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Microglial responses to dopamine in a cell culture model of Parkinson's disease

Diego Mastroeni^a, Andrew Grover^a, Brian Leonard^a, Jeffrey N. Joyce^a, Paul D. Coleman^a, Brooke Kozik^b, Denise L. Bellinger^b, and Joseph Rogers^a

^a Sun Health Research Institute, 10515 West Santa Fe Drive, Sun City, AZ 85372

^b Department of Pathology and Human Anatomy, Loma Linda University School of Medicine, Loma Linda, CA 92350

Abstract

Activated microglia appear to selectively attack dopamine (DA) neurons in the Parkinson's disease (PD) substantia nigra. We investigated potential mechanisms using culture models. As targets, human SH-SY5Y cells were left undifferentiated, or were differentiated with retinoic acid (RA) or RA plus brain-derived neurotrophic factor (RA/BDNF). RA/BDNF-treated cells were immunoreactive for tyrosine hydroxylase and the DA transporter, took up exogenous DA, and released DA after K⁺ stimulation. Undifferentiated and RA-treated cells lacked these characteristics of a DA phenotype. Co-culture of target cells with human elderly microglia resulted in elevated toxicity in DA phenotype (RA/BDNF) cells. Lipopolysaccharide plus K⁺-stimulated DA release enhanced toxicity by 500-fold. DA induced microglial chemotaxis in Boyden chambers. Spiperone inhibited this effect. Cultured human elderly microglia expressed mRNAs for D1–D4 but not D5 DA receptors. The microglia, as well as PD microglia in situ, were also immunoreactive for D1–D4 but not D5 DA receptors. These findings demonstrate that activated microglia express DA receptors, and suggest that this mechanism may play a role in the selective vulnerability of DA neurons in PD.

Keywords

Microglia; dopamine; dopamine receptor; Parkinson's disease; substantia nigra; chemotaxis

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopamine (DA) neurons of the substantia nigra pars compacta and the ensuing depletion of DA in the striatum. Although the pathogenic mechanisms responsible for targeting DA neurons remain unclear, many reports have suggested that the inflammatory cascade, in general, and microglia, in particular, may play a role in the selective vulnerability of PD substantia nigra DA neurons (Kim and Joh, 2006; Lui, 2006; Teismann and Schulz, 2004). These studies range from animal and culture models of PD, where inflammatory mechanisms are consistently observed (Kim

Corresponding Author/Reprint Requests: Joseph Rogers, Ph.D., Sun Health Research Institute, P.O. Box 1278, Sun City, AZ 85372. Tel: 623-876-5466. FAX: 623-876-5461. joseph.rogers@sunhealth.org.

Disclosure Statement

There are no actual or potential conflicts of interest that the authors have.

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and Joh, 2006; Lui, 2006; Teismann and Schulz, 2004), to epidemiologic surveys, where anti-inflammatory drug use appears to be associated with decreased PD risk (Chen et al., 2003; Chen et al., 2005; Hernan, 2006).

Although these findings are reminiscent of the much larger literature on inflammation and Alzheimer's disease (AD), which has, to date, produced few if any successes in treatment trials (McGeer et al., 2006), PD may actually provide a more realistic target for investigating whether or not brain inflammation can be pathogenic as opposed to a mere mechanism for the clearance of detritus. That is, PD is primarily characterized by changes in a clearly-defined, highly vulnerable neuron type, neurotransmitter system, and set of brain structures. By contrast, AD entails pathology in many different neuron types, from pyramidal cells to basal forebrain projection neurons, many different brain regions, from the frontal to the occipital poles, and many different neurotransmitter systems, from glutamate to acetylcholine.

Microglia are cells of mesodermal origin that appear to have many properties in common with peripheral macrophages. They are capable of movement through nervous tissue (Kokovay and Cunningham, 2005), are activated in multiple neurologic disorders (Jack et al., 2005; Kim and Vellis, 2005; Minagar et al., 2002; Nelson, 2002), including PD (Kim and Joh, 2006; Lui, 2006; Teismann and Schulz, 2004), and can act as phagocytes (Jack et al., 2005; Nicoll et al., 2006; Rogers et al., 2002). The substantia nigra is reported to have one of the highest densities of microglia in brain (Kim, 200; Lawson, 1990), amounting to some 12% of the total cells there and more than twice the concentrations observed in cortex and white matter (Lawson, 1990). In PD, activated microglia characteristically cluster around nigral DA neurons, especially those with dystrophic morphology (Akiyama and McGeer, 1989; Kim and Joh, 2006; Lui, 2006; McGeer et al., 1988; Teismann and Schulz, 2004). Moreover, many previous studies have found that activating nigral microglia—for example by injecting lipopolysaccharide (LPS) into or near the substantia nigra—results in a permanent, selective depletion of nigral DA (Arimoto et al., 2003; Gao et al., 2002; Herrera et al., 2005; Hunter et al., 2007; Irvani et al., Peng et al., 2005; Zhou et al., 2005), and that such effects can be mitigated by treatment with anti-inflammatory agents (Hunter et al., 2007; Peng et al., 2005).

