly, the cerebral hemispheres of newborn Sprague—Dawley rats were removed and meninges were carefully dissected off. The basal ganglia and midbrain were removed and the remaining cortical tissue dissociated with Dispase (Life Technologies, Gaithersburg, MD). Cells were grown and treated with Mn salts in minimal essential medium (MEM), supplemented with 10% horse serum (AGM). The cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. The media was changed twice weekly. Northern blots were performed after 3 weeks in culture, when the cells have formed a confluent monolayer. Immunocytochemically, >95% of the cells stained positively for the astrocytic marker glial fibrillary acidic protein (GFAP). Cell viability was measured by the trypan blue exclusion method (50% (v/v) of 0.4% staining solution).

Northern Blot

Following a 6 h exposure in media (see above for composition) containing 0, 10, 50, 100 or 300 μM $MnCl_2$, $MnPO_4$, or $MnSO_4$, the total RNA was isolated from astrocyte cultures with a monophase phenol and guanidine isothiocyanate solution (RNAStat-60, Tel-Test Laboratories, Friendswood, TX). RNA (10 μg) was separated by denaturing agarose electrophoresis and transferred to nitrocellulose membranes (Nytran SuPerCharge, Schleicher and Schuell, Keene, NH) by capillary action in 10x SSC overnight. Membranes were cross-linked with an ultraviolet cross-linker to immobilize RNA. Each blot was probed for MT, GLAST, and tau-T.

Blots were pre-hybridized in Ultrasensitive Hybridization Buffer (Ambion Inc., Austin, TX) at 45 °C (MT) or 50 °C (GLAST and tau-T) for 1 h, followed by overnight hybridization with approximately 10^6 cpm/ml of [alpha-^{32}P] dCTP-labeled random primed cDNA probe (RadPrime DNA Labeling System, GibcoBRL, Life Technologies). GLAST cDNA probe was obtained from Dr. Michael B. Robinson (University of Pennsylvania); MT cDNA probe from Dr. Robert Andersen (University of California, Los Angeles) and tau-T cDNA was made according to the procedure of Smith et al. (1992). Membranes were washed two to three times in 2 x SSC/0.1 % SDS at 45 °C (MT) or 50 °C (GLAST and tau-T) for 20 min. Membranes were exposed to Kodak Biomax MR film, at —80°C with intensifying screens for 2-3 days. The autoradiograms were quantified by densitometry scanning in conjunction with the TINA v2.09e computer program (Raytest USA Inc., Wilmington, NC). To correct for total RNA level, the blots were stripped in 0. 1 x SSC/0.1% SDS/ 40 mM Tris buffer and probed for 28s rRNA in 50% de-ionized formamide, 5 x Denhardt's solution, 10% dextran sulfate, 0.1% SDS, 4x SSC, 100 μg/ml salmon sperm DNA, 20 mM Tris, pH 8.0 as described by Barbu and Dautry (1989) to determine relative levels of the MT, GLAST and tau-T mRNAs. Films were digitized and band density was determined using the Tina v2.09e computer program (Raytest USA Inc., Wilmington, NC).

Statistical Analyses

All experiments were conducted in four astrocyte preparations with two to three replicates each. The data were analyzed with the GraphPad InStat version 3.02 for Windows (GraphPad Software, San Diego, CA). Two-way analysis of variance was used to test for effects of Mn salt and concentration on GLAST, MT and tau-T mRNA expression. Tukey's HSD post-hoc test was used to evaluate mean differences. One-way analysis of variance was used to test for a concentration-dependent effect on mRNA levels within each Mn salt group. Dunnett's procedure was used to determine a difference from control mRNA levels. The alpha level for the analyses was $P < 0.05$.

