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Manganese Exposure is Cytotoxic and Alters Dopaminergic and GABAergic Neurons within the Basal Ganglia

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Abstract

Manganese is an essential nutrient, integral to proper metabolism of amino acids, proteins and lipids. Excessive environmental exposure to manganese can produce extrapyramidal symptoms similar to those observed in Parkinson's disease (PD). We used *in vivo* and *in vitro* models to examine cellular and circuitry alterations induced by manganese exposure. Primary mesencephalic cultures were treated with 10–00μM manganese chloride ($MnCl_2$) which resulted in dramatic changes in the neuronal cytoskeleton even at subtoxic concentrations. Using cultures from mice with red fluorescent protein (RFP) driven by the tyrosine hydroxylase (TH) promoter, we found that dopaminergic neurons were more susceptible to manganese toxicity. To understand the vulnerability of dopaminergic cells to chronic manganese exposure, mice were given IP injections of $MnCl_2$ for 30 days. We observed a 20% reduction in TH-positive neurons in the substantia nigra pars compacta (SNpc) following manganese treatment. Quantification of Nissl bodies revealed a widespread reduction in SNpc cell numbers. Other areas of the basal ganglia were also altered by manganese as evidenced by the loss of GAD67 in the striatum. These studies suggest that acute manganese exposure induces cytoskeletal dysfunction prior to degeneration and that chronic manganese exposure results in neurochemical dysfunction with overlapping features to PD.

Keywords

manganese; neurotoxicity; dopamine; Parkinson's disease; striatum; substantia nigra

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INTRODUCTION

Manganese (Mn^{2+}) is a naturally occurring essential element with an environmental prevalence second only to iron. Manganese is crucial for maintaining proper cellular function and contributes to biological processes including maintenance of redox status, ensuring appropriate protein conformation, modulating ion and energy homeostasis and signal transduction (Takeda 2003; Aschner and Aschner 2005; Keen et al. 2005; Liu et al. 2006). Dietary intake is the largest source of manganese in the human body under normal circumstances, but airborne manganese particulates comprise the most prevalent source of excessive manganese exposure (Dobson et al. 2004; Fitsanakis et al. 2006b). Manganese is used in numerous industries including welding, mining, steel production, and formulating gasoline additives. Chronic manganese overexposure results in a neurological irreversible phenomenon referred to as manganism (Dorman et al. 2002; Dobson et al. 2003; Dorman et al. 2004; Aschner et al. 2005; Fitsanakis et al. 2006b). The factors that influence vulnerability to manganese and onset of manganism remain ill defined. The motor symptoms of the disorder are, however, strikingly similar to those observed in PD (Aschner and Aschner 1991; Lee 2000; Finkelstein et al. 2007). Dystonia and movement disorders have also been described in case reports of adults and children receiving prolonged total parental nutrition when the gastrointestinal tract is nonfunctional because of an interruption in continuity or because absorptive capacity is impaired (Kafritsa et al. 1998; Nagatomo et al. 1999; Takagi et al. 2001; Hsieh et al. 2007).

Manganese is capable of having direct actions on neurons and glia within the central nervous system. Manganese is readily transported into the brain, either as a free ion species or as a nonspecific protein-bound species (Aschner and Gannon 1994). Transport into a variety of tissues and cells occurs by way of the non-specific divalent metal transporter-1 (Aschner et al. 1999; Fitsanakis et al. 2006a), which belongs to the family of natural resistance-associated macrophage proteins. This protein is expressed broadly across the brain early in postnatal development (Siddappa et al. 2002; Wang et al. 2002). When complexed with transferrin, manganese is transported by transferrin receptors (Aschner and Aschner 1991). The citrate transporter has also been invoked to transport Mn^{2+} (Crossgrove et al. 2003). Candidates for the transport of the Mn^{2+}-citrate complex include members of the organic anion transporter polypeptide or ATP-binding cassette superfamilies. Finally, an additional Mn^{2+} transporter is ZIP-8, a member of the solute carrier-39 (He et al. 2006). ZIP-8 is a Mn^{2+}/HCO_3^- symporter; an HCO_3^- gradient across the plasma membrane is invoked as the driving force for Mn^{2+} uptake.

We have previously shown that primary astrocytic cultures are highly vulnerable to manganese and undergo apoptotic cell death involving mitochondrial dysfunction (Yin et al. 2008), a finding which is consistent with the work of Maynard and Cotzias who demonstrated preferential sequestration of this element in the mitochondria (Maynard and Cotzias 1955; Gunter et al. 2006). Bioenergetic studies have shown that neurons are even more intensely dependent upon intact mitochondria for respiration. The majority of mitochondria are located in dendrites (Wongriley 1989; Hertz and Peng 1992) where the density of excitatory inputs necessitates a high respiratory capacity due to the need to maintain Na^+/K^+ gradients during neural activation (Erecinska and Silver 1989; Hertz 2008). If mitochondria are an essential target organelle of manganese, one would predict that changes in neural processes may proceed nuclear or somal dysfunction and thus may contribute to circuit level dysfunction. Indeed, cytoskeletal changes may be essential to mediating neurodegeneration in PD and other neurological disorders. Tau, tubulin and neurotransmitter releasing proteins have been well/extensively documented in these disorders (Masliah et al. 1996; Billingsley and Kincaid 1997; Ovadi et al. 2004; Siman et al.

2004; Andreadis 2005; Cappelletti et al. 2005; Cuadrado-Tejedor et al. 2005; Willis et al. 2005; Heredia et al. 2006; Esposito et al. 2007; Fulga et al. 2007).

The purpose of this work was to extend our previous studies in astrocytes to dopaminergic neurons and *in vivo* systems to address the neurotoxic potential of manganese and to determine if nigrostriatal pathways are uniquely vulnerable to manganese exposure. The ability to define mechanisms of toxicity and cellular features, which increase cellular vulnerability, would enhance our ability to treat manganism and potentially provide essential insight into the vulnerability of the basal ganglia in this and other disorders.

EXPERIMENTAL PROCEDURES

Antibodies

Rabbit polyclonal tyrosine hydroxylase antibody (TH, Chemicon, 1:1000) was used to identify dopaminergic neurons *in vitro*. Antibodies to β-tubulin (Sigma, 1:1000), synapsin-1 and tau-1 (Chemicon, 1:1000) were used to identify cytoskeletal and synaptic protein distribution. For staining of brain sections, mouse monoclonal α-glutamic acid decarboxylase 67 (GAD67, Chemicon, 1:2000, #MAB5406) and mouse monoclonal α-tyrosine hydroxylase (TH) (1:8000, Sigma, St. Louis, MO) were used. All secondary antibodies for primary culture work were purchased from Molecular Probes (Eugene, OR), while the brain section secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Bisbenzimide (Hoechst 33342; Sigma) was used as a nuclear counterstain.

