Manganese exposure alters extracellular GABA, GABA receptor and transporter protein and mRNA levels in the developing rat brain

By: Joel G. Anderson, Steve C. Fordahl, Paula T. Cooney, Tara L. Weaver, Christa L. Colyer, Keith M. Erikson

Anderson, J.G., Fordahl, S.C., Cooney, P.T., Weaver, T.L., Colyer, C.L., and Erikson, K.M. (2008) Manganese exposure alters extracellular GABA, GABA receptor and transporter protein and mRNA levels in the developing rat brain. Neurotoxicology Nov; 29(6):1044-53.

Abstract:

Unlike other essential trace elements (e.g., zinc and iron) it is the toxicity of manganese (Mn) that is more common in human populations than its deficiency. Data suggest alterations in dopamine biology may drive the effects associated with Mn neurotoxicity, though recently γ-aminobutyric acid (GABA) has been implicated. In addition, iron deficiency (ID), a common nutritional problem, may cause disturbances in neurochemistry by facilitating accumulation of Mn in the brain. Previous data from our lab have shown decreased brain tissue levels of GABA as well as decreased ^{3}H-GABA uptake in synaptosomes as a result of Mn exposure and ID. These results indicate a possible increase in the concentration of extracellular GABA due to alterations in expression of GABA transport and receptor proteins. In this study weanling-male Sprague–Dawley rats were randomly placed into one of four dietary treatment groups: control (CN; 35 mg Fe/kg diet), iron-deficient (ID; 6 mg Fe/kg diet), CN with Mn supplementation (via the drinking water; 1 g Mn/l) (CNMn), and ID with Mn supplementation (IDMn). Using *in vivo* microdialysis, an increase in extracellular GABA concentrations in the striatum was observed in response to Mn exposure and ID although correlational analysis reveals that extracellular GABA is related more to extracellular iron levels and not Mn. A diverse effect of Mn exposure and ID was observed in the regions examined via Western blot and RT-PCR analysis, with effects on mRNA and protein expression of GAT-1, $GABA_A$, and $GABA_B$ differing between and within the regions examined. For example, Mn exposure reduced GAT-1 protein expression by approximately 50% in the substantia nigra, while increasing mRNA expression approximately four-fold, while in the caudate putamen mRNA expression was decreased with no effect on protein expression. These data suggest that Mn exposure results in an increase in extracellular GABA concentrations via altered expression of transport and receptor proteins, which may be the basis of the neurological characteristics of manganism.

Keywords: Manganese, GABA, Rat, Microdialysis, Brain

Article:

1. Introduction

Manganese (Mn), an essential nutrient functioning as a critical cofactor for many key enzymes in the body (Hurley and Keen, 1987), is required for proper immune function, regulation of metabolism, reproduction, digestion, bone growth, and blood clotting (see review by Aschner et al., 2005). However, exposure to high concentrations of Mn is known to result in neurotoxicity. Manganese neurotoxicity, or manganism, initially characterized by a psychiatric disorder resembling schizophrenia, shares neuro- pathologies with several clinical disorders, in particular Parkinson's disease (Pal et al., 1999). Manganese neurotoxicity is associated with the accumulation of Mn in iron-rich, dopaminergic regions of the brain, specifically areas of the basal ganglia (Aschner et al., 2005). Manganism is most often associated with occupational exposure of welders, miners, and steel workers to chronic high levels of airborne particulate Mn (Pal et al., 1999; Mergler et al., 1994), though cases from exposure to contaminated drinking water have been reported as well (Wasserman et al., 2006; Kondakis et al., 1989).

Iron deficiency (ID) has become a clear risk factor for Mn accumulation (Davis et al., 1992; Erikson et al., 2002; Finley, 1999; Kwik-Uribe et al., 2000). A common nutritional problem through-out the world, ID is associated with cognitive and behavioral problems (Beard, 2001), potentially disturbing neurochemistry by facilitating accumulation of Mn in the brain, as iron (Fe) status is known to affect absorption of Mn, regardless of Mn concentrations in the body (Chandra and Shukla, 1976; Shukla et al., 1976). Manganese most likely competes with Fe for transport via divalent metal transporter 1 (DMT-1) (Gunshin et al., 1997), a transporter of various divalent metals. DMT-1 mRNA expression is known to increase in cases of ID (Gunshin et al., 2001; Burdo et al., 1999), and has recently become of interest as a potential transport protein for Mn (Garrick et al., 2003; Roth and Garrick, 2003). While most research has focused on the effects of Mn neurotoxicity on the metabolism of dopamine (DA) due to locomotor effects, alterations in the metabolism of other neurotransmitters, such as γ-aminobutyric acid (GABA), have been noted (Garcia et al., 2006, 2007; Li, 1998).

GABA is the most abundant inhibitory neurotransmitter in the adult brain (Beleboni et al., 2004; Olsen and DeLorey, 1999) and is found in the medium spiny neurons of the striatum, mediating the dopaminergic activity in this region (Ade et al., 2008). Cortical glutamate afferents project into the striatum where, in concert with GABA and DA, motor behaviors are controlled (Carlsson and Carlsson, 1990). Li (1998) found alterations in GABA metabolism during ID, though no absolute changes in GABA concentration were detected. Gwiazda et al. (2002) found a significant 16% increase in striatal GABA concentrations resulting from cumulative low-dose Mn exposure (14.4mg Mn/kg body weight i.p./ week, over five weeks) in a pre-Parkinsonian rat model. More recent studies have shown a significant increase in tissue levels of GABA in rats exposed to a high Mn diet at post-natal day 21 (Garcia et al., 2006), but not at earlier time points (Garcia et al., 2007), as well as a marginally significant ($p < 0.1$) decrease in pallidal GABA concentrations in monkeys exposed to $MnSO_4$, with a 20% decrease in caudate and putamen of Mn exposed monkeys compared to controls (Struve et al., 2007). Erikson et al. (2002) observed an inverse correlation between Mn and GABA concentration in the caudate putamen of ID male rats. However, none of these studies examined extracellular levels of GABA, which would reflect what is happening at the synaptic level and can be associated with behaviors more relevantly than tissue levels. While various microdialysis studies have observed the effect of Mn on extracellular DA concentrations (Vidal et al., 2005) or the relationship between GABA and DA (Expósito et al., 1999; Galindo et al., 1999), the effect of dietary Mn exposure on extracellular GABA concentrations has not been examined.