In order to begin to understand the cellular and molecular bases for these phenomena, we have attempted to develop cell culture models using human elderly microglia obtained from rapid autopsies and, as potential targets for the microglia, human cells that express some of the characteristics of DA neurons, including expression of tyrosine hydroxylase and the DA transporter, uptake of DA, and release of DA after K⁺ stimulation. Such cells were derived by treating the human SH-SY5Y line with retinoic acid (RA) followed by brain-derived neurotrophic factor (BDNF) (Encinas et al., 2000). Vehicle-treated, undifferentiated SH-SY5Y cells and SH-SY5Y cells treated with RA alone served as controls. After characterization, target cells were co-incubated with microglia to investigate cytotoxic mechanisms and their specificity for the DA phenotype. Subsequent experiments examined DA receptor expression by microglia, and the ability of DA to induce microglial chemotaxis.

2. Materials and Methods

2.1 Tissue samples for microglia cultures

Human microglia cultures were derived from rapid (< 4 hours) autopsies of superior temporal gyrus of PD (mean age = 75 ± 3 years old) and normal elderly control (NC) (mean age = 78 ± 4 years old) subjects. All autopsy cases were volunteer participants in the Sun Health Research Institute Tissue Bank, and had signed Institutional Review Board-approved consent forms prior to death. Antemortem, the subjects received thorough annual neurologic examinations by board-certified neurologists, and their diagnoses were confirmed at autopsy by a board-certified neuropathologist. There were no material or statistical differences in the viability or

responses of the microglia from PD and NC cases, and the culture data have been pooled across groups in this report.

2.2 Human elderly microglia cultures

Human elderly microglia were isolated and maintained in culture as previously described (Lue et al., 1996, 2001; Walker et al., 2005), using methods similar to those originally developed by Kim (1985). The cultured microglia expressed antigenic, morphologic, and other criteria of microglia, and were approximately 98% pure, confirming previous studies (Lue et al., 1996, 2001; Walker et al., 2005).

2.3 RA-, RA/BDNF-, and vehicle-treated cells from SH-SY5Y cultures

SH-SY5Y is a thrice-cloned subline of the human neuroblastoma cell line SK-N-SH. The SH-SY5Y cells employed in the present experiments (American Tissue Culture Collection, Manassas, VA) were grown to confluence in DME/F12 (Gibco) supplemented with 10% FBS and 0.1% gentamycin. After sub-culturing into 6- and 12-well plates (Corning Costar, Lowell, MA), the cells were subjected to one of three differentiation protocols. Vehicle-treated, undifferentiated (UNDIFF) cells were grown in DME/F12 supplemented with 10% FBS and 0.1% gentamycin and were replenished with fresh medium every three days. Parallel SH-SY5Y cultures were treated similarly, but with 10 μ M RA (Sigma, St. Louis) in 2% FBS for three days, followed by replenishing with fresh medium containing 10 μ M RA in 0.5% FBS for another three days (RA cells). As previously reported, SH-SY5Y cells treated with such a protocol exhibit several characteristics of cholinergic neurons, including expression of ChAT and VMAT (Pahlman et al., 2005; Presgraves et al., 2004). An additional, parallel set of SH-SY5Y cells were further differentiated by exposing them to RA, as described above, followed by treatment with 50ng/ml BDNF (Peprotech, Rocky Hill, NJ) in 0.5% FBS for three days (RA/BDNF cells) (Encinas et al., 2000). RA/BDNF cultures were replenished with fresh 50ng/ml BDNF in 0.5% FBS for three more days, and were subsequently found to exhibit several characteristics of DA neurons, including expression of tyrosine hydroxylase and the DA transporter, as well as uptake and K⁺-stimulated release of DA (see below).

2.4 DA uptake

To assay DA uptake, UNDIFF, RA, and RA/BDNF cells in 4-well chamber slides (30,000 cells/well) were incubated with vehicle (serum free medium) or 50 μ M DA plus 1mM L-glutathione for 1 hour at 37°C. Glutathione was included because its antioxidant properties have been reported to protect cells from damage by reactive oxygen species generated by exogenous DA in culture paradigms similar to those used here (Grima et al., 2003). Uptake of DA was assayed using the glyoxylic acid condensation method (de la Torre and Surgeon, 1976).³⁴ Briefly, following three washes with PBS, the cells were incubated for 5 min at 4°C in sucrose-potassium phosphate-glyoxylic acid buffer (pH 7.4) containing 1% (w/v) glyoxylic acid, 3.2% (w/v) KH₂PO₄, 6.8% (w/v) sucrose. Chamber slides were then dried and incubated for 5 min at 80°C. After immersion under a coverslip with one drop of mineral oil, fluorescence of the cells was viewed under a confocal microscope.