Cytotoxicity of Manganese in Primary Mesencephalic Cultures

To study the effect of manganese on dopaminergic neurons, primary mesencephalic culture cells from rats at embryonic day 14 were plated on poly-L-ornithine-covered coverslips. Cultures were prepared as previously described (McLaughlin et al. 1998b). In brief, mesencephalon was isolated and incubated in trypsin at room temperature for 30min. Tissue was dissociated in 10ml plating medium containing DMEM, 10% F12 nutrients, 10% bovine calf serum (Hyclone, Logan, UT), 100U/ml penicillin, and 100μg/ml streptomycin. Cells were counted with a hemocytometer, diluted to 300,000 cells/ml and 2 ml of this stock was placed in each well of a 6-well plate. Typically viability following dissociation is > 95% by trypan blue exclusion, and neuronal cell number is maintained during the *in vitro* period (<5% loss over 2 weeks, unpublished observations). After two days *in vitro*, cells were treated for 24hr with 1–2μM cytosine arabinoside to prevent glial proliferation, and then transferred to Neurobasal media containing B27 supplement and 10μM β-mercaptoethanol. Mesencephalic cells were maintained for two weeks in culture after which they were treated with 10μM – 800μM $MnCl_2$ in low serum media free from antioxidants and phenol red for 24hr (Musiek et al. 2006). The chosen $MnCl_2$ concentrations are based on estimates from the literature. For example, weekly injections of $MnCl_2$ over a 3-month period in the caudate-putamen (CPu) and globus pallidus (GP) of monkeys (0, 2.25, 4.5, and 9g) produce dose-related clinical signs, which are more severe in the higher dose range (Suzuki et al. 1975). At the highest dose, the $MnCl_2$ concentration is increased 12-fold in the CPu and 9-fold in the GP. The “physiological range” (no symptoms) is ~75–100μM, and clinical signs increase in frequency and severity above this level, suggesting this concentration is near or at the threshold of toxicity. Upon treatment termination, cells were returned to the incubator, and neuronal viability was determined 20–24hr after exposure using a lactate dehydrogenase (LDH)-based *in vitro* toxicology assay kit (Sigma-Aldrich, St. Louis, MO). Media samples (40μl) were analyzed spectrophotometrically (490:630), according to the manufacturer's protocol, to obtain a measure of cytoplasmic LDH release from dead and dying neurons (McLaughlin et al. 2003). Cells were derived from 7 distinct culturing sessions. Statistical differences between control and treated cells were determined with one-way randomized

ANOVA design. When the overall test of significance ($p<0.05$) leads to a rejection of the null hypothesis, *post hoc* Newman-Keuls comparison was performed. Statistical analyses were performed with Graphpad Instat software (GraphPad Software, La Jolla, CA).

Fluorescent Staining

Following $MnCl_2$ treatment, cells were fixed in 3% paraformaldehyde for 30min. Non-specific binding of antigens was inhibited by incubating cells in blocking buffer (3% bovine serum albumin with 0.2% NP-40) for 1hr at room temperature. After blocking buffer immersion, cells were incubated with primary antibodies made in blocking buffer at 4°C overnight, followed by a 1hr incubation with appropriate conjugated secondary antibodies in 1% bovine serum albumin. Hoescht staining was performed as previously described by placing coverslips in 0.1mg/ml bisbenzamide for 10min prior to mounting coverslips on microscope slides (McLaughlin et al. 1998a).

Image Analysis

The AxioVision imaging program (Carl Zeiss, Thornwood NY) was used for morphological analysis of tau, β-tubulin and synapsin. Quantification of fluorescence intensity, threshold areas, and the number of cells and nuclei were established using Metamorph 4.0 software (Universal Imaging Corporation). For statistical analyses, comparisons were performed by a one-way ANOVA, followed by Bonferroni comparison test. All data are derived from averages of multiple coverslips per condition derived from at least 3 separate culturing conditions.

Cell Counts of Cultures from TH:RFP Transgenic Animals

Mesencephalic cultures were generated from a transgenic mouse expressing red fluorescent protein (RFP) under the control of the tyrosine hydroxylase (TH) promoter (Zhang et al. 2004). Mice were a generous gift from D. McMahon (Vanderbilt University). RFP-expressing midbrain dopaminergic neurons were isolated from E13 mice initially identified as TH:RFP by visualizing retinal expression of TH:RFP construct using a dissecting microscope equipped with a DsRed filter set. Identification was confirmed by PCR amplification using published primer sets (Zhang et al. 2004).

Primary murine cultures were generated as described above except that they were plated on plastic coverslips with embossed labeled grids for repeated identification of neurons and pre- and post-exposure cell counts as previously described (McLaughlin et al. 1998b). Precounts were done 12–14 days after dissociation. The following day, cells were exposed to $MnCl_2$ for 24hr as described above. The experiments were terminated when medium was removed, and phosphate-buffered saline (PBS; pH 7.4) containing 0.5% Trypan Blue was added to the cultures for 5min. Coverslips were then washed twice with PBS and fixed in 4% paraformaldehyde made in PBS. Adherent TH^+ and TH^- cells excluding Trypan Blue were counted and data was expressed as percentages of the pre-exposure numbers obtained in the same squares.

In Vivo Manganese Treatment

All experiments were run under the oversight of Vanderbilt University IACUC of the and NIH guidelines. Male mice (approximately eight weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and acclimated to a Vanderbilt University vivarium for two weeks. Mice were fed with ad lib water and Purina Lab Diet 5001 (~10 ppm Mn and 35 ppm Fe). For immunohistochemistry studies, twelve $C57BL/6^J$ mice (six in each group) were given intraperitoneal (IP) injections of $MnCl_2$ (5 mg/kg/day) or saline vehicle control daily for 30 days.

Immunohistochemistry on In Vivo $MnCl_2$ Exposed Mice

On day 31 of $MnCl_2$ or control treatment, mice were anesthetized with sodium pentobarbital and transcardially perfused with 60–75mL of 4% paraformaldehyde. Brains were then removed and post-fixed at 4°C overnight. Cryoprotection was performed using a series of sucrose solutions in phosphate-buffered saline (10%, 20%, and 30%). Series of coronal sections were cut on a freezing microtome at 40μm and stored at −20°C.

Sections were treated with Tris-glycine (0.1M glycine, pH 7.4) to reduce non-specific labeling, blocked in 4% Blotto (Nestlé Carnation dry milk) in phosphate-buffered saline (PBS, pH 7.5), and incubated for 72hr with the appropriate primary antibody using previously published protocols (Stanwood et al. 2001; Stanwood et al. 2005). Triton X-100 (0.2%) was added to blocking solutions for all antibodies except GAD67. Following primary antibody incubation, sections were washed five times, and then incubated in biotinylated secondary antibodies for 60 min a t R T (Jackson Immunoresearch, West Grove PA, 1:1000 dilution). Sections were washed again and then avidin-biotin-perioxidase amplification (Vectastain ABC, Vector Labs) and 3,3'-diaminobenzidine reactions were used to visualize protein labeling. Sections were mounted on poly-L-lysine-coated slides, dehydrated in an ethanol series, and then coverslipped using DPX (Electron Microscopy Sciences, Hatfield PA). Sections from $MnCl_2$-treated and control mice were processed in parallel to minimize variability in the quantity of immunostaining between groups, and negative controls, which omitted incubation with primary antibody, presented no specific labeling. An additional series of sections were mounted onto slides and stained with 0.5% cresyl-violet for labeling of all cell bodies.

