

## 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  $\gamma$ -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<sub>EC</sub>) 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<sub>EC</sub> 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 <sup>3</sup>H-Taurine uptake after 3-min exposure in primary rat astrocytes. These data suggest that Mn increases GABA<sub>EC</sub> 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.

### **Article:**

#### **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 and 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 and Anderson et al., 2009). Manganese neurotoxicity shares similarities with the neurodegenerative disorder Parkinson's disease (Beuter et al., 1994, Calne et al., 1994 and Pal et al., 1999), though the two are clinically distinct (Calne et al., 1994, Pal et al., 1999 and 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  $\gamma$ -aminobutyric acid (GABA) (Anderson et al., 2007a, Anderson et al., 2007b, Anderson et al., 2008, Garcia et al., 2006, Garcia et al., 2007 and Gwiazda et al., 2002).

With the intriguing findings that striatal extracellular GABA ( $\text{GABA}_{\text{EC}}$ ) concentrations are higher due to Mn-exposure (Anderson et al., 2008), and uptake of  $^3\text{H}$ -GABA is attenuated by Mn-exposure in striatal synaptosomes ([Anderson et al., 2007a] and [Anderson et al., 2007b]) 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, nipecotic 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] and [Krogsgaard-Larsen et al., 2000]). We can, therefore, test GAT function by measuring the increase in  $\text{GABA}_{\text{EC}}$  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  $\text{GABA}_A$  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 and 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 ( $\text{Tau}_{\text{EC}}$ ) in the striatum is unknown. Therefore, we sought to determine if Mn-exposure effects  $\text{Tau}_{\text{EC}}$  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 ( $\text{Gly}_{\text{EC}}$ ) 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.

## MATERIALS AND METHODS

### *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., 2007a, Anderson et al., 2007b and Anderson et al., 2008): control (CN; 35 mg Fe/kg, 10 mg Mn/kg diet and d.i. water) and Mn-exposed (Mn; control diet and 1 g Mn (as  $MnCl_2$ )/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 h/day, with the lights off between 1800 and 600 h and room temperature maintained at  $25 \pm 1$  °C. The University of North Carolina at Greensboro Animal Care and Use Committee approved all of the animal procedures.

### *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%  $CO_2$  at 37 °C. Manganese treatments were delivered using 0, 100, or 300  $\mu M$  Mn in the form of  $MnCl_2$ . These dose concentrations are based on previous studies in non-human primates reporting clinical symptoms of Mn neurotoxicity at brain concentrations of 300  $\mu M$ , while 100  $\mu M$  concentrations appeared to be asymptomatic (Suzuki et al., 1975). For this reason 300  $\mu M$  was used to examine the effect of toxic Mn accumulation on taurine uptake, while 100 and 300  $\mu M$  were used for the mRNA experiments to examine if there is a change in expression of *Scl6a6* from moderate non-symptomatic levels (100  $\mu M$ ) to known toxic accumulation (300  $\mu M$ ).

### *<sup>3</sup>H-Taurine*

Uptake of tritiated taurine (<sup>3</sup>H-Taurine) was measured as described by Erikson and Aschner (2002). Astrocytes (cultured for 3–4 weeks, seeded  $2 \times 10^5$  in 6-well plates, and grown to confluence) were incubated overnight at 37 °C with treatment media containing 0 or 300  $\mu M$   $MnCl_2$ . The next day, cells were washed 3 $\times$  with HEPES buffer [122 mM NaCl, 3.3 mM KCl, 0.4 mM  $MgSO_4$ , 1.3 mM  $CaCl_2$ , 1.2 mM  $KH_2PO_4$ , 10 mM glucose, and 25 mM N-2-hydroxyethylpiperazine N'-2-ethansulfonic acid, pH 7.4] and incubated for 1, 3, or 6 min with HEPES buffer containing 0.5  $\mu Ci$  <sup>3</sup>H-Taurine (GE Healthcare Life Sciences, Piscataway, NJ). The reaction was stopped by aspirating the tritiated HEPES 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 1 mL RIPA lysis buffer (99 mL 1 $\times$  PBS, 1 mL non-idet 40, 0.1 g sodium dodecyl sulfate, 0.5 g sodium deoxycholate, pH 7.4) and 750  $\mu L$  aliquots were used

for  $\beta$ -counting with a Beckman LS 3801 liquid scintillation analyzer (Beckman Instruments). The remaining 250  $\mu$ L was used for protein determination using the bicinchoninic assay (BCA, Pierce Chemicals).

