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Manganese exposure inhibits the clearance of extracellular GABA and influences taurine homeostasis in the striatum of developing rats

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Abstract

Manganese (Mn) accumulation in the brain has been shown to alter the neurochemistry of the basal ganglia. Mn-induced alterations in dopamine biology are fairly well understood, but recently more evidence has emerged characterizing the role of γ -aminobutyric acid (GABA) in this dysfunction. The purpose of this study was to determine if the previously observed Mn-induced increase in extracellular GABA (GABA_{EC}) was due to altered GABA transporter (GAT) function, and whether Mn perturbs other amino acid neurotransmitters, namely taurine and glycine (known modulators of GABA). Extracellular GABA, taurine, and glycine concentrations were collected from the striatum of control (CN) or Mn-exposed Sprague-Dawley rats using *in vivo* microdialysis, and the GAT inhibitor nipecotic acid (NA) was used to probe GAT function. Tissue and extracellular Mn levels were significantly increased, and the Fe:Mn ratio was decreased 36-fold in the extracellular space due to Mn exposure. NA led to a 2-fold increase in GABA_{EC} of CNs, a response that was attenuated by Mn. Taurine responded inversely to GABA, and a novel 10-fold increase in taurine was observed after the removal of NA in CNs. Mn blunted this response and nearly abolished extracellular taurine throughout collection. Striatal taurine transporter (Slc6a6) mRNA levels were significantly increased with Mn exposure, and Mn significantly increased ³H-Taurine uptake after 3-minute exposure in primary rat astrocytes. These data suggest that Mn increases GABA_{EC} by inhibiting the function of GAT, and that perturbed taurine homeostasis potentially impacts neural function by jeopardizing the osmoregulatory and neuromodulatory functions of taurine in the brain.

Keywords

manganese; GABA; taurine; neurotoxicity; microdialysis; nipecotic acid

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1. Introduction

An essential trace element and a cofactor for several enzymes (Hurley and Keen, 1987), manganese (Mn) is involved in immune function, regulation of metabolism, reproduction, digestion, bone growth, and blood clotting (see review by Aschner et al., 2005). While frank manganese deficiency has not been clinically observed in humans, Mn toxicity, in particular Mn neurotoxicity, is of concern (Aschner et al., 2005; Dobson et al., 2004). A recent study suggests that high levels of Mn in drinking water ($>300 \mu\text{g/L}$) are associated with reduced intellectual function in children (Wasserman et al., 2006) likely due to altered neurochemistry (Garcia et al., 2006; Anderson et al., 2008, 2009). Manganese neurotoxicity shares similarities with the neurodegenerative disorder Parkinson's disease (Beuter et al., 1994; Calne et al., 1994; Pal et al., 1999), though the two are clinically distinct (Calne et al., 1994; Pal et al., 1999; Perl and Olanow, 2007). Due to the similarities of Mn neurotoxicity with Parkinson's disease, most research in the area of Mn neurotoxicity has focused on its effect on the biology of dopamine. Recently it has become clear that alterations in the biology of other neurotransmitters are involved in the etiology of Mn neurotoxicity, with the most evidence concerning γ -aminobutyric acid (GABA) (Anderson et al., 2007, 2008; Garcia et al., 2006; 2007; Gwiazda et al., 2002).

With the intriguing findings that striatal extracellular GABA (GABA_{EC}) concentrations are higher due to Mn-exposure (Anderson et al., 2008), and uptake of ^3H -GABA is attenuated by Mn-exposure in striatal synaptosomes (Anderson et al., 2007) despite no significant effect of Mn on GABA transporter (GAT) protein and mRNA levels (Anderson et al., 2008); we hypothesize that the functioning of the transporter is altered by Mn exposure leading to attenuation of GABA reuptake. Thus, we designed our current experiment to pharmacologically probe the functioning of GAT by administering a known uptake inhibitor, nipepicotic acid (NA). NA has a high binding affinity for human GAT-1 and rat GAT-1 and -2, decreasing astrocyte and neuronal GABA uptake (Krogsgaard-Larsen 1980; Krogsgaard-Larsen et al., 2000). We can, therefore, test GAT function by measuring the increase in GABA_{EC} concentrations in the striatum of Mn-exposed rats by comparing them to the controls. The use of NA is also advantageous because it does not block the transport of other amino acids neurotransmitters, most notably taurine (del Olmo et al., 2004).

Taurine is an abundant non-essential amino acid in the brain formed from cysteine. Traditionally, brain taurine is thought to function as an osmoregulator in cells (cell volume regulation), but has also been implicated in neuromodulation, possibly functioning as a neurotransmitter. Data exist suggesting that taurine functions as an anxiolytic agent (Kong et al., 2006) and interacts with the GABA_A receptor (Jia et al., 2008). These data make sense given that it has long been recognized that taurine and GABA are structurally similar and may share transporters in the brain.