Cell Counts of Sections and Statistical Analyses

Slides were coded so that the investigator was blinded to the treatment groups and sections were imaged using a Zeiss Axioplan II microscope to capture color images of sections using a high-resolution color CCD camera (Axiocam HR). Cells were counted in the 8-bit format, using the Image J program (NIH), bilaterally, from at least 3 nonadjacent sections per region. Labeled cells were counted and diameters were measured in 20x images to allow for correction of profile size by the method of Abercrombie (Abercrombie 1946).

Profiles of Nissl body distribution were counted in the SNpc and SNpr. Profiles of TH-immunoreactive cells were counted in the VTA and in the SNpc. GAD67-immunoreactive cells were analyzed in the anterior cingulate cortex, dentate gyrus, dorsomedial caudate putamen, and SNpr. Brain regions were identified using regional, cytoarchitectural and laminar landmarks. Three to six images from areas of the ventral midbrain (including the VTA, SNpc, and SNpr) were merged together using the automerge feature of Adobe Photoshop (version 9.02). For all regions of interest, at least four images were analyzed for each animal derived from at least 2 non-adjacent sections. Immunopositive cell densities were calculated as mean ± S.E.M and statistical analyses were performed using Student's *t*-tests.

RESULTS

Our first experiments examined the relative vulnerability of mesencephalic cells to manganese by exposing primary cultures to increasing concentrations of the metal continuously for 24hr. We used neuron-enriched cultures (50% neurons) that were dissected mid-gestation to promote survival of TH-positive cell populations. Our cultures typically had 5–10% TH-positive neurons with the other population of neurons (identified with MAP2 staining) expressing the GABAergic marker GAD67 (data not shown, but similar to (Zeevalk et al. 1995). Cell death was assessed by measuring LDH release by dead and dying

cells into the exposure media and by visually inspecting cells for signs of cell death including loss of neurites, soma shrinkage and presence of cellular debris in media. We found that $MnCl_2$ induced cell death in a concentration-dependent manner with an LD_{50} of 909μM (Figure 1A). Representative photomicrographs were taken at the end of the 24hr exposure period and demonstrate the many phase-bright neurons (Figure 1B) in control cultures, which grow on top of a bed of glia. With increasing concentrations of $MnCl_2$, soma volume shrinkage resulting in a stippled, less smooth appearance (800μM; Figure 1C), and extensive neuronal cell death can be seen at 3mM $MnCl_2$ (Figure 1D).

Next, we chose to focus on the consequences of pathophysiologically relevant concentrations of chronic manganese exposure at 100–800μM (Aschner et al. 1999). As demonstrated above, these concentrations were not overtly neurotoxic, but could alter cell structure in ways that might influence the circuitry of the basal ganglia and contribute to manganese-induced motor dysfunction. For this work, we surveyed the changes in the structural protein tau, the synapse specific marker synapsin and the cytoskeletal marker tubulin. Neurons were treated with vehicle (Figure 2 A1–3), 100μM $MnCl_2$ (Figure 2 B1–3) or 800μM $MnCl_2$ (Figure 2 C1–3). We observed that increasing manganese concentrations lead to a progressive loss of cohesive tau staining and an increase in cytoskeletal abnormalities as reflected by the >60% decrease in tau-positive neurites (Figure 2D). Similarly, synapsin was altered with both 100μM $MnCl_2$ (Figure 3 B1–4) and 800μM $MnCl_2$ exposure (Figure 3 C1–4), resulting in a ~75% decrease in synapsin staining (Figure 3D). However, as in Figure 2, no qualitative changes in nuclei were evident from our Hoechst stain suggesting that preapoptotic asymmetric chromatin formations were not induced and changes were confined to regions outside the nucleus.

Mesencephalic cultures contain both GABAergic and dopaminergic populations although based on the symptomology of manganism, there appears to be a stronger dopaminergic dysfunction. To test this hypothesis we used a combination of *in vitro* and *in vivo* systems. We first stained manganese-exposed cultures for TH, the rate-limiting step in dopamine biosynthesis and a specific marker of dopaminergic neurons. We observed that manganese exposure resulted in shortening of TH-positive neurites with increasing exposure (data not shown). We next used cultures derived from TH:RFP reporter mice and counted TH-positive and TH-negative neurons before and after exposure to $MnCl_2$. Cells containing this dopamine synthetic enzyme comprised 12 ± 3% of the total neuronal population. Manganese induced a similar concentration-dependent toxicity (data not shown) as in the cultures from the non-transgenic animals. The TH-positive cells were, however, more vulnerable to $MnCl_2$ at both 300μM and 800μM concentrations than the other neuron populations in the mesencephalic cultures. This was evidenced by cell counts performed before and after $MnCl_2$ exposure where we observed a progressive increasing in death of TH-positive neurons between concentrations of 300μM to 1mM, which was significantly greater than that of TH-negative neurons (Figure 4). These data also confirm that the effects of manganese were not simply to reduced reporter expression (i.e. TH promoter activity), because in that event one would predict the number of non-dopaminergic cells to increase concomitantly with the loss of TH^+ neurons.

We next moved to an *in vivo* model of chronic manganese exposure to determine if systemic administration of manganese in any way recapitulated alterations in the direct and indirect circuits of the basal ganglia that have been observed in PD and PD models. Animals were given IP injections (5 mg/kg body mass, intraperitoneal) of $MnCl_2$ or vehicle daily for 30 days. Coronal brain sections were stained with TH antibody and cell counts were performed. Representative photomicrographs of control (Figure 5A) or manganese treated (Figure 5B) animals are shown at the level of the SN. Cell counts revealed a significant decrease in TH-

positive cells in the SN but not in the adjacent ventral tegmental area (VTA) (Figure 5C; n=6; $p < 0.05$ unpaired t test).

To determine if the loss of TH staining was caused by a loss of the dopamine synthetic enzyme itself or by a loss of cellular viability, we undertook cell counts of the SN and VTA (Figure 6). We found that the loss of TH staining was associated with cell loss in the SN as there was a 20% decrease in cresyl violet-stained cell numbers in this region, which was not evident in the VTA (Figure 6C).

Our final experiments were designed to evaluate the consequences of chronic manganese exposure on the basal ganglia by evaluating the GABA synthetic enzyme, glutamic-acid-decarboxylase (GAD67), staining within this circuit. We observed appreciable GAD67 loss in the CPu which receives the dopaminergic projections of the SNpc in manganese treated animals (Figure 7B) compared to controls (Figure 7A). GAD67 immunoreactivity was not altered in the SNpr, which was consistent with our *in vitro* findings suggesting that dopaminergic dysfunction occurs prior to GABAergic dysfunction in this region. We also observed a loss of GAD67 staining in the GP in manganese-treated animals compared to controls (Figures 7C and D). Cell counts of several other regions within and outside the basal ganglia showed no significant difference (Figure 7E), suggesting that the loss of GAD-positive cells was limited to the striatum (STR) and globus pallidus (GP), but not in the dentate gyrus or anterior cingulate cortex (n=6; $p < 0.05$ unpaired *t* test).

DISCUSSION

Manganese concentrations present in mammalian cells can be estimated from the literature. *In vivo* chronic low-dose Mn exposure leads to accumulation of Mn in the striatal and globus pallidus at concentrations as high as 200 μM (Erikson and Aschner 2002). In addition, weekly injections of Mn over a 3-month period in striatum and GP of monkeys (0, 2.25, 4.5, and 9 gm), produce dose-related clinical signs, with increased severances at the higher dose range (Suzuki *et al.* 1975). At the highest dose (9 gm), the Mn concentration is increased 12-fold in the striatum and 9-fold in the GP compared to conrtrols. The “physiological range” (no symptoms) approximates Mn levels at 75–100 μM, and clinical signs increase in frequency and severity above this level. Accordingly, for the purpose of the present study, Mn at 100 μM was deemed to be represent a threshold level of toxicity. Concentrations of Mn below this level were deemed non-toxic, while Mn at 800 μM was deemed to represent a toxic levels. Accordingly, we used a low (10μM) and minimally toxic concentrations (100–800μM) in our acute studies. We note, however, that we did directly measure manganese concentrations within the cells. It has been shown previously that cultured cortical astrocytes and undifferentiated PC12 cells are capable of accumulating manganese intracellularly over a period of hours to days. In contrast, however, neuronally-derived NT2 cells and nerve growth factor-differentiated PC12 cells appear to only minimally accumulate Mn^{2+} above the media concentration (Gunter et al. 2005; Gunter et al. 2006).

Mn induces mitochondrial respiratory dysfunction *in vitro*, promotes free radical production and inhibits the antioxidant system by depleting glutathione and glutathione peroxidase (Liccione and Maines 1988; Gavin et al. 1992; Chen and Liao 2002; Weber et al. 2002; Stredrick et al. 2004; Erikson et al. 2006; Erikson et al. 2007; Zhang et al. 2007; Zhang et al. 2008). Activation of oxidative stress-sensitive kinases and transcription factors including NF-κB have also been observed in cell lines exposed to manganese (Zhang et al. 2007; Prabhakaran et al. 2008; Yin et al. 2008). Expression changes and inflammatory responses in glial cells can be observed following bath application of manganese at concentrations as low as 10 μM (Moreno et al. 2008).

In the present study, we sought to identify changes in neuronal cytoarchitecture associated with manganese exposure and to determine if these changes are more pronounced in mesencephalic cells. Neurites are rich in mitochondria, which produce the most reactive oxygen species in cells and thus are likely early targets of redox stress. Tau plays an essential role in the stabilization of the microtubule tracks and mutations in tau have been linked to PD pathology. Both tau and synapsin have been shown to be altered in PD and PD models as well as by environmental and genetic stress (Fortin et al. 2005; Ansari et al. 2008), but neither has been evaluated following manganese treatment. Our data support the hypothesis that acute manganese exposure induces early and profound changes in neurite length and integrity at concentrations that are not overtly neurotoxic (100μM). This would suggest that circuit level dysfunction caused by the loss of dopaminergic projections to the striatum may be an early consequence of manganese exposure. Mechanistic overlap between PD and manganese toxicity is also suggested by the recent observation that PARK9 can provide protection both to α-synuclein mutation and manganese exposure (Gitler et al. 2009) and that the divalent metal transporter-1 (DMT1), a transporter of manganese, contributes to neurodegeneration and effects inherent to PD-related cell death (Salazar et al. 2008).

Microtubules play obligatory functions in the maintenance of cellular transport, but overexpression of the tubulin gene or tubulin depolymerization by toxins cause significant damage to the cell, especially in projection neurons with long axons, such as the dopaminergic neurons of the nigrostriatal pathway (Ren et al. 2003). Failure to transport sufficient dopamine containing vesicles via microtubules to synaptic terminals early in PD decreases synaptic plasticity in the striatum (Brown et al., 2005). An inherited form of PD is caused by a mutation in the E3 ubiquitin ligase Parkin that binds to α- and β-tubulins and increases their ubiquitination and subsequent proteasomal degradation. Phosphorylation of Parkin results in the loss of Parkin's ability to ubiquitinate damaged proteins thereby preventing their proteasomal degradation, leading to the accumulation of tubulin and other misfolded proteins. Parkin mutations also result in the formation of Lewy bodies consisting of β-tubulin and α-synuclein and are linked to the onset of autosomal recessive juvenile PD (Ren et al. 2003). The loss of synapsin staining observed with manganese treatment is consistent with data supporting an essential role for appropriate manganese content in maintaining neurotransmission and synaptic function (Takeda et al. 2002, 2003) and the role of PD associated proteins in regulating synaptic function (Murphy et al. 2000; Sidhu et al. 2004b; Sidhu et al. 2004a). While the underlying signaling pathways remain ill-defined, manganese has been shown to interact with dopamine-reactive quinones and other dopamine specific species, which may contribute to the *in vitro* pathology of manganese-induced damage in dopaminergic cells. Recent work has shown that manganese toxicity is indeed enhanced by dopamine preexposure in mesencephalic cultures (Prabhakaran et al. 2008), although specific changes in the dopaminergic vs. GABAergic cell populations in these cultures were not evaluated. Our data strongly support a preferential loss of TH-positive neurons in mesencephalic cultures at concentrations of manganese which are not overtly toxic to other populations.

The endogenous pools of manganese have been studied extensively in an effort to determine if the concentration of manganese in discreet anatomical sites correlates with regional injury. Magnetic resonance imaging has demonstrated preferential manganese sequestration in the striatum, GP, and SN of primates (Calne et al. 1994; Shinotoh et al. 1995). In rodent studies, however, there are discrepancies in the literature. Brenneman and colleagues reported that rat CPu and GP do not preferentially accumulate manganese after excess exposure (Brenneman et al. 1999). In other works, accumulation in these regions could be promoted by placing animals on diets deficient in iron implicating iron deficiency as a risk factor for manganese accumulation (Kim et al. 2005).

The neuropathological changes we observed using chronic exposure suggest that manganese toxicity is not limited to dopaminergic neurons, but also occurs in the globus pallidus and striatum. These findings overlap with the pathological changes observed in the scant number of post mortem human tissue samples from manganese cases, where morphological alterations range from no gross changes to massive atrophy and gliosis which appears to be most consistently observed in the internal segment of the globus pallidus. Loss of neurons in the SN has been reported albeit less consistently, but there is a clear need to obtain more specimens, correlate changes with the mode of Mn^{2+} exposure and resulting motor and cognitive dysfunction as well as measure the manganese levels in tissue (McKinney et al. 2004; Perl and Olanow 2007).