2.5 DA release

To assay DA release, UNDIFF, RA, or RA/BDNF cells in 6-well plates (300,000 cells/well) were incubated with DA, as above, washed 3X with PBS, and exposed to serum free medium or a 10% (v/v) high K⁺ Ringer's solution (1,283mM NaCl, 60mM KCl, 13.5mM CaCl₂-2H₂O, 20mM MgCl₂, 20mM Na₂HPO₄) in serum free medium. This protocol has been previously shown to induce release of DA from DA neurons after their prior incubation with exogenous DA (Gross et al., 1999; Shalaby et al., 1983). Concentrations of DA released into

the supernatant were determined using standard HPLC-EC methods, as previously described (Rice et al., 2002).

2.6 Assays of cell damage and cell death

Twelve days after seeding, microglial cultures were exposed to 10µg/ml LPS (Sigma) or vehicle. Twenty-four hours later, the cultures were washed with 1X HBSS (Irvine Scientific) and trypsinized with trypsin 1X EDTA (Gibco). Suspended cells were pelleted by centrifugation for 10 min at 200g. The supernatant was aspirated and the cells were resuspended in medium for undiluted transfer at approximately 20,000 microglia/well into 6-well plates coated with both poly-L-lysine (Sigma) and collagen IV (Sigma). These wells had previously been plated with RA, RA/BDNF, or UNDIFF cells at approximately 15,000 cells/well, permitting subsequent evaluation of cell damage and death in the target cells after exposure to LPS-treated or untreated microglia. In addition, parallel experiments sought to evaluate the effects of DA uptake and release by the target cells on microglia-induced toxicity. Here, UNDIFF, RA, or RA/BDNF cells were incubated for 2 hours with either vehicle (serum free medium) or 50µM DA plus 1mM L-glutathione, washed 3× with HBSS, co-cultured with microglia, and immediately exposed to either serum free medium or a 10% (v/v) high K⁺ Ringer's solution in serum free medium to release DA (Gross et al., 1999; Shalaby et al., 1983). All experimental conditions were evaluated in triplicate. It is important to note that with these methods the target cells were never directly exposed to LPS, and the microglia were never directly exposed to growth factors or DA, with the exception of DA potentially released by the target cells after K⁺ stimulation. At baseline, 24, 48, and 96 hours after exposure to the various experimental conditions, cell viability was assayed in two ways. First, RA, RA/BDNF, and UNDIFF cells were counted under phase contrast optics using an eyepiece reticule divided into a 10 × 10 square of 100 smaller counting squares. For each well in each condition, 20 random fields were selected at 20× objective magnification. The number of cells with perikarya falling entirely within the center-most small counting square was counted, and the mean over the 20 fields was recorded. To accommodate for slight differences in the numbers of cells plated per well, all cell counts were normalized to percent of baseline. Cell counts were performed blind to experimental condition. Lactic acid dehydrogenase (LDH) release provided a second measure of cell viability. Cell-free culture supernatants were collected from the wells at baseline, and at 24, 48, and 96 hours after exposure to the various experimental treatments. Cytotoxicity detection kits (Roche Applied Science, Indianapolis) were then applied to assay LDH release. The manufacturer's protocol, which is based on LDH-catalyzed conversion of lactate to pyruvate and the formation of fluorescent formazan dye, was followed without modification. Formazan fluorescence emissions at 500 nm were recorded for each well and condition. The LDH data were corrected for cell density based on concurrent cell counts, as described above.

To assess the effects of target cell phenotype and microglial activation, two 3-way factorial ANOVAs were initially performed, with microglial LPS exposure (LPS or vehicle) as the first factor, target cell type (UNDIFF, RA, or RA/BDNF) as the second factor, and hours of co-incubation with microglia (24, 48, or 96 hours) as the third factor. LDH release was the dependent variable in the first ANOVA, and cell counts were the dependent variable in the second ANOVA. Specific comparisons were then tested based on a priori hypotheses that were backed by significant interaction terms in the overall ANOVAs. To assess the effects on the target cells of high K⁺ stimulation, as well as incubation with LPS-activated microglia, a 3-way ANOVA was also run, followed by specific comparisons based on a priori hypotheses and significant interaction terms in the overall ANOVA. The main effects factors were microglial LPS stimulation (LPS or vehicle), target cell type (UNDIFF, RA, or RA/BDNF), and high K⁺ stimulation (K⁺ or vehicle).

