Synergistic dopaminergic neurotoxicity of manganese and lipopolysaccharide: differential involvement of microglia and astroglia

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Abstract

Overexposure to manganese is known to cause damage to basal ganglial neurons and the development of movement abnormalities. Activation of microglia and astrocytes has increasingly been associated with the pathogenesis of a variety of neurological disorders. We have recently shown that microglial activation facilitates manganese chloride ($MnCl_2$, 10–300 μM)-induced preferential degeneration of dopamine neurons. In this study, we report that combinations of $MnCl_2$ (1–30 μM) and endotoxin lipopolysaccharide (LPS, 0.5–2 ng/ml), at minimally effective concentrations when used alone, induced synergistic and preferential damage to dopamine neurons in rat primary neuron-glia cultures. Mechanistically, $MnCl_2$ significantly potentiated LPS-induced release of tumor necrosis factor-alpha and interleukin-1 beta in microglia but not in astroglia. $MnCl_2$ and LPS were more effective in inducing the formation of reactive oxygen species and nitric oxide in microglia than in astroglia. Furthermore, $MnCl_2$ and LPS-induced free radical generation, cytokine release and dopamine neurotoxicity was significantly attenuated by pretreatment with potential anti-inflammatory agents minocycline and naloxone. These results demonstrate that the combination of manganese overexposure and neuroinflammation is preferentially deleterious to dopamine neurons. Moreover, these findings not only shed light on the understanding of manganese neurotoxicity but may also bear relevance to the potentially multifactorial etiology of Parkinson's disease.

Keywords

neuroinflammation; neurotoxicity; dopamine; microglia; astroglia; free radical

Introduction

Excessive exposure to manganese (Mn), an essential trace metal important in mediating a variety of biological processes, in miners and smelters can result in significant neuronal damage to the basal ganglia and the development of movement disorders called manganism (Aschner *et al.* 2007, Cersosimo & Koller 2006). Clinically, classic cases of manganism overlap with that of idiopathic Parkinson's disease (IPD) in the presentation of rigidity and bradykinesia but differ in the intensity of tremor and response to levodopa therapy (Calne *et al.* 1994, Cersosimo & Koller 2006, Pal *et al.* 1999). Pathologically, neuronal damage in manganism is most prominent in globus pallidus and less severe in striatum and other structures of the basal ganglia (Aschner et al. 2007, Perl & Olanow 2007).

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A number of studies have associated occupational overexposure to Mn with increased incidence and/or decreased onset age of developing Parkinsonism (Gorell *et al.* 1997, Gorell *et al.* 1999, Mergler *et al.* 1994, Racette *et al.* 2001, Racette *et al.* 2005) although increased PD incidence has not been consistently associated with welding (Fored *et al.* 2006, Marsh & Gula 2006). Furthermore, several studies strongly suggest that environmental exposure to elevated levels of Mn through airborne, dietary and other routes contributes to IPD development (Finkelstein & Jerrett 2007, Lucchini *et al.* 2007, Powers *et al.* 2003, Tan *et al.* 2003).

IPD represents the vast majority of PD cases and cardinal symptoms (i.e., rigidity, bradykinesia, rest tremor and posture instability) do not start to appear before the 5–6th decade of life and a massive degeneration of the nigrostriatal dopaminergic (DA) pathway. Development of IPD most likely is a result of complex interactions among multiple factors that include the innate vulnerability of the nigrostriatal DA pathway to oxidative damage, exposure to environmental toxicants and potential genetic predisposition superimposed over decades of the aging process (Di Monte 2003, Klein & Schlossmacher 2007, Logroscino 2005, Manning-Bog & Langston 2007, Sulzer 2007). Neuroinflammation has increasingly been recognized to be a key contributor to DA neurodegeneration based on observations that include the presence of inflammatory markers in the SN of postmortem PD brains, reduced PD incidence in users of certain nonsteroidal anti-inflammatory drugs, neuroprotection achieved with anti-inflammatory agents in animal PD models and recapitulation of pathological PD features in animals with immuno-stimulators such as endotoxin lipopolysaccharide (LPS) (Chen *et al.* 2005, Liu 2006, McGeer & McGeer 2008, Przedborski 2007, Dutta *et al.* 2008). Activation of microglia and astroglia, main players of neuroinflammation, can lead to overproduction and accumulation of various proinflammatory and neurotoxic factors that include cytokines tumor necrosis factor-alpha (TNFα) and interleukin-1 beta (IL-1β), reactive oxygen species (ROS), reactive nitrogen species (RNS) such as nitric oxide (NO), and lipid mediators that impact DA neurons to induce and/or exacerbate DA neurodegeneration (Liu 2006, Liu *et al.* 2003).

Activation of microglia significantly facilitates the Mn-induced preferential degeneration of DA neurons (Zhang *et al.* 2009) that are vulnerable to Mn neurotoxicity (Stanwood *et al.* 2009). Mn-stimulated ROS and RNS generation primarily in microglia and astroglia respectively are key mediators of the DA neurotoxicity (Zhang *et al.* 2007, Zhang *et al.* 2009). Several groups have also reported that Mn potentiates LPS and/or cytokine-induced glial iNOS upregulation and microglial release of TNFα and IL-6 (Bae *et al.* 2006, Barhoumi *et al.* 2004, Chang & Liu 1999, Filipov *et al.* 2005, Moreno *et al.* 2008, Spranger *et al.* 1998). However, the impact of the Mn and inflammation-enhanced glial activation on neurons and the relative contribution of microglia and astroglia to neurotoxicity remain undefined.

In this study, using various primary glial and neuronal cultures, we determined the combined neurotoxicity of Mn and LPS. Combinations of Mn and LPS were used at concentrations that, when used alone, were ineffective or minimally effective in causing neuronal damage (Gao *et al.* 2002b, Zhang et al. 2009). Mechanistically, the capacity of microglia and astroglia to produce ROS, RNS, and proinflammatory cytokines in response to treatment with Mn and LPS was determined. The contribution of Mn and LPS-induced glial activation to neurodegeneration was determined using minocycline and naloxone, agents that are known to modulate glial activation (Kim & Suh 2009, Liu & Hong 2003c).

Materials and methods

Primary microglia and astroglia cultures

Primary microglia and astroglia were prepared from mixed glia cultures which were prepared from brains of 1-day-old Fisher F344 rats as described (Liu & Hong 2003b, Liu *et al.* 2001, Zhang et al. 2007). Microglia and astroglia were seeded at 5×10^4 cells/well in 96-well in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and grown overnight (microglia) or to near confluence (astroglia) before treatment.

Primary neuron-glia, neuron-enriched, neuron-microglia and neuron-astroglia cultures

Primary neuronal and neuron-glia cultures were prepared as described (Gao *et al.* 2002a, Liu *et al.* 2000, Qin *et al.* 2005, Zhang et al. 2009). Briefly, mixed neuron-glia cultures were prepared by seeding, in poly-D-lysine coated 24-well plates, cells (5×10^5/well) isolated from gestation day 14 Fisher F344 rat mesencephalon and maintaining cultures at 37°C and 5% CO_2 in MEM containing 10% FBS and 10% horse serum (HS). Seven-day old cultures were used for treatment. Neuron-enriched cultures were prepared by adding cytosine β-D-arabinocide (10 μM) 2 days after seeding the neuron-glia cultures to suppress glial proliferation. Cultures were used for treatment 5 days later. Neuron-microglia cultures were prepared by adding microglia (10^5 cells/well) to the neuron-enriched cultures 1 day before treatment. Neuron-astroglia cultures were prepared by adding L-leucine methyl ester (0.5 mM) to neuron-glia cultures to eliminate microglia 3 days before treatment.

Treatment

Stock solutions of LPS (*E. coli* 0111:B4) and $MnCl_2$ were prepared in de-ionized and distilled water (Gao et al. 2002b, Zhang et al. 2009). For treatment, equal volumes of treatment media (MEM-2% FBS and 2% HS) containing vehicle (0.2% water), $MnCl_2$ or LPS were added to the cultures (0.1 and 1 ml/well final volume for 96 and 24-well plates respectively). For the conditioned media experiment, microglia cultures (1.2×10^5/well in 48-well plates) were treated for 24 hr with vehicle control or 30 μM $MnCl_2$ and 2 ng/ml LPS in DMEM-2% FBS (300 μl/well). Supernatants (250 μl/well) were removed and added (250 μl/well) to neuron-enriched cultures containing 750 μl/well MEM-2% FBS and 2% HS.

Immunocytochemistry (ICC)

ICC analysis was performed as described (Gao et al. 2002b, Liu et al. 2000, Zhang et al. 2009). DA neurons, neurons in general, microglia and astroglia were recognized with monoclonal antibodies against tyrosine hydroxylase (TH), neuron-specific nuclear protein (Neu-N), complement type-3 receptor antibody (OX-42) and glial fibrillary acidic protein (GFAP) respectively. ICC images were recorded with a Micropublisher microscope CCD camera using the QCapture Suite software.

Assessment of neurotoxicity

Neurotoxicity was assessed using a combination of structural and functional parameters as described (Gao *et al.* 2002a, Liu et al. 2000, Zhang et al. 2009). For DA neurons, the number of TH-immunoreactive (TH-ir) neurons was counted and neurite length of individual TH-ir neurons was measured. DA uptake was determined using [³H]DA and DA uptake inhibitor mazindol. Degeneration of neurons in general was determined by counting the number of Neu-N-ir neurons (Gao et al. 2002b, Zhang et al. 2009). Uptake of gamma-aminobutyric acid (GABA) was determined using [³H]GABA and GABA uptake blocker, NO-711.

Measurement of NO, ROS, TNFα and IL-1β

The amounts of nitrite and cytokines (TNFα and IL-1β) in the supernatants were determined using the Griess reagent and enzyme-linked immunosorbent assays respectively (Liu et al. 2000, Zhang et al. 2009). ROS production was determined using 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) as described (Zhang et al. 2009).

Statistical Analysis

Data were analyzed for statistical significance with ANOVA followed by Bonferroni/Dunn post hoc analysis using the StatView program (SAS Institute, Cary, NC). A p value of < 0.05 was considered statistically significant.

Results

Mn and LPS induce synergistic and preferential degeneration of DA neurons

When neuron-glia cultures were treated for 7 days with combinations of $MnCl_2$ and LPS at low concentrations (Gao et al. 2002b, Zhang et al. 2009), the combinations were more effective than individual agents in damaging DA neurons (Fig. 1). While 1–10 μM $MnCl_2$ or 0.5 ng/ml LPS were ineffective when used alone, combinations of 3–10 μM $MnCl_2$ with 0.5 ng/ml LPS caused a significant loss of TH-ir neuron (Fig. 1a). Significant potentiation of DA neurotoxicity was also observed for combinations of 3–30 μM $MnCl_2$ with 2 ng/ml LPS and 30 μM $MnCl_2$ with 0.5 ng/ml LPS (Figs. 1a & 1b). The combination of 1 μM $MnCl_2$ with 0.5 ng/ml LPS, while incapable of causing significant loss of TH-ir neurons (Fig. 1a), caused significant degeneration in TH-ir neurites and reduction in [^{3}H]DA uptake capacity (Fig. 1c). In contrast, in neuron-enriched cultures, treatment with the combination of 30 μM $MnCl_2$ with 2 ng/ml LPS did not result in any cooperative DA neurotoxicity (Fig. 1d).

The combination of 30 μM $MnCl_2$ and 2 ng/ml LPS was not equally effective in causing the loss of TH-ir neurons and that of neurons in general (i.e, Neu-N-ir neurons, Fig. 2a). Compared to a 44% loss of TH-ir neurons (Fig. 1a), the same treatment resulted in a statistically insignificant 9% reduction in Neu-N-ir neurons (Fig. 2b). In addition, combinations of $MnCl_2$ and LPS were significantly more effective in damaging TH-ir neurites and reducing DA uptake capacity of DA neurons than reducing GABA uptake capacity of non-DA neurons (Fig. 2c). For example, 30 μM $MnCl_2$ and 2 ng/ml LPS caused a 63% and 75% the reduction in DA uptake capacity and TH-ir neurite length respectively but only a 27% reduction in GABA uptake capacity (Fig. 2c).

Effect of Mn and LPS on the production of proinflammatory and neurotoxic factors in neuron-glia cultures

Glial activation and release of cytokines TNFα and IL-1β and free radicals are known to contribute to neurodegeneration (Liu 2006). In neuron-glia cultures treated for 7 days with 1–30 μM $MnCl_2$, no significant release of TNFα or IL-1β was detected (Fig. 3). However, $MnCl_2$, in a concentration-dependent manner, augmented the LPS-induced release of TNFα or IL-1β. In cultures treated with 0.5 ng/ml and 2 ng/ml LPS, significant augmentation of TNFα release was observed with ≥ 3 μM and ≥ 10 μM $MnCl_2$ respectively (Fig. 3a). Significant enhancement of 0.5 ng/ml and 2 ng/ml LPS-stimulated IL-1β release was observed for ≥ 10 μM and 30 μM $MnCl_2$ respectively (Fig. 3b). Different from a lack of effect on stimulating TNFα or IL-1β release, $MnCl_2$ caused a modest increase in NO production with significant elevation observed with 30 μM $MnCl_2$ (Fig. 3c). Moreover, significant enhancement of 0.5 ng/ml and 2 ng/ml LPS-induced nitrite production was observed for ≥ 3 μM and 10 μM $MnCl_2$ respectively (Fig. 3c).

Microglia and astroglia are differentially involved in the Mn and LPS-induced DA neurodegeneration

In analysis of the contribution of microglia and astroglia to the Mn and LPS-induced neurotoxicity, we treated astroglia-neuron and microglia-neuron cultures for 7 days with 30 μM $MnCl_2$ and 2 ng/ml LPS. TH-ir neurons in the microglia-neuron cultures were found to be significantly more vulnerable than those in the astroglia-neuron cultures to the Mn and LPS-induced neurotoxicity (Fig. 4a). The magnitude of reduction in TH-ir neurons in the microglia-neuron cultures (Fig. 4a) was less than that observed in the mixed neuron-glia cultures (Fig. 1a). The elevated susceptibility of neurons in the microglia-neuron cultures to Mn and LPS toxicity prompted us to collect conditioned media from microglia treated for 1 day with 30 μM $MnCl_2$ and 2 ng/ml LPS. When the conditioned media were used to treat neuron-enriched cultures (6 days), significant loss of TH-ir neurons was observed while vehicle-treated microglia-conditioned media had no effect (Fig. 4b). The degree of reduction in TH-ir neurons observed in neuron-enriched cultures treated with Mn and LPS-conditioned media (Fig. 4b) was less than that observed in Mn and LPS-treated neuron-microglia cultures (Fig. 4a).

Next, we directly compared the effect of Mn and LPS on the production of cytokines TNFα and IL-1β, NO and ROS in microglia and astroglia cultures. First, in astroglia cultures, compared to the vehicle-treated control cultures, no significant release of TNFα and IL-1β was detected following treatment with $MnCl_2$ (1–30 μM), LPS (0.5 and 2 ng/ml), or the combinations of $MnCl_2$ and LPS for 24 (Figs. 5a & 5b), 48 or 72 hr (data not shown). In microglia cultures, although $MnCl_2$ (1–30 μM; 24 hr) alone did not cause significant release of TNFα or IL-1β, $MnCl_2$ markedly augmented the LPS-induced TNFα and IL-1β release (Figs. 5c & 5d). Significant augmentation of TNFα release was observed with the combinations of 0.5 ng/ml LPS and ≥1 μM $MnCl_2$ and 2 ng/ml LPS and ≥3 μM $MnCl_2$; and IL-1β release with 0.5 ng/ml LPS and 10–30 μM $MnCl_2$ and 2 ng/ml LPS and 30 μM $MnCl_2$ (Figs. 5c & 5d). Second, compared to the control cultures, no significant NO production was observed in astroglia or microglia cultures treated with 1–30 μM $MnCl_2$ for up to 72 hr (Figs. 6a & 6c). However, the combinations of $MnCl_2$ and LPS were more effective than either agent alone in causing NO production in both astroglia and microglia. At 10 and 30 μM, $MnCl_2$ significantly augmented the LPS (2 ng/ml; 72 hr)-induced astroglial NO production from 130% to 205% and 256% of control and LPS (0.5 ng/ml, 24 hr)-induced microglial NO production from 526% to 723% and 843% of control respectively (Figs. 6a & 6c). Third, $MnCl_2$ (1–30 μM; 24 hr) caused a more robust production of ROS in microglia than in astroglia (Figs. 6b & 6d). In astroglia, significant augmentation of LPS (2 ng/ml)-induced ROS production was observed for 10 and 30 μM $MnCl_2$ and in microglia significant enhancement of LPS (0.5 ng/ml)-induced ROS production with 1–30 μM $MnCl_2$ (Figs. 6b & 6d).

Blockade of proinflammatory and neurotoxic factor production protects DA neurons from Mn and LPS-induced degeneration

Minocycline and naloxone have been shown to inhibit glial activation and afford neuroprotection (Dutta et al. 2008, Liu 2006). In this study, neuron-glia cultures were pretreated with 2 and 10 μM minocycline or naloxone prior to treatment for 7 days with the combination of 30 μM $MnCl_2$ and 2 ng/ml LPS. Minocycline and naloxone significantly reduced TH-ir neuron loss induced by $MnCl_2$ and LPS (Fig. 7). At equal concentrations, minocycline appeared to be slightly more effective than naloxone in affording neuroprotection. The production of TNFα, IL-1β and NO was significantly reduced by minocycline and naloxone in neuron-glia cultures treated for 7 days with 30 μM $MnCl_2$ and 2 ng/ml LPS (Fig. 8a). In addition, $MnCl_2$ and LPS-induced microglial and astroglial ROS production was significantly reduced by minocycline or naloxone (Fig. 8b).

Discussions

In this study, we have demonstrated, for the first time, that Mn (1–30 μM MnCl$_2$) and LPS (0.5–2 ng/ml) work in concert to induce synergistic and preferential degeneration of DA neurons in mesencephalic neuron-glia cultures (Figs. 1 & 2). Microglia and astroglia are both involved in the Mn and LPS-induced neurotoxicity with microglia possibly playing a larger role than astroglia (Fig. 4). The more prominent role for microglia may be a result of their robust and synergistic production of proinflammatory cytokines including TNFα and IL-1β and free radicals including ROS and RNS in response to the combined stimulation of Mn and LPS (Figs. 5 & 6). While astroglia do not seem to respond to Mn, LPS or the combination of both to produce more TNFα and IL-1β than the basal levels, Mn and LPS induce astroglia to produce more ROS and RNS than that by either agent alone albeit at a subdued intensity compared to microglia (Figs. 5 & 6). The contribution of glial activation to the Mn and LPS-induced synergistic neurotoxicity is supported by the observation that minocycline or naloxone affords neuroprotection (Fig. 7) through blockade of glial production of proinflammatory and neurotoxic factors (Fig. 8).

Difference in primary site of neuronal damage and response to levodopa therapy indicates that excessive exposure to Mn-induced manganism is distinct from IPD (Aschner et al. 2007, Huang 2007, Perl & Olanow 2007). Excessive exposure to Mn by itself does not seem to result in PD development. However, the combination of Mn and other contributing factors may contribute to DA neurodegeneration in PD. In the present study, we show that Mn and inflammogen LPS work in concert to induce DA neurodegeneration in primary neuron-glia cultures. While the plasma Mn content in healthy individuals are at the low to mid nanomolar levels (Zheng *et al.* 1998), levels of Mn in the range of 4.4–334 μM have been reported for various brain regions, in particular the basal ganglia in brains of accidentally exposed human subjects and experimental animals (rodents and non-human primates) (Erikson *et al.* 2004). While low micromolar concentrations of Mn by itself exhibit little neurotoxicity [(Stanwood et al. 2009, Zhang et al. 2009) and this study], combinations of Mn (1–30 μM) with LPS (0.5–2 ng/ml) that by itself is very modestly effective (Gao et al. 2003a, Gao et al. 2002b) result in synergistic DA neurotoxicity (this study). While the potential relevance of environmental Mn exposure and episodic systemic infections and consequential neuroinflammation to PD development remains to be proven (Brydon *et al.* 2008, Teeling & Perry 2009), PD may represent a collection of highly similar pathological abnormalities caused by long-term interactions among intrinsic (the vulnerability of the SN DA pathway and aging), genetic and various combinations of environmental factors. Analogous to Mn and inflammation, cooperative DA neurotoxicity has been reported for combinations of herbicide paraquat and the Mn-containing fungicide maneb, pesticide dieldrin and neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), LPS and MPTP or 6-hydroxydopamine, and LPS and parkin gene deletion (Frank-Cannon *et al.* 2008, Gao et al. 2003a, Gao *et al.* 2003b, Ling *et al.* 2004a, Ling *et al.* 2004b, Richardson *et al.* 2006, Thiruchelvam *et al.* 2000).

DA neurotoxicity of Mn as well as LPS is dependent upon the concentrations of Mn or LPS. In the neuron-glia cultures, at low micromolar concentrations, Mn is more effective in damaging DA neurons than non-DA neurons and with increasing concentrations (high micromolar and low millimolar concentrations), non-DA neurons begin to be markedly affected (Milatovic et al. 2009, Stanwood et al. 2009, Zhang et al. 2009). For example, 1 mM MnCl$_2$ induced >90% loss of TH-ir neurons compared to ~35–60% of Neu-N-ir neurons in cultures (Stanwood et al. 2009, Zhang et al. 2009). In animals, Mn-induced loss of TH-ir neurons is paralleled by a loss of Nissl-stained neuronal cell bodies in the SN (Stanwood et al. 2009), indicative of the destruction of TH-expressing neurons. Similarly, LPS-induced DA neurotoxicity involves the degeneration of TH-ir and Neu-N-ir neurons in

cultures and in the SN with preferential DA neurotoxicity observed at lower LPS concentrations (Gao et al. 2002b, McCoy *et al.* 2006). The combination of low concentrations of Mn ($\leq$30 μM) and LPS ($\leq$2 ng/ml) seem to preferentially target DA neurons. While 30 μM $MnCl_2$ and 2 ng/ml LPS resulted in a 63% reduction in DA uptake and 44% loss of TH-ir neurons, the same treatment only caused a 27% reduction in GABA uptake but not a statistically significant loss of Neu-N-ir neurons (Figs. 1 & 2). The loss of uptake capacity could also be due, at least in part, to glia-originated ROS and RNS since transporters of neurotransmitters are known targets of free radicals (Fleckenstein *et al.* 2007).

The preferential DA neurotoxicity observed in this study may be due to the innate vulnerability of DA neurons and the ability of Mn and LPS to stimulate glial production of deleterious factors. DA neurons are known to be particularly susceptible to oxidative and nitrosative stress (Jenner & Olanow 1996). In this study, the combination of low concentrations of Mn and LPS stimulate glial cells to produce ROS, RNS and cytokines TNFα and IL-1β, factors that are known to cause the demise of DA neurons (Liu 2006). The formation of more damaging intermediates such as peroxynitrite from ROS and RNS may render DA neurons more vulnerable to Mn and LPS-induced toxicity. It should be pointed out that the complete repertoire of factors that mediate the glial activation-enhanced Mn neurotoxicity will not only include ROS, NO, TNFα and IL-1β, but also IL-6 and prostaglandin E_2, as well as additional and yet to be identified factors (Filipov et al. 2005, Milatovic *et al.* 2009).

While LPS depends on the activation of glial cells to induce neurotoxicity (Liu *et al.* 2002, Zhang et al. 2009), the involvement of glia in Mn neurotoxicity appears to be concentration-dependent. Direct comparison using cell culture models indicates that at 1–30 μM, $MnCl_2$ is only effective in causing DA neurotoxicity in the presence of glia and at 1 mM, $MnCl_2$ appears to be equally toxic regardless of glial presence (Zhang et al. 2009). Therefore, it is reasonable to speculate that Mn-induced neurodegeneration involves both glia-mediated and direct neurotoxicity and the degree of glial participation is dependent upon the concentrations of Mn. At low and perhaps more environmentally relevant doses, especially in combination with other agents such as glia-activating agents, Mn-induced neurotoxicity may involve a significant contribution from glial cells.

