Microglial Activation Induced by Neurodegeneration

A PROTEOMIC ANALYSIS

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Neuroinflammation mediated by microglial activation appears to play an essential role in the pathogenesis of Parkinson disease; however, the mechanisms by which microglia are activated are not fully understood. Thus, we first evaluated the effects of two parkinsonian toxicants, manganese ethylene bisdithiocarbamate (Mn-EBDC) and 1-methyl-4-phenylpyridine (MPP⁺), on microglial activation as well as associated dopaminergic (DAergic) neurotoxicity in primary cell culture systems. The results demonstrated that, when rat primary mesencephalic neuron-enriched or neuron-microglia mixed cultures were treated with Mn-EBDC at 2–8 μM or MPP⁺ at 0.25–5 μM, respectively, for 7 days, both toxicants were capable of inducing DAergic neurodegeneration as well as activating microglia via a mechanism secondary to DAergic neurodegeneration. Furthermore, activated microglia subsequently enhanced DAergic neurotoxicity induced by Mn-EBDC or MPP⁺. Detailed scrutiny of neuron-microglia interactions identified a fraction of the conditioned media derived from a DAergic cell line treated with Mn-EBDC or MPP⁺ that potently activated microglia. To further define potential mediators leading to microglial activation secondary to neurodegeneration, we utilized a quantitative proteomic technique termed SILAC (for stable isotope labeling by amino acids in cell culture) to compare the protein profiles of MPP⁺-treated cellular fraction that mediated microglial activation as compared with controls. The search revealed numerous novel proteins that are potentially important in neurodegeneration-mediated microglial activation, a process believed to be critical in Parkinson disease progression. Molecular & Cellular Proteomics 4: 1471-1479, 2005.

Microglia, the resident immune cells of the central nervous system, have been indicated to play central roles in the initiation and/or aggravation of neurodegeneration in a number of neurodegenerative disorders, including Parkinson disease (PD) (1–4). However, what triggers microglial activation in PD and other neurodegenerative diseases remains to be determined.

It is generally accepted that microglia can be activated directly by products of microorganisms (5, 6), environmental toxicants (7), and protein aggregates like amyloid β aggregates (8) or indirectly after neurodegeneration is induced (9). Carvey et al. (10) have demonstrated that exposure to lipopolysaccharide, the major component of bacterial walls, during early life could constitute a potential mechanism leading to dopaminergic (DAergic) cell death and subsequent development of PD. On the other hand, the research group led by Hong has proposed that parkinsonian toxicants can be divided into three major categories with respect to microglial activation and DAergic neurodegeneration: 1) those activating microglia directly followed by induction of neurotoxicity, 2) those activating microglia by damaging neurons first, and 3) those activating microglia and causing neurodegeneration simultaneously (11). The corresponding prototypes of these toxicants are lipopolysaccharide, 1-methyl-4-phenylpyridine (MPP⁺), the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and rotenone, respectively (11).

Other environmental toxicants that have been linked to PD development include paraquat and manganese ethylene bisdithiocarbamate (Mn-EBDC) (11–14). Several groups, including us, have demonstrated that Mn-EBDC induces selective DAergic neurodegeneration both in vivo and in vitro. In addition, the pathogenesis appears to involve mitochondrial and proteasomal dysfunction with increased oxidative stress (11, 12, 14, 15). It is not clear, however, whether Mn-EBDC, the active component of the widely used fungicide maneb (11, 12), could produce DAergic neurotoxicity via microglial activation.

The other major issue that remains to be investigated is the mechanisms by which DAergic neurodegeneration, regard-

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less of etiology, activates microglia, thereby resulting in progressive neurodegeneration and disease progression. In a recent study, we have demonstrated that aggregated α-synuclein potently activates microglia via a mechanism that involves phagocytosis of aggregated α-synuclein by microglia (16). This observation is very similar to, although more potent than, that induced by aggregated amyloid β (8). The second potential culprit that might lead to microglial activation following neurodegeneration in PD is neuromelanin released from damaged neurons (17), but direct nigral injection of human neuromelanin in rats failed to produce such effects (18). Other potential endogenous mediators of microglial activation include tissue-type plasminogen activator and matrix metalloproteases (19), although their roles in PD are largely unexplored.

Thus, in the current study, with various defined culture systems, we tried to address two issues: 1) whether parkinsonian toxicant Mn-EBDC-mediated DAergic neurodegeneration also involves microglial activation and 2) what are the potential mechanisms mediating neurodegeneration-mediated microglial activation.

EXPERIMENTAL PROCEDURES

Primary Neuron-enriched and Microglial Cultures—All experiments related to animals were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee at the University of Washington.

Primary rat ventral mesencephalic neuron-enriched cultures were prepared by following our previously published protocol with minor modifications (16). Briefly, ventral mesencephalic tissues were dissected from embryonic day 13/14 Sprague-Dawley rats (Charles River, Wilmington, MA) and then treated with papain (15 units/ml, Worthington Biochemical Corp., Lakewood, NJ). After mechanical trituration by pipetting, the cells were seeded to 24-well (5 × 10^5/well) culture plates precoated with poly-D-lysine (20 ng/ml) and laminin (Collaborative Biomedical Products, Bedford, MA) and maintained in 0.5 ml/well Neurobasal medium (Invitrogen) supplemented with 1% B27, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂, 95% air, and the medium was changed 1 and 4 days later, respectively. On reaching confluence (10–14 days), the microglia were separated from astrocytes by shaking the flasks. The enriched microglia were >98% pure as determined by counting OX-42-immunoreactive (IR), GFAP-IR, and NeuN-IR cells.

Neuron-Microglia Interaction—To study the interaction of microglia with DAergic neurons, microglia and primary mesencephalic neuron-enriched cultures were co-cultured in 24-well culture plates either directly or separated by a microporous membrane (polyester Transwell inserts; pore size, 0.4 μm; Corning, Corning, NY). In brief, the purified microglia were plated into 7-day-old primary mesencephalic neuron-enriched cultures, and the treatments were started 4 h after the addition of microglia. The co-cultures were treated with different concentrations of either Mn-EBDC or MPP⁺ (Sigma) for 7 days in Neurobasal medium supplemented with 1% B27, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C. Mn-EBDC was synthesized as described previously (14, 22), aliquoted, and kept under nitrogen until use. Stock solutions were prepared freshly in Me₂SO.

To study the interaction of microglia with immortalized DAergic neurons, a rodent mesencephalic neuronal cell line, MES 23.5 (MES), with many features of DAergic neurons (15, 23) was cultured in Dulbecco’s modified Eagle’s medium/F-12 medium containing 1% N-2 supplement, 2% fetal bovine serum, and 50 μg/ml streptomycin at 37 °C in a 5% CO₂, humidified incubator. Cells were plated onto 0.005% poly-D-lysine-precoated 96-well plates or 100-mm dishes (cell density for both, 1.56 × 10^5/cm²) 24 h before treatment.

Assessment of Neurotoxicity—To evaluate the viability of primary cultured DAergic neurons, [3H]DA and [3H]GABA uptake assays were performed as described previously (24). [3H]DA (43.0 Ci/mmol) and [3H]GABA (90 Ci/mmol) were obtained from PerkinElmer Life Sciences. Cultures were washed twice with Krebs-Ringer buffer containing 16 mM NaH₂PO₄, 119 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM EDTA, and 5.6 mM glucose (pH 7.4). For [3H]DA and [3H]GABA uptake the cultures were incubated for 15 min at 37 °C with 25 nM [3H]DA (a total of 1 μM DA) and 100 nM [3H]GABA (a total of 5 μM GABA) in Krebs-Ringer buffer, respectively. After being washed three times with ice-cold Krebs-Ringer buffer, the cells were collected in 0.4 ml of 1 N NaOH, and radioactivity was counted with a liquid scintillation counter (LS6500 multipurpose scintillation counter, Beckman Coulter). Nonspecific uptake was determined in parallel wells that received both the tritiated tracer and 10 μM mazindol for [3H]DA uptake or 10 μM NO-711 and 1 mM β-alanine for [3H]GABA uptake (25, 26).

