Published in final edited form as:

Neurotoxicology. 2011 March ; 32(2): 215–226. doi:10.1016/j.neuro.2010.11.003.

Manganese exposure induces microglia activation and dystrophy in the substantia nigra of non-human primates

Tatyana Verina¹, Samara F Kiihl², Jay S Schneider³, and Tomás R Guilarte^{1,*}

Tatyana Verina: tverina@jhsp.h.edu; Samara F Kiihl: samara.kiihl@gmail.com; Jay S Schneider: Jay.Schneider@jefferson.edu; Tomás R Guilarte: trguilarte@columbia.edu

¹Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

²Department of Biostatistics, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

³Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA

Abstract

Chronic manganese (Mn) exposure produces neurological deficits including a form of parkinsonism that is different from Parkinson's disease (PD). In chronic Mn exposure, dopamine neurons in the substantia nigra (SN) do not degenerate but they appear to be dysfunctional. Further, previous studies have suggested that the substantia nigra pars reticulata (SNr) is affected by Mn. In the present study, we investigated whether chronic Mn exposure induces microglia activation in the substantia nigra pars compacta (SNc) and SNr in *Cynomolgus macaques*. Animals were exposed to different weekly doses of Mn (3.3–5.0, 5.0–6.7, 8.3–10 mg Mn/kg body weight) and microglia were examined in the substantia nigra using LN3 immunohistochemistry. We observed that in control animals, LN3 labeled microglia were characterized by a resting phenotype. However, in Mn-treated animals, microglia increased in number and displayed reactive changes with increasing Mn exposure. This effect was more prominent in the SNr than in the SNc. In the SNr of animals administered the highest Mn dose, microglia activation was the most advanced and included dystrophic changes. Reactive microglia expressed increased iNOS, L-ferritin, and intracellular ferric iron which were particularly prominent in dystrophic compartments. Our observations indicate that moderate Mn exposure produces structural changes on microglia, which may have significant consequences on their function.

Keywords

microglia; dystrophy; activation; manganese; substantia nigra; iNOS; ferritin; ferric iron - neurotoxicity

*Corresponding author: Tomás R. Guilarte, Ph.D., Neurotoxicology & Molecular Imaging Laboratory, Department of Environmental Health Sciences, Johns Hopkins University, Bloomberg School of Public Health, 615 North Wolfe Street, Room E6622, Baltimore, Maryland 21205, Phone: (410) 955-2485, FAX: (410) 502-2470.

Present address: Tomás R. Guilarte, Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, 600 Haven Avenue, B1-108, New York, NY 10032, Phone: 212-305-3959, Fax: 212-305-3857

Conflict of interest statement: The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Chronic exposure to moderate levels of manganese (Mn) from environmental, occupational or in certain clinical conditions such as cirrhosis, leads to cognitive, psychiatric and motor function deficits, with the latter resembling some features of idiopathic Parkinson's disease (PD) (Banta and Markesbery 1977; Barbeau 1984; Aschner et al., 2005; Martin, 2006; Perl and Olanow, 2007; Burton and Guilarte, 2009; Guilarte, 2010). Following exposure, Mn accumulates throughout the living brain with some selectivity for basal ganglia nuclei with the highest accumulation occurring in the globus pallidus (Dorman et al., 2006; Guilarte et al., 2006).

Idiopathic PD is the result of degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) (Braak et al., 2003). However, a loss of DA neurons has not been a common feature in autopsy samples from humans or non-human primates exposed to Mn (Perl and Olanow, 2007; Burton and Guilarte, 2009; Guilarte, 2010). The latter is consistent with neuroimaging studies showing that there is a lack of an effect of Mn exposure on DA transporter levels in the caudate and putamen (striatum) (Guilarte et al., 2008; Selikhova et al., 2008; Sikk et al., 2010; Guilarte, 2010). Dopamine transporters are a commonly used marker of dopaminergic terminal integrity (Brooks et al., 2003). Neuroimaging data from some occupational exposed cases show reduced levels of DA transporter (Huang et al., 2003; Kim et al., 2002). However, in these cases, one cannot rule out the possibility that these individuals had idiopathic PD with coincidental Mn exposure.

Although chronic Mn exposure does not appear to cause dopamine neuron degeneration, recent Positron Emission Tomography (PET) studies in non-human primates have demonstrated that elevated brain Mn levels result in a marked decrease of *in vivo* dopamine release (Guilarte et al., 2006; 2008). These studies suggest that the effect of Mn on dopaminergic neurons is not degeneration or a decrease in tissue dopamine levels but rather it appears to be associated with impairment of dopamine release. These findings are consistent with the fact that L-dopa therapy is not effective in Mn-exposed humans with motor abnormalities (Koller et al., 2004; Perl and Olanow 2007; Guilarte, 2010), a therapy that is effective in idiopathic PD subjects (Lees, 1986).

Exposure to heavy metals is known to alter metal-ion homeostasis in the brain and to induce oxidative stress and neuroinflammation (Riederer et al. 1992; Campbell et al., 2004; Guilarte et al., 2008a). Iron-mediated oxidative damage is known to play a central role in the pathogenesis of neurodegenerative disorders (Riederer et al., 1989; Youdim et al., 1999; Dexter et al., 1991; Connor et al., 1992). Iron brain content is increased in the brains of patients with idiopathic PD and other neurodegenerative diseases (Melega et al., 2007; Riederer et al., 1989; Griffiths and Grossman, 1993; Griffiths et al., 1999; Sofic et al., 1988; Jellinger et al., 1990; Sofic et al., 1991). It is likely that an elevated Mn level may alter iron homeostasis in the brain leading to increased oxidative stress (Verity, 1999), particularly within the SN where the concentrations of iron as well as DA and lipids are high (Riederer et al., 1992).

Microglia participate in the maintenance of brain iron homeostasis and prevent iron-dependent oxidation by sequestering iron within ferritin (Cheepsunthorn et al., 1998; Connor et al., 1994; Han et al., 2002; Zhang et al., 2006). Ferritin consists of functionally distinct heavy (H) and light (L) chain subunits: H-ferritin transforms soluble ferrous (Fe^{2+}) into ferric (Fe^{3+}) iron which in turn is stored by L-ferritin (Levi et al., 1992). The ratio of H/L subunits is brain region- and cell type-specific (Connor and Menzies, 1995; Connor et al., 1995a; Connor J., 1996). That is, oligodendrocytes contain a mixture of H/L ferritins, while microglia contain mainly L-ferritin, and, therefore, play a major role in iron storage (Connor

et al., 1994). Being a highly dynamic cellular population, microglia rapidly respond to any pathological condition by a complex transformation into an activated state and accompanied by the induction of reactive oxygen and nitrogen species (Streit, 1999; Hanisch and Kettenmann, 2007; Colton and Gilbert, 1987; Corradin et al., 1993). It has been suggested that these highly oxidizing radicals may impair the iron binding capacity of ferritin and cause damage to microglia (Agrawal et al., 2001; Biemond et al., 1984).

In the current study, we examined the effect of chronic Mn exposure on microglia in the SNc and SNr of *Cynomolgus macaques*. We report that Mn induces a microglia response manifested by reactive and dystrophic changes and by increased accumulation of intracellular ferric iron, L-ferritin and iNOS expression. These findings indicate that chronic Mn exposure produces microglia activation and dystrophy most prominently in the SNr of non-human primates and these changes may be produced by iron-mediated oxidative stress mechanism.