We chose to look at the taurine/GABA relationship in the striatum because it is a known region for Mn accumulation (Erikson et al., 2005; Liu et al., 2000) and because the GABAergic medium spiny neurons of the striatum help orchestrate dopaminergic activity in the basal ganglia (Ade et al., 2008), where dysfunction is known to contribute to movement abnormalities during Mn neurotoxicity (Carlsson and Carlsson, 1990). Microdialysate fractions collected from the striatum of rats revealed that taurine release was higher than glutamate and glycine, and that overall the striatum is very rich in taurine (Molchanova et al., 2004). To date, however, the effect of Mn exposure on extracellular taurine (Tau_{EC}) in the striatum is unknown. Therefore, we sought to determine if Mn exposure effects Tau_{EC} concentrations in the striatum possibly as it relates to GABA biology.

In addition to GABA and taurine, we felt it was prudent to examine the effect of Mn on another amino acid neurotransmitter, glycine. Glycine is an abundant inhibitor neurotransmitter, similar to GABA, and it is known that taurine is a glycine receptor agonist (Xu et al., 2006). Although previous studies have not shown Mn to have an effect on extracellular glycine (Gly_{EC}) levels in the striatum (Takeda et al., 2003), it is possible that glycine levels may be affected due to potential alterations in GABA or taurine concentrations driven by NA or Mn exposure.

Within the brain, astrocytes are the primary cells that maintain the composition of the extracellular fluid (Wang and Bordey, 2008). It is logical, therefore, that disturbances in GABA_{EC}, Gly_{EC} and Tau_{EC} caused by Mn exposure could be due to astrocyte dysfunction. Thus, our final goal of this study was to examine the effect of Mn exposure on amino acid biology in primary astrocyte cultures.

2. Materials and Methods

2.1 Animals

Male weanling (post-natal day 21) Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) ($n=8$ for microdialysis study; $n=6$ for PCR gene expression and metal analysis studies) were randomly divided into two dietary treatment groups used in previous studies (Anderson et al., 2007, 2008): control (CN; 35 mg Fe/kg, 10 mg Mn/kg diet & d.i. water) and Mn-exposed (Mn; control diet & 1 g Mn (as MnCl₂)/L d.i. water). Diets were obtained from Bio-Serv (Frenchtown, NJ) and certified for metal content. Rats had free access to food and water 24 hr/day, with the lights off between 1800 and 600 h and room temperature maintained at $25 \pm 1^\circ\text{C}$. The University of North Carolina at Greensboro Animal Care and Use Committee approved all of the animal procedures.

2.2 Cell Cultures

Rat primary cortical astrocyte cultures were purchased from Invitrogen (Carlsbad, CA) and certified for purity with > 95% staining positive for the astrocytic marker glial fibrillary acidic protein (GFAP). Cells were grown in Dulbecco's Modified Eagle Media (D-MEM) with 15% fetal bovine serum (FBS), and maintained in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Manganese treatments were delivered using 0, 100, or 300 μM Mn in the form of MnCl₂. These dose concentrations are based on previous studies in non-human primates reporting clinical symptoms of Mn neurotoxicity at brain concentrations of 300 μM , while 100 μM concentrations appeared to be asymptomatic (Suzuki et al., 1975). For this reason 300 μM was used to examine the effect of toxic Mn accumulation on Taurine uptake, while 100 and 300 μM were used for the mRNA experiments to examine if there is a change in expression of Scl6a6 from moderate non-symptomatic levels (100 μM) to known toxic accumulation (300 μM).

2.3 ³H-Taurine

Uptake of tritiated taurine (³H-Taurine) was measured as described by Erikson and Aschner (2002). Astrocytes (cultured for 3–4 weeks, seeded 2×10^5 in 6-well plates, and grown to confluence) were incubated overnight at 37°C with treatment media containing 0 or 300 μM MnCl₂. The next day, cells were washed 3X with HEPES buffer [122 mM NaCl, 3.3 mM KCl, 0.4 mM MgSO₄, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, and 25 mM N-2-hydroxyethylpiperazine N'-2-ethansulfonic acid, pH 7.4] and incubated for 1, 3, or 6 minutes with HEPES buffer containing 0.5 μCi ³H-taurine (GE Healthcare Life Sciences, Piscataway, NJ). The reaction was stopped by aspirating the tritiated HEPES and washing the cells 4X 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 1 mL RIPA lysis buffer (99 mL 1X PBS, 1 mL

Nonidet 40, 0.1 g sodium dodecyl sulfate, 0.5 g sodium deoxycholate, pH 7.4) and 750 μ L aliquots were used for β -counting with a Beckman LS 3801 liquid scintillation analyzer (Beckman Instruments). The remaining 250 μ L was used for protein determination using the bicinchoninic assay (BCA, Pierce Chemicals).