A recent study by this lab found a significant decrease in ^{3}H-GABA uptake correlating with increased Mn concentrations and ID in rat striatal synaptosomes (Anderson et al., 2007a), and we hypothesized that this was due to altered expression of GABA transporter (GAT-1). It is known that ID affects dopamine transporter (DAT) number and function (Erikson et al., 2000), and that it is a member of the solute carrier-6 (SLC-6) transporter family like the GAT-1 protein (Gether et al., 2006). The DAT has also been shown to respond to Mn and to play a role in Mn transport during toxicity (Anderson et al., 2007b; Erikson et al., 2005; Ingersoll et al., 1999). Therefore, we hypothesize that increased Mn and/or decreased Fe in the brain will cause decreased GAT-1 expression leading to elevated extracellular GABA and overall altered GABA biology, including GABA receptors, in the basal ganglia.

In the brain, the inhibitory actions of GABA are mediated by ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. While $GABA_A$ receptors occur post-synaptically, $GABA_B$ receptors are present pre-synaptically as autoreceptors, post-synaptically, and extra-synaptically as heteroreceptors, taking on a more neuromodulatory role. Upregulation of peripheral type benzodiazepine receptors in globus pallidus has been shown in rats exposed to Mn (Hazell et al., 2003) and in cultured primary rat astrocytes (Hazell et al., 1999). Additionally, Mn is known to affect GABA by dose-dependently increasing binding at $GABA_B$ receptors (Kerr and Ong, 1995). Alterations in neurotransmitter receptor expression can affect expression of transport proteins of the respective neurotransmitter via regulation by autoreceptors. This is well characterized in mice lacking functional D_2 receptors, which consequently experience a significant downregulation of the DAT (Dickinson et al., 1999). Similar effects on GAT-1 could be possible through action at $GABA_B$ autoreceptors (Zahniser and Doolen, 2001).

Based upon previous data from our lab showing decreased tissue levels of GABA (Erikson et al., 2002) and decreased ^{3}H-GABA uptake (Anderson et al., 2007a) as a result of dietary Mn exposure and ID, coupled with the fact that behaviors associated with Mn neurotoxicity suggest altered GABA metabolism, we hypothesize that dietary Mn exposure could potentially lead to an increase in the concentration of extracellular GABA, most likely as a result of altered GABA transporter and/or receptor expression. To test this hypothesis, we developed this study: (1) to establish alterations in the neuromodulatory extracellular concentrations of GABA in response to dietary Mn exposure and ID; (2) to examine alterations in the protein and mRNA expression of the transport and receptor proteins of GABA resulting from dietary Mn exposure and ID.

2. Materials and methods

2. 1. Animals

Male 21-day-old Sprague–Dawley rats (Harlan Sprague– Dawley, Indianapolis, IN) (n = 24 for the microdialysis studies; n = 24 for protein and mRNA analysis) were randomly divided into four dietary treatment groups as in previous studies (Anderson et al., 2007a,b): control (CN; 35 mg Fe/kg, 10 mg Mn/kg diet & d.i. water); control Mn-exposed (CNMn; control diet & 1 g Mn (as MnCl2)/l d.i. water); iron-deficient (ID; 4 mg Fe/kg, 10 mg Mn/kg diet & d.i. water); and iron-deficient/Mn exposed (IDMn; ID diet & 1 g Mn/l d.i. water). Diets were obtained from Bio-Serv (French-town, NJ) and certified for metal content. Rats had free access to food and water 24 h/day, and the lights were turned off between 1800 and 600 h. Room temperature was maintained at 25 ± 1 °C. The University of North Carolina at Greensboro Animal Care and Use Committee approved all of the animal procedures.

2.2. 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 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 returned to shoebox cages with Tek-Fresh bedding (Harlan, Indianapolis, IN) and monitored daily until microdialysis experiments began.

2.3. 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 perfused with artificial CSF (155 mM Na^{+}, 0.83 mM Mg^{2+},2.9 mM K^{+}, 132.76 mM Cl^{-}, 1.1 mM Ca^{+}, pH 7.4) for 1 h at a flow rate of 1 μl/min. After perfusion, the flow rate was adjusted to 0.5 μl/min and 30 min fractions were collected in microtubes for a total of 2 h (4 samples per rat) in a refrigerated fraction collector (CMA Microdialysis, Acton, MA). Samples were stored at -80 °C until analysis of the dialysate fraction. To quantify levels of GABA from the microdialysate, fractions were analyzed for GABA content using capillary electrophoresis with laser induced fluorescence detection (CE-LIF) (Biorad Biofocus 2000, Hercules, CA, with 488 nm diode laser/590 nm emission filter). Rats were then returned to their home cage and, the following day, were sacrificed, brains removed, and probe placement verified post mortem. Brains were dissected into five regions (caudate putamen, globus pallidus, substantia nigra, hippocampus, and cerebellum) for metal, protein, and mRNA analyses (see below). Regions were selected based on the known heterogeneous accumulation of metals in response to alterations in dietary Fe and Mn levels (Anderson et al., 2007a,b; Erikson et al., 2002) and the density of GABAergic neurons.

2.4. CE-LIF analysis

A protocol by Chen et al. (2001) allowing for detection of amino acids and biogenic amines at nanomolar concentrations was modified to accommodate the needs of this study. 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 μl 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 GABA 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 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 a calibration curve for GABA constructed each day of sample analysis using three points with a concentration range of 50 nM to 1 μM GABA. GABA (Sigma) and homoserine standard solutions used for construction of calibration curves were prepared in artificial cerebral spinal fluid with the same composition as that used in the microdialysis studies. A representative limit of detection for this method is 6.9 nM GABA (based on 3σ, where σ represents the standard deviation of the background) with a linear dynamic range of 3.64 decades. The ratio of GABA peak height to homoserine peak height for each sample was used to determine the concentration of GABA based on the calibration curve response.

2.5. Metal analysis

Tissue Mn and Fe concentrations were measured with graphite furnace atomic absorption spectroscopy (Varian AA240, Varian, Inc., USA). Blood samples were collected at the end of the experiment into heparinized tubes, cooled to 4 °C, and centrifuged in a clinical centrifuge for 15 min to separate cells from plasma. Plasma was frozen at -80 °C until analyzed for Mn and Fe. Equal volumes of plasma and 0.5% Triton-X were vortexed for 30 s before being centrifuged at 12,000 x g for 10 min. The supernatant was removed and an aliquot of 50 μl brought to 1 ml total volume in 2% nitric acid and analyzed for Mn and Fe content. Brain regions were digested in ultra-pure nitric acid (1: 10, w/v dilution) for 48–72 h in a sand bath (60 °C). An aliquot of 50 μl of digested tissue was brought to 1 ml total volume with 2% nitric acid and analyzed for Mn and Fe. Microdialysate samples were pooled from individual animals and diluted (1:2 dilution in 2% nitric acid) to a final volume of 60 μl prior to metal analysis. Bovine liver (NBS Standard Reference Material, USDC, Washington, DC) (10 μg Mn/g; 184 μg Fe/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).

