

Morphinan Neuroprotection: New Insight into the Therapy of Neurodegeneration

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ABSTRACT: Neuro-inflammation plays a pivotal role in numerous neurodegenerative disorders, such as Parkinson's disease (PD). Traditional anti-inflammatory drugs have limited therapeutic use because of their narrow spectrum and severe side effects after long-term use. Morphinans are a class of compounds containing the basic morphine structure. The following review will describe novel neuroprotective effects of several morphinans in multiple inflammatory disease models. The potential therapeutic utility and underlying mechanisms of morphinan neuroprotection are discussed.

KEY WORDS: morphinan, naloxone, dextromethorphan (DM), 3-hydroxymorphinan (3-HM), neuroprotection, neuro-inflammation, microglia, astroglia

ABBREVIATIONS

3-MM: 3-methoxymorphinan
A β : β -amyloid peptide
AD: Alzheimer's disease
AMPA: alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid
CNS: central nervous system
COX-2: cyclooxygenase-2
CPK-5: 3-allyloxy-17-methylmorphinan
CPK-6: 3-cyclopropylmethoxy-17-methylmorphinan
CSF: cerebrospinal fluid
DA: dopamine

DDC: diethyldithiocarbamate
 DM: dextromethorphan
 DMPO: 5,5-dimethyl-1-pyrroline *N*-oxide
 DOPAC: 3,4-dihydroxyphenylacetic acid
 DT: dextrophan
 EPR: electron paramagnetic resonance
 GBS: Guillain-Barré syndrome
 GGF: glycine-glycine-phenylalanine
 GSH-Px: glutathione peroxidase
 5-HIAA: 5-hydroxyindolacetic acid
 3-HM: 3-hydroxymorphinan
 HVA: homovanillic acid
 I/R: ischemia/reperfusion
 IL-1: interleukin-1
 iNOS: inducible nitric oxide synthases
 iROS: intracellular reactive oxygen species
 KA: kainic acid
 LPS: lipopolysaccharide
 MDA: malonyldialdehyde
 MES: maximal electroshock convulsions
 MNP: maximal negative peak
 MPP⁺: 1-methyl-4-phenylpyridinium
 MPTP: 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine
 MS: multiple sclerosis
 NADPH: nicotinamide adenine dinucleotide phosphate
 NMDA: *N*-methyl-D-aspartate
 NO: nitric oxide
 NSAIDs: non-steroidal anti-inflammatory drugs
 PCP: phencyclidine
 PD: Parkinson's disease
 PGE₂: prostaglandin E₂
 PMA: phobol-12-myristate-13-acetate
 ROS: reactive oxygen species
 SCEP: spinal cord-evoked potentials
 SCI: spinal cord injury
 SEP: somatosensory-evoked potentials
 SN: substantia nigra
 SNpc: substantia nigra pars compacta
 SOD: superoxide dismutase
 TNF α : tumor necrosis factor α

I. INTRODUCTION

Morphinans, which are a series of compounds structurally similar to morphine, including naloxone and naltrexone, are neuroprotective in a variety of neurodegenerative disease animal models. However, the mechanism underlying their neuroprotective ef-

fects is not clear. Morphine has been used for the treatment of spinal cord injury (SCI), and its effect was believed to be mediated through opioid receptors.¹⁻⁴ However, opioid receptor antagonists, such as naloxone or naltrexone, have also been reported to be neuroprotective in the SCI and brain ischemia animal models.^{5,6} Thus, it is questionable whether morphine- and naloxone-induced neuroprotection are mediated through opioid receptors. Moreover, many publications also indicate that dextromethorphan (DM) exerts neuroprotective effects in various brain and SCI models.^{7,8} The neuroprotective effect of DM has been attributed to its *N*-methyl-D-aspartate (NMDA) receptor antagonist property.

We have been interested in elucidating the possibility that novel mechanisms, which are not mediated through either opioid or NMDA receptors, may be associated with morphinan-induced neuroprotection. In this review, we propose that anti-inflammatory effects of morphinans may underlie their neuroprotective effects. We will first briefly review the current concept of receptor-mediated neuroprotection of morphinans and then focus on the anti-inflammatory effect of morphinans as a potential major mechanism for neuroprotection.

II. ROLE OF NMDA RECEPTOR IN MEDIATING MORPHINAN-INDUCED NEUROPROTECTION

II.A. DM Protects Neurons Against Cerebral Ischemia

The first evidence for a neuroprotective action of DM was reported by Choi,⁹ who demonstrated that DM offered protection for primary neurons exposed to the excitotoxin glutamate.⁹ Shortly thereafter, it was shown in several *in vivo* models of ischemic brain injury that treatment with DM could protect the brain against infarction and related pathophysiological and functional consequences of injury.^{7,10-13} More recently, several *in vitro*^{14,15} and *in vivo* studies¹⁶⁻¹⁸ have confirmed the neuroprotective actions of DM and have offered critical insights into its possible cellular mechanism of

action. In particular, comprehensive studies undertaken with the rodent focal ischemia/reperfusion (I/R) injury model showed the potent actions of DM to decrease the volume of cerebral infarction and improve functional recovery as a postinjury therapy.¹⁸ Interestingly, novel analogs of DM have been described as antagonists of glutamate-induced excitotoxic calcium signals in neurons,^{14,18} and one such analog—namely, the (+)-3-amino-17-methylmorphinan derivative AHN649—is an effective and safe neuroprotective agent possessing a similar potency, but improved safety profile, when compared to DM.¹⁸ Although the aforementioned reports indicated that the neuroprotective effect of DM on cerebral ischemia is attributable to its NMDA antagonist property; research in our laboratory showed strong evidence that the neuroprotective effect of DM against inflammation-related neurodegeneration is mediated through its anti-inflammatory effect (see Section VIII).

II.B. Neuroprotection of DM and its Metabolite on Spinal Cord Injury (SCI)

With the development of experimental SCI models and recent advances in SCI research, many therapeutic regimens, such as anti-inflammatory drugs,^{19,20} immunosuppressive drugs,²¹ and receptor blockers²⁰ have been studied in animals. An *in vivo* study showed that a chronic allodynia-like response to mechanical stimulation was observed in rats after severe spinal cord ischemia.⁸ This allodynia-like response was not relieved by most conventional analgesics used for treating chronic neuropathic pain.²² Hao and Xu²² found that systemic DM relieved the mechanical allodynia-like response in a dose-dependent fashion. They also observed increased spontaneous motor activity in the absence of severe motor impairment at analgesic doses. It is well known that the analgesic effect of most NMDA antagonists is associated with severe side effects, with psychomimetic effects being the most common. However, DM is known for its safety record and can be used for treating the chronic pain associated with SCI.²²

Glutamate is a potent and rapidly acting neurotoxin on cultured spinal neurons, supporting the involvement of excitotoxicity mediated through NMDA receptors in acute SCI.²² Regan and Choi²³ found that exposure of mixed spinal cord neuron-glia cultures to glutamate for 5 minutes produced widespread acute neuronal swelling followed by neurodegeneration over the next 24 hours. By 14–20 days, 80–90% of the neuronal population was destroyed by a 5-minute exposure to glutamate. Both acute neuronal swelling and late neurodegeneration were effectively blocked by dextrophan (DT), the metabolite of DM.

The spinal cord and the brain are particularly vulnerable to free radical oxidation following traumatic insults because of their high lipid content²⁴ and poor iron-binding capacity.²⁵ In traumatic SCI, the lesion results not only from the direct (primary) physical trauma but also from the indirect (secondary) injury, associated with ischemia, edema, increased excitatory amino acids (EAAs), and oxidative damage to the tissue from reactive oxygen species (ROS),¹⁹ which in turn contribute to lipid peroxidation.^{19,20,24,26,27} The levels of the lipid peroxidation products, including malonyldialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), can be measured in monitoring the degree of lipid peroxidation. It has been found that DM and DT²⁸⁻³⁰ block Ca²⁺ entry into neurons as a result of both NMDA-antagonist^{11,28,29,31-33} and voltage-gated Ca²⁺ influx-inhibiting effects. Topsokal et al.²⁵ found that DM was rather effective against spinal cord trauma at 120 minutes, which might have stemmed from the high plasma concentrations of DM at 60–120 minutes, as reported previously.²⁹ Kato³¹ used DT to protect the spinal cord from ischemia.

II.C. Neuroprotective Effect of DM Through its Anticonvulsant Effect

Several studies revealed that DM has a significant anticonvulsant effect.³⁴⁻³⁶ DM was found to reduce the seizures and mortality and decreased the cell loss in the CA1 and CA3 areas of the hippocampus in a

dose-dependent manner. One interpretation for the neuroprotective effect of DM through its anticonvulsant effect is that it reduces the excitotoxicity exerted on the neurons by glutamate through the inhibition of NMDA receptors. DM has also been shown to attenuate kainic acid (KA)-induced increases in AP-1 binding activity and C-Jun/FRA expression in the hippocampus, collectively suggesting that DM is an effective antagonist of KA and a potent protectant for convulsants.³⁴

Recently, new derivatives of DM also showed a promising anticonvulsant effect.^{37,38} Kim et al.³⁸ investigated the effect of a series of synthesized DM analogs (that were modified in positions 3 and 17 of the morphinan ring system) on maximal electroshock convulsions (MES) in mice. They found that DM, DT, 3-allyloxy-17-methylmorphinan (CPK-5), and 3-cyclopropylmethoxy-17-methylmorphinan (CPK-6) had anticonvulsant effects against MES, while 3-methoxymorphinan (3-MM), and 3-hydroxymorphinan (3-HM) did not show any anticonvulsant effects. They found that DM, DT, CPK-5, and CPK-6 were high-affinity ligands to sigma 1 receptors, while they all had low affinity to sigma 2 receptors. DT had relatively higher affinity for the phencyclidine (PCP) sites than DM. By contrast, CPK-5 and CPK-6 had very low affinities for PCP sites, suggesting that PCP sites are not required for their anticonvulsant actions. Their results suggest that the new morphinan analogs are promising anticonvulsants that are devoid of PCP-like behavioral side effects, and their anticonvulsant actions may be, in part, mediated via sigma 1 receptors.³⁶

Another study indicated that the anticonvulsant effects of the morphinans partially involve the L-type calcium channel and that DM is a more potent anticonvulsant than DT in both KA- and BAY K-8644-induced seizure models.³⁵ BAY K-8644 is an L-type Ca^{2+} channel agonist of the dihydropyridine class, which is recognized as a potent convulsant agent that can also potentiate seizures induced by KA. The anticonvulsant effect of a low dose of DM was reversed by BAY K-8644 in this model. In contrast, BAY K-8644 did not significantly affect an anticonvulsant effect

from a higher dose of DM and DT. Furthermore, DM appeared more efficacious than DT in attenuation of KA- and BAY K-8644-induced seizures by decreasing KA-induced AP-1 DNA-binding activity and fos-related antigen-immunoreactivity, as well as reducing neuronal loss in the hippocampus.

III. ROLE OF OPIOID RECEPTOR-MEDIATED MORPHINAN NEUROPROTECTION

III.A. Naloxone Protects Neurons Against Cerebral Ischemia

Koc et al.³⁹ studied the effect of naloxone on focal cerebral ischemia induced by middle cerebral artery occlusion with the transorbital approach in a rabbit model. Animals receiving naloxone treatment showed improvement in neurological outcome. In addition, naloxone significantly reduced the infarct size and edema compared to controls.⁴⁰ It was suggested that the attenuation of the disturbance of cellular functions following cerebral I/R via restoration of mitochondrial activities or energy metabolism is the mechanism of the neuroprotective effect of naloxone. Chen et al.⁴¹ found that both pretreatment and post-treatment with naloxone by intracerebroventricular infusion significantly reduced cortical infarct volumes. Pretreatment with naloxone reduced ischemia-induced suppression of the extracellular pyruvate level and enhancement of the lactate/pyruvate ratio, as well as cerebral I/R-induced increases of endogenous catalase, glutathione peroxidase, and manganese SOD activities.

Furthermore, effects of naloxone on the somatosensory-evoked potentials (SEP) were studied in cat brains during focal cerebral ischemia by Ding et al.⁴² They found that naloxone can improve the electrical activity of neurons in the ischemic region of the brain. Gunnarsson et al.⁴³ have shown that naloxone stereospecifically enhanced the SEP without changes in cortical blood flow. The high dose of naloxone needed to enhance the SEP suggested that the attenuation was mediated by low-affinity opioid receptors (δ or κ). The same model was used to study

the effect of naloxone-methobromide, a quaternary derivative of naloxone with selective peripheral action when injected intravenously. Only naloxone changed the amplitude of SEP significantly compared to the control. However, there was a tendency for a delayed effect of naloxone-methobromide on SEP, possibly indicating that the substance slowly passed the blood–brain barrier.

