Published in final edited form as:

Neurobiol Aging. 2012 February ; 33(2): 423.e27–423.e36. doi:10.1016/j.neurobiolaging.2010.09.025.

The effects of age and LPS-mediated peripheral inflammation on numbers of central catecholaminergic neurons

P.R. Mouton^{a,f,g}, B. Kelley-Bell^a, D. Tweedie^d, E.L. Spangler^a, E. Perez^a, O.D. Carlson^c, R.G. Short^c, R. deCabo^a, J. Chang^a, D.K. Ingram^e, Y. Li^b, and N.H. Greig^b

^a Laboratory of Experimental Gerontology, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD

^b Laboratory of Neurosciences, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD

^c Laboratory of Clinical Investigations, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD

^d MedStar Health Research Institute, Baltimore, MD

^e Pennington Brain Research Center, Louisiana State University, Baton Rouge, LA

^f Byrd Alzheimer's Center and Research Institute, University of South Florida College of Medicine, Tampa, FL

^g Stereology Resource Center, Chester, MD

Abstract

Parkinson's disease (PD), an age-related movement disorder, is characterized by severe catecholaminergic neuron loss in the substantia nigra pars compacta-ventral tegmental area (SN_{PC}-VTA) and locus coeruleus (LC). To assess the stability of these central catecholaminergic neurons following an acute episode of severe inflammation, 6 to 22 month-old C57/Bl6 mice received a maximally tolerated dose of LPS followed by euthanasia two hours later to assay peak levels of peripheral and central cytokines; and, 14 weeks later for computerized stereology of tyrosine hydroxylase-immunopositive (TH+) neurons in the SN_{PC}-VTA and LC. Two hours after LPS, cytokine levels varied in an age-related manner, with the greatest peripheral and central elevations in old and young mice, respectively. Severe inflammation failed to cause loss of TH+ neurons in SN_{PC}-VTA or LC; however, there was an age-related decline in these TH+ neurons in LPS-treated and control groups. Thus, unknown mechanisms in the B6 mouse brain appear to protect against catecholaminergic neuron loss during an acute episode of severe inflammation, while catecholaminergic neuron loss occurs during normal aging.

Send correspondence to: Peter R. Mouton, Ph.D., Department of Pathology & Cell Biology, Byrd Alzheimer's Center and Research Institute at the University of South Florida College of Medicine, Room 311, 4001 E Fletcher Ave, Tampa, Florida 33613-4808; 813-396-0662 (office); 410-490-2036 (cell); 866-262-4901 (fax); pmouton@health.usf.edu.

Disclosure Statement No actual or potential conflicts of interest are present for any of the authors and the work presented in this report.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Age-related Neuron Loss; Parkinsonism; Neuroinflammation; Computerized stereology; Stereologer; Substantia nigra; Ventral Tegmental Area

1. Introduction

Parkinson's disease (PD) is a progressive, age-related neurodegenerative disease that is clinically characterized by a variety of movement disorders, including resting tremor, bradykinesia, cogwheel rigidity and postural instability (Fahn, 2003). Between 20 and 40% of patients with PD also manifest progressive dementia in the form of attention abnormalities, impairments with planning and concept formation, and deficits in learning and memory (Aarsland et al., 2003). Currently, no biomarkers or therapies exist to assist in early diagnoses or modify the course of the disease.

Patients with PD have increased levels of tumor necrosis factor alpha (TNF- α) and interleukin-1 β (IL-1 β) and interferon-gamma in the SN and cerebrospinal fluid (CSF) (Mogi et al. 1994, 1996 Mogi et al. 2000; Hunot et al 1999). Postmortem examination of brains from patients with PD reveals loss and atrophy of tyrosine hydroxylase (TH)-immunopositive (TH+) catecholaminergic neurons in the substantia nigra pars compacta (SN_{PC})-ventral tegmental area (VTA) of the ventral midbrain and the locus coeruleus (LC) in the pons that increases in severity as a function of disease duration (Chay-Palay and Asan, 1989; Pakkenberg et al., 1991; Møller et al., 1992; German et al., 1992; Ma et al., 1997; Rudow et al., 2008). During normal aging, design-based stereology studies of the brains of human and non-human primates report significant loss and compensatory hypertrophy of catecholaminergic neurons in SN_{PC} and VTA (Siddiqi et al., 1999; Cabello et al., 2002; Rudow et al., 2008), while the numbers and size of catecholaminergic neurons in LC remain stable (Mouton et al., 1994; Ohm et al., 1997).

A range of cellular and animal studies has strongly linked the idiopathic onset of PD to a wide variety of toxins, bacterial infections, and pesticides. Macrophage-mediated inflammation in the peripheral and central nervous systems, with subsequent death of catecholaminergic neurons through production of free radicals (reactive oxygen and nitrogen species), cytokines, and lipid metabolites, is thought to play an important role in the neuron loss associated with PD. An experimental model that includes loss of central TH+ neurons would be especially valuable for the development of therapeutic approaches for the management of patients afflicted with PD. Existing models for PD-like neuropathology use exposure to toxins such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine (6-OHDA), rotenone, and the combination of paraquat and maneb (for reviews, Betarbet et al., 2002; Orth and Tabrizi 2003). The bacterial endotoxin, lipopolysaccharide (LPS), has been used extensively for *in vivo* and *in vitro* studies of neuroinflammation in PD and a variety of other conditions (Kim et al., 2000; Sanlioglu et al., 2001; Gayle et al., 2002; Shibata et al., 2003; see Dutta et al., 2008 for review). Binding of the endotoxin LPS to LPS binding protein (LPB), one of several key extracellular-cell surface proteins vital for the initiation of the host innate immune response, stimulates immune cells to synthesize and release a host of cytokines (Gioannini and Weiss 2007; Miyake, 2007; Schumann et al., 1990). Studies carried out *in vitro* indicate that LPS-activated macrophages and microglial cells stimulate multiple signaling pathways of the innate immune system, including the up-regulation and release of proinflammatory cytokines, including TNF- α , IL-1 β , and IL-6, anti-inflammatory cytokine IL-10, and reactive oxygen species (ROS) (Dentener et al., 1993; Dutta et al., 2008; Medvedev et al., 2000; Sanlioglu et al., 2001). A number of *in-vivo* studies have found evidence of central

inflammation and neurodegeneration following intranigral injections of LPS into the rodent brain (Herrera et al., 2000; Lu et al., 2000; Arai et al., 2004; Koprich et al, 2008), with one group reporting significant, progressive loss of central catecholaminergic neurons in the SN following a single systemic injection of LPS to young mice (Qin et al., 2007). To date, however, no studies have used systemic LPS injection, in combination with TH immunocytochemistry and rigorous stereology methods, to assess whether catecholaminergic neuron loss occurs in the brains of aged rodents following a single severe episode of peripheral and central inflammation.

To investigate whether mechanisms exist in the mouse brain to protect against inflammation-mediated death of central catecholaminergic neurons, we administered a single intraperitoneal (i.p.) dose of LPS to young, middle-aged, and aged C57Bl/6NIA (B6) mice. Pilot studies for this experiment identified the maximally tolerated LPS dose (0.3 mg/kg, i.p.) that was lethal to about 40% of aged B6 mice. At the time of the peak TNF- α response in plasma, i.e., 2 hrs after LPS injection, this maximally tolerated dose of LPS induced age-related changes in peripheral and central pro- and anti-inflammatory cytokines. Despite widespread stimulation of inflammatory markers following LPS treatment, there was no loss of TH+ neurons in SN_{PC}-VTA or LC, suggesting that mechanisms in the mammalian brain protect against inflammation-mediated death of central catecholaminergic neurons. Independent of LPS treatment, however, there was significant loss of TH+ neurons in SN_{PC}/VTA during normal aging.

2. METHODS

2.1. Subjects

Animals for these studies included C57/Bl6Nia (B6) mice aged 6 mos (n=16), 14–16 mos (n=16) and 22 mos (n=22) from the colony at the NIA in Baltimore, MD; and 3 month old F344 rats (n=6) from the Taconic Farms in Germantown, MD. After entry in the study, mice and rats were housed at the NIA vivarium under standard conditions with *ad libitum* access to food and water and in a 12-hr light dark cycle (lights on at 0600 hr). These experiments were carried out with the explicit approval of the Animal Care and Use Committee at the National Institute on Aging (NIA), according to guidelines set forth by the National Institutes of Health.

2.2. Selection of LPS doses

LPS was ordered as a single batch (Batch 00007794, Calbiochem, Gibbstown NJ) in a quantity sufficient for all experiments. As it is harvested from bacteria and sold as batches with non-standardized potency, pilot studies were initially undertaken to determine a maximally tolerated dose of LPS in middle-aged mice, with the expectation of higher susceptibility in mice from the aged group. Four doses of LPS (0.3, 0.6, 1.0 and 2.0 mg/kg) were administered as single systemic (i.p.) injections to mice in the middle-aged group (14–15 mos), with dose-dependent mortality as follows: 0% (0.3 mg/kg), 17% (0.6mg/kg), 91% (1.0 mg/kg) and 91% (2.0 mg/kg). Thereafter, the 0.3mg/kg LPS dose was administered to the young (3–6 mo) and aged (20–22) mouse groups and found to be sublethal to young mice (0%) and highly lethal to aged mice (36%). Thus, on the basis of high mortality observed in the aged group of B6 mice, 0.3 mg/kg LPS was selected as the maximally tolerated dose for further studies. All mice received a single LPS challenge, and no injected mice were utilized in subsequent studies.

2.3. TNF- α time course

To characterize the acute time-dependent action of LPS to induce TNF- α protein levels in plasma, young F344 rats were anaesthetized (50 mg/kg sodium pentobarbital, 1 ml/kg vol.

i.p., n=6) and the right femoral artery cannulated with PE 50 tubing to permit blood sampling. Compared to mice, rats allow for multiple blood collection at quantities sufficient for analysis of TNF- α protein by enzyme linked immunosorbant assay (ELISA). LPS was administered (1 mg/kg i.p., E. coli 055:B5; Sigma, St. Louis, MO) and thereafter serial blood samples were collected over a subsequent 4 hr period. Plasma was derived immediately by centrifugation (8,000 G $\times$ 5 min), frozen at -80°C and subsequently analyzed for TNF- α by ELISA. This pilot study identified the time to peak response (Tmax) at 2 hours following LPS injection, which identified the optimal time-point for quantification of pro- and anti-inflammatory cytokine protein and mRNA in plasma and brains for subsequent experiments. The chosen post-LPS time, 2 hours, is in accord with previous studies in mice (Qin et al., 2007).

2.4. Tissue processing

Mice were anesthetized with Isoflurane (Abbot Laboratories, Abbott Park, IL) and then transcardially perfused with 10 ml ice cold 1x phosphate buffered saline (PBS), followed by 24 ml of ice cold 4% paraformaldehyde (PFA, Sigma, St. Louis, MO). Brains were removed and stored overnight in 4% PFA, moved to 30% sucrose for 48 hrs and then flash frozen in liquid nitrogen. Serial frozen sections through the entire SN_{PC}-VTA and LC were cut on a freezing microtome at an instrument setting of 50 μm and then stored in a cryopreservative buffer consisting of 30% glycerol/30% ethylene glycol in 40 mM sodium phosphate at -20°C until immunostaining.

2.5. TH immunohistochemistry

For stereological analysis of TH+ neurons, systematic-random samples of sections through the SN_{PC}-VTA and LC were collected (8–12 sections per region per brain). Sections were washed with 1x Tris buffered saline (TBS; Quality Biological inc, Gaithersburg, MD) and endogenous peroxidases quenched by 1% hydrogen peroxide for 30 min at room temperature (RT), followed by rinses with 1x TBS and incubation with 0.3% Triton-X. Sections were removed and blocked with 5% normal goat serum in 1x TBS and then incubated with 1:2000 rabbit anti-tyrosine hydroxylase (Santa Cruz, CA) overnight at 4°C . Following primary incubation, sections were washed with 1x TBS and incubated in biotinylated anti-rabbit secondary antibody (1:400; Vector Laboratories, Burlingame, CA) with normal goat serum in 1x TBS for 90 min. at RT. Afterward, sections were washed with 1x TBS and then reincubated in ABC solution (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 90 min at RT. For color development, sections were washed in 1x TBS and treated with 3-diaminobenzidine [(DAB); 10 mg DAB in 40 ml 1x TBS] as the chromogen with nickel chloride amplification. Finally, sections were mounted on Poly-L lysine coated slides, counterstained with cresyl violet and coverslipped.

2.6. Stereology methods

Total number of TH+ neurons in two reference spaces (SN/VTA and LC) were quantified using the optical fractionator method (West et al., 1991), with assistance from the *Stereologer* system (Stereology Resource Center, Chester, MD), as previously reported (Long et al., 1998 Mouton et al., 2002a; O'Neil et al., 2007). Reference spaces on each section were outlined under low magnification (5x), and TH+ neurons counted at high magnification (63x oil immersion), with a guard volume of 2 μm to avoid artifacts on the cut surface of sections. For counts of total neuron number, the following sampling fractions were used: section sampling fraction (ssf, number of sections sampled divided by the total number of sections); area sampling fraction (asf, area of the sampling frame divided by the area of the xy sampling step); and thickness sampling fraction (tsf, height of the disector divided by the section thickness). The counting criteria were TH immunoreactivity in the cytoplasm of cells with a neuronal phenotype, including a clear nuclear membrane and

distinct nucleolus. To assess whether LPS or aging might be associated with reduced TH expression in catecholaminergic neurons, sections through each reference space were examined at high magnification for the presence of cells with a neuronal phenotype of TH⁺ neurons, but without TH immunoreactivity in cytoplasm. Sampling of x-y locations within each reference space was continued to a high level of sampling stringency, i.e., mean coefficient of error (CE, Gundersen et al., 1999) per group in the range of 0.10 to 0.15.

2.7. Assay of cytokines in mouse plasma samples and hippocampus

Concentrations of cytokines (IL-1 β , IL-6, IL-10 and TNF- α) were determined using Millipore's MilliplexTM Mouse Cytokine bead-based kit (catalog # MPXMCYTO-70K). For all analyses, a lyophilized standard of 10,000 pg/ml was diluted with 250 μ l deionized water. Five-fold serial dilutions of this maximum standard provided five standards with concentrations ranging from 2000 to 3.2 pg/ml. Homogenates of thawed left hippocampus from the mouse brain were prepared in 1.5 ml Eppendorf tubes that contained 100 μ l of BioPlex cell lysis buffer (kit # 171-304011), according to the protein extraction method of Datta and Opp (2008). The cell lysis buffer was used as a matrix solution for the background, standards, and quality controls. Duplicate assays of cytokine expression were measured using the BioPlex System (Bio-Rad Laboratories, Hercules, CA). The resulting cytokine levels were analyzed using the BioPlex Manager 4.0 software with five-parameter logistic regression curve and, finally, expressed as pg/ml relative to the amount of protein in each sample.

2.8. Quantitative RT-PCR

Total RNA was extracted from mouse hippocampal tissue using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA quantity and purity were assessed using Nanodrop (Thermo Scientific, Wilmington, DE) and complementary deoxyribonucleic acid (cDNA) was synthesized using SuperScript II reverse transcriptase with random hexamers (Invitrogen, Carlsbad, CA). Quantitative PCR was performed in duplicate, using the iTaq SYBR Green supermix with ROX (Biorad, Hercules, CA). The final reaction volume of 20 μ l consisted of the pre-made reaction mix, 1 μ M primer pair and 8 μ l cDNA (2 or 6 ng) in water. Reaction conditions for qPCR for the Step One Plus qPCR System (Applied Biosystems, Foster City, CA) were as follows: 95 °C for 10 min and 45 cycles of 95 °C for 30 s (denaturation step) and 60 °C for 45 s (combined annealing and extension steps). A melting curve analysis was performed by denaturation at 95°C for 15 s, annealing at 60 °C for 1 min and at a melting rate of 0.3 °C/s to 95 °C. Primer sequences were as follows: forward 5'-CCCTCACA CTGATCATCTTC-3' and reverse 5'-GCTACGACGTGGGCTACAG-3' for TNF- α (Primer bank number 7305585a1 (Spandidos et al., 2008; Wang and Seed, 2003), Gene Bank accession no. NM_013693, 61 bp); forward 5'-GCAACTGTTCTGAACTCAACT-3' and reverse 5'-ATCTTTTGGGGTCCGTCAACT-3' for IL1 β (Spandidos et al., 2008; Wang and Seed, 2003); Primer bank number 6680415a1; Gene Bank accession no. NM_008361, 89 bp); forward 5'-TCCTTCCTACCCCAATTTCC-3' and reverse 5'-GCCACTCCTTCTGTGACTCC-3' for IL6 (Gene Bank accession number NM_031168, 63 bp) and forward 5'-TAGCAGAGATCACCAATGCC-3' and reverse 5'-GGCAGCAAGCCATGTATTTA-3' for tubulin (Gene Bank accession number NM_011654.2, 87 bp). The relative levels of transcript were quantified using the standard curve method. Levels of mRNA expression are reported relative to the expression of the tubulin gene, i.e., IL-1B/tubulin.

2.9. Statistical analyses

Testing for significant group differences in numbers of TH⁺ neurons was carried out using two-way analysis of variance (ANOVA), with posthoc testing by the Student-Newman-Keuls method. The one-way ANOVA with a Tukey posthoc test was carried out for testing

group difference in cytokine levels in the brain and periphery, and Grubb's test on the qPCR data with outliers subsequently removed. Levels of statistical significance were set at $p \leq 0.05$.

3. RESULTS

These experiments tested whether an acute episode of severe neuroinflammation and/or normal aging lead to loss of central catecholaminergic neurons in the brains of B6 mice. Since non-immunologically challenged mice have low levels of peripheral and central cytokines, we quantified central and peripheral responses after stimulation of young, middle-aged and aged B6 mice with a single, maximally tolerated dose of LPS (i.p.). Two hrs after LPS injection, i.e., the interval for peak responses of TNF- α protein (Figure 1), mice of all age groups were sacrificed for quantification of peripheral and central levels of pro-inflammatory (TNF- α , IL-1 β , IL-6) and anti-inflammatory (IL-10) cytokine proteins by Luminex xMAP technology; and, mRNAs by quantitative-RT-PCR analysis. To assess age-related effects of inflammation on catecholaminergic neurons in two brain regions (SN_{PC}-VTA and LC), a second cohort of LPS- and vehicle-treated young, middle-aged, and old mice were sacrificed at 14 weeks after treatment. Brains from these mice were removed and processed for TH immunocytochemistry to estimate the total numbers of catecholaminergic neurons in SN_{PC}-VTA and LC using the optical fractionator method (West et al., 1991).

3.1. Effects of systemic LPS administration on cytokine levels in plasma

Figure 1 illustrates the time-dependent change in plasma TNF- α protein levels following a single LPS injection in adult rats. Plasma levels of TNF- α protein rose sharply following LPS injection to peak levels between 90 to 120 min, and then declined at subsequent time points. Unchallenged control animals showed levels of TNF- α protein below the detection threshold of ELISA (Datta et al., 2008). Based on these findings, subsequent studies of age-dependent comparisons of cytokine responses were performed at 2 hrs following LPS treatment.

To induce acute inflammation, young, middle-aged and aged B6 mice received a single i.p. injection of LPS (0.3 mg/kg, i.p.), and age-matched controls from the same cohorts received equivalent volumes of buffered saline (vehicle). Mice were euthanized 2 hrs thereafter, and plasma prepared from whole blood obtained by cardiac puncture. Brain tissue samples were collected for analysis of proinflammatory (TNF- α , IL-1 β , IL-6) and anti-inflammatory (IL-10) cytokine protein and mRNA levels. The detailed results of these studies are outlined below and illustrated in Table 1. Levels of pro- and anti-inflammatory cytokines were readily detectable in LPS-challenged mice, but below the level of detection in unchallenged mice. Since the percent elevation of challenged compared to unchallenged littermates cannot be determined, comparisons are reported between LPS-challenged mice in different age groups.

TNF- α protein—There was an age-related increase in plasma TNF- α protein at 2 hrs following LPS administration, with levels in the aged group significantly higher than that in young ($p = 0.006$) and middle-aged mice ($p = 0.004$).

IL-6 protein—An age-related increase in plasma IL-6 protein was observed 2 hrs after systemic LPS administration. Levels of IL-6 were significantly increased in both the middle-aged ($p < 0.001$) and the aged groups ($p < 0.006$) compared to the young group.

IL-1 β protein—Compared to the young group, there was an LPS-mediated increase in IL-1 β protein in the middle-aged group ($p < 0.034$), but not the aged group, two hrs after LPS treatment.

IL-10 protein—LPS induced age-related elevations in IL-10 levels, with the greatest effect in the young group compared to the aged group ($p < 0.025$, $n=6$). For mice in the aged group, IL-10 values did not significantly differ from those in the middle-aged group.

3.2. Effects of acute LPS treatment on mRNA and protein levels of central cytokines

To assess the age-related effects of acute inflammation on the central immune system, young, middle-aged and aged B6 mice received a single i.p. injection of LPS, and were euthanized 2 hrs later. Hippocampal tissue was collected for analysis of proinflammatory (TNF- α , IL-1 β , IL-6) and anti-inflammatory (IL-10) cytokine mRNA and protein levels and mRNA levels of TNF- α , IL-1 β , IL-6 were analyzed by quantitative RT-PCR. These data are showed in Table 2 and outlined below.

3.2.1 Acute effects of systemic LPS on central cytokine mRNA levels

TNF- α mRNA: There was a significant age-dependent difference in levels of LPS-induced elevations in TNF- α mRNA in the hippocampus 2 hrs following LPS injection ($p < 0.03$, $n=6$). Post hoc testing confirmed a significantly lower value for the aged group relative to that of middle-aged mice ($p < 0.05$, $n=6$).

IL-1 β mRNA: There was a significant age related change in IL-1 β mRNA levels in hippocampus after LPS administration ($p = 0.007$, $n=6$). Post hoc testing showed that values for mice in the aged group were decreased relative to the middle-aged group ($p < 0.01$, $n=6$).

IL-6 mRNA: Changes in IL-6 mRNA were similarly altered in response to LPS in an age-dependent manner. Levels for both young and aged groups were lower relative to that for the middle-aged mice ($p < 0.005$, $n=6$).

3.2.2 Acute effects of systemic LPS on central cytokine protein levels

Central TNF- α protein levels: ANOVA revealed a significant age-related effect on LPS-induced TNF- α protein levels in the hippocampus at 2 hrs following injection ($p = 0.018$, $n=6$). Posthoc testing confirmed a lower induction of TNF- α in the aged mice relative to the middle-aged and young mice ($p < 0.05$, $n=6$).

Central IL-6, IL-1 β protein levels: There was a strong trend in favor of an age-related decrease in LPS-induced response, with aged mice showing lower IL-1 β values than young mice ($p = 0.051$; $n=6$ for young and middle aged, $n=4$ for aged). No differences were observed for the induction of IL-6 measured for any of the groups following LPS stimulation ($p < 0.2$, $n=6$).

Central IL-10 protein levels: LPS-mediated induction of IL-10 was significantly higher for mice in the middle-aged group relative to the young ($p = 0.02$; $n=6$ for young and middle-aged mice, $n=4$ for aged mice).

3.3. Stereology of central catecholaminergic neurons

To assess whether acute peripheral inflammation leads to loss of central catecholaminergic neurons, mice were treated with a single i.p. injection of LPS and euthanized 14 weeks later. Systematic-random sets of TH-immunostained sections were collected in an unbiased manner through SN/VTa from young, middle-aged, and old mice, and through LC of aged

mice treated with saline or LPS. For all groups of LPS-treated mice, computerized stereology was used to quantify mean total number of TH+ neurons. Results of these studies are shown in Table 3, and outlined below.

3.3.1. Total TH+ neuron number in SN_{PC}-VTA—A 2×3 ANOVA was performed with age and treatment as the independent variable. As shown in Table 3, there was no LPS-mediated effect in any age groups, though numbers of TH+ neurons in SN_{PC}-VTA were significantly reduced in aged mice compared to young mice in both saline and LPS-treated groups. Young mice treated with saline had a mean total number of 7092 (SEM 2018) TH+ neurons compared to 4168 (SEM 608) for aged saline-treated controls ($p < 0.02$). A posthoc Student-Newman-Keuls test confirmed a significant decrease in the mean total number of TH+ cells in the SN_{PC}/VTA of aged mice compared to young mice ($p = 0.02$). This 40% reduction in TH+ neurons in saline-treated control mice paralleled a 38% reduction in mean total number of TH+ neurons in SN_{PC}-VTA of LPS-treated mice in the young [mean 9986 (SEM 1403)] vs. aged [mean 6215 (SEM 1306); $p = 0.02$] groups. There were no differences in TH+ neuron numbers for the middle-aged mice versus young or aged mice in either the saline or LPS-treated groups.

3.3.2. Total number TH+ neuron in LC from aged mice treated with LPS—Mean total number of TH+ neurons in the LC was quantified 14 weeks after LPS treatment. There were no differences in total numbers of TH+ neurons in LC in brains of aged mice treated with either saline [mean 1372 (SEM 186) cells] or LPS (mean 1150 (SEM 140) cells; $p = 0.4$; $n=5$ per group].

3.3.3. Examination of catecholaminergic neurons for loss of TH immunoreactivity—High magnification examination of TH-immunostained sections was carried out to address the possibility that reduced expression of TH protein confounded the stereology estimates of total numbers of TH+ neurons in SN_{PC}/VTA and LC. Careful examination of TH-immunostained sections through SN_{PC}/VTA and LC revealed that this was no likely the case, since no cells were found with a cellular phenotype consistent with catecholaminergic neurons, but lacking cytoplasmic TH immunoreactivity.

4. DISCUSSION

The causes of PD and parkinsonism-like disorders include both inherited and non-familial mechanisms, with environmental etiologies for the latter largely unknown. An acute episode of severe inflammation is thought to play a role in the loss of catecholaminergic neurons in at least some cases of PD. To test the hypothesis that brain mechanisms exist in the brains of the aging mouse to protect central catecholaminergic neurons following severe peripheral inflammation, we treated young, middle-aged, and aged C57Bl/6NIA (B6) mice with a maximally tolerated intraperitoneal (i.p.) dose of LPS or saline. Two hours later, LPS treatment caused severe age-related alterations in peripheral and central levels of proinflammatory and anti-inflammatory cytokines, in accord with similar studies in young (8-week old) C57/Bl mice (Qin et al., 2007). In the present study, 14 weeks after LPS treatment there were no differences in total numbers of TH+ neurons in either SN_{PC}/VTA or LC. However, at this same time-point we found significant reductions in total numbers of TH+ neurons in the SN_{PC}/VTA in the aged compared to young group for mice treated with either LPS or saline.

Recent work by Banks and Robinson (2010) suggests that brain uptake of circulating LPS is so low that most effects of peripherally administered LPS are likely mediated through LPS receptors located outside the brain. Following systemic injection, LPS binds to its receptor and enters capillary endothelial cells where it increases permeability of the blood brain

barrier in a mass-selective manner. This bound complex triggers a series of signaling events that stimulate proinflammatory and anti-inflammatory cytokine responses in the peripheral and central nervous systems (Schumann et al., 1990; Medvedev et al, 2000; Singh and Jiang, 2004). Recently, Qin et al., (2007) reported that 10 mos following systemic injections of either LPS (strain O111:B4, Calbiochem, San Diego, CA) or TNF- α to young adult mice stimulated dramatic TNF- α levels in the brain and a robust loss of TH+ neurons in the SN_{PC}. This study suggests that peripheral LPS administration activates peripheral macrophages to migrate into the CNS and stimulate central microglia to chronically produce proinflammatory cytokines, which cause death of dopaminergic neurons in the nigrostriatal pathway (Qin et al., 2007).

To further explore this hypothesis along the dimension of aging, we designed experiments to treat young, middle-aged, and aged B6 mice with sufficient LPS to cause severe acute inflammatory responses in the periphery and brain. Since individual sources of commercially available LPS vary in their potency, we used the same strain of male B6 mice as Qin et al., (2007) from the National Institute on Aging in Baltimore, MD to determine the maximally tolerated dose that stimulated severe inflammatory responses in mice across a range of 6 to 20+ months of age, without exceeding 50% mortality in the oldest group. Second, to define the time of peak TNF- α plasma protein secretion the time-dependent action of LPS induction of TNF- α protein synthesis and release was evaluated in F344 rats, which allow for multiple blood collection at sufficient quantities for analysis of cytokines. These studies showed that the dose of 0.3 mg/kg resulted in about 40% mortality in the oldest group of mice (20+ mos), with 0% mortality in either of the two younger groups (6 to 15 mos); and, the peak plasma TNF- α protein level occurs at 90 to 120 min after LPS administration (Figure 1), which is similar to Qin et al., (2007). Thus, for these studies we used 0.3 mg/kg as the maximally tolerated dose of LPS. A single systemic injection of this maximally tolerated dose of LPS to young, middle-aged, and aged B6 mice caused significant age-related effects on TNF- α , IL-1 β , and IL-10 protein levels in plasma, with older mice showing higher levels of pro-inflammatory cytokines than younger mice. In response to the same LPS-mediated induction of peripheral cytokines, younger mice showed higher levels of the same cytokines in brain than older mice.

To test the hypothesis that mechanisms exist to protect catecholaminergic neurons from damage following severe peripheral inflammation, we chose two populations of TH+ neurons that undergo severe neuron loss in PD, the LC and SN-VTA. For the LC, these counts were only done in the aged group on the assumption that these TH+ neurons in LC would be the most vulnerable in aged mice. The effects of LPS and aging on numbers of central catecholaminergic neurons were determined using well-established methods of unbiased stereology (West et al., 1991; Saper et al., 1996; Coggeshall and Lekan 1996; Evans et al., 2004; Howard and Reed 2005; Mouton 2002, 2009; Mouton and Gordon 2009). To assess moderate to large age-related differences in total numbers of TH+ neurons in the SN_{PC}-VTA and LC, the present study used statistical power in the range of 5 to 10 mice per group and a high sampling stringency within each mouse, i.e., coefficient of error (CE) 50% or less than the observed biological variance for each group. To exclude that possibility that the observed results on catecholaminergic neurons in SN_{PC}/VTA and LC were the results of reduced TH expression, rather than changes in neuron number loss *per se*, we carefully examined TH-immunostained sections from mice treated with LPS or vehicle (saline) at high magnification. This analysis did not show neurons in the SN_{PC}/VTA or LC regions with a neuronal phenotype that lacked TH immunoreactivity in cytoplasm; thus, loss of TH expression and phenotype, rather than changes in number of TH+ neurons, provides an unlikely explanation for the results in this study.

These results support the view that neuroprotective mechanisms exist in the brains of young, middle-aged, and aged mice that prevent age-related loss of TH⁺ neurons in SN_{PC}/VTA or LC up to 14 weeks following severe systemic inflammation. The possibility remains that LPS-induced death of catecholaminergic neurons would develop after an interval longer than 14 weeks following LPS treatment. Qin et al. (2007) reported that a single systemic injection of LPS reduces the number of TH⁺ neurons in the SN by 23% after seven months, which progressed to 47% at ten months after LPS injection. Due to the advanced age of the aged mice in the present study, i.e., 20+ mos at the time of euthanasia, a longer time course between treatment and sacrifice would have resulted in unacceptably high levels of morbidity and mortality.

The underlying cause of age-related TH⁺ neuron loss in SN_{PC}/VTA and LC in patients afflicted by PD remains unknown. However, experimental animal models with PD-like characteristics provide significant insights into the pathogenesis of PD (Arai et al., 2004; Gayle et al., 2002; Iravani et al., 2002; Kim et al., 2000; Koprich et al., 2008; Mogi et al., 1996; Shibata et al., 2003; Sulzer, 2007; Thiruchelvam et al., 2003; Wilms et al., 2007). Central inflammation caused by direct LPS injection directly into brains of rats and mice causes damage to TH⁺ neurons in SN and microgliosis, as evidenced by qualitative and semiquantitative morphometry (Arai et al., 2004; Herrera et al., 2000; Iravani et al., 2002; Lu et al., 2000; Zhang et al., 2005). When injected directly into the brains of different aged rats, LPS reduced the density of TH⁺ neurons in the SN as a function of increasing age; furthermore, young rats recovered from LPS-induced motoric impairment after 4 weeks, while older rats showed no recovery (Zhang et al., 2005). Peripheral administration of MPTP causes a movement disorder similar to PD, including significant loss of catecholaminergic neurons in the brains of young drug abusers, non-human primates and B6 mice (Langston and Ballard 1983; Burns et al., 1984; Ricaurte et al., 1987; Saitoh et al., 1987). Unilateral injection of the chemical, 6-hydroxydopamine (6-OHDA) into the rat brain causes TH⁺ neuron loss in the SN, reduced dopamine levels in striatum, and classical circling behavior (Berger et al., 1991; Cadet et al., 1991). Age enhances the toxic effects of 6-OHDA and MPTP exposure (Ricaurte et al., 1987; Irwin et al., 1993; Sauer et al., 1994); however, neither of these compounds are environmental neurotoxicants and their effects are reversible (Bedard et al., 1986; Date et al., 1990; Saitoh et al., 1987), thus limiting their potential to more closely model a progressive disorder such as PD.

Using the same methods as in the present study, Chan et al. (1997) estimated about 5500–6000 TH⁺ SN_{PC} neurons per side in untreated, wild-type B6 mice about 2–3 months old; however, that study limited their reference space to the SN_{PC}. Here we estimated about 7000–10,000 neurons per side in the six month-old mice for TH⁺ neurons in SN_{PC} combined with the ventral tegmental area (i.e., SN_{PC}-VTA). Our finding of progressive, age-related loss of TH⁺ neurons in B6 mice agrees with previous reports in humans (Cabello et al., 2002; Rudow et al., 2008) and non-human primates (Siddiqi et al. 1999), while other studies find stability of these catecholaminergic neurons during normal aging (McCormack et al. 2004; Scott et al., 2007). Among the possible bases for these discrepancies in the literature include the definitions of what constitutes “aging.” The study by Scott et al. (2007), for example, reported age-related stability in TH⁺ neurons in SN of Sprague-Dawley rats, though in that study “older” rats were 7 mos of age, which falls within the age-range considered young for the majority of investigations, including the present study. Another source of variability in published studies arises from the range of different methods for sampling tissue and determination of neuron loss, e.g., unbiased stereology using the three-dimensional disector probe vs. so-called assumption- and model-based counting methods. Prior to the advent of unbiased stereology approaches, e.g., optical fractionator (West et al., 1991), variations in sampling and quantification methods frequently reported discrepant results, such as age-related stability (McNeill and Koek 1990) and loss (Tatton et al., 1991)

for TH⁺ neurons in the SN of the B6 mouse brain. Even using the same methods of design-based stereology, discrepant results may arise from biological variation between different species of experimental animals. The study by McCormack et al. (1994) reported age-related stability in number of TH⁺ neurons in SN of squirrel monkeys, while Siddiqi et al. (1999) found age-related losses in the same population of neurons in brains of rhesus monkeys, which raises the possibility of biological effects, i.e., strain differences, as an explanation for variability in study results. For the above rationale, comparisons of studies designed to assess age-related changes in numbers of catecholaminergic neurons should consider the full range of possible differences in study methodology, including age, species, strain, reference spaces, and techniques for sampling and parameter estimation.

In conclusion, the present study support the existence of presently unknown mechanisms in the brains of B6 mice, and possibly in human brains, which protect central catecholaminergic neurons during an acute episode of severe peripheral inflammation. Understanding these mechanisms could lead to the development of therapeutic strategies to prevent or delay the onset of PD in aging human populations. Finally, our findings of age-related loss of central catecholaminergic neurons in mice, in parallel with similar reports in non-human primates and humans, deserves further study to clarify the extent to which age-related loss of SN_{PC}/VTA neurons may help to understand the neurobiological basis for physiological, motoric, and cognitive changes that occur during normal aging of the mammalian brain.

Acknowledgments

The authors would like to thank Christopher Quigley his assistance with data collection for the stereology studies. This work was supported in part by the Intramural Research Program of the National Institute on Aging, National Institutes of Health and by extramural grants from the National Institute on Mental Health (MH076541-04). A portion of the support was also provided through a R & D contract with MedStar Health Research Institute. Animal studies were performed in accordance with approved protocols, in compliance with the National Institutes of Health's Guidelines for Animal Experimentation.

Abbreviations

PD	Parkinson's disease
SN_{PC}	Substantia nigra pars compacta
VTA	Ventral tegmental area
LC	Locus coeruleus
TNF-α	Tumor necrosis factor alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-10	Interleukin-10
LPS	Lipopolysaccharide
TH	Tyrosine hydroxylase
i.p	Intraperitoneal
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate buffered saline
cDNA	Complementary deoxyribonucleic acid

QPCR	Quantitative polymerase chain reaction
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
6-OHDA	6-hydroxydopamine
mRNA	messenger ribonucleic acid
α-syn	alpha-synuclein

References

- Aarsland D, Andersen K, Larsen JP, Lolk A, Kragh-Sorensen P. Prevalence and characteristics of dementia in Parkinson disease: an 8-year prospective study. *Arch Neurol*. 2003; 60:387–392. [PubMed: 12633150]
- Arai H, Furuya T, Yasuda T, Miura M, Mizuno Y, Mochizuki H. Neurotoxic effects of lipopolysaccharide on nigral dopaminergic neurons are mediated by microglial activation, interleukin-1 β , and expression of caspase-11 in mice. *J Neurochem*. 2004 Sep; 86(5):1201–12. 2003.
- Banks WA, Robinson SM. Minimal penetration of lipopolysaccharide across the murine blood-brain barrier. *Brain Behav Imm*. 2010; 24:102–109.
- Bedard PJ, Di Paolo T, Falardeau P, Boucher R. Chronic treatment with L-DOPA, but not bromocriptine induces dyskinesia in MPTP-parkinsonian monkeys. Correlation with [3H]spiperone binding. *Brain Res*. 1986; 379:294–299. [PubMed: 3488796]
- Berger K, Przedborski S, Cadet JL. Retrograde degeneration of nigrostriatal neurons induced by intrastratial 6-hydroxydopamine injection in rats. *Brain Res Bull*. 1991; 26:301–307. [PubMed: 1826461]
- Betarbet R, Sherer TB, Greenamyre JT. Animal models of Parkinson's disease. *Bioessays*. 2002; 24:308–18. [PubMed: 11948617]
- Burns RS, Markey SP, Phillips JM, Chiueh CC. The neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in the monkey and man. *Can J Neurol Sci*. 1984; 11:166–168. [PubMed: 6608980]
- Cabello CR, Thune JJ, Pakkenberg H, Pakkenberg B. Ageing of substantia nigra in humans: cell loss may be compensated by hypertrophy. *Neuropathol Appl Neurobiol*. 2002; 28:283–91. [PubMed: 12175340]
- Cadet JL, Last R, Kostic V, Przedborski S, Jackson-Lewis V. Long-term behavioral and biochemical effects of 6-hydroxydopamine injections in rat caudate-putamen. *Brain Res Bull*. 1991; 26:707–713. [PubMed: 1933391]
- Chan P, Di Monte DA, Langston JW, Janson AM. (+) MK-801 does not prevent MPTP-induced loss of nigral neurons in mice. *J Pharmacol Exp Ther*. 1997; 280:439–446. [PubMed: 8996226]
- Chan-Palay V, Asan E. Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. *J Comp Neurol*. 1989; 287:373–392. [PubMed: 2570794]
- Coggeshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Com Neurol*. 1996; 364:6–15.
- Date I, Felten SY, Felten DL. Cografts of adrenal medulla with peripheral nerve enhance the survivability of transplanted adrenal chromaffin cells and recovery of the host nigrostriatal dopaminergic system in MPTP-treated young adult mice. *Brain Res*. 1990; 537:33–39. [PubMed: 1982243]
- Datta SC, Opp MR. Lipopolysaccharide-induced increases in cytokines in discrete mouse brain regions are detectable using Luminex xMAP technology. *J Neurosci Methods*. 2008; 175:119–124. [PubMed: 18771691]
- Dentener MA, Von Asmuth EJ, Francot GJ, Marra MN, Buurman WA. Antagonistic effects of lipopolysaccharide binding protein and bactericidal/permeability-increasing protein on

- lipopolysaccharide-induced cytokine release by mononuclear phagocytes. Competition for binding to lipopolysaccharide. *J Immunol.* 1993; 151:4258–4265. [PubMed: 8409400]
- Dutta G, Zhang P, Liu B. The lipopolysaccharide Parkinson's disease animal model: mechanistic studies and drug discovery. *Fundam Clin Pharmacol.* 2008; 22:453–464. [PubMed: 18710400]
- Evans, SM.; Janson, AM.; Nyengaard, JR. Quantitative methods in neuroscience: a neuroanatomical approach. Oxford University Press; New York: 2004.
- Fahn S. Description of Parkinson's disease as a clinical syndrome. *Ann N Y Acad Sci.* 2003; 991:1–14. [PubMed: 12846969]
- Gayle DA, Ling Z, Tong C, Landers T, Lipton JW, Carvey PM. Lipopolysaccharide (LPS)-induced dopamine cell loss in culture: roles of tumor necrosis factor-alpha, interleukin-1beta, and nitric oxide. *Brain Res Dev Brain Res.* 2002; 133:27–35.
- German DC, Manaye KF, White CL, Woodward DJ, McIntire DD, Smith WK, Kalaria RN, Mann DM. Disease-specific patterns of locus coeruleus cell loss. *Ann Neurol.* 1992; 32:667–676. [PubMed: 1449247]
- Gioannini TL, Weiss JP. Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunol Res.* 2007; 39:249–260. [PubMed: 17917069]
- Gundersen HJG, Jensen EBV, Kieu K, Nielsen J. The efficiency of systematic sampling in stereology – reconsidered. *J Microsc.* 1999; 193:199–211. [PubMed: 10348656]
- Herrera AJ, Castano A, Venero JL, Cano J, Machado A. The single intranigral injection of LPS as a new model for studying the selective effects of inflammatory reactions on dopaminergic system. *Neurobiol Dis.* 2000; 7:429–447. [PubMed: 10964613]
- Howard, CV.; Reed, MG. Unbiased stereology: three dimensional measurement in microscopy. Taylor & Francis; London: 2005.
- Hunot S, Dugas N, Faucheux B, Hartmann A, Tardieu M, Debre P, Agid Y, Dugas B, Hirsch EC. FcepsilonRII/CD23 is expressed in Parkinson's disease and induces *in vitro* production of nitric oxide and tumor necrosis factor-alpha in glial cells. *J Neurosci.* 1999; 19:3440–3447. [PubMed: 10212304]
- Iravani MM, Kashefi K, Mander P, Rose S, Jenner P. Involvement of inducible nitric oxide synthase in inflammation-induced dopaminergic neurodegeneration. *J Neurosci.* 2002; 110:49–58.
- Irwin I, DeLanney LE, Langston JW. MPTP and aging. Studies in the C57BL/6 mouse. *Adv Neurol.* 1993; 60:197–206. [PubMed: 8420136]
- Kim WG, Mohny RP, Wilson B, Jeohn GH, Liu B, Hong JS. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain: role of microglia. *J Neurosci.* 2000; 20:6309–6316. [PubMed: 10934283]
- Koprach JB, Reske-Nielsen C, Mithal P, Isacson O. Neuroinflammation mediated by IL-1beta increases susceptibility of dopamine neurons to degeneration in an animal model of Parkinson's disease. *J Neuroinflammation.* 2008; 5:8. [PubMed: 18304357]
- Langston JW, Ballard PA. Parkinson's disease in a chemist working with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *N Engl J Med.* 1983; 309:310–315. [PubMed: 6602944]
- Langston JW. Aging and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced degeneration of dopaminergic neurons in the substantia nigra. *Brain Res.* 1987; 10:43–51. [PubMed: 3493827]
- Long JM, Kalehua AN, Muth NJ, Hengemihle JM, Jucker M, Calhoun ME, Ingram DK, Mouton PR. Stereological estimation of total microglia number in mouse hippocampus. *J Neurosci Methods.* 1998; 84:101–108. [PubMed: 9821640]
- Lu X, Bing G, Hagg T. Naloxone prevents microglia-induced degeneration of dopaminergic substantia nigra neurons in adult rats. *J Neurosci.* 2000; 97:285–291.
- Ma SY, Rottta M, Rinne JO, Collan Y, Rinne UK. Correlation between neuromorphometry in the substantia nigra and clinical features in Parkinson's disease using disector counts. *J Neurol Sci.* 1997; 151:83–7. [PubMed: 9335015]
- McCormack AL, Di Monte DA, Delfani K, Irwin I, DeLanney LE, Langston WJ, Janson AM. Aging of the nigrostriatal system in the squirrel monkey. *J Comp Neurol.* 2004; 471:387–95. [PubMed: 15022260]

- McNeill TH, Koek LL. Differential effects of advancing age on neurotransmitter cell loss in the substantia nigra and striatum of C57BL/6N mice. *Brain Research*. 1990; 521:107–117. [PubMed: 1976411]
- Medvedev AE, Kopydlowski KM, Vogel SN. Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J Immunol*. 2000; 164:5564–5574. [PubMed: 10820230]
- Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol*. 2007; 19:3–10. [PubMed: 17275324]
- Mogi M, Harada M, Kondo T, Riederer P, Inagaki H, Minami M, Nagatsu T. Interleukin-1 beta, interleukin-6, epidermal growth factor and transforming growth factor-alpha are elevated in the brain from parkinsonian patients. *Neurosci Lett*. 1994; 180:147–150. [PubMed: 7700568]
- Mogi M, Harada M, Narabayashi H, Inagaki H, Minami M, Nagatsu T. Interleukin (IL)-1 beta, IL-2, IL-4, IL-6 and transforming growth factor-alpha levels are elevated in ventricular cerebrospinal fluid in juvenile Parkinsonism and Parkinson's disease. *Neurosci Lett*. 1996; 21:13–6. [PubMed: 8809836]
- Møller A. Mean volume of pigmented neurons in the substantia nigra in Parkinson's disease. *Acta Neurol Scand Suppl*. 1992; 137:37–39. [PubMed: 1414260]
- Mouton PR, Pakkenberg B, Gundersen HJG, Price DL. Absolute number and size of pigmented locus coeruleus neurons in the brains of young and aged individuals. *J Chem Neuroanat*. 1994; 7:185–190. [PubMed: 7848573]
- Mouton, PR. Principles and practices of unbiased stereology: an introduction for bioscientists. The Johns Hopkins University Press; Baltimore: 2002a.
- Mouton PR, Long JM, Lei DL, Howard V, Jucker M, Calhoun ME, Ingram DK. Age and gender effects on microglia and astrocyte numbers in brains of mice. *Brain Res*. 2002b; 956:30–35. [PubMed: 12426043]
- Mouton, PR.; Gordon, M. Stereological and image analysis techniques for quantitative assessment of neurotoxicology. In: Harry, GJ.; Tilson, HA., editors. *Neurotoxicology*. 3. Taylor and Francis; Philadelphia: 2009a. Target Organ Toxicology Series
- Mouton, PR. Applications of unbiased stereology to neurodevelopmental toxicology. In: Wang, C.; Slikker, W., editors. *Developmental Neurotoxicology Research: Principles, Models, Techniques, Strategies and Mechanisms*. John Wiley & Sons; Hoboken: 2009.
- Ohm TG, Busch C, Bohl J. Unbiased estimation of neuronal numbers in the human nucleus coeruleus during aging. *Neurobiol Aging*. 1997; 18:393–9. [PubMed: 9330970]
- O'Neil JN, Mouton PR, Tizabi Y, Ottinger MA, Lei DL, Ingram DK, Manaye KF. Catecholaminergