

Manganese Causes Differential Regulation of Glutamate Transporter (GLAST) Taurine Transporter and Metallothionein in Cultured Rat Astrocytes

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Abstract:

Neurotoxicity due to excessive brain manganese (Mn) can occur due to environmental (air pollution, soil, water) and/ or metabolic aberrations (decreased biliary excretion). Manganese is associated with 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], it is likely that in pathological conditions it can reach 100–500 μM . Amino acid (e.g. aspartate, glutamate, taurine), as well as divalent metal (e.g. zinc, manganese) concentrations are regulated by astrocytes in the brain. Recently, it has been reported that cultured rat primary astrocytes exposed to Mn displayed decreased glutamate uptake, thereby, increasing the excitotoxic potential of glutamate. Since the neurotoxic mechanism(s) Mn employs in terms of glutamate metabolism is unknown, a primary goal of this study was to link altered glutamate uptake in Mn exposed astrocytes to alterations in glutamate transporter message. Further, we wanted to examine the gene expression of metallothionein (MT) and taurine transporter (tau-T) as markers of Mn exposure. Glutamate uptake was decreased by nearly 40% in accordance with a 48% decrease in glutamate/aspartate transporter (GLAST) mRNA. Taurine uptake was unaffected by Mn exposure even though tau-T mRNA increased by 123%. MT mRNA decreased in these Mn exposed astrocytes possibly due to altered metal metabolism, although this was not examined. These data show that glutamate and taurine transport in Mn exposed astrocytes are temporally different.

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

Article:

INTRODUCTION

Manganese (Mn) is an essential nutrient and is important for cellular functioning. However, excess Mn is known to cause neurotoxicity. 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] levels upon manganese intoxication. A study performed almost three decades ago, examined [Mn] 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] reached 264 μM , while globus pallidus [Mn] peaked at 334 μM . In rats, after Mn dosing, [Mn] can reach up to 200 μM depending upon brain region examined and dosing regimen ([Ingersoll et al., 1999](#); [Lai et al., 1999](#) and [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. 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, 1971](#); [Westergaard et al., 1995](#); [Ottersen et al., 1992](#)). Therefore, an attenuation of this pathway could be detrimental to neurons dependent on this astrocytic function.

It has been estimated that 80% of 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 and glutamate/aspartate transporter (GLAST) are the prominent astrocytic transporters (Danbolt, 2001).

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 oxidants by metallothionein (MT). 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 cells (Aschner, 1996). MT is important for cellular metabolism of metals, particularly in the brain where neural tissue is sensitive to any changes. 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, both taurine uptake and tau-T gene expression in astrocytes exposed to Mn are unknown.

Given the critical role of astrocytes in maintaining the composition of the extracellular fluid, the goals of this study were (1) to link altered glutamate uptake in Mn exposed cultured astrocytes to alterations in transporter metabolism (GLAST mRNA levels) and (2) to examine the gene expression of MT-I and tau-T as markers of Mn exposure.

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 in minimal essential medium (MEM), supplemented with 10% horse serum (AGM). The cultures were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37 °C. The media was changed twice weekly. ³H-aspartic acid uptake experiments and 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).

³H-Aspartic Acid and ³H-Taurine Uptake

Uptake of [2,3, -³H]-D-aspartate and [1,2, -³H] taurine were measured as described by Aschner et al. (1993). D-Aspartate is a non-metabolizable glutamate analogue that shares the same transporter as glutamate (Drejer et al., 1982). Astrocytes (grown on six well plates for 3–4 weeks) were incubated overnight at 37°C with AGM

containing 0, 100, 250 or 500 μM MnCl_2 . The next day, cells were washed 3 times with HEPES buffer and incubated 1 min with HEPES buffer containing 0.5 μCi [2,3,- ^3H]-D-aspartate. The reaction was stopped by aspirating the buffer and washing the cells 4 times with cold (4°C) 290 mM mannitol buffer, containing 0.5 mM calcium nitrate to maintain cell adhesion to the substrate. Cells were solubilized in 2 ml 1 M NaOH, and aliquots (750 μl) were neutralized for 0-counting with a Beckman LS 3801 liquid scintillation analyzer (Beckman Instruments) and for protein determination (50 μl) with the bicinchonic assay (BCA, Pierce Chemicals).

Northern Blot

Following an overnight (18 h) exposure to 0, 100, 250 or 500 μM MnCl_2 , the total RNA was isolated from astrocyte cultures with a monophasic 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 10 x SSC overnight. Membranes were cross-linked with an ultraviolet cross-linker to immobilize RNA. Each blot was probed for MT, GLAST and taurine transporter (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 [α - ^{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 Taurine Transporter cDNA was made according to [Smith et al., 1992](#). Membranes were washed 2–3 times in 2x SSC/0.1% SDS at 45°C (MT) or 50°C (GLAST and tau-T) for 20 min. Membranes were exposed to Kodak Bio-max 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, 4 x SSC, 100 $\mu\text{g}/\text{ml}$ 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 five astrocyte preparations 1–2 replicates each (northern analysis), or 3–6 replicates each (uptake experiments). The data were analyzed with the GraphPad InStat version 3.02 for Windows, (GraphPad Software, San Diego, CA). One-way analysis of variance was carried out to test for concentration-dependent effects. Dunnett's procedure was used to evaluate mean differences compared to control (0 μM MnCl_2 treatment). The alpha level for the analyses was set at $P < 0.05$.

RESULTS

^3H -Aspartic Acid and ^3H -Taurine Uptake

Overnight incubation with medium containing both 250 and 500 μM MnCl_2 significantly decreased the 1 min ^3H -aspartate uptake in astrocytes by 24 and 38%, respectively, ($P = 0.041$) ([Fig. 1](#)) compared to control (0 μM MnCl_2). However, no concentration of MnCl_2 affected astrocyte ^3H -taurine uptake ($P = 0.54$) ([Fig. 2](#)).

Northern Analysis

Exposure to 500 μM MnCl_2 for 18 h significantly decreased astrocytic GLAST mRNA (48%; $P = 0.037$) ([Fig. 3](#)) and MT mRNA (55%; $P = 0.012$) ([Fig. 4](#)) and increased tau-T mRNA (124%; $P = 0.008$) ([Fig. 5](#)) compared to control. All three mRNAs changed with 100 and 250 μM MnCl_2 exposure, however, these changes were not significantly different from the control astrocyte mRNA expression levels.

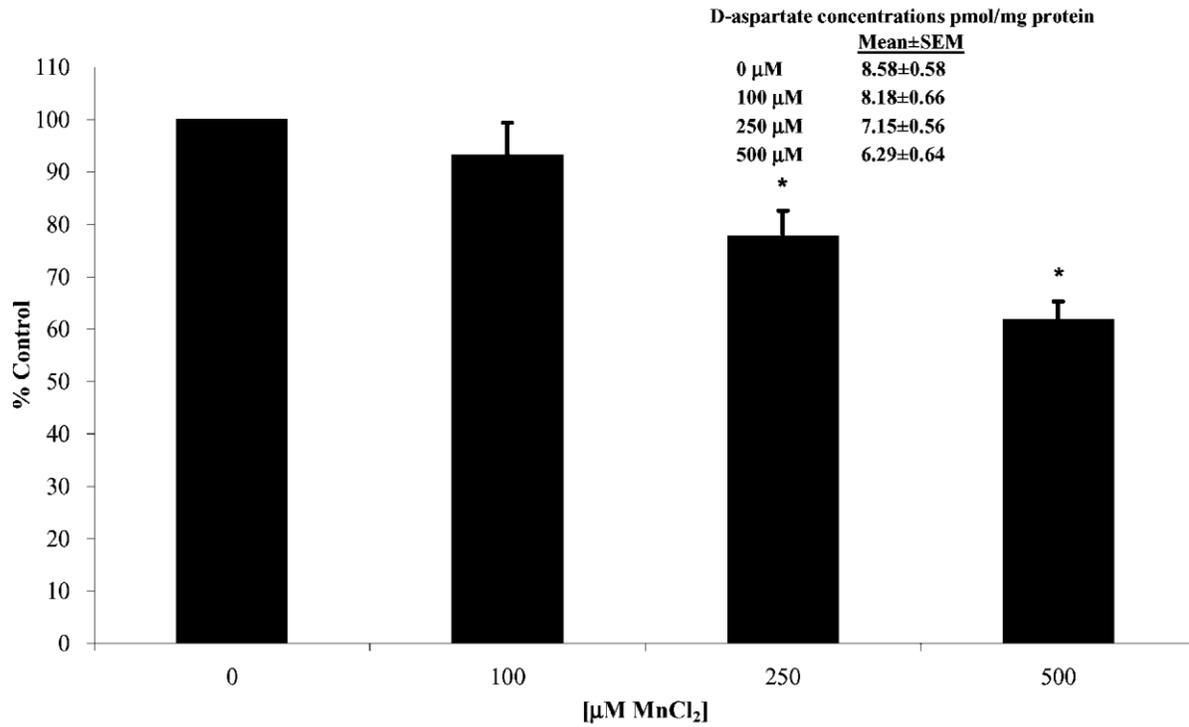


Fig. 1. One-minute uptake of ³H-D-aspartic acid in astrocytes exposed overnight to MnCl₂. Data are Mean ± S.E.M. for five independent astrocyte preparations with 3–6 replicates each per MnCl₂ concentration (*n* = 24, per concentration). Both 250 and 500 μM MnCl₂ exposure led to a significant decrease in ³H-aspartate uptake compared to 0 μM MnCl₂, indicated by asterices “*”.

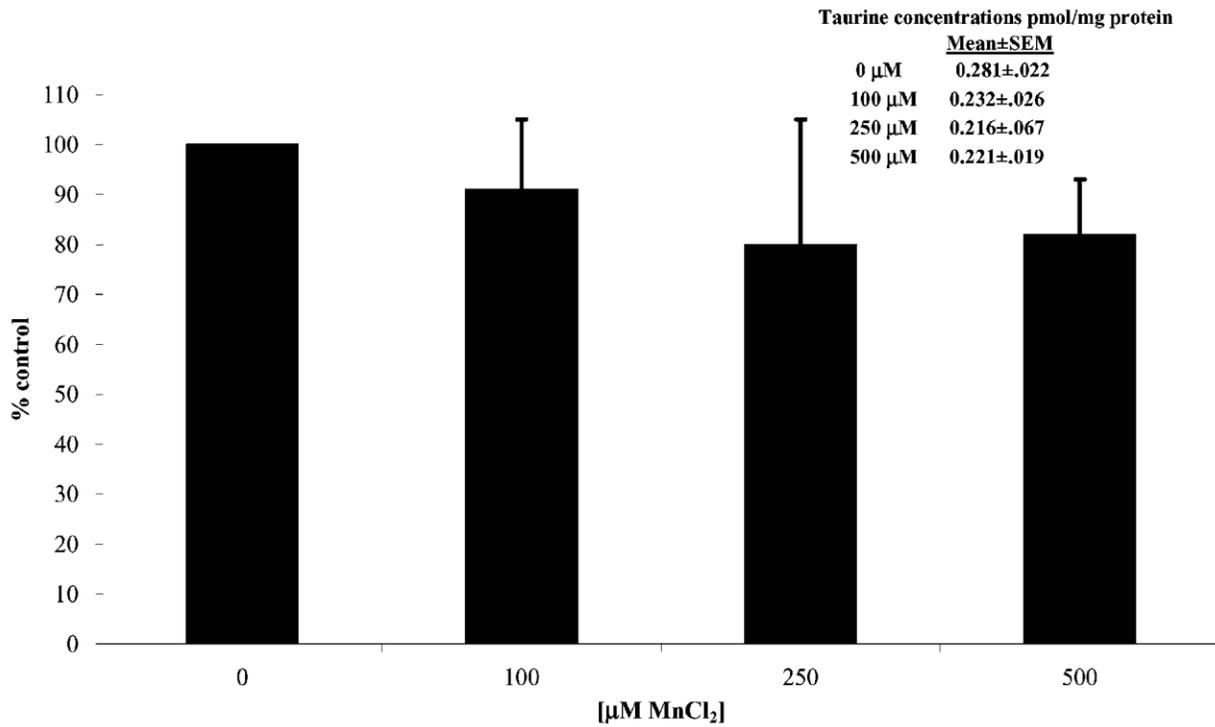


Fig. 2. One-minute uptake of ³H-aurine in astrocytes exposed overnight to MnCl₂. Data are Mean ± S.E.M. for five independent astrocyte preparations with 3–6 replicates each per MnCl₂ concentration (*n* = 21, per concentration). There was no significant effect of MnCl₂ exposure on ³H-aurine uptake.

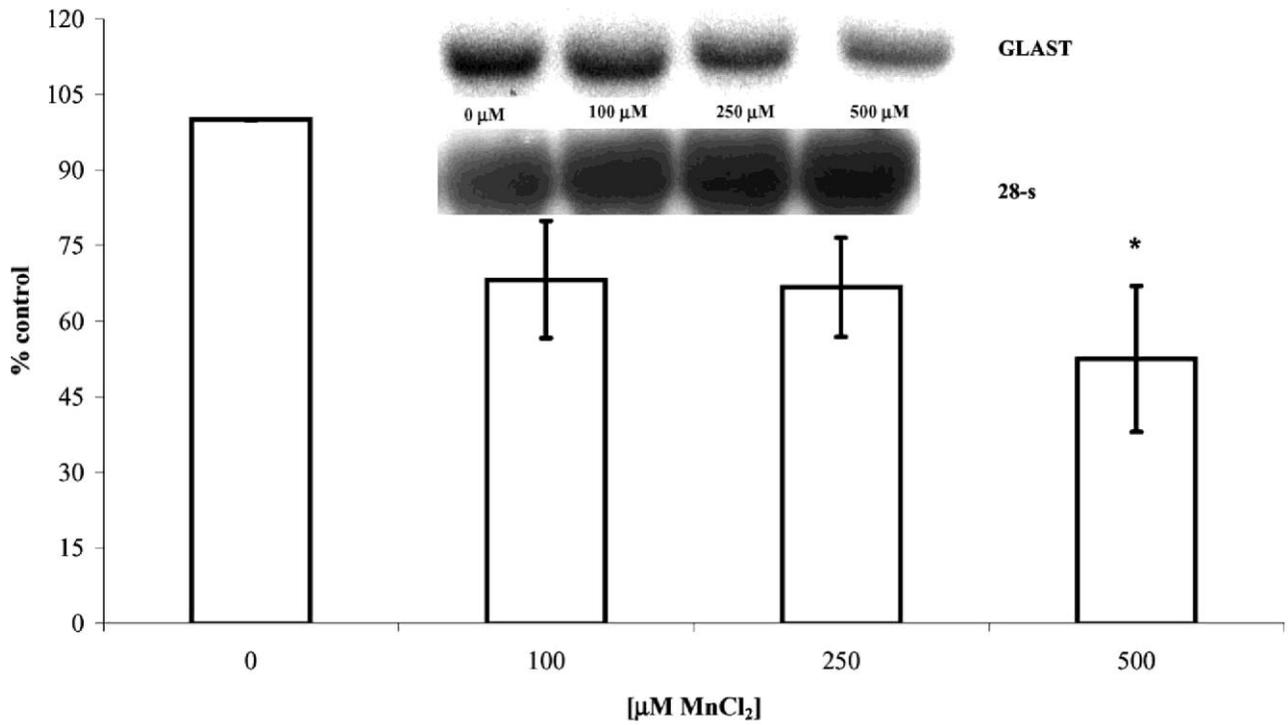


Fig. 3. GLAST mRNA expression in astrocytes exposed overnight to MnCl_2 . Data are Mean \pm S.E.M. for five independent astrocyte preparations with 1–2 replicates per MnCl_2 concentration. Specifically, 0 μM , $n = 8$; 100 μM , $n = 9$; 250 μM , $n = 7$; 500 μM , $n = 8$. Astrocytes incubated in media containing 500 μM MnCl_2 had significantly lower GLAST mRNA levels compared to control as indicated by asterisk “*”. Northern blot shown is a representative one. All were standardized with 28-S rRNA (representative blot shown).

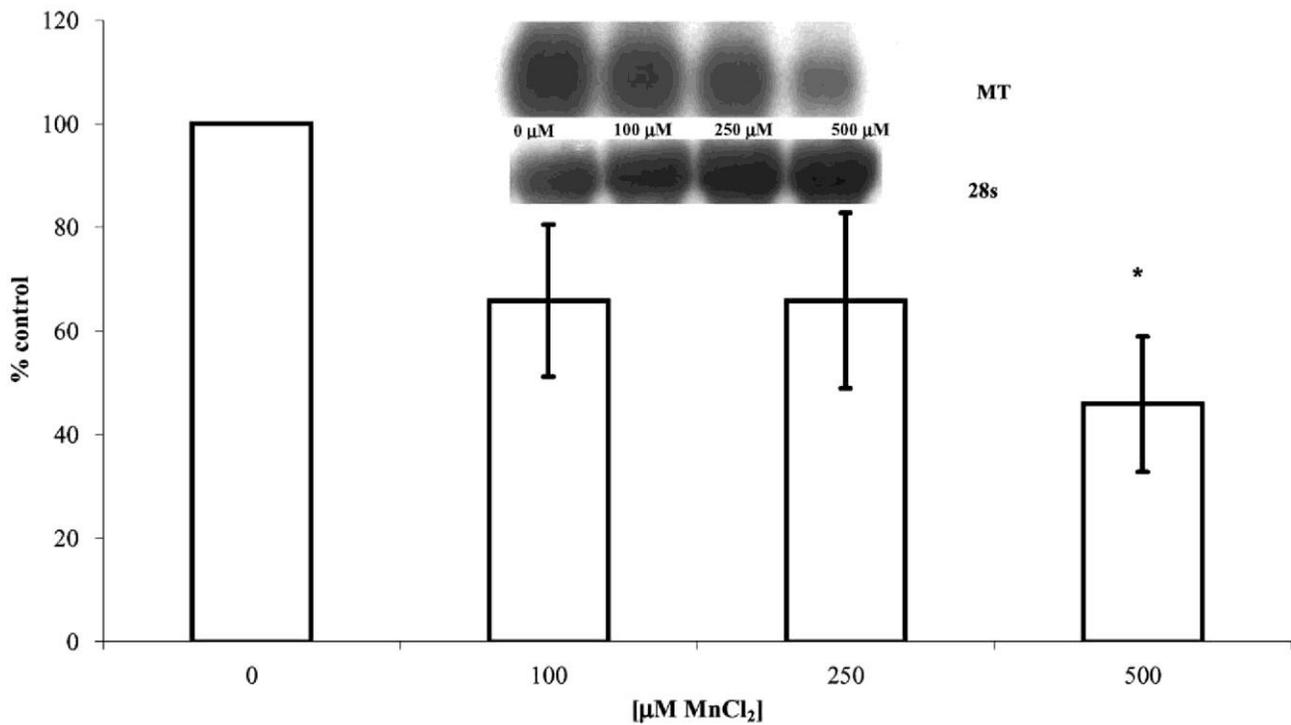


Fig. 4. MT mRNA expression in astrocytes exposed overnight to MnCl_2 . Data are Mean \pm S.E.M. for five independent astrocyte preparations with 1–2 replicates per MnCl_2 concentration. Specifically, 0 μM , $n = 8$; 100 μM , $n = 9$; 250 μM , $n = 7$; 500 μM , $n = 8$. Astrocytes incubated in media containing 500 μM MnCl_2 had significantly lower MT mRNA levels compared to control as indicated by asterisk “*”. Northern blot shown is a representative one. All were standardized with 28-S rRNA (representative blot shown).

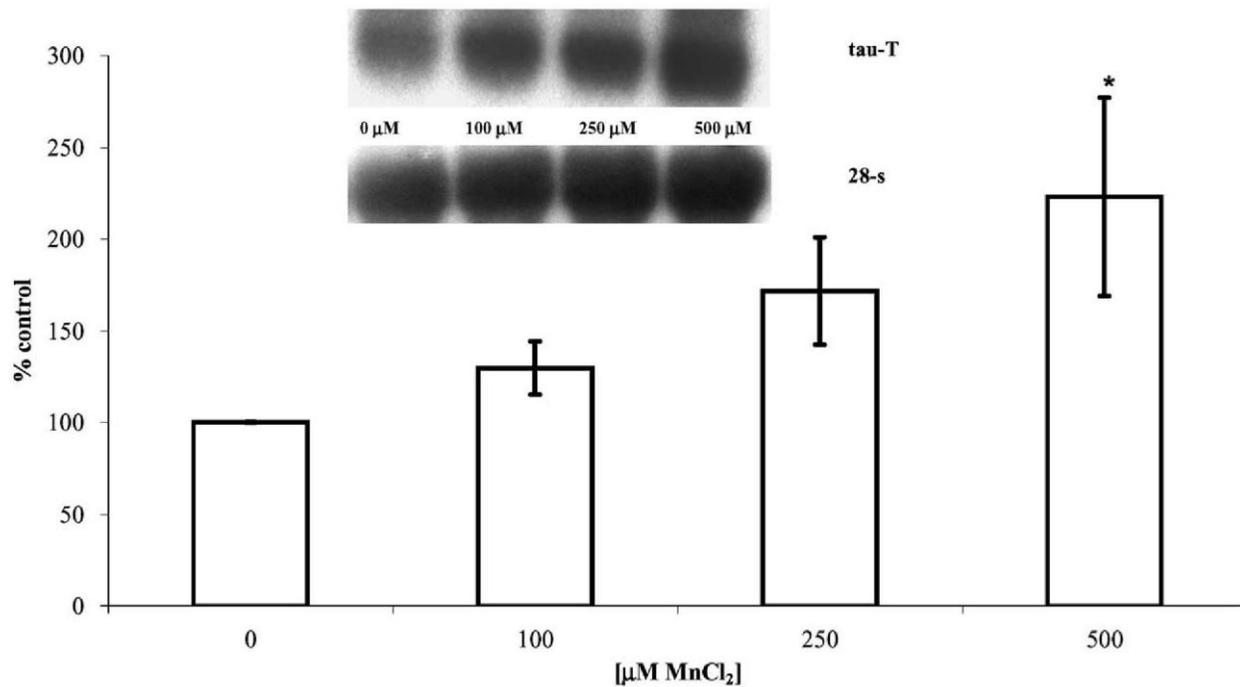


Fig. 5. tau-T mRNA expression in astrocytes exposed overnight to MnCl₂. Data are Mean ± S.E.M. for five independent astrocyte preparations with 1–2 replicates per MnCl₂ concentration. Specifically, 0 μM, *n* = 8; 100 μM, *n* = 9; 250 μM, *n* = 7; 500 μM, *n* = 8. Astrocytes incubated in media containing 500 μM MnCl₂ had significantly higher tau-T mRNA levels compared to control as indicated by asterisk “*”. Northern blot shown is a representative one. All were standardized with 28-S rRNA (representative blot shown).

DISCUSSION

This study demonstrates variable responses in astrocyte gene expression and amino acid uptake in response to potentially toxic concentrations of Mn. Exposure to 500 μM MnCl₂ led to decreased mRNA levels of GLAST and MT, but the same concentration of manganese increased tau-T mRNA levels. Furthermore, aspartate uptake was significantly attenuated in Mn exposed astrocytes (250 and 500 μM) (Fig. 1), whereas, taurine uptake remained unchanged (Fig. 2). Both taurine uptake and tau-T mRNA data demonstrate that changes in aspartate uptake and GLAST, MT mRNA are specific to Mn exposure and not frank cytotoxicity.