2.6. Protein extraction

Protein was extracted from the brain tissue samples for Western blot analysis from animals that did not undergo surgery to ensure no changes in expression due to surgery. Tissue samples were sonicated in 500 μl of RIPA lysis buffer (1% Nonidet 40, 1% SDS, 0.5% sodium deoxycholate, 1 mM NaF, 2 mM β-glycerolphosphate, 1 mM sodium orthovanadate, and 1 x protease inhibitor cocktail (Sigma, St. Louis, MO) in 1 x PBS) on ice until completely homogenized. Homogenates were incubated on ice for 20 min before being centrifuged at 12,000 x g for 2 0 min at 4 °C. Supernatant was then transferred to new tubes and total protein concentration determined by BCA assay (Pierce, Rockford, IL) before proceeding with Western analysis.

2.7. Western blot analysis

Western blot analysis was conducted to examine the effects of the dietary treatment on expression of GAT-1, $GABA_A$, and $GABA_B$ proteins in vivo. Protein samples (20 μg) were combined with 4 x LDS sample buffer (Invitrogen, Carlsbad, CA) containing 5% β-mercaptoethanol and heated at 70 °C in a heat block for 10 min. Samples were then loaded onto a 4–12% Bis-Tris pre-cast mini gel (Invitrogen, Carlsbad, CA) and electrophoretically separated under denaturing conditions in 1 x MOPS buffer containing 1% antioxidant

(Invitrogen, Carlsbad, CA). Proteins were transferred to a PVDF membrane (Millipore, Billerica, MA) before blocking with 5% BSA. Membranes were probed overnight at 4 °C with primary antibody (mouse monoclonal anti-β-actin, Santa Cruz Biotech, Santa Cruz, CA) (rabbit polyclonal anti-$GABA_{B(R2)}$ subunit; rabbit polyclonal anti-GAT-1, C-terminus; rabbit poly-clonal anti-$GABA_{A(\alpha 1)}$; Chemicon, Temecula, CA) for the protein of interest in 5% BSA. Membranes were rinsed in 1 x TBST (10 mM Tris, pH 7.4,150 mM NaCl, 0.05% Tween 20) and probed for 2 h at room temperature with an HRP-conjugated secondary antibody (goat anti-rabbit, Chemicon, Temecula, CA) (goat anti-mouse, Santa Cruz Biotech, Santa Cruz, CA) in 5% BSA. Membranes were then rinsed several times in 1 x TBST before incubation in ECL solution (PerkinElmer, Waltham, MA) and exposure to radio-graphic film (Pierce, Rockford, IL). Membranes were also probed for β-actin to verify equal loading and for image analysis. This housekeeping protein was chosen for comparison over GAPDH, which has been shown to be affected by Mn concentrations (Hazell, 2002). Films were analyzed using image analysis software (Image J, NIH, Bethesda, MD), with the amount of the target protein from each sample standardized to the amount of β-actin from the sample.

2.8. RNA isolation and cDNA synthesis

Total RNA was isolated from brain regions for quantitative PCR analysis from animals that did not undergo surgery to ensure no changes in mRNA expression as a result of surgery. Tissue samples were stored in 1 ml of RNAlater® solution (Ambion Inc., Austin, TX) and stored at —20 °C until analysis. Total RNA was isolated utilizing the ToTALLY RNATM kit (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 from total RNA was performed using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions.

2.9. Quantitative PCR

Quantitative real-time PCR analysis was utilized to determine expression of GAT-1, $GABA_A$, and $GABA_B$. Triplicate aliquots of cDNA were analyzed on 96-well plates using expression assays for the genes of interest obtained from Applied Biosystems (Foster City, CA). Values of cDNA expression were normalized relative to the expression of β-actin analyzed from the same sample on the same plate and reported as percent of control.

2.10. Statistical analysis

Statistical analyses were conducted using SPSS v14. Data were examined for normality of distribution using a one-sample Kolmogorov-Smirnov test and presence of outliers by boxplot analysis. Data were analyzed using analysis of variance, with Dunnett's post hoc analysis conducted to assess difference from controls when $p < 0.05$. Pearson's correlational analyses were conducted to examine relationships between extracellular concentrations of GABA, Mn, and Fe.

3. Results

3.1. Microdialysis analysis

GABA concentrations were increased in CNMn and ID versus CN (Fig. 1A). For analysis, we excluded the IDMn group because only two animals survived surgery. Though the difference in GABA concentrations was not statistically significant ($p = 0.06$), the observation does denote an obvious biological response to the dietary protocol. Concentrations of Mn in dialysate samples were significantly increased ($p < 0.001$) in CNMn and ID versus CN (Fig. 1B). No correlation was found between the extracellular concentrations of GABA and Mn (data not shown). Concentrations of Fe in dialysate samples were significantly decreased ($p = 0.023$) in CNMn and ID versus CN (Fig. 1C). An inverse correlation was observed between the extracellular concentrations of GABA and Fe, with GABA concentrations decreasing with increased Fe (Fig. 1C, inset), though the correlation was not statistically significant ($p = 0.08$). Being that there is a trend towards significance ($p < 0. 10$), it does suggest an effect of Fe levels on extracellular GABA concentrations. The statistical significance of this experiment is most likely affected by the small sample size. Only two animals from the IDMn group survived the surgery, with microdialysis samples being collected. Of the other three treatment groups, samples were collected from four out of the six animals. This is not uncommon given the 50% success rate associated with

this procedure (Nelson et al., 1997), due either to animals succumbing to surgery or removing the cannula before sample collection.