2. Materials and methods

2.1. Manganese administration and animal care

Young adult male *Cynomolgus macaques*, 5-6 years of age were used in this study. All animal studies were reviewed and approved by the Johns Hopkins and the Thomas Jefferson University Animal Care and Use Committees. Animals received injections of manganese sulfate into the saphenous vein under 1-3% isoflourane anesthesia once or twice a week. Animals were exposed to different weekly doses of Mn (3.3–5.0, 5.0–6.7, 8.3–10 mg Mn/kg BW) (Table 1) and several of their characteristics have been described in several publications (Guilarte et al., 2006;2006a;2008;2008a;Schneider et al., 2006;2009). All animals were euthanized by ketamine injection (20–30 mg/kg BW) followed by an overdose of pentobarbital (100 mg/kg BW) and the brains were harvested. We should note that brain tissue samples from control and Mn-treated animals has been used extensively in previous studies and thus SN tissue was not available from all of the animals. In the current study, 10 out of a total of 13 Mn-treated animals were available and 4 out of 6 controls.

2.2. Tissue preparation

Brains were removed from the skull, embedded into agarose and cut into 4 mm slabs in the coronal plane. Alternate brain tissue slabs from the right hemisphere were processed for immunohistochemistry in two different ways for different purposes. The first slab (in a rostral to caudal direction) and every other slab was fixed in 4% paraformaldehyde, further embedded in paraffin and cut into 5 µm thick sections which were used in the other experiments not presented in this study. Alternate tissue slabs (starting from the second frontal slab) were immersed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for 28 hrs, cryoprotected with 20% glycerol, 0.5% DMSO in PB, and frozen at -80°. 50 µm thick sections were cut using a freezing microtome, stored in cryoprotecting solution at -20°C, and utilized for various immunohistochemical studies as needed. In the current study, two or three sections from anatomically comparable midbrain region were selected from each animal and processed free-floating for detection of microglia.

2.3. Immunohistochemical, immunofluorescent and histochemical labeling

In order to visualize resident microglia in the brain of non-human primates, the following microglia markers were tested: mouse-anti-human CD11b (Serotec), anti-human HLA-DR, alpha-chain (Dako), mouse-anti-human HLA class II (DR) antigen clone LN3 (Novocastra), mouse-anti-rat CD11b (Serotec). From these antibodies, in our hands, only the LN3 antibody from Novocastra produced high quality and specific microglia labeling allowing detailed analysis of cellular morphology. The HLA-DR antigen is expressed in both resting

and reactive microglia (Miles and Chou, 1988). Tissue sections were processed free-floating using the following procedure. Sections were pre-treated with 3% H₂O₂ and 10% methanol in Tris-buffered saline (TBS) for 20 min, rinsed and incubated with 5% normal horse serum and 0.2% Triton X-100 in TBS for 1 hr and then with primary antibodies made against the human HLA class II (DR) antigen clone LN3 (Novocastra, 1:500) for 48-66 hrs at 4°C followed by incubation with horse-anti-mouse secondary biotinylated IgG (1:250, Vector, 1.5 hr, at room temperature) and the avidin-biotin-peroxydase complex solution (1:170, 1 hr at room temperature). The reaction product was visualized with 0.25 mg/ml 3,3'-diaminobenzidine (DAB) and 0.03% H₂O₂. Sections were mounted on slides, stained with cresyl violet, dehydrated in graded ethanols and coverslipped using DPX mounting media. After examination, some LN3-labeled brain sections were rehydrated and processed for Perl's Prussian blue staining to visualize ferric iron (Parmley et al., 1978). This dual staining procedure yielded high quality labeling allowing analysis of intracellular iron associated with LN3-positive cells.

For immunofluorescence dual labeling, sections were processed using the following combinations of antibodies: LN3 (Novocastra, 1:500) and iNOS (Calbiochem 1:1000), LN3 and ferritin (L-chain) (Sigma, 1:1000). Sections were incubated in a cocktail of primary antibodies 48-66 hrs at 4°C followed by incubation with corresponding fluorophore-conjugated secondary antibodies (Jackson Immunoresearch, 1:200). Sections were mounted on slides, coverslipped using ProLong mounting media containing DAPI (Molecular Probes) and analyzed using an LSM510-Meta confocal microscope (Carl Zeiss Microimaging GmbH).

2.4. Evaluation of microglia

Microglia were assessed in the SNc and SNr in control and Mn-exposed animals at the level of the third cranial nerve both qualitatively and quantitatively. Sections were examined using an Olympus BX51 microscope with a motorized (Proscan II, Prior, Rockland, MA, USA) stage allowing x, y and z axes control, and linked to an Olympus DP70 video camera and a Visiopharm Integrator System (Visiopharm, Denmark). High resolution imaging and contrast microscopy using oil immersion and $\times 40$ (numerical apertures 1.00) and $\times 100$ (numerical aperture 1.35) was implemented for analysis of microglia throughout the section thickness with respect to their 3D structure. The SNc and SNr boundaries were outlined on the monitor screen using an $\times 4$ objective and a total magnification factor $\times 140$. Cell counts within the outlined boundaries were obtained using an $\times 40$ objective and a total magnification factor $\times 1400$. Cell number was obtained per mm².

LN3-positive cells were categorized based on morphological criteria for resting, activated and dystrophic microglia, similar to those described by Streit and colleagues (Streit and Sparks, 1997; Streit et al., 2004; Streit, 2006). The following cell categories were counted: (a) resting microglia represented by cells with fine ramified processes; (b) cells with morphological characteristics of activated microglia including cytoplasmic hypertrophy, clustering, and short cytoplasmic processes; (c) dystrophic microglia represented by reactive cells with elements of dystrophic morphology such as beading, spheroid swellings, de-ramification, and atrophy. We also examined whether the reactive state of microglia correlated with the presence of intracellular ferric iron deposits as visualized by Perl's staining. For this purpose, LN3-positive cells with and without iron deposits were counted separately. Simultaneously, iron-containing LN3-positive cells were further categorized based on the size of their intracellular iron deposits: (a) small -single deposits ranging approximately from 0.5 to 3 μm^2 ; (b) medium – single or multiple deposits ranging approximately 4 to 10 μm^2 ; (c) large – single or multiple deposits ranging approximately from 11 to 40 μm^2 in size.

2.5. Statistical analysis

Statistical analysis was performed using R, version 2.9.0 (R Development Core Team, 2009). Animals were classified according to the Mn brain tissue levels that generally correlated with the treatment doses and allowed us to use Mn exposure as a continuous variable. If an animal was considered an outlier, but not an influential point, it was used in the analysis since it did not influence the model fit. When an animal was considered an outlier and an influential point, it was excluded from the statistical analysis. Data were analyzed using different statistical models (Agresti, 2003); (1) The total densities of LN3-positive cells were estimated using a Poisson regression model with total cell count as response variable, reference area (mm^2) as an offset, and Mn brain tissue level ($\mu\text{g/g}$ tissue) and structure as explanatory variables; (2) The association between the reactive state of LN3-positive cells and Mn brain tissue level was performed using logistic regression. The following cell categories were analyzed: 1) resting cells (R) or 2) activated (A) and dystrophic (D) cells combined (AD) to represent reactive microglia. The proportions of R and AD cells were compared across Mn exposure individually for each the SNc and SNr; (3) The association between Mn level and the presence of intracellular iron in LN3-positive cells was analyzed using a logistic regression. The proportions of cells with iron deposits (Fe) and without iron deposits (NoFe) were compared across Mn exposure individually for each the SNc and SNr; (4) The association between LN3 cell reactivity state and the presence of intracellular iron was analyzed using logistic regression. The observed proportion of Fe and NoFe cells were compared across reactivity states for the following cell categories: resting cells (R), activated cells (A), and dystrophic cells (D).