DAergic neurotoxicity of MES cells was evaluated by the Live-Dead assay (Molecular Probes) with two fluorescent indicators of membrane integrity (26). After treatment with Mn-EBDC or MPP⁺ for 3
days, MES cells were administered a solution containing both calcein acetoxyethyl ester (4 μM) and ethidium homodimer-1 (1 μM) and incubated for 45 min at 37 °C before being viewed under epifluorescence optic fluorescence (calcein) and rhodamine (ethidium homodimer-1) filters (excitation/emission 485 nm/530 nm and 530 nm/645 nm, respectively; Spectra Max Gemini, Molecular Devices Inc., Sunnyvale, CA). In addition to Live-Dead assay, remaining viable cells were also counted manually to correlate with the results obtained with Live-Dead assay.

Biochemical Fractionation of Conditioned Media—MES cells were plated onto 0.005% poly-d-lysine precoated 100-mm dishes (cell density, 1.56 × 10^5/cm^2) 24 h before treatment with Mn-EBDC at 4 μM or MPP⁺ at 25 μM for 3 days. The conditioned media were carefully collected and centrifuged at 260 × g for 12 min at 4 °C to concentrate the cell debris and very large aggregates. The supernatants were again centrifuged at 4000 × g for 30 min at 4 °C to obtain the second pellet and the residual supernatant. Both pellets were resuspended in culture medium. Aliquots of three fractions (normalized to equal cell numbers at the time of harvesting) were then incubated with microglia-enriched cultures for 24 h before the extent of microglial activation was evaluated.

Determination of Microglial Activation—The extracellular prostaglandin E2 (PGE2) level was measured as an index for microglial activation. PGE₂ monoclonal enzyme immunoassay kit was obtained from Cayman Chemical Co. Assays were performed following the manufacturer’s specifications. Microglial activation was also assessed by examining microglial morphology after immunohistochemical stain with Ox-42 antibody.

Proteomic Analyses—To search for the potential mediators of microglial activation induced by Mn-EBDC- or MPP⁺-induced neurotoxicity, a newly developed quantitative proteomic technique termed SILAC (for stable isotope labeling with amino acids in cell culture) was used (27). Essentially two groups of MES cells were grown in identical culture media except for one essential amino acid, L-arginine: the first media contains the “light” (L-[13C6]Arg isotope), and the other contains the "heavy" form (L-[12C6]Arg isotope; Cambridge Isotope Laboratories, Andover, MA). After being cultured for at least five generations (to achieve near 100% incorporation of arginine (27, 28), L-[13C6]Arg- and L-[12C6]Arg-labeled MES cells were treated with 25 μM MPP⁺ or saline, respectively, for another 3 days. A 150-μg aliquot of membrane proteins isolated from the 260 × g fraction was taken from each group of cells (29), combined, and resolved by 8–16% SDS-PAGE. After electrophoresis, Coomassie Brilliant Blue-stained protein bands were cut and in-gel trypsin (Promega, Madison, WI)-digested (22, 30). Proteins/peptides were separated by LC followed by identification with MS/MS using a proteomic system (LCQ DECA XP PLUS, ThermoElectron, San Jose, CA). Data search was accomplished using Sequest (ThermoElectron) against the mouse rat protein IPi (for International Protein Index) database version 3.01. At least two peptides were required for any protein identification. PeptideProphet and ProteinProphet software were used to enhance the accuracy of protein identification by calculating the probability of a protein that is likely to be identified correctly; this process also takes percentage of peptide coverage of the particular protein into account. Relative protein abundance between experimental groups was calculated using an algorithm termed the automated statistical analysis of protein abundance ratio. All of these methods are used routinely in our laboratory (22, 30, 31).

Statistics—The obtained data were evaluated by one-way analysis of variance and Newman-Keuls test using a commercially available computer software program (Prism 3.0, GraphPad, San Diego, CA). Repeated measures were performed at least three times in all experiments. p < 0.05 was accepted as significant.

RESULTS
We began our investigation on toxic effects of Mn-EBDC, the active component of the widely used pesticide maneb, and MPP⁺, the active component of a classical parkinsonian toxicant MPTP, on DAergic neurons (Fig. 1). Rat primary mesencephalic neuron-enriched cultures were treated for 7 days with 2–8 μM Mn-EBDC or 0.25–5 μM MPP⁺, respectively. [3H]DA uptake and the number of TH-IR neurons were measured to assess neurotoxicity to DAergic neurons. As shown in Fig. 1, A and B, both Mn-EBDC and MPP⁺ induced significant DAergic neurotoxicity in primary neuron-enriched cultures with apparent morphological alterations of remaining DAergic neurons, including loss of the number and length of neurites and the inconsecutive TH immunoreactivity of the remainder of the neurites. Moreover Mn-EBDC- and MPP⁺-mediated neurotoxicity appeared to be dose-dependant (Fig. 1, C and D) with the EC₅₀ being about 3.0 and 0.5 μM for Mn-EBDC and MPP⁺, respectively. Notably both Mn-EBDC- and MPP⁺-mediated neurotoxicity showed relative selectivity toward DAergic neurons as [3H]GABA uptake was largely preserved in both cultures, especially at lower concentrations (<4 μM and 1 μM for Mn-EBDC and MPP⁺, respectively). This relative DAergic selectivity was further confirmed by counting the number of surviving TH-IR and NeuN-IR neurons in Mn-EBDC- or MPP⁺-treated cultures (data not shown). It should be noted that our results on MPP⁺ are largely consistent with observations made by Gao et al. previously (7).

Next we investigated whether the presence of microglia would influence Mn-EBDC- or MPP⁺-mediated DAergic toxicity. In this study, a series of primary microglial cells varying in their percentage were added into mesencephalic neuron-enriched cultures treated with either 2 μM Mn-EBDC or 0.5 μM MPP⁺ for 7 days. These concentrations were chosen to make sure that the dosages used were specific for DAergic neurons (Fig. 1). As shown in Fig. 2, the addition of microglia significantly increased the sensitivity of DAergic neurons to both neurotoxicants positively correlated with the percentage of microglia added to the neuron-enriched cultures.

It is noteworthy that activated microglia in neuron-glia cocultures appeared to be clustering around degenerating DAergic neurons, especially degenerating neurites or axons (Fig. 3A). This phenomenon is consistent with an early notion that degenerating neurons may be the trigger for microglial activation (32). To further study the potential mediators of microglial activation secondary to neurodegeneration, we repeated the experiments in the following three conditions: 1) neuron-enriched cultures without microglia added, 2) neuron-enriched cultures directly mixed with 20% microglia, and 3) mixed neuron-microglia cultures with microglia and neurons being separated with a microporous membrane (pore size, 0.4 μm). All cultures were treated with Mn-EBDC at 2 μM or MPP⁺.
at 0.5 μM for 7 days with DAergic toxicity assessed by determining [3H]DA uptake. As seen in Fig. 3B, it is obvious that Mn-EBDC- or MPP⁺-mediated toxicity was significantly and sufficiently attenuated by the porous membrane. From these results, it was concluded that microglial activation followed by aggravation of DAergic neurotoxicity induced by Mn-EBDC or MPP⁺ requires either direct contact of neurons with microglia or mediation via high molecular weight molecules and aggregates.

To further narrow down the potential mediators of microglial activation induced by parkinsonian toxicants, we switched our system to immortalized DAergic MES cells as the percentage of DAergic neurons in primary mesencephalic cultures is typically too low (usually <5% of total neurons) for more defined mechanistic studies. In this study, MES cells were treated with Mn-EBDC at 4 μM or MPP⁺ at 25 μM for 3 days before the conditioned media were fractioned into three parts by centrifugations: 260 g pellet, 4000 g pellet, and 4000 g supernatant. As shown in Fig. 4, treatment of Mn-EBDC or MPP⁺ for 3 days produced substantial neurotoxicity (Fig. 4A). Notably these results were very similar to what we observed previously (15). Next all three fractions were applied to primary microglia-enriched cultures for 24 h, and the PGE₂ concentration was measured in the conditioned media as an index for the extent of microglial activation. Remarkably the 260 g pellet fraction was most potent in mediating microglia activation (Fig. 4B), suggesting again that direct cell contact or very large aggregates, including cell debris, are primarily responsible for neuronal death-mediated microglia activation.

