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Neuromelanin Activates Microglia and Induces Degeneration of Dopaminergic Neurons: Implications for Progression of Parkinson's Disease

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Abstract In Parkinson's disease (PD), there is a progressive loss of neuromelanin (NM)-containing dopamine neurons in substantia nigra (SN) which is associated with microgliosis and presence of extracellular NM. Herein, we have investigated the interplay between microglia and human NM on the degeneration of SN dopaminergic neurons. Although NM particles are phagocytized and degraded by microglia within minutes in vitro, extracellular NM particles induce microglial activation and ensuing production of superoxide, nitric oxide, hydrogen peroxide (H_2O_2), and pro-inflammatory factors. Furthermore, NM produces, in a microglia-depended manner, neurodegeneration in primary ventral midbrain cultures. Neurodegeneration was effectively attenuated with microglia derived from mice deficient in macrophage antigen complex-1, a microglial integrin receptor involved in the initiation of phagocytosis. Neuronal loss was also attenuated with microglia derived from mice deficient in phagocytic oxidase, a subunit of NADPH oxidase, that is responsible for superoxide and H_2O_2 production, or apocynin, an NADPH oxidase inhibitor. In vivo, NM injected into rat SN produces microgliosis and a loss of tyrosine hydroxylase neurons. Thus, these results show that extracellular NM can activate microglia, which in turn may induce dopaminergic neurodegeneration in PD. Our study may have far-reaching implications, both pathogenic and therapeutic.

Keywords Substantia nigra · Neuroinflammation · Microglia · Neurodegenerative diseases

Wei Zhang and Kester Phillips have equally contributed to this study.

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Abbreviations

COX2	Cyclooxygenase 2
DA	Dopamine
DCF	Dichlorofluorescein
GABA	γ-Aminobutyric acid
GFAP	Glial filrillary acidic protein
H_2O_2	Hydrogen peroxide
IL	Interleukin
iNOS	Inducible NO synthase
iROS	Intracellular ROS
Mac-1	Macrophage antigen complex-1
Mac-$1^{+/+}$	Wild-type Mac-1 mice
Mac-$1^{-/-}$	Knockout Mac-1 mice
MIP-1α	Macrophage inflammatory protein-1α
NM	Neuromelanin
NO	Nitric oxide
PD	Parkinson's disease
PHOX	Phagocytic oxidase
PHOX$^{+/+}$	PHOX wild-type mice
PHOX$^{-/-}$	PHOX-deficient mice
PMA	Phorbol ester myristate
ROS	Reactive oxygen species
SN	Substantia nigra
TH	Tyrosine hydroxylase
TH-ir	TH immunoreactive
TNF-α	Tumor necrosis factor-α

Introduction

Parkinson's disease (PD) is a common neurodegenerative disease, characterized by disabling motor abnormalities which include tremor, muscle stiffness, paucity of voluntary movements, and postural instability (Fahn 2003). Its main neuropathological feature is the loss of the nigrostriatal dopamine (DA)-containing neurons, whose cell bodies are in the substantia nigra (SN) pars compacta and nerve terminals in the striatum (Dauer and Przedborski 2003). Except for a handful of inherited cases related to known gene defects, PD is a sporadic condition of unknown pathogenesis (Fahn 2003). Yet, epidemiological studies suggest that inflammation increases the risk of developing PD (Chen et al. 2003) and experimental models of PD show that inflammatory factors may contribute to SN pars compacta DA neuronal death (Gao et al. 2002; Liberatore et al. 1999; Wu et al. 2002). Among inflammatory mediators capable of promoting neurodegeneration there are microglial-derived reactive species such as superoxide and nitric oxide (NO) as well as a host of toxic chemokines and cytokines (for review see Block et al. 2007).

SN pars compacta dopaminergic neurons containing neuromelanin (NM) degenerate to a greater extend in PD than dopaminergic neurons not containing NM (Hirsch et al. 1988) and free extracellular NM and microgliosis are two additional features of PD neuropathology (Langston et al. 1999; McGeer et al. 1988).

We reported that addition of human NM to microglial cultures activates positive chemotaxis and stimulates the release of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and NO. This process involves nuclear factor-κB activation by NM via phosphorylation/degradation of the inhibitor protein κB and the p38 mitogen-activated protein kinase transactivation pathway (Wilms et al. 2003). We have also shown that human NM injected into rat SN induces microglia activation and degeneration of dopaminergic neurons but the mechanism involved is still unknown (Zecca et al. 2008). As NM can induce microglial activation, chemotaxis, and proliferation, NM released from dying neurons is a strong candidate for an agent that induces chronic inflammation in SN. NM is insoluble, contains high levels of reactive iron, and in PD can exist in large amounts and for long durations in the extracellular space (Zecca et al. 1994, 2002).