3. Results

3.1. Qualitative evaluation of the effect of Mn exposure on microglia in the SNc and SNr

In control animals, LN3-positive cells were sparsely distributed throughout the SNc (Figure 1A, E) and SNr (Figure 2A, E). Cells usually had small perinuclear cytoplasmic volume and fine ramified processes typical for resting microglia (Figure 1A, E). LN3-positive cells were evenly distributed throughout the brain neuropil and frequently localized in close proximity to neurons that were visualized by Nissl staining (Figure 1A).

In Mn-exposed animals, LN3-positive cells were increased in number and displayed morphological characteristics typical of activated microglia. The number of activated microglia varied among individual animals within the same Mn treatment group, but overall, was greater with higher Mn exposure (also see results of the quantitative analysis in Figure 3). That is, in the SNc of animals that received 3.3–5.0 mg Mn/kg BW, there were no obvious changes in LN3 cell appearance with the exception of a single animal exhibiting small microglia patches. Occasionally, more intensely labeled cells with slightly thicker processes were observed (Fig 1B, F). In the SNc of animals that received 5.0–6.7 and 8.3–10 mg Mn/kg BW (Figure 1C–D, G–H), LN3-positive cells were increased in number and displayed mild to severe cytoplasmic hypertrophy, fluffy appearance, and short and thickened cytoplasmic processes (Figure 1D, H). Some cells had rod-like morphology and were fused via their long processes into elongated aggregates (Figure 1C, G).

In the SNr of most animals treated with 3.3–5.0 mg Mn/kg BW, LN3-positive cells did not display notable reactive changes (Figure 2B). Only a single animal in this treatment group displayed sparse small patches of hypertrophic microglia with thicker processes. In animals that received 5.0–6.7 and 8.3–10 mg Mn/kg BW weekly dose, LN3-positive cells displayed a range of reactive changes including microglia aggregates, hypertrophic cytoplasm and bushy appearance (Figure 2 C–D, F–L). Animals administered 8.3–10 mg Mn/kg, had the most advanced reactive changes in microglia morphology representing signs of what has been

termed by Streit and colleagues as “microglial dystrophy” (Streit, 2008; Streit, 2006; Conde and Streit, 2006; Flanary et al., 2007). This included the presence of abundant bulbous cytoplasmic swellings (spheroids), beading, and atrophic changes (Figure 2 F, I-L). Spheroidal swellings were detected either adjacent to the nuclear compartment (Figure 2F, K; see Figure, Supplemental Digital Content 1) and/or to the distal aspects of cell processes (Figure 2K arrowhead and 4G). LN3 positive processes around apoptotic bodies were occasionally observed (Figure 2G).

3.2. Quantitative analysis of the effect of Mn exposure on microglia in the SNc and SNr

Statistical analysis revealed that the density of LN3-positive cells increased significantly with Mn brain tissue content (p-value < 0.0001) (Figure 3A). Although the density of LN3-labeled cells in the SNr appeared greater than in SNc, the differences between the structures were not significant (p-value=0.41). We should note that one animal (ID 7839, see Table 1) from the high Mn group (8.3-10 mg Mn/kg BW weekly doses) did not display a pattern of microglia activation that was typical for this treatment group. Such observations are not uncommon as there is individual variability in the neuropathological response amongst animals to the same Mn treatment dose (Verity, 1999 and our own observations). This case was considered as an outlier in the statistical model fit, but was not an influential point and therefore it was included in the analysis.

Analysis of the distribution of the microglia phenotype (or reactivity state) across Mn exposure revealed a highly significant association between microglia reactivity and the level of Mn exposure. The proportions of resting cells (R) and reactive cells (represented by the combination of activated and dystrophic cells (AD)) were compared across Mn exposures for the SNc and SNr. One animal (ID 000-1099, Table 1) had much higher cell counts than the other animals in the group exposed to 5.0-6.7 mg Mn/kg and for statistical purposes was excluded from the analysis as it was considered an outlier. Nevertheless, this particular animal expressed a much higher degree of microglia activation than the other animals in the same treatment group. Figure 3B shows the observed proportion of R and AD cells across Mn exposure. Data for the SNc and SNr were analyzed individually, since the interaction between Mn exposure and structure was significant (p-value < 0.0001). For the SNc, the odds of having AD cells for animals exposed to 3.3-5.0 mg Mn/kg were 3.7 times that for controls (95% CI (1.3-10.8), p-value 0.012); the odds of having AD cells for animals exposed to 5.0-6.7 mg Mn/kg was 1.1 times that for controls (95% CI (0.4-3.1), p-value 0.810); the odds of having AD cells for animals exposed to 8.3-10 mg Mn/kg was 15.4 times that for controls (95% CI (6.7-35.3), p-value <0.0001). These data indicate that the population of microglia consisting of reactive AD cells in the SNc increased with Mn exposure, but remained small compared to the population of resting cells (See Figure 3B). Although the effect did not reach statistical significance in the analysis for the medium Mn exposure group (5.0-6.7 mg Mn/kg), one must take in consideration that the animal with the highest observed microglia counts was excluded from the statistical analysis and potentially diminished the estimated effect in this Mn treatment group.

For the SNr, the odds of having AD cells in animals exposed to 3.3-5.0 mg Mn/kg was 9.3 times the odds for controls (95% CI (4.2-20.6), p-value <0.0001); the odds of having AD cells in animals exposed to 5.0-6.7 mg Mn/kg was 14.6 times the odds for controls (95% CI (6.8-31.2), p-value <0.0001); and the odds of having AD cells in animals exposed to 8.3-10 mg Mn/kg was 103.2 times the odds for controls (95% CI (48.6-219.6), p-value <0.0001). These data indicate that the population of microglia consisting of reactive AD cells in the SNr markedly increased with Mn exposure. It was slightly elevated for the 3.0-5.0 and 5.0-6.7 mg Mn/kg exposure groups, and became predominant relative to the population of resting cells in the 8.3-10.0 mg Mn/kg treatment group (see Figure 3B).

These results show that with increased Mn exposure, (a) the number of LN3-positive cells in both the SNc and SNr increases as a quadratic function of Mn brain tissue content; (b) the fraction of reactive microglia increases and becomes predominant in the SNr of animals that received the highest Mn dose; and (c) the effects are more pronounced in the SNr compared to the SNc.

3.3. Visualization of ferric iron using Perl's staining in the SN

To assess the presence of ferric iron in microglia, LN3-labelled sections were rehydrated and processed for Perl's staining (Parmley et al., 1978). Bright blue-green ferric iron deposits were clearly associated with LN3-positive cells (Figure 4 and See Figure Supplemental Digital Content 1) as well as with small LN3-negative cells that may be representative of oligodendrocytes. In the SNc, large neurons (presumably DA neurons) visualized with Nissl stain did not exhibit ferric iron staining (See Panel A and B in Figure Supplemental Digital Content 2). In the SNc and SNr of control animals, ferric iron staining in microglia appeared as small punctate deposits (0.5 to $3\mu\text{m}^2$ in size) localized either within the perinuclear space or within fine processes (See an example in Figure 4C). In Mn-treated animals, there were clear regional differences in intracellular ferric iron distribution. While in the SNc, intracellular iron deposits were either non-detectable or insignificant in microglia (see A and B, Figure Supplemental Digital Content 2), in the SNr, they were much more abundant (see C, Figure Supplemental Digital Content 2) and appeared to correlate with the level of microglia activation (Figure 4). That is, in animals administered 3.3–5.0 mg Mn/kg, there was no obvious differences in ferric iron distribution in microglia (Figure 4D) compared to controls (Figure 4C, also see results of the quantitative analysis in Figure 5). Ferric iron staining was more notable in animals administered 5.0–6.7 mg Mn/kg BW (Figure 4E) and it was the most prominent in animals that received the 8.3–10 mg Mn/kg exposure (Figure 4B, F–H, see C, Figure Supplemental Digital Content 1). In the SNr of these animals, microglia expressed single or multiple intracellular ferric iron deposits reaching up to $40\mu\text{m}^2$ in area. Examination of LN3-labeled sections before and after Perl's staining confirmed that ferric iron was associated with bulbous spheroids (see Panel B, Figure Supplemental Digital Content 1) and frequently appeared as a core inside the cell with hypertrophic cytoplasm (Figure 4F). Furthermore, spheroids with a clear content had pale ferric iron staining, while “filled” LN3-positive spheroids were always intensely stained for ferric iron (see B, Figure Supplemental Digital Content 1; Figure 4G).