### *Stereotaxic surgery*

After 5 weeks of dietary treatment and 1 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.

### *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<sup>+</sup>, 0.83 mM Mg<sup>2+</sup>, 2.9 mM K<sup>+</sup>, 132.76 mM Cl<sup>-</sup>, 1.1 mM Ca<sup>+</sup>, pH 7.4) for 1 h at a flow rate of 1  $\mu$ L/min. After perfusion, the flow rate was adjusted to 0.5  $\mu$ L/min and 30 min 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 and Anderson et al., 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, Anderson et al., 2009, Beard et al., 1994, Chen et al., 1995 and Nelson et al., 1997). The microdialysate samples analyzed were collected at 0, 60, 120, 180, and 240-min time-points with NA administration (100  $\mu$ M in aCSF) just prior to the 60-min 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.

### *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 and Anderson et al., 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  $\mu$ L of microdialysate sample was derivatized at 40 °C by the addition to 100 nmol ATTO-TAG™ FQ fluorogenic reagent (Molecular Probes, Eugene, OR) and 10  $\mu$ L 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  $\mu$ L using HPLC grade methanol (G.J. Chemical Company, Newark, NJ). After a minimum reaction time of 90 min, 1  $\mu$ L 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/s. Uncoated silica capillary (Polymicro, Arizona) with an i.d. of 25  $\mu$ m, o.d. of 361  $\mu$ m, 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 0.1–5  $\mu$ M. 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.

### *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<sup>®</sup> solution (Ambion Inc., Austin, TX) and kept at –80 °C until analysis. Astrocytes were cultured in 6-well plates, then treated for 24 h with media containing 0, 100, or 300  $\mu$ M Mn. Astrocytes were harvested in 500  $\mu$ L denaturation solution (Ambion Inc., Austin, TX). Tissue and cell culture RNA isolation were performed using the ToTALLY RNA™ system (Ambion 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.

### *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<sup>®</sup> Gene Expression assays (Applied Biosystems, Foster City, CA). Values of cDNA expression were normalized relative to the expression of  $\beta$ -actin (Rn00667869\_m1, Chr. 12 – 12047070–12050040) analyzed from the same sample on the same plate and reported as percent of control.

### 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 h 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 ultra-pure nitric acid and used as an internal standard for analysis (final concentration 5 µg Mn/L; 92 µg Fe/L; 10 µg Cu/L).

### Statistical analysis

Data were analyzed using SPSS v14 for Windows (Microsoft, Redmond, WA). Metal, baseline microdialysis, and <sup>3</sup>H-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 <sup>3</sup>H-Taurine uptake changes, and significance between Mn-exposed versus control mRNA expression of Scl6a6. A *p*-value of <0.05 was considered significant.

## RESULTS

### 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 and Fitsanakis et al., 2008).

**Table 1:** Brain tissue and extracellular metal concentrations.

	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*

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.

\**p* < 0.05 versus control according to paired-sample *t*-test analysis.