RESULTS

GLAST mRNA

The overall treatment effect of each Mn salt on astrocytic GLAST expression is shown in Fig. 1. $MnCl_2$ exposure significantly decreased GLAST expression relative to $MnPO_4$ ($P < 0.0001$). No significant differences in GLAST expression were noted between $MnCl_2$ and $MnSO_4$. With two of the Mn salts tested, there was a concentration-dependent decrease in GLAST mRNA levels. Exposure to 300 μM $MnCl_2$ significantly decreased GLAST expression ($P < 0.01$); both 100 and 300 μM $MnSO_4$ exposure also decreased GLAST expression ($P <$

0.05, Fig. 1). No concentration of $MnPO_4$ altered GLAST mRNA compared to control, nor was there a significant interaction between concentration and Mn salt main effects.

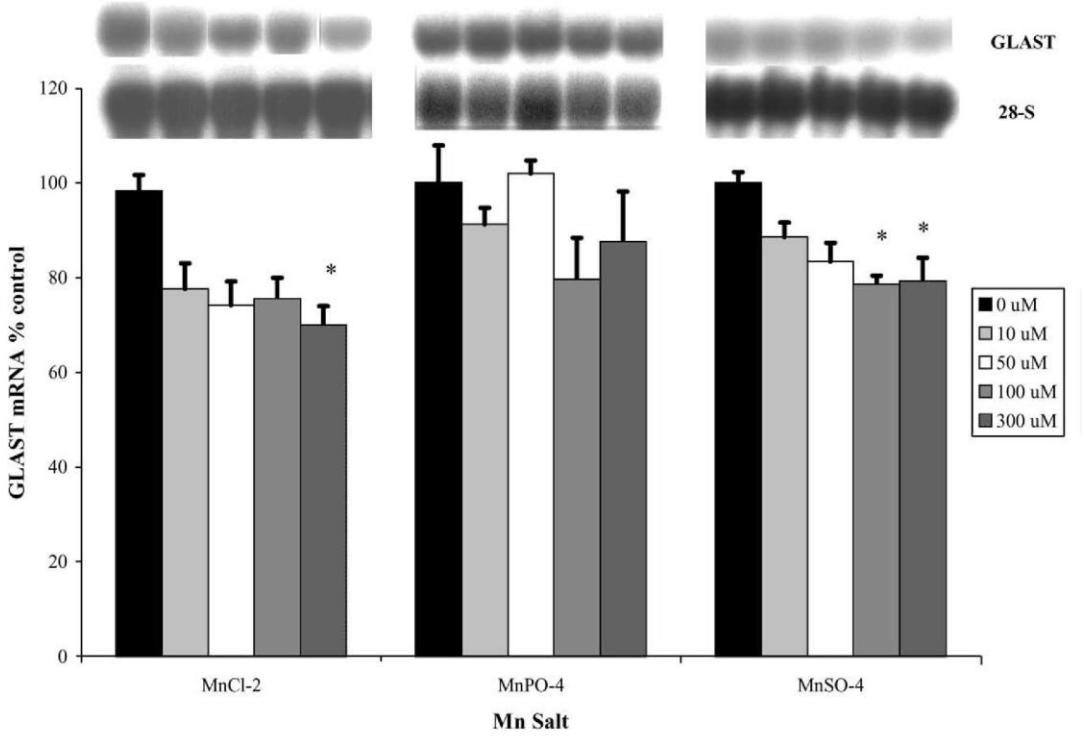

Fig. 1. GLAST mRNA expression in astrocytes exposed to $MnCl_2$, $MnPO_4$, or $MnSO_4$ for 6 h. Data are mean ± S.E.M. for four astrocyte preparations with two to three replicates per Mn concentration. Astrocytes incubated in media containing 300 μM $MnCl_2$ and 100 and 300 μM $MnSO_4$ had significantly lower GLAST mRNA levels compared to their respective control levels as indicated by asterices "*". Representative Northern blots are shown. All were standardized with 28s rRNA (representative blot shown).

