Inhibition of DAT function attenuates manganese accumulation in the globus pallidus

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Abstract:
Manganese (Mn) is an essential nutrient, though exposure to high concentrations may result in neurotoxicity characterized by alterations in dopamine neurobiology. To date, it remains elusive how and why Mn targets dopaminergic neurons although recently the role of the dopamine transporter has been suggested. Our primary goal of this study was to examine the potential roles of the monoamine transporters, dopamine transporter (DAT), serotonin transporter (SERT), and norepinephrine transporter (NET), in neuronal Mn transport. Using striatal synaptosomes, we found that only inhibition of DAT significantly decreased Mn accumulation. Furthermore, weanling rats chronically exposed to Mn significantly accumulated Mn in several brain regions. However, rats receiving the specific DAT inhibitor GBR12909 (1 mg/kg bw, three times/week; 4 weeks) had significantly lower Mn levels only in the globus pallidus compared to saline-treated rats ($p < 0.05$). Our data show that inhibition of DAT exclusively inhibits Mn accumulation in the globus pallidus during chronic exposure.
Keywords: Dopamine transporter; Manganese; Rat; Neurotoxicity

Article:
1. Introduction
Manganese (Mn) is an essential nutrient and functions as a critical cofactor for many key enzymes involved in cellular processes (Hurley and Keen, 1987). It is required for proper immune function, regulation of metabolism, reproduction, digestion, and bone growth (Aschner et al., 2005). However, exposure to high concentrations of Mn is known to result in neurotoxicity. Mn neurotoxicity, or manganism, shares neuropathologies with several clinical disorders, in particular Parkinson's disease (Pal et al., 1999; Aschner et al., 2005). Manganism is characterized by the accumulation of Mn in iron-rich, dopaminergic brain regions, specifically the basal ganglia (Aschner et al., 2005). The globus pallidus within the basal ganglia is a target region for Mn accumulation during Mn toxicity (Verity, 1999; Erikson et al., 2002, 2004).

Most research in the area of Mn neurotoxicity has focused on its effect on the metabolism of dopamine. Why and by what means Mn specifically accumulates in brain regions rich in dopamine remains unclear. The neurotoxins 1-methyl-4- phenylpyridium (MPP+) and paraquat are known to be selectively lethal to dopaminergic neurons due to the fact that they are transported by the dopamine transporter (DAT) (Petzinger et al., 2006; Yang and Tiffany-Castiglioni, 2005). Ingersoll et al. (1999) examined the effect on Mn uptake by inhibiting DAT. Cocaine, a DAT inhibitor, and reserpine, which decreases extracellular dopamine concentrations by inhibiting vesicular reuptake in the synapse, were administered to rats injected with Mn intrathecally. A significant decrease in Mn accumulation in the ventral pallidum was seen in rats treated with both. This suggests a potential role for the DAT and dopamine metabolism in the transport of Mn. However, both cocaine and reserpine are known to affect other neurotransmitters, most notably serotonin and norepinephrine (Ritz et al., 1990). Therefore, it is unclear if this decrease in ventral pallidum Mn concentrations is directly related to the DAT being inhibited or is related to the inhibition of the serotonin transporter and/or the norepinephrine transporter.

A recent study by this lab (Erikson et al., 2005) examined the potential role of the DAT in Mn accumulation in the brain using a knockout mouse model. Homozygous DAT-KO and WT mice were exposed to an acute dose

of 50mg/kg of $MnCl_2$ via intraperitoneal injection. After 15 min, brains were removed for metal analysis. No difference in basal Mn levels was seen between the DAT-KO mice and the WT mice. However, there was a significant 40% decrease in Mn accumulation in the striatum of the DAT-KO mice receiving Mn injection compared to the WT mice receiving Mn injection. This suggests that the DAT may not play a central role in normal Mn transport in the brain, but may become relevant in a toxicological paradigm in terms of Mn exposure.

While the effect of Mn toxicity on dopamine metabolism has been the primary focus, the effect of Mn toxicity on the metabolism of the biogenic amines norepinephrine (NE) and serotonin (5-HT) has not been as extensively studied. One group exposed PC12 cells to Mn pyrophosphate which resulted in more than a 70% decrease in cellular serotonin levels (Reaney and Smith, 2005). Another lab found an increase in norepinephrine in the brain stem of rats treated with $MnCl_2$, but norepinephrine concentrations were decreased in the hippocampus (Autissier et al., 1982). While the effect of Mn exposure on NE and 5-HT concentrations has been examined, the role(s) of the NET and SERT during Mn toxicity is virtually unknown.