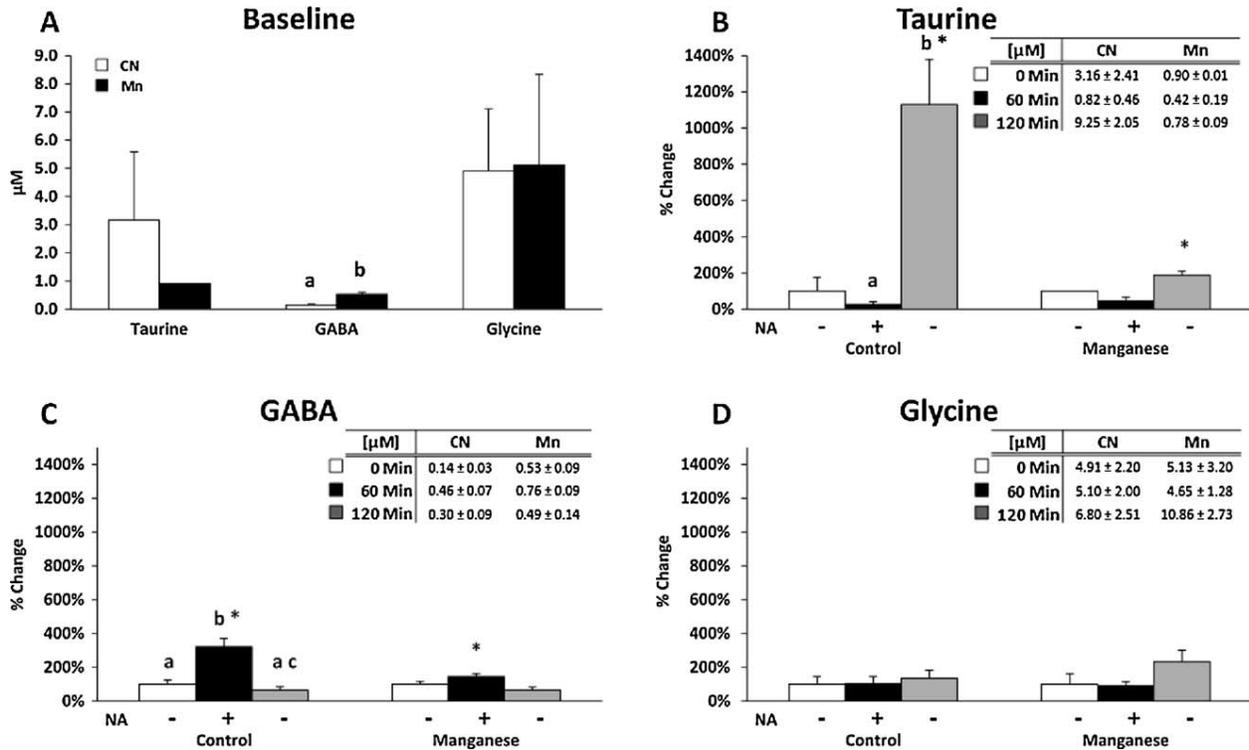
\*\**p* ≤ 0.001 versus control according to paired-sample *t*-test analysis.

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).

### 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 (Fig. 1). Mn-exposure, however, did significantly increase ( $p = 0.017$ ) baseline GABA concentrations over control (Fig. 1A), corroborating our previous findings (Anderson et al., 2007a, Anderson et al., 2007b and Anderson et al., 2008). While GABA<sub>EC</sub> 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 (Fig. 1C). Administration of NA caused a significant 228% increase in the GABA<sub>EC</sub> levels of the control ( $p = 0.015$ ) but not in the Mn-exposed group ( $p = 0.233$ ) (Fig. 1C). After the removal of NA and a 60-min perfusion with aCSF, GABA levels returned to baseline and remained unchanged at the 180- and 240-min time-points (data not shown).



**Fig. 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 min, and 120 min. Nipecotic acid (NA) was administered prior to the 60-min time-point. Graph values are expressed as percent change  $\pm$  SEM; inset data are  $\mu$ 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-min 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.

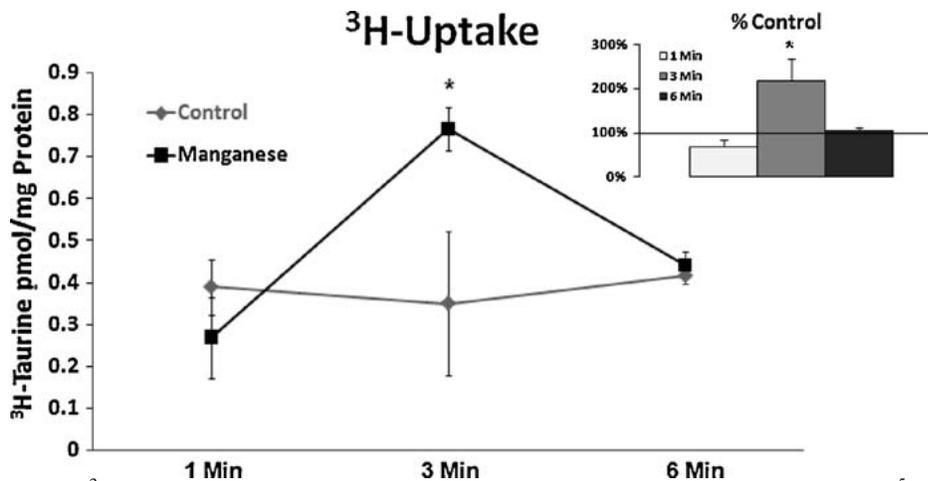
No significant difference in baseline taurine levels was found between control and Mn-exposed animals (Fig. 1A). In control animals administration of NA caused a modest 75% decline in  $\text{Tau}_{\text{EC}}$  from baseline, followed by a significant ( $p = 0.010$ ) 1000% increase after removal of NA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

$\text{Gly}_{\text{EC}}$  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 (Fig. 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  $6.9 \pm 1.7$  nM,  $24 \pm 5$  nM, and  $42 \pm 21$  nM, respectively, with linear dynamic ranges of 3.6 decades, 3.1 decades, and 3.3 decades, respectively.