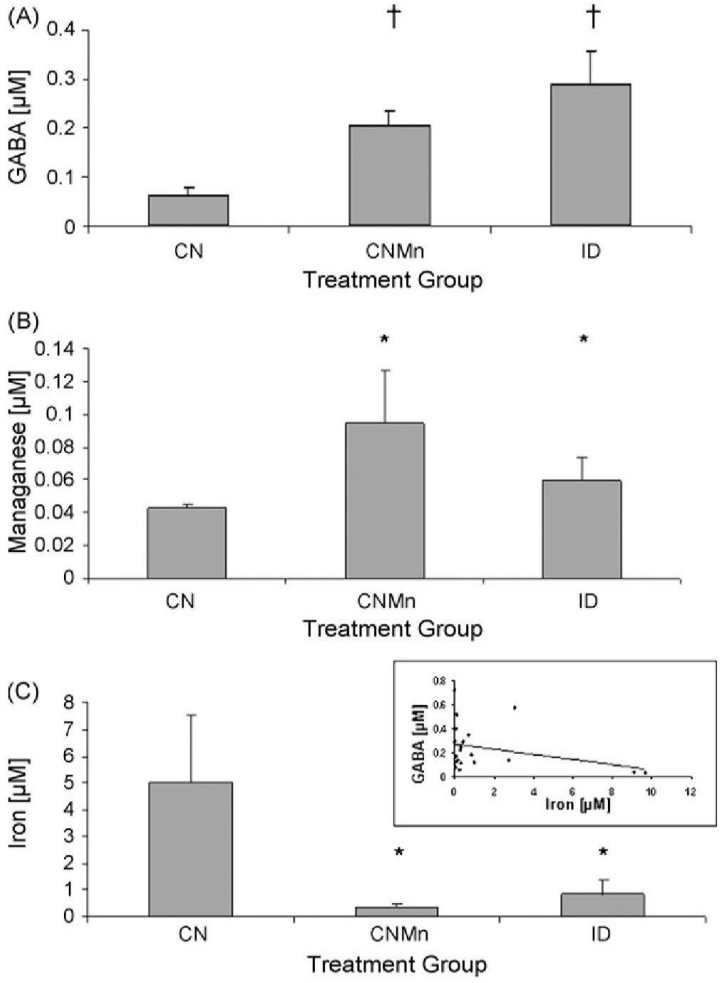

Fig. 1. Microdialysate analysis. Mean concentrations ± S.E.M. are shown for (A) extracellular GABA, (B) manganese, and (C) iron in microdialysate samples from the striatum after six weeks of dietary treatment. *Inset*: Correlational analysis of extracellular Fe and GABA concentrations ($R = -0.32$; $p = 0.08$). $^{*}p < 0.001$; $^{\dagger}p < 0.10$.

3.2. Plasma manganese and iron

Plasma Mn concentrations were significantly increased in those animals receiving Mn supplementation versus those animals receiving deionized water alone (Fig. 2A) ($p = 0.02$). A significant decrease in plasma Fe concentrations was observed in animals receiving the ID diet versus the CN diet (Fig. 2B) ($p = 0.007$).

3.3. Brain manganese and iron

In all brain regions, Mn concentration was significantly increased in the Mn-exposure group versus those animals that did not receive Mn supplementation (Fig. 3A) ($p < 0.05$). A heterogeneous response was observed in the brain in regard to Fe levels, with a general decrease in Fe concentration in those animals receiving the ID diet versus animals receiving the CN diet, though the only significant decrease was seen in the globus pallidus (Fig. 3B) ($p < 0.05$).

3.4. Western blot analysis

Elevated brain Mn and ID caused an approximate 50% decrease in GAT-1 protein expression in both the hippocampus and substantia nigra (Fig. 4A). In the hippocampus, GAT-1 protein expression was significantly decreased in CNMn (41%) and ID (47%) versus CN ($p < 0.05$). Similarly, in the substantia nigra, there was a significant decrease in expression of GAT-1 in CNMn (68%), ID (48%), and IDMn (44%) versus CN ($p = 0.03$). No significant change in GAT-1 protein expression was observed in the globus pallidus, caudate

putamen, or cerebellum. The dietary treatment had no effect on β-actin protein levels. Representative blots for GAT-1 and β-actin for each region are shown in Fig. 4C, with each band representing an individual animal.

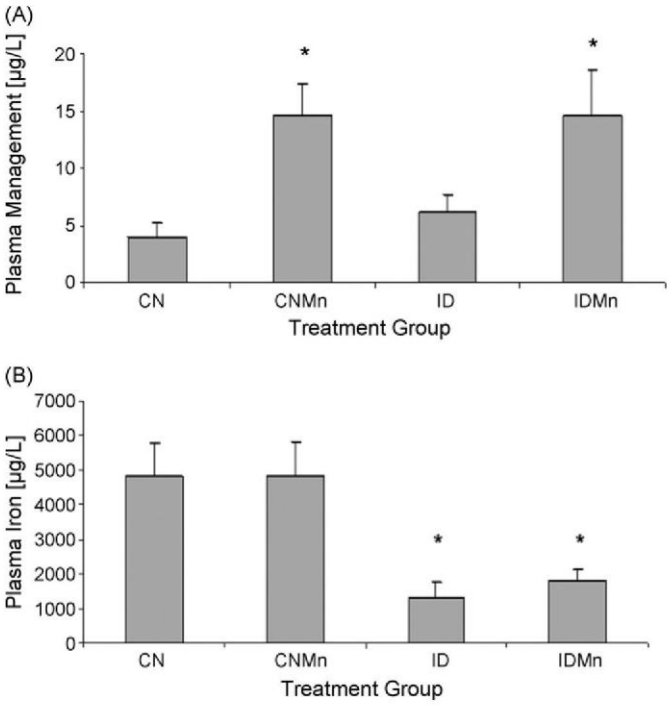

Fig. 2. Plasma metal concentrations at six weeks. (A) Plasma manganese concentrations were significantly increased in those animals receiving manganese supplementation versus those animals receiving deionized water alone ($p = 0.02$). (B) A significant decrease in plasma iron concentration was observed in animals receiving the ID diet versus the CN diet ($p = 0.007$).

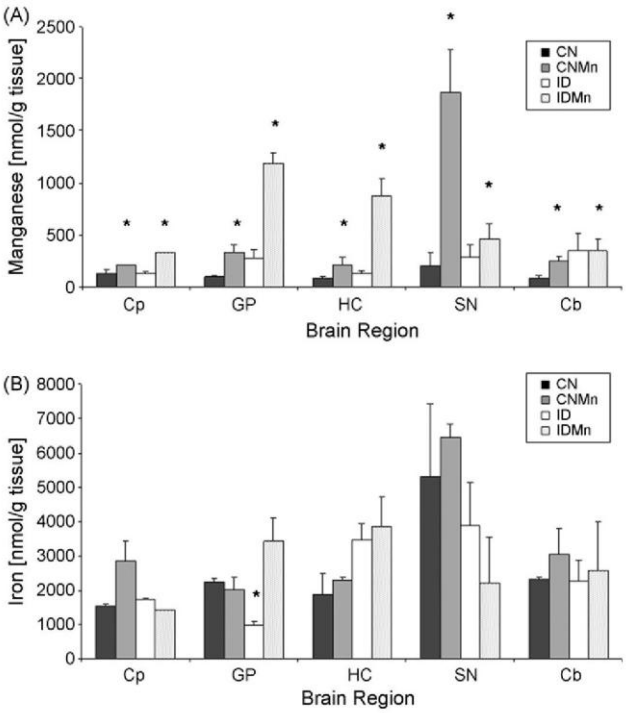

Fig. 3. Brain metal concentrations at six weeks. Mean concentrations ± S.E.M. are shown for manganese (A) and iron (B) for caudate putamen (Cp), globus pallidus (GP), hippocampus (HC), substantia nigra (SN), and cerebellum (Cb). CN is represented in black, CNMn in gray, ID in white, and IDMn in dotted area. $^{*}p < 0.05$ according to Dunnet's post hoc analysis.