2. Naloxone is Protective in SCI

Studies have shown that naloxone influences the pathophysiology of SCI.⁴⁴ Naloxone has been subjected to rigorous testing to determine its ability to protect compromised but viable cellular elements and improve the functional outcome of survivors, thus potentially serving as a useful agent for this purpose.⁴⁵ Kuniyama et al.⁴⁶ studied the effect of naloxone on EAAs in cerebrospinal fluid (CSF) in patients undergoing thoracoabdominal aortic surgery. In patients with SCI, CSF levels of glutamate and glycine continued to increase for as long as 72 hours postoperatively, and were significantly more elevated than those without SCI. Postoperative maximum levels of CSF glutamate and glycine were also significantly higher in patients with postoperative SCI than those without SCI. Naloxone significantly decreased the CSF levels of glutamate and aspartate and the postoperative maximum level of CSF aspartate.⁴⁶

The effects of naloxone on behavioral recovery following unilateral peripheral vestibular deafferentation (unilateral labyrinthectomy, UL) in guinea pigs was first investigated by Dutia et al.⁴⁷ Naloxone was found to significantly reduce the frequency of spontaneous nystagmus relative to controls, suggesting that naloxone can reduce the oculomotor effects of UL in a dose-dependent fashion. The influence of naloxone on spinal cord conduction and edema formation after trauma, measured by spinal cord evoked potentials (SCEP) and water content of the cord, respectively, was investigated in a rat model.⁴⁸ Pretreatment with naloxone inhibited the immediate postinjury decrease of the rostral maximal negative

peak (MNP) amplitude without any significant effect on latency changes. Measurement of water content in the traumatized spinal cord segment showed a significant reduction by naloxone compared to controls.

IV. INFLAMMATION-MEDIATED NEURODEGENERATIVE DISEASES

Increasing evidence indicates that inflammation plays a pivotal role in numerous neurodegenerative disorders, such as Parkinson's disease (PD),⁴⁹⁻⁵¹ Alzheimer's disease (AD),⁵² multiple sclerosis (MS),^{53,54} stroke,⁵⁵⁻⁶¹ prion disease,⁶²⁻⁶⁷ HIV dementia,⁶⁸⁻⁷³ Pick's disease,^{74,75} demyelination,^{76,77} and Guillain-Barré syndrome (GBS).⁷⁸ The pathology and clinical manifestations of neurodegenerative diseases often develop over years, indicating a progressive process. This gradual accumulation of damage across time suggests a large potential treatment window, offering a therapeutic hope to alter the disease course. While there are few treatment approaches professing to slow the progression of neurodegenerative disease, current research suggests that anti-inflammatory drugs can slow or even halt the propagation of neuronal death.⁷⁹⁻⁸¹ Here, we introduce morphinans as an ideal class of neuroprotective compounds that attenuate the inflammation linked to the ongoing vicious cycle of neurodegeneration and that possess multiple neuroprotective characteristics.

Traditionally, the central nervous system (CNS) was perceived as an immunologically privileged structure devoid of immune cell influence. Current evidence indicates that the CNS is under constant immune surveillance in both normal physiological conditions and pathological circumstances. Studies of both humans and animals reveal that neuro-inflammation participates in the pathogenesis and progression of numerous neurological diseases.⁸²⁻⁹¹

PD is an age-related chronic and ultimately devastating neurodegenerative disorder in the CNS characterized by a selective, progressive, and massive loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNpc) and subsequent deple-

tion of the neurotransmitter DA in the striatum, which leads to major clinical and pathological abnormalities.^{92,93} To date, both the cause and the detailed mechanism of DA neuron death in PD are unknown.

Research by McGeer et al.⁸³ was the first to suggest roles of neuro-inflammation in the pathogenesis of PD. In their report, a large population of reactive microglia, the resident phagocyte in the brain, staining positive for the human leukocyte antigen (HLA)-DR, was discovered in the substantia nigra (SN) of PD patients. In another study, the premise of an immunological response in PD was further supported by analysis of postmortem brains from PD patients, where there was clear evidence of microglia activation in the SN.⁹³ Several pro-inflammatory cytokines have been detected in postmortem brains of PD patients, and there is evidence of oxidative stress, which also lends strong support to the association of microglial activation and PD.^{93,94} Furthermore, cases of immunological insults to the brain have recently been linked to the onset of PD.⁵¹ Specifically, reports have correlated early-life brain injury, fetal brain inflammation, and viruses or infectious reagents with the later onset of PD.⁵⁰ Thus, in addition to the massive loss of DA neurons in the SNpc, PD is also characterized by a conspicuous (localized) glial overactivation, which is evidenced by elevated levels of cytokines and upregulation of inflammation-associated factors, such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthases (iNOS).

V. ROLE OF MICROGLIA IN INFLAMMATION-MEDIATED NEURODEGENERATION

Increasing evidence supports microglia as the pivotal cell type mediating neuro-inflammatory damage.⁹⁵⁻¹⁰⁸ Microglia are derived from the myeloid cell lineage, thus possessing many characteristics required for the innate immune response. Microglia engage in phagocytic functions to remove damaged and foreign cells and are further involved in immunological surveillance by secreting pro-inflammatory factors, such as prostaglan-

dins, tumor necrosis factor α (TNF α), interleukin-1 (IL-1), and free radicals, such as superoxide and nitric oxide (NO). Recent work in our laboratory has shown that much of the microglia-mediated neurotoxicity is through the release of superoxide and other reactive oxygen species (ROS) generated from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.^{87,109-114}

Microglia serve the role of immune surveillance under normal physiological condition.⁸⁶ However, in pathological circumstances, microglia easily become activated and produce an array of neurotoxic factors, including cytokines and ROS. It is the overactivated microglia that govern the disease state, where the consequent accumulation of neurotoxic factors is deleterious to neurons. In PD, DA neurons in the SNpc are especially vulnerable to oxidative damage because of their reduced antioxidant capacity and potential defect in mitochondrial function.¹¹⁵ Because the SN is particularly rich in microglia,^{90,116} the activation of microglia and subsequent release of neurotoxic factors contribute to DA neurodegeneration associated with PD.

Even without a direct immune trigger (such as LPS) stimulating the pro-inflammatory response required for the DA neuronal death, microglia activation is also implicated in the case of DA neuronal death caused directly by neurotoxins, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Specifically, the initial death of the neuron itself serves as a potent immunological stimulus, resulting in slow and persistent microglia activation, so called "reactive microgliosis."¹¹⁵ Because the presence of neurons inhibits the microglia-induced inflammatory response, the death of neurons not only activates microglia, but also removes some of the anti-inflammatory factors essential for maintaining microglial inflammatory homeostasis.¹¹⁷ Regardless of the trigger, once passing the stimulus threshold, microglia can enter a self-propelling cycle of inflammation, resulting in slow and persistent neuronal death, commonly referred to as *reactive microgliosis*. Thus, microglia are fundamental to the progression of DA neurodegeneration as both a potential trigger for neurotoxicity and the engine

driving the immunotoxic neuronal death resulting from reactive microgliosis.

In addition to the individual contributions to the neurotoxicity, a variety of factors probably work in concert to cause the synergistic DA neurotoxicity, which is particularly relevant to the complex neuro-inflammatory mechanisms of the *in vivo* PD process. For example, NO and superoxide can interact with each other and form more toxic intermediates such as peroxynitrite.⁵¹

VI. CURRENT THERAPEUTIC STRATEGIES FOR NEURODEGENERATIVE DISORDERS

Traditional anti-inflammatory drugs have also been studied for years for their possible therapeutic utility against neurodegenerative disorders. Dexamethasone is a steroidal anti-inflammatory drug (SAID) shown to inhibit microglial activation and reported to alleviate the neurodegenerative process induced by lipopolysaccharide (LPS).¹¹⁸ However, it will be not clinically useful for long-term therapy because of its severe side effects.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have also been studied for their potential therapeutic effect on PD.¹¹⁹ Much epidemiological and limited clinical evidence suggests that NSAIDs can impede the onset and slow the progression of neurodegenerative diseases. However, these drugs strike only at the periphery of the inflammatory reaction.⁵² Specifically, effects of NSAIDs on the CNS consist of a partial and limited spectrum of action on selective neurotoxic factors. For example, COX-2 inhibitors emerged as a novel agent for attenuating MPP⁺-elicited depletion of DA in the striatum and MPTP-induced loss of DA neurons in the SNpc.

However, other results raise concerns about the mechanism of NSAIDs' action. For example, corticosterone and aspirin only partially block the inflammatory factors (prostaglandin E₂ [PGE₂], TNF α , NO) and represent an unsuccessful therapeutic approach for neurodegenerative diseases. Hence, much better results might be obtained if drugs were

identified that could inhibit the microglial activation or the production of an array of proinflammatory and neurotoxic factors in the brain, or if combinations of drugs were aimed at different inflammatory targets as effective therapy.

Anti-inflammatory drugs designed to attenuate microglial activation hold the promise of a two-fold therapeutic benefit. First, there is the possibility of blocking the initial immunological insult that triggers the inflammatory cascade event resulting in neuronal death. Second, there is the promise of further neuroprotection by slowing down or halting the perpetuated and self-propelling reactive microgliosis, regardless of the initial neurotoxic event.

Morphinans are a series of compounds structurally similar to morphine (Fig. 1) but lacking the E ring found in the naturally occurring opioids, as well as the 6-OH and the 7,8-double bond. This work proposes that morphinan compounds, including naloxone, DM (3-methoxy-17-methylmorphinan), and its analog 3-HM (Fig. 1) are innovative and neuroprotective agents for inflammation-related neurological disorders through inhibition of microglial activation-mediated neurotoxicity.

VII. NALOXONE IS NEUROPROTECTIVE

Endogenous opioid peptides are reported to exert their physiological effects through interaction with their respective opioid receptors. These peptides are important in regulating the development of neurons and in modulating a variety of cellular activities, such as the immune response, respiration, ion channel action, and nociceptive/analgesic effects.¹²⁰ Naloxone is a potent nonselective antagonist of the classic G-protein-linked opioid receptors, which are widely expressed on the cells in the CNS and peripheral nervous system. Naloxone has a similar affinity for the μ -type opioid receptor, as does morphine.¹²¹ The capacity of naloxone as an opiate receptor antagonist is stereospecific: only (–)-naloxone is effective, while the (+)-enantiomer is considered inert at opioid receptors.^{122,123}

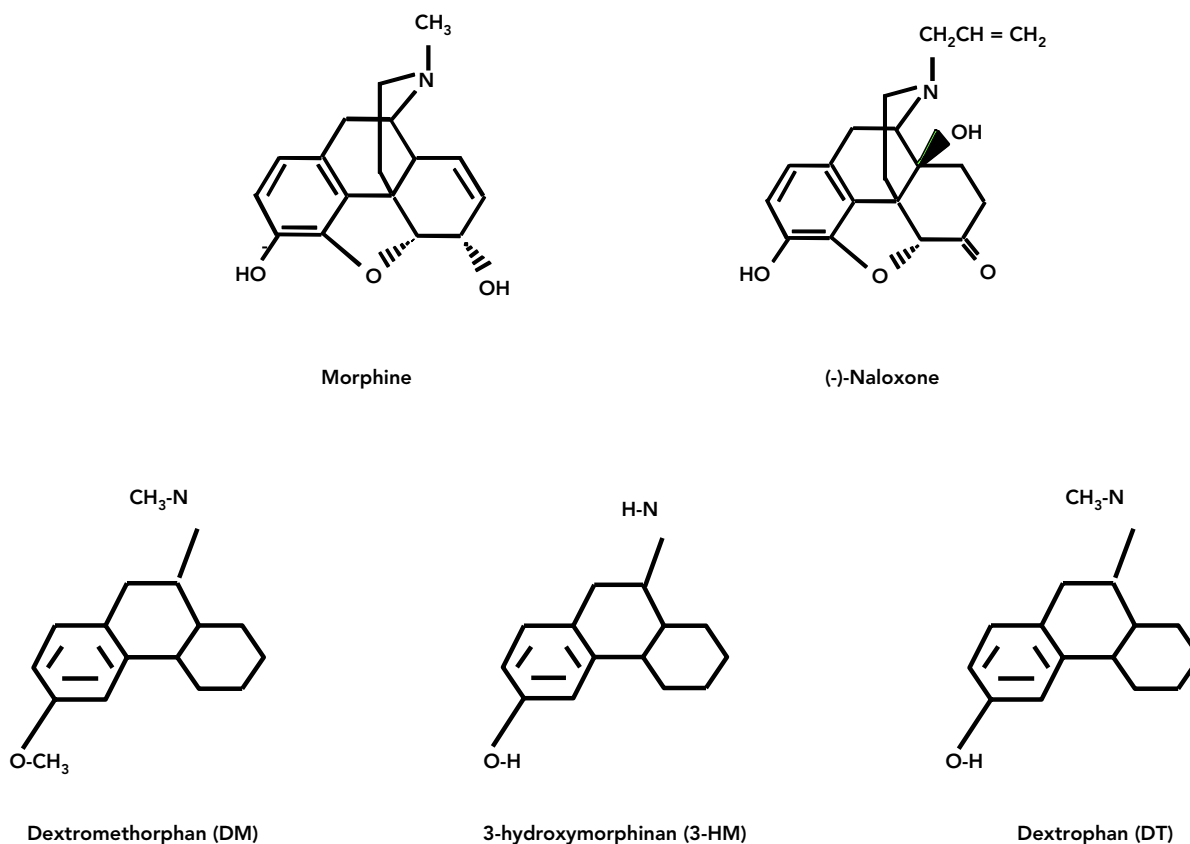


FIGURE 1. Structure of morphine and morphinans. DM is 3-methoxy-17-methylmorphinan; 3-HM (3-hydroxymorphinan), an *O*- and *N*-demethylated analog of DM.