### *<sup>3</sup>H-Taurine uptake*

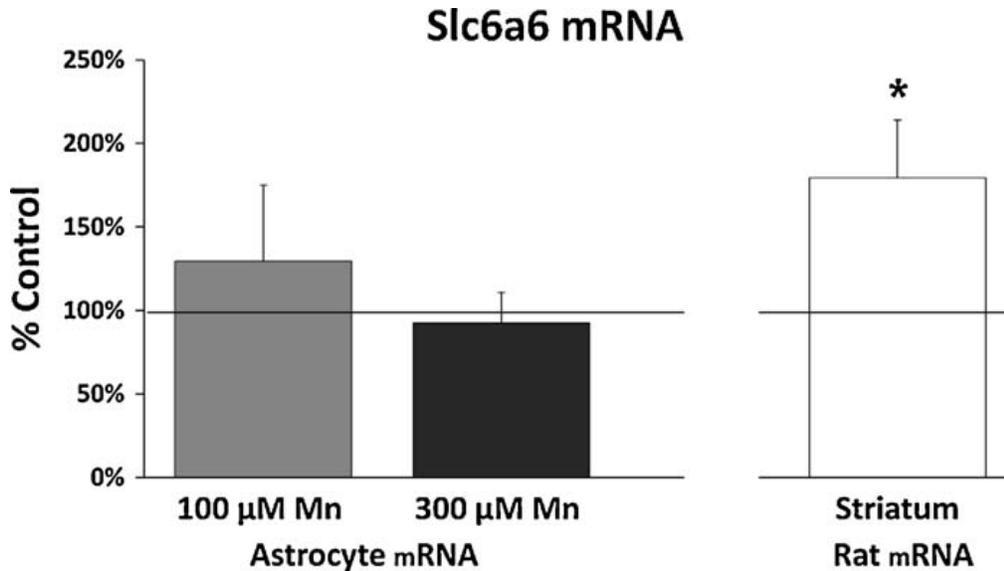
Mn-exposure results in increased <sup>3</sup>H-Taurine uptake in astrocytes. After observing the unique effects of Mn-exposure on  $\text{Tau}_{\text{EC}}$  in the striatum of rats *in vivo*, we decided to examine the effect of Mn-exposure on <sup>3</sup>H-Taurine uptake in primary rat astrocytes *in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



**Fig. 2:** <sup>3</sup>H-Taurine uptake in primary astrocytes. Primary astrocytes, seeded  $2 \times 10^{-5}$  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 24 h cultures were exposed to <sup>3</sup>H-Taurine for 1, 3, or 6 min and analyzed for <sup>3</sup>H-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 <sup>3</sup>H-Taurine uptake was observed after 3 min 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.

