Manganese is an essential trace element required for normal development and bodily functions. However, exposure of the brain to excessive amounts of manganese results in neurotoxicity. Although previous studies examining manganese neurotoxicity have focused on neuronal injury, especially direct injury to dopaminergic neurons, the effects of manganese-induced neurotoxicity on glial cells have not been reported. The current study was designed to examine the effect of manganese on microglial activation, and the underlying mechanism of manganese-induced dopaminergic neuronal injury in vivo. We established an animal model of manganism by intrastriatal injection of MnCl₂·4H₂O into male Sprague-Dawley rats. One day after administration of manganese, a few microglial cells in the substantia nigra (SN) were activated, although the number of tyrosine hydroxylase (TH)-immunoreactive neurons in the SN was unaffected. Seven days after administration of manganese, a marked reduction in the number of TH-immunoreactive neurons was observed in the SN, and the majority of microglial cells were activated. We found that manganese upregulated inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF-α) gene expression, as well as iNOS, TNF-α, and interleukin-1β (IL-1β) protein levels in the SN. Furthermore, treatment with minocycline, an inhibitor of microglial activation, attenuated microglial activation and mitigated IL-1β, TNF-α, and iNOS production as well as dopaminergic neurotoxicity induced by manganese. These results suggested that dopaminergic neurons could be damaged by manganese neurotoxicity, and that the activated microglial cells and their associated activation products played an important role in this neurodegenerative process.

Key Words: manganese; microglia; dopaminergic neuron; inducible nitric oxide synthase; tumor necrosis factor α; interleukin-1β.

Manganese is an essential trace element that is widely distributed and is required for normal lipid, protein, and carbohydrate metabolism, and serves as a cofactor for various enzymes such as arginase and glutamine synthetase (Carl et al., 1993; Keen et al., 2000). However, the brain is susceptible to an excess of manganese, and accumulation of manganese can lead to a neurodegenerative disorder known as manganism (Hudnell, 1999; Iregren, 1999). Chronic occupational exposure to high levels of inhalable manganese (>1–5 mg Mn/m³) is the most frequently observed cause of manganese-induced neurotoxicity. Health risks of exposure to manganese have also been associated with the use of manganese in the production of steel, aluminum cans, fungicides, fertilizers, and electronics and as a contrast agent in medical diagnostics.

Chronic manganism can cause some extracortical spin tract symptoms, which are similar to Parkinson’s disease (PD). The exact mechanisms of manganese neurotoxicity are still being elucidated. Previous studies on manganese neurotoxicity have focused on oxidative stress (HaMai et al., 2001; Kitazawa et al., 2002) and its interaction with other essential trace elements (Aschner et al., 1999; Lai et al., 1999; Zheng, 2001). However, several recent reports have suggested that inflammation contributes to the progressive nature of neurodegeneration (Kim and Joh, 2006; McGeer and McGeer, 2008).

Inflammation is an underlying component of a diverse range of neurodegenerative diseases and their associated neuropathologies, and increasing evidence suggests that microglia are a key factor mediating this process. Microglia, the standby cells for immune defense in the central nervous system, monitor the brain environment (Nimmerjahn et al., 2005). One characteristic feature of microglial cells is their rapid activation in response to even minor pathological changes in the central nervous system. In the activated state, microglia can serve diverse beneficial functions essential to neuron survival (Streit, 2002). However, under other circumstances, overactivated microglia can induce significant and highly detrimental neurotoxic effects by the excess production of a large number of factors such as inducible nitric oxide synthase (iNOS), tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β) (Lee et al., 1993; Liu et al., 2002).

Recently, it has been suggested that manganese neurotoxicity involves activation of microglia. Together with exposure to microglial activators, such as bacterial lipopolysaccharide or interferon gamma, manganese could induce sustained production of neurotoxic nitric oxide or proinflammatory factors

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via activation of microglial cells (Chang and Liu, 1999; Chen et al., 2006). Consequently, neurons adjacent to the activated microglia could be injured. However, little is known about the effects of manganese on microglial activation in vivo. The present study was designed to investigate the role of manganese in microglial activation and their associated activation products, and the relationship between manganese-induced dopaminergic neuronal injury and microglial activation in vivo. These findings may provide further insight into the potential mechanisms of manganese neurotoxicity.