Regarding the overlap between our neurochemical and pathological changes and PD, there appears to be a larger discrepancy. GABA is the major inhibitory neurotransmitter in the brain and the highest concentrations of GABA are found in the internal segment of the globus pallidus and the substantia nigra pars reticulata, which receive inputs from the striatum (caudate nucleus and putamen). Changes in GAD or loss of GABA content are not present in the post mortem striatum in PD, although the changes at circuit level of GABAergic tone will be altered by the hallmark loss of dopamine in early stages of the disease. While the changes in GAD-immunoreactive neuron number in the STR and GP were relatively subtle in our animals chronically exposed to manganese, they were statistically significant. In this regard chronic Mn exposure in this model differs from PD. Similarities between PD and manganism include the presence of generalized bradykinesia and widespread rigidity. Dissimilarities between PD and manganism were also recognized, notably the following in manganism: (a) a less frequent resting tremor, (b) more frequent dystonia, (c) a particular propensity to fall backward, (d) failure to achieve a sustained therapeutic response to levodopa, and (e) failure to detect a reduction in fluorodopa uptake by positron emission tomography (PET; for further details see (Calne et al. 1994). Other rodent models of manganese exposure have also pointed to alterations in GABA and GAD in CPu and GP (Gwiazda et al. 2002; Tomás-Camardiel et al. 2002).

Based on our data, we conclude th at acute *in vitro* manganese exposure is neurotoxic and produces more profound cytoarchitectural dysfunction and death in TH-positive neurons than other neuronal populations. In our chronic *in vivo* manganese exposure paradigm, we also observed cell loss in the TH-rich SN, but damage was not solely restricted to this region. The loss of GAD-positive cells further downstream in the basal ganglia circuitry may be caused by the loss of chemical, electrical or physical support due to neurite dysfunction or may be an independent neurotoxic event. Given that culture studies require isolation of embryonic tissue, it is also possible that developmental issues contribute to selective vulnerabilities (see also Erecinska et al. 2005). On net, our data provide some explanation for the motor manifestations of manganese intoxication but do not support a model of selective dopaminergic dysfunction *in vivo*. These data suggest that circuit level influences are essential to mediating manganese induced degeneration in the adult brain.

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Abbreviations

BSA bovine serum albumin

CPu	caudate putamen
DMEM	Dulbecco's Modified Eagle Medium
GAD67	glutamic acid decarboxylase, MW 67kDa
GP	globus pallidus
LDH	lactate dehydrogenase
Mn^{2+}	manganese
$MnCl_2$	manganese chloride
PD	Parkinson's Disease
RFP	red fluorescent protein
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
TH	tyrosine hydroxylase
VTA	ventral tegmental area

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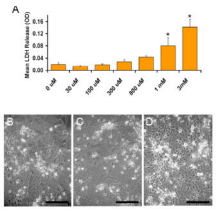


Figure 1. Chronic Manganese is Cytotoxic to Primary Mesencephalic Cultures

Mature primary mesencephalic neurons were exposed to increasing concentrations of $MnCl_2$ for 24hr, at which time cell death was assessed. A) Lactate dehydrogenase release by dead and dying cells was assessed in the media and revealed that manganese induces cell death in a dose-dependant manner. Data represent the mean ± S.E.M. of 7 independent experiments, each performed in triplicate wells. Representative photomicrographs were taken following 24hr exposure to B) control conditions, C) 800μM $MnCl_2$ or D) 3mM $MnCl_2$. *$p<0.05$ vs. control by one-way ANOVA. Scale bar = 200 μm.

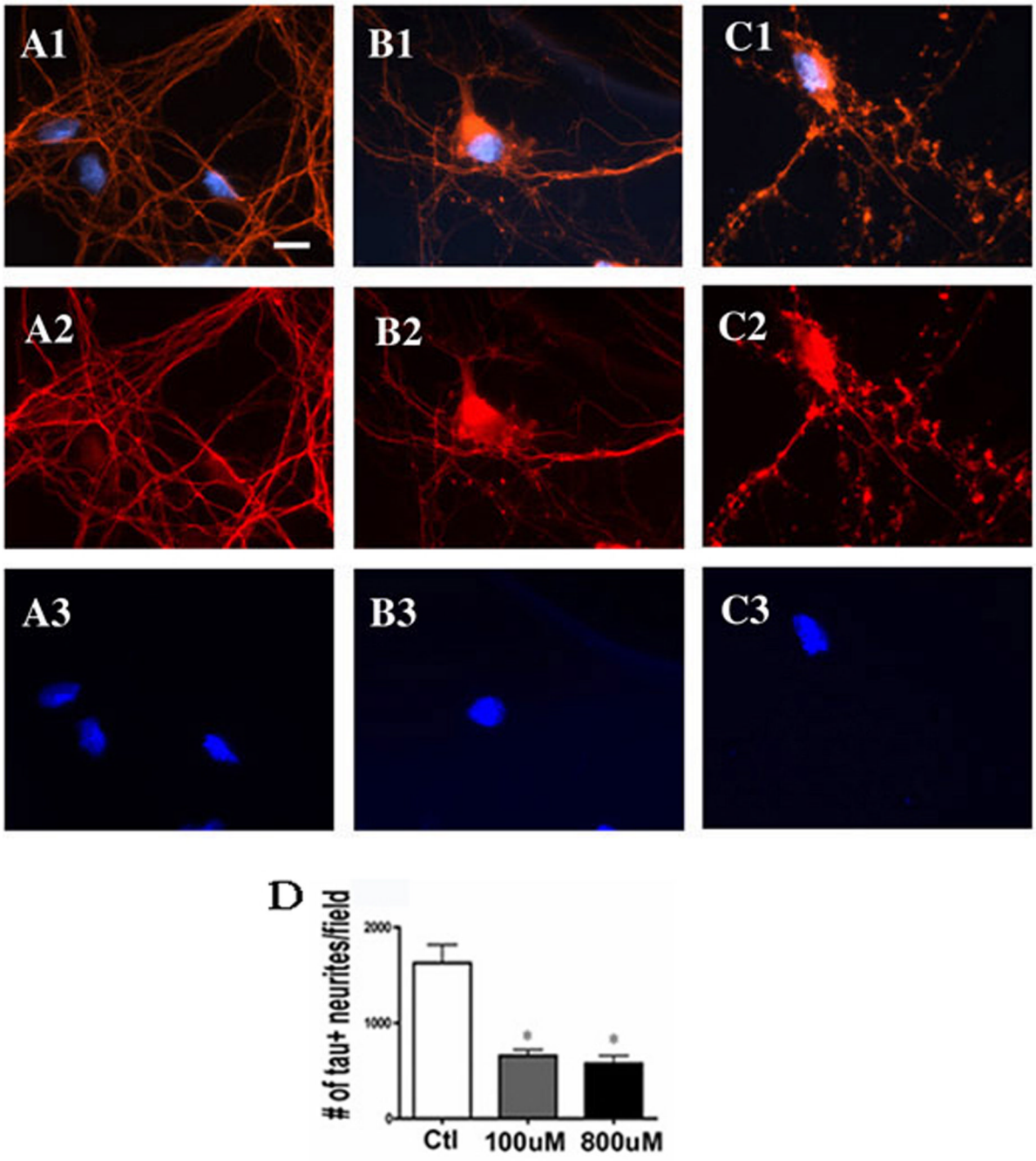


Figure 2. Profound Changes in Neural Structure Occur at Subtoxic Exposures to Manganese

Mature primary mesencephalic neurons were exposed to increasing concentrations of $MnCl_2$ for 24hr, at which time Immunofluoroscent detection of tau (red) and nuclei (blue) was performed. Cultures were treated with vehicle (A1–3), 100μM $MnCl_2$ (B1–3) or 800μM $MnCl_2$ (C1–3). Results show that while both concentrations of $MnCl_2$ were not toxic, they did lead to a dose dependent increase in cytoskeletal abnormalities, including a significant loss of architectural complexity, process retraction and beading (D) following 24hr exposure. Data represent the mean ± StDev *$p < 0.05$ one way analysis of variance with Bonferroni correction. Scale bar = 10 μm.