### Gene expression of taurine transporter

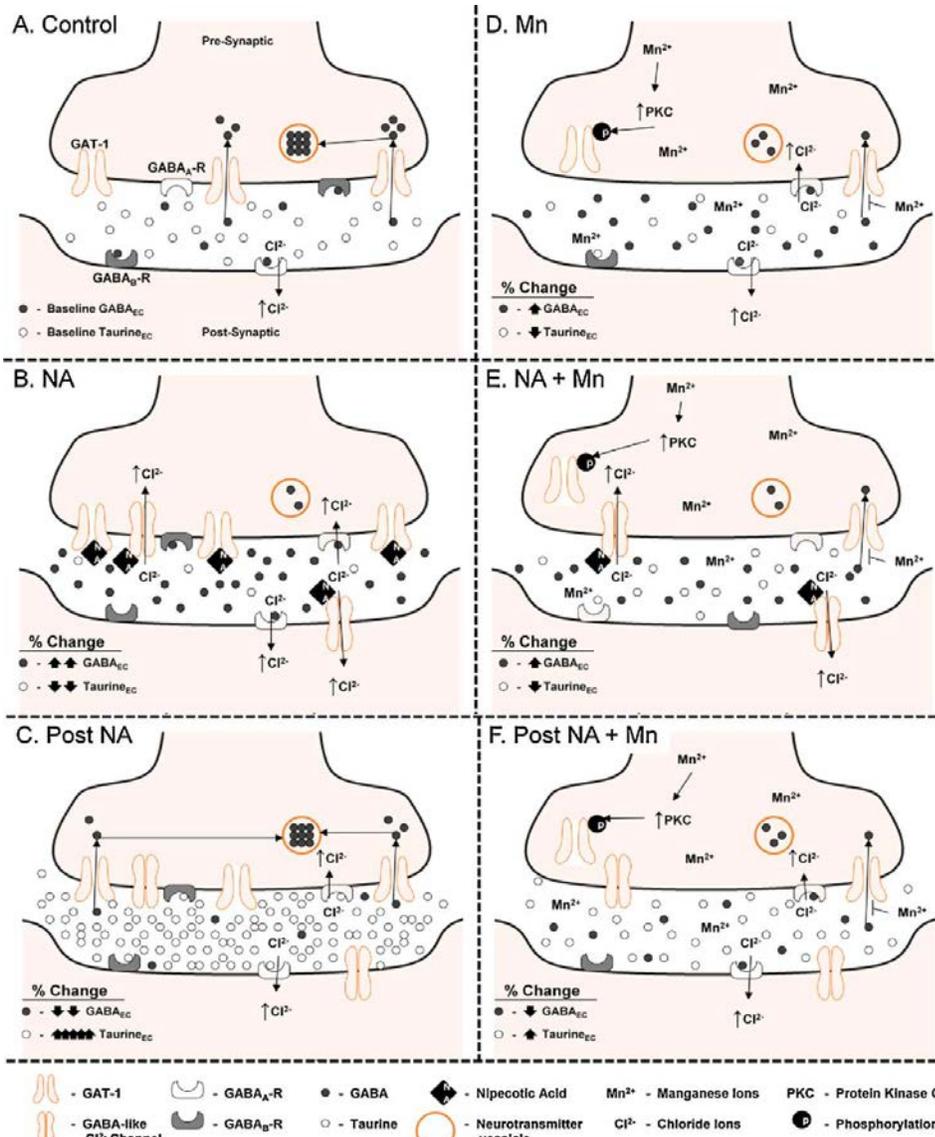
Mn-exposure increased taurine transporter gene expression in the rat brain, but not cultured astrocytes. Quantitative RT-PCR analysis was conducted on primary astrocytes and striatal brain tissue to determine whether or not taurine transporter (Slc6a6) gene expression reflected the observed Mn-induced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



**Fig. 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  $\mu$ M MnCl<sub>2</sub>. 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.

### 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 and 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<sub>EC</sub> alters taurine but not glycine homeostasis. Specifically, we observed a 10-fold increase in Tau<sub>EC</sub> upon removal of NA in the control animals but not in the Mn-exposed, implicating a critical neurotransmitter function of Tau<sub>EC</sub> that Mn alters (discussed in more detail below and in Fig. 4).



**Fig. 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<sub>EC</sub> and Tau<sub>EC</sub> 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<sub>EC</sub> binds to GABA<sub>A</sub> receptors (GABA<sub>A</sub>-R) allowing chloride ion (Cl<sup>-</sup>) movement for inhibitory hyperpolarization of post-synaptic neurons, while pre-synaptic binding to GABA<sub>B</sub> receptors (GABA<sub>B</sub>-R) regulates GABA release (Kamisaki et al., 1993) through slow G-protein-linked inhibitory tone via GABA<sub>B</sub> activation (Chen and van den Pol, 1998). GAT-1 functions normally to clear excess GABA from the synapse as Tau<sub>EC</sub> modulates pre- and post-synaptic transmission ([Namima et al., 1982] and [Namima et al., 1983]). (B) Administration of NA, a GAT specific inhibitor, blocks GABA reuptake substantially increasing GABA<sub>EC</sub> while decreasing Tau<sub>EC</sub>. Additionally, NA activates GABA<sub>A</sub>-like Cl<sup>-</sup> channels (Barrett-Jolley, 2001) in addition to GAT binding, increasing the pre- and post-synaptic inhibitory tone. (C) Upon removal of the NA, GABA<sub>EC</sub> returns to normal; however, a 10-fold increase in Tau<sub>EC</sub> ensues. We speculate that the taurine efflux is a compensatory response to regulate GABA release through GABA<sub>B</sub> activation (Chen and van den Pol, 1998). Elevated Tau<sub>EC</sub> may also function to stabilize the inhibitory tone achieved in the striatum due to increased GABA<sub>A</sub> activation ([del Olmo et al., 2000] and [Jia et al., 2008]). Tau<sub>EC</sub> 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<sub>B</sub> expression is decreased (Anderson et al., 2008) and GABA reuptake via GAT-1 is attenuated, resulting in higher GABA<sub>EC</sub> and lower Tau<sub>EC</sub> (Fig. 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 López-Colomé, 2001). This internalization decreases synaptic density of GAT-1 and attenuates GABA reuptake. Additionally, decreased GABA<sub>B</sub> expression alters auto-receptor feedback resulting in significantly higher GABA<sub>EC</sub> 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<sub>EC</sub> coupled with decreased auto-receptor expression results in less GABA<sub>A</sub> and GABA<sub>B</sub> activation leading to reduced inhibitory tone compared to control. (F) Removal of NA in Mn-exposed rats led to GABA<sub>EC</sub> returning to pre-NA levels (panel D); however, the taurine efflux was absent compared to control (refer to Fig. 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<sub>EC</sub>, compared to the normal feedback regulation and significant rise (228%) in GABA<sub>EC</sub> 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.