Increased Mn and ID were associated with a significant increase in protein expression of $GABA_A$ in globus pallidus (CNMn 75%; ID 300%; IDMn 300%; p = 0.012) and substantia nigra (ID 200%; IDMn 200%; p = 0.045) versus CN (Fig. 5A). $GABA_A$ expression was significantly increased in hippocampus in CNMn (72%) versus CN (p = 0.03) (Fig. 5A). No significant difference in $GABA_A$ protein expression was observed in caudate putamen or cerebellum. Representative blots for $GABA_A$ and β-actin for each region are shown in Fig. 5C, with each band representing an individual animal.

Iron deficiency and Mn exposure were associated with a decrease in $GABA_B$ protein in globus pallidus (IDMn 46%; p = 0.03), substantia nigra (ID 41%; IDMn 54%; p = 0.02), and hippocampus (ID 70%; IDMn 55%; p = 0.027) (Fig. 6A). No significant change in $GABA_B$ protein expression was observed in caudate putamen or cerebellum. Representative blots for $GABA_B$ and β-actin for each region are shown in Fig. 6C, with each band representing an individual animal.

3.5. Quantitative PCR analysis

Increased Mn significantly decreased mRNA expression of GAT-1 in the hippocampus (CNMn 52%; IDMn 67%; p = 0.023) and caudate putamen (IDMn 54%; p = 0.020) (Fig. 4B). In the substantia nigra, mRNA expression of GAT-1 was significantly increased three-fold in ID and over five-fold in IDMn versus CN (p = 0.003) (Fig. 4B). A two-fold increase in GAT-1 expression was observed in the globus pallidus in the Mn-exposed groups versus CN (Fig. 4B). This increase, though not statistically significant (p = 0.080), does indicate a physiological response to the presence of Mn. The dietary treatment had no effect on β-actin mRNA levels.

In substantia nigra, $GABA_A$ mRNA expression was significantly decreased in CNMn (67%) and ID (64%) versus CN (p = 0.006) (Fig. 5B). No significant changes in mRNA expression of $GABA_A$ were observed in any of the other regions analyzed (Fig. 5B). Expression of $GABA_B$ receptor mRNA was significantly decreased in

the hippocampus (CNMn 59%; ID 45%; IDMn 43%; $p = 0.04$), substantia nigra (CNMn 92%; ID 90%; IDMn 94%; $p < 0.001$) and caudate putamen (CNMn 55%; IDMn 57%; $p = 0.003$) versus CN (Fig. 6B). No significant changes in mRNA expression were observed in globus pallidus, substantia nigra, or cerebellum.

4. Discussion

We report for the first time that increased dietary Mn (via drinking water; 100 times more concentrated than in the diet) causes elevated extracellular GABA and altered expression of GABA receptors and transporters, at both the protein and mRNA level. Extracellular GABA concentrations increased in response to Mn exposure and the ID diet (Fig. 1A). Correlational analyses revealed an inverse correlation between extracellular GABA and Fe concentrations (Fig. 1C, inset) with no correlation between extracellular GABA and Mn, suggesting GABA concentrations are altered as a result of the lowered extracellular Fe caused by Mn exposure and the ID diet. In addition, dietary Mn exposure was shown to alter expression of GABA receptor and transport proteins and mRNA *in vivo*, with a varied effect observed across the brain regions examined (Figs. 4–6). These data suggest that altered tissue levels of GABA (Erikson et al., 2002) and decreased uptake of GABA (Anderson et al., 2007a) due to Mn exposure may be the result of altered expression of its transport protein resulting in elevated extracellular GABA concentrations.

As in earlier studies (Anderson et al., 2007a,b), our dietary protocol led to perturbations in systemic levels of Mn and Fe. The addition of Mn in the drinking water significantly increased the levels of Mn in the plasma (Fig. 2A), while the ID diet lowered the plasma concentration of Fe (Fig. 2B). In the brain, Mn accumulated in a diverse fashion across the regions examined, with increased levels of Mn found in those animals receiving Mn supplementation via the drinking water (Fig. 3A). In general, Fe was depleted in those animals receiving the ID diet; however, the only statistical difference in Fe levels in the brain was observed in the globus pallidus (Fig. 3B) similar to a previous study from our lab (Erikson et al., 2004). This varied response in Fe levels was observed in our previous studies, at both four weeks (Anderson et al., 2007b) and six weeks (Anderson et al., 2007a) of dietary treatment, with Fe levels varying between and within regions. Additionally, when examining the Fe:Mn ratio in the brain, a reduction in brain Fe levels in these animals is apparent, with the ID diet causing a significant reduction in the Fe:Mn ratio in most regions (data not shown).