To explore potential candidates mediating microglial activation induced by parkinsonian toxicants such as Mn-EBDC and MPP⁺, we finally used a nonbiased proteomic technique to verify the potential mediators identified in the previous section.
to determine the protein profiles in the membranous fraction of the 260 \times g \text{ pellet} after MES cells were treated with \text{MPP}^{+}. This approach was taken for the following two reasons. 1) Unlike \text{MPP}^{+}, \text{Mn-EBDC} also directly activated microglia, particularly at high concentration (data not shown), which would introduce ambiguity in data interpretation with respect to the mechanisms by which microglia are activated. 2) The proteome of neurons is very complex, and all current MS technology is biased toward abundant proteins; thus, focusing on only one fraction would enhance our ability to discover low abundance yet more interesting proteins.

To perform the proteomic experiments, MES cells were cultured in light and heavy SILAC reagents, respectively, for 3 days before being treated with \text{MPP}^{+} at 25 \mu M for another 3 days. Then the membranous fractions were isolated from the 260 \times g \text{ pellets} of the conditioned media from control and \text{MPP}^{+}-treated MES cells, combined together, and then subjected to further fractionation using an SDS-PAGE method. LC-MS/MS analysis of 10 PAGE fractions identified a total of 621 proteins in the membranous protein fraction. A detailed protein list is provided in Supplemental Appendix I, and the functional classes of proteins are illustrated in a pie chart (Fig. 5).
| I. Decreased in MPP \(^+\) vs. vehicle \(>100\%\) | II. Decreased in MPP \(^+\) vs. vehicle \(>50\% \text{ but } <100\%\) |
|-----------------------------------------------|-----------------------------------------------|
| 1. Similar to progesterone-induced blocking factor 1 (IPI00373533) | 21. Development- and differentiation-enhancing factor 2 (IPI00365595 and IPI00355808) |
| 2. Hypothetical zinc finger (IPI002262857 and IPI00222566) | 22. Ensembl_locations (Chr-bp): 3-97389423, olfactory receptor MOR245-8 (IPI00210799 and IPI00312426) |
| 3. Similar to 26 S proteasome non-ATPase regulatory subunit 11 (IPI00370382 and IPI00222515) | 23. Vacular ATP synthase subunit F (IPI00198291, IPI00388886, and IPI00365861) |
| 4. Similar to KIAA0788 protein (IPI00373118) | 24. Thioredoxin-like protein 2 (IPI00315595, IPI00403599, and IPI00310100) |
| 5. Similar to ninein (IPI00365822) | 25. Clathrin coat assembly protein AP17 (IPI00118022 and IPI00198371) |
| 6. ADP-ribosylation factor 1 (IPI00221613, IPI00221614, IPI00331953, and IPI00231674) | 26. CD3e antigen (IPI00127644) |
| 7. B-cell-stimulating factor 3, similar to neurotrophin-1/B-cell-stimulating factor-3 (IPI00317193 and IPI00361845) | 27. RIKEN cDNA 2810036L13 (IPI00121760, IPI00360055, and IPI00264565) |
| 8. Prostatic acid phosphatase precursor (IPI00394000, IPI00201376, IPI00135015, and IPI00394635) | 28. Aldolase A (IPI00231734 and IPI00221402) |
| 9. Very large inducible GTPase-1 (IPI00227871) | 29. 2210415M14Rik protein (IPI001323470) |
| 10. Serologically defined colon cancer antigen 1 isoform a (IPI00227669) | 30. Similar to phosphoserine phosphatase (IPI00188112 and IPI00117146) |
| 11. Similar to KIAA0266 gene product (IPI00370044) | 31. Proteasome subunit \(\alpha\) type 6 (IPI00191501 and IPI00131845) |
| 12. RIKEN cDNA 1110007C09 (IPI00315974) | 32. Ensembl_locations (Chr-bp): 17-90853438 (IPI00227669) |
| 13. MKI/A1824 protein (IPI00340504) | 33. Ensembl_locations (Chr-bp): 10-88200227 (IPI00190407 and IPI00390786) |
| 14. Hypothetical grpe protein homolog (IPI00205198 and IPI00171083) | 34. Development- and differentiation-enhancing factor 2 (IPI00365595 and IPI00355808) |
| 15. 2400001E08Rik protein (IPI001323470) | 35. Ensembl_locations (Chr-bp): 3-97389423, olfactory receptor MOR245-8 (IPI00210799 and IPI00312426) |
| 16. UPF0120 protein DKFZp564C186 homolog (IPI00125382) | 36. Vacular ATP synthase subunit F (IPI00198291, IPI00388886, and IPI00365861) |
| 17. Gag-Pol polyprotein (IPI00224370 and IPI00108206) | 37. Thioredoxin-like protein 2 (IPI00315595, IPI00403599, and IPI00310100) |
| 18. Proteasome subunit \(\alpha\) type 6 (IPI00191501 and IPI00131845) | 38. Clathrin coat assembly protein AP17 (IPI00118022 and IPI00198371) |
| 19. Serologically defined colon cancer antigen 1 isoform a (IPI00227669) | 39. CD3e antigen (IPI00127644) |
| 20. Similar to KIAA0266 gene product (IPI00370044) | 40. similar to phosphoserine phosphatase (IPI00188112 and IPI00117146) |

| III. Increased in MPP \(^+\) vs. vehicle \(>100\%\) | IV. Increased in MPP \(^+\) vs. vehicle \(>50\% \text{ but } <100\%\) |
|-----------------------------------------------|-----------------------------------------------|
| 33. Thioredoxin-like, thioredoxin-related protein (IPI00318203 and IPI00266281) | 52. Ubiquitin-activating enzyme E1 1 (IPI00123313) |
| 34. Non-POU domain-containing, octamer-binding protein (IPI00205912 and IPI00320016) | 53. Peripherin (IPI00230444, IPI00129527, IPI00326582, IPI00230445, and IPI00204217) |
| 35. Hypothetical protein (IPI00123129) | 54. Histocompatibility 13 (IPI00364738, IPI00192553, IPI00408103, IPI00228575, IPI00228576, and IPI00112072) |
| 36. Vesicle-associated membrane protein 3 (IPI00132276 and IPI00210971) | 55. Hypothetical protein (IPI001373680) |
| 37. Similar to COP9 complex subunit 4 (IPI00193349 and IPI00131871) | |
TABLE I—continued

| No. | Protein Name and Accession Numbers |
|-----|-----------------------------------|
| 56  | GTP cyclohydrolase I precursor (IPI00205214 and IPI00114691) |
| 57  | Vimentin (IPI00227299) |
| 58  | ATP-binding cassette subfamily E member 1 (IPI00193816 and IPI00322869) |
| 59  | 2500002N19Rik protein, 0610016L08Rik protein (IPI00110885 and IPI00121079) |
| 60  | Hypothetical protein (IPI00153660) |
| 61  | ATP-binding cassette, subfamily F, member 2 (IPI00116825 and IPI00213162) |
| 62  | 4F2 cell surface antigen heavy chain (IPI001114641) |
| 63  | Similar to Txndc1 protein (IPI00365626) |

5). Notably a significant portion of these proteins (about 17%) has no known functions, i.e. they are novel. It should be emphasized, however, that because none of the biochemical methods could obtain absolutely pure membranous factions, it is quite possible that some of these proteins may not actually associate with cell membranes. Thus, the more realistic advantage of fractionation of the 260 × g pellet is that we could enrich the proteins that are low in abundance, thereby allowing us to discover novel proteins that are low in abundance but demonstrate significant changes between two experimental conditions. In this regard, our method identified 38 proteins that either increased or decreased by more than 100% in MPP⁺⁻-treated cells as compared with controls. Another 26 proteins changed >50% but <100% as compared with controls (Table I).

DISCUSSION

Using various well defined culture systems, we demonstrated that microglia were actively involved in Mn-EBDC- and MPP⁺⁻-induced selective DAergic neurodegeneration. Furthermore, we showed that microglial activation secondary to DAergic neurodegeneration induced by Mn-EBDC or MPP⁺ was at least partially mediated by either direct cell contact or factors with high molecular weights. Finally, using quantitative proteomics, we identified numerous candidate proteins that could potentially contribute to microglial activation induced by neurodegeneration.