To define the role of extracellular NM in the neurodegenerative process of PD and its interplay with microglia, we have tested the effects of human-purified NM on both the activation of microglia and on the survival of neighboring dopaminergic neurons in both in vitro and in vivo model systems. Our study shows that while NM particles are phagocytized and degraded by microglia in vitro, extracellular NM particles induce microglial activation and the ensuing production of superoxide, NO, hydrogen peroxide (H_2O_2), and pro-inflammatory factors. We also report that NM produces neurodegeneration in primary ventral midbrain cultures that relies on the presence of microglia and that both the macrophage antigen complex-1 (Mac-1)—a microglial integrin receptor involved in the initiation of phagocytosis—and phagocytic oxidase (PHOX), a subunit of NADPH oxidase—the multicomplex enzyme responsible for oxygen burst—are instrumental in the NM-related neurotoxic effects. In vivo, NM injected into rat SN produces microgliosis and a loss of tyrosine hydroxylase (TH) neurons. We conclude that our results provide compelling evidence that extracellular NM is able to activate microglia, which in turn may exacerbate dopaminergic neurodegeneration. We believe that by providing insights into the molecular underpinning of NM-induced microglial activation and microglial neurotoxic mediators, our study may have far-reaching implications, both pathogenic and therapeutic.

Methods

Isolation of NM from SN

NM was prepared from human SN and characterized as reported (Zecca et al. 2004). This study was approved by

the Ethical Committee of the Italian National Research Council—Institute of Biomedical Technologies (Segrate, Milano, Italy) and was carried out in agreement with the Policy of Italian National Research Council.

Animals

Time pregnant Fisher 344 rat, PHOX-deficient mice ($PHOX^{-/-}$) and wild-type mice ($PHOX^{+/+}$), knockout Mac-1 mice ($Mac\text{-}1^{-/-}$), and wild-type mice ($Mac\text{-}1^{+/+}$) were purchased from Jackson Laboratories (Bar Harbor, ME, USA).

Cultures

Primary Postnatally Derived Cultures

Postnatal culture techniques were adapted from the methods as detailed in the study of Staal et al. 2006 and from http://cumc.columbia.edu/dept/neurology/sulzer/download.html.

Primary Enriched Microglia and Astroglia Cultures

Enriched microglia and astroglia were prepared according to the previously published method (Zhang et al. 2005).

Primary Embryonically Derived Cultures

Embryonically derived primary mesencephalic neuron-glia cultures (*embryonic cultures*) were prepared according to the previously published method (Zhang et al. 2005) from the brains of embryonic day 14 of Fisher 344 rats, 13 ± 0.5 days of $Mac\text{-}1^{+/+}$ and $Mac\text{-}1^{-/-}$, $PHOX^{+/+}$, and $PHOX^{-/-}$, respectively, following previously described protocols (Zhang et al. 2005).

Time Lapse Videos

Videos were acquired over the course of 10 h using Olympus IX81 microscope with a digitized stage controlled by Metamorph Software (Molecular Devices). Cells were maintained in the culture dish on a heated platform at a constant temperature of 37°C in Tyrode medium. Images were recorded at 2 min intervals for the 10-h duration using a 20× objective with differential interference contrast optics at ten positions per dish.

Immunocytochemistry

For embryonic cultures, immunocytochemical staining was performed according to the previously published method (Zhang et al. 2005). TH immunoreactive (TH-ir) and total neurons were recognized with anti-TH and anti-Neu N antibodies (Calbiochem, USA), respectively, whereas activated microglia was detected with the monoclonal antibody against the CR3 complement receptor (OX-42) antibody (BD Pharmingen, USA), which recognizes the CR3 receptor.

Measurement of Oxidative Stress

H_2O_2 production. Measurement was done as described (Berger et al. 2002).

Proinflammatory factors: extracellular superoxide anion, intracellular reactive oxygen species (iROS) and nitrite. These proinflammatory factors were measured according to the previously published method (Zhang et al. 2005).

RT-PCR and Real-Time PCR Analysis

The levels of expression of the selected genes, including inducible NO synthase (iNOS), TNFα, cyclooxygenase 2 (COX2), IL-1β, macrophage inflammatory protein-1α (MIP-1α), and the catalytic subunit of the PHOX (gp91), were quantified using real-time RT-PCR analysis (Liu et al. 2005). Microglia were seeded at the density of 2.5×10^5/well for 24 h and treated with 1, 2.5, and 5 μg/ml of NM for 4 h. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA), followed by purification with RNeasy columns. The forward and reverse primers for selected genes were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequences of the primers were:

β-actin	TCCTCCTGAGCGCAAGTACTCT (F), GCTCAGTAACAGTCCGCCTAGAA (R);
iNOS	ACATCAGGTCGGCCATCACT (F), CGTACCGGATGAGCTGTGAATT (R);
TNFα	TCGTAGCAAACCACCAAGCA (F), CCCTTGAAGAGAACCTGGGAGTA (R);
COX2	CCAGCAGGCTCATACTGATAGGA (F), GCAGGTCTGGGTCGAACTTG (R);
IL-1β	CTGGTGTGTGACGTTCCCATTA (F), CCGACAGCACGAGGCTTT (R);
MIP-1α	GCTTGAGCCCCAGAACATTC (F), GATGTGGCTACTTGGCAGCAA (R);
gp91	CCTGCAGCCTGCCTGAATT (F), AAGGAGAGGAGATTCCGACACA (R)

Total RNA was reversely transcribed with MuLV reverse transcriptase and oligo-dT primers, and subjected to real-time PCR analysis using SYBR green PCR master mix. The relative differences in expression between groups were determined using cycle time (Ct) values as follows: the Ct values of the interested genes were first normalized with β-actin of the same sample, and then the relative differences between control and treatment groups were

calculated and expressed as relative increases setting control as 100%. Assuming that the Ct value is reflective of the initial starting copy and that there is 100% efficiency, a difference of one cycle is equivalent to a twofold difference in starting copy. Standard curve analysis was performed and used for the calculation.

In Vivo Experiments

A stereotaxic injection of 0.85 mg/ml human NM (4 µl) in phosphate buffer was performed into the left SN of anaesthetized rats at the following coordinates: AP = +0.24; Lat = +0.16; DV = −0.26; nosebar set at −0.24 (Paxinos and Watson 1982); controls received vehicle only. At 10 days after NM injection into the SN, animals were perfused with 4% paraformaldehyde, the brains were removed, post-fixed overnight in the same fixative, frozen, and sectioned at 30 µm. Sections were immunostained with antibodies against TH for TH-ir neurons detection (1:1000, Calbiochem), against Iba-1 for microglia (1:1000; DAKO, USA), against glial fibrillary acidic protein (GFAP, 1:1000; DAKO) for astrocytes and for γ-aminobutyric acid (GABA) neurons (1:1000; Chemicon, USA). TH-immunostained sections were counter-stained with cresyl violet. The loss of TH-ir neurons was determined by stereological counting (Tieu et al. 2003).

Statistical Analysis

All values are expressed as the mean ± SEM. Differences among means were analyzed using one- or two-way ANOVA with time, treatment, or genotype as the independent factors. When ANOVA showed significant differences, pair-wise comparisons between means were tested by Newman–Keuls post-hoc testing. Proportions were compared by either Chi-square or Exact Fisher test. In all analysis, the null hypothesis was rejected at the $P = 0.05$ level.

Results

NM Activation of Microglia Induces Oxyradical Stress

To determine whether microglia can be activated by NM, primary microglia were purified from embryonic and postnatal brains then cultured with and without the presence of purified human NM for 4 h and then harvested to measure gene expression of selected pro-inflammatory and neurotoxic factors. This experiment revealed that the abundance of transcripts for several such factors including TNF-α, IL-1β, and iNOS were increased in a dose-dependent manner (Supplementary Fig. 1a–c). We confirmed that the microglial-derived NO production was increased upon exposure to human NM by measuring nitrite production (Supplementary Fig. 2). NM was also found to enhance the gene expression of MIP-1α, COX2, and gp91, the catalytic subunit of the PHOX (Supplementary Fig. 3a–c). Thus, these data indicate that, under the present experimental conditions, NM did activate microglia, yielding a wide range of released factors and upregulation of multiple mechanisms involved in inflammation.

The major source of microglial ROS is thought to be PHOX, a key enzyme in phagocytic cells responsible for the generation of the respiratory burst. In our cell model system, NM did enhance the production of microglial-derived superoxide, as measured by electron spin resonance (Fig. 1a), as well as secondary oxidants such as H_2O_2 production (Fig. 1b). This H_2O_2 production was inhibited in microglial cultures by the PHOX antagonist, apocynin (100 µM), and enhanced by the PHOX activator, phorbol ester myristate (PMA; 100 µM) (Fig. 1b). H_2O_2 synthesis was also blocked by the PHOX inhibitor, diphenylene iodonium (5 µM), but this compound proved neurotoxic in our protocols and was not used in neuronal experiments (data not shown).

To further examine the involvement of PHOX, we used the fluorogenic compound dichlorofluorescein (DCF) diacetate. While DCF diacetate provides a relatively non-specific measure of iROS, it has the advantage of being selectively retained in cytosol. In addition, its conversion by oxidation to its fluorescent derivative DCF is thought to specifically indicate iROS activity. NM induced microglial DCF production in a dose-dependent manner (Supplementary Fig. 4a). DCF fluorescence in response to NM was significantly less in microglia derived from PHOX$^{-/-}$ that lack the catalytic gp91 subunit of NADPH-oxidase and are unable to generate extracellular superoxide in response to immunological stimulus (Fig. 1c). Thus, our results indicate that the enhanced microglial ROS production following NM exposure is NADPH-oxidase dependent.

To confirm that the enhanced iROS was indeed due to microglial activation, we used embryonic microglial cultures derived from Mac-1$^{-/-}$. These cultures produced far less DCF than wild-type microglia (Fig. 1d). Consistently, NM-induced iROS in wild-type microglia was blocked by an inhibitor of phagocytosis, cytochalasin D (Supplementary Fig. 4b). We thus conclude that NM activation of microglia causes ROS production, including superoxide, H_2O_2, and iROS.

Phagocytosis and Degradation of NM by Microglia

Because the potency of extracellular NM to activate neighboring microglia and promote neurodegeneration may depend on how long it may last in the neuropil, we then examined whether microglia can phagocytize and degrade

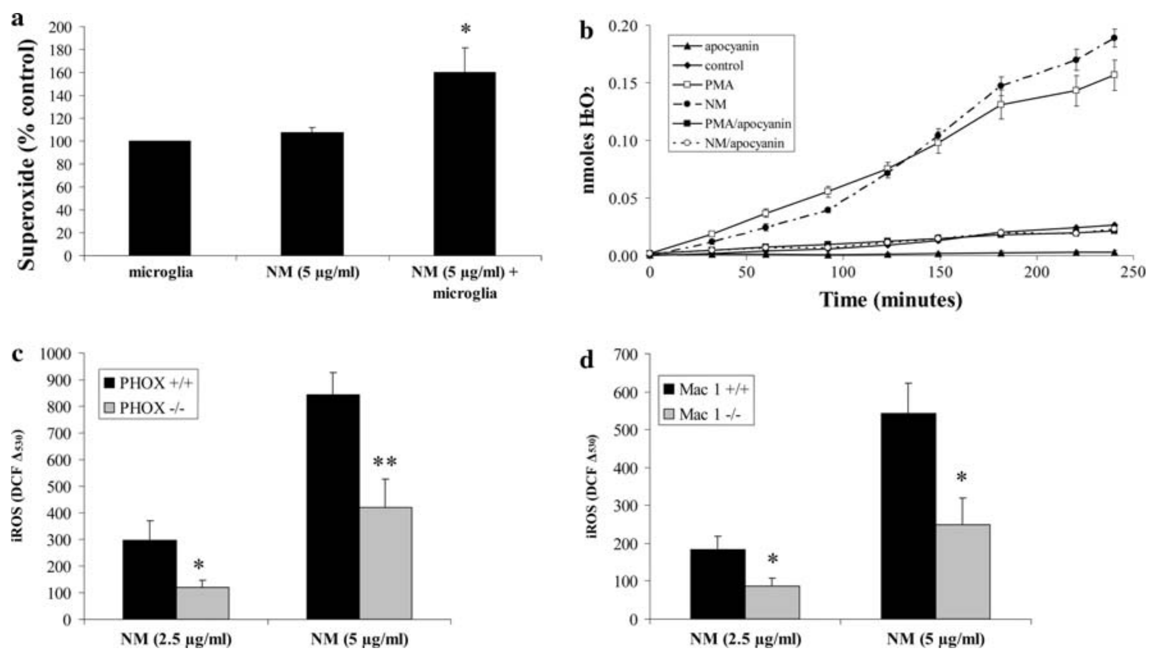

Fig. 1 Microglia activated by NM produce iROS. **a** NM enhances microglial superoxide production, as measured by electron spin resonance (mean ± SEM; $n = 3$; * $P < 0.05$). **b** Measurement of H_2O_2 production from microglial cultures over time indicate that NM (5 μg/ml) elicits H_2O_2 production. This is effectively blocked by the PHOX inhibitor, apocynin (100 μM). H_2O_2 synthesis is triggered by PMA (100 μM), a known PHOX activator (mean ± SEM; $n = 3$). **c**, **d** DCF fluorescence in response to NM, as an indicator of iROS production. **c** PHOX$^{-/-}$ mice produce less iROS in response to NM (mean ± SEM; $n = 8$; * $P < 0.05$; ** $P < 0.01$). **d** Mac-1$^{-/-}$ microglia produce less iROS in response to NM (mean ± SEM; $n = 6$; * $P < 0.05$)

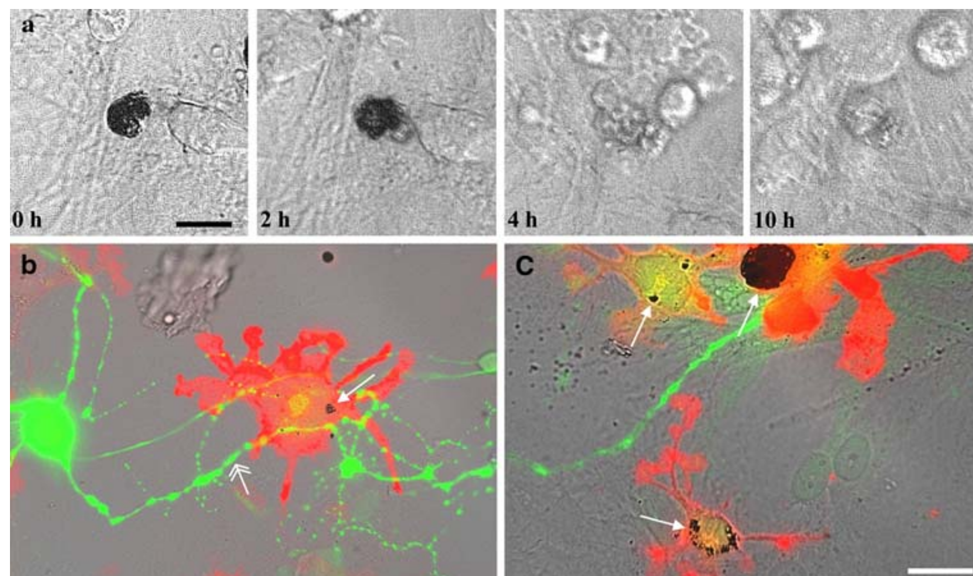

Fig. 2 Microglia phagocytose and degrade NM. **a** Series of differential contrast images of microglial phagocytosis and degradation of a large particle (~14 nm diameter) of NM in ventral midbrain/astrocyte/microglial co-culture at 2 h intervals excerpted from a video http://www.sulzerlab.org/videos/NMmicrogliaDIC.mov. Image acquisition commenced 15 min after addition of NM for 10 h. A microglial cell to the right of the particle at 0 h is in contact with the particle at 2 h. At 4 h, the pigment appears to be absent or nearly so and additional microglia have arrived at the scene. Scale bar = 20 μm. For an example of uptake of NM by microglial filipodia and subsequent NM degradation, see http://www.sulzerlab.org/videos/NMfilopodia.mov. **b**, **c** The co-cultures prepared as above were fixed 72 h after addition of NM and immunolabeled for dopaminergic neurons by TH (*green*) and activated microglia by OX-42 (*red*). Both panels illustrate activated microglia in close contact with neuronal processes and swollen neurites. Examples of NM particles that have been phagocytosed are indicated by the *single-headed arrows*. The *double-headed arrow* indicates an example of a swollen neurite varicosity, which is several-fold larger than typical varicosities in these neurons (average ~1.2 μm, Pothos et al. 1998) which can also be observed. The swollen varicosities are an indicator of toxicity (Cubells et al. 1994). Scale bar = 20 μm. (Color figure online)

this pigment. To visualize the potential interaction between NM and microglia, we prepared time-lapse videos of differential interference contrast optics, which provided clear images of microglial morphology. We seeded NM (5 μg/ml) on postnatal microglia/neuron co-cultures and at 15 min after seeding, acquired images every 2 min for 10 h. The microglia were highly motile, extended filopodia onto NM particles, took up NM via phagocytosis, and degraded the NM (see stills from a video in Fig. 2a). We thus conclude that microglia are capable of breaking down NM.

To characterize these processes, we followed 35 extracellular NM particles that ranged in size from 2 to 14 μm in diameter. Of these, 18 were phagocytosed during the 10 h. Microglial filopodia were observed to touch the particles at varying times throughout the recording, and often apparent encounters did not lead to phagocytosis. For those particles that were phagocytosed, some appeared to be grabbed by filopodia and delivered to the microglial cell body for phagocytosis; for an example, see http://www.sulzerlab.org/videos/NMfilopodia.mov. In other cases, the cell body migrated to or with the particle prior to complete or partial engulfment, sometimes apparently activating, fusing with, or intimately interacting with nearby microglia during the phagocytosis and breakdown; see http://www.sulzerlab.org/videos/NMmicrogliaDIC.mov.

Engulfment of the particles appeared complete by 4 min after filopodial contact for 14 of the 18 particles, whereas 3 particles required 6 min, and the largest particle required 10 min. There were also instances, particularly for larger particles, when only a portion was apparently sheared off by the microglia. We do not know whether the variability in phagocytosis and degradation is a result of the state of the microglia, NM, or other factors. We were able to observe 7 of the 18 phagocytized particles to determine if there was a complete loss of pigment under differential interference contrast optics. Of the seven, three were completely degraded (at 34, 173, and 284 min after phagocytosis was initiated), one appeared to be only partly degraded by the end of the experiment (285 min after phagocytosis), and three particles apparently did not undergo degradation.

To evaluate whether Mac-1 receptor is involved in phagocytosis, Mac-1$^{+/+}$, and Mac-1$^{-/-}$ cultures were treated with NM. Wild-type microglia phagocytized a higher fraction of NM particles (86%: 345/399) than Mac-1$^{-/-}$ microglia (65%; 187/287) (Chi-square $= 42.33$; $P < 0.001$).

To better observe fragmentation and breakdown of NM, we used bright field optics, which resolve cellular morphology as well as differential interference contrast optics, but more clearly show the state of the pigment. An example of NM disappearance in bright field video (0.2 frame/min) of NM added to microglial culture is available from http://www.sulzerlab.org/videos/NMbrightfield.mov. For those particles that were degraded, the disappearance generally occurred in 4–8 min. In postmortem human brain, NM granules are observed in the cell body of microglia cells in the SN of PD patients (Langston et al. 1999; McGeer et al. 1988) where microglia are very aged as compared to those of newborn rat. We thus examined whether microglia cells aged in vitro still effectively degraded NM. We observed that while older microglia (1 month post-plating) rapidly phagocytosed NM, they were comparatively poor at degrading the pigment. Indeed, by combining the results from two independent experiments, we found that 56% (18/32) of the 10–20-day-old cultured microglia did degrade NM, whereas only 22% (10/46) of ≥1-month-old cultured microglia degraded NM ($P < 0.05$, Fisher's exact test).

To better resolve the phagocytosis of NM by microglia, activation of microglia, and damage caused by microglia to neurons, we fixed and immunolabeled postnatal co-cultures during NM degradation. Figure 2b, c shows dopaminergic neurons immunolabeled for TH (green) and microglia immunolabeled using OX-42, which recognizes activated microglia (red). The arrows point to phagocytosed NM; a particle of over 10 nm in diameter can be observed in Fig. 2b. The activated microglia are often observed to be in close contact with neuronal processes, and swollen neurites, which indicate neurotoxicity, can be observed in the vicinity of these apparent points of contact (Fig. 2c).

NM-Mediated Neurotoxicity Involves Mac-1 Receptor and PHOX

Next, we tested the possibility that NM can provoke neurodegeneration through microglial activation. We exposed mixed neuron/glia co-cultures prepared from E14/15 rat embryos to different concentration of NM and assessed the ventral midbrain dopaminergic neuron survival. We scored neurotoxicity by counting cells immunolabeled for TH, a synthetic enzyme for DA. After 10 days incubation, NM produced significant decrease in the numbers of TH-ir neurons in a dose-dependent manner (Fig. 3a). In a separate experiment, when NM was added to neuron-enriched cultures with no microglia present, the damage of dopaminergic neurons was minimal (data not shown). This result suggests a critical role of microglia in mediating NM-induced neurotoxicity.

Consistent with a role for microglial activation in neurotoxicity, the number of TH-ir neurons in embryonic cultures exposed to Mac-1$^{-/-}$ microglia and NM were greater than with microglia derived from Mac-1$^{+/+}$ (Fig. 3b). We conclude that the Mac-1 receptor interacts with NM via intracellular signaling to stimulate microglia-mediated neurotoxicity.

Finally, we tested whether neurotoxicity associated with microglial activation involved PHOX. Microglia derived

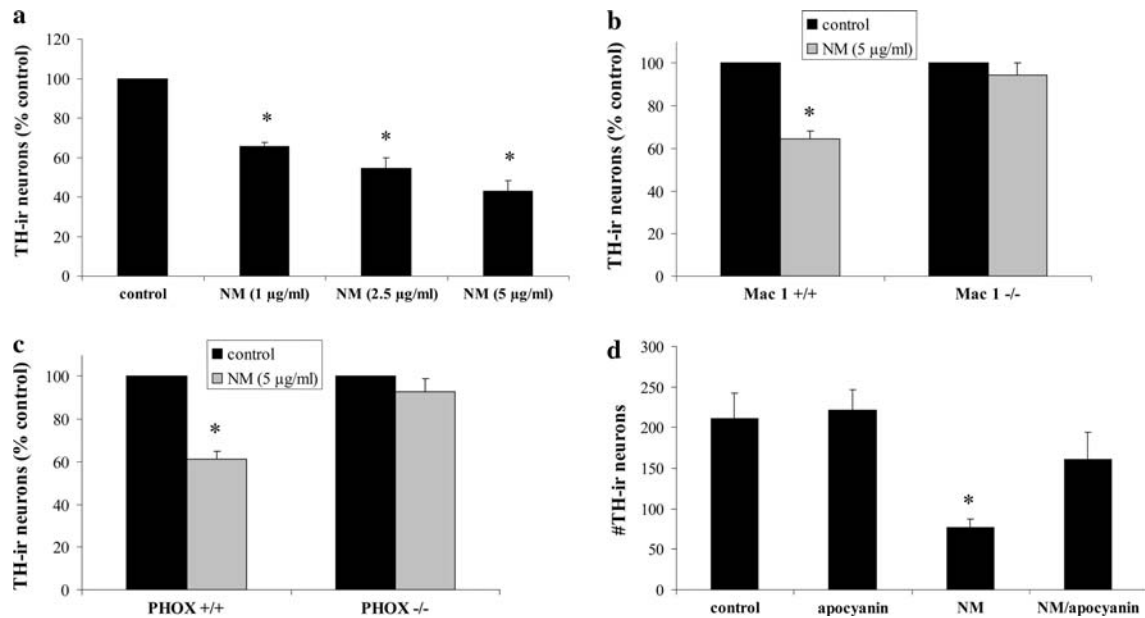

Fig. 3 Neuronal death induced by NM-activated microglia. **a** NM is toxic to dopaminergic neurons in primary mesencephalic mixed neuron-glia cultures. Rat primary mixed mesencephalic neuron-glia cultures were seeded in 24-well plates at 5×10^5/well and treated with 1–5 µg NM/ml for 10 days. Effects of NM on the dopaminergic neurons in mixed neuron-glia cultures were assessed by counting the number of dopaminergic neurons after TH staining. Results are expressed as a percentage of the vehicle-treated control cultures (mean ± SEM; $n = 3$; * $P < 0.001$). **b** Microglia derived from Mac-$1^{-/-}$ mice exhibit less NM neurotoxicity (mean ± SEM; $n = 4$; * $P < 0.001$). NM toxicity in co-cultures is inhibited in microglia derived from $PHOX^{-/-}$ mice (**c**; mean ± SEM; $n = 4$; * $P < 0.001$) and by the PHOX inhibitor, apocynin (100 µM) (**d**; NM 5 µg/ml; mean ± SEM; $n = 7$; * $P < 0.01$)

from $PHOX^{-/-}$ mice produced significantly less NM-stimulated neurotoxicity than did $PHOX^{+/+}$ (Fig. 3c) while the addition of apocynin partially inhibited NM neurotoxicity (Fig. 3d). Thus, it appears that the activation of this enzyme is in part responsible for neurotoxicity of NM and microglia.

NM Induces Microgliosis and TH Neuron Loss in Rat SN

To examine whether extracellular NM could also exert neurotoxic effects on dopaminergic neurons in vivo, adult rats received a stereotaxic injection of NM in the SN and their brains were analyzed 10 days later. In the injection site of NM, a marked reduction of TH-ir neuron number was consistently observed compared to vehicle-injected control animals (Fig. 4a, b). In saline-injected rats, the number of TH-ir neurons did not differ from that of contralateral intact side of rats injected with NM. Notably, in saline-treated rats, 73 ± 1% of neurons were TH-ir ($n = 5$), whereas in NM-treated rats, only 33 ± 1% of neurons were TH-ir ($n = 3$, $P < 0.001$, two-tailed t-test). Thus, 55% of SN DA neurons either died or lost TH activity. The total number of neurons (sum of TH-ir and Nissl stained neurons) indicated that 38% of total SN neurons died as a consequence of NM injection.

A robust microgliosis, as evidenced by Iba-1 immunostaining, was also observed in SN along the area of NM injection (Fig. 4c). The injection of vehicle produced minimal microglia response (Fig. 4d). The area of SN showing the highest microglial response corresponds to the area having the highest loss of TH neurons. The astrocytes immunostaining with GFAP showed a moderate astrocytosis in SN along the NM injection area (Fig. 4e) and almost none in the site of vehicle injection (Fig. 4f). In sections immunostained for GABA, neuron numbers on the NM injection did not differ from those of vehicle controls (Fig. 4g, h). These data indicate that NM injection in rat SN causes a strong microgliosis, a mild astrocytosis, and a selective reduction of TH-ir neurons.

Discussion

This study provides significant support to the hypothesis that NM released from dying neurons can activate microglia, thereby promoting the degeneration of neighboring neurons (Zecca et al. 2003). Importantly, and in contrast with prior PD models, this study examined the effects of adding NM to cell cultures containing microglia and neurons and injecting NM into rat SN rather than treating cells or animals with exogenous toxins, such as 1-methyl-4-phenylpyridinium

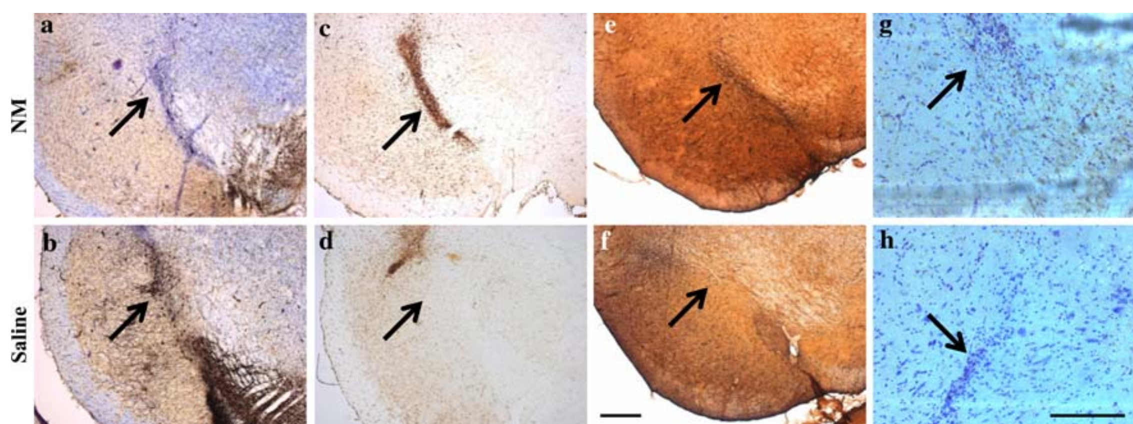

Fig. 4 Immunostaining in the rat SN 10 days after NM injection. Rats received either phosphate buffered saline or NM at 1 µl/min for 4 min, then the total amount of NM injected into the rat SN was 3.4 µg. The needle was left in place an additional 2 min. *Arrows* indicate the site of injection within the SN. Representative TH immunostaining of the SN in a NM-injected rat (**a**), and in a vehicle-injected rat (**b**). Representative SN Iba-1 stained microglia (**c**, **d**), GFAP-stained astrocytes (**e**, **f**), and GABA neurons (**g**, **h**) from a NM-injected rat (**c**, **e**, **g**) and from a vehicle-injected rat (**d**, **f**, **h**) are also shown. Scale bar: (**a**–**f**) = 440 µm; (**g**, **h**) = 175 µm

ion, 6-hydroxydopamine, l-buthionine-[S,R]-sulfoximine, rotenone, or bleomicyn, that are used in other PD models (for review see Dauer and Przedborski 2003; Hattori and Sato 2007; Shimohama et al. 2003). Our cellular system moreover reproduces several morphological and biochemical characteristics present in SN of PD patients. The in vivo experiment here reported reproduces the cellular conditions occurring in SN of PD patients with extracellular NM, activated microglia, and neuronal loss. Both in vitro and in vivo systems may thus provide reliable models for studying potential interventions for the chronic phase of PD neuronal death.

NM is a highly insoluble and degradation resistant compound composed of oxidized catecholamines, lipids, peptides, and metals that are resident within autophagic/lysosomal organelles of catecholaminergic neurons. We have suggested that NM synthesis is neuroprotective since this process removes excess cytosolic catechols and its derivatives and chelates toxic metals (Sulzer et al. 2000; Zecca et al. 1994). NM accumulates over a lifetime in normal individuals because neuronal lysosomes lack the ability to break it down efficiently, a mechanism that may be further compromised during aging (Sulzer et al. 2008). PD patients, however, loose NM pigment in the SN and locus coeruleus during the course of their disease, an observation that is explained by the microglial degradation pathway introduced here. It is interesting that in our cultures, younger microglia (10–20 days after plating) degrade NM within 30 min of phagocytosis, whereas in older microglia (4 weeks) the ability to phagocytose NM is preserved but the degradation of NM is reduced. The decreased capacity to degrade NM could be due to lower production of H_2O_2 consequent to a reduced PHOX activity. The behavior of aged microglia may be more similar to that of microglia in SN of PD patients where non-degraded NM granules are present in microglial cell bodies (Banati et al. 1998; Langston et al. 1999; McGeer et al. 1988). An additional reason for a reduced microglial degradation of NM in PD may be that NM organelles remaining after SN cell death in PD are associated with additional components not present in the purified NM used here. Moreover, microglia derived from healthy young rats may be more reactive than those resident in older and diseased subjects.

In addition to the previously reported production of NO, TNF-α, and IL-6, and our present findings of enhanced prostaglandin E2 (data not shown) and MIP-1α, we showed that NM induced production of superoxide, a precursor of reactive species like H_2O_2 and indirectly hypochlorite and hydroxyl peroxide. The production of superoxide is dependent on the translocation of cytosolic subunits to the plasma membrane. We observed that NM induced expression of gp91 and H_2O_2, and iROS by microglia in a dose-dependent manner. As these processes were effectively blocked by PHOX inhibitors and much reduced in PHOX$^{-/-}$ microglia that lack the gp91 subunit and Mac-1$^{-/-}$ microglia, the data confirm a key role of this Mac-1/PHOX activity in the formation of NM-triggered microglial iROS. The release of inflammatory molecules by NM-activated microglia would be expected to contribute to neuronal degeneration by synergizing with cytotoxic agents like H_2O_2, peroxynitrite, and proinflammatory cytokines. These inflammatory molecules can diffuse through brain tissue to cause vascular reaction with efflux of blood leukocytes including macrophages into the brain parenchyma, further exacerbating, and extending inflammation around blood vessels. Notably, neuropathological studies have demonstrated the presence of vascular damage in SN of PD (Farkas et al. 2000). Thus, it is likely that

microglia-associated neurodegeneration produces molecules which can induce vascular release of inflammatory molecules and leukocytes which in turn can produce neuronal damage. This results in a self-propelling cycle of neuroinflammation and neurodegeneration.

In our cellular model, we introduced a large amount of NM in order to produce rapid and strong microglial activation and extensive neuronal death in 10 days, whereas in PD the NM is slowly released by dying neurons over years. Although we used the same components present in the SN of PD patients, we reproduced in 10 days in culture a neuronal loss of about 40% that requires years in human brain. It is remarkable that this acceleration requires only a level of extraneuronal NM in cultures slightly higher than that present in human SN. This concept is further supported by the fact that increasing the amount of NM in the microglia–neuron cultures yields an increased release of ROS, NO, and inflammatory factors along with increased damage to neurons. In our in vivo experiments, the injection of human NM into rat SN produced after 10 days an intense microgliosis and loss of a high number of TH neurons.

The amount of NM injected (3.4 μg) was selected based on results from previous study, where we found an extensive microglial activation and neuronal death in rat SN injected with such an amount of NM (Zecca et al. 2008). This amount of NM was calculated to generate a tissue concentration of NM, slightly higher than that we observed in human SN (Zecca et al. 2002) since it allows to induce a significant microglial activation and neurodegeneration within 1–2 weeks. In our previous study (Zecca et al. 2008), we used as control an injection of gold microparticles suspension to produce a mechanical effect similar to that of NM particles but without the chemical components of NM. The results observed in our studies are specifically due to the chemical components of NM.

The astrocytosis observed in these conditions is a typical response to neuronal damage. It is noteworthy that GABA neurons were not affected either directly by the injected NM or indirectly via microglia activation. This sparing of GABA neurons in presence of an extensive loss of TH neurons makes our in vivo system a promising candidate model of PD. In our in vivo context, a localized high concentration of extracellular NM was produced with injection of human NM into rat SN. With this procedure, we obtain in 10 days a localized neuronal loss whose degree is similar to that taking place during years in the entire SN of PD subjects. Obviously, further experiments will be necessary for a full validation of this concept/setup as an animal model of PD.

The present data demonstrate that extracellular NM in the absence of microglia is not itself toxic for neurons. However, once released from dying neurons, NM activates microglia, in part due to recognition by the Mac-1 integrin receptor to further promote neurotoxity. As the treatment with apocynin that inhibited PHOX-dependent iROS also reduced neurotoxicity, a portion of the toxicity due to NM microglial activation is due to the activity of this enzyme. Significant work will be required to determine which of the many other potential additional pathways activated by NM in microglia cause neurotoxicity. Notably, the activated microglia appear to make extensive contact with neurons, and it may be that the toxicity is highly localized, perhaps by release of toxic compounds at zones of tight adhesion. If so, this process, which is not in itself selective for catecholamine neurons, may induce chronic neuronal death particular to the SN and locus coeruleus, since these catecholaminergic nuclei, which are severely damaged in PD, are also those with the highest NM content in the brain. There are several factors contributing to the high vulnerability of DA neurons of SN in PD. It is worth noting that SN has the highest density of microglia in the brain (Kim et al. 2000). Moreover in DA neurons of SN there is a high concentration of DA which can be oxidized to generate reactive/toxic quinones. The low glutathione content corresponding to low antioxidant ability and the high load of organic toxins and toxic metals into NM further make DA neurons of SN particularly vulnerable (Zecca et al. 2004).

Considering that a continuous and high release of NM by dying neurons of SN occurs in PD, and that NM is insoluble and stable for long periods in the extracellular space these results suggest that extraneuronal NM can induce a sustained microglia activation and ensuing neurodegeneration in PD.

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