3.4. Quantitative analysis of the effect of Mn exposure on intracellular ferric iron in microglia in the SNc and SNr

The presence of intracellular ferric iron deposits in microglia was significantly correlated with the level of Mn exposure. The proportion of cells with iron deposits (Fe) and without iron deposits (NoFe) was compared across Mn exposure individually for the SNc and SNr, since the interaction between Mn exposure and region was significant ($p\text{-value}=0.0003$). One animal (ID 6697; see Table 1) in the 8.3–10 mg/kg group was an outlier and was excluded from the statistical analysis. Figure 5A shows the observed proportion of Fe and NoFe LN3-positive cells across Mn exposure groups. Statistical analysis indicated that for the SNc, the odds of having Fe deposits in microglia in animals exposed to 3.3–5.0 mg Mn/kg was 2.4 times the odds for controls (95% CI (1.3–4.4), $p\text{-value}$ 0.0045); the odds of having Fe deposits in animals exposed to 5.0–6.7 mg Mn/kg was 2.3 times the odds for controls (95% CI (1.5–3.7), $p\text{-value}=0.0003$); and the odds of having Fe deposits in animals exposed to 8.3–10 mg Mn/kg was 13.3 times the odds for controls (95% CI (8.1–21.6), $p\text{-value}<0.0001$). For the SNr, the odds of having Fe deposits in microglia in animals exposed to 3.3–5.0 mg Mn/kg was 1.6 times the odds for animals controls (95% CI (1.2–2.2), $p\text{-value}=0.0009$); the odds of having Fe deposits in animals in exposed to 5.0–6.7 mg Mn/kg was 2.9 times the odds for controls (95% CI (2.3–3.7), $p\text{-value}<0.0001$); and the odds of

having Fe deposits in animals exposed to 8.3-10 mg Mn/kg was 8.0 times the odds for controls (95% CI (6.1-10.4), p -value<0.0001). These results indicate that within each structure, the proportion of LN3-positive cells containing ferric Fe progressively increased with Mn exposure relative to the fraction of cells without iron deposits (Figure 5A).

Analysis on the association between the intracellular Fe deposit and microglia reactivity state was performed using logistic regression. The statistical model treated the presence of Fe as the response variable and the state of microglia reactivity as the exploratory variable, controlling for the region. There was no statistical significant interaction between the region and microglia reactivity state (p -value=0.33). Figure 5B shows the observed proportion of Fe and NoFe LN3-positive cells across microglia reactivity states. The odds of having Fe deposits in activated cells was 1.8 times the odds for resting cells (95% CI (1.3-2.3), p -value<0.0001); the odds of having Fe deposits for dystrophic cells was 75 times the odds for resting cells (95% CI (10-564), p -value<0.0001). The odds of having intracellular Fe deposits in microglia in SNr was 3.6 times the odds for the SNc (95% CI (3.1-4.2), p -value<0.0001).

These data indicate that, in contrast to resting and activated cells, the proportion of Fe-containing cells with a dystrophic phenotype was predominant and showed a close association between the presence of intracellular ferric Fe and microglia dystrophy. We should note that the data reflect the presence or absence of intracellular ferric Fe deposits in microglia and does not take in account actual amount of intracellular Fe that could not be considered in this analysis. However, we also analyzed the average size of intracellular Fe deposits (in μm^2). In resting microglia, the size of Fe deposits did not exceed 3 μm^2 , while in microglia with an activated and in particularly with a dystrophic phenotype observed only in Mn-exposed animals, reached up to 40 μm^2 .

3.3. Colocalization of LN3 with ferritin and iNOS in the SNr

We next examined the level of L-ferritin staining in microglia using confocal microscopy and immunofluorescence double-labeling for L-ferritin and LN3. Based on the co-localization of these markers, the following cell types were observed in the SNr: (1) LN3-negative cells that labeled for L-ferritin and most likely represent oligodendrocytes, (2) LN3-positive cells negative for L-ferritin, and (3) LN3-positive cells that co-label with L-ferritin (Figure 6). In control animals, LN3-positive cells with fine processes did not show significant co-localization with L-ferritin (Figure 6 A-D). In Mn-treated animals, ferritin was expressed in microglia with activated and dystrophic phenotypes. In animals administered the 5.0-6.7 mg Mn/kg dose, ferritin was detectable in the perinuclear space and processes of microglia that appeared less ramified (Figure 6 E-H). In animals administered 8.3-10 mg Mn/kg, ferritin appeared to be increased and was particularly abundant within the hypertrophic soma and dystrophic compartments of clustered microglia (Figure 6 I-L and M-P).

We also examined whether activated microglia in Mn-treated animals expressed inducible nitric oxide synthase (iNOS) using double labeling for LN3 and iNOS. Microglial cells without signs of dystrophy had non-detectable or low level of iNOS (Figure 7 A-D). However, iNOS labeling was prominent in LN3-positive cells with dystrophic morphology and localized particularly within bulbous spheroids (Figure 7 E-H).

4. Discussion

In this communication, we report that chronic exposure to Mn induces a region-specific and dose-dependent pattern of microglia activation in the SN of non-human primates. Prominent microglia activation was observed in the SNr of animals that received the highest Mn dose

(8.3-10 mg Mn/kg). The microglia response was manifested by reactive morphological transformation, including signs of dystrophy, and by abnormally high intracellular accumulation of ferric iron and increased expression of the oxidative stress marker iNOS. Furthermore, the magnitude of ferric iron deposit in microglia correlated with their reactive state, being the highest within dystrophic cells. These findings suggest that an excessive accumulation of ferric iron in microglia is associated with signs of dystrophy in this non-human primate model of Mn neurotoxicity.

The SN is one of the brain regions characterized by a high tissue iron concentration (Beard et al., 1993; Hill and Switzer). Compared to the SNc, the SNr has been shown to have a higher iron content in normal subjects in MRI studies (Bartzokis et al., 1999), human postmortem studies (Jellinger et al., 1990; Sofic et al., 1991), and studies of hemiparkinsonian monkeys (Ren et al., 2003). The distribution of iron in the brain is associated with regions rich in transferrin receptors, such as the globus pallidus and SNr. It has been proposed that Mn may displace iron from its transferrin-binding sites and increase iron-mediated oxidative stress (Verity, 1999). In the normal non-human primate brain, the SNr has also been shown to be one of the brain nuclei displaying high levels of divalent metal transporter-1 (Huang et al., 2004) which transports Mn into cells (Gunshin et al., 1997). In addition, the SN has a relatively high density of microglia, comprising up to 12% of the cellular population (Lawson et al., 1990; Kim et al., 2000). Together, these findings are consistent with our observation of increased microglia reactivity in the SNr of Mn-exposed non-human primates. The pattern of Mn-induced microglia activation in the SN is not uniform and is more pronounced in the SNr, a region with higher tissue iron content, suggesting a role of microglia iron in the susceptibility of the SNr to Mn neurotoxicity.