The goal of the present study was to examine the potential role of the biogenic amine transporters DAT, NET, and SERT in Mn accumulation in the male rat brain. Due to the homology in the amino acid sequence of these transporters, their sodium dependence of their uptake mechanisms, and the fact that how and where Mn interacts with these proteins in unknown (Borowsky and Hoffman, 1995), striatal synaptosomes were utilized to screen for potential effects of inhibiting these biogenic amine transporters on Mn accumulation in the striatum, a target region for Mn neurotoxicity. The results of these experiments lead to in vivo studies using both dietary and pharmacological intervention to examine the effects on Mn accumulation on the developing male rat brain.

2. Materials and methods

2. 1. Animals

Weanling male Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN) and maintained on a modified AIN-93-G diet (BioServ, Frenchtown, NJ) containing 35 mg/kg Fe and 10 mg/kg Mn. Rats had free access to food and water and were housed in hanging cages in the animal facility in a typical light:dark cycle (06:00–18:00 h light on) with a room temperature of 25 °C. Animal care was in accordance with the University of North Carolina at Greensboro Animal Care and Use Committee.

2.2. Synaptosome isolation

The striata from the brains of control rats were removed and synaptosomes were obtained from this region. The brain regions were homogenized using a Teflon/glass homogenizer in 20 volumes of ice cold 0.32 M sucrose-HEPES, pH 7.4. The homogenate was then centrifuged at 2000 x *g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 20,000 x *g* for 15 min at 4 °C. The pellet was resuspended in 10ml fresh ice cold Krebs-Ringer-HEPES (KRH) buffer [118.4 mM NaCl, 1.18 mM $MgSO_4$,4.7 mM KCl, 1.2 mM KH_2PO_4, 10.0 mM HEPES, 5.6 mM dextrose (pH 7.4)] containing 10 μM pargyline (Sigma, St. Louis, MO). An aliquot of 200 pl of the synaptosomes was placed in a tube with 50 μl KRH buffer.

2.3. In vitro studies

Using striatal synaptosomes, we examined the effect of inhibiting three biogenic amine transporters on Mn transport. We used GBR12909 (K_i = 1 nM), desipramine (K_i = 3.2 μM), and fluoxetine (K_i = 0.6 μM) to block DAT, NET, and SERT, respectively. All drugs were purchased from Sigma.

To establish an appropriate working concentration for each of the transporter inhibitors, dose–response experiments were conducted using ^{3}H-dopamine, ^{3}H-norepinephrine, and ^{3}H-serotonin. A 200 μl aliquot of synaptosomes was incubated either in the presence or absence of a biogenic amine transporter inhibitor at various concentrations (0. 1–100 μM) for 5 min at 37 °C in a water bath. A 10 μl aliquot of the respective tritiated neurotransmitter (50 nM) (NEN, Boston, MA) was added to each tube and tubes were incubated for 15 min at 37 °C. The reaction was stopped by adding ice cold KRH buffer to each tube followed by rapid filtration through a GF/F Whatman fiberglass filter on a Millipore sampling manifold. Filters were washed twice with ice

cold KRH, placed in scintillation vials with 5 ml scintillation cocktail, and counted on a Beckman LS scintillation counter (Beckman, Fullerton, CA). An additional set of tubes, one for each treatment group, was incubated on ice to determine non-specific uptake. Protein analysis was performed utilizing the BCA method (Pierce, IL).

To assess Mn concentration in the striatal synaptosomes, a 200 μl aliquot of synaptosomes was incubated with or without 500 μM $MnCl_2$ in the presence or absence of each of the biogenic amine transporter inhibitors at the ED50 dose (1 μM GBR1 2909; 10 μM fluoxetine; 100 μM desipramine) for 15 min at 37 °C in a water bath. The reaction was stopped by adding ice cold KRH buffer to each tube followed by rapid filtration through a GF/F Whatman fiberglass filter on a Millipore sampling manifold. After filtration, the filters were digested in 500 μl ultra-pure nitric acid for 48 h. The supernatant was removed and Mn concentration was determined by GFAAS.

2.4. Iron and manganese analysis

Tissue Mn and iron (Fe) concentrations were measured with graphite furnace atomic absorption spectroscopy (GFAAS) (Varian AA240, Varian, Inc., USA). Tissue was digested in ultra-pure nitric acid (1: 10, w/v, dilution) for 48–72 h in a sandbath (60 °C). An aliquot of 100 μl of digested tissue was brought to 1 ml total volume with 2% nitric acid and analyzed for Mn and Fe as outlined previously (Erikson and Aschner, 2006).

2.5. In vivo studies

Because synaptosomal studies showed a direct interaction between DAT and Mn accumulation, we sought to examine the effect of DAT blockade in vivo. Weanling male Sprague–Dawley rats (n = 24) (Harlan, Indianapolis, IN) were randomly placed into one of four dietary treatment groups: control (CN), iron-deficient (ID), control with Mn supplementation (via the drinking water; 1 g Mn/l) (CNMn), and ID with Mn supplementation (IDMn) (Fig. 1). The CN diet consisted of a modified AIN-93-G diet (BioServ) containing 35 mg/kg Fe and 10 mg/kg Mn. The ID diet consisted of a modified AIN-93-G diet (BioServ) containing 6 mg/kg Fe and 10 mg/kg Mn. Two independent experiments with two separate sets of animals were conducted (n = 24 for each experiment). Within each dietary group, one-half of the rats received intraperitoneal injections of GBR12909 (Sigma), 1 mg/kg (ED-50), three times/week while the other half received saline injections of equal volume. Dietary and pharmacological intervention lasted for 4 weeks. Brains were dissected into five regions, known for accumulating Mn to varying degrees: caudate putamen (CP), globus pallidus (GP), hippocampus (HC), substantia nigra (SN), and cerebellum (CB).

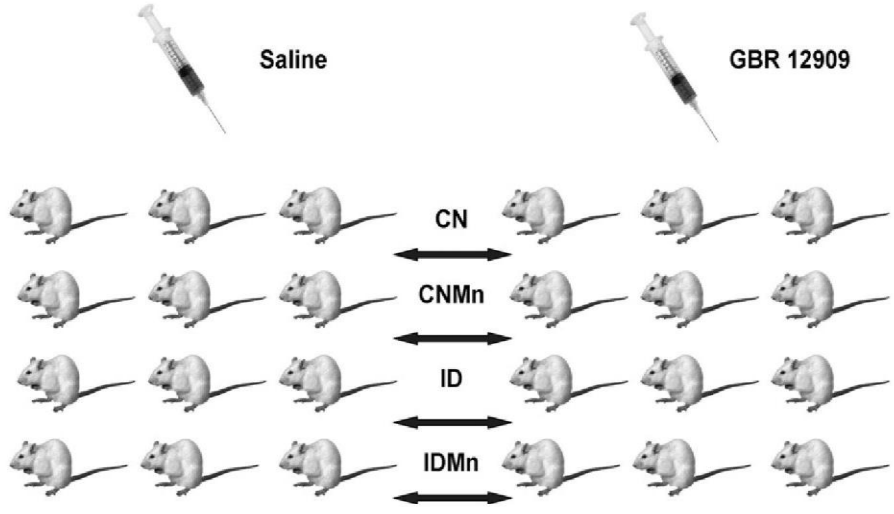

Fig. 1. Experimental design of in vivo studies. Weanling male Sprague–Dawley rats were randomly placed into one of four dietary treatment groups: control (CN), iron-deficient (ID), control with Mn supplementation (via the drinking water; 1 g Mn/l) (CNMn), and ID with Mn supplementation (IDMn). Two independent experiments with two separate sets of animals were conducted (n = 24 for each experiment; n = 48 total). Within each dietary group, one-half of the rats received intraperitoneal injections of GBR12909, 1 mg/kg (ED-50), three times/week while the other half received saline injections of equal volume. Dietary and pharmacological intervention lasted for 4 weeks.