MATERIALS AND METHODS

Animals, treatment, and dissection. Male Sprague-Dawley (SD) rats weighing 210–240 g were used in all experiments. The animals were housed in stainless-steel cages in a temperature-controlled, 12/12 light/dark room, and given free access to food and drinking water. Rats were anaesthetized with sodium pentobarbital (40 mg/kg ip) and placed in a stereotaxic frame with the nose bar set at −2.4 mm. The subsequent stereotaxic injections of 1 μl of either MnCl₂·4H₂O or 0.9% NaCl were performed within the striatum at the following stereotaxic co-ordinates: 1 mm anterior-posterior, 3 mm lateral, 4 mm ventral to the dura. The volumes were infused for 5 min and the needle remained in situ for 5 min before being slowly withdrawn. For manganese intoxication, rats received intrastral injections of MnCl₂·4H₂O (1 μmol/μl). For minocycline treatment, rats were administered minocycline (120 mg/kg per day in 5% sucrose; Sigma-Aldrich, St Louis, MO) by oral gavage before, during and after manganese administration. We treated SD rats with minocycline daily for 4 or 10 days. On day 3, rats were injected with manganese. Control rats received intrastral injections of saline only. Rats (n = 8–10 per group; saline, Mn, and Mn-minocycline) were killed on days 1 and 7 after the injection, and their brains were used for morphological and biochemical analyses. All procedures involving animal studies were in accordance with the guidelines of and therefore approved by the local Animal Care and Use Committee.

Immunohistochemistry. Rats were perfused through the heart under deep anesthesia with 150–200 ml of 4% paraformaldehyde in phosphate buffer, pH 7.4. The brains were removed rapidly from decapitated rats and cooled immediately in ice-cold 0.9% NaCl. The mesencephalon was divided into two parts with a cut from the ventral side perpendicular to the long axis of mesencephalon exactly at the caudal border eminence. The SN then easily identified and dissected. The samples were frozen in liquid nitrogen until analysis. The primer sequences used in this study were as follows: for rat iNOS, 5′-ACAACGTGGAGAAAAAACCAGTTG-3′ (forward) and 5′-ACAGCTC-CGGGCATCGAAGACC-3′ (reverse); for rat TNF-α, 5′-CCCTACACTCA-GATCATCATTCTCAA-3′ (forward) and 5′-TCTAAAGTCTGGGCAGTT-GACCTC-3′ (reverse); and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-ACACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCAC-CACCCTGTGGCCTGA-3′ (reverse). PCR amplification was performed for 26 cycles for iNOS, TNF-α, and GAPDH. After amplification, products were resolved by 1.5% agarose gel electrophoresis and stained with ethidium bromide, and then the radioactivity was quantified using a computerized analysis system (Bio-Rad PhosphorImager system, Hercules, CA).

Enzyme-linked immunosorbent assay. The SN samples were collected 1 and 7 days after the manganese or saline injection. The SN samples were obtained as described previously. TNF-α and IL-1β levels in the SN were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (eBioscience, San Diego, CA). Data are expressed as pg/g.

Statistical analysis. All values are expressed as the mean ± SD. Differences between means were analyzed using a two-tail Student’s t-test. In all analyses, the null hypothesis was rejected at the 0.05 level.

RESULTS

Effect of Manganese on the Nigral Microglia

To determine whether manganese can induce microglial activation, we examined the expression of OX42, a specific marker for microglia, by immunostaining. Resting microglial cells are highly branched with small cell bodies. In the activation state, the length of the branches is reduced, and the cell body is enlarged. One day after administration of manganese, the majority of microglial cells were in the resting state, with only a few activated cells visible (Fig. 1B). However, 7 days after manganese administration, there was a dramatic change in the morphology of the OX42-immunoreactive cells. The number of activated microglial cells was significantly increased in the SN (Fig. 1D). In control animals, activated microglia were not observed on days 1 and 7 following saline administration (Figs. 1A and 1C).

Effect of Manganese on Nigral Dopaminergic Neurons

We next measured the effect of manganese neurotoxicity on dopaminergic neurons by immunostaining with an antibody against TH. One day after manganese administration, no detectable difference in TH-immunoreactivity was observed in the SN between the saline-treated controls and the manganese-treated rats (Figs. 2A–C). However, 7 days after manganese treatment, the TH-immunoreactivity was significantly decreased in the cell
bodies of neurons in the SN of manganese-treated rats compared with the controls (Figs. 2D–F). Manganese caused more than a 70% reduction in the number of SN dopaminergic neurons.