VII.A. Naloxone Protects DA Neurons in Lipopolysaccharide (LPS)-Induced Inflammation-Related PD Model In Vivo and In Vitro

Inflammation-mediated DA neurodegeneration in the rat SN and in primary mesencephalic mixed neuron-glia cultures resulting from the targeted injection and treatment with LPS have been serving as widely accepted and useful *in vivo* and *in vitro* models, respectively. These models can be used to gain further insight into the pathogenesis and therapy of PD.

Microglia, the first line of defense in the brain, produce free radicals such as superoxide, contributing to neurodegeneration. Chang et al.¹²⁴ studied the effects of naloxone on the production of superoxide

from the murine microglial cell line, BV2, stimulated with LPS as measured by electron paramagnetic resonance (EPR). The production of superoxide triggered by phobol-12-myristate-13-acetate (PMA) resulted in SOD-inhibitable, catalase-uninhibitable 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) hydroxyl radical adduct formation. LPS enhanced the production of superoxide and triggered the formation of the non-heme iron/nitrosyl complex. Cells pretreated with naloxone showed significant reduction of superoxide production by 35%. However, the relationship between the neuroprotective effects of naloxone and microglial activation was not elucidated in this study.

Recent work from our laboratory demonstrated

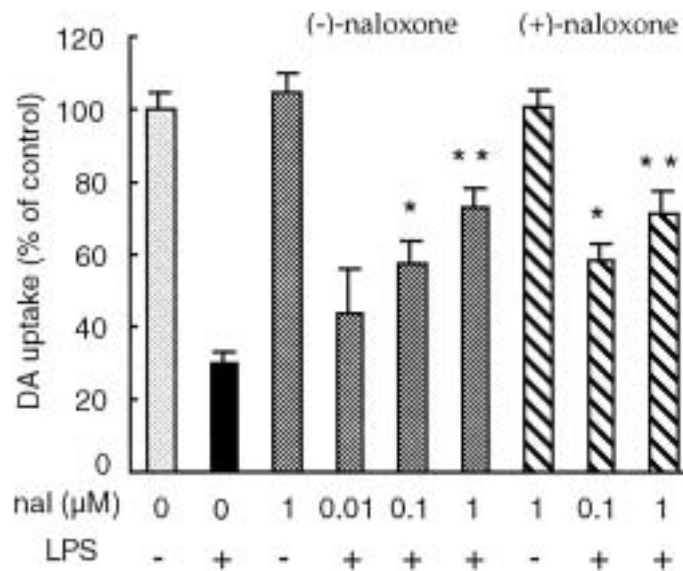


FIGURE 2. Comparison between naloxone stereoisomers for their effect on LPS-induced reduction of [^3H]dopaminergic uptake in midbrain cultures. Cells were pretreated with the indicated concentrations of naloxone isomers for 30 minutes, followed by LPS treatment for 24 hours. The dopaminergic uptake assay was performed. *nal*, naloxone; * $p < .005$, ** $p < .001$ compared with the LPS-treated cultures. (From *J Pharmacol Exp Ther* 2000; 293(2):607–617.)

that naloxone holds promise as a neuroprotective agent through the inhibition of neuro-inflammation characterized by microglial activation.^{120,125} Treatment of rat mesencephalic mixed neuron-glia cultures with LPS activated microglia to release pro-inflammatory and neurotoxic factors ($\text{TNF}\alpha$, NO, IL-1 β , and superoxide), which subsequently caused damage to midbrain DA neurons. The LPS-induced DA neurodegeneration was significantly reduced by naloxone (Fig. 2). The underlying mechanism for this protective effect of naloxone on DA neurons was shown to be related to the inhibition of the activation of microglia (Fig. 3) and their release of NO, $\text{TNF}\alpha$, and IL-1 β , and most importantly superoxide free radical (Fig. 4). Both naloxone and its opioid receptor inactive stereoisomer (+)-naloxone protected DA neurons with equal potency (Fig. 2). These results demonstrate that the underlying mechanism(s) of the neuroprotective effect of naloxone may be closely related to its ability to interfere with activation of

microglia and their production of pro-inflammatory and neurotoxic factors and that inhibition of microglial generation of superoxide free radical best correlates with the neuroprotective effect of naloxone isomers.¹²⁰

In the *in vivo* model of inflammation-mediated neurodegeneration, injection of LPS via an osmotic minipump into the rat SN led to the activation of microglia and degeneration of DA neurons: microglial activation was observed as early as 6 hours, and loss of DA neurons was detected 3 days after LPS injection.¹²⁶ Furthermore, the LPS-induced loss of DA neurons in the SN was time- and LPS concentration-dependent. Systemic infusion of either (-)-naloxone or (+)-naloxone inhibited the LPS-induced activation of microglia and significantly reduced the LPS-induced loss of DA neurons in the SN (Fig. 5). These *in vivo* results, combined with previous observations in the mesencephalic neuron-glia cultures, confirmed

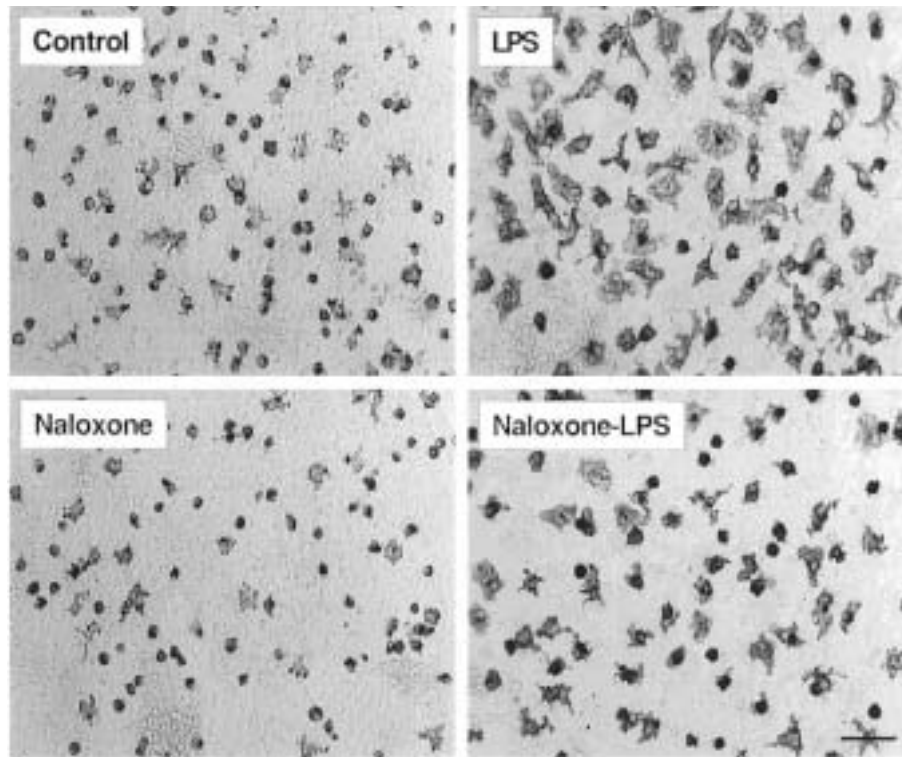


FIGURE 3. Immunocytochemical analysis for the effect of naloxone on LPS-induced morphological changes in OX-42-immunoreactive microglia. Cultures were pretreated with naloxone (1 μ M) for 30 minutes before treatment with 100 ng/mL LPS for 24 hours. Cells were fixed and stained with antibody OX-42. Scale bar, 100 μ m. In the control cultures, a significant portion of the OX-42-positive microglia were small in size. Following treatment with LPS, the microglia became activated with a greatly enlarged cell body and the characteristic shapes of activated microglia. Although naloxone alone did not significantly alter the appearance of the microglia, naloxone significantly inhibited the LPS-induced activation of microglia, as demonstrated by the return of the OX-42-positive cells to the morphology of untreated cells. (From *J Pharmacol Exp Ther* 2000; 293(2):607–617.)

that naloxone protected DA neurons against inflammation-mediated degeneration through inhibition of microglial activation and their release of pro-inflammatory and cytotoxic factors (NO, TNF α , IL-1 β , and most importantly superoxide free radical).¹²⁵ Thus, these studies demonstrate that both stereoisomers of naloxone attenuate the inflammation-mediated DA neurodegeneration in an opioid receptor-independent manner by inhibiting microglial activation both in vivo¹²⁰ and in vitro.¹²⁵

VII.B. Naloxone Protects DA Neurons Against β -Amyloid (A β) Peptide-Induced AD in an In Vitro Model

To determine whether naloxone exerts neuroprotective effects beyond LPS models of neurotoxicity, we have also used the other models of neurotoxin-induced neurodegeneration. For this purpose, we have determined the protective effect of naloxone and its isomers on A β (1-42)-induced neurodegeneration.¹²⁷ Pretreatment of either cortical or mesencephalic neuron-glia cultures with

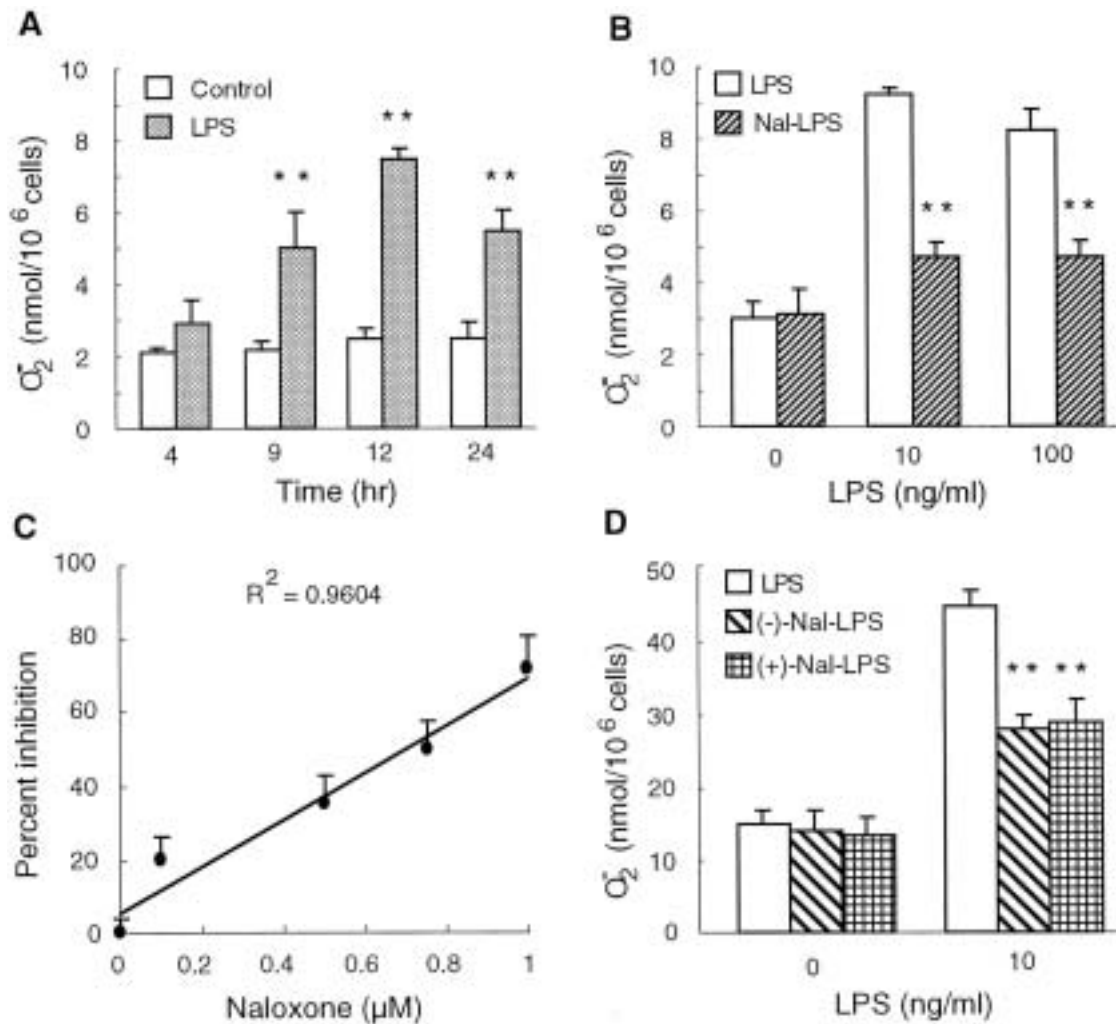


FIGURE 4. Effect of naloxone on LPS-induced generation of superoxide. Mesencephalic cultures (**A** and **B**) were treated with naloxone and/or LPS, and the treated cells were further stimulated with PMA for 90 minutes. Superoxide generation, measured as the SOD-inhibitable reduction of ferricytochrome c, was performed. **A**, time course for superoxide generation induced by 10 ng/mL LPS. $**p < .005$ compared with time-matched controls. **B**, effect of naloxone (nal) on LPS-induced generation of superoxide. Mesencephalic cultures were pretreated with 1 μ M naloxone for 30 minutes followed by treatment with the indicated doses of LPS for 12 hours. $**p < .005$ compared with the LPS-treated cultures. Similar results were obtained when supernatants from LPS- and/or naloxone-treated cultures were directly assayed for reduction of ferricytochrome c. **C**, naloxone concentration-dependent inhibition of LPS-induced superoxide formation. Mesencephalic cultures were pretreated with indicated concentrations of naloxone for 30 minutes, followed by treatment with the indicated doses of LPS for 12 hours. **D**, effect of naloxone on LPS-induced superoxide generation in rat microglia-enriched cultures. Enriched microglia (10^5 cells) were cultured in 24-well plates for 24 hours and then treated with the indicated naloxone isomers (1 μ M) followed by LPS (10 ng/mL) for 12 hours. Cells were then transferred to 96-well plates and superoxide generation was determined. $**p < .005$ compared with LPS-treated cultures. (From *J Pharmacol Exp Ther* 2000; 293(2):607–617.)