2.6. Statistical analysis

The data were analyzed using the SPSS v13, statistical analysis package (SPSS, Inc., Chicago, IL). Multiple factor analysis of variance was used to test for between subject effects (level of Fe, level of Mn, and drug treatment). When the overall significance resulted in rejection of the null hypothesis ($p < 0.05$), Dunnett's procedure was used to evaluate treatment means compared to control means.

3. Results

3. 1. Synaptosomal uptake of Mn

To screen for biogenic amine transporters with a potential role in Mn accumulation, striatal synaptosomes were utilized. To establish a working concentration for each of the transporter inhibitors, striatal synaptosomes were treated with various concentrations (0.1–100 μM) of the individual inhibitors before being incubated with the respective tritiated neurotransmitter. Treatment with all three drugs significantly inhibited the uptake of the respective tritiated neurotransmitter in striatal synaptosomes at the ED50 dose ($p < 0.05$) (Fig. 2).

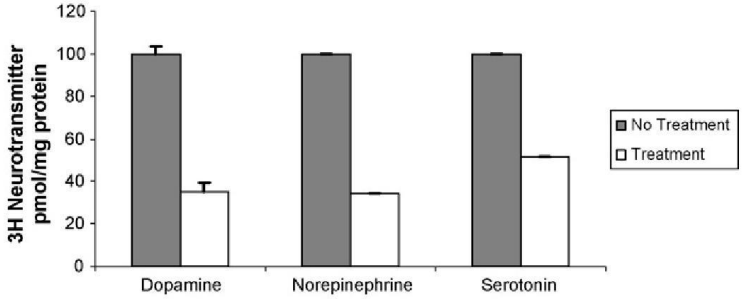

Fig. 2. Mean uptake of ^{3}H-dopamine is shown for striatal synaptosomes incubated in the absence (gray) or presence (white) of biogenic amine transporter inhibitors for dopamine (DA), norepinephrine (NE), or serotonin (5HT) at the ED50 dose (1 μM GBR12909; 100 μM desipramine; 10 μM fluoxetine) for 5 min.

To examine the effect of each of the inhibitors on Mn accumulation, synaptosomes were incubated with $MnCl_2$ in the presence of each of the inhibitors (1 μM GBR; 10 μM fluoxetine; 100 μM desipramine). Only treatment with GBR12909 significantly decreased accumulation of Mn compared to synaptosomes not treated with GBR12909 ($p < 0.001$) (Fig. 3). No significant difference was seen in the amount of Mn accumulation in those samples treated with either desipramine or fluoxetine in the presence of Mn compared to those samples without Mn treatment (Fig. 3). Unexpectedly, a significant increase in Mn accumulation was seen in synaptosomes treated with GBR12909 in which Mn was not added ($p < 0.05$) (Fig. 3).

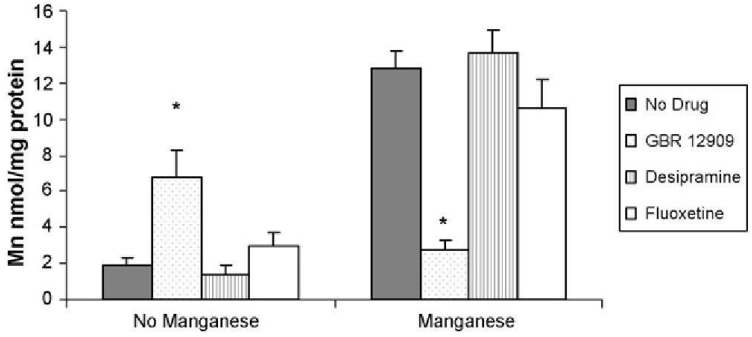

Fig. 3. Manganese concentration [μg/l] is shown for striatal synaptosomes incubated in the absence or presence of 0.5 mM manganese for 5 min. Synaptosomes either received no treatment (gray), 1 μM GBR12909 (dotted), 100 μM desipramine (striped), or 10 μM fluoxetine (white). The mean concentration is shown with SEM. $^{*}p$-value < 0.001.

3.2. In vivo studies

3.2. 1. Body weight and hematocrit

Rats receiving the CN diet gained significantly more weight than rats receiving the ID diet ($p < 0.05$) and hematocrit was significantly lower in rats receiving the ID diet compared to the CN diet ($p < 0.05$) indicating

that iron deficiency anemia had been induced. Treatment with GBR12909 had no effect on body weight or hematocrit levels versus those animals receiving saline injections (Table 1).