In the aging brain and in neurodegenerative disorders, iron progressively accumulates in selective brain regions including the SN (Sipe et al. 2002; Zecca et al., 2004; Benarroch, 2009). For example, iron is increased in PD and in aging (Melega et al., 2007; Sofic et al., 1988; Riederer et al., 1989; Griffiths et al., 1999). Dysregulation in iron homeostasis in this region involves the iron regulatory protein ferritin (Riederer et al., 1989; Jellinger et al., 1990; Dexter et al., 1991; Gerlach et al., 1994; Mann et al., 1994; J.R. Connor et al., 1995; Thompson et al., 1999; Mehlhase et al., 2006; Lopes et al., 2008). Alterations in ferritin in activated microglia have been shown to increase the susceptibility of microglia to dystrophy and affect their ability to maintain iron storage in Alzheimer's disease (Mehlhase et al., 2005; Lopes et al., 2008). With aging, the microglial response is described as being more robust (Conde and Streit, 2006; Flanary et al., 2007; Sierra et al., 2007) and accompanied by increased production of reactive radicals (Kyrkanides et al., 2001), microglia functional deficits and dystrophic changes (Lopes et al., 2008).

Exposure to Mn also alters brain iron metabolism (Zheng et al., 1998; Verity, 1999; Zheng et al., 1999; Zheng and Zhao, 2001) that leads to increased oxidative stress (Desole et al., 1994; Dobson et al. 2003; Erikson et al. 2007). Increased free radicals may affect the iron-sequestering capacity of ferritin in Mn-induced reactive microglia and lead to the release of free iron, which in turn may cause damage to microglia themselves and promote microglia dystrophy and loss of their neuroprotective function. In microglia, ferritin is mostly represented by the L-subunit (which does not have ferroxidase activity) (Levi et al., 1992) and this may partially explain the inability of microglia to maintain iron-storage capacity during increasing oxidative stress conditions.

In summary, we provide evidence that chronic Mn exposure leads to progressive activation of microglia in the SN of non-human primates. Mn-induced reactive and dystrophic changes in microglia morphology were prominent in the SNr and were similar to those described in the aging brain and in neurodegenerative disorders (Streit et al., 2004; Lopes et al., 2008).

Dystrophic changes in microglia were associated with increased accumulation of ferric iron and iNOS expression. Our findings suggest that the mechanisms related to Mn neurotoxicity in the SNr may involve Mn-induced alterations in microglia iron metabolism leading to reactive transformation of microglia and iron-mediated oxidative stress that may have a damaging effect to microglia themselves. Ultimately, Mn-induced microglia activation and dystrophy may alter their neuroprotective capacity, resulting in neuronal dysfunction in this brain region. It is important to note that this study has some limitations. First, although it is the largest cohort of non-human primates that have been exposed to Mn and followed using behavioral, neuroimaging and detailed neuropathological studies, the total number of animals that could be used in the present study was 10 Mn-treated animals and 4 controls. Secondly, because of limitations in tissue availability, the analysis of Mn effects on microglia in the SN was performed at a specific level (at the level of the third cranial nerve). Despite these limitations, we believe that the results presented are important and represent a starting point from which a more comprehensive and detailed analysis could be performed in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIEHS grant number ES010975 to TRG.

References

- Agrawal R, Sharma PK, Rao GS. Release of iron from ferritin by metabolites of benzene and superoxide radical generating agents. *Toxicol* 2001;168:223–30.
- Agresti, A. *Categorical Data Analysis*. 2d. Hoboken, NJ: John Wiley and sons Inc; 2002.
- Aschner M, Erikson KM, Dorman DC. Manganese dosimetry: species differences and implications for neurotoxicity. *Crit Rev Toxicol* 2005;35:1–32. [PubMed: 15742901]
- Banta RG, Markesbery WR. Elevated manganese levels associated with dementia and extrapyramidal signs. *Neurology* 1977;27:213–6. [PubMed: 557755]
- Barbeau A. Manganese and extrapyramidal disorders (a critical review and tribute to Dr George C Cotzias). *Neurotoxicol* 1984;5:13–35.
- Bartzokis G, Cummings JL, Markham CH, Marmarelis PZ, Treciokas LJ, Tishler TA, Marder SR, Mintz J. MRI evaluation of brain iron in earlier- and later-onset Parkinson's disease and normal subjects. *Magn Reson Imag* 1999;17:213–22.
- Beard JL, Connor JD, Jones BC. Brain iron: location and function. *Prog Food Nutr Sci* 1993;17:183–221. [PubMed: 7901870]
- Benarroch EE. Brain iron homeostasis and neurodegenerative disease. *Neurol* 2009;72:1436–1440.
- Biamond P, Van Eijk HG, Swaak AJG, Koster JF. Iron mobilization from ferritin by superoxide derived from stimulated polymorphonuclear leukocytes: possible mechanism in inflammation diseases. *J Clin Invest* 1984;73:1576–9. [PubMed: 6327764]
- Braak H, Del Tredici K, Rub U, de Vos RAI, Jansen Steur ENH, Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 2003;24:197–211. [PubMed: 12498954]
- Brooks DJ, Frey KA, Marek KL, Oakes D, Paty D, Prentice R, Shults CW, Stoessl AJ. Imaging end points for monitoring neuroprotection in Parkinson's disease. *Ann Neurol* 2003;53 3:S110–8. discussion S118–9. [PubMed: 12666103]
- Burton NC, Guilarte TR. Manganese neurotoxicity: lessons learned from longitudinal studies in nonhuman primates. *Environ Health Perspect* 2009;117:325–32. [PubMed: 19337503]