2.7 Chemotaxis

Microglial chemotaxis was evaluated using single well blind well chemotaxis chambers (Boyden chambers) (Neuroprobe, Gaithersburg, MD) (Marra et al., 1999). The manufacturer's suggested procedures were employed throughout. Briefly, 200µl of test solution was placed in the lower chamber, and 100µl of microglial suspension containing approximately 16,000 cells was placed in the upper compartment. A 5µm polycarbonate filter (Neuroprobe) separated the two compartments. Test solutions were 100nM monocyte chemoattractant protein-1 (MCP-1) (Peprotech, Rocky Hill, NJ), 100nM DA (Sigma), 100nM DA plus 10nM spiperone (Sigma), or vehicle. MCP-1 is a potent monocyte chemokine to which microglia respond (Hayashi et al., 1995; Platten et al., 2003), and served as a positive control. Vehicle alone (serum free medium) served as a negative control. Spiperone is a D1/D2 receptor antagonist with higher affinity for D2 receptors (Seeman and Van, 1994). The chambers were incubated in 7% CO₂ at 37°C for 45 min. At the end of incubation, filters were wiped twice with a cotton swab and stained with calcein AM (Chemicon). Cells were counted using a Wallac multilabel counter, then fixed and visualized under a confocal microscope.

2.8 Immunocytochemical characterization of microglial cultures

Cultures were washed in Dulbecco's phosphate buffered saline (DPBS) (Gibco), fixed with 4% paraformaldehyde, incubated at 4°C for 15 min, washed again, and exposed to a 1% peroxidase block for 35 min. After an additional wash the cells were blocked in 3% goat serum/bovine serum albumin (BSA) (Sigma) for 45 min at room temperature, washed, and incubated with primary antibody in DPBS with 0.25% goat serum for 2 hours at 4°C. All washes were done three times with DPBS for 10 min each. Primary antibodies and dilutions were 1:1000 mouse monoclonal anti-LN3 (ICN Biomedical, Costa Mesa, CA), a marker for the major histocompatibility complex type II that is expressed by activated microglia (Maat-Schieman et al., 1994; Perlmutter et al., 1992); 1:1000 mouse monoclonal anti-CD11b (Caltag Laboratories, Burlingame, CA), another well-established marker for activated microglia (Akiyama and McGeer, 1990; Denker et al., 2007); 1:1000 rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (Dako, Carpinteria, CA), an astrocyte marker; 1:500 rabbit polyclonal anti-tyrosine hydroxylase (Chemicon International, Temecula, CA), 1:500 rabbit polyclonal anti-D1 DA receptor (Chemicon International); 1:500 rabbit polyclonal anti-D2 DA receptor (Novus Biologicals, Littleton, CO); 1:500 rabbit polyclonal anti-D3 DA receptor (Chemicon International), 1:500 rabbit polyclonal anti-D4 DA receptor (Chemicon International), or 1:500 rabbit polyclonal anti-D5 DA receptor (Abcam, Cambridge, MA). Vector ABC kits (Vector Laboratories, Burlingame, CA) were used for bright field microscopy. The chromagen label was 125µl of a 10 mg/ml diaminobenzidine (DAB) (Sigma) solution in 500 mM Tris buffer plus 500µl saturated nickel ammonium sulfate. Incubations during chromagen development were no longer than 4 min, and were followed by two quick rinses in 50 mM Tris to stop the reaction. Fluorophore-conjugated secondary antibodies appropriate for species were employed for single- and double-label fluorescence microscopy. Secondary antibodies and dilutions were 1:1000 goat anti-mouse or goat anti-rabbit Alexa 488 (Molecular Probes, Carlsbad, CA) or 1:1000 goat anti-mouse or goat anti-rabbit Alexa 568 (Molecular Probes).

2.9 Immunohistochemistry of tissue sections

Substantia nigra and striatum sections were cut at 20 and 40µm from fixed blocks of PD and NC samples, then double-immunoreacted for markers of microglial activation and DA receptors. Antibody sources and dilutions were the same as for immunocytochemistry (see above). Sections were blocked in 1% H₂O₂ for 30 min at room temperature, washed 3 × 10 min in PBST, blocked in 3% goat serum for 1 hour at room temperature, washed 2 × 10 min, and incubated overnight at 4°C with primary antibodies. Following 3 × 10 min washes in PBST, the sections were incubated in species appropriate secondary antibodies using Vector ABC kits

(Vector Laboratories). For double-label experiments, DAB plus saturated nickel, as above, was applied first to visualize the first primary antibody, and DAB alone was applied second to visualize the second primary antibody.

2.10 RT-PCR for D1–D5 DA receptor mRNAs

Microglia were cultured from human brain autopsy tissues as described above, with seeding in 12-well plates at approximately 500,000 cells/well. After 48 hours, total RNA was isolated using TRIzol Reagent (Invitrogen). RNA precipitation was enhanced with RNase-free glycogen (Invitrogen) and genomic DNA was degraded by treatment with RNase-free DNase (Invitrogen). For each DA receptor subtype, cDNA was generated from 8µl RNA using Superscript II kits (Invitrogen). All RT-PCR employed Quiagen reagents and primers that were designed for DA receptor subtypes D1–D5 (genomic nomenclature DRD1–DRD5) (Table 1). Reaction conditions were held constant for all DA receptor subtypes using 35 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min. RT-PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide (0.5µg/ml) on an Alpha Innotech digital imaging system. The experiments were performed in triplicate. A quantity standard (Low Mass Ladder, Invitrogen) was also run on the gel. Intensity of PCR and Mass Ladder bands was assessed by densitometry using AlphaEaseFC software, and the estimated mass of each PCR product was calculated.