MT mRNA

The overall treatment effect of each Mn salt on astrocytic MT-1 mRNA expression is shown in Fig. 2. $MnCl_2$ exerted the most potent effect significantly reducing MT gene expression compared to both $MnPO_4$ and $MnSO_4$ species ($P < 0.0001$). Within the $MnCl_2$ group, 50, 100 and 300 μM exposure significantly ($P < 0.01$) lowered MT expression com-pared to control. Within the $MnPO_4$ group, 100 and 300 μM exposure significantly ($P < 0.05$) lowered MT expression compared to control. Within the $MnSO_4$ group, 300 μM exposure significantly ($P < 0.05$) lowered MT expression compared to control. There was no significant interaction between concentration and Mn salt-type.

Tau-T mRNA

The tau-T mRNA expression was not affected by any of the Mn salts exposed to various concentrations for 6 h (Fig. 3).

DISCUSSION

This study demonstrates variable responses in astrocyte gene expression in response to potentially toxic concentrations of Mn with three different salt complexes. The main effect of Mn species was consistent with pharmacokinetic experiments where the solubility of Mn species has been shown to be an important determinant (Dorman et al., 2001). According to the Merck Index, the solubility of the three Mn complexes are as follows: $MnCl_2 > MnSO_4 \gg MnPO_4$. The present study shows that astrocytic gene expression is dependent upon Mn species and that the effect on GLAST mRNA was determined by Mn-salt solubility. For example, $MnPO_4$ did not alter GLAST mRNA whereas $MnCl_2$ significantly decreased expression, as did $MnSO_4$, albeit to a lesser extent (Fig. 1). Additionally, MT mRNA levels were profoundly decreased by $MnCl_2$ exposure compared to both $MnSO_4$ and $MnPO_4$ (Fig. 2).