The decreased uptake of D-aspartate by astrocytes exposed to Mn corroborates an earlier study by Hazell and Norenberg (1997). Since astrocytes take up about 80% of extracellular glutamate (Aschner et al., 2001), any decrease in this uptake process can have profound neurotoxicological implications. Therefore, the excitotoxic effect of increased extracellular glutamate associated with Mn-induced uptake inhibition, might represent a neurotoxic mechanism of manganese (Stokes et al., 2000; DeSole et al., 1997; Miele et al., 2000; Montes et al., 2001). Most literature on Mn neurotoxicity and neurotransmitter metabolism focuses on alterations in the dopamine system (Verity, 1999). However, this study lends credence to the possibility of alterations in the glutamatergic system, as well as Y-amino butyric acid (GABA) systems, particularly in striatum where cortical glutamatergic afferents converge. Altered glutamatergic and GABAergic functioning can contribute to altered striatal dopamine metabolism (Page et al., 2001; Castro and Zigmond, 2001). Therefore, the neurotoxic effects of Mn on striatal dopamine may be indirectly mediated via abnormal striatal glutamate and/or GABA metabolism.

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 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 extra-cellular glutamate levels (Davis et al., 1998). Decreased glutamate uptake observed in Mn-exposed astrocytes is, therefore, potentially due to decreased functional GLASTs. While the protein levels of GLAST were not measured in the present

study, mRNA levels were indicative of decreased message due to manganese treatment (Fig. 3), 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, it was quite unexpected to note decreased MT-I expression in Mn-treated astrocytes (Fig. 4). A number of explanations can account for this effect. First, increased intracellular [Mn] 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 proven to interfere with divalent metal metabolism (Zheng et al., 1999). A second potential explanation is that decreased expression of MT-I mRNA upon Mn exposure is not associated with changes in functional MT protein levels, which have not been measured in the present study.

Our study shows that astrocytes, exposed to Mn, have varying amino acid uptake capacities. This differential effect is evidenced by a decline in glutamate but not taurine uptake. When linking uptake of these amino acids to their respective transporters, the decreased glutamate uptake is congruent with GLAST expression. However, the unchanged taurine uptake is in contrast to the significant increase in tau-T mRNA (Fig. 5). One explanation of this inconsistency is that the 18 h exposure time to manganese was adequate for tau-T transcription, but not translation. Alternatively, the tau-T transporter protein may be present in these cells, but incorporation into the membrane is altered (i.e. decreased functional transporter). Protein kinase C (PKC) has been implicated in regulating tau-T, with increased PKC activity (increased phosphorylation) causing decreased taurine uptake in astrocytes (Tchoumkeu-Nzouessa and Rebel, 1996) and retinal cells (Loo et al., 1996). Unfortunately, no data are available on the effect of Mn on PKC activity, and the possibility that PKC regulates tau-T expression will have to await additional studies. Similarly, data on Mn-induced tau-T expression are lacking.

It has been reported that hyperosmotic medium increases tau-T mRNA levels in astrocytes (Bitoun and Tappaz, 2000). However, it is unlikely that Mn is causing a hyperosmotic condition in the experiments presented herein. Therefore, a more plausible explanation of our data is that the astrocytes are “gearing up” for increased taurine transport, but that the time frame used in this study was too short to measure a change in the functional capacity of tau-T. Indeed, we previously observed this phenomena in ethanol treated astrocytes where 24 h exposure did not effect taurine uptake, but 96 h exposure increased its uptake (Aschner et al., 2001). Taken collectively, these data support the fact that taurine and glutamate transport in astrocytes are temporally different.

In conclusion, our data indicate that decreased glutamate uptake due to Mn exposure in astrocytes is linked to decreased GLAST mRNA. The experiments also support the hypothesis that manganese exposure might lead to exaggerated excitotoxic response due to diminished glutamate uptake by astrocytes. Further-more, the decreased MT-I mRNA and increased tau-T mRNA demonstrate a differential response by astrocytes to Mn exposure. Future studies will focus on measuring MT and tau-T protein levels and their role in Mn neurotoxicity.

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