- Campbell A, Becaria A, Lahiri DK, Sharman K, Bondy SC. Chronic exposure to aluminum in drinking water increases inflammatory parameters selectively in the brain. *J Neurosci Res* 2004;75:565–72. [PubMed: 14743440]
- Cheepsunthorn P, Palmer C, Connor JR. Cellular distribution of ferritin subunits in postnatal rat brain. *J Comp Neurol* 1998;400:73–86. [PubMed: 9762867]
- Colton CA, Gilbert DL. Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett* 1987;223:284–8. [PubMed: 2822487]
- Conde JR, Streit WJ. Microglia in the aging brain. *Neuropathol Exp Neurol* 2006;65:199–203.
- Connor JR, Menzies SL, St Martin SM, Mufson EJ. A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. *J Neurosci Res* 1992;31:75–83. [PubMed: 1613823]
- Connor JR, Boeshore KL, Benkovic SA, Menzies SL. Isoforms of ferritin have a specific cellular distribution in the brain. *J Neurosci Res* 1994;37:461–465. [PubMed: 8021970]
- Connor JR, Menzies SL. Cellular management of iron in the brain. *J Neurol Sci* 1995;134(Suppl):33–44. [PubMed: 8847543]
- Connor JR, Snyder BS, Arosio P, Loeffler DA, LeWitt P. A quantitative analysis of isoferritins in select regions of aged, parkinsonian, and Alzheimer's disease brains. *J Neurochem* 1995a;65:717–4. [PubMed: 7616228]
- Connor JR, Menzies SL. Relationship of iron to oligodendrocytes and myelination. *Glia* 1996;2:83–93. [PubMed: 8776576]
- Corradin SB, Muel J, Donini SD, Quattrocchi E, Ricciardi-Castagnoli P. Inducible nitric oxide synthase activity of cloned murine microglial cells. *Glia* 1993;7:255–62. [PubMed: 7681038]
- Desole MS, Miele M, Esposito G, Migheli R, Fresu L, De Natale G, Miele E. Dopaminergic system activity and cellular defense mechanisms in the striatum and striatal synaptosomes of the rat subchronically exposed to manganese. *Arch Toxicol* 1994;68:566–70. [PubMed: 7998823]
- Dexter DT, Carayon A, Javoy-Agid F, Wells FR, Daniel SE, Lees AJ, Jenner P, Marsden CD. Alterations in the levels of iron, ferritin and other trace metals in Parkinson's disease and other neurodegenerative diseases affecting the basal ganglia. *Brain* 1991;114:1953–75. [PubMed: 1832073]
- Dobson AW, Weber S, Dorman DC, Lash LK, Erikson KM, Aschner M. Oxidative stress is induced in the rat brain following repeated inhalation exposure to manganese sulfate. *Biol Trace Elem Res* 2003;93:113–26. [PubMed: 12835496]
- Dorman DC, Struve MF, Wong BA, Dye JA, Robertson ID. Correlation of brain magnetic resonance imaging changes with pallidal manganese concentrations in rhesus monkeys following subchronic manganese inhalation. *Toxicol Sci* 2006;92:219–27. [PubMed: 16638924]
- Erikson KM, Dorman DC, Lash LH, Aschner M. Manganese inhalation by rhesus monkeys is associated with brain regional changes in biomarkers of neurotoxicity. *Toxicol Sci* 2007;97:459–66. [PubMed: 17347134]
- Eriksson H, Magiste K, Plantin LO, Fonnum F, Hedstrom KG, Theodorsson-Norheim E, Kristensson K, Stalberg E, Heilbronn E. Effects of manganese oxide on monkeys as revealed by a combined neurochemical, histological and neurophysiological evaluation. *Arch Toxicol* 1987;61:46–52. [PubMed: 3439874]
- Flanary BE, Sammons NW, Nguyen C, Walker D, Streit WJ. Evidence that aging and amyloid promote microglial cell senescence. *Rejuvenation Res* 2007;10:61–74. [PubMed: 17378753]
- Gerlach M, Ben-Shachar D, Riederer P, Youdim MB. Altered brain metabolism of iron as a cause of neurodegenerative diseases? *J Neurochem* 1994;63:793–807. [PubMed: 7519659]
- Griffiths PD, Crossman AR. Distribution of iron in the basal ganglia and neocortex in postmortem tissue in Parkinson's disease and Alzheimer's disease. *Dementia* 1993;4:61–65. [PubMed: 8358514]
- Griffiths PD, Dobson BR, Jones GR, Clarke DT. Iron in the basal ganglia in Parkinson's disease. An in vitro study using extended X-ray absorption fine structure and cryo-electron microscopy. *Brain* 1999;122:667–73. [PubMed: 10219780]
- Guilarte TR. Manganese and Parkinson's disease: a critical review and new findings. *Env Hlth Perspect* 2010;118:1071–80.

- Guilarte TR, Chen MK, McGlothan JL, Verina T, Wong DF, Zhou Y, Alexander M, Rohde CA, Syversen T, Decamp E, Koser AJ, Fritz S, Gonczi H, Anderson DW, Schneider JS. Nigrostriatal dopamine system dysfunction and subtle motor deficits in manganese-exposed non-human primates. *Exp Neurol* 2006;202:381–90. [PubMed: 16925997]
- Guilarte TR, McGlothan JL, Degaonkar M, Chen MK, Barker PB, Syversen T, Schneider JS. Evidence for cortical dysfunction and widespread manganese accumulation in the nonhuman primate brain following chronic manganese exposure: a 1H-MRS and MRI study. *Toxicol Sci* 2006a;4:351–8.
- Guilarte TR, Burton NC, McGlothan JL, Verina T, Zhou Y, Alexander M, Pham L, Griswold M, Wong DF, Syversen T, Schneider JS. Impairment of nigrostriatal dopamine neurotransmission by manganese is mediated by pre-synaptic mechanism(s): implications to manganese-induced parkinsonism. *J Neurochem* 2008;107:1236–47. [PubMed: 18808452]
- Guilarte TR, Burton NC, Verina T, Prabhu VV, Becker KG, Syversen T, Schneider JS. Increased APLP1 expression and neurodegeneration in the frontal cortex of manganese-exposed non-human primates. *J Neurochem* 2008a;105:1948–59. [PubMed: 18284614]
- Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 1997;388:482–488. [PubMed: 9242408]
- Han J, Day JR, Connor JR, Beard JL. H- and L- ferritin subunit mRNA expression differs in brains of control and iron-deficient rats. *J Nutr* 2002;132:2769–74. [PubMed: 12221243]
- Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci* 2007;10:1387–94. [PubMed: 17965659]
- Hill J, Switzer R. The regional distribution and cellular localization of iron in the rat brain. *Neurosci* 1984;11:595–603.
- Huang CC, Weng YH, Lu CS, Chu NS, Yen TC. Dopamine transporter binding in chronic manganese intoxication. *J Neurol* 2003;250:1335–39. [PubMed: 14648150]
- Huang E, Ong WY, Connor JR. Distribution of divalent metal transporter-1 in the monkey basal ganglia. *Neurosci* 2004;128:487–96.
- Jellinger K, Paulus W, Grundke-Iqbal I, Riederer P, Youdim MB. Brain iron and ferritin in Parkinson's and Alzheimer's diseases. *J Neural Transm Park Dis Dement Sect* 1990;2:327–40. [PubMed: 2078310]
- Kim WG, Mohny RP, Wilson B, Jeohn GH, Liu B, Hong JS. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. *J Neurosci* 2000;20:6309–16. [PubMed: 10934283]
- Kim Y, Kim JM, Kim JW, Yoo CI, Lee CR, Lee JH, Kim HK, Yang SO, Chung HK, Lee DS, Jeon B. Dopamine transporter density is decreased in parkinsonian patients with a history of manganese exposure: what does it mean? *Mov Dis* 2002;17:568–575.
- Koller WC, Lyons KE, Truly W. Effect of levodopa treatment for parkinsonism in welders: A double-blind study. *Neurology* 2004;62:730–3. [PubMed: 15007122]
- Kyrkanides S, O'Banion MK, Whiteley PE, Daeschner JC, Olschowka JA. Enhanced glial activation and expression of specific CNS inflammation-related molecules in aged versus young rats following cortical stab injury. *J Neuroimmunol* 2001;9:269–77. [PubMed: 11585630]
- Lawson LJ, Perry VH, Dri P, Gordon S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neurosci* 1990;39:151–70.
- Lees AJ. L-dopa treatment and Parkinson's disease. *Q J Med* 1986;59:535–47. [PubMed: 3529159]
- Levi S, Yewdall SJ, Harrison PM, Santambrogio P, Cozzi A, Rovida E, Albertini A, Arosio P. Evidence of H- and L-chains have co-operative roles in the iron-uptake mechanism of human ferritin. *Biochem J* 1992;288:591–6. [PubMed: 1463463]
- Lopes KO, Sparks DL, Streit WJ. Microglial dystrophy in the aged and Alzheimer's disease brain is associated with ferritin immunoreactivity. *Glia* 2008;56:1048–60. [PubMed: 18442088]
- Mann VM, Cooper JM, Daniel SE, Srai K, Jenner P, Marsden CD, Schapira AH. Complex I, iron, and ferritin in Parkinson's disease substantia nigra. *Ann Neurol* 1994;36:876–81. [PubMed: 7998774]
- Martin CJ. Manganese neurotoxicity: connecting the dots along the continuum of dysfunction. *Neurotoxicol* 2006;27:347–9.