Table 1
Mean body weight and hematocrit

Group	Body weight (g)	Hematocrit
CN	196.9 ± 24.2[a]	0.47 ± 0.02[a]
CN with GBR treatment	201.1 ± 25.1[a]	0.43 ± 0.08[a]
CNMn	192.0 ± 23.3[a]	0.43 ± 0.04[a]
CNMn with GBR treatment	179.5 ± 1.83[a]	0.42 ± 0.04[a]
ID	168.1 ± 13.0[b]	0.15 ± 0.05[b]
ID with GBR treatment	148.2 ± 6.52[b]	0.15 ± 0.08[b]
IDMn	116.8 ± 12.7[b]	0.19 ± 0.05[b]
IDMn with GBR treatment	109.5 ± 7.27[b]	0.16 ± 0.05[b]

Mean body weight (g) and hematocrit are given for each treatment group ± S.D.
Means in a column not sharing a superscript are significantly different ($p < 0.05$).

3.2.2. Manganese

Mn concentrations were significantly higher in supplemented groups versus non-supplemented groups in most brain regions ($p < 0.05$) (Fig. 4). Treatment with GBR12909 significantly decreased accumulation of Mn in the GP of supplemented rats ($p < 0.001$) and there was a trend toward significance in the HC of CN rats ($p = 0.07$) (Fig. 5).

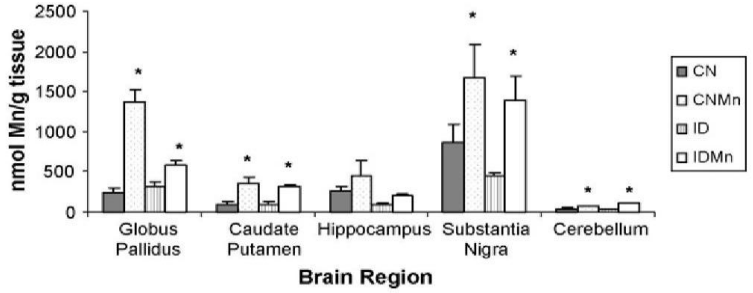

Fig. 4. Manganese concentration [nmol/g tissue] is shown for all brain regions: globus pallidus (GP), caudate putamen (CP), hippocampus (HC), substantia nigra (SN), and cerebellum (CB). Diets were CN (gray), CNMn (dotted), ID (striped), and IDMn (white). Mn supplementation led to a significant increase in brain regional Mn concentrations as indicated by the asterisk. The mean concentration is shown with SEM. *p-value < 0.05.

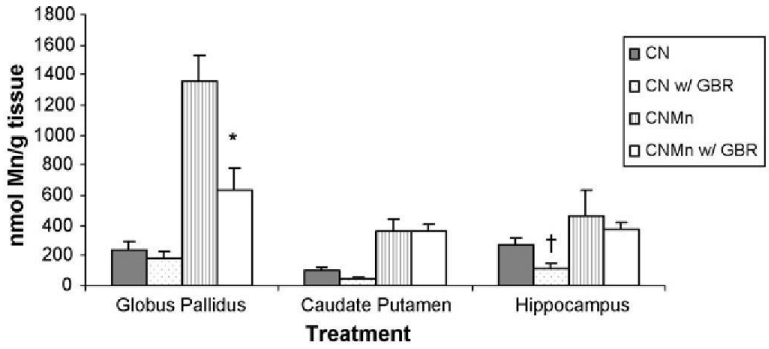

Fig. 5. Manganese concentrations [nmol/g tissue] are shown for CN and CNMn groups with and without 1 mg/kg GBR for three representative brain regions: globus pallidus (GP), caudate putamen (CP), and hippocampus (HC). Diets were CN (gray), CN w/ GBR (dotted), CNMn (striped), and CNMn w/ GBR (white). The mean concentration is shown with SEM. *p-value < 0.001; †p-value = 0.07.

3.2.3. Iron

Overall Fe concentrations were significantly different between CN and ID groups ($p < 0.05$); however, Fe concentrations were not statistically lower in ID groups versus CN groups within individual brain regions (Fig. 6). Treatment with GBR12909 had no effect on Fe accumulation in the brain.