2.4 Stereotaxic Surgery

After five weeks of dietary treatment and one week prior to microdialysis experiments, rats were anesthetized with ketamine-HCl (80 mg/kg) and xylazine (12 mg/kg) and maintained on a heating pad at 37° C. The heads of the rats were shaved and wiped with a 5% povidone-iodine solution to reduce risk of infection. Sterile instruments and gloves were used throughout the surgical procedure. The rats were secured in the stereotaxic frame and an incision was made perpendicular to the bregma. A guide cannula (CMA/12, CMA Microdialysis, Acton, MA) was implanted into the striatum using the following coordinates: 2.4 mm lateral to the midline, 7.5 mm anterior to the lambda. The cannula was lowered to a depth of 2.5 mm, positioning it in the medial area of the striatum (Paxinos and Watson, 1998). Anchoring screws were utilized to maintain the position of the cannula before being cemented into place using dental adhesive. Animals were given 0.9% sterile saline (0.5 mL/kg body weight, i.p.) to reduce fluid lost while under anesthesia and to aid in recovery time. Animals were also given the xylazine reversal agent Antisedan (Atapimazole) (0.1 mg/kg body weight, i.p.) (Allivet, Hialeah, FL) to reduce recovery time. Animals were returned to shoebox cages with Tek-Fresh bedding (Harlan, Indianapolis, IN) and monitored daily until microdialysis experiments began.

2.5 Microdialysis

During week six of the dietary protocol, a microdialysis probe (CMA/12 Elite, CMA Microdialysis, Acton, MA) was inserted into the guide cannula and the rat was perfused with artificial cerebral spinal fluid (aCSF) (155 mM Na⁺, 0.83 mM Mg²⁺, 2.9 mM K⁺, 132.76 mM Cl⁻, 1.1 mM Ca⁺, pH 7.4) for one hour at a flow rate of 1 μ L/min. After perfusion, the flow rate was adjusted to 0.5 μ L/min and 30 minute fractions were collected in microtubes for a total of four and a half hours (9 samples per rat) in a refrigerated fraction collector (CMA Microdialysis, Acton MA). This protocol has been used successfully in previous studies with stable neurotransmitter recovery in the dialysate (Anderson et al., 2008; 2009). Probe recoveries measured using *in vitro* standards for GABA, taurine, and glycine were averaged for each amino acid over all probes; however, because tissue diffusion may affect *in vivo* probe recovery no correction was made for total recovery as in previous studies (Anderson et al., 2008; 2009; Beard et al., 1994; Chen et al., 1995; Nelson et al., 1997). The microdialysate samples analyzed were collected at 0, 60, 120, 180, and 240-minute time-points with NA administration (100 μ M in aCSF) just prior to the 60-minute collection. This time course identifies baseline values (0 min), the response of extracellular amino acid concentrations to decreased GAT function (60 min), their recovery after removal of NA and re-perfusion with aCSF (120 min), and renormalization (180 and 240 min). Samples were stored at -80° C until analysis of the dialysate fraction. Rats were then returned to their home cage, and, the following day, were euthanized, brains removed, and probe placement verified post mortem.

2.6 CE-LIF analysis

A protocol by Chen et al. (2001) allowing for detection of amino acids and biogenic amines at nanomolar concentrations, modified to accommodate the needs of our previous studies (Anderson et al., 2008, 2009), was utilized in the current study as well. The advantages of applying CE analysis to neuroactive compounds include minimal required sample volumes, speed of analysis, and high separation efficiency (Powell and Ewing, 2005). Briefly, on the day of sample analysis, 5 μ L of microdialysate sample were derivatized at 40 C by the addition to 100 nmol ATTO-TAGTM FQ fluorogenic reagent (Molecular Probes, Eugene, OR) and 10

μL of a 10 mM borate (Fisher, Fair Lawn, NJ)/ 25 mM KCN (Fluka) solution (pH 9.18). The total sample volume was adjusted to 20 μL using HPLC grade methanol (G.J. Chemical Company, Newark, NJ). After a minimum reaction time of 90 min., 1 μL of an FQ derivatized homoserine (Sigma, St. Louis, MO) internal standard solution was added to the derivatized microdialysate sample and analyzed. CE-LIF conditions leading to high efficiency peaks for microdialysate samples were 10 kV for 10 min with sample injections at 10 psi/sec. Uncoated silica capillary (Polymicro, Arizona) with an i.d. of 25 μm, o.d. of 361 μm, and effective/total lengths of 25.4/30.0 cm was used. The run buffer was 15 mM sodium borate (Fisher), pH 9.0, with 45 mM sodium dodecyl sulfate (Pierce, Rockford, IL), 5 mM sodium cholate (Anatrace, Maumee, OH), and 4% (v/v) 2-propanol (Fisher). Three replicates were analyzed for each sample, with calibration curves for neurotransmitters of interest constructed each day of sample analysis using three points with a concentration range of 0.1 μM to 5 μM. GABA (Sigma), glycine (Sigma), taurine (Sigma), and homoserine standard solutions used for construction of calibration curves were prepared in ACSF with the same composition as that used in the microdialysis studies. The ratio of neurotransmitter peak height to internal standard (homoserine) peak height for each sample was used to determine the concentration of the neurotransmitter based on the calibration curve response.

