Manganese-Induced Neurotoxicity: The Role of Astrogial-Derived Nitric Oxide in Striatal Interneuron Degeneration

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Chronic exposure to excessive manganese (Mn) is the cause of a neurodegenerative movement disorder, termed manganism, resulting from degeneration of neurons within the basal ganglia. Pathogenic mechanisms underlying this disorder are not fully understood but involve inflammatory activation of glial cells within the basal ganglia. It was postulated in the present studies that reactive astrocytes are involved in neuronal injury from exposure to Mn through increased release of nitric oxide. C57Bl/6 mice subchronically exposed to Mn by intragastric gavage had increased levels of Mn in the striatum and displayed diminutions in both locomotor activity and striatal DA content. Mn exposure resulted in neuronal injury in the striatum and globus pallidus, particularly in regions proximal to the microvasculature, indicated by histochemical staining with fluorochrome and cresyl fast violet. Neuropathological assessment revealed marked perivascular edema, with hypertrophic endothelial cells and diffusion of serum albumin into the perivascular space. Immunofluorescence studies employing terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate (DUTP)-biotin nick-end labeling revealed the presence of apoptotic neurons expressing neuronal nitric oxide synthase (NOS), choline acetyltransferase, and enkephalin in both the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus. In contrast, soma and terminals of dopaminergic neurons were morphologically unaltered in either the striatum and globus pallidus.

Key Words: astrocytes; manganese; nitric oxide; neurodegeneration; apoptosis; dopamine; basal ganglia; movement disorders; inflammation; blood-brain barrier.

Manganese (Mn) is an essential nutrient that functions as a cofactor in numerous enzymes critical to metabolic and redox homeostasis in the central nervous system, including glycosyltransferase, pyruvate decarboxylase, glutamine synthetase, and superoxide dismutase (Gonzalez-Zulueta et al., 1998; Keen et al., 2000; Kemmerer et al., 1931; Wedler et al., 1982). However, overexposure to Mn causes neurotoxicity resulting in manganism or Mn-induced parkinsonism (Couper, 1837; Huang et al., 1989; Kawamura et al., 1941; Mergler et al., 1994). This condition is distinct from idiopathic Parkinson’s disease (PD) in both etiology and pathology, particularly in the notable preservation of dopaminergic soma in the substantia nigra pars compacta (SNpc; Olanow, 2004). In addition to occupational exposure, such as in the manufacturing of dry batteries, steel, aluminum, welding metals, and organochemical fungicides (Keen and Leach, 1987; Keen et al., 2000), individuals receiving total parenteral nutrition (Bertinet et al., 2000) and patients with chronic liver failure are at a higher risk of Mn intoxication (Hauser et al., 1994; Krieger et al., 1995). Further concerns regarding the risk for excessive exposure are raised by the high levels of Mn in soy-based infant formulas (Krachler and Rossipal, 2000; Lonnerdal, 1994).

Early-stage manganism is characterized by psychiatric symptoms, including emotional liability, mania, compulsive or violent behavior, hallucinations, disturbance of sleep, and eating and sexual disturbances, but few, or subtle, motor effects (Rodier, 1955). A later, or established, phase is dominated by motor symptoms such as bradykinesia, rigidity, and dystonia (Rodier, 1955); however, it is noteworthy that patients can develop the motor deficits of manganism without having experienced any preceding psychiatric symptoms (Huang et al., 1989). Finally, it has been documented that neurologic functions continue to deteriorate long after cessation of exposure to Mn (Huang et al., 1993).

Neurochemical changes in human and animal Mn intoxication include a severe reduction in dopamine (DA) levels in the caudate nucleus, putamen, and substantia nigra (SN), as well as a distinct reduction of noradrenaline in the hypothalamus (Autissier et al., 1982). Although the globus pallidus is a primary target in Mn neurotoxicity, data from both primates and humans indicate that the striatum is also vulnerable. Positron emission tomography (PET) studies examining 18F-6-fluoro-L-DA uptake in patients suffering from manganism (Shinotoh et al., 1997) and in Mn-intoxicated monkeys.
(Shinotoh et al., 1995) indicated decreased D2 DA receptor density in the striatum with intact DA transporter function on presynaptic terminals, suggesting postsynaptic injury relative to the nigrostriatal dopaminergic system. In addition, studies by Kessler et al. (2003) demonstrated markedly reduced striatal postsynaptic D2 receptor density by 18F-methylspiperone PET imaging in an advanced case of chronic manganism.

Pathologic changes in human manganism mirror sites of neurochemical deficits, particularly in the globus pallidus, where marked neuronal loss and astrocytosis occur, particularly in the medial segment. A less severe degeneration occurs in the putamen, the caudate nucleus, and substantia nigra pars reticulata (SNpr; Yamada et al., 1986). Although data from nonhuman primates and humans demonstrate the vulnerability of the striatum and globus pallidus to Mn, the specific neuronal subtypes that degenerate have not been reported. Moreover, the possible contribution of astrogliosis in neuronal degeneration remains poorly understood. To date, rodent studies have yielded variable results (Brenneman et al., 1999; Newland, 1999), and there are only a limited number of studies reporting mouse models of manganism. It is postulated in this study that specific neuronal subtypes within the striatal-pallidal system are selectively vulnerable to Mn neurotoxicity and that astrocyte-derived nitric oxide (NO) plays a role in the observed neuronal degeneration. To test this hypothesis, we developed a mouse model of manganism utilizing a gastric gavage-dosing regimen to mimic human dietary exposure to moderate doses of Mn. The findings represent, to our knowledge, the first data on specific interneurons vulnerable to Mn in the striatal-pallidal system.

MATERIALS AND METHODS

Materials. All chemical reagents were obtained from Sigma Chemical Co. (St. Louis, MO). C57Bl/6 mice were obtained from Harlan (Indianapolis, IN). Primary antibodies to tyrosine hydroxylase (TH), anti-choline acetyltransferase (ChAT), and enkephalin (ENK) were from Chemicon (Temecula, CA). Primary antibodies to glial fibrillary acidic protein (GFAP) and nitric oxide synthase (NOS) 1 were from Santa Cruz Biotechnology (Santa Cruz, CA), primary antibodies to 5-3-nitrotyrosine (3-NTyr) were from Upstate (Charlottesville, VA), and primary antibodies to dynorphin (DYN) were from Serotec (Oxford, United Kingdom). Anti-mouse serum albumin was from Sigma Chemical Co. Horseradish peroxidase–conjugated secondary antibodies and diaminobenzidine reagents were part of the Vectastain ABC kit from Vector Labs (Burlingame, CA). Terminal transferase recombinants were from Roche Molecular Biochemicals (Indianapolis, IN). AlexaFluor-488–labeled dUTP and AlexaFluor-568–labeled secondary antibodies were from Molecular Probes (Eugene, OR).

Animal exposure model. Twelve-week-old female C57Bl/6 mice were housed in microisolator cages (four animals per cage) and kept on 12-h light/dark cycles with access to laboratory chow and water ad libitum. Littermates from timed pregnant dams were paired in control and Mn-exposed groups and received either 0.9% normal saline or MnCl2·6H2O (100 mg/kg) in sterile deionized water by gastric gavage once daily for 8 weeks (n = 19 animals per group total over two repetitions of the study). The amount of Mn delivered was adjusted for the molar concentration in the hexahydrate form so as to achieve a precise dose of 100 mg/kg. This dose was selected based upon pilot studies indicating that 100 mg/kg was the minimum sufficient dose to induce measurable neurologic deficits in exposed mice during the course of subchronic exposure by intragastric gavage; lower doses required longer than 8 weeks to produce measurable deficits (data not shown). All procedures were performed under the supervision of the Animal Care and Use Committee at the Colorado State University and were approved prior to onset of the studies.

Locomotor activity. Locomotor activity was determined in each animal at 0, 2, 4, 6, and 8 weeks during the gavage regimen in xz-planes using VersaMax infrared beam activity chambers (Accuscan Instruments, Inc., Columbus, OH). Multiple behavioral parameters pertaining to basal ganglia function were collected and analyzed using VersaDat software (Accuscan Instruments, Inc.), including total distance traveled, center time, margin time, horizontal activity, vertical activity, rearing activity, time moving, and time resting (Miller et al., 2001). Treated and control animals were paired in the same activity monitor during each recording session. Animals were monitored for 20 min, and the data were binned in 1-min increments. Data from 0–5 min were averaged for statistical analysis using Prism software (v4.0a, Graphpad Software, Inc., San Diego, CA). Differences between control and Mn-treated groups were analyzed using a paired, two-tailed t-test, with the level of significance set at p < 0.05.

Determination of tissue catecholamine levels and Mn. The content of total striatal DA, 3,4-dihydroxyphenylacetic acid (DOPAC), γ-aminobutyric acid (GABA), and 5-hydroxytryptamine (serotonin; 5-HT) was determined by
high-performance liquid chromatography using electrochemical detection as described (Champney et al., 1992). For the determination of Mn levels in tissue, brains were rapidly dissected, and regions corresponding to the pallidum-striatum or SN were removed. Tissue blocks were snap frozen in liquid nitrogen and saved at −80°C for analysis by inductively coupled plasma mass spectrometry as described (Melnyk et al., 2003). Differences between control and Mn-treated groups were analyzed using a paired, two-tailed t-test, with the level of significance set at \( p < 0.05 \).

Immunohistochemistry and fluorojade staining. All mice were deeply anesthetized by ip injection with 50 mg/kg pentobarbital and perfused intracardially with 4% paraformaldehyde in 0.1M NaKPO₄ buffer (pH 7.4). The brains were collected and kept in cold 0.1M NaKPO₄ buffer. Immunohistochemistry (IHC) of paraffin-embedded, 10-μm coronal serial sections through the SN, striatum, and globus pallidus was performed as described (Harlan et al., 2001) with primary antibodies to TH (1:400), GFAP (1:400), 3-NTyr (1:200), or mouse serum albumin (1:400). Sections were developed using a horseradish peroxidase–conjugated secondary antibody and diaminobenzidine reagents (Vectastain ABC kit, Vector Labs). Fluorojade staining of paraffin-embedded sections was performed as described (Schmued et al., 1997).

**FIG. 2.** Locomotor activity in C57Bl/6 mice subchronically exposed to Mn. Female C57Bl/6 mice were exposed to saline or MnCl₂ (100 mg/kg/day) by oral gavage once daily for 8 weeks. Mice were paired in recording chambers (control and treated), and the activity in x,y,z-axes was quantified for 20 min. Data from the first 5 min were binned and analyzed for total distance traveled (A), margin time (B), horizontal activity (C), and vertical activity (D). Values represent mean ± SEM.* \( p < 0.05 \) compared to control. Control: \( n = 19 \); Mn: \( n = 19 \).

**Immunofluorescence and TUNEL staining.** Immunofluorescence was performed on fixed, paraffin-embedded, 10-μm coronal serial sections through the striatum and globus pallidus. Sections were first examined for TUNEL-positive cells using terminal deoxynucleotidyl transferase (TdT) reagents as per the manufacturer’s instructions and visualized with AlexaFluor-488–labeled dUTP. All slides included a control section lacking primary antibody and TdT to ensure specificity of staining. Following TUNEL labeling, sections were incubated with antineuronal NOS1(1:200), anti-ChAT (1:50), anti-ENK (1:300), anti-DYN (1:50) antibodies, and then with AlexaFluor-568–labeled secondary antibody. Cells were imaged by fluorescence microscopy as described below.

**Fluorescence microscopy.** Fluorescence images of fluorojade-stained brain tissue were acquired using a Zeiss Axiovert 200M microscope equipped with a ×63 1.4 numerical aperture oil immersion objective and Hamamatsu ORCA-ER–cooled charge-coupled device camera (Optical Elements Corporation, Dulles, VA). Samples were excited using a Sutter DG-4 xenon source at 490 nm/475 nm for TUNEL staining and at 555 nm/590 nm for antibody staining. Colocalization of GFAP and NOS2 staining was performed by 3-dimensional (3-D) deconvolution imaging using Slide Book software (v. 4.1, Intelligent Imaging Innovations, Inc., Denver, CO). Images were acquired every 0.5 μm across a 12-μm z-series and analyzed by no-neighbor...
deconvolution. The deconvolved images were extrapolated into a single, composite 2-D image to illustrate morphological details across the series of optical planes.

RESULTS

Body Weight and Mn Concentrations

To examine the role of glial activation in neuronal injury from Mn, we exposed mice subchronically for 8 weeks to MnCl₂ by intragastric gavage and evaluated neurologic and neuropathologic indices of injury. As shown in Figure 1A, there was no change in body weight during the exposure regimen in either control or Mn-treated mice. However, there was a significant ($p < 0.05$) increase in Mn levels in the striatum of treated mice compared to control mice ($0.70 \pm 0.10$ ppm control vs. $1.81 \pm 0.44$ ppm; Fig. 1B). Mn levels were also elevated in the SN ($0.72 \pm 0.08$ ppm control vs. $1.25 \pm 0.26$ ppm treated), although with a less rigorous degree of statistical significance ($p < 0.09$).

Behavioral Testing and Catecholamine Levels

Neurobehavioral and neurochemical parameters were evaluated to determine the functional consequences of Mn exposure in C57Bl/6 mice. The dose and time selected for treatment (100 mg/kg for 8 weeks) was based upon preliminary studies and selected to produce only a moderate degree of dysfunction in the animals. Upon evaluation of locomotor performance in open-field activity chambers (Fig. 2), Mn-treated animals displayed a decrease in the total distance traveled (Fig. 2A), relative to saline-treated controls, and an increase in margin time (amount of time spent within 1 cm of the margin of the

FIG. 3. Striatal catecholamines in C67Bl/6J mice exposed to Mn. Female C57Bl/6 mice were exposed to saline or MnCl₂ (100 mg/kg/day) by oral gavage once daily for 8 weeks. At the cessation of treatment, animals were evaluated for striatal DA (A), DOPAC (B), DOPAC/DA (C), and GABA (D) levels by high-performance liquid chromatography using electrochemical detection. Values represent mean ± SEM. *$p < 0.05$ compared to control. Control: $n = 19$; Mn: $n = 19$. 524 LIU ET AL.
chamber; Fig. 2B). Horizontal activity (number of movements in the xy-plane) and vertical activity (movements in the z-plane) in Mn-treated mice were not significantly different from those of the controls (Figs. 2C and 2D). The total content of selected catecholamines in the striatum was determined by liquid chromatography at the termination of the study following evaluation of locomotor activity (Fig. 3). Total striatal DA was decreased in Mn-treated mice by 47.3%, relative to saline-treated controls (Fig. 3A; DA control, \(57.95 \pm 3.118\) ng/mg protein, \(n = 4\); DA Mn-treated, \(30.56 \pm 4.256\), \(n = 5\)). However, neither levels of DOPAC were not altered (Fig. 3B) nor was the DOPAC/DA ratio (Fig. 3C). Levels of GABA (Fig. 3D) and 5-HT (data not shown) were similarly unaltered.

**Assessment of Neuronal Injury and Blood-Brain Barrier Integrity**

Neuronal injury within the basal ganglia was assessed by cresyl fast violet staining for Nissl substance and by histochemical staining with fluorojade to detect irreversible neurodegeneration (Fig. 4). Additionally, IHC staining for serum albumin was used to assess the effect of Mn exposure on the integrity of the blood-brain barrier (BBB). Neurons in control

**FIG. 4.** Nigrostriatal injury in C57Bl/6 mice exposed to Mn. At the cessation of subchronic exposure to Mn, mice were evaluated for histopathological changes in the striatum and globus pallidus by cresyl violet stain for Nissl substance (A, control; B, +Mn) and fluorojade (C, control; D, +Mn) and IHC staining for serum albumin (E, control; F, +Mn). Images were acquired by light (A, B, E, and F) or fluorescence (C and D) microscopy (scale bar = 10 \(\mu\)m). Arrowheads in (F) indicate sites of perivascular edema. Representative sections from striatum are presented, but similar changes were observed in globus pallidus. Control, \(n = 8\) mice processed for histopathology over two repetitions of study; +Mn, \(n = 7\) mice processed for histopathology over two repetitions of study. Scale bar = 10 \(\mu\)m.
animals displayed rounded, open nuclei by cresyl violet staining (Fig. 4A), and there were no evident fluorojade-positive cells in either the globus pallidus or the striatum, although slight background staining of white matter tracts within striosomes was noted (Fig. 4C). The microvasculature in control animals was intact with patent endothelium and no evidence of edema by either cresyl violet (Fig. 4A) or albumin staining (Fig. 4E). In contrast, neuronal injury was evident in Mn-treated mice by the appearance of condensed, pyknotic cells of neuronal phenotype in cresyl violet–stained sections, particularly in perivascular regions (Fig. 4A). Moreover, there was prominent edema surrounding striatal and pallidal capillaries, with hypertrophy of endothelial cells and apparent loss of vascular integrity. Fluorojade staining revealed the presence of dying neurons and, surprisingly, reactive endothelial cells within edematous vessels (Fig. 4D). A compromise in BBB function was confirmed by localization of albumin in the perivascular space (Fig. 4F). IHC staining for TH was performed to determine if injury within the striatal-pallidal system was associated with direct loss of dopaminergic neurons (Fig. 5). However, no loss of dopaminergic terminals in the striatum (Figs. 5A and 5B) or soma in the SN (Figs. 5C and 5D) was detected, and dopaminergic neurons were morphologically unaltered between control and Mn-treated mice (Figs. 5E and 5F).

Identification of Vulnerable Interneurons

To determine the identity of neurons vulnerable to Mn within the basal ganglia, dual-label immunofluorescence studies were performed in sections from the striatum and globus pallidus using TUNEL staining for apoptotic cells in conjunction with antibodies for specific neuronal subtypes. Specific subtypes of pallidal and striatal interneurons examined were those expressing NOS1, ChAT, ENK, and DYN. Representative images of control and TUNEL-positive neurons are presented in Figure 6. As is evident from the composite images of TUNEL staining and specific neuronal markers in Figures 6A–6C, no apoptotic cells were detected in saline-treated control mice. In Mn-treated mice, apoptosis was detected in NOS1 (Figs. 6D–6F), ChAT (Figs. 6G–6I), and ENK (Figs. 6J–6L) interneurons but not in DYN interneurons (data not shown). TUNEL-positive neurons that expressed NOS1 and ENK were observed almost exclusively in the striatum, whereas dying ChAT-positive neurons were found in the globus pallidus. Neurons with TUNEL-positive nuclei evinced a condensed cellular morphology with loss of axons and dendritic arbors, consistent with an apoptotic phenotype. It was noted that many of the apoptotic interneurons in both the pallidum and striatum were located proximally to capillaries.

Astrogial Activation and NO Production

The association of activated glial cells with regions of neuronal injury was examined by IHC staining for GFAP, a marker for astrocytes, and for 3-NTyr protein adducts, an indicator of NO production and ONOO⁻ (peroxynitrite) formation. Activation of microglial cells was examined by IHC for isolectin-B4 binding, but no difference in activation of this cell type was detected between control and treated animals (data not shown). A pronounced activation of astroglia was detected in both the striatum and globus pallidus of Mn-treated mice, particularly along the medial segment of the striatum and lateral segment of the pallidum (Figs. 7A and 7B). In contrast to astrocytes in control mice (Fig. 7C), activated astrocytes in Mn-treated animals were hypertrophic and contained large fiber bundles that stained intensely for GFAP (Fig. 7D). Activation of astroglia was noted particularly in areas proximal to capillaries. Staining for 3-NTyr protein adducts revealed a pattern of NO formation that overlapped areas of astroglial activation in Mn-treated mice in both the striatum and globus pallidus (Figs. 7G and 7H). Background levels of NO formation in control mice were extremely low in both brain regions (Fig. 7G). High-power examination of these brain regions revealed that cells labeled for 3-NTyr adducts were consistently
those with a neuronal phenotype, many of which were small and pyknotic (Fig. 7H, inset).

The source of NO leading to the formation of 3-NTyr adducts in neuronal cells was examined using double-label immunofluorescence staining for GFAP and NOS2. High-power fluorescence images of sections stained for GFAP and NOS2 revealed normal astrocyte morphology in control animals with a complete lack of expression of NOS2 in astrocytes or any other cell type (Figs. 8A–8D). In contrast, marked activation of astrocytes was observed in Mn-treated animals, which stained intensely for GFAP that colocalized with NOS2 (Figs. 8E–8H). Most, but not all, astrocytes expressing NOS2 were located proximally to capillaries. Although not all astrocytes in Mn-treated mice expressed NOS2, no other cell type was found that expressed this protein, as evidenced by spatial colocalization under high-power magnification.

DISCUSSION

Lesions of the basal ganglia in humans lead to various motor disturbances that range from hypokinesia (e.g., PD) to hyperkinesia (e.g., hemiballismus and chorea) and psychotic disorders such as schizophrenia. The striatum is the major recipient structure of neuronal efferents in the basal ganglia; the internal segment of the globus pallidus and the pars reticulata of the SN are the two major output nuclei (Saka et al., 2002). These brain regions are severely affected in manganism, but precise cellular mechanisms underlying the known pathological effects of Mn have remained elusive. The present studies identify populations of interneurons in the striatal-pallidal system vulnerable to Mn and provide insight into the possibility of gliovascular dysfunction as an important contributing factor to the underlying pathology.

Selective increases in Mn levels within the basal ganglia are observed in both rodents and humans after overexposure (Erikson et al., 2002; Nagatomo et al., 1999) consistent with the approximate twofold increase in striatal levels of Mn noted in our model (Fig. 1). Increased levels of Mn in the SN showed a trend towards statistical significance \((p < 0.09)\). We attribute this \(p\) value to the relatively small sample size \((n = 4\) in Mn-treated group), as well as the small size of the SN in the mouse, which lends variability to analytical determination of Mn content. The extent of Mn accumulation in the striatum-pallidum of treated mice in this model suggests that selective

FIG. 6. Identification of vulnerable striatal-pallidal interneurons. Apoptotic cells identified by TUNEL were colabeled with specific antibodies to distinguish among various neuronal subtypes. TUNEL-positive cells were labeled with AlexaFluor-488-labeled dUTP (green), and neuronal markers were labeled with an AlexaFluor-568-labeled secondary antibody (red). Following TUNEL, tissue sections were incubated with anti-NOS1, anti-ChAT, or anti-ENK antibody and visualized by fluorescence microscopy using a Zeiss Axiovert 200M inverted microscope and Planapochromat \( \times 63 \) oil immersion 1.4 Numerical aperture objective and detected using a Hamamatsu ORCA-ER–cooled charge-coupled device camera. (A–C) Representative images of sections from control mice stained for TUNEL-positive cells and NOS1, ChAT, and ENK. (D–F) Representative TUNEL-positive NOS1+ interneuron in the striatum after Mn treatment. (D) NOS1+ interneuron; (E) TUNEL-positive nucleus; (F) merged image of TUNEL-positive nucleus of NOS1+ interneuron. (G–I) Representative TUNEL-positive ChAT+ interneuron in the striatum after Mn treatment. (G) ChAT+ interneuron; (H) TUNEL-positive nucleus; (I) merged image of TUNEL-positive nucleus of ChAT+ interneuron. (J–L) Representative TUNEL-positive ENK+ projection neuron in the striatum after Mn treatment. (J) ENK+ projection neuron; (K) TUNEL-positive nucleus; (L) merged image of TUNEL-positive nucleus of ENK+ projection neuron. Scale bar = 10 \( \mu \)m.
accumulation occurs in these brain regions even at moderate absorbed doses over a longer period of time; high-dose, short-term studies of mice injected with Mn observe two- to threefold greater accumulation just 4–7 days after dosing with 100 mg/kg by ip injection in C57Bl/6 mice and Sprague Dawley rats (Dodd et al., 2005; Hazell et al., 2003).

Elevated brain Mn in exposed mice correlated with hypoactivity (decrease in total distance traveled) as well as development of anxiety and novelty-seeking behavior (increased margin time; Figs. 2A and 2B). Moreover, these changes in behavioral indices were associated with a drop in striatal DA of just under 50% (Fig. 3A). This degree of diminution in catecholamine content mimics the early-established phase of manganism (Aupeissier et al., 1982) and indicates that the behavioral parameters altered in this model may be useful for assessing early neurologic dysfunction following Mn overexposure in mice. A trend towards an increased DA/DOPAC ratio was observed (Fig. 3), suggesting that increased turnover may be responsible for decreased striatal DA in this model. Similar findings were reported in Mn-exposed baboons, where inhibition of the synaptic DA transporter was cited as a mechanism involved in the depletion of striatal DA (Chen et al., 2006).

The striatum receives excitatory input from the cortex and dopaminergic input from the SNpc and projects to the internal segment of the globus pallidus and the SNpr through direct and indirect inhibitory pathways (Dimova et al., 1993; Saka et al., 2002). Dopaminergic input to the striatum from the SNpc differentially affects striatal interneurons based on the subtype of DA receptor expressed (Gerfen et al., 1990). DYN-positive projecting neurons of the direct pathway express D1 DA receptors and activate the direct pathway, whereas ENK-positive interneurons express D2 DA receptors and activate the indirect pathway in response to DA (Gerfen and Young, 1988). Activation of the indirect pathway results in a reduction of inhibitory output from the internal pallidum and, therefore, disinhibition of thalamocortical neurons and facilitation of movement. By contrast, activation of the indirect pathway increases the tonic inhibitory output of the internal pallidum and suppresses movement (Wichmann and DeLong, 1996). Although interneurons comprise only a small percentage of the overall number of neurons in the striatum, they are highly interconnected with the GABAergic output nuclei and exert a profound influence on the coordination of motor signals by the basal ganglia (Saka et al., 2002).

The identification of specific interneurons in the striatal-pallidal system vulnerable to Mn suggests several pathways by which neurological function may be inhibited in Mn-treated animals. The appearance of TUNEL-positive nuclei and evident nuclear condensation are consistent with an apoptotic phenotype in striatal interneurons expressing NOS1, ChAT, and ENK (Fig. 6) at this early stage of Mn intoxication. Loss of ENK interneurons would be expected to reduce inhibition of the globus pallidus through the indirect pathway, thereby increasing tonic inhibitory output and inhibiting corticothalamic motor circuits. Interneurons expressing DYN and ENK receive dopaminergic input from the SN through D1 and D2 DA receptors, respectively (Gerfen and Young, 1988), but only ENK interneurons of these two subtypes were found to be injured in Mn-treated mice (Fig. 6). Likewise, loss of ChAT interneurons,
which can excite ENK interneurons (Dimova et al., 1993; Gauchy et al., 1991), would be expected to elicit a similar effect. The hypokinesia noted in Mn-treated mice in this study (Fig. 2) is consistent with such a mechanism involving loss of function within the indirect pathway and disinhibition of GABAergic output from the internal pallidum. The studies in Figure 6 identifying apoptotic ChAT and ENK neurons in Mn-treated mice further support this mechanism.

Both NOS1 and ChAT interneurons modulate the activity of striatal projection neurons of the direct pathway (Saka et al., 2002). Striatal projection neurons that receive dopaminergic, cholinergic, or nitrinergic inputs also synapse with glutamatergic projecting neurons from the cortex (Sancesario et al., 2004). It has been noted that corticostriatal glutamatergic neurotransmission is dramatically overactive in models of parkinsonism (Calabresi et al., 1999), possibly as a compensatory mechanism due to loss of DA (Sancesario et al., 2004). NOS1, ChAT, and ENK interneurons possess ionotropic and metabotropic glutamate receptors (Kosinski et al., 1999) and could thereby be rendered vulnerable to injury from overactive glutamatergic inputs. In this regard, it is noteworthy that pharmacologic inhibition of metabotropic glutamate receptors is protective against Mn neurotoxicity, implicating an excitotoxic mechanism in the etiology of manganism (Brouillet et al., 1993). Other investigators suggest that deafferentation of dopaminergic inputs or loss of DA could directly result in retrograde injury and loss of NOS1-expressing spiny interneurons (Sancesario et al., 2004). In either case, damage to NOS1 interneurons would diminish activation of the direct pathway, thereby contributing to hypokinesia, as observed in our model. Other investigators have reported that the neuropeptide somatostatin, expressed by NOS1 interneurons, is reduced in the striatum in experimental parkinsonism (Bolam et al., 2000), underscoring the importance of this population of interneurons to the manifestation of motor dysfunction.

The location and morphology of dying cells identified by fluorojade staining (Figs. 4C and 4D) suggest that endothelial cells of the striatal microvasculature may be an important target in Mn neurotoxicity, with subsequent disruption of the BBB. The appearance of serum albumin within edematous regions of the perivascular space in Mn-treated mice (Figs. 4E and 4F) also indicates disruption of normal BBB function. This may explain why neuronal injury and astrocytosis occurred to a large extent proximal to vessels. Astrocytes and endothelial cells have unique and complex interactions in regulation of the BBB and cerebral vascular tone that are vulnerable to disruption by neurotoxicants. Pathologic changes noted in endothelial cells, such as striking fluorojade staining, a histologically reactive phenotype, and perivascular albumin leakage, would profoundly affect astroglia, which extend foot processes to surround the cerebral microvascular capillaries. Recent studies (reviewed in Koehler et al., 2006) highlight the importance of astrocyte calcium waves and NO effusion in modulating cerebral vascular tone by inducing release of arachidonic acid metabolites by endothelial cells. The profound cerebrovascular edema noted in pallidal-striatal capillaries in Mn-exposed mice thus suggests that endothelial-astroglial interactions may represent an early site of dysfunction in Mn neurotoxicity.

Increased staining for GFAP and 3-NTrp in Mn-treated mice (Fig. 7), as well as colocalization of GFAP and NOS2 (Fig. 8), suggests that astrocytosis and astrocyte-derived NO may be involved in neurodegeneration. This hypothesis is further supported by recent studies from our laboratory demonstrating the requirement for astroglial-derived NO in neuronal apoptosis following exposure of cocultured astrocytes and neurons to Mn and inflammatory cytokines (Liu et al., 2005). These observations
raise the intriguing possibility that death or dysfunction of endothelial cells in the striatal microvasculature might trigger astrocytosis and NOS2 expression, predisposing neurons to injury in these brain regions during Mn intoxication. Because Mn also disrupts ATP production (Zhang et al., 2003) and glutamate uptake (Aschner et al., 2001) in astrocytes, associated neurons in the basal ganglia could further be rendered vulnerable to excitotoxic injury.

Collectively, these studies demonstrate that subchronic exposure of mice to moderate levels of Mn by intragastric gavage recapitulates both neurologic and pathologic features of manganism. In addition to the globus pallidus, the striatum is a direct target of Mn in mice, consistent with reports in humans exposed to Mn (Kim et al., 1999). The neuropathological changes in these nuclei precede overt loss of dopaminergic neurons and occur at a level of striatal DA considered subclinical in Parkinson patients. Dysfunction of specific interneurons in early-Mn intoxication is associated with loss of BBB integrity and marked astrogliosis that profoundly affects striatal-pallidal function. These observations suggest reconsideration of Mn neurotoxicity as a lesion that is fundamentally gliovascular in origin.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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**REFERENCES**