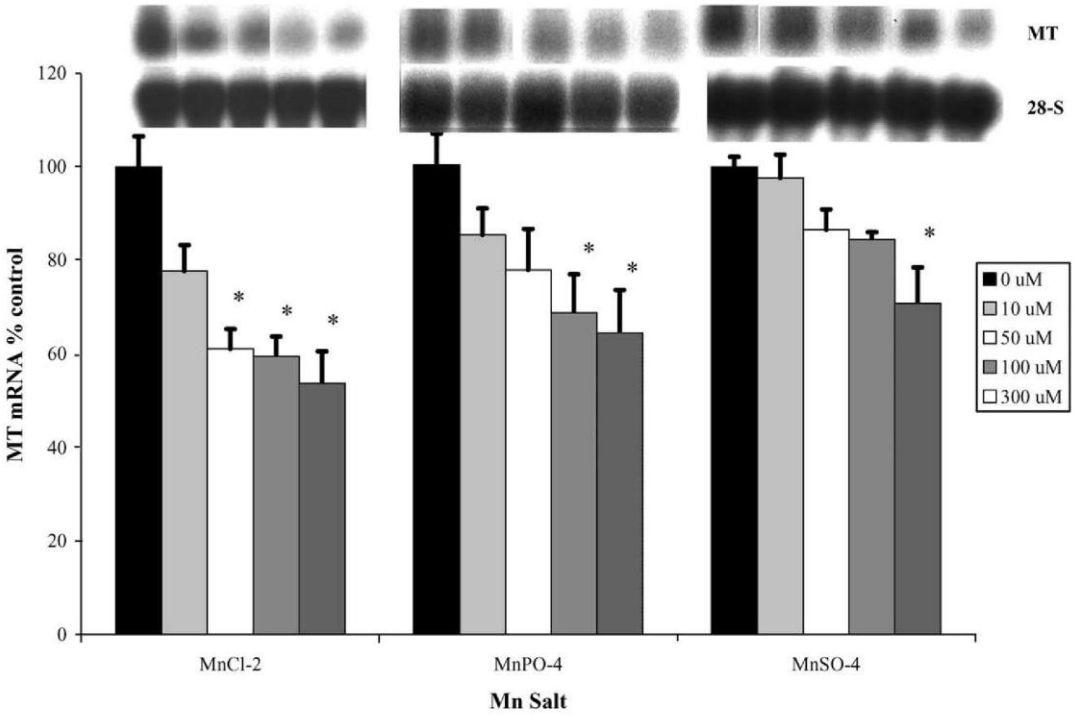

Fig. 2. MT mRNA expression in astrocytes exposed to $MnCl_2$, $MnPO_4$, or $MnSO_4$ for 6 h. Data are mean ± S.E.M. for four astrocyte preparations with two to three replicates per Mn concentration. Astrocytes incubated in media containing 50, 100 and 300 μM $MnCl_2$, 100 and 300 μM $MnPO_4$, and 300 μM $MnSO_4$ had significantly lower MT mRNA levels compared to their respective control levels as indicated by asterices "∗". Representative Northern blots are shown. All were standardized with 28s rRNA (representative blot shown).

Studies conducted by Vitarella et al. (2000) showed that manganese phosphate (as hureaulite) and Mn_3O_4 are relatively insoluble in simulated lung lining fluids, while $MnSO_4$ is considerably more soluble in biological fluids and that solubility influenced lung clearance rates. Dorman et al. (2001) confirmed that brain delivery of inhaled Mn is likewise influenced by particle solubility. Manganese is more rapidly cleared from the rat lung following inhalation of a soluble form of Mn (e.g. $MnSO_4$) when compared with considerably less soluble Mn particles like the phosphate or tetroxide forms (Dorman et al., 2001). Moreover, animals exposed to high levels (3 mg Mn/m^3) of the soluble sulfate form have significantly higher brain (striatal) manganese concentrations when compared with levels achieved following exposure to the insoluble manganese tetroxide or phosphate forms.

GLAST is the most prevalent glutamate transporter in cultured astrocytes (Kondo et al., 1995). It is, therefore, probable that the decreased glutamate uptake observed in Mn-exposed astrocytes (Hazell and Norenberg, 1997; Erikson and Aschner, in press) represents decreased GLAST expression. Glutamate transporters are rapidly synthesized, and a cytosolic pool of these proteins is available for plasma membrane insertion as functional proteins in response to changes in extracellular glutamate levels (Davis et al., 1998). Decreased glutamate uptake observed in manganese-exposed astrocytes is, therefore, potentially due to decreased functional glutamate transporters (e.g. GLAST). While the protein levels of GLAST were not measured in the present study, mRNA levels were indicative of decreased message due to Mn treatment (Fig. 1), potentially leading to decreased protein levels as well.

MT is important for normal cellular homeostasis including gene regulation, adaptation to stress, and metal metabolism (Cherian et al., 1997;Dunn et al., 1987; Durnam and Palmiter, 1987). MT-I gene expression increases in response to elevated intracellular zinc as well as other divalent metals (Katakai et al., 2001). Thus, the decrease in MT-I expression in Mn-treated astrocytes was quite unexpected (Fig. 2). A number of explanations can account for this effect. Increased intracellular Mn concentration might diminish the content of other divalent metals (e.g. zinc, copper), thereby exerting feedback inhibition, and decreasing MT mRNA expression. This is consistent with a recent report that decreased hepatic copper levels are associated with reduced MT-I expression (Aoyama et al., 2001). To date, it is unknown what, if any, effect Mn has on the

metabolism of other metals in astrocytes. However, in the brain, increased Mn has been shown to interfere with divalent metal metabolism (Zheng et al., 1999). Another potential explanation is that decreased expression of MT-I mRNA upon Mn exposure is associated with increased MT protein levels (inhibiting MT mRNA synthesis) or post-transcriptional changes that increase the degradation process of this mRNA.