2.7 RNA isolation and cDNA synthesis

Total RNA was isolated from astrocyte monolayers and the striatum of control and Mn exposed rats for quantitative PCR analysis. Tissue samples were stored in 1 mL of RNeasy lysis solution (Qiagen Inc., Austin, TX) and kept at -80° C until analysis. Astrocytes were cultured in 6-well plates, then treated for 24 hrs with media containing 0, 100, or 300 μM Mn. Astrocytes were harvested in 500 μL RNeasy lysis solution (Qiagen Inc., Austin, TX). Tissue and cell culture RNA isolation was performed using the RNeasy spin system (Qiagen Inc., Austin, TX), following manufacturer's instructions. RNA concentration and purity were determined by spectrophotometric analysis before carrying out cDNA synthesis. Synthesis of cDNA was performed using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA), following manufacturer's instructions.

2.8 Quantitative PCR

Quantitative real-time PCR analysis was utilized to determine differential mRNA expression between control and Mn treated tissue or cell samples of the solute carrier family taurine transporter Slc6a6 (Applied Biosystems, Foster City, CA; Rn00567962_m1, Chr. 4 - 125875817 – 125945795). Triplicate aliquots of cDNA were analyzed on 96-well plates using TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA). Values of cDNA expression were normalized relative to the expression of β-actin (Rn00667869_m1, Chr. 12 - 12047070 – 12050040) analyzed from the same sample on the same plate and reported as percent of control.

2.9 Metal analyses

Mn, Fe, and copper (Cu) concentrations were measured with graphite furnace atomic absorption spectrometry (Varian AA240, Varian, Inc., USA). Brain tissue from the striatum was digested in ultra-pure nitric acid (1:10 w/v dilution) for 48–72 hours in a sand bath (60° C). A 50 μL aliquot of digested tissue was brought to 1 mL total volume with 2% nitric acid for analysis. The extracellular striatal samples obtained via microdialysis were not diluted due to the small volume (20 μL) and the likelihood that this biological compartment has a low concentration of metals. Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) (10 μg Mn/g; 184 μg Fe/g; 80 μg Cu/g) was digested in ultrapure nitric acid and used as an internal standard for analysis (final concentration 5 μg Mn/L; 92 μg Fe/L; 10 μg Cu/L).

2.10 Statistical Analysis

Data were analyzed using SPSS v14 for Windows (Microsoft, Redmond, WA). Metal, baseline microdialysis, and ^3H -Taurine uptake data were analyzed using paired samples *t*-tests to examine the difference between Mn treated samples and controls. Independent sample *t*-tests were used to examine time-point percent change differences in the microdialysis data, time-point ^3H -Taurine uptake changes, and significance between Mn-exposed versus control mRNA expression of Scl6a6. A *p*-value of < 0.05 was considered significant.

3. Results

3.1 Manganese and Iron concentrations

Mn exposure resulted in significant alterations in compartmental metal concentrations

—As expected, tissue Mn levels were significantly higher in Mn exposed rats versus control ($p = 0.001$) (Table 1). Cu levels were slightly increased with Mn exposure, and no appreciable difference was observed in Fe levels between the two groups; however, there was a significant reduction ($p = 0.002$) in the Fe:Mn ratio in the Mn exposed group (Table 1). Examining Fe and Mn as a ratio may portray metal toxicities more accurately. The use of an Fe:Mn ratio has recently emerged as a reliable diagnostic criteria for metal neurotoxicities, as levels of one divalent cation may alter the availability or functionality of the other (Chua and Morgan, 1996; Cowan et al., 2009; Fitsanakis et al., 2008).

Collected fractions of microdialysate were analyzed for Fe and Mn to assess changes in extracellular metal levels as a consequence of oral Mn exposure. Extracellular Mn within the striatum was significantly increased in the Mn exposed rats, while Fe levels significantly decreased, compared to controls ($p = 0.021$ and 0.001 , respectively) (Table 1). Differences in extracellular metal concentrations between Mn exposed and control groups revealed a significant ($p = 0.020$), 36-fold, decrease in the extracellular Fe:Mn ratio due to increased Mn (Table 1).