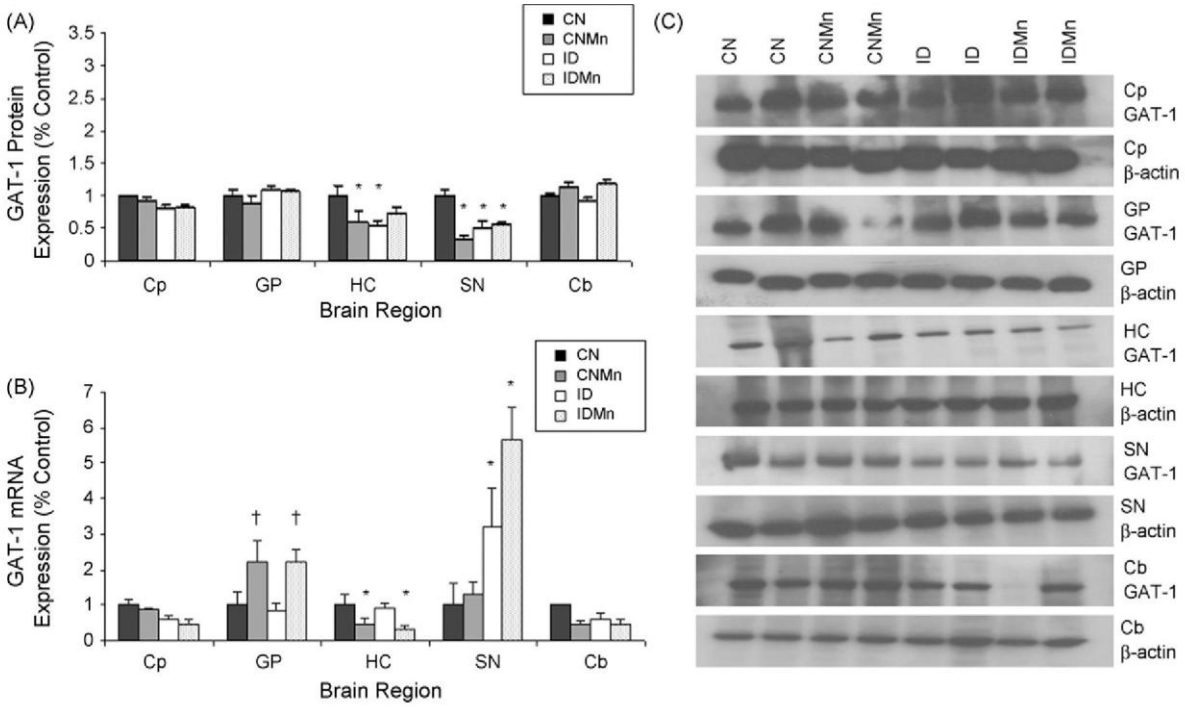

Fig. 4. Effect of dietary treatment on GAT-1 protein and mRNA expression. Mean expression as percentage of control ± S.E.M. for GAT-1 (A) protein and (B) mRNA relative to β-actin are shown for caudate putamen (Cp), globus pallidus (GP), hippocampus (HC), substantia nigra (SN), and cerebellum (Cb). CN is represented in black, CNMn in gray, ID in white, and IDMn in dotted area. (C) Representative blots for GAT-1 and β-actin for each region are shown, with each band representing an individual animal. $^{*}p < 0.05$ according to Dunnet's post hoc analysis.

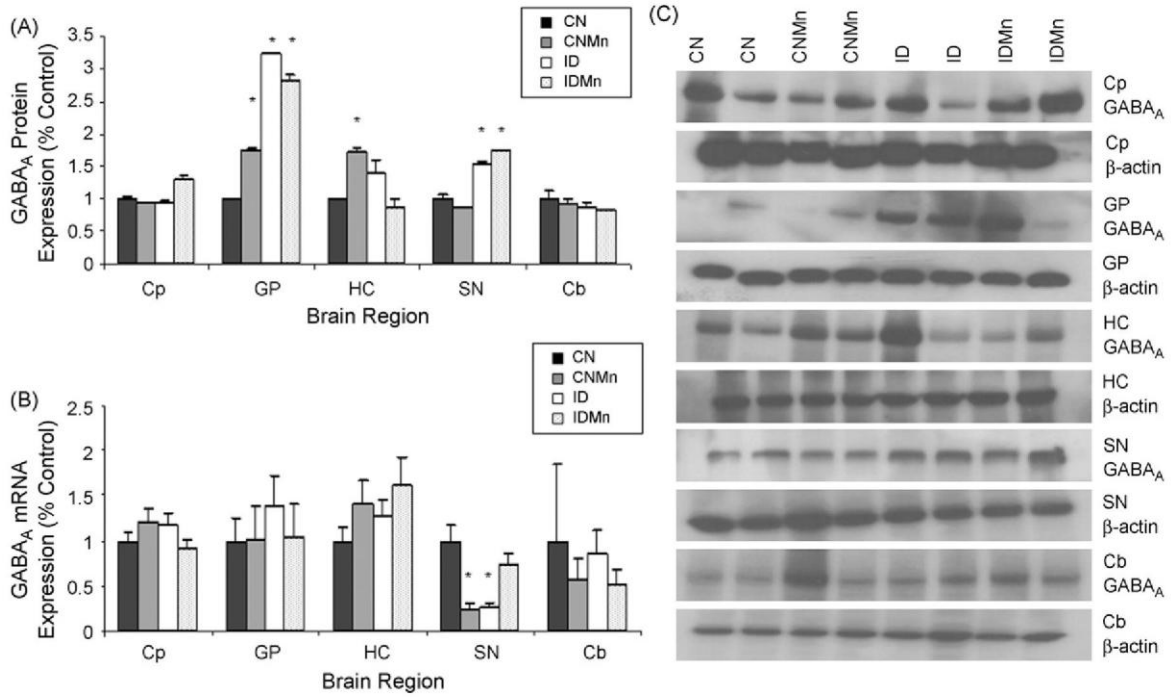

Fig. 5. Effect of dietary treatment on $GABA_A$ protein and mRNA expression. Mean expression as percentage of control ± S.E.M. for $GABA_A$ (A) protein and (B) mRNA relative to β-actin are shown for caudate putamen (Cp), globus pallidus (GP), hippocampus (HC), substantia nigra (SN), and cerebellum (Cb). CN is represented in black, CNMn in gray, ID in white, and IDMn in dotted area. (C) Representative blots for $GABA_A$ and β-actin for each region are shown, with each band representing an individual animal. $^*p < 0.05$ according to Dunnet's post hoc analysis.