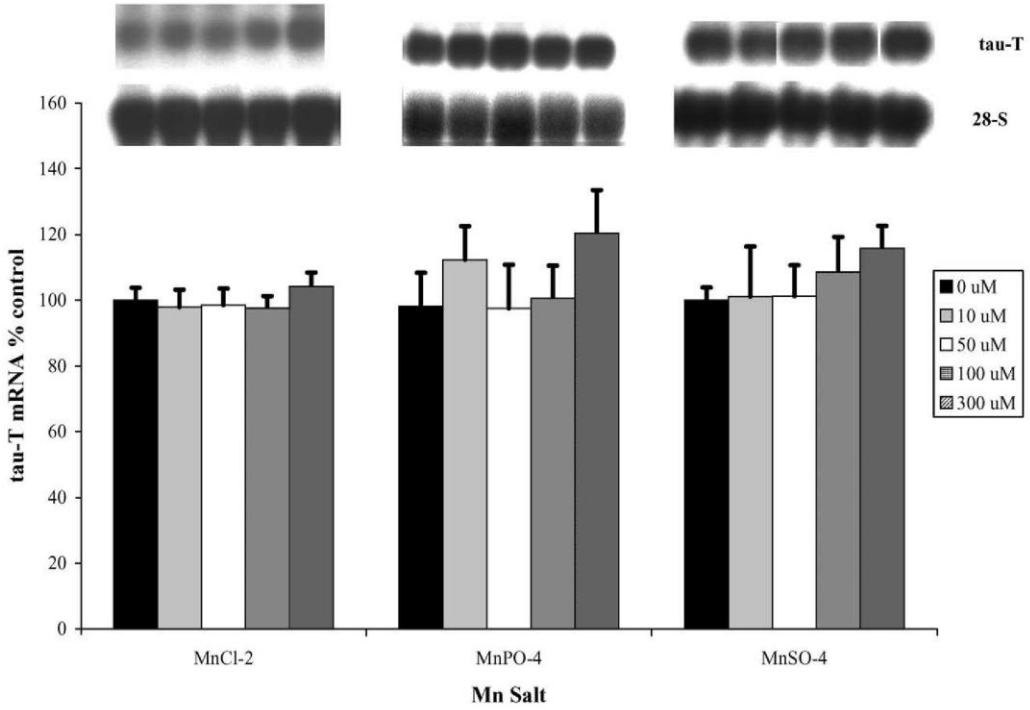

Fig. 3. The tau-T mRNA expression in astrocytes exposed to $MnCl_2$, $MnPO_4$, or $MnSO_4$ for 6 h. Data are mean ± S.E.M. for four astrocyte preparations with two to three replicates per Mn concentration. There was no significant effect of Mn on tau-T mRNA expression after a 6 h exposure. Representative northern blots are shown. All were standardized with 28s rRNA (representative blot shown).

Our laboratory has shown that astrocytes exposed to $MnCl_2$ for 18 h, significantly increased tau-T mRNA levels. In this study, astrocytes exposed to $MnCl_2$, $MnSO_4$, and $MnPO_4$ for 6 h did not have altered tau-T mRNA levels. Our previous study suggested that taurine and glutamate metabolism in astrocytes are regulated quite differently (Erikson and Aschner, in press). GLAST mRNA decreased in response to Mn exposure as did glutamate uptake. Conversely, tau-T mRNA increased in response to 18 h $MnCl_2$ exposure, but taurine uptake did not change. However, in astrocytes exposed to ethanol for 24 h there was no effect on taurine uptake, but 96 h exposure increased its uptake (Aschner et al., 2001). Thus, while glutamate metabolism responds immediately to Mn exposure, taurine metabolism is only altered after long-term exposure (>18 h).

In conclusion, our data indicate that 6 h Mn exposure significantly decreases astrocytic GLAST and MT mRNA expression. This effect is salt-dependent, with $MnCl_2$, and to a lesser extent $MnSO_4$, decreasing both mRNAs significantly. $MnPO_4$ decreased astrocytic MT mRNA but did not affect GLAST mRNA expression. Therefore, in terms of overall effect on astrocytic gene expression, the following ranking is apparent: $MnCl_2 \gg MnSO_4 > MnPO_4$. These data are consistent with the original hypothesis that the neurotoxicity associated with Mn exposure is salt species-dependent, with solubility and by inference, intracellular Mn concentrations, representing the most critical determinant of neurotoxicity.

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