3. Results

3.1 Characterization of human elderly microglia cultures

Consistent with previous studies, microglial cultures derived from rapid (<4 hours) autopsies of human elderly neocortex were approximately 98% pure. The cultures exhibited no detectable immunoreactivity for non-microglial markers (e.g., GFAP, an astrocyte marker), but were clearly immunoreactive for LN3, a major histocompatibility class II (MHCII) marker for activated monocytes and microglia (Maat-Schieman et al., 1994; Perlmutter et al., 1992) (Fig. 1A, 1B). Microglial cultures also were immunoreactive for CD11b, a beta II integrin marker for macrophages and microglia (Akiyama and McGeer, 1990; Denker et al., 2007) (Fig. 1C, 1D).

3.2 Characterization of target cells

To provide targets for microglial responses, SH-SY5Y neuroblastoma cells were vehicle-treated and left undifferentiated (UNDIFF cells), treated with RA alone (RA cells), or treated with RA followed by BDNF (RA/BDNF cells). UNDIFF cells typically grew in compact, aggregated clusters with thread-like neurites, as observed in earlier studies (Encinas et al., 2000; Presgraves et al., 2004). RA and RA/BDNF cells exhibited classic characteristics of differentiated neurons in culture, including bright phase somas and elongated neuritic processes that in many cases appeared to form a network. This process extension was most marked in RA/BDNF cells (Fig. 2).

RA/BDNF cells also exhibited several DA phenotypic properties. They were immunoreactive for tyrosine hydroxylase and the DA transporter (Fig. 3), and, consistent with expression of the DA transporter, appeared able to take up exogenous DA and to release DA after high K⁺ stimulation (Gross et al., 1999; Shalaby et al., 1983) (Fig. 4). UNDIFF and RA cultures exhibited little to no evidence of DA transporter immunoreactivity, tyrosine hydroxylase immunoreactivity, DA uptake, or DA release (Figs. 3, 4).

3.3 Effects of culturing microglia with target cells

To assess the specificity of microglial responses to the target cells, as well as the effects of treatment of the microglia with LPS, a classic stimulant of microglial activation (Arimoto et al., 2003; Gao et al., 2002; Herrera et al., 2005; Hunter et al., 2007; Irvani et al., Peng et al., 2005; Zhou et al., 2005), LPS-treated or untreated human elderly microglia were co-cultured with the target cells. Importantly, LPS was applied to microglia and growth factors were applied to target cells prior to co-culture. The cultures were also washed prior to co-culture, so that microglia were never directly exposed to growth factors and target cells were never directly exposed to LPS. Assays of toxicity in the co-cultures were performed at baseline and 24, 48, and 96 hours thereafter. Cell viability, as measured by LDH release, was significantly decreased as a function of prior microglial LPS exposure ($F_{\text{LPS}} = 10.5$, $P = 0.002$), time of co-incubation with microglia ($F_{\text{hours}} = 192.3$, $P < 0.001$), and target cell type ($F_{\text{target}} = 12.4$, $P < 0.001$). However, the effects of microglial LPS exposure were not uniform, but differed significantly as a function of the target cell with which the LPS-stimulated microglia were co-incubated ($F_{\text{LPS} \times \text{target}} = 14.4$, $P < 0.001$). In particular, as a percentage of baseline, LDH release after exposure to LPS-activated microglia resulted in significantly greater toxicity in RA/BDNF cells than RA ($F = 4.6$, $P = 0.05$) or UNDIFF cells ($F = 21.4$, $P < 0.001$) (Fig. 5A). Inspection of the cultures revealed many dystrophic target cells, with no evidence of damage to microglia, suggesting that LDH release was due to target cell toxicity. Although microglia were initially seeded randomly into all cultures, clustering of microglia around dystrophic RA/BDNF cells was particularly marked, especially when microglia had been previously activated with LPS (Fig. 5B).

Virtually identical results were obtained with estimates of cell death as the dependent measure. Cell loss was significantly increased as a function of prior microglial LPS exposure ($F_{\text{LPS}} = 10.1$, $P = 0.003$), time of co-incubation with microglia ($F_{\text{hours}} = 163.2$, $P < 0.001$), and target cell type ($F_{\text{target}} = 90.1$, $P < 0.001$). Again, the effects of microglial LPS exposure were not uniform, but differed significantly as a function of the target cell phenotype ($F_{\text{LPS} \times \text{target}} = 5.5$, $P = 0.007$). In particular, cell death after incubation with LPS-activated microglia was significantly greater in RA/BDNF cells than RA ($F = 7.6$, $P < 0.014$) or UNDIFF cells ($F = 63.9$, $P < 0.001$).