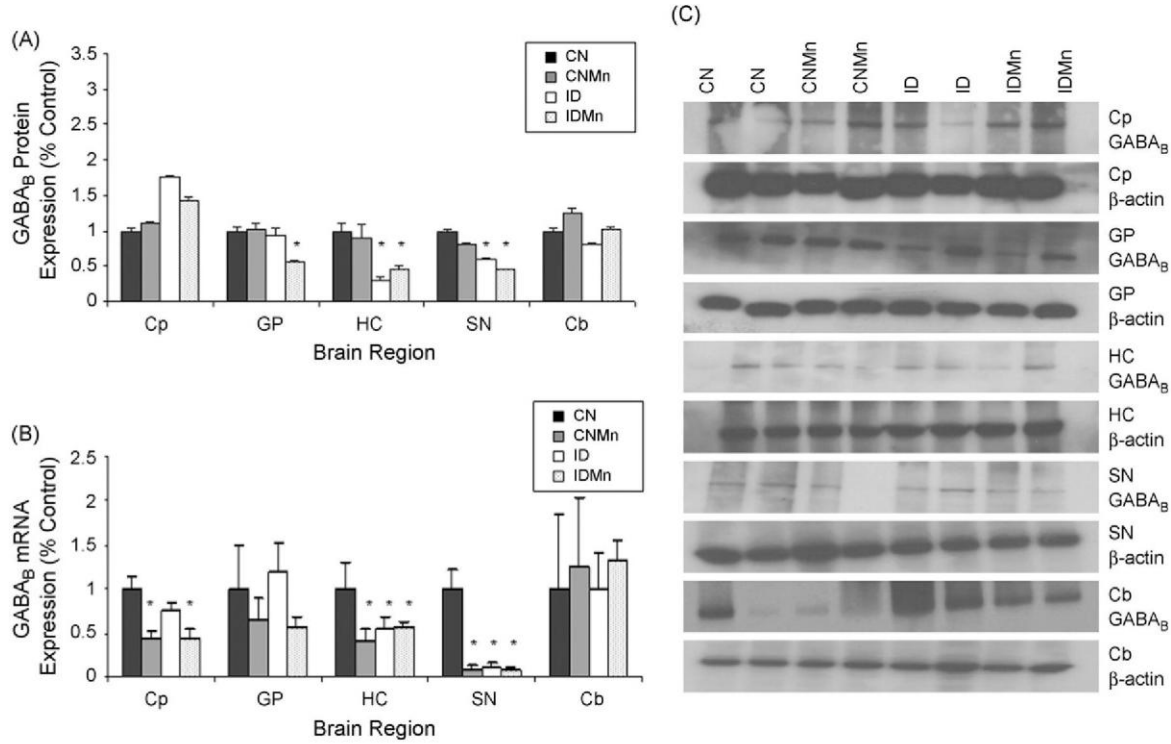

Fig. 6. Effect of dietary treatment on $GABA_B$ protein and mRNA expression. Mean expression as percentage of control ± S.E.M. for $GABA_B$ (A) protein and (B) mRNA relative to β-actin are shown for caudate putamen (Cp), globus pallidus (GP), hippocampus (HC), substantia nigra (SN), and cerebellum (Cb). CN is represented in black, CNMn in gray, ID in white, and IDMn in dotted area. (C) Representative blots for $GABA_B$ and β-actin for each region are shown, with each band representing an individual animal. $^*p < 0.05$ according to Dunnet's post hoc analysis.

Extracellular GABA concentrations in the caudate putamen were increased in response to Mn exposure and ID (Fig. 1A); and while this difference was not statistically significant (p = 0.06), it does indicate a significant physiological response to changes in regional metal levels (e.g., high Mn = 3-fold increase; low Fe = 4- fold increase compared to CN). An important question arose from these findings; is the elevated GABA due to increased extracellular Mn or decreased extracellular Fe? To answer this question, we measured both Mn and

Fe concentrations in the microdialysis samples and correlated them with the GABA concentrations. Manganese levels were significantly increased in those animals receiving Mn supplementation and the ID diet (Fig. 1B); however, no correlation was found between extracellular Mn and GABA concentrations, contrary to our original hypothesis. Concentrations of extracellular Fe were significantly decreased in those animals receiving the ID diet as well as in the CNMn animals versus CN (Fig. 1 C). This finding is interesting given that plasma Fe levels in the CNMn animals were normal. However, the Fe found in the plasma represents a more labile pool than that found in the CSF. An inverse correlation between extracellular GABA and Fe concentrations was also observed, with GABA concentration increasing with decreased Fe, suggesting that decreased Fe (due to both dietary deficiency and Mn-exposure) affects the levels of extracellular GABA. Iron deficiency affects GABA synthesis via glutamate decarboxylase (GAD) (Li, 1998) and a reduction in GABA brain tissue concentrations has been shown in rats fed a marginally ID diet for eight weeks (Shukla et al., 1989). Low tissue neurotransmitter levels may be the result of decreased uptake of the respective neurotransmitter (Gainetdinov et al., 1998), increasing the extracellular concentrations, a scenario which might be occurring in the current study.

The observed increase in extracellular GABA concentrations could potentially mediate the locomotor effects seen in Mn neurotoxicity, such as hyperkinesia, ataxia, and dystonia. GABAergic neurons in the striatum receive dopaminergic terminals from the substantia nigra (Smith and Bolam, 1990), in turn modulating the dopaminergic functioning in the striatum (Galindo et al., 1999). Increased extracellular levels of GABA in the striatum could affect the activity of the GABA projection neurons to the substantia nigra (Koós and Tepper, 1999), leading to dysregulation of striatal DA release via the nigrostriatal pathway and increased inhibition, causing hypokinetic activity, a hallmark of Mn neurotoxicity (Fig. 7). Normandin et al. (2004) observed decreases in locomotor activity in Mn-exposed young adult rats, while motor deficits were also observed in a study utilizing a pre-Parkinsonian rat model and cumulative low-dose Mn exposure (Gwiazda et al., 2002). In both of these studies, DA was not altered, suggesting that changes in GABA may precede and facilitate changes in DA during manganism.

A major component of our hypothesis is that increased extracellular GABA due to Mn-exposure is caused by alterations in GAT-1. While protein expression of GAT-1 was not decreased in caudate putamen, mRNA expression was decreased (Fig. 4B), indicating modulation of GAT-1 and a possible transition occurring in that region. Expression of GAT-1 mRNA was significantly decreased in hippocampus as well, while a two-fold increase was observed in the globus pallidus and a three and five-fold increase observed in substantia nigra (Fig. 4B). While the two-fold increase in GAT-1 mRNA expression in globus pallidus was not statistically significant ($p = 0.08$), it does indicate a physiological response to the dietary protocol within this region, perhaps as a mechanism to maintain adequate levels of GAT-1 protein. It should be noted that the current results were obtained after six weeks of dietary treatment. However, this represents only a snapshot of the modification occurring in the globus pallidus over the course of the treatment period as a result of increased Mn. Protein levels of GAT-1 in the globus pallidus may have been attenuated earlier than six weeks, with the increases in mRNA observed at six weeks the result of a compensatory mechanism. In our previous study, decreases in ^{3}H-GABA uptake were observed after four weeks of dietary treatment in relation to Fe levels (Anderson et al., 2007a), indicating the likelihood of perturbations in GAT-1 at an earlier time. The increase seen in mRNA expression of GAT-1 in substantia nigra as a result of Mn exposure could potentially be a physiological response to compensate for decreases in GAT-1 protein in that region by increasing transcription. These changes in GAT-1 expression in the substantia nigra and globus pallidus could affect extracellular concentrations of GABA in the caudate putamen via nigrostriatal projections (Fig. 7).