Consistent with previous observations made by us and others, Mn-EBDC and MPP⁺ produced significant DAergic selective neurotoxicity in primary mesencephalic culture as well as in immortalized MES cells (15, 32–34). More importantly, Mn-EBDC- and MPP⁺⁻-mediated neurotoxicity was enhanced by the addition of microglia to the cultures, concurring with the hypothesis that microglial activation initiated either directly by offending factors or secondary to neuronal cell death could contribute to further neurodegeneration. With respect to direct activation of microglia, various environmental toxicants and infectious agents that release a variety of neurotoxic proinflammatory mediators have been hypothesized to trigger or aggravate neurodegeneration in PD in several parkinsonian models (35–37). There is also epidemiological evidence to support this hypothesis (38), although direct evidence for involvement of infectious agents remains to be established (39).

Although direct microglial activation by various agents is worth pursuing, the current study was more focused on microglial activation secondary to neuronal death, an issue directly related to potential mechanisms of disease progression. A number of factors have been proposed to mediate microglial activation after neuronal cell death in PD, including neuromelanin (17) and aggregated α-synuclein (16). However, in this study, the cellular fraction isolated from the conditioned media (i.e. the 260 × g pellet) that potently activated microglia had no increase in aggregated α-synuclein (Western blot data not shown) as compared with controls. To search for potential mediators to guide us in future research, we used a quantitative proteomic approach comparing the membranous fraction of the 260 × g pellet isolated from the conditioned media between MPP⁺⁻-treated MES cells and those of controls. This approach identified more than 600 proteins in this fraction that can be classified into several major classes, including functions related to cytoskeleton, signal transduction, metabolism, and ubiquitin proteasomal system (UPS). Notably there is a significant portion of proteins that has no known functions (Fig. 5). It should be emphasized that although it is quite possible that all of these proteins are somehow linked to the membrane, contaminants from other cellular compartments cannot be ruled out given that sensitive MS technology could detect trace amount of proteins. Another caveat associated with proteomics is that proteins could be misidentified due to the current incomplete database; thus, validation needs to be performed on the proteins of interest before extensive biological experiments are pursued.

In contrast to cataloging proteins, quantitative proteomics is advantageous in that it identifies proteins with changes in relative abundance that are biologically more interesting. Among the 621 proteins identified, 63 of them displayed significant difference in relative abundance in MES cells treated with MPP⁺ as compared with vehicle-treated controls (Table I). Although there is still a significant portion of proteins without known functions, quite a few identified proteins are appealing, e.g. those related to UPS, cytoplasmic membranous proteins that might be involved in cell-cell contact, or molecular trafficking and those related to signal transduction/ regulation of cellular redox stages.

Proteins related to UPS include proteosome subunit F, α2, and ubiquitin-activating enzyme E1 1. UPS dysfunction has been demonstrated in PD patients as well as parkinsonian animals. Although it is possible that these proteins are just caught with protein aggregates that ultimately activated mi-
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croglia, their potential role in the direct activation of microglia might merit further pursuit. In fact, accumulating evidence has suggested that UPS plays a pivotal role in microglial function as well (40). The second major category of interesting proteins, e.g. clathrin coat assembly protein AP17, is related to cytoplasmic membrane protein or lipid trafficking. Although detailed mechanisms remain to be defined, clathrin-mediated endocytosis has been clearly implicated in microglial activation in a number of settings, including Alzheimer disease (40). The last category of intriguing proteins, e.g. thioredoxin-like protein 2, is related to regulation of cellular redox states. It has been known that thioredoxin plays a cytoprotective role against oxidative stress and alleviates MPTP-mediated neurotoxicity (41). In fact, it has been reported that MPP+ suppresses thioredoxin expression in PC12 cells (42). A decreased level of this protein in the conditioned media derived from MES cells treated with MPP+ is in line with this observation. Finally its role in microglial activation may also need to be investigated further.

In summary, we have demonstrated that microglia are actively involved in Mn-EBDC- and MPP+-induced selective DAergic neurodegeneration. In addition, with a nonbiased proteomic study, we have identified a number of novel proteins that might be involved in microglial activation secondary to DAergic neurodegeneration.

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