3.2 Extracellular concentrations of Taurine, GABA, and Glycine

Extracellular amino acid concentrations are differentially altered by Mn-exposure

—Baseline levels of taurine and glycine were more abundant than GABA in the extracellular space, though Mn does not have a statistically significant effect on their levels compared to control (Figure 1). Mn-exposure, however, did significantly increase ($p = 0.017$) baseline GABA concentrations over control (Figure 1A), corroborating our previous findings (Anderson et al. 2007, 2008). While GABA_{EC} was more concentrated in the striatum of Mn-exposed rats, the rise in NA-induced GABA levels was not as profound in Mn-exposed versus control rats (Figure 1C). Administration of NA caused a significant 228% increase in the GABA_{EC} levels of the control ($p = 0.015$) but not in the Mn-exposed group ($p = 0.233$) (Figure 1C). After the removal of NA and a 60-minute perfusion with aCSF, GABA levels returned to baseline and remained unchanged at the 180- and 240-minute time-points (data not shown).

No significant difference in baseline taurine levels were found between control and Mn-exposed animals (Figure 1A). In control animals administration of NA caused a modest 75% decline in Tau_{EC} from baseline, followed by a significant ($p = 0.010$) 1000% increase after removal of NA at the 120 minute time-point (Figure 1B). The decrease in Tau_{EC} was similar in the Mn-exposed group due to NA administration; however, no rise in Tau_{EC} was observed after removal of NA, as observed in the control rats (Figure 1B). In control and Mn-exposed animals, taurine levels returned to and maintained levels similar to baseline at the 180- and 240-minute time-points (data not shown).

Gly_{EC} levels were similar in control and Mn-exposed groups, and no significant percent changes were observed between time-points within either control or Mn groups (Figure 1D).

Limits of detection of the CE-LIF method employed for each neurotransmitter were found by serial dilution of derivatized standards until no discernable analyte peak could be obtained. Accordingly, limits of detection for GABA, glycine, and taurine were 6.9 ± 1.7 nM, 24 ± 5 nM, and 42 ± 21 nM, respectively, with linear dynamic ranges of 3.6 decades, 3.1 decades, and 3.3 decades, respectively.

3.3 ³H-Taurine Uptake

Mn exposure results in increased ³H-Taurine uptake in astrocytes—After observing the unique effects of Mn-exposure on Tau_{EC} in the striatum of rats *in vivo*, we decided to examine the effect of Mn exposure on ³H-Taurine uptake in primary rat astrocytes *in vitro*. Primary astrocytes exposed to Mn revealed a slight (30%) decrease in taurine uptake after 1 minute, followed by a significant (219%) increase after 3 minutes ($p = 0.034$) (Figure 2). Six minute ³H-Taurine retention in Mn-exposed cells was similar to that of controls. Uptake of ³H-Taurine in control cells remained consistent around 0.4 pmol/mg protein at each time-point (Figure 2 inset). To examine whether or not 24 hr Mn exposure had an effect on taurine transporter expression in astrocytes, we next evaluated Mn-induced alterations in the taurine transporter, Slc6a6.

3.4 Gene expression of Taurine transporter

Mn exposure increased taurine transporter gene expression in the rat brain, but not cultured astrocytes—Quantitative RT-PCR analysis was conducted on primary astrocytes and striatal brain tissue to determine whether or not taurine transporter (Slc6a6) gene expression reflected the observed Mn-induced alterations in Tau_{EC} and ³H-uptake. Chronic Mn exposure caused a significant ($p = 0.045$) increase in striatal Slc6a6 mRNA levels compared to control (Figure 3). Alternatively, acute Mn exposure (100 and 300 μ M Mn) had relatively little effect on astrocyte Slc6a6 mRNA levels (Figure 3).

4. Discussion

The purpose of this study was to examine the effect of Mn on GAT-mediated GABA uptake. Knowing that glycine and taurine are important amino acid neurotransmitters that are known to modulate GABA neurochemistry (Namima et al., 1982; Hernandez and Troncone, 2009), it was logical that we measure them in the dialysate too. We found that GAT function is attenuated by Mn exposure, and that the resulting increase in GABA_{EC} alters taurine but not glycine homeostasis. Specifically, we observed a 10-fold increase in Tau_{EC} upon removal of NA in the control animals but not in the Mn-exposed, implicating a critical neurotransmitter function of Tau_{EC} that Mn alters (discussed in more detail below and in Figure 4).

Mn, Fe, and Cu levels were analyzed in the striatum of Mn exposed rats and non-exposed controls to ascertain homeostatic changes due to Mn accumulation. As expected, Mn exposure led to significant Mn accumulation in the striatum (Table 1). Striatal Cu levels were slightly higher with Mn exposure, but tissue Fe levels were unaffected. These data are consistent with striatal Fe levels reported in previous studies with Mn exposure (Anderson et al., 2009; Erikson et al., 2004; Fitsanakis et al., 2008). However, there was a near three-fold decrease in the Fe:Mn ratio with Mn exposure, suggesting altered metal homeostasis. While tissue levels of Fe remained relatively unchanged, extracellular Fe was significantly decreased by Mn accumulation, with a 36-fold drop in the Fe:Mn ratio. Previously, Mn-driven decreases in extracellular Fe have been positively correlated with extracellular norepinephrine levels and inversely associated with GABA_{EC} (Anderson et al., 2008, 2009), but no significant changes

in tissue Fe levels were observed. The disparity between tissue and extracellular Fe:Mn ratios suggest the synaptic environment may be subject to drastic changes in metal homeostasis. Moreover, these changes may leave the extracellular compartment vulnerable compared to striatal tissue, in which Fe and Mn levels appear to be more tightly regulated.