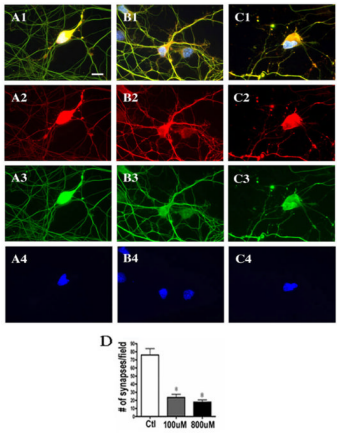


Figure 3. Synapsin and Tubulin Staining are Decreased with Subtoxic Manganese Exposures

Primary mesencephalic neurons were exposed to increasing concentrations of $MnCl_2$ for 24hr, at which time immunofluoroscent detection of tubulin (green), synapasin (red) and nuclei (blue) was performed. Cultures treated with vehicle (A1–4), 100μM $MnCl_2$ (B1–4) or 800μM $MnCl_2$ (C1–4) had increasing cytoskeletal abnormalities as evidenced by lack of continuity of neural processes and failure to discreetly localize the synapsin phosphoproteins which regulate transmitter release and trafficking. The loss of synapsin-positive cells was statistically significant at both concentrations of manganese used (D) as quantified by cell counting in at least four independent experiments using Metamorph software. Data represent the mean ± StDev *$p < 0.05$ one way analysis of variance with Bonferroni correction. Scale bar = 10 μm.

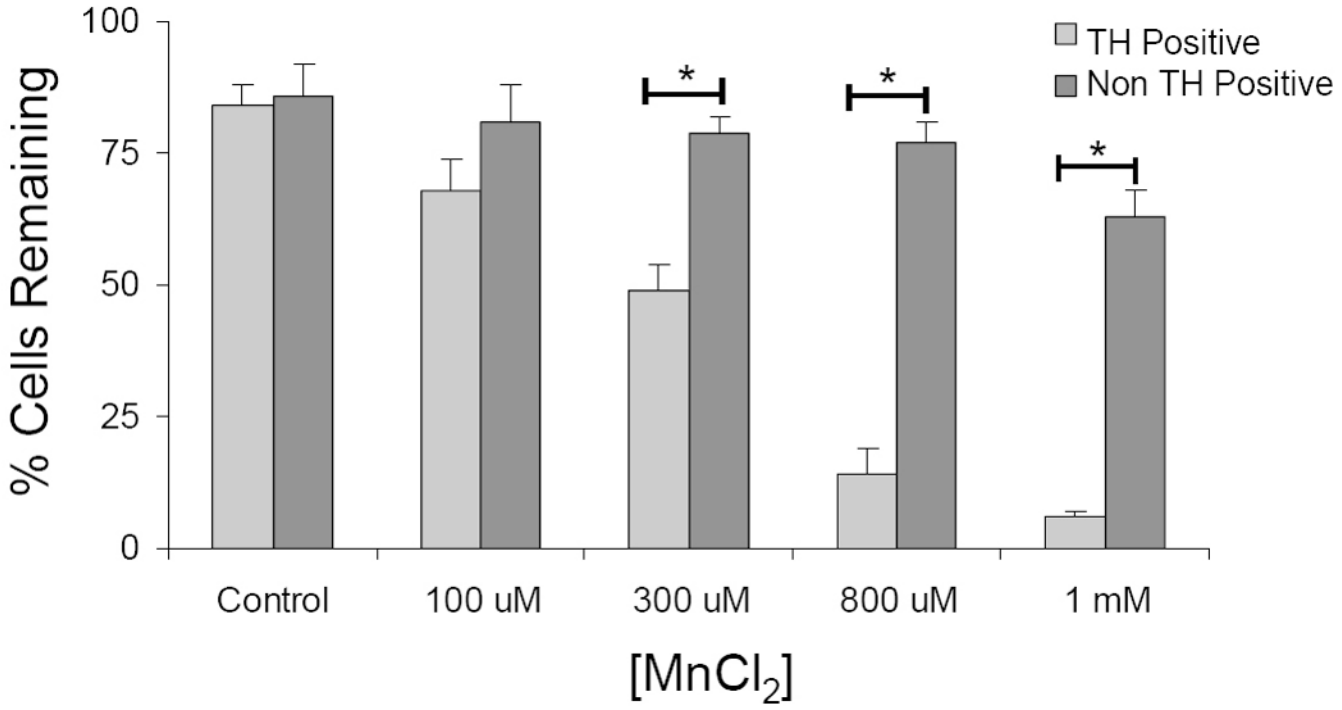
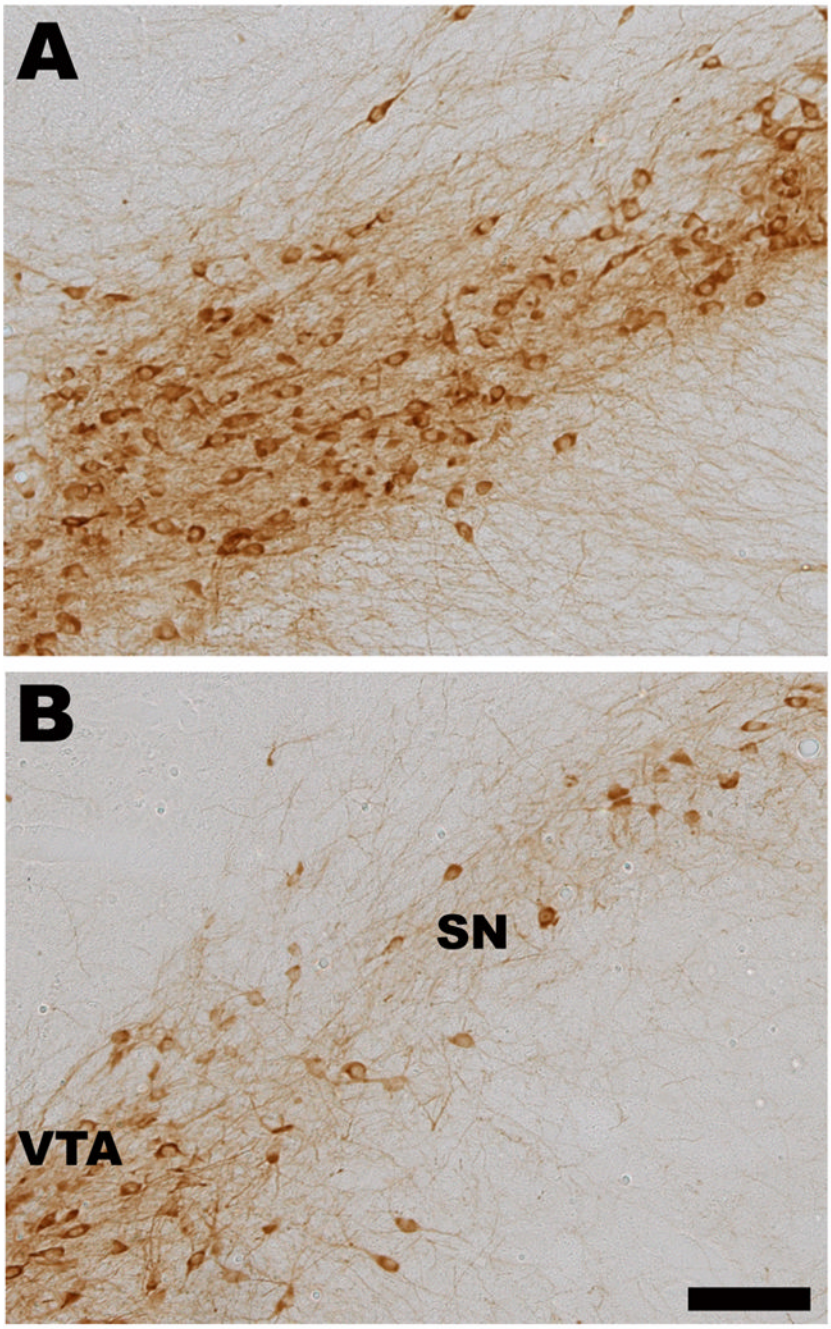