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 and Fitsanakis et al., 2008). However, there was a near 3-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<sub>EC</sub> (Anderson et al., 2008 and Anderson et al., 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<sub>EC</sub> and GAT function because it is a known region for Mn accumulation (Erikson et al., 2005 and Liu et al., 2000), and due to its high density of GABAergic cell bodies (Oertel and Mungnaini, 1984). Mn-exposure has been associated with increased GABA<sub>EC</sub> concentrations in the striatum, and decreased <sup>3</sup>H-GABA uptake has been reported in striatal synaptosomes (Anderson et al., 2007a, Anderson et al., 2007b and Anderson et al., 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<sub>EC</sub> 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<sub>EC</sub>; however, in Mn-exposed rats NA only increased GABA<sub>EC</sub> 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<sub>EC</sub> 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 (Latchoumycandane et al., 2005), and PKC activation has been demonstrated to decrease  $^3\text{H}$ -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 López-Colomé, 2001). This could explain why Anderson et al. (2008) found increased  $\text{GABA}_{\text{EC}}$  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 auto-receptor (e.g.,  $\text{GABA}_{\text{B}}$  or  $\text{GABA}_{\text{A}}$ ). 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 and Namima et al., 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  $\text{Tau}_{\text{EC}}$  in the striatum, an effect that was absent in the Mn-exposed rats. Changes in  $\text{Tau}_{\text{EC}}$  coincided inversely with alterations in GABA (Fig. 1A and 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  $\text{GABA}_{\text{EC}}$  concentrations. Taurine binding to  $\text{GABA}_{\text{A}}$  receptors (del Olmo et al., 2000 and Jia et al., 2008) and  $\text{GABA}_{\text{B}}$  auto-receptors (Kamisaki et al., 1993, Namima et al., 1982 and Namima et al., 1983), may help to regulate GABA release. Enhanced taurine efflux observed in control animals after NA administration could functionally decrease GABA release by activating  $\text{GABA}_{\text{A}}$  and  $\text{GABA}_{\text{B}}$  receptors thereby normalizing  $\text{GABA}_{\text{EC}}$ . Additionally, there is evidence that NA activates  $\text{GABA}_{\text{A}}$ -like ion channels (Barrett-Jolley, 2001). Taurine efflux may be an adaptive response to facilitate  $\text{GABA}_{\text{A}}$  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 Fig. 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  $^3\text{H}$ -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 (Fig. 2). Uptake of  $^3\text{H}$ -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  $\mu\text{M}$  Mn. Similar results were found by Erikson and Aschner (2002), with Slc6a6 expression significantly increasing in astrocytes only when exposed to 500  $\mu\text{M}$  Mn. It is important, however, to remember that taurine transport may also occur via volume-sensitive organic osmolyte anion channels (VSOACs) (Mongin et al., 1999). VSOACs allow the transport of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , 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  $\text{Cl}^-$  influx due to  $\text{GABA}_{\text{A}}$  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 (Fig. 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 h) 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 and 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<sub>EC</sub> and Tau<sub>EC</sub> are indeed influenced by Mn accumulation and altered GAT function. Moreover, Mn virtually abolished Tau<sub>EC</sub> and dramatically blunted the taurine rebound observed during the post-NA recovery period (Fig. 2 and Fig. 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.

#### CONFLICT OF INTEREST STATEMENT

*The authors have no conflict of interest to declare.*

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