We specifically selected the striatum to examine the effect of Mn on GABA_{EC} and GAT function because it is a known region for Mn accumulation (Erikson et al., 2005; Liu et al., 2000), and due to its high density of GABAergic cell bodies (Oertel and Munglani, 1984). Mn exposure has been associated with increased GABA_{EC} concentrations in the striatum, and decreased ³H-GABA uptake has been reported in striatal synaptosomes (Anderson et al., 2007, 2008). While these studies reported little effect on GAT protein and mRNA levels with Mn exposure, the implications of these data on GAT functionality prompted us to pharmacologically probe GAT function with NA. We hypothesized that Mn exposure alters GAT function as indicated by the attenuation of increased GABA_{EC} concentrations in the presence of NA. Results from the microdialysis experiment indicate that this is indeed the case. NA in the striatum of control rats caused a 228% increase in GABA_{EC}; however, in Mn-exposed rats NA only increased GABA_{EC} by 43%. The use of NA in the current study identifies GAT as a target for Mn toxicity, and provides an explanation for the observed increase in GABA_{EC} with Mn accumulation. Exactly how Mn regulates GAT function warrants further investigation; however, decreased GAT function may be regulated through protein kinase C (PKC) activation. Mn exposure has been shown to activate PKC in N27 mesencephalic cells (Lathoumyrandane et al., 2005), and PKC activation has been demonstrated to decrease ³H-GABA transport by GAT (Sato et al., 1995). Moreover, phosphorylation of GAT-1 via PKC (Mandela and Ordway, 2006) may lead to internalization of GABA transporters (Gadea and Lopez-Colome, 2001). This could explain why Anderson et al. (2008) found increased GABA_{EC} despite no decrease in GAT-1 protein levels (as detected by western blot analysis which would measure both plasma membrane and internalized GAT-1 levels). It is also possible that Mn may alter some sort of feedback mechanism such as an autoreceptor (e.g., GABA_B or GABA_A). Being that taurine is a known modulator of GABA receptors (del Olmo et al., 2000; Kamisaki et al., 1993; Jia et al., 2008; Namima et al., 1982, 1983), perturbations in taurine biology may play a role in GABA homeostasis.

A novel finding from our study was that control rats responded to cessation of NA with a 10-fold increase of Tau_{EC} in the striatum, an effect that was absent in the Mn-exposed rats. Changes in Tau_{EC} coincided inversely with alterations in GABA (Figure 1A, B), presumably due to its role in osmoregulation. These results suggest that alterations in GABA uptake may dictate taurine release due to a hyperosmotic environment. Alternatively, the 10-fold increase in taurine after removal of NA may be a compensatory response to help decrease GABA_{EC} concentrations. Taurine binding to GABA_A receptors (del Olmo et al., 2000; Jia et al., 2008) and GABA_B autoreceptors (Kamisaki et al., 1993; Namima et al., 1982, 1983), may help to regulate GABA release. Enhanced taurine efflux observed in control animals after NA administration could functionally decrease GABA release by activating GABA_A and GABA_B receptors thereby normalizing GABA_{EC}. Additionally, there is evidence that NA activates GABA_A-like ion channels (Barrett-Jolley, 2001). Taurine efflux may be an adaptive response to facilitate GABA_A activation compensating for the loss of inhibitory tone due to NA cessation. Whether the taurine response observed in the control rats is due to its role in regulating striatal neurochemistry or through a secondary osmoregulatory effect, the lack of this response in the Mn-exposed rats may have profound consequences (See Figure 4).