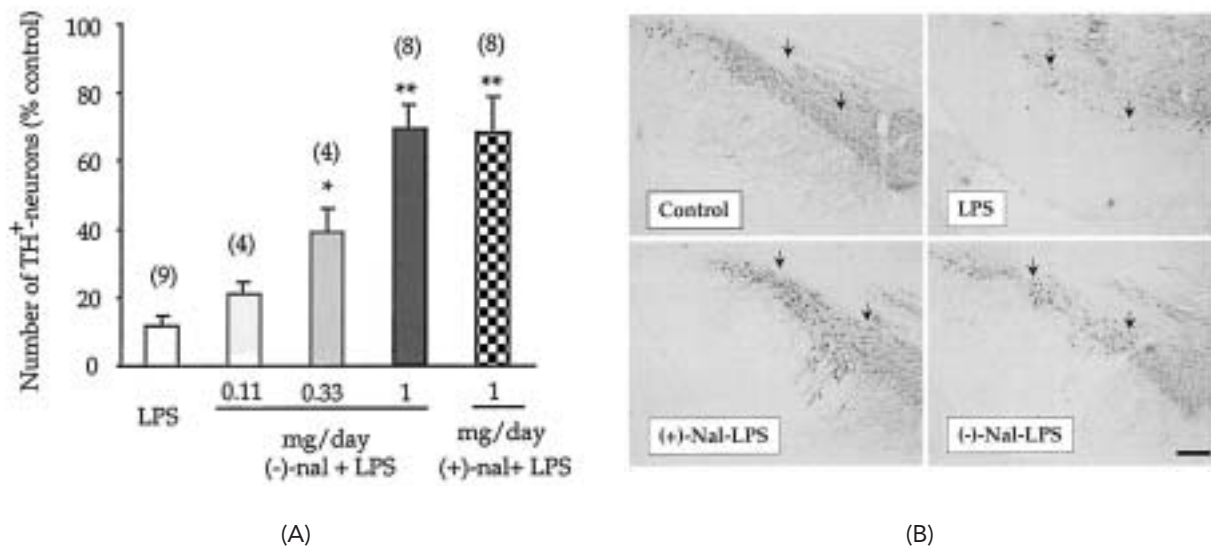


FIGURE 5. Effect of naloxone isomers on the LPS-induced loss of TH-immunoreactive neurons in the SN. **A**, rats were infused systemically with 0.11 to 1.0 mg/day (-)-naloxone or 1 mg/day (+)-naloxone. Infusion was initiated 24 hours before the injection of LPS. Five days after LPS (2.5 μ g) injection, brains were harvested and sectioned. Each or every other of the 24 sections was immunostained with an anti-TH antibody, and the number of TH-immunoreactive neurons in the SN was counted. Results were expressed as a percentage of the number of TH-positive neurons of the side of brain injected with saline. Numbers in parentheses indicate the number of animals used for each group. * $p < .01$ and ** $p < .001$ compared with the group treated with LPS only. **B**, immunohistochemical analysis of the effect of naloxone isomers on LPS-induced degeneration of TH-immunoreactive neurons in the SN. Brains were obtained from rats infused with 1 mg/day of either naloxone isomer followed by the injection of LPS (2.5 μ g; 5 days). The brain sections were immunostained with an anti-TH antibody. Shown herein are representative brain sections. Arrow, TH-immunoreactive neurons. Scale bar, 250 μ m. (From *J Pharmacol Exp Ther* 2000; 295(1):125–132.)

1–10 μ M (-)-naloxone prior to treatment with 0.1–3 μ M A β (1–42) afforded significant neuroprotection, as judged by DA uptake (Fig. 6), immunocytochemical analysis, and cell counting. More importantly, (+)-naloxone, the ineffective enantiomer of (-)-naloxone in binding opioid receptors, was equally effective in affording neuroprotection. Mechanistically, inhibition of A β (1–42)-induced superoxide production from microglia underlay the neuroprotective effect of naloxone stereoisomers (Fig. 7).

Moreover, a neuroprotective effect comparable to that of naloxone and inhibition of A β (1–42)-induced superoxide production was also achieved

with naloxone methiodide, a charged analog with quaternary amine, suggesting that the site of action for naloxone isomers is at the cell surface of microglia. However, the specific site of action for naloxone on microglia remains undescribed.¹²⁷

These results demonstrated that naloxone isomers, through mechanisms independent of the traditional opiate receptors, were capable of inhibiting A β (1–42)-induced microglial activation and degeneration of both cortical and mesencephalic neurons. Combined with our previous findings with inflammagen-induced neurodegeneration, naloxone analogs, especially (+)-naloxone, may have potential therapeutic efficacy for

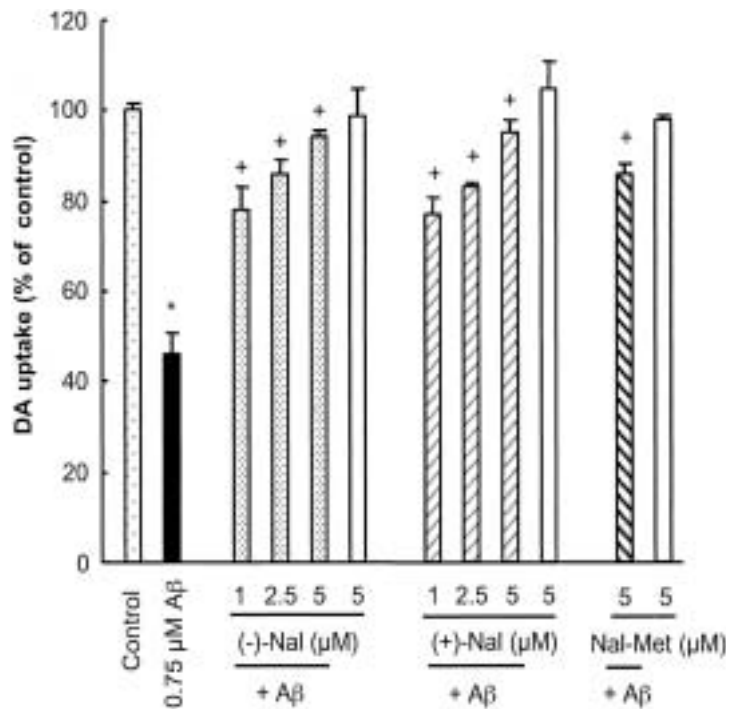


FIGURE 6. Effect of naloxone analogs on AB (1-42)-induced decrease in DA uptake in mesencephalic neuron-glia cultures. Cultures were pretreated with the indicated concentrations of (-)-naloxone, (+)-naloxone, or naloxone methiodide for 30 minutes before treatment for 9 days with 0.75 μM AB (1-42). Afterward, DA uptake was performed. **AB**, AB (1-42); **(-)-Nal**, (-)-naloxone; **(+)-Nal**, (+)-naloxone; **(-)-Nal-Met**, (-)-naloxone methiodide. * $p < 0.05$ compared with the control cultures; +, $p < 0.05$ compared with the AB(1-42)-treated cultures. (From *J Pharmacol Exp Ther* 2002; 302(3): 1212–1219.)

the treatment of both PD and AD. Thus, the seeming lack of selectivity in its neuroprotective effect may actually suggest a broader spectrum of efficacy in combating various inflammation-related neurodegenerative disorders.

VII.C. Femtomolar Concentrations of Naloxone Are Neuroprotective

We have previously reported that femtomolar concentrations of the opiate peptide dynorphin,¹⁻¹⁷ a κ -receptor agonist, protect mesencephalic DA neurons from microglia-mediated neurotoxicity through a mechanism that was independent of the traditional

opiate receptors.¹²⁸ This opiate peptide can be reduced to the smallest biologically active fragment required for DA neuroprotection, glycine-glycine-phenylalanyl (GGF).

Qin et al.¹³² found that GGF and naloxone at femtomolar concentrations showed similar dose response and efficacy when these compounds were compared for their ability to inhibit microglial activation and confer DA neuroprotection from LPS in vitro (Fig. 8). Furthermore, femtomolar concentrations of both GGF and naloxone were shown to attenuate microglial response to LPS by inhibition of NADPH oxidase activation. While GGF is a component of dynorphin, which binds to the κ receptor, GGF peptide fragment is unable to bind the κ -receptor.

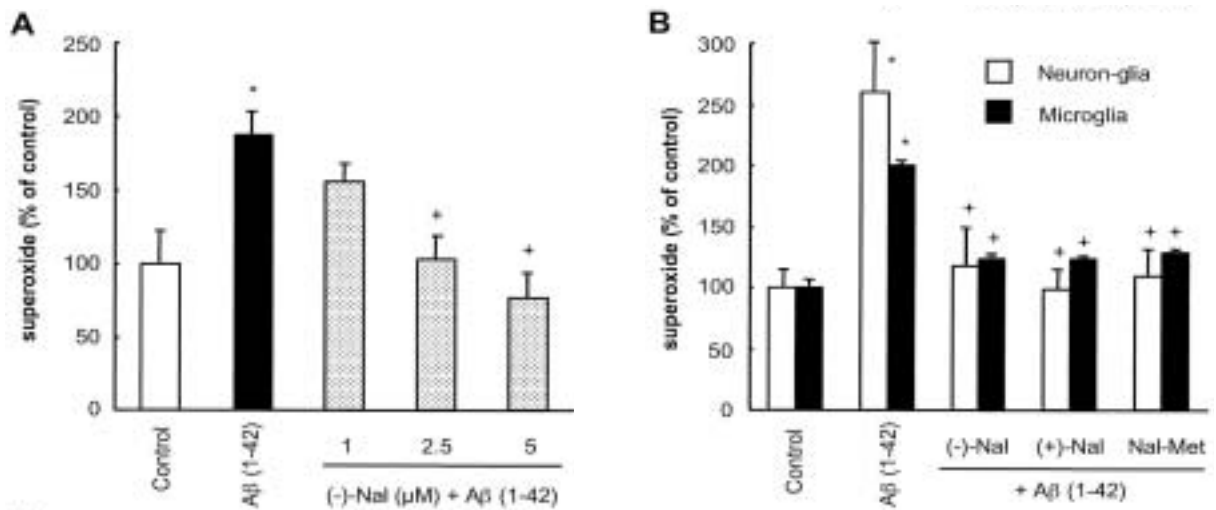


FIGURE 7. Effect of naloxone analogs on AB (1-42)-induced production of superoxide. **A**, mesencephalic neuron-glia cultures were pretreated for 30 minutes with the indicated concentrations of (-)-naloxone prior to stimulation with 0.3 μ M AB (1-42). **B**, mesencephalic neuron-glia cultures or microglia-enriched cultures were pretreated for 30 minutes with a 5 μ M concentration of the indicated naloxone analogs prior to stimulation with 0.3 μ M AB (1-42). Superoxide generation was then measured. $p < 0.05$ compared with the control cultures; +, $p < 0.05$ compared with the AB (1-42)-treated cultures. (From *J Pharmacol Exp Ther* 2002; 302(3):1212–1219.)

Naloxone, a nonspecific opiate antagonist, is also neuroprotective at femtomolar concentrations, indicating a femtomolar-acting mechanism independent of the traditional opiate receptor. Pharmacophore analysis of dynorphin peptides and naloxone revealed common chemical properties (hydrogen bond acceptor group, hydrogen bond donor group, positive ionizable group, and hydrophobic group) of these femtomolar-acting compounds. These results support that GGF and naloxone share similar effects, common chemical properties, and mechanisms at femtomolar doses, suggesting that naloxone is a femtomolar mimetic for dynorphin, independent of the traditional opiate receptor.

NADPH oxidase is involved in the neuroprotective action of naloxone at both micromolar and femtomolar concentrations; however, the detailed mechanism is not yet clear. We speculate that both micromolar and femtomolar concentrations of naloxone and GGF inhibit the enzyme activity of NADPH oxidase by

binding to the different sites of action on the gp91 subunit. Research is ongoing in our lab to test this hypothesis.