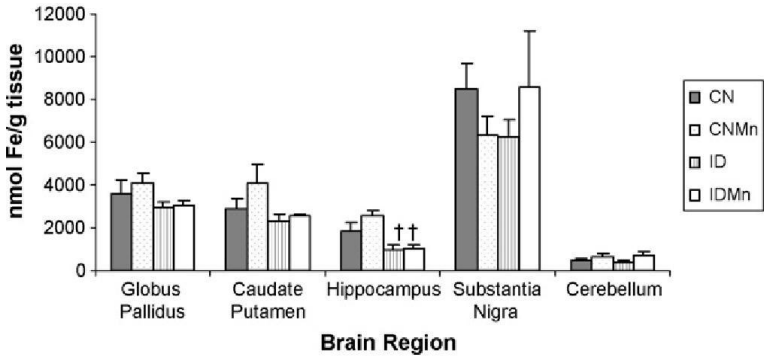

Fig. 6. Iron concentration [nmol/g tissue] is shown for all brain regions: globus pallidus (GP), caudate putamen (CP), hippocampus (HC), substantia nigra (SN), and cerebellum (CB). Diets were CN (gray), CNMn (dotted), ID (striped), and IDMn (white). The mean concentration is shown with SEM. $^{\dagger}p$-value = 0.13.

4. Discussion

In this study, both in vitro and in vivo data suggest a potential role for the DAT in the transport of Mn into the globus pallidus during toxic exposure. Initially, striatal synaptosomes were utilized to screen three biogenic amine transporters as potential Mn transporters: DAT, NET, and SERT. Previously, the DAT had been implicated as a putative Mn transporter (Ingersoll et al., 1999). However, the potential role of other monoamine transporters in the striatum in Mn transport, specifically norepinephrine and serotonin, remained elusive. Furthermore, this previous study administered Mn directly into the central nervous system bypassing any systemic or blood brain barrier mechanisms involved in Mn intoxication. Therefore, we sought to study the role(s) of biogenic amine transporters in brain Mn accumulation due to chronic, dietary exposure.

Before we launched into the animal intensive studies, we evaluated the efficacy of specific uptake inhibitors for DAT, NET, and SERT on blocking Mn transport into striatal synaptosomes. Synaptosomes were used because of their relative simplicity and their proven sensitivity to manipulation of neurotransmitter uptake. Dissociation from other interacting neuronal systems is advantageous in this case in order to provide direct interpretation of results and allow us to plan the dietary studies in a precise fashion.

Synaptosomes were isolated from the striatum, as Mn is known to accumulate in the Fe and dopamine-rich regions of the basal ganglia (Aschner et al., 2005). GBR12909 was used to specifically inhibit DAT; desipramine was used to inhibit NET, and fluoxetine to inhibit SERT (Fig. 2). Only those synaptosomes treated with the specific DAT inhibitor, GBR12909, significantly blocked Mn accumulation in vitro (Fig. 3). No effect was seen in those synaptosomes treated with either desipramine or fluoxetine. These data suggest that DAT is involved in Mn accumulation in the striatum during toxic expo-sure and agree with findings from previous studies (Ingersoll et al., 1999; Erikson et al., 2005). In addition, these in vitro observations suggest NET and SERT are not involved in Mn transport during toxicity in the striatum. For the purposes of this study the striatum was used to isolate synaptosomes, as it is a target region for Mn accumulation (Aschner et al., 2005). However, the striatum is a region of the brain less rich in NET and SERT relative to DAT. Whether or not these biogenic amine transporters play a role in Mn transport in noradrenergic or serotonergic areas of the brain remains unclear, however these brain regions are not typically involved in Mn neurotoxicity like the striatum. To definitively eliminate the potential role of NET and SERT in Mn accumulation in the brain, experiments using synaptosomes from more noradrenergic and serotonergic regions need to be conducted.

In those synaptosomes incubated in the absence of Mn, treatment with GBR12909 significantly increased Mn accumulation (Fig. 3). Though unexpected, this observation may indicate upregulation of other potential Mn transport mechanisms found in the striatum such as DMT- 1 (Roth and Garrick, 2003; Burdo et al., 1999), a transporter with an expanding role in regulating Mn concentrations (Erikson et al., 2004), voltage-gated calcium channels (Yokel and Crossgrove, 2004) or even the transferrin receptor (Connor et al., 2001; Malecki et al., 1999). Additionally, since Mn concentrations were not decreased by drug treatment under normal conditions, this supports data from a previous study that DAT may only be involved in Mn transport during toxic conditions (Erikson et al., 2005).