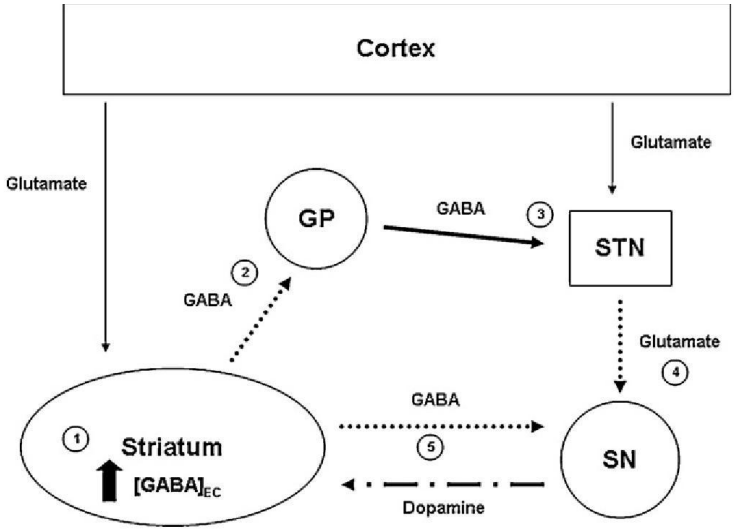

Fig. 7. GABA biology during Mn overload. This simple schematic of the basal ganglia represents the potential consequences of the increased extracellular GABA concentrations in the striatum due to alterations of Mn and Fe status observed in the current study. (1) Increased extracellular GABA concentrations in the striatum would reduce the activity of the GABA striatopallidal projection neurons (2) (dotted line). This reduction in activity would (3) increase the GABAergic inhibitory firing from the globus pallidus (GP) to the subthalamic nucleus (STN) (heavy black line), in turn (4) decreasing the excitatory glutamatergic firing from this region to the substantia nigra (SN) (dotted line). (5) Decreased glutamatergic excitation in the substantia nigra, along with decreased GABAergic inhibition from the striatonigral projection neurons (dotted line) and decreased protein expression of GAT-1 and $GABA_B$, would lead to a dysregulation of dopaminergic firing to the striatum (alternating line). This decrease in GABAergic firing to the substantia nigra may also contribute to the dopaminergic alterations observed during ID (Nelson et al., 1997; Erikson et al., 2000, 2001).

Decreased protein expression of GAT-1 in hippocampus and substantia nigra may also be the result of protein degradation and recycling via protein kinase C (PKC). Protein kinase C is known to regulate GAT-1 via phosphorylation (Mandela and Ordway, 2006), causing internalization of these proteins, degradation, and recycling (Gadea and López-Colomé,2001). An acute Mn treatment (300 μM) has been shown to activate caspase-3 leading to activation of PKCδ in N27 mesenchephalic cells (Latchoumycandane et al., 2005). A similar response was seen in those cells incubated for 72 h with a lower dose of Mn (50 μM), replicating chronic exposure. This 300 μM dose represents a physiologically relevant dose similar to that achieved in the current study. Additionally, expression of GAT-1 may be inhibited through effects on pre-synaptic $GABA_B$ autoreceptors, which can play a part in regulating expression of GAT-1 (Zahniser and Doolen, 2001). Decreases in these $GABA_B$ autoreceptors could result in a general decrease of GAT-1 density in that region.

As anticipated, $GABA_B$ protein expression was significantly decreased in globus pallidus, substantia nigra, and hippocampus in those animals receiving the ID diet and Mn supplementation (Fig. 6A). Additionally, expression of $GABA_B$ mRNA was attenuated in the hippocampus, substantia nigra, and caudate putamen of the same animals (Fig. 6B). While Mn and Fe are not known to have specific actions on the transcription of $GABA_B$, Mn has been shown to bind to various forms of DNA structure, leading to conformational changes that might potentially affect the efficiency of gene transcription (Kennedy and Bryant, 1986). Protein expression of $GABA_B$ may also be attenuated through post-translational processes, such as recycling and degradation, mediated by regulatory kinases that may be affected by excess Mn. Decreases in both mRNA and protein expression of $GABA_B$ in hippocampus might indicate neuronal loss in that region as a result of Mn exposure (Sloot et al., 1994) as Mn has been shown to activate caspase-3 (Latchoumycandane et al., 2005), a mediator of apoptosis. A similar scenario might be the case in the substantia nigra.

Decreases in $GABA_B$ expression due to Mn exposure and increased extracellular GABA may result in marked changes in behavior, particularly related to anxiety-like behaviors. $GABA_B$ deficient mice have ‘panic’ attacks

and are more anxious in anxiety related behavioral screens (Mombereau et al., 2005). Similarly, $GABA_B$ knockout mice display spontaneous seizures, hyperalgesia, hyperlocomotion, and memory impairments (Emson, 2007). Although both manganism and ID share many of these characteristics (Pal et al., 1999; Aschner et al., 2005) with these mice models, it should be noted that changes in $GABA_A$ receptors might play a role.

The change in mRNA expression in substantia nigra might represent interactions of Mn with DNA, causing a decrease in gene transcription efficiency (Hazell et al., 2003). The lack of changes in $GABA_A$ mRNA expression in the other regions suggests that the alterations observed in $GABA_A$ protein expression result from effects on post-translational processes. One possible mechanism for the increase seen in $GABA_A$ protein expression could be through increased trafficking of the receptor to the plasma membrane via phosphorylation of $GABA_A$ subunits by Akt (Wang et al., 2003) and since treatment of microglial cells with 500 μM Mn has been shown to activate Akt (Bae et al., 2006).

The changes in protein and mRNA expression observed in the hippocampus and substantia nigra are particularly interesting given these two regions were observed to have the most impact from our dietary protocol. The hippocampus plays a vital role in memory and learning, with the majority of the neurons present in the hippocampus GABAergic (Gadea and López-Colomé, 2001). Manganese has also been shown to generate lesions in the hippocampus (Sloot et al., 1994) and impaired memory and learning scores have been observed in children exposed to high levels of manganese from the drinking water (Wasserman et al., 2006). The dramatic changes in expression of protein and mRNA levels in the substantia nigra, a region association with Parkinson's disease and a target region for Mn accumulation (Aschner et al., 2005), lend credence to the hypothesis of a role for GABA in the effects on DA in this region during manganism. Recall that GABA neurons project from the striatum to the substantia nigra, inhibiting the dopaminergic output from the substantia nigra (Fig. 7). The differences in protein and mRNA expression observed in this study present a potential mechanism(s) to explain behaviors associated with Mn neurotoxicity.

The novel results of this study illustrate the complex mechanisms involved in Mn neurotoxicity. The alterations observed in protein and mRNA expression of GABA receptor and transport proteins were not universal, with a diverse effect of Mn exposure on these two indices not only from region to region, but, in some cases, within the same region. Future studies to tease out the pathways involved in these shifts in expression are warranted. These alterations in receptor and transporter expression are important in light of the effects observed in animal models lacking GAT-1 (tremor and anxiety) and $GABA_B$ (anxiety and nervousness) (Emson, 2007; Chiu et al., 2005; Mombereau et al., 2005), which are similar to symptoms of Mn neurotoxicity. Given the current findings, the increased levels of extracellular GABA in response to Mn exposure and ID are most likely due to decreased uptake of GABA rather than an increase in GABA release. Continuing studies in our lab will pharmacologically probe the effect of ID and Mn exposure on these extracellular GABA levels in order to fully understand the neurobiological consequences of Mn toxicity, especially as they relate to dietary exposures.

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