VII.D. Naloxone Protects Against Brain Ischemia

The pathogenesis of cerebral I/R involves cytokine/chemokine production, inflammatory cell influx, astrogliosis, cytoskeletal protein degradation, and breakdown of the blood–brain barrier.⁴¹ (-)-Naloxone is able to reduce infarct volume and has been used as a therapeutic agent for cerebral I/R injuries. After cerebral I/R, the neuronal damage was strongly associated with gliosis, inflammatory cell infiltration, cytokine/chemokine overproduction, and matrix metalloproteinase-9 activation. (-)-Naloxone pretreatment suppresses post-ischemia-induced inflammation and neuronal damage. Therefore, (-)-naloxone administration might be an effective therapeutic intervention for

reducing ischemic injuries in which the anti-inflammatory mechanism may be involved.⁴¹

VIII. DM IS NEUROPROTECTIVE

Based on our findings that naloxone had significant neuroprotective effect on DA neurons mediated through a non-opioid receptor mechanism, we

speculated that another morphinan compound DM, which shares the basic morphine-like structure with naloxone, might also show neuroprotective effect in a NMDA receptor-independent fashion. Thus, studies were conducted to test this hypothesis, and we found that DM and its analogs are protective of the DA neurons against inflammation-related neurodegeneration through a mechanism similar to that of naloxone.

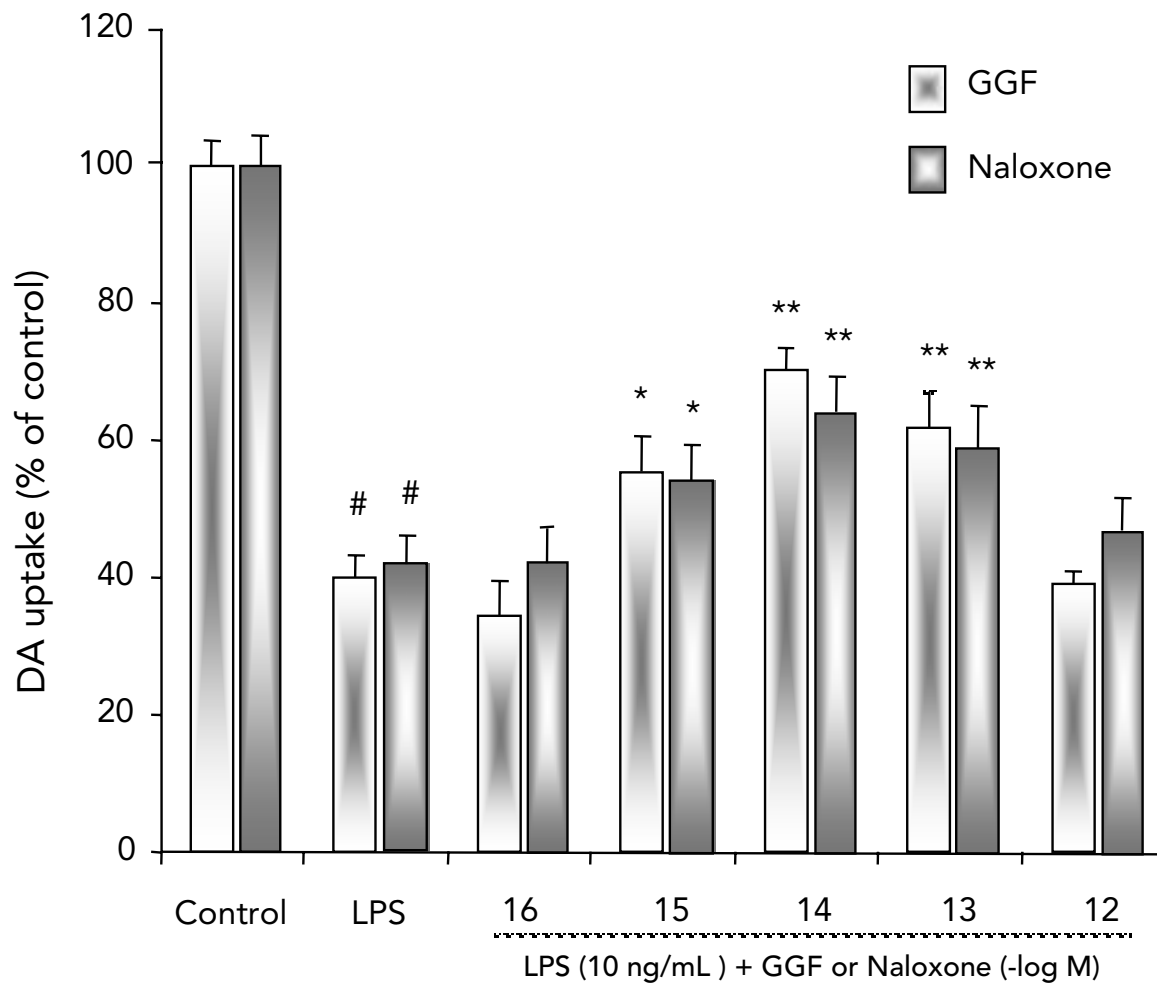


FIGURE 8. Femtomolar concentrations of a tripeptide, GGF, and naloxone protect DA neurons from LPS-induced neurotoxicity. DA neurotoxicity was measured at 7 days post-treatment using the [³H] DA uptake assay. * $p < 0.05$, ** $p < 0.01$ compared to LPS treatment. (From FASEB J 2005; 19(6):550–557.)

VIII.A. DM Protects Against LPS-Induced DA Neurodegeneration In Vitro

First, we have reported that DM protected DA neurons against inflammation-mediated degeneration.¹²⁹ Our novel finding was that 1–10 μM DM protected DA neurons against LPS (10 ng/mL)-induced reduction of DA uptake functionally in rat primary mixed mesencephalic neuron-glia cultures (Fig. 9). DM (10 μM) significantly attenuated the LPS-induced reduction in the number of DA neurons (Fig. 9). Morphologically, in LPS-treated cultures, in addition to the reduction of abundance of DA neurons, the dendrites of the remaining DA neurons were significantly less elaborate than those of controls. In cultures pretreated with DM (10 μM) before LPS stimulation, DA neurons were significantly more numerous and the dendrites less affected. Significant neuroprotection was observed in

cultures with DM added up to 60 minutes after the addition of LPS. Thus, DM significantly protects DA neurons not only with pretreatment but also with post-treatment.

It is well known that 1-methyl-4-phenylpyridinium (MPP⁺), the active component of MPTP, damages DA neurons directly. Pretreatment of neuron-enriched cultures with DM (10 μM) did not significantly alter the magnitude of the MPP⁺-induced reduction of DA uptake, suggesting that the neuroprotective effect of DM was mediated through microglia.

Activated microglia secrete a variety of pro-inflammatory and neurotoxic factors, including ROS and cytokines. We found that DM dose-dependently decreased the production of superoxide, NO and TNF α either in neuron-glia or microglia-enriched cultures after LPS treatment. So the neuroprotective

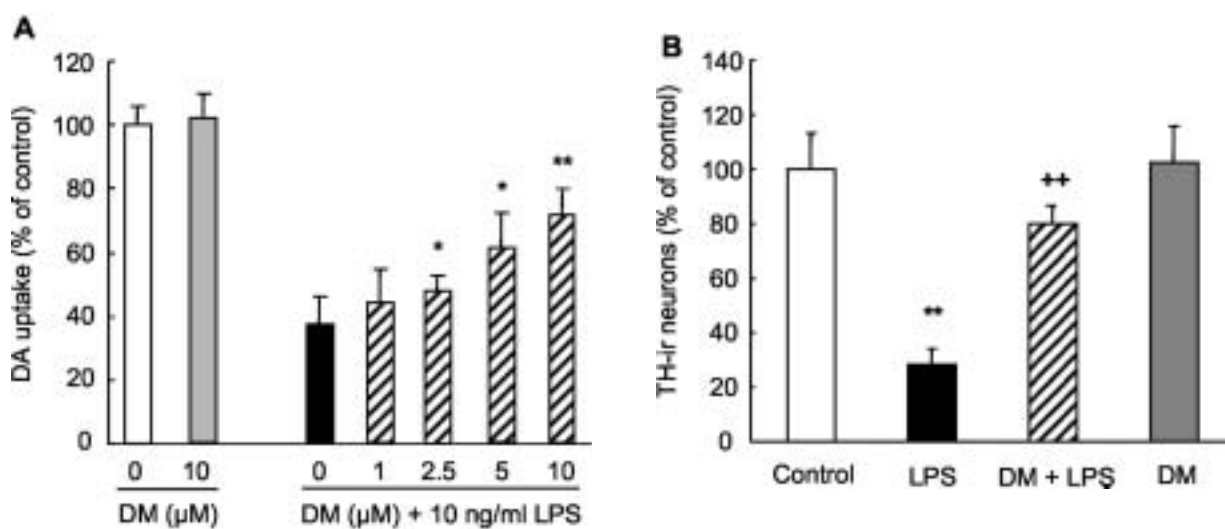


FIGURE 9. Effect of DM on LPS-induced degeneration of DA neurons in mesencephalic neuron-glia cultures. Cultures were treated with vehicle alone, 10 μM DM alone, or pretreated for 30 minutes with indicated concentrations of DM before treatment with 10 ng/mL LPS. Seven days later, neurotoxicity was assessed by DA uptake (A) or counting of TH-ir neurons after immunostaining with an anti-TH antibody (B). Results in (A) are expressed as a percentage of the control cultures. ** $p < 0.001$; * $p < 0.01$ compared with the control cultures. Results in (B): ** $p < 0.005$ compared with the control cultures; ++, $p < 0.005$ compared with the LPS-treated cultures. (From *J Pharmacol Exp Ther* 2003; 305(1): 212–218.)

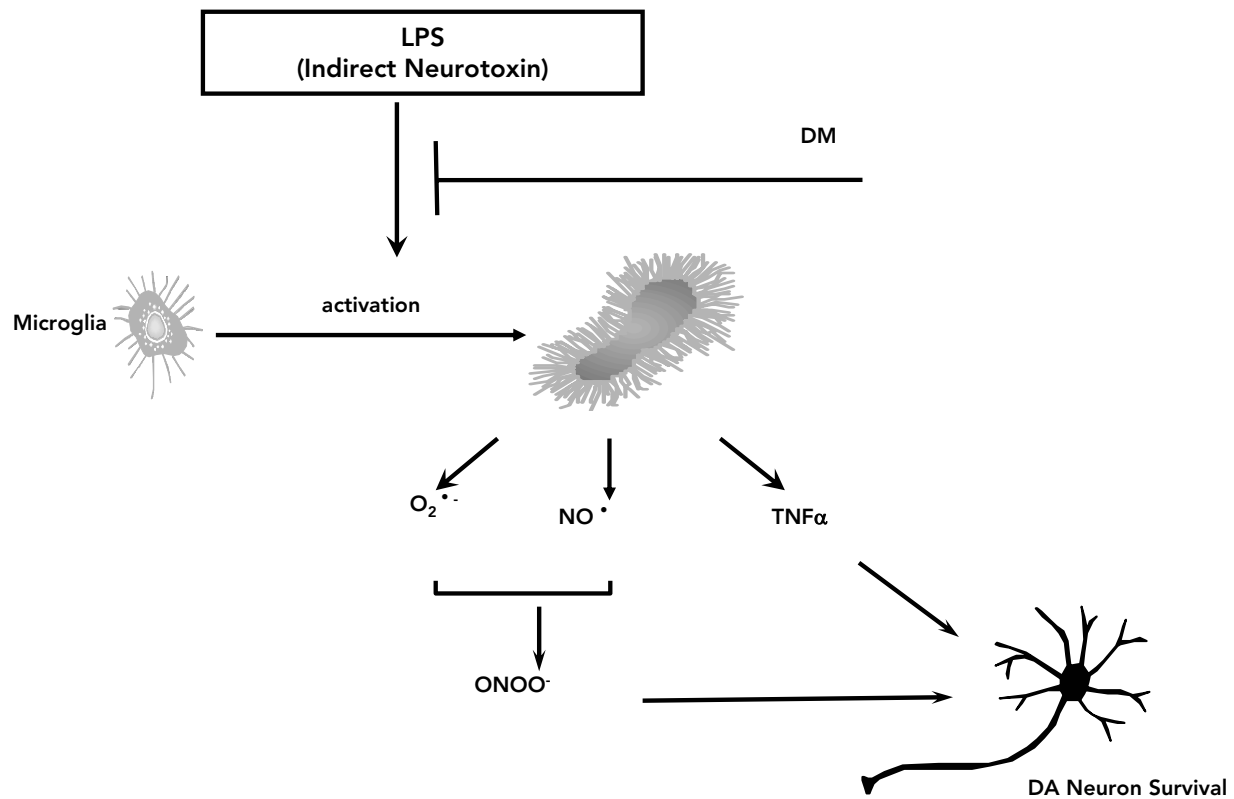


FIGURE 10. Proposed mechanism of DM's neuroprotection in in vitro LPS model. Inflammagen LPS stimulates microglia to produce an array of neuro-inflammatory and neurotoxic factors including superoxide, NO, and TNF α . The neuroprotective effect of DM on DA neurons against LPS-induced neurotoxicity in primary mesencephalic neuron-glia cultures is directly associated with its ability to inhibit microglial activation and subsequent release of superoxide, NO, and TNF α , thus promoting the survival of DA neurons.

effect of DM on DA neurons did not appear to involve NMDA receptors, but rather directly related to its ability to inhibit the microglial activation with the release of superoxide, NO and TNF α , among which inhibition of superoxide production by DM was most pronounced (Fig. 10).

The mechanism of the neuroprotective effect of DM in this PD model is associated with the inhibition of microglial activation but not with its NMDA receptor antagonist property. We have examined several NMDA receptor antagonists, including MK801, AP5, and memantine, in the LPS in vitro PD model (unpublished observations). Results from these stud-

ies indicate that among these compounds tested there was no correlation between the affinity of NMDA receptor antagonist activity and the potency of the neuroprotection on DA neurons. On the contrary, a better correlation was found between the anti-inflammatory potency and the neuroprotection. These results suggest that the DA neuroprotection provided by DM in the inflammation-related neurodegenerative models is not mediated through the NMDA receptor. This conclusion is not in conflict with the previous reports, indicating that NMDA receptor blockade is associated with the neuroprotective effect of DM in acute glutamate-induced excitotoxicity models.

VIII.B. DM Protects DA Neurons from MPTP-Induced Damage Through Inhibition of Reactive Microgliosis

Recent animal studies from our laboratory revealed that DM also exerted potent protection for DA neurons in the subchronic MPTP mice model, in which the underlying mechanisms for neuroprotective activity of DM were also investigated using wild-type and NADPH oxidase-deficient mice.¹⁰⁹ We first found that C57 wild-type mice that received daily MPTP (15 mg free base/kg body weight s.c.) injections exhibited significant loss of DA neurons in the SNpc. However,

the MPTP-elicited neuronal loss was significantly attenuated in those mice receiving daily injections of DM (10 mg/kg body weight).

NADPH oxidase is the primary enzyme producing extracellular superoxide in microglia, and we speculate that NADPH oxidase may contribute to MPTP-induced neurotoxicity.^{87,109-114} We found that NADPH oxidase-deficient mice exhibited significant resistance to MPTP-induced DA neurodegeneration compared to wild-type mice. In addition, the neuroprotective effect of DM was only observed in wild-type mice, but not in NADPH oxidase-deficient mice (Fig. 11). These results provide strong evidence

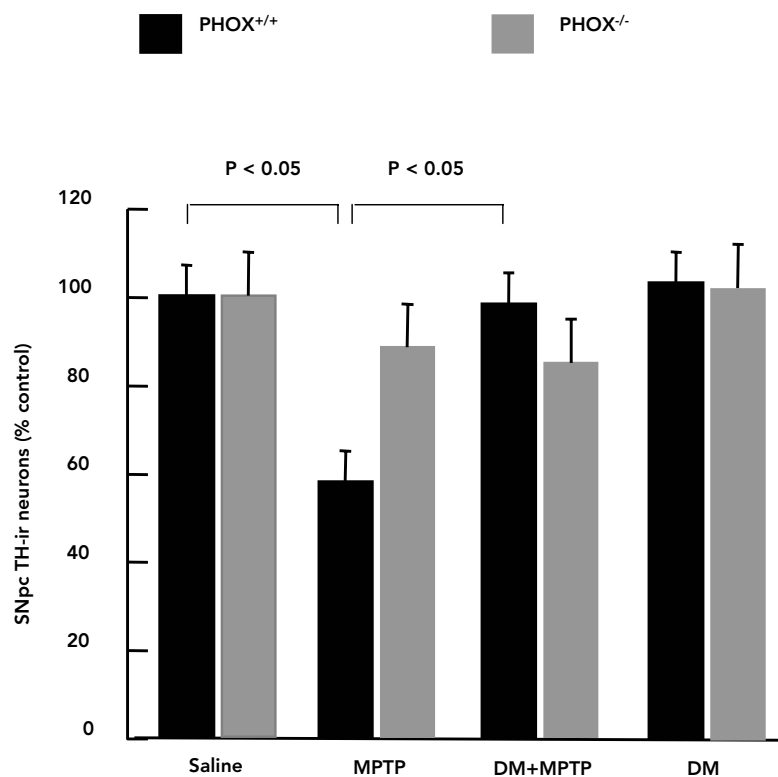


FIGURE 11. Lack of neuroprotective effect of DM in NADPH oxidase-deficient mice. Wild-type or NADPH oxidase-deficient mice received saline, DM (10 mg/kg body weight s.c.), and/or MPTP (15 mg free base/kg body weight s.c.) injections. Six days after the last MPTP injection, mice were sacrificed and brain sections were stained for TH-ir neurons. Eight mice were used for each group. The differences were analyzed using a multi-factorial ANOVA; a difference of $p < 0.05$ was considered significant. (From FASEB J 2004; 18(3):589–591.)

indicating that NADPH-oxidase is a critical target mediating DM's neuroprotective activity in the MPTP *in vivo* model.

We have reported that the reactive microgliosis is associated with the MPTP-induced DA neurodegeneration,¹¹² and we further propose that the inhibition of reactive microgliosis underlie the neuroprotective effect against MPTP toxicity by DM. The initial damage of dead neurons elicited by MPTP induces reactive microgliosis, which in turn activates NADPH oxidase through the translocation of cytosolic subunits to the cell membrane and generation of superoxide in wild-type mice. The neuroprotective effect of DM is attributed to its blockade of reactive microgliosis induced by DA neuronal death through the inhibition of both extracellular superoxide and intracellular reactive oxygen species (iROS). However, in NADPH-deficient mice, reactive microgliosis fails to occur because of the lack of gp91 membrane-binding subunit, and hence DM can not exert its neuroprotective action (Fig. 12).

3. Femtomolar Concentrations of DM are Neuroprotective

Similar to naloxone, femtomolar concentrations of DM also significantly decreased LPS-induced production of NO, TNF α , PGE₂, and superoxide free radicals in primary microglia-enriched and mesencephalic neuron-glia cultures. The important role of superoxide in this ultra-low-concentration phenomenon was further demonstrated through DM's failure to show a neuroprotective effect in neuron-glia cultures from NADPH oxidase-deficient mice. These results reveal that the protective action of femtomolar concentrations of DM is also glia mediated and that inhibition of the production of superoxide plays a pivotal role in the neuroprotective action of DM. In addition, the mechanism of DM in reducing NO and PGE₂ is through inhibition of activity of iNOS and COX2 (Guorong Li et al.¹³³). Similar to the initial findings reported with femtomolar concentrations of naloxone, DM is also likely a femtomolar-acting GGF mimetic.

VIII.D. 3-HM Is Neurotrophic to DA Neurons and Neuroprotective Against LPS-Induced Neurotoxicity

In order to find more potent neuroprotective agents, structure-activity studies were performed. After screening a series of DM analogs, 3-HM, a DM analog missing methyl groups at the *O* and *N* sites, emerged as a novel candidate for the treatment of PD. Our study showed that 3-HM was more potent in neuroprotection against LPS-induced neurotoxicity than its parent compound, DM.¹³⁰

3-HM was first found to be neuroprotective against LPS-induced DA neurotoxicity and was also neurotrophic to DA neurons in primary mixed mesencephalic neuron-glia cultures (Fig. 13).¹³⁰ The neurotrophic effect of 3-HM was glia dependent, in that 3-HM failed to show any protective effect in neuron-enriched cultures. We subsequently demonstrated that it was the astroglia and not the microglia that contributed to the neurotrophic effect of 3-HM. This conclusion was based on the reconstitution studies, in which we added different percentages of microglia (10–20%) or astroglia (40–50%) back to the neuron-enriched cultures and found that 3-HM was neurotrophic after the addition of astroglia, but not microglia.

Furthermore, 3-HM-treated astroglia-derived conditioned media exerted a significant neurotrophic effect on DA neurons (Fig. 14). It appeared likely that 3-HM caused the release of certain neurotrophic factor(s) from astroglia, which in turn was responsible for the neurotrophic effect of 3-HM. As may be expected, there is also a considerable glial reaction in the SNpc in PD that can potentially be protective in addition to the detrimental effect on DA neurons. Glial cells, especially astroglia, protect stressed DA neurons through production of neurotrophic factor(s) that counteract oxidative stress. One potential therapeutic avenue will be to stimulate astroglia to produce these neurotrophic factors to rescue damaged or dying neurons. Currently, the identity of the neurotrophic factor(s) released from astroglia induced by 3-HM is being undertaken.

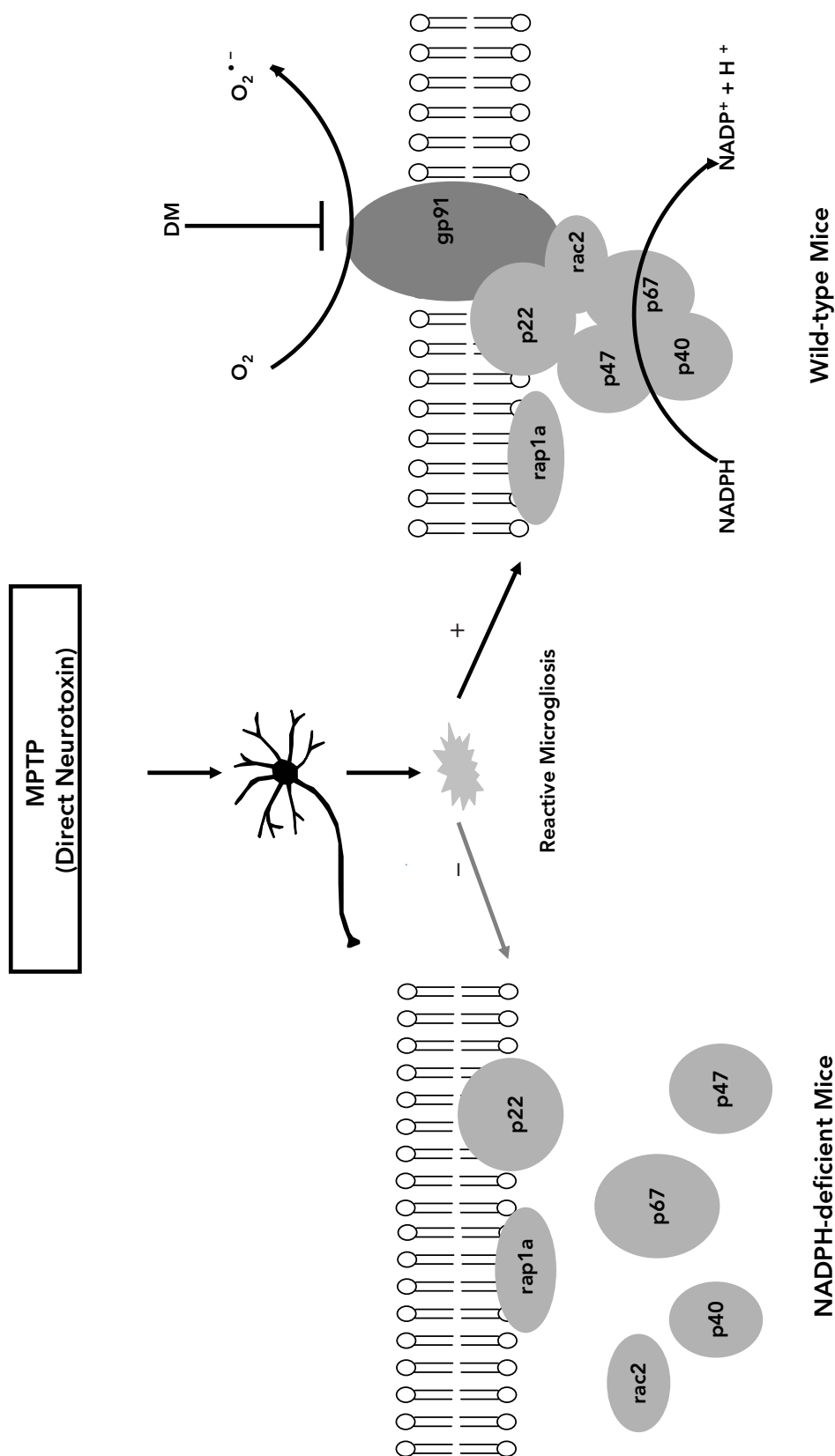


FIGURE 12. Mechanism for the neuroprotective effect of DM against MPTP-induced DA neurodegeneration in vivo. In wild-type mice, the damaged DA neurons directly induced by MPTP produce secondary microglial activation (reactive microgliosis), which triggers the translocation of cytosolic subunits of NADPH oxidase to the cell membrane and binds to the gp91 catalytic subunit, resulting in production of superoxide. The neuroprotective action of DM is attributed to its effective blockade of the reactive microgliosis induced by DA neuronal death through inhibiting the production of superoxide. In contrast, reactive microgliosis fails to induce the production of superoxide in NADPH-deficient mice because of the lack of the gp91 catalytic subunit. DM fails to show the neuroprotective effect as a result of the loss of its active binding site.

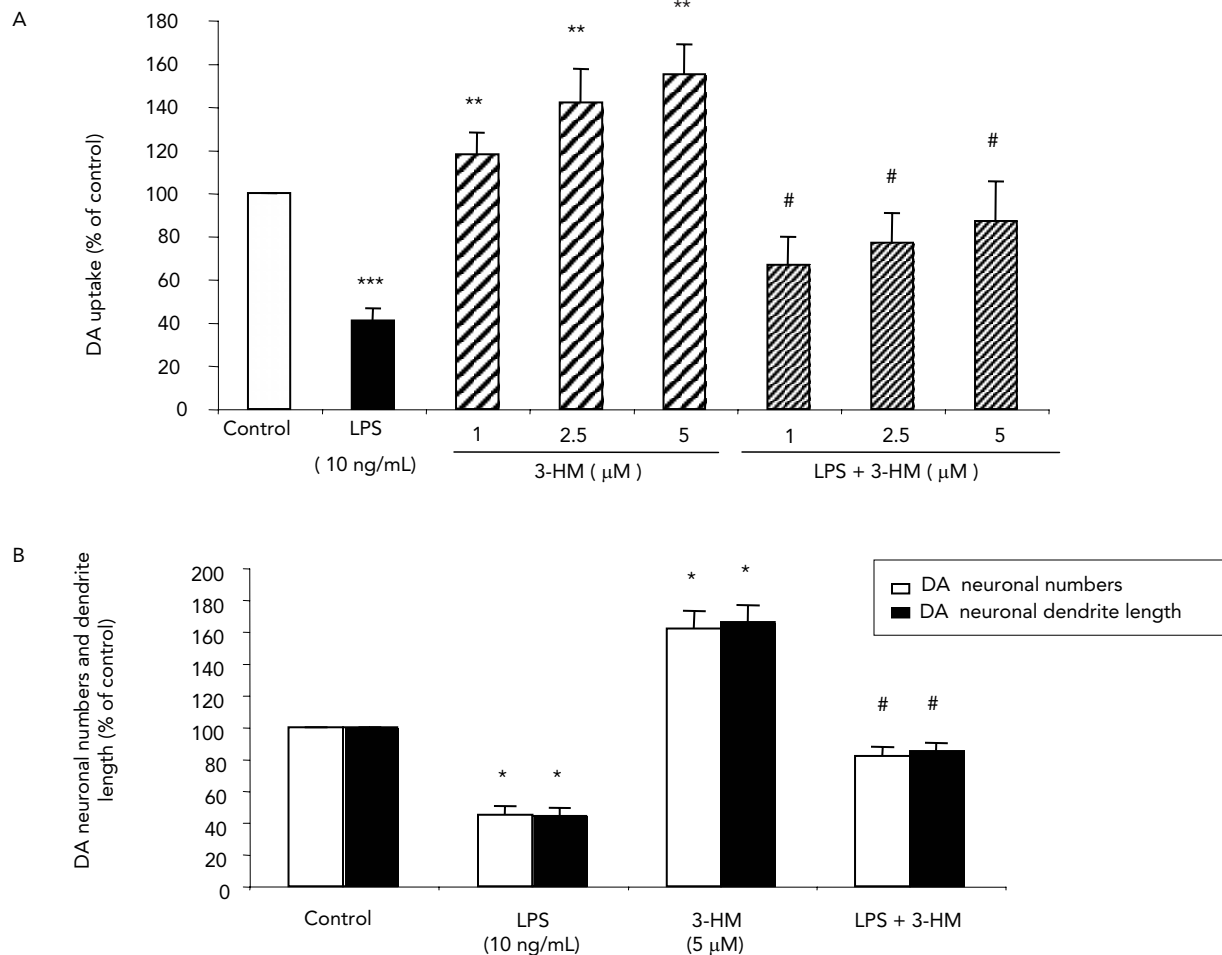


FIGURE 13. 3-HM is neurotrophic to DA neurons and is also neuroprotective against LPS-induced neurotoxicity. Rat primary mesencephalic neuron-glia cultures seeded in 24-well plates at 5×10^5 /well were pretreated with 1–5 μM 3-HM for 30 minutes before addition of 10 ng/mL LPS. Seven days later, LPS-induced DA neurotoxicity was quantified by [^3H] DA uptake assay (A), immunocytochemical analysis, including TH-ir neuron counts and dendrite length measurements (B). The differences were analyzed using a multifactorial ANOVA; a difference with $p < 0.05$ was considered significant. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, compared with corresponding vehicle-treated control cultures, # $p < 0.05$ compared with LPS-treated cultures. (From FASEB J 2005; 19(3):395–397.)

In addition to the neurotrophic effect, the anti-inflammatory mechanism was also important for the neuroprotective activity of 3-HM because the more microglia that were added back to neuron-enriched cultures, the more pronounced the LPS-induced neurotoxicity and the more significant the neuroprotective effect exerted by 3-HM. The anti-inflam-

atory mechanism of 3-HM was attributed to its inhibition of LPS-induced production of an array of pro-inflammatory and neurotoxic factors, including NO, $\text{TNF}\alpha$, PGE_2 , and ROS.

Thus, 3-HM provides potent neuroprotection by acting on two different cell targets: a neurotrophic effect mediated by astroglia and an anti-inflammatory

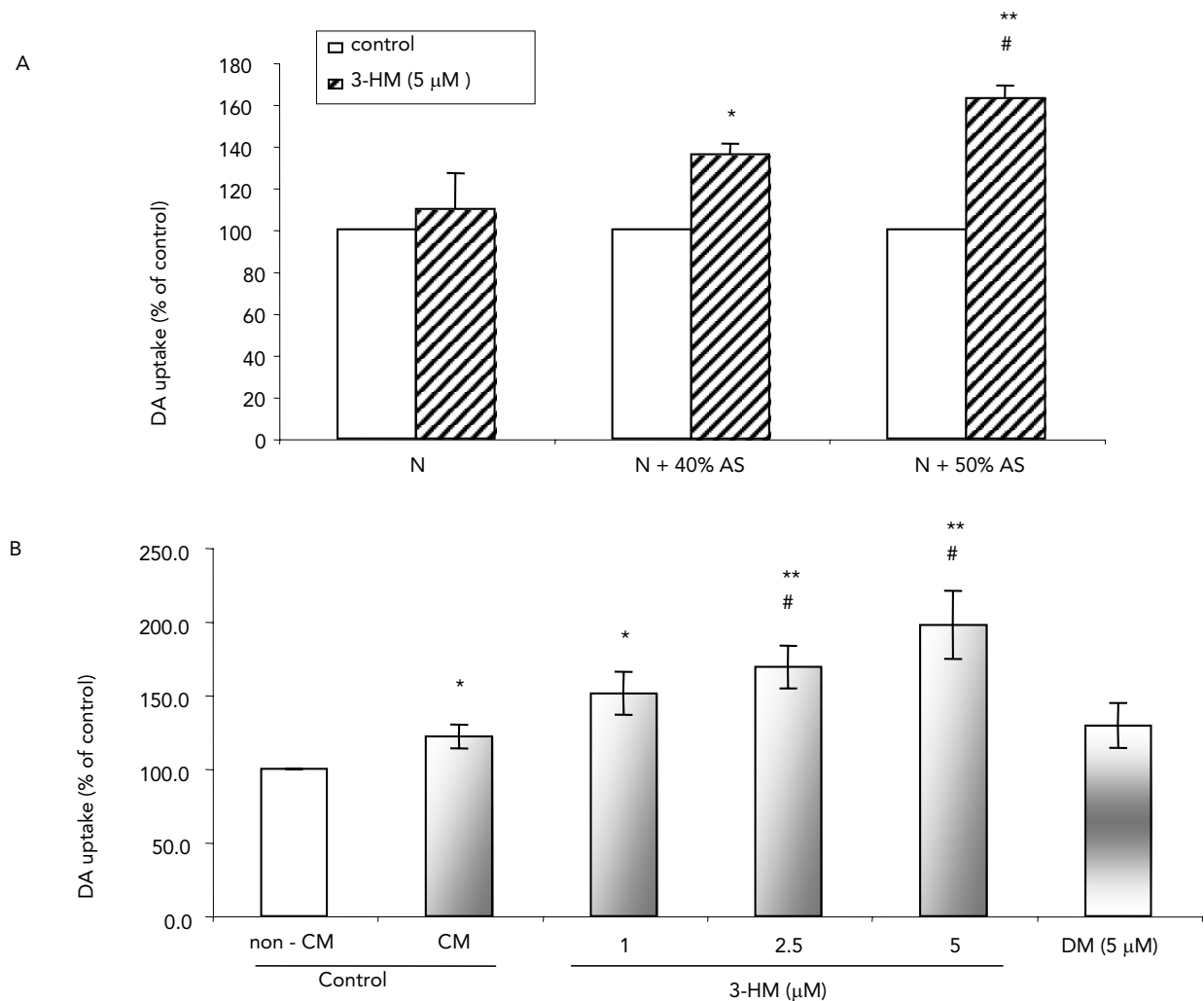


FIGURE 14. Astroglia are the contributors to the neurotrophic effect of 3-HM. 40% and 50% (2×10^5 /well and 5×10^5 /well) of astroglia were added back to the neuron-enriched cultures and treated with $5 \mu\text{M}$ 3-HM. [^3H]DA uptake was performed 10 days after treatment. Results were expressed as percentage of vehicle-treated control cultures. The differences were analyzed using a multifactorial ANOVA; a difference with $p < 0.05$ was considered significant. * $p < 0.05$, ** $p < 0.001$ compared with corresponding vehicle-treated control cultures. # $p < 0.05$ compared with 40% astroglia added back cultures (A). Astroglia conditioned media increase the DA uptake capacity in the neuron-enriched cultures. Astroglia-enriched cultures were pretreated with 3-HM (1–5 μM) 24 hours after initial seeding. Conditioned media were collected 24 hours later and added to the neuron-enriched cultures. Ten days after adding the conditioned media, the [^3H] DA uptake measurements were performed. Results were expressed as a percentage of the vehicle-treated non-conditioned control cultures and were the mean \pm S.E.M. from four independent experiments in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared with the vehicle-treated non-conditioned control cultures, # $p < 0.05$ compared with the vehicle-treated conditioned control cultures. **CM**, conditioned medium; **non-CM**, non-conditioned medium (B). (From FASEB J 2005; 19(3):395–397.)

effect mediated by inhibition of microglial activation. The higher potency of 3-HM is attributed to its additional neurotrophic effect in addition to the anti-inflammatory mechanism shared by both DM and 3-HM (Fig. 15).

5. 3-HM Protects DA Neurons Against MPTP-Elicited Degeneration In Vivo and In Vitro

To continue this line of research, we investigated the neuroprotective property of 3-HM in another PD model. We found that 3-HM provided neuroprotection in the MPTP model, in both in vivo and in vitro studies. In

the in vitro system, using primary mixed mesencephalic neuron-glia cultures, 1–5 μM 3-HM significantly and dose-dependently attenuated MPTP-induced, or its active component MPP⁺-induced, reduction in DA uptake; 1–5 μM 3-HM alone resulted in a significantly high capacity of DA uptake compared with controls, confirming its neurotrophic effect.

In vivo studies showed that administration of 3-HM (5 mg/kg body weight, s.c., twice daily) significantly reduced MPTP (15 mg free base/kg body weight, s.c., once daily)-induced loss of DA neurons in the SNpc, which is similar to the effect exerted by DM. In the striatum, significant depletion of DA and its metabolites 3,4-dihydroxyphenylacetic acid

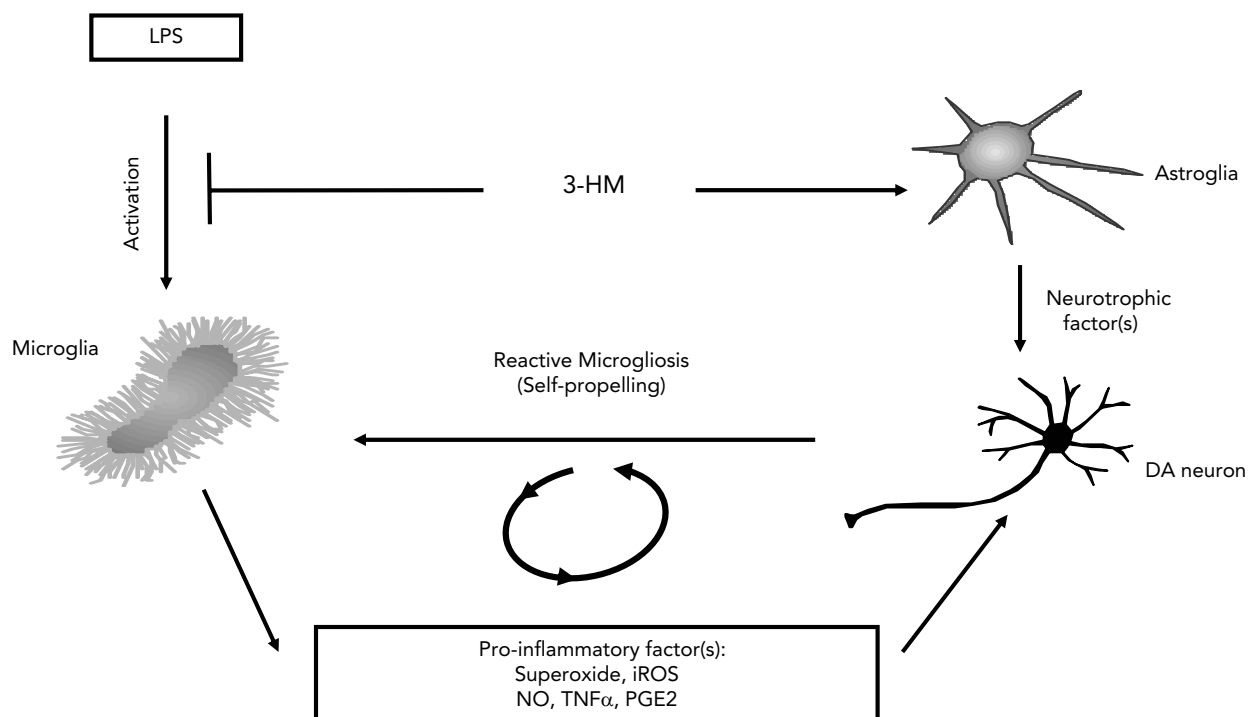


FIGURE 15. Dual mechanisms of the protective effect of 3-HM in LPS-induced DA neurotoxicity in primary mesencephalic neuron-glia cultures. 3-HM provides potent neuroprotection through dual mechanisms by acting on two different targets: first is a neurotrophic effect mediated by astroglia, which may produce and release neurotrophic factor(s) and promote the survival of DA neurons after LPS challenge; second is an anti-inflammatory activity mediated by the inhibition of microglial activation and its subsequent generation of a variety of pro-inflammatory and neurotoxic factors, including superoxide, iROS, NO, TNF α , and PGE $_2$. Both the neurotrophic and anti-inflammatory effects will prevent the vicious cycle, which occurs with the participation of microglial activation, release of pro-inflammatory factors, the death of DA neurons, and reactive microgliosis.

(DOPAC) and homovanillic acid (HVA), as well as serotonin and its metabolite 5-hydroxyindolacetic acid (5-HIAA), were observed in MPTP-treated mice compared with controls. The levels of all of these biogenic amines were attenuated in the lesioned mice that received 3-HM administration (Fig. 16). Compared to DM, the advantage of 3-HM is the neurotrophic effect, which may enhance the sprouting of DA terminal fibers of the striatum, accelerate the formation of DA-containing vesicles, assist the

resynthesis of DA after the lesion, and eventually reestablish the return of DA to normal function.

In addition to the neurotrophic effect, the anti-inflammatory effect exerted by 3-HM resulting from the inhibition of microgliosis generated from the damaged DA neurons induced by MPTP/MPP⁺ is another important mechanism of 3-HM. We have found that 3-HM inhibited the reactive microgliosis in primary mesencephalic neuron-glia cultures after MPTP/MPP⁺ treatment (unpublished observations).

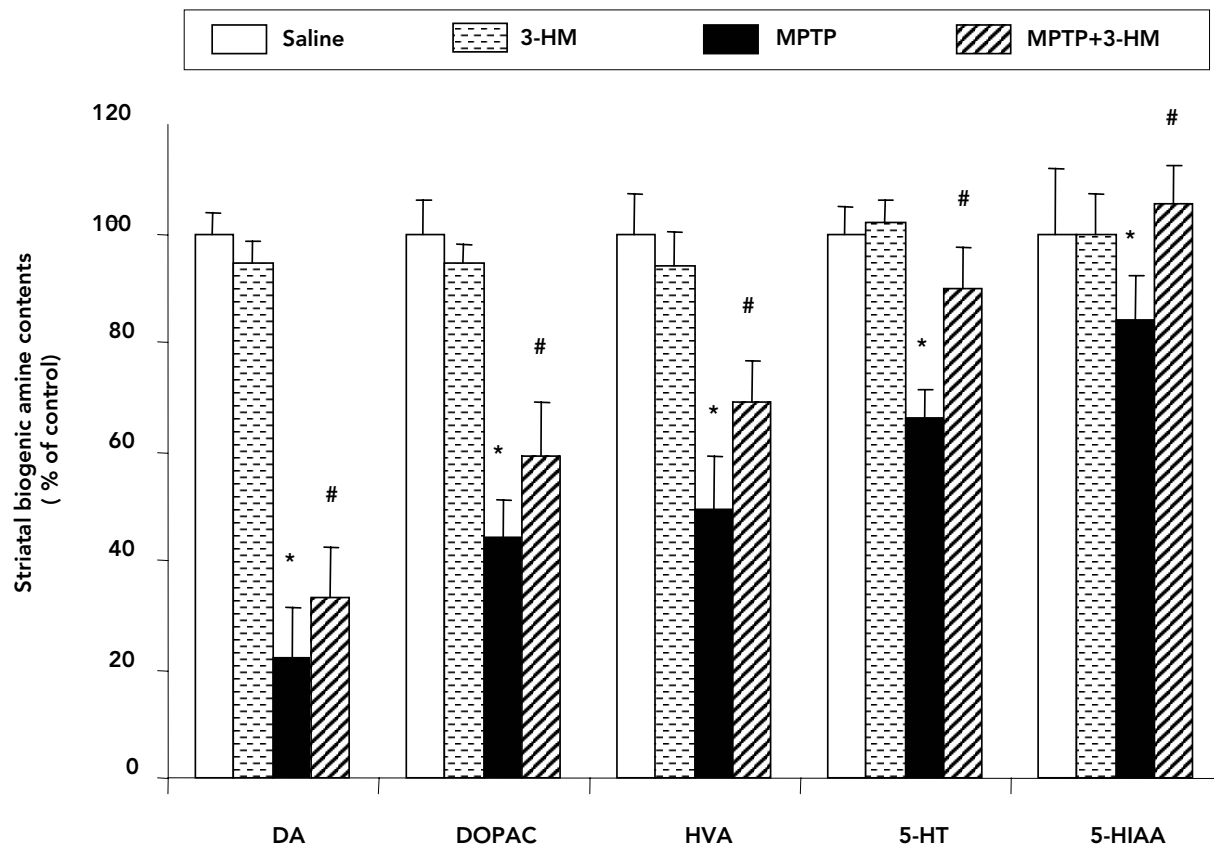


FIGURE 16. 3-HM attenuated the depletion of DA, 5-HT, and their metabolites in the striatum in MPTP in vivo model. C57BL/6J mice were injected with vehicle (saline), MPTP (15 mg free base/kg body weight, s.c., once daily), 3-HM (5 mg/kg body weight, s.c., twice daily), or 3-HM plus MPTP for 6 consecutive days. 12 days after MPTP and 3-HM injections, mice were sacrificed and striatal tissues were harvested. The levels of DA, 5-HT and their metabolites were determined with HPLC analysis and expressed as ng/100 mg of wet tissue, respectively. * $p < 0.05$ compared with saline-injected control mice; # $p < 0.05$ compared with MPTP-injected mice.

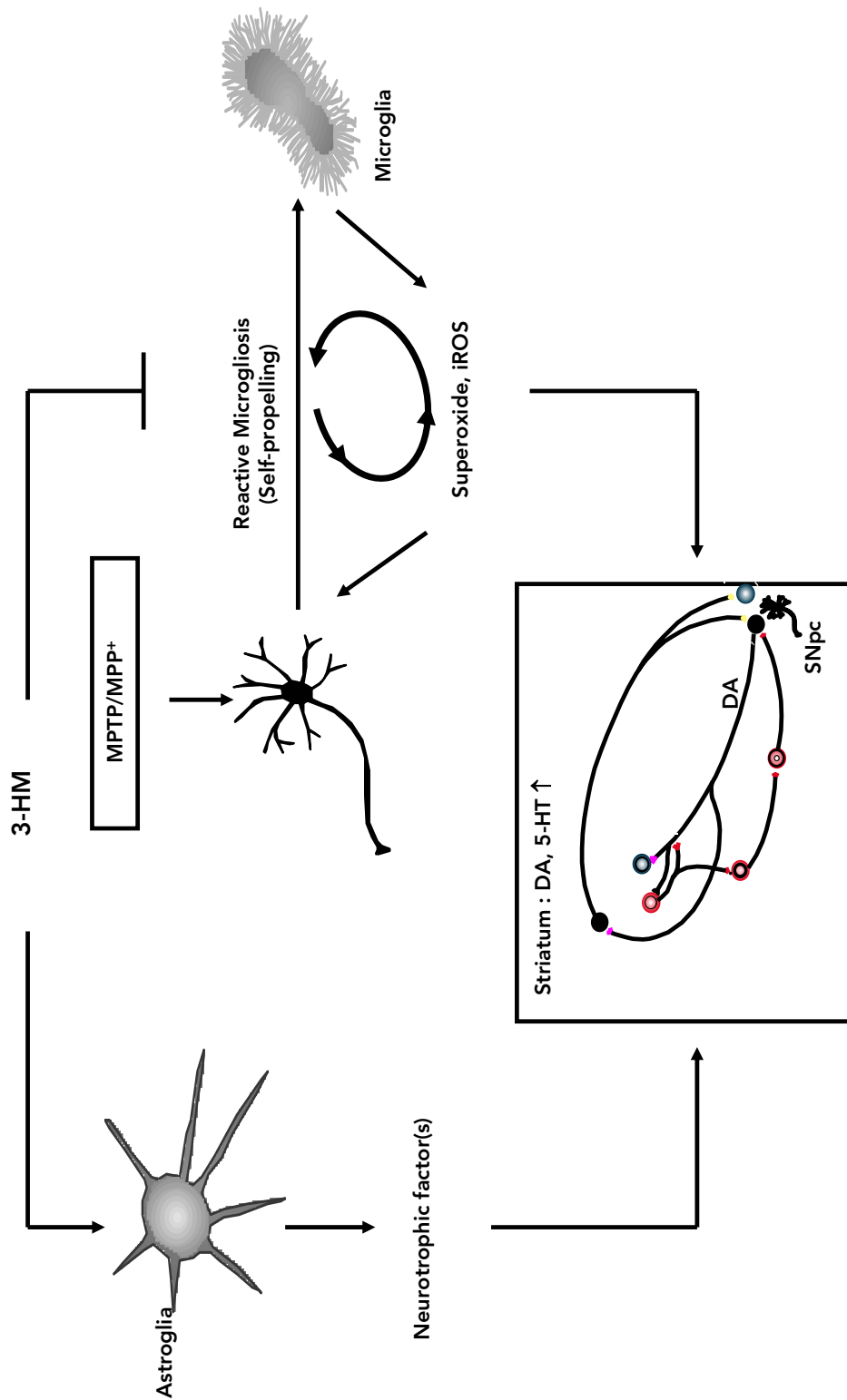


FIGURE 17. Dual mechanisms of protective effect of 3-HM in MPTP-induced DA neurotoxicity in both in vivo and in vitro model. The potent neuroprotection on 3-HM of the entire nigrostriatal pathway in MPTP PD model resulted from two important functions of neurotrophic effect and reduction of reactive microgliosis by acting on two different cell targets—astroglia and microglia, respectively. On the one hand, 3-HM promotes astroglia to generate and secrete neurotrophic factor(s); on the other hand, 3-HM inhibits the dying or dead DA neurons (directly caused by MPTP/MPP⁺ exposure)-elicited secondary microglial activation (reactive microgliosis), which is characterized by the production of superoxide and iROS, producing further DA neuronal death (self-propelling process). The dual actions are beneficial both in reducing DA neuronal degeneration in the SNpc and in restoring the depletion of biogenic amines, including DA and its metabolites DOPAC and HVA, and serotonin and its metabolite 5-HIAA, thus providing a complete neuroprotection on the entire nigrostriatal pathway.

Thus, administration of 3-HM in MPTP in vivo and in vitro PD models is beneficial both in reducing DA neuronal degeneration in the SNpc and restoring the depletion of biogenic amines in the striatum through dual functions of neurotrophic effect and reduction of reactive microgliosis, thus providing significant neuroprotection (Fig. 17).

In contrast to our finding that DM exerted neuroprotection through the inhibition of microglial overactivation, other studies supported a possible neurotrophic mechanism of DM. Vaglini et al.¹³¹ reported that DM prevented the diethylthiocarbamate (DDC) enhancement of MPTP toxicity in mice. From a histological analysis, they found that the depletion of DA neurons induced by the combined DDC + MPTP treatment was completely prevented by DM. DM was also effective in preventing the toxicity of glutamate in mesencephalic cell cultures, evaluated via the [³H] DA uptake. DM showed the same pattern of protection as nicotine and MK801, which increased the fibroblastic growth factor in the striatum, indicating that DM might function through the same mechanism of neurotrophic action.

IX. CONCLUDING REMARKS

Neuro-inflammation is critical for the pathogenesis of an array of neurodegenerative disorders. Anti-inflammation therapy thus may be an effective strategy to slow down or even halt the progression of the neurodegeneration. In this review, we present evidence that morphinan compounds, including naloxone, DM, and their analogs, offer potentially potent neuroprotection in multiple inflammatory disease models both by exerting a neurotrophic effect and by inhibiting microglial activation associated with the production of a host of pro-inflammatory and neurotoxic factors, including NO, TNF α , PGE₂, extra-cellular superoxide, and iROS. Thus, morphinans may offer a new therapeutic direction as promising compounds for the treatment of neuro-inflammatory diseases.

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