Figure 4. Tyrosine Hydroxylase Expression Decreases with Increasing Manganese Exposures

Mesencephalic cultures from transgenic TH:RFP mice were exposed to manganese and the number of TH-positive cells was counted. Data are expressed as a percent loss of TH-positive or TH-negative cells compared to preexposure cell counts in the same quadrants. Data represent the mean + SEM of 4 independent experiments, each performed in at least duplicate. Data was analyzed by two-tailed unpaired *t*-test. *$p<0.05$.



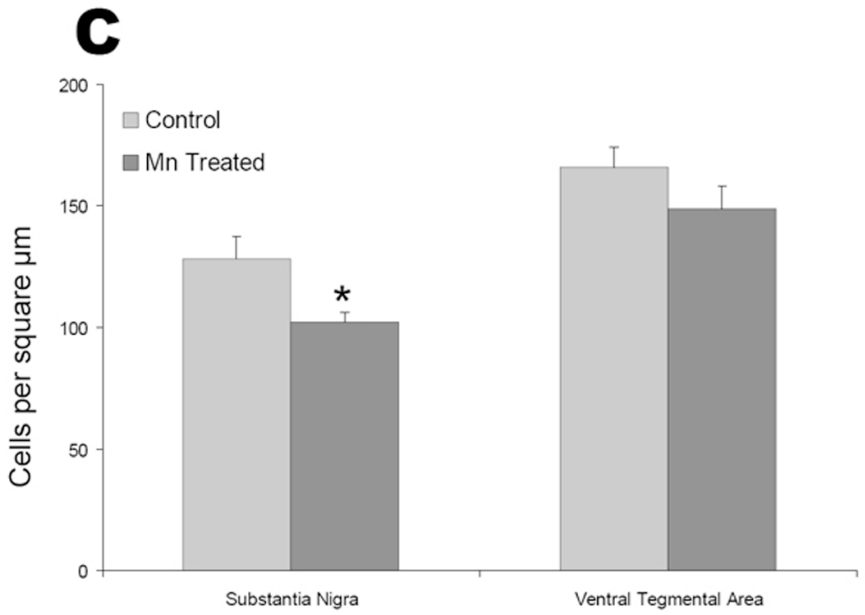


Figure 5. Chronic Manganese Exposure Decreases Tyrosine Hydroxylase Immunoreactive Neurons *in Vivo*

Mice were exposed to IP injections (5 mg/kg body mass) of $MnCl_2$ or vehicle daily for 30 days. Coronal brain sections were stained with TH antibody and cell counts were performed. Photomicrographs of control (A) or $MnCl_2$ treated (B) animals are shown at the level of the ventral midbrain. A particular robust example of TH cell loss following chronic $MnCl_2$ is depicted in panel B. C) Cell counts revealed a significant decrease in TH positive cells in the SN but not in the VTA (n=6; $p < 0.05$, *t* test). Scale bar = 100 µm. SN = substantial nigra, VTA = ventral tegmental area, cp = cerebral peduncle, ml = medial lemniscus.

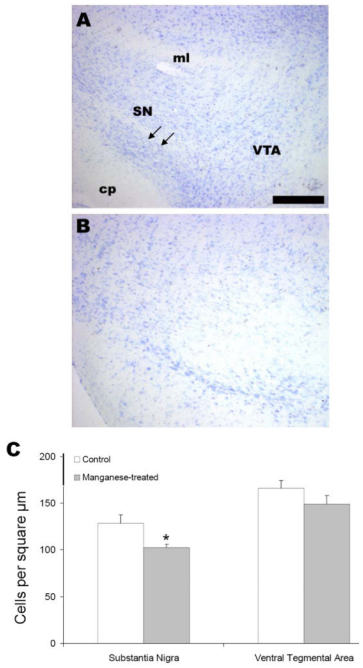
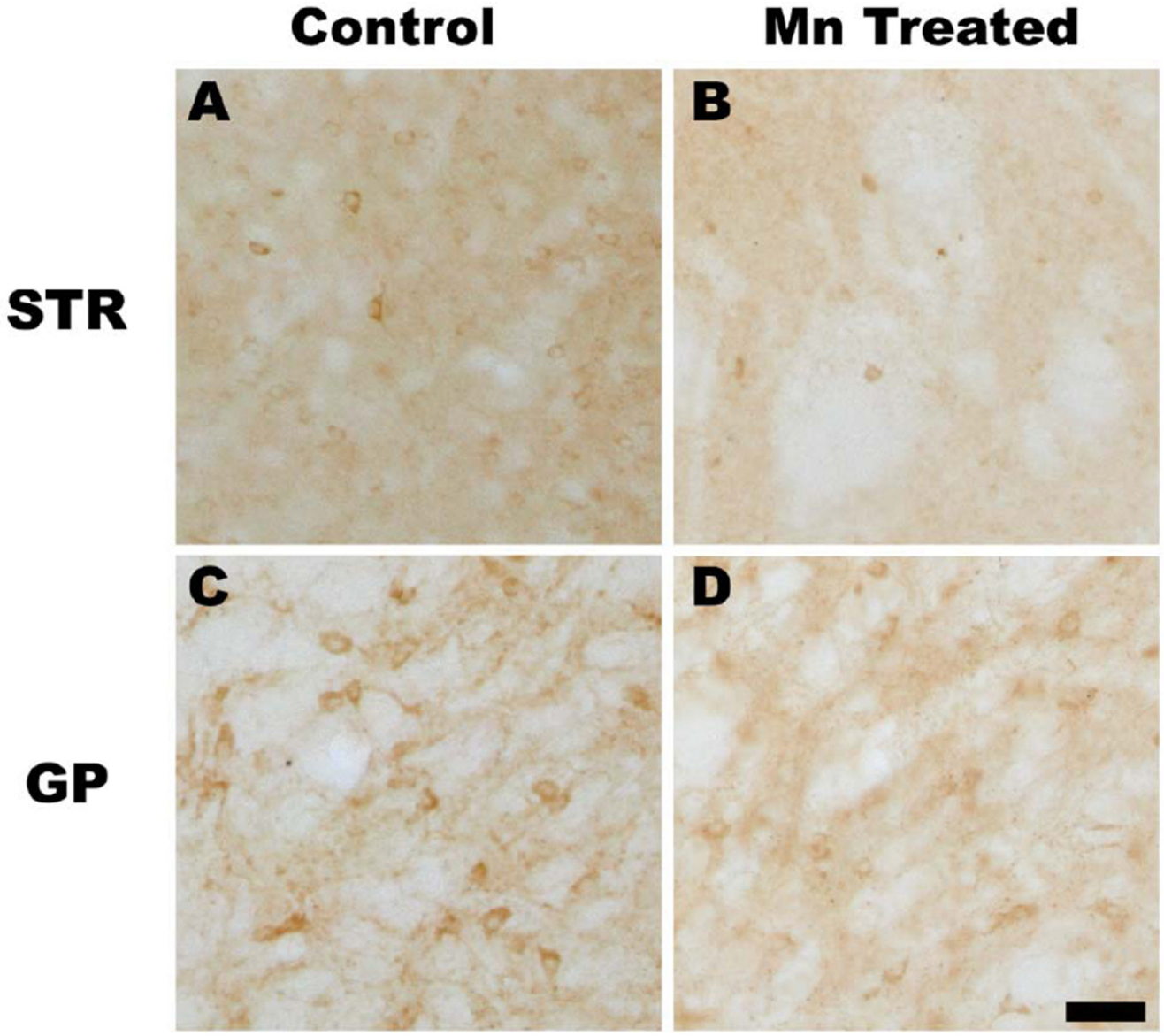


Figure 6. Chronic Manganese Exposure Induces Cell Loss in the Substantia Nigra

Mice were exposed to IP injections (5 mg/kg body mass) of $MnCl_2$ or vehicle daily for 30 days. Coronal sections of brain were Nissl stained to identify cells and counts were performed within the SN and VTA. Representative photomicrographs of control (A) or manganese treated (B) animals are shown at the level of the SN. Note the lack of tightly compacted cell bodies (arrows in sections of vehicle-treated mice) in $MnCl_2$ treated animals. Cell counts (C) reveal a 20% decrease in total cell density in the SN, which is not evident in the VTA. (n=6; * $p < 0.05$ unpaired *t* test.



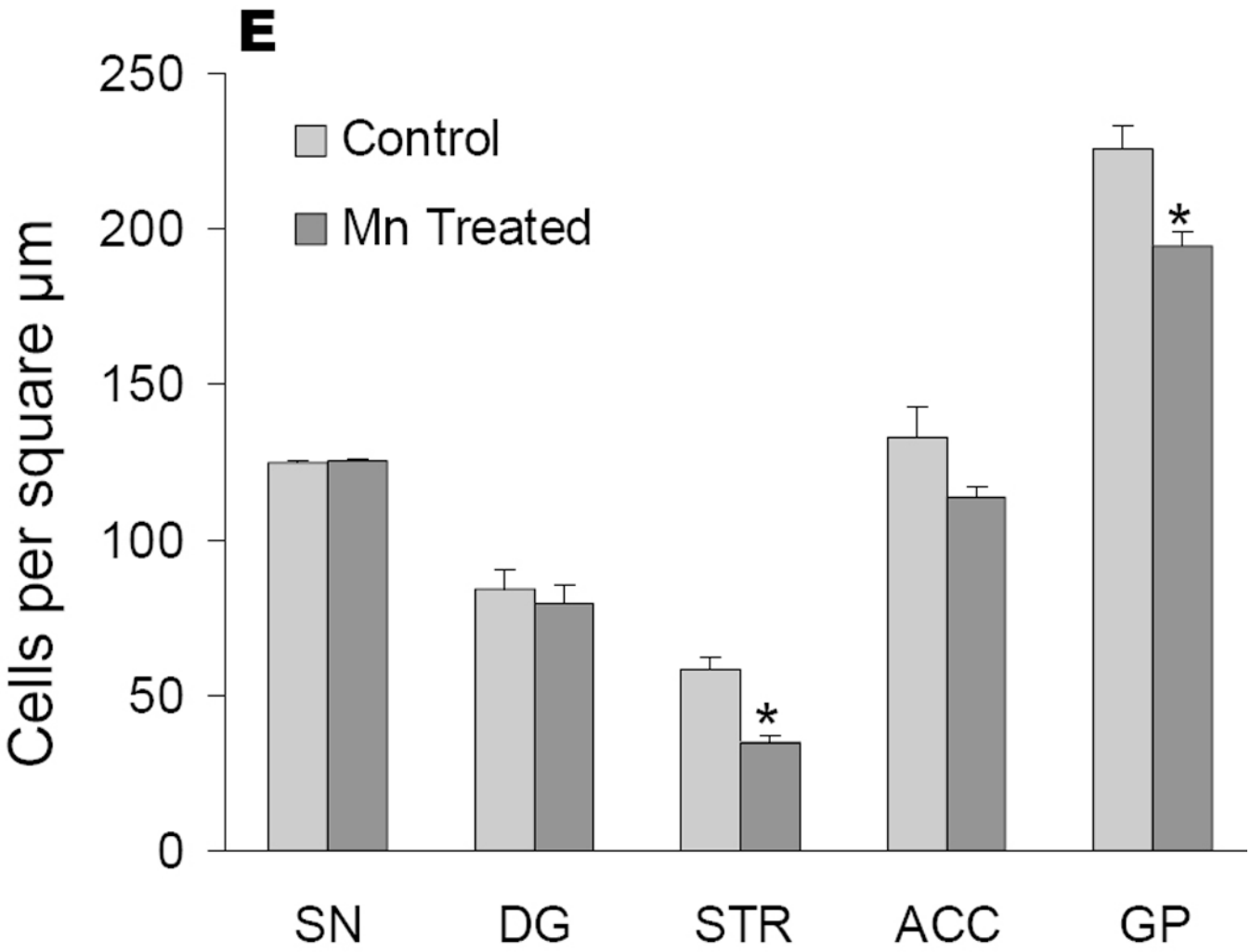


Figure 7. Chronic Manganese Exposure Decreases GAD Expression *in Vivo*

Mice were exposed to IP injections (5 mg/kg body mass) of $MnCl_2$ or vehicle daily for 30 days. Coronal sections of brain were stained with a glutamic-acid-decarboxylase (GAD) reactive antibody and cell counts were performed. Representative photomicrographs from the striatum from control (A) or manganese treated (B) animals are shown. Immunoreactivity of GAD was also decreased in the globus pallidus (control, C) in manganese treated animals (D). Cell counts revealed that the loss of GAD-positive cells was limited to the striatum (STR) and globus pallidus (GP) and was not observed in the substantia nigra (SN), dentate gyrus (DG) or anterior cingulate cortex (ACC) (n=6; $p < 0.05$, unpaired *t* test).