3.4 Effects of DA exposure and release on target cell viability

As previously noted, RA/BDNF cells, but not RA or undifferentiated cells, were able to take up exogenous DA and to release it after high K^+ stimulation. When the target cells were exposed to DA prior to co-incubation with LPS-treated or untreated microglia, but DA release was not stimulated, LDH measures of toxicity were again significantly increased as a function of microglial exposure to LPS ($F_{\text{LPS}} = 9.2$, $P = 0.006$) and target cell type ($F_{\text{target}} = 14.8$, $P < 0.001$). However, exogenous DA exposure followed by stimulation of the target cells with high K^+ to release DA significantly exacerbated this effect ($F_{K^+} = 15.9$, $P < 0.001$). As shown by the significant interaction terms, these results owed almost entirely to the exquisite sensitivity of RA/BDNF cells to stimulated DA release ($F_{K^+ \times \text{target}} = 14.9$, $P < 0.001$), especially under conditions of co-incubation with LPS-activated microglia ($F_{K^+ \times \text{target} \times \text{LPS}} = 9.0$, $P < 0.001$), where the LDH toxicity measure increased some 500-fold (Fig. 6).

Cultured rodent microglia have been reported to possess potassium channels that mediate a number of microglial properties, including chemotaxis, through regulation of integrin (Natile-McMenemy, Elfenbein, and Deleo, 2007) and inducible nitric oxide synthase expression (respiratory burst) (Chang et al., 2000). Although the high extracellular potassium concentration (60 mM) used in the present experiments represents a standard method for eliciting DA release (Gross et al., 1999; Shalaby et al., 1983), it is therefore possible that this method also triggered a microglial respiratory burst that contributed to the enhanced cell death

that was observed. However, this cannot have been the sole effect of potassium exposure, as significant neurotoxicity was observed only in DA phenotype neurons, not undifferentiated or partially differentiated cells. If a potassium-stimulated microglial respiratory burst were the sole mechanism for toxicity, then undifferentiated and partially differentiated neurons should also have been affected.

3.5 Microglial chemotaxis

In order to begin to evaluate mechanisms that might underlie the increased co-localization of microglia with RA/BDNF cells, the enhanced toxicity observed when RA/BDNF-treated, DA phenotype cells were co-incubated with activated microglia, and the exacerbation of these effects under conditions of prior DA exposure and high K^+ -stimulated DA release, the possibility that DA itself might mediate microglial responses was investigated. As a starting point, microglial migration in standard Boyden chemotaxis chambers (Marra et al., 1999) was assayed. Monocyte chemoattractant protein-1 (MCP-1), a potent stimulator of microglial chemotaxis (Hayashi et al., 1995; Platten et al., 2003), was used as a positive control, and vehicle (serum free medium) was used as a negative control. DA and DA plus spiperone, a DA receptor antagonist with higher affinity for D2, D3, and D4 DA receptors (Seeman and Van, 1994), were the test solutions. Over all conditions, significant microglial chemotaxis was observed ($F = 294.3$, $P < 0.001$). MCP-1, the positive control, induced 3-fold more migration than the serum free medium control ($t = 17.5$, $P = 0.003$). DA alone induced 4-fold more migration than the serum free medium control ($t = 33.9$, $P < 0.001$) and, in fact, stimulated significantly greater migration than MCP-1 ($t = 5.8$, $P = 0.03$). Moreover, in the presence of the DA receptor antagonist spiperone, there was significant inhibition of DA-induced microglial chemotaxis ($t = 23.5$, $P = 0.002$) (Fig. 7).

3.6 Microglial expression of DA receptors and DA receptor mRNAs

Enhanced chemotaxis of microglia to DA and its inhibition by spiperone suggested that microglia might express DA receptors. Replicating an earlier pilot study (not shown), RT-PCR for D1–D5 DA receptor mRNAs in human elderly microglia cultures revealed clear bands at the appropriate amplicon sizes for D1–D4 (DRD1: 151 bp, DRD2: 199 bp, DRD3: 828 bp, DRD4: 186 bp). By contrast, a band for D5 mRNA at its appropriate amplicon size (305 bp) was barely detectable, and was significantly less intense than the bands for D1–D4 DA receptor mRNA (Fig. 8). Validation with immunocytochemistry and immunohistochemistry also showed clear immunoreactivity for D1–D4 but not D5 DA receptors both in human elderly microglial cultures and in substantia nigra and striatum sections from PD and NC autopsy cases (Fig. 9).

4. Discussion

In the present study, significantly enhanced vulnerability to microglia-mediated attack was observed in cells with characteristics of a DA phenotype compared to cells that lacked these characteristics. Toxicity, as measured by LDH release and by cell counts, was further enhanced by LPS activation of the microglia and by incubation with and release of DA by the target cells, effects that appeared to be synergistic. In particular, the LDH toxicity measure increased some 500-fold in K^+ -stimulated DA phenotype (RA/BDNF) neurons after co-incubation with LPS-activated microglia. DA also potently stimulated microglial chemotaxis in Boyden chambers, an effect inhibited by spiperone. Immunocytochemistry, immunohistochemistry, and RT-PCR revealed not only the expression of DA receptors and DA receptor mRNAs by human elderly microglia, but also the same patterns of expression: D1–D4 DA receptor immunoreactivity and mRNAs were clearly evident, whereas D5 DA receptor immunoreactivity and mRNAs were equivocal at best.