- Melega WP, Lacan G, Harvey DC, Way BM. Methamphetamine increases basal ganglia iron to levels observed in aging. *Neuroreport* 2007;18:1741–45. [PubMed: 17921879]
- Mehlhase J, Sandig G, Pantopoulos K, Grune T. Oxidation-induced ferritin turnover in microglial cells: role of proteasome. *Free Radic Biol Med* 2005;38:276–85. [PubMed: 15607911]
- Mehlhase J, Gieche J, Widmer R, Grune T. Ferritin levels in microglia depend upon activation: Modulation by reactive oxygen species. *Biochim Biophys Acta - Molecular Cell Res* 2006;1763:854–9.
- Miles JM, Chou SM. A new immunoperoxidase marker for microglia in paraffin section. *J Neuropath Exp Neurol* 1988;47:579–87. [PubMed: 2459314]
- Parmley RT, Spicer SS, Alvarez CJ. Ultrastructural localization of nonheme cellular iron with ferrocyanide. *J Histochem Cytochem* 1978;26:729–41. [PubMed: 712049]
- Perl DP, Olanow CW. The neuropathology of manganese-induced Parkinsonism. *J Neuropathol Exp Neurol* 2007;66:675–82. [PubMed: 17882011]
- R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing; Vienna, Austria: 2009.
- Ren MQ, Ong WY, Wang XS, Watt F. A nuclear microscopic and histochemical study of iron concentrations and distribution in the midbrain of two age groups of monkeys unilaterally injected with MPTP. *Exp Neurol* 2003;184:947–54. [PubMed: 14769387]
- Riederer P, Sofic E, Rausch WD, Schmidt B, Reynold GP, Jellinger K, Youdim MBH. Transition metals, ferritin, glutathione, and ascorbic acid in parkinsonian brains. *J Neurochem* 1989;52:515–20. [PubMed: 2911028]
- Riederer P, Dirr A, Goetz M, Sofic E, Jellinger K, Youdim MB. Distribution of iron in different brain regions and subcellular compartments in Parkinson's disease. *Ann Neurol* 1992;32(Suppl):S101–4. [PubMed: 1510366]
- Schneider JS, Decamp E, Koser AJ, Fritz S, Gonczi H, Syversen T, Guilarte TR. Effects of chronic manganese exposure on cognitive and motor functioning in non-human primates. *Brain Res* 2006;1118:222–31. [PubMed: 16978592]
- Schneider JS, Decamp E, Clark K, Bouquio C, Syversen T, Guilarte TR. Effects of chronic manganese exposure on working memory in non-human primates. *Brain Res* 2009;1258:86–95. [PubMed: 19133246]
- Selikhova M, Fedoryshyn L, Matviyenko Y, Komnatska I, Kyrilchuk M, Krolicki L, Friedman A, Taylor A, Jäger HR, Lees A, Sanotsky Y. Parkinsonism and dystonia caused by the illicit use of ephedrone—a longitudinal study. *Mov Disord* 2008;23:2224–31. [PubMed: 18785245]
- Sierra A, Gottfried-Blackmore AC, McEwen BS, Bulloch K. Microglia derived from aging mice exhibit an altered inflammatory profile. 2007;55:412–24.
- Sipe JC, Lee P, Beutler E. Brain Iron Metabolism and Neurodegenerative Disorders. *Dev Neurosci* 2002;24:188–196. [PubMed: 12401958]
- Sofic E, Riederer P, Heinsen H, Beckmann H, Reynolds GP, Hebenstreit G, Youdim MB. Increased iron (III) and total iron content in postmortem substantia nigra of parkinsonian brain. *J Neural Transm* 1988;74:199–205. [PubMed: 3210014]
- Sofic E, Paulus W, Jellinger K, Riederer P, Youdim MB. Selective increase of iron in the substantia nigra zona compacta of parkinsonian brains. *J Neurochem* 1991;56:978–82. [PubMed: 1704426]
- Streit, WJ.; Graeber, MB. *Progr Histochem Cytochem*. Vol. 31. Stuttgart: Gustav Fischer; 1996. Microglia: a pictorial.
- Streit WJ, Sparks DL. Activation of microglia in the brains of humans with heart disease and hypercholesterolemic rabbits. *J Mol Med* 1997;75:130–8. [PubMed: 9083930]
- Streit, WJ. *CNS injures: cellular responses and pharmacological strategies*. Boca raton: CRS Press; 1999. Role of macrophages and microglia in the injured CNS; p. 81-9.
- Streit WJ, Sammons NW, Kuhns AJ, Sparks DL. Dystrophic microglia in the aging human brain. *Glia* 2004;45:208–12. [PubMed: 14730714]
- Streit WJ. Microglial senescence: does the brain's immune system have an expiration date? *Trends in Neurosci* 2006;29:506–51.

- Streit WJ, Miller KR, Lopes KO, Njie E. Microglial degeneration in the aging brain--bad news for neurons? *Front Biosci* 2008;13:3423–38. [PubMed: 18508444]
- Thomson AM, Rogers JT, Leedman PJ. Iron-regulatory proteins, iron-responsive elements and ferritin mRNA translation. *Int J Biochem Cell Biol* 1999;31:1139–52. [PubMed: 10582343]
- Verity MA. Manganese neurotoxicity: a mechanistic hypothesis. *NeuroToxicology* 1999;20:489–97. [PubMed: 10385907]
- Yamada M, Ohno S, Okayasu I, Okeda R, Hatakeyama S, Watanabe H, Ushio K, Tsukagoshi H. Chronic manganese poisoning: a neuropathological study with determination of manganese distribution in the brain. *Acta Neuropathol* 1986;70:273–8. [PubMed: 3766127]
- Youdim MB, Grunblatt E, Mandel S. The pivotal role of iron in NF-kappa B activation and nigrostriatal dopaminergic neurodegeneration: prospects for neuroprotection in Parkinson's disease with iron chelators. *Ann N Y Acad Sci* 1999;890:7–25. [PubMed: 10668410]
- Zecca L, Youdim MB, Riederer P, Connor JR, Crichton RR. Iron, brain ageing and neurodegenerative disorders. *Nat Rev Neurosci* 2004;5:863–73. [PubMed: 15496864]
- Zhang X, Surguladze N, Slagle-Webb B, Cozzi A, Connor JR. Cellular iron status influences the functional relationship between microglia and oligodendrocytes. *Glia* 2006;54:795–804. [PubMed: 16958088]
- Zheng W, Ren S, Graziano JH. Manganese inhibits mitochondrial aconitase: a mechanism of manganese neurotoxicity. *Brain Res* 1998 Jul 20;799(2):334–42. [PubMed: 9675333]
- Zheng W, Zhao Q. Iron overload following manganese exposure in cultured neuronal, but not neuroglial cells. *Brain Res* 2001;897:175–9. [PubMed: 11282372]
- Zheng W, Zhao Q, Slavkovich V, Aschner M, Graziano JH. Alteration of iron homeostasis following chronic exposure to manganese in rats. *Brain Res* 1999;833:125–32. [PubMed: 10375687]

Figure 1. Manganese-induced changes in microglia morphology in the SNc

(A, E) In the SNc of control animals, LN3-positive cells were characterized by small perinuclear cytoplasmic volume and long, highly ramified processes. (B, F) In animals administered 3.3-5.0 mg Mn/kg BW, some LN3-positive cells had more intense labeling and increased cytoplasmic volume. (C, G) Example of fused rod-like cells (arrows) lacking lateral processes and displaying a distinctive bipolar structure in an animal administered 5.0-6.7 mg Mn/kg BW. (D, H) Example of activated microglia in an animal administered 8.3-10 mg Mn/kg BW: (D) intensely labeled hypertrophic cell with short thick processes (arrow); (H) cell with hypertrophic cytoplasm, bushy appearance, short processes, and an

“empty” spheroidal swelling (arrowhead) adjacent to cell soma. Scale bar 40 μm (A-D), 20 μm (E-F).

Figure 2. Manganese-induced changes in microglia morphology in the SNr

(A, E) Examples of resting microglia with fine ramified processes in the SNr of control animals. As it is shown in (A), LN3-labeled cells appear evenly distributed throughout the tissue. (B) Example of more intensely labeled ramified microglia in an animal exposed to 3.3-5.0 mg Mn/kg BW. (C, D, F, G) Examples of activated microglia in animals administered 5.0-6.7 mg Mn/kg BW: (C) cluster of microglia with intense cytoplasmic labeling and thicker processes; (F,G) areas with increased microglia density contain hypertrophic cells some of which have an “empty” spheroidal swelling adjacent to the nuclear compartment (F, arrow); other hypertrophic cells have processes engulfing a pyknotic body (G, arrow). (H-L) Examples of reactive microglia in animals administered 8.3-10 mg Mn/kg BW: (H) a hypertrophic cell with thick “fluffy” processes; (I-L) a range of microglia dystrophic changes including aggregation (I), atrophy (J), cytoplasmic swelling (arrow in K), beading (arrowhead in K), and disintegration of distal processes (L, arrow). Scale bar 40 μ m (A-D), 20 μ m (E-F).

Figure 3. Effect of manganese exposure on microglia number in the SNc and SNr
 (A) Microglia cell count (cells/mm²) increased as a quadratic function of Mn tissue content (μg/g tissue) (p-value < 0.0001). Although the number of LN3-labeled cells in the SNr appeared greater than in SNc, the differences between the structures were not significant (p-value=0.41). (B) The distribution of microglia phenotype (or reactivity state) correlated with the level of Mn exposure. The figure shows the observed proportion of resting cells (R) and activated and dystrophic cells combined (AD) across Mn exposure. The interaction was significant (p-value < 0.0001), and the treatment effect was interpreted separately for each structure. In both the SNc and SNr, the proportion of AD cells increased with Mn exposure. However, in the SNr, the fraction of AD cells was greater than in the SNc and became predominant in animals exposed to the highest level of Mn. C – controls; LM – 3.3-5.0 mg Mn/kg BW weekly doses; MM - 5.0-6.7 mg Mn/kg BW; HM - 8.3-10 mg Mn/kg BW.

Figure 4. Effect of Mn exposure on accumulation of ferric iron in microglia in the SNr
 (A, C) low (A) and high (C) magnification images show that in normal control brain, small punctate deposits of ferric iron as visualized by Perl's stain are infrequently seen within ramified microglia (arrow in panel C). (B) Low magnification image from an animal administered 8.3-10 mg Mn/kg, shows prominent iron deposits within activated microglia. (D-H): High magnification images of dual LN3 and ferric iron labeling in Mn-exposed animals. In an animal administered 3.3-5.0 mg Mn/kg, LN3-positive cell expressed small amounts of intracellular ferric iron (D, arrows). (E) In an animal administered 5.0-6.7 mg Mn/kg BW, the size and number of intracellular ferric iron deposits appear to be increased (arrows). (F-H): Intracellular iron deposits were particularly large and abundant in an animal

administered 8.3-10 mg Mn/kg BW. Note an intensely stained ferric iron deposit forming a core inside a cell with hypertrophic cytoplasm (F, arrows). Ferric iron deposits were also localized within spheroidal swellings at the distal end of cytoplasmic processes (G, large arrow) or occupied the entire perinuclear cytoplasm of some reactive microglial cells (H, arrows). Scale bars (A-B) 100 μm , (C-H) 20 μm .

Figure 5. Effect of manganese exposure on microglia intracellular iron deposits in the SNc and SNr

(A) Analysis of the intracellular ferric iron distribution in microglia across Mn exposure shows an association between the presence of intracellular iron within LN3 cells and the level of Mn exposure. The proportion of cells with iron (Fe) and without iron (NoFe) was compared across Mn levels for the SNc and SNr. The interaction between Mn exposure and brain region was significant (p -value=0.0003). There is a significant increase in the proportion Fe to NoFe across Mn levels within both the SNc and SNr when compared to control. In the SNr of animals exposed to 8.3-10 mg Mn/kg, the proportion of cells containing Fe was predominant. (B) Overall for the SNc and SNr, the combined data show that the presence of intracellular iron was highly correlated with microglia reactivity state. That is, among resting (R) and activated (A) cells, the proportion of Fe-containing cells was minor; however, the vast majority of dystrophic (D) cells had intracellular Fe deposits. C – controls; LM – 3.3-5.0 mg Mn/kg BW weekly doses; MM – 5.0-6.7 mg Mn/kg BW; HM – 8.3-10 mg Mn/kg BW.

Figure 6. Co-labeling of LN3 and L-ferritin in the SNr

(A-D): In control animals, there is no appreciable L-ferritin co-labeling in LN3-positive cells with ramified morphology. A ferritin-positive LN3-negative cell (arrowhead), most likely, is an oligodendrocyte. (E-H): Example of LN3 and L-ferritin dual labeling in an animal administered 5.0-6.7 mg Mn/kg. Note that L-ferritin is expressed in activated microglia. (I-P) Examples of LN3 and L-ferritin co-labeling in an animal administered 8.3-10 mg Mn/kg. Note that L-ferritin expression is increased in reactive microglia aggregated in clusters. L-ferritin labeling was particularly intense within spheroidal swellings (arrows in J-L). DAPI= blue; LN3= red; ferritin= green. Panels D, H, L, P are composite images. Scale bar 10 μ m.

Figure 7. Co-labeling of LN3 and iNOS in the SNr of a manganese-exposed animal

Panels A-D show an LN3-positive cell with morphological features of a resting microglia that does not seem to express a significant level of iNOS (arrow) in an animal exposed to 8.3-10 mg Mn/kg. Panels E-H demonstrate increased iNOS expression selectively in the bulbous swellings of LN3-positive cells with dystrophic phenotype (arrowhead) in the SNr of the same animal. DAPI= blue; iNOS= red; LN3= green. Panels D, H are composite. Scale bar 10 μm.

Table 1

Dosing regimen of manganese exposed animals

Animals exposed to 3.3-5.0 mg Mn/kg BW weekly doses received a single full-dose injection per week. Animals exposed to 5.0-6.7 and 8.3-10.0 mg Mn/kg BW weekly doses received two half-dose injections per week (*). Animals marked with term "bolus" received a single full-dose injection on the first week of dosing, but then were administered two half-dose injections on the subsequent weeks. Animals ID 6697 and 7426 had shorter duration of exposure and received lower cumulative Mn doses because they developed severe movement abnormalities. Additional animals that are not shown in the table were used as controls (ID 6770, 123-193, 001-1167, and 6499).

Animal ID	Dose Level (MnSO ₄ , mg/kg)	Dose Level (Mn, mg/kg)	Tissue Mn (µg/g tissue)	Dosing Interval	Exposure Duration (weeks)	Cumulative MnSO ₄ (mg/kg)	Cumulative Mn (mg/kg)
144T	10-15	3.5-5.0	0.63	1/week	50	515	171.7
107-705	10-15	3.5-5.0	0.56	1/week	42	500	166.7
3154	10-15	3.5-5.0	0.55	1/week	45	515	173.8
9093	15-20	5.0-6.7	0.91	2/week*	59	770	250.8
7469	15-20	5.0-6.7	0.36	2/week*	32	525	170.7
000-8001	15-20	5.0-6.7	1.15	2/week*	34	535	173.9
001-1099	15-20	5.0-6.7	1.08	2/week*	32	535	173.9
7839	25-30	8.3-10.0	1.06	2/week*	38	640	218.3
6697	25-30	8.3-10.0	2.74	Bolus, 2/week*	7	206	68.3
7426	25-30	8.3-10.0	1.61	Bolus, 2/week*	15	340	113.3