We used an iron-deficient diet because it has been shown to facilitate Mn accumulation in the brain, especially in the basal ganglia (Kwik-Uribe et al., 1999; Erikson et al., 2002, 2004). However, iron deficiency is known to alter dopamine transporter functioning (Erikson et al., 2000); so, while we treated the ID rats with GBR identically to the rats fed the control diet, we did not see an effect of the drug on blocking the uptake of Mn into any brain region (data not shown). Iron (Fe) accumulated in a heterogeneous fashion across all brain regions. Those animals receiving the ID diet had significantly lower concentrations of Fe overall, but there was no statistically significant difference within each brain region when compared to control (Fig. 6). In our previous studies (Erikson et al., 2002), the dietary intervention was conducted for 6 weeks rather than the 4 weeks in this study. Thus, the degree of iron deficiency was less severe resulting in anemia but not significant Fe depletion within the brain regions, which is known to result in Mn accumulation (Kwik-Uribe et al., 1999; Erikson et al., 2002). Importantly, we observed no effect of GBR12909 treatment on Fe accumulation which corroborates our previous study that showed that mice lacking a functioning DAT had altered accumulation of Mn but not Fe (Erikson et al., 2005).

As in previous studies (Erikson et al., 2002, 2004), Mn accumulated diversely in the brain and Mn concentrations were significantly higher in those animals receiving Mn supplementation in most brain regions (Fig. 4). Treatment with GBR1 2909 significantly attenuated Mn accumulation in the globus pallidus, but not the caudate putamen of Mn-exposed (CNMn) rats (Fig. 5). We found this surprising in that we expected the effects of treatment with GBR12909 to lie in a dopamine-rich brain region such as the caudate putamen (Erikson et al., 2005). However, it should be noted that these data are a collection of two separate animal experiments performed in the same manner (i.e., pharmacological and dietary treatments) and both experiments resulted in this finding that the effects of DAT blockade on inhibiting Mn accumulation are specifically unique to the globus pallidus.

We suggest two possible scenarios for why DAT blockade leads to decreased Mn accumulation in the globus pallidus (a GABA-ergic region) but not in the caudate putamen (a dopaminergic region). First, the effect of DAT blockade in the globus pallidus which has some dopaminergic input (Debeir et al., 2005; Gauthier et al., 1999) is directly leading to decreased transport of Mn into this region. While this is a plausible scenario, it is difficult to accept that blockade of the DAT located in the globus pallidus is leading to a 60% decrease in Mn concentration in supplemented animals. It could be that DAT blockade in this brain region causes an alteration in DMT- 1 functioning thereby decreasing regional Mn transport, but this remains to be elucidated. A more likely scenario, albeit speculative, is that during Mn toxicity, the DAT facilitates Mn transport into striatal neurons (caudate putamen) and the Mn accumulates in the globus pallidus via axonal transport (Murayama et al., 2006; Sloot and Gramsbergen, 1994). Thus, blockade of the DAT in the caudate putamen attenuates this accumulation of Mn in striatal neurons due to chronic Mn exposure ultimately causing decreased Mn concentrations in the globus pallidus. In a previous study using a DAT-KO mouse model, acute Mn exposure lead to a decrease in Mn accumulation in the caudate putamen (Erikson et al., 2005). A blockade of DAT in the caudate putamen might potentially lead to a buildup of Mn in that region under chronic exposure conditions as in this study.

In conclusion, these data suggest that during Mn toxicity, the DAT is involved in the facilitation of the specific accumulation of Mn into the globus pallidus. Whether the DAT is directly involved or indirectly is yet to be determined. Potentially, Mn may not be transported directly by DAT, but rather its trans-port may be affected

by interaction(s) of the DAT protein with other putative Mn transporters, such as DMT- 1 (Burdo et al., 1999), monocarboxylic acid transporters or voltage-gated calcium channels (Yokel and Crossgrove, 2004). Ongoing studies in our lab will help to further elucidate the specific role of DAT in Mn transport.

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