Because Mn alters GAT function and GAT transport proteins are in the solute carrier protein family Slc6 (shared by the taurine transporter, Slc6a6), it is reasonable to assume that Mn may influence taurine movement by altering the function of the taurine transporter. In cultured astrocytes we measured ³H-Taurine uptake and found that while Mn initially decreased taurine

uptake by 30%, it was followed by a significant 219% increase in uptake before normalizing to control levels (Figure 2). Uptake of ^3H -Taurine in control cells remained consistent around 0.4 pmol/mg protein at each time-point, suggesting that fluctuations in taurine uptake *in vitro* is probably due to osmoregulation or altered transporter kinetics. Taurine transporter Slc6a6 mRNA levels were not altered in cultured astrocytes exposed to 100 or 300 μM Mn. Similar results were found by Erikson and Aschner (2002), with Slc6a6 expression significantly increasing in astrocytes only when exposed to 500 μM Mn. It is important, however, to remember that taurine transport may also occur via volume-sensitive organic osmolyte anion channels (VSOAC) (Mongin et al., 1999). VSOACs allow the transport of Na^+ , K^+ , Cl^- , and organic osmolytes (e.g. taurine) under conditions of cell shrinkage or swelling (Lang, 2007). Without directly inhibiting Slc6a6, controlling osmolarity, and taking into account Cl^- influx due to GABA_A activation we cannot confirm the functionality of Slc6a6 in the presence of Mn. Interestingly, Slc6a6 mRNA levels are increased in the striatum of Mn exposed rats, compared to control (Figure 3). The contradiction of *in vivo* and *in vitro* Slc6a6 expression may be a product of chronic (6 week) exposure to Mn *in vivo* versus acute (24 hr) exposure *in vitro*. Additionally, Slc6a6 gene expression is increased in the absence of sufficient taurine, and decreased when taurine is in excess (Bitoun and Tappaz, 2000; Lambert, 2004). Therefore, it is likely that altered taurine homeostasis in the striatum due to Mn exposure influences striatal Slc6a6 expression similarly.

Collectively, our data show that the GABA_{EC} and Tau_{EC} are indeed influenced by Mn accumulation and altered GAT function. Moreover, Mn virtually abolished Tau_{EC} and dramatically blunted the taurine rebound observed during the post NA recovery period (Figures 2B and 4), indicating a serious disconnect in taurine homeostasis in the Mn-exposed rat striatum. Moving forward, it is essential to understand the effect of Mn toxicity on taurine movement in the brain. Characterizing the role of Mn on GABA and taurine may help depict the multifaceted etiology of Mn neurotoxicity, and provide insight into some of the behavioral changes observed with Mn accumulation.

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Figure 1. Extracellular Amino Acids Concentrations

Microdialysate fractions from the striatum of control (n=4) and Mn-exposed (n=4) rats were analyzed for taurine, GABA, and glycine concentrations at baseline, 60 minutes, and 120 minutes. Nipecotic Acid (NA) was administered prior to the 60-minute time-point. Graph values are expressed as percent change $\pm$ SEM; inset data are μ M concentrations $\pm$ SEM. A) Baseline concentrations of each amino acid at onset of sample collection (0 Min) in both control and Mn-exposed rats. Percent change in amino acid concentration was calculated from baseline (100%) to post-NA administration, then post-NA to 120-minute recovery period for B) Taurine, C) GABA, and D) Glycine to observe the effect of Mn on extracellular amino acid levels. Superscript letters denote significant within-group differences (bars with different letters are significantly different from one another, bars that share a letter are not significant from one another), while * denote significance between groups. a, b, c, and * = $p < 0.05$ via independent samples t -test within or between groups.

Figure 2. ^3H -Taurine Uptake in Primary Astrocytes

Primary astrocytes, seeded 2×10^5 in 6-well plates ($n=6$) then grown to confluence, were cultured with either Mn-treated ($300 \mu\text{M MnCl}_2$) or control media. After 24hrs cultures were exposed to ^3H -Taurine for 1, 3, or 6 minutes and analyzed for ^3H -Taurine retention. The inset represents percent change in uptake due to Mn exposure expressed as percent control $\pm$ SEM. A significant ($p = 0.034$) increase in ^3H -Taurine uptake was observed after 3mins of exposure in the Mn treated astrocytes versus control.

* = $p < 0.05$ via independent samples t -test between Mn and control treatment groups at each time-point.

Figure 3. Taurine Transporter, Slc6a6, mRNA Levels

Quantitative RT-PCR results for taurine transporter, Slc6a6, in Mn exposed astrocytes (n=6) and the striatum of Mn exposed rats (n=6). Values are expressed as percent control $\pm$ SEM, with control values representing no Mn exposure. Minimal alterations in Slc6a6 levels were observed in astrocytes treated with either 100 or 300 μ M MnCl₂. Mn exposure did, however, significantly increase Slc6a6 mRNA levels within the caudate putamen, versus matched control animals.

* = $p < 0.05$ via independent samples T-Test between Mn and control treatment groups.