Microglia and astroglia are the main players of the neuroinflammatory process (Liu & Hong 2003c). Results from this study indicate that microglia and astroglia exhibit differential contribution to the Mn and LPS-induced generation of neurotoxic factors. Mechanistically, several groups have reported the involvement of members of the mitogen-activated protein kinases (MAPKs) and transcription factors in the Mn-potentiated production of neurotoxic factors in activated glia. For example, Barhoumi et al has shown that increased mitochondrial ROS production and activation of nuclear factor kappa B (NFκB) mediate Mn enhancement of LPS-induced iNOS upregulation in C6 astrocytic cells (Barhoumi et al. 2004). In cytokine-activated astroglia, Mn potentiation of iNOS expression is mediated through increased production of cGMP that leads to activation of MAPK and NFκB (Moreno et al. 2008). In N9 microglial cells, MAPK and NFkB activation is involved in Mn potentiation of LPS-induced TNFα and IL-6 release and iNOS upregulation (Crittenden & Filipov 2008, Filipov et al. 2005). Members of MAPKs are also involved, at least in part, in Mn-stimulated microglial release of hydrogen peroxide (Zhang et al. 2007). The precise mechanisms of action underlying the differential contribution of microglia and astrocytes to Mn and LPS-induced neurotoxicity remain to be further delineated. In addition, the potent effect of LPS at 0.5 ng/ml on glial production of free radicals and cytokines (Gao et al. 2002b) warrant future studies to determine the interaction of even lower and potentially subclinical doses of LPS with Mn.

The Mn and LPS-induced glial production of cytokines and free radicals and DA neurotoxicity is markedly reduced by minocycline and naloxone (Figs. 7 & 8). Minocycline, a tetracycline derivative and naloxone, a non-selective opioid receptor antagonist, have been shown to inhibit glial activation induced by LPS and neurotoxicants such as MPTP and 6-OHDA and afford neuroprotection (Dutta et al. 2008,Liu & Hong 2003c,Liu & Hong 2003a). In particular, minocycline and naloxone have been shown to be effective inhibitors of microglial ROS production (Liu et al. 2000,Wu *et al.* 2002). In addition, minocycline has been shown to possess anti-inflammatory and protective effects for a variety of disorders of the central nervous and peripheral systems (Kim & Suh 2009,Rempe *et al.* 2007).

In summary, this study demonstrates the synergistic DA neurotoxicity of Mn, an occupational and potential environmental neurotoxicant and LPS, an inflammatory stimulus. This study has identified differential involvement of microglia and astroglia in the combined Mn and LPS neurotoxicity with TNFα, IL-1β, ROS and RNS as key mediators. Continued delineation of the underlying molecular and cellular mechanisms of action will further advance our understanding of the contribution of combinations of multiple occupational and/ or environmentally relevant agents to the development of disorders of the brain and peripheral systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations used

DA	dopamine
DCF	dichlorodihydrofluorescein
DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
HS	horse serum
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acidic protein
ICC	immunocytochemistry
IL-1β	interleukin-1beta
LPS	lipopolysaccharide
MEM	minimum essential medium
MnCl$_2$	manganese chloride
Neu-N	neuron-specific nuclear protein
NO	nitric oxide
PD	Parkinson's disease
RNS	reactive nitrogen species

| **ROS** | reactive oxygen species |
| **TH** | tyrosine hydroxylase |
| **TNFα** | tumor necrosis factor-alpha |

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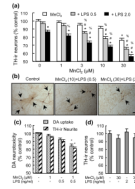
Fig. 1.
DA neurotoxicity induced by combined treatment with $MnCl_2$ and LPS. Rat primary mesencephalic neuron-glia cultures (a–c) or neuron-enriched cultures (d) were treated for 7 days with vehicle control (0.2% water), indicated concentrations of $MnCl_2$ or LPS alone, and combinations of $MnCl_2$ and LPS. Following treatment, the cultures were either immunostained for TH-ir neurons (a–d) or subject to measurement of [^{3}H]DA uptake capacity (c). The number of TH-ir neurons (a & d) and neurite length of TH-ir neurons (c) were determined. Results are expressed as a percentage of the vehicle-treated control cultures and are mean ± SEM of three (d) or five (a & c) independent experiments performed in triplicate. *p < 0.05 compared to vehicle-treated control cultures, [#]p < 0.05 compared to corresponding LPS-treated cultures, [%]p < 0.05 compared to corresponding $MnCl_2$-treated cultures. In (b), the numbers in parentheses of indicate 10 or 30 μM and 0.5 or 2 ng/ml respectively. ICC images (b) are from a representative experiment. Arrowheads: TH-ir neurons. Scale bar: 100 μm.
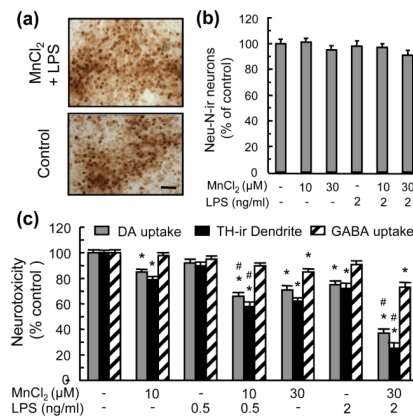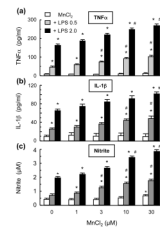
Fig. 2.
Preferential DA neurotoxicity induced by $MnCl_2$ and LPS. Neuron-glia cultures were treated for 7 days with vehicle, $MnCl_2$ or LPS alone, and combinations of $MnCl_2$ and LPS. Cultures were then immunostained for counting Neu-N-ir neurons (b) and measurement of TH-ir neurites (c), or assayed for [^{3}H]DA and [^{3}H]GABA uptake (c). For ICC analysis (a), cultures were treated with vehicle (Control) or 30 μM $MnCl_2$ and 2 ng/ml LPS ($MnCl_2$ + LPS), scale bar: 50 μm. Results are expressed as a percentage of the vehicle-treated control cultures and are mean ± SEM of four (b) or five (c) experiments performed in triplicate. *$p < 0.05$ compared to vehicle-treated control cultures, [#]$p < 0.05$ compared to corresponding $MnCl_2$ or LPS-treated cultures.

Fig. 3.
Effect of MnCl₂ and LPS on the release of TNFα and IL-1β and production of NO in
neuron-glia cultures. Supernatants from cultures treated for 7 days with vehicle, MnCl₂ or
LPS alone, and combinations of MnCl₂ and LPS were collected and the levels of TNFα,
IL-1β, and nitrite were determined. Results are mean ± SEM of five experiments performed
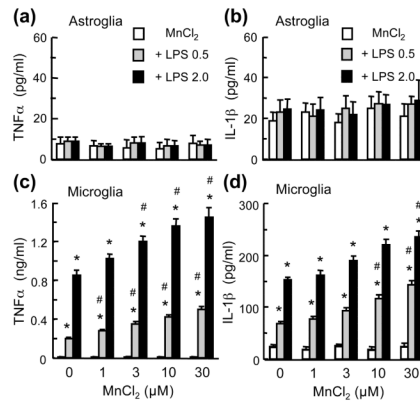in triplicate. *p< 0.05 compared to vehicle-treated control cultures, #p < 0.05 compared to
corresponding MnCl₂ or LPS-treated cultures.
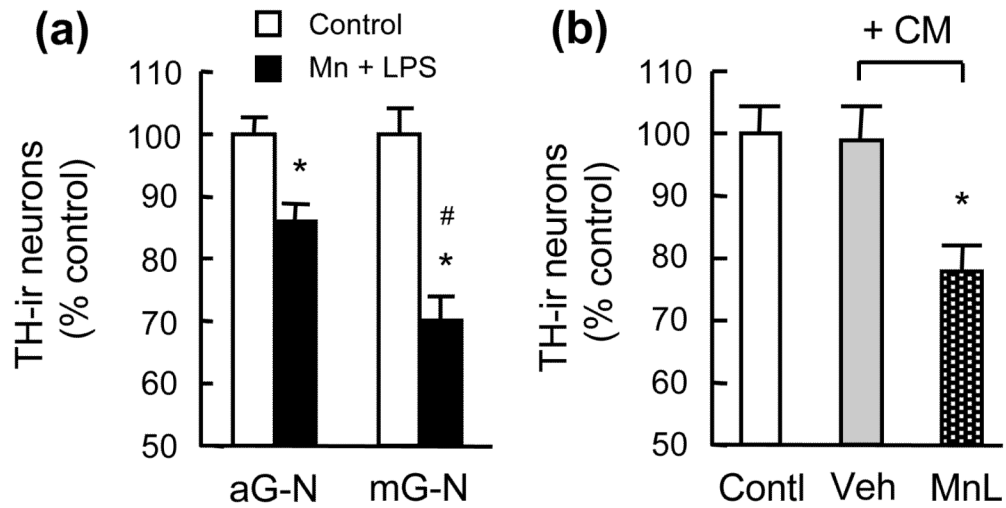
Fig. 4.
Characterization of glial dependence for MnCl$_2$ and LPS-induced DA neurotoxicity. (a). Astroglia-neuron (aG-N) or microglia-neuron (mG-N) cultures were treated for 7 days with vehicle and 30 μM MnCl$_2$ and 2 ng/ml LPS (Mn+LPS). (b). Neuron-enriched cultures were treated for 6 days with treatment media alone (Contl) or treatment media containing conditioned media (CM) collected from microglia treated for 24 hr with vehicle (Veh) or 30 μM MnCl$_2$ and 2 ng/ml LPS (MnL). Following treatment, cultures were immunostained and the number of TH-ir neurons determined. Results are mean ± SEM of four experiments performed in triplicate. *p< 0.05 compared to vehicle-treated control cultures, #p < 0.05 compared to MnCl$_2$+LPS-treated astroglia-neuron cultures.

Fig. 5.
Comparison of the effect of $MnCl_2$ and LPS on TNFα and IL-1β release in microglia and astroglia. Astroglia (a & b) microglia (c & d) cultures were treated for 24 hr with vehicle, $MnCl_2$ or LPS alone, and the combinations of $MnCl_2$ and LPS. The levels of TNFα (a & c) and IL-1β (b & d) in the culture supernatants were determined. Results are mean ± SEM of four to six experiments performed in triplicate. *p< 0.05 compared to vehicle-treated control cultures, #p < 0.05 compared to corresponding $MnCl_2$ or LPS-treated cultures.
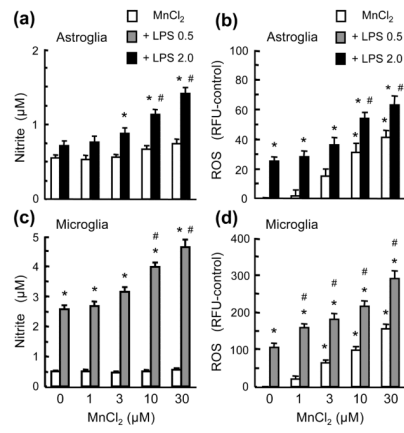
Fig. 6.
Effect of MnCl$_2$ and LPS on NO and ROS production in microglia and astroglia. Astroglia (a & b) microglia (c & d) cultures were treated for 24 hr (b–d) or 72 hr (a) with vehicle, MnCl$_2$ or LPS alone, and the combinations of MnCl$_2$ and LPS and production of NO and ROS was determined. Results are mean ± SEM of four to six experiments performed in triplicate. *p< 0.05 compared to vehicle-treated control cultures, #p < 0.05 compared to corresponding MnCl$_2$ or LPS-treated cultures.
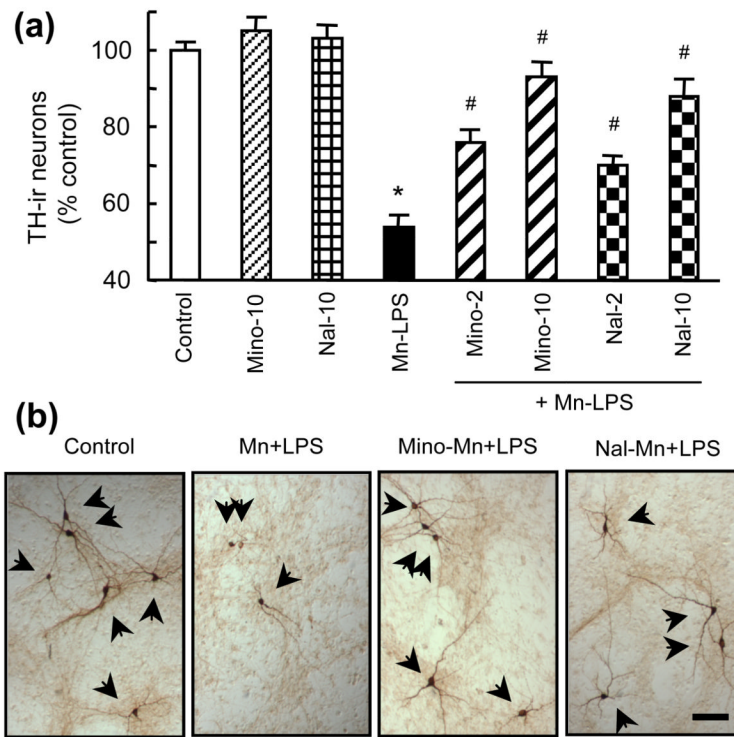
Fig. 7.
Effect of naloxone and minocycline on the $MnCl_2$ and LPS-induced DA neurodegeneration. Neuron-glia cultures were treated for 7 days with vehicle, 10 μM minocycline (Mino-10) or naloxone (Nal-10) alone, the combination of 30 μM $MnCl_2$ and 2 ng/ml LPS (Mn-LPS), or pretreated for 30 min with 2 or 10 μM minocycline (Mino-2, Mino-10) or naloxone (Nal-2, Nal-10) prior to treatment with 30 μM $MnCl_2$ and 2 ng/ml LPS (+ Mn-LPS). Cultures were then immunostained for TH-ir neurons and the number of TH-ir neurons was determined. Results in (a) are mean ± SEM of five experiments performed in triplicate. *$p < 0.05$ compared to vehicle-treated control cultures, #$p < 0.05$ compared to $MnCl_2$ and LPS-treated cultures. ICC images (b) are from cultures treated with vehicle (control), 30 μM $MnCl_2$ and 2 ng/ml LPS (Mn+LPS), and pretreated with 10 μM minocycline or naloxone prior to treatment with $MnCl_2$ and LPS (Mino-Mn+LPS or Nal-Mn+LPS). Bar: 100 μm.
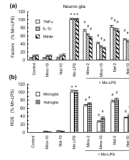
Fig. 8.
Effect of minocycline and naloxone on the MnCl$_2$ and LPS-induced production of
neurotoxic factors. (a). The levels of TNFα, IL-1β, and nitrite in the supernatants from
cultures treated as described for Fig. 7a were determined. (b). Microglia and astroglia
cultures were treated for 24 hr with the same combinations of agents as described for Fig. 7a
and ROS production was determined. Results in are mean ± SEM of five (a) or four (b)
experiments performed in triplicate (a) or quadruplicate (b). *p< 0.05 compared to vehicle-
treated control cultures, #p < 0.05 compared to MnCl$_2$ and LPS-treated cultures.