In neurons, signal transduction from activated dopamine receptors is transmitted through Gs-type (D1 family) or Gi-type (D2 receptor family) g-proteins to influence adenylate cyclase activity and intracellular cAMP levels (Thompson, Burnham, and Cole, 2005). Whether this is also the case in microglia remains to be demonstrated. The present results do, however, clearly demonstrate that activated human elderly microglia express DA receptors, and suggest that this mechanism might play a role in the selective vulnerability of DA neurons in PD. It is also conceivable that microglial responses to DA could help explain DA toxicity after long term treatment with L-DOPA (Kostrzewa et al., 2002), although direct effects of L-DOPA on DA neurons have been reported as well (Falkenburger and Schulz, 2006).

Although one previous study has demonstrated functional expression of D1-like and D2-like DA receptors in rodent neonate microglia cultures and brain slices (Farber et al., 2005), the expression of DA receptors and DA receptor mRNAs by human elderly microglia in culture, as well as microglial immunoreactivity for DA receptors in substantia nigra and striatum tissue sections, is a novel and somewhat unexpected finding. Why microglial DA receptor mRNAs and protein have not been reported in previous studies could owe to several factors. First, the slender morphology of microglia and their proximity to neurons and neurites often make it difficult to discriminate microglial from neuronal expression of surface antigens without a specific microglial counterstain, which would not typically be considered in a DA receptor experiment. Second, many early studies of DA receptor distributions employed autoradiographic techniques, where discrimination of a signal on microglia would be difficult to discern, and more recent studies have often focused on PET and SPECT analyses, neither of which would reveal microglial labeling. Differences in fixation and postmortem intervals (here, all under 4 hours) could be factors as well.

Microglial DA receptors may enhance toxic responses to DA phenotype neurons by facilitating chemotactic targeting, phagocytic activation, or both, as there is precedent for both mechanisms in other cell types. For example, DA selectively induces migration and homing of CD8⁺ T cells via D3 receptors (Watanabe et al., 2006), and also stimulates macrophage phagocytosis at doses as low as 10⁻¹⁵M (Roy and Rai, 2004). If, as we suspect, microglial DA receptor expression is coupled to microglial activation under inflammatory conditions, then selective vulnerability of DA neurons would be likely to follow a wide range of suggested etiologies for PD, almost all of which are known to stimulate inflammation. That is, the induction of inflammation is known to occur after repeated head trauma (Xue and Del Bigio, 2003), pesticide exposure (Gao et al., 2003), 6-OHDA administration (Akiyama and McGeer, 1989), MPTP intoxication (Kokovay and Cunningham, 2005), Lewy body formation (Su et al., 2007), or brain infection (Wang, Rumbaugh, and Nath, 2006). Such inflammation might then upregulate microglial DA receptor expression, providing a final common pathway for selective migration to and attack on neurons that secrete DA. Alternatively, the release of large amounts of DA into the extracellular space by deteriorating DA neurons (e.g., as the result of inflammatory attack or other causes such as failures of energy metabolism, Lewy bodies, or genomic alterations) could direct selective targeting of DA neurons by DA receptor-bearing microglia as a secondary response. Investigating these alternatives, elucidating the signaling pathways and specific DA receptor subtypes that are involved, and determining whether or not blocking them might have therapeutic value in PD, are challenges we look forward to in future.

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Fig. 1.

Immunoreactivity for LN3 (A, B) (green fluorophore) and CD11b (C, D) (red fluorophore), classic markers for microglia and microglial activation in cultures of human elderly microglia (DAPI counterstain for DNA). Primary antibody deletion and/or prior absorption of antibodies with antigens gave uniformly negative immunohistochemical and immunocytochemical results both in this experiment and all others reported here. As was found in the present experiments, partial activation of human elderly microglia commonly occurs when the cells are placed into culture, presumably due to the rigors of cell isolation and maintenance in vitro, the prior state of the microglia in vivo, or other factors.

Fig. 2.
Morphology of SH-SY5Y cells 7 days after treatment with vehicle only (UNDIFF), RA only (RA), or RA followed by BDNF (RA/BDNF). (Phase contrast, 20×).

Fig. 3. Immunoreactivity for the DA transporter (DAT) (upper panels) and tyrosine hydroxylase (TH) (bottom panels) in vehicle-treated (UNDIFF), RA-treated (RA), and RA/BDNF-treated (RA/BDNF) cells. (DAB chromagen, bright field, 10 $\times$).

Fig. 4.

DA uptake and release by the cultures. (Upper three panels) Fluorescence histochemistry (glyoxylic acid condensation method) for DA uptake by UNDIFF (vehicle-treated) (10×), RA-treated (10×), and RA/BDNF-treated (40×) cells after 30 min exposure to 50µM exogenous DA plus 1mM L-glutathione. (Bottom panel) DA release detected by HPLC-EC assays of culture supernatants. Cultures were exposed to 50 or 100 µg/ml exogenous DA for 30 min, washed 3×, then stimulated with 10% (v/v) high K⁺ Ringer's solution. DA release was near background for UNDIFF and RA cells, and was significantly less than that for RA/BDNF cells (RA/BDNF vs UNDIFF at 50 and 100 µg/ml DA: $t = 56.5$, $P < 0.001$ and $t = 42.6$, $P < 0.001$, respectively; RA/BDNF vs RA at 50 and 100 µg/ml DA: $t = 44.7$, $P < 0.001$, and $t = 34.8$, $P < 0.001$, respectively). Comparisons were made over three independent HPLC-EC runs. A fourth run with materials that had been frozen and thawed twice was excluded from the analysis because of low values, although significance levels were still less than 0.001 when this run was included.

Fig. 5.

Effects of microglia co-culture with UNDIFF, RA, and RA/BDNF cells. (A) Toxicity, as measured by LDH release, after 96 hours co-incubation. (B) Nearly identical results were obtained using cell counts as the measure of toxicity, as suggested by the representative photomicrographs shown here of microglia and target cells after co-culture for 96 hours. Darker cells are LN3 immunoreactive, DAB-stained activated microglia. Lighter, unstained cells with a more flattened morphology are the target cells. (Phase contrast with LN3 immunocytochemistry, 10 $\times$).

Fig. 6.

Prior exposure of the target cells to 50mM DA, followed by stimulated release with high K⁺ Ringer's, dramatically exacerbated toxicity in RA/BDNF-treated cells, especially when microglia were previously activated with LPS. This effect was again statistically significant in quantitative LDH release assays (A) and visually apparent in representative photomicrographs (B), where loss of the target cells (tan, neutral red-stained cells) and preservation of LN3 immunoreactive microglia (darker, DAB-stained cells) was especially obvious when RA/BDNF was used for differentiation to a DA phenotype and LPS exposure plus stimulated DA release were used as the treatment conditions (bright field, 20 $\times$).

Fig. 7. Microglial chemotaxis in Boyden chambers in response to vehicle (serum free medium), 100nM MCP-1, 100 nM DA, or 100nM DA plus 10 nM spiperone. Significance levels after t-test comparisons are as shown in figure.

Fig. 8.

Dopamine receptor mRNA expression by cultured human elderly microglia. A) Representative gel image of D1 (DDR1) through D5 (DDR5) DA receptor mRNAs. B) Densitometry of bands in panel A. Replicating an earlier pilot experiment, D1–D4 DA receptor mRNAs (DRD1–DRD4) showed clear bands at appropriate amplicon sizes and were significantly elevated compared to D5 DA receptor mRNA (DRD5) ($P < 0.001$, all comparisons, by t-test). (Kb+: base pair standard; –ve: vehicle control; LML: Low Mass Ladder).

Fig. 9.

Photomicrographs of double immunostaining with the microglia marker LN3 and monoclonal antibodies to human D1 through D5 DA receptors. Confocal fluorescence images are of human elderly microglia cultures with a green fluorophor for the various DA receptors and a red fluorophor for LN3 (60×). Bright field images are of PD striatum with a purple chromagen (DAB plus nickel) for the various DA receptors and a brown chromagen (DAB alone) for LN3 (40×). Positive microglial immunoreactivity for D1, D2, D3, and D4 DA receptors was detected both in culture and in striatum sections, but D5 immunoreactivity was absent in microglia cultures and highly equivocal in striatum sections. Similar findings were obtained in the nigra and in NC cases (not shown). Notably, whereas the D5 DA receptor antibody did not unequivocally label microglia, it did prominently stain neuronal/neuritic elements in cortex of the same patients (not shown), suggesting that the failure to label nigral and striatal microglia with the D5 antibody was not due to inadequate techniques or defective reagents.

Table 1

Primer Sequences

mRNA	Forward	Reverse
DRD1	5'-GAAGTCCACATTCCAAGCTC-3'	5'-GGTGGTGACAGGAGATTCTC-3'
DRD2	5'-TCTGGACGTCATGATGTGC-3'	5'-GTCTGCGTTATTGAGTCCG-3'
DRD3	5'-TAGTAGTGAGCCTGGCTGTG-3'	5'-GCCAGCAGACAATGAAGG-3'
DRD4	5'-CCACCAACTCCTTCATCG-3'	5'-CGCACAGGTTGAAGATGG-3'
DRD5	5'-GGCAGTTCGCTCTATACCA-3'	5'-GCAGAACGCTCCAAAGG-3'