Figure 4. Working Model for Mn-induced GABA and Taurine Alterations

The dynamic shifts in neurotransmitter concentrations observed in response to nipecotic acid (NA) (panels A, B, and C) are mitigated by Mn (panels D, E, and F). We hypothesize this lack of response in Mn-exposed rats is driven by decreased GABA transporter (GAT-1) function. **(A)** The control panel displays $GABA_{EC}$ and Tau_{EC} under normal conditions, representing baseline microdialysis measurements. All percent change (% change) comparisons in subsequent panels are based on the % change from baseline levels, represented in the control panel. Under normal conditions $GABA_{EC}$ binds to $GABA_A$ receptors ($GABA_A-R$) allowing chloride ion (Cl^{2-}) movement for inhibitory hyperpolarization of postsynaptic neurons, while presynaptic binding to $GABA_B$ -receptors ($GABA_B-R$) regulates GABA release (Kamisaki et al., 1993) through slow G-protein-linked inhibitory tone via $GABA_B$ activation (Chen and van den Pol, 1998). GAT-1 functions normally to clear excess GABA from the synapse as Tau_{EC} modulates pre- and post-synaptic transmission (Namima et al., 1982; 1983). **(B)** Administration of NA, a GAT specific inhibitor, blocks GABA reuptake substantially increasing $GABA_{EC}$ while decreasing Tau_{EC} . Additionally, NA activates $GABA_A$ -like Cl^{2-} channels (Barrett-Jolley, 2001) in addition to GAT binding, increasing the pre- and post-synaptic inhibitory tone. **(C)** Upon removal of the NA, $GABA_{EC}$ returns to normal; however, a 10-fold increase in Tau_{EC} ensues. We speculate that the taurine efflux is a compensatory response to regulate GABA release through $GABA_B$ activation (Chen and van den Pol, 1998). Elevated Tau_{EC} may also function to stabilize the inhibitory tone achieved in the striatum due to increased $GABA_A$ activation (del Olmo et al., 2000; Jia et al., 2008). Tau_{EC}

slowly returns to control levels over the next two hours (data not shown), indicating the acute nature of this response. **(D)** During Mn-exposure, GABA_B expression is decreased (Anderson et al., 2008) and GABA reuptake via GAT-1 is attenuated, resulting in higher GABA_{EC} and lower Tau_{EC} (Figure 1B and C) compared to control. We hypothesize that this alteration in GAT-1 function is regulated by Mn activation of protein kinase C (PKC) (Latchoumycandane et al., 2005) causing phosphorylation of GAT-1 and subsequent internalization (Gadea and Lopez-Colome, 2001). This internalization decreases synaptic density of GAT-1 and attenuates GABA reuptake. Additionally, decreased GABA_B expression alters auto-receptor feedback resulting in significantly higher GABA_{EC} compared to normal conditions. **(E)** Mn exposure significantly attenuates the effects of NA, potentially via decreased synaptic availability of GAT-1. The attenuated rise in GABA_{EC} coupled with decreased auto-receptor expression results in less GABA_A and GABA_B activation leading to reduced inhibitory tone compared to control. **(F)** Removal of NA in Mn-exposed rats led to GABA_{EC} returning to pre-NA levels (Panel D); however, the taurine efflux was absent compared to control (refer to Figure 1B). We interpret the dramatically decreased taurine efflux observed with Mn exposure as a function of altered auto-receptor biology (Anderson et al., 2008). Altered feedback regulation in conjunction with a modest rise (40%) in GABA_{EC}, compared to the normal feedback regulation and significant rise (228%) in GABA_{EC} observed in control rats, leads to an uncoupling of the taurine efflux response due to Mn-exposure. Our studies suggest that Mn exposure disrupts extracellular conditions within the striatum, altering neurochemical coordination with other brain regions. Elucidating the mechanism involved in this response will further the development of pharmacological therapies aimed at susceptible populations.

Table 1
Brain tissue and extracellular metal concentrations

Compartmental metal concentrations represented in the striatum of Mn exposed rats. Extracellular metal concentrations represent Mn and Fe levels measured in microdialysate fractions of extracellular fluid collected from the rat striatum (n=4). Striatal Mn, Fe, and Cu levels represent metal concentrations of brain tissue (n=6). The Fe:Mn ratio depicts metal homeostasis changes due to Mn accumulation. A significant increase in extracellular Mn accompanied by significant decreases in both Fe levels and the Fe:Mn ratio were observed in rats exposed to Mn treatment. No significant changes in tissue Fe levels were observed; however, a significant reduction in the Fe:Mn ratio indicates altered metal homeostasis.

	Extracellular (μM)			Ratio
	Mn	Fe	Cu	Fe:Mn
Control	0.023 ± 0.006	4.071 ± 0.510	-	5044:1
Manganese	0.104 ± 0.030 [*]	1.526 ± 0.304 [‡]	-	137:1 [*]
	Striatum (nmol/mg Protein)			Ratio
	Mn	Fe	Cu	Fe:Mn
Control	0.185 ± 0.029	3.896 ± 0.106	0.793 ± 0.085	25:1
Manganese	0.477 ± 0.059 [‡]	3.329 ± 0.407	0.975 ± 0.089	9:1 [*]

^{*} $p < 0.05$,

[‡] $p \leq 0.001$ versus control according to paired-sample *t*-test analysis.