**Effect of Manganese on Nigral iNOS, TNF-α, and IL-β Expression**

Gene expression was measured by RT-PCR to determine whether manganese could upregulate TNFα and iNOS mRNA in the SN. As shown in Figure 3, there was no significant induction of iNOS mRNA in manganese-treated rats one day after manganese administration. However, TNF-α mRNA levels were increased in manganese-treated rats compared with control rats at the same time point. Seven days after manganese administration, iNOS and TNF-α mRNA levels in the SN were higher in the manganese-treated rats than in the controls.

In control rats, iNOS-immunoreactivity was not detected in the SN by immunostaining on days 1 and 7 after saline treatment (Figs. 4A and 4C). One day after manganese administration, a similar pattern of iNOS staining was observed in treated and control rats (Fig. 4B). However, administration of manganese resulted in a large increase in iNOS-immunoreactivity by 7 days post-treatment (Fig. 4D). We also found increased protein expression of TNF-α and IL-β in manganese-treated rats compared with control rats at both day 1 (Figs. 5A and 5C) and day 7 (Figs. 5B and 5D) postmanganese treatment.

**Minocycline Blocks the Activation of Microglia Induced by Manganese**

We next examined whether minocycline, an inhibitor of microglial activation, had a neuroprotective role against manganese-induced neurotoxicity in dopaminergic neurons. Rats were administered orally with minocycline daily for 4 and 10 days. On day 3, rats were injected with manganese and their brains were collected and analyzed on days 1 and 7 after administration of manganese. As shown in Figure 6B, a few OX42-immunoreactive active microglia were observed in the SN one day after manganese treatment. Seven days after treatment, an increased number of OX42-positive activated microglia were observed (Fig. 6E). The OX42-immunostaining observed in rats receiving both manganese and minocycline...
after days 1 and 7 (Figs. 6C and 6F) were similar to those observed on day 1 after administration of manganese alone. Rats receiving both manganese and minocycline exhibited minimal microglial activation in the SN. No activated microglia were observed in control rats (Figs. 6A and 6D) on days 1 and 7 after saline injection.

Minocycline Blocks Manganese-Induced Expression of iNOS, TNF-α, and IL-β

We examined whether several deleterious factors known to be associated with activated microglia were involved in the protective effect of minocycline against manganese-induced neurotoxicity. No marked difference in iNOS-immunoreactivity was observed between all treated groups one day after treatment (Figs. 7A–C). However, the marked induction of iNOS-immunoreactivity observed 7 days postmanganese treatment (Fig. 7E) was blocked by minocycline (Fig. 7F). As described earlier, manganese caused an increase in the protein expression of TNF-α and IL-β. Minocycline attenuated this manganese-induced increase in TNF-α protein levels at day 7 (Fig. 8D) and IL-β protein levels at both day 1 (Fig. 8A) and 7 (Fig. 8B).

Minocycline Attenuates Manganese-Induced Dopaminergic Neurodegeneration

To investigate the neuroprotective effects of minocycline on manganese-induced dopaminergic neuronal loss in vivo, we quantified TH-positive neurons in the SN by immunohistochemistry. There was no difference in the number of TH-positive neurons in the SN of all treated groups one day after treatment (Fig. 9D). However, 7 days postmanganese injection, manganese treatment reduced the number of TH-positive neurons by approximately 70% compared with saline-treated controls (Fig. 9F), whereas minocycline significantly attenuated the manganese-mediated loss of TH-immunoreactive neurons by approximately 60% (Fig. 9G).

FIG. 3. Manganese modulates the gene expression of iNOS and TNF-α. Total RNA was extracted from the rat SN on days 1 and 7 after saline or manganese treatment, and subjected to RT-PCR analysis for the detection of iNOS, TNF-α, and GAPDH mRNA levels. GAPDH was used as the internal control.

FIG. 4. Manganese enhances iNOS protein levels. Rats were killed on days 1 (A) and 7 (C) after saline injection, and on days 1 (B) and 7 (D) after manganese injection. iNOS protein expression was determined by immunohistochemical staining. Scale bar indicates 200 μm.

DISCUSSION

Although manganese has been found to be involved in many biological systems, the primary basis for its neurotoxicity remains unknown. Results from this study suggest that manganese can lead to selective dopaminergic neuron loss, and that activated microglial cells and their activation products might play an important role in this process.

Previous studies have implicated that microglial activation was involved in the pathogenesis of neurodegenerative diseases (Budka, 1991; McGeer et al., 1987). In chronic neurodegenerative diseases, microglial activation is an early event that often precedes neuronal death. Numerous reactive microglia cells have been observed in the SN of patients with PD or with other Parkinsonian syndromes (McGeer et al., 1988). Similar observations have been reported in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)–induced model of PD (Członkowska et al., 1996).

In the present study, we used an animal model of manganese made by the rat with a single unilateral injection of MnCl₂ into the caudate-putamen complex to examine the possible relationship between microglial activation and the loss of dopaminergic neurons induced by manganese in vivo. The neurons of the striato-nigral system in the caudate putamen send projecting fibers to the SN, and the axons of the neurons located in the SN pars compacta run along the medial forebrain bundle and terminate in the dorsal striatum. Atsushi and Sloot (Sloot and Gramsbergen, 1994; Takeda et al., 1998) showed that manganese is subjected to axonal transport in the GABAergic and/
or dopaminergic nigrostriatal pathways. In order to make more selective destructions of the nigrostriatal dopaminergic pathway, the caudate putamen has been targeted as the site of toxin injection in many recent studies.

Although we found that one day after manganese treatment most microglia were present in the resting state, by day 7 the majority of microglial were in the active phase. Furthermore, the loss of dopaminergic neurons on day 7 was greater than that observed one day after exposure to manganese. These results indicated that microglial cells become active during the process of neurodegeneration. We propose that manganese can lead to microglial activation, and that activation of microglial cells

FIG. 5. Manganese potentiates TNF-α and IL-1β content. Rats were killed and their SN was collected on days 1 (A, C) and 7 (B, D) after saline or manganese administration. IL-1β (A, B) and TNF-α (C, D) protein expression levels were determined by ELISA. Data are expressed as the mean ± SD, where n = 3 independent experiments. *p < 0.05 compared with control groups.

FIG. 6. Minocycline blocks the manganese-induced activation of microglia. Rats were administered orally with minocycline (120 mg/kg) daily during the experiment. On day 3, rats were injected with manganese. The samples were collected on days 1 and 7 after the manganese injection. Samples were stained with an antibody against OX42 to evaluate the level of microglial activation in the treated groups. (A, D) Microglia in the SN after saline treatment for 1 and 7 days, respectively; (B, E) microglia in the SN after manganese treatment for 1 and 7 days, respectively; (C, F) microglia in the SN after pretreatment with minocycline for 3 days followed by manganese treatment then one (C) or 7 days (F) post-treatment with minocycline. Solid arrows indicate resting microglia, and hollow arrows show active microglia. Scale bar indicates 200 μm.
might be involved in the manganese-induced selective loss of dopaminergic neurons.

Previously, inflammation has been viewed as a passive response to neuronal damage. In response to certain cues such as brain injury, microglia become activated (Nimmerjahn et al., 2005). However, increasing reports demonstrate that inflammation actively contributes to neuronal death and damage. An uncontrolled or chronic inflammatory response may cause irreversible tissue damage, which then fuels a self-propelling cycle of neuronal death. In the brain, inflammatory mediators

![Image of Figure 7](image_url)

**FIG. 7.** Minocycline attenuates manganese-induced expression of iNOS. Rats were administered orally with minocycline (120 mg/kg) daily during the experiment. On day 3, rats were injected with manganese. The samples were collected on days 1 and 7 after the manganese injection. Samples were stained with an antibody against iNOS to evaluate the level of iNOS protein expression in the treated groups. (A, D) iNOS-immunoreactivity in the SN after saline treatment for 1 and 7 days, respectively; (B, E) iNOS-immunoreactivity in the SN after manganese treatment for 1 and 7 days, respectively; (C, F) iNOS-immunoreactivity in the SN after pretreatment with minocycline for 3 days followed by manganese treatment then 1 (C) or 7 days (F) post-treatment with minocycline. Scale bar indicates 200 μm.

![Image of Figure 8](image_url)

**FIG. 8.** Minocycline attenuates manganese-induced expression of deleterious factors. Rats were administered orally with minocycline (120 mg/kg) daily during the experiment. On day 3, rats were injected with manganese. The samples were collected on days 1 and 7 after the manganese injection. (A, B) Changes in IL-1β expression on days 1 (A) and 7 (B). (C, D) Changes in TNF-α expression on days 1 (C) and 7 (D). Data are expressed as the mean ± SD, where n = 3 independent experiments. *p < 0.05 compared with control groups. #p < 0.05 compared with manganese-treated groups.
released mainly by microglia are implicated in the pathogenesis of neurological diseases (Minghetti and Levi, 1998; Streit, 2000).

Our results indicate that manganese significantly increased production of the microglial activation products, iNOS, IL-1β, and TNF-α, on days 1 and 7 after administration of manganese. In addition, we found that the increased production of IL-1β and TNF-α in the SN occurred before the increase in iNOS expression. It has been reported that IL-1 stimulates the release of neurotoxins such as nitric oxide from glia (Boje and Arora, 1992). An increase in nitric oxide, via increased expression of iNOS, has also been associated with PD-like pathology (Tieu et al., 2003). TNF-α and IL-1β are also clearly involved in neurodegeneration, because high levels of TNF-α and IL-1β have been detected in the SN (Imamura et al., 2003) and striatum (Mogi et al., 1994) of PD patients. TNF-α initiates a sequence of events associated with neuronal apoptosis and neurological damage and, like IL-1β, increases activity of the JUN amino terminal kinase/stress-activated protein kinase, caspasas and nitric oxide (Liu et al., 1996; Wright et al., 1999; Zhang et al., 1996). Moreover, TNF-α directly induces glutamate release from activated microglia leading to excitotoxic neuronal death (Takeuchi et al., 2006).

In particular, dopaminergic neurons are susceptible to the deleterious effects of microglial activation under special conditions. The most characteristic feature of microglial cells is their rapid activation in response to even minor pathological changes in the central nervous system. In the activated state, microglia can serve diverse beneficial functions essential to neuron survival. However, under other circumstances, microglia become overactivated and can induce significant and highly detrimental neurotoxic effects by the excess production of a large array of factors such as iNOS, TNF-α, and IL-1β. Dopaminergic neurons possess reduced antioxidant capacity, as evidenced by low intracellular glutathione, which renders dopaminergic neurons more vulnerable to oxidative stress and microglial activation relative to other cell types (Loeffler et al., 1994). In addition, the SN contains 4.5 times as many microglia compared with other regions of the brain, suggesting that the localization of microglia in the SN predisposes them to vulnerability to immunological insults (Kim et al., 2000).

We next applied minocycline, an inhibitor of microglial activation, to examine the neurotoxic effects of microglial activation on manganese-induced dopaminergic neuronal loss in vivo. Minocycline, a semisynthetic second-generation tetracycline, is an antibiotic that possesses superior penetration through the brain-blood barrier. It has been reported to be neuroprotective against dopaminergic neurotoxicity in the MPTP mouse model of PD (Wu et al., 2002). The neuro-protection afforded by minocycline is thought to be associated with its ability to inhibit microglial activation, thereby reducing the levels of cytotoxic factors released by microglia. In this study, minocycline prevented the manganese-induced activation of microglial cells and the associated increased release of iNOS, IL-1β, and TNF-α, as well as mitigated nigral dopaminergic neurotoxicity. In addition to dramatic morphologic alternations, another feature of microglial cells upon activation is the upregulated activation product. So although there were not obvious morphologic changes of microglial cells at 1 day after manganese-minocycline treatment, the decreased

**FIG. 9.** Minocycline prevents the manganese-induced loss of dopaminergic neurons. Rats were administered orally with minocycline (120 mg/kg) daily during the experiment. On day 3, rats were injected with manganese. The samples were collected on days 1 and 7 after the manganese injection. Samples were stained with an antibody against TH to determine the number of dopaminergic neurons in the SN. (A, E) TH-immunoreactivity in the SN after saline treatment for 1 and 7 days, respectively; (B, F) TH-immunoreactivity in the SN after manganese treatment for 1 and 7 days, respectively; (C, G) TH-immunoreactivity in the SN after pretreatment with minocycline for 3 days followed by manganese treatment then 1 (C) or 7 days (G) post-treatment with minocycline. (D, H) Comparison of the TH-immunoreactivity data after days 1 (D) and 7 (H). *p < 0.05 compared with control groups. #p < 0.05 compared with manganese-treated groups. Scale bar indicates 500 µm.
levels of cytotoxic factors at this time point were due to the use of minocycline as a specific inhibitor of microglial activation. These results suggest that minocycline induced neuroprotection against manganese-induced neurotoxicity by blocking the activation of microglia.

In conclusion, we show that manganese-induced direct activation of microglia and increased release of microglial activation products, which preceded neuronal cell death. These findings support the hypothesis that microglial activation may play a role in the selective vulnerability of dopaminergic neurons. Mediation of microglial activation may provide a therapeutic strategy for slowing the neurodegenerative process. It should be noted that this study examined only the relationship between microglial activation and loss of dopaminergic neurons: further studies are required to elucidate the underlying molecular mechanisms associated with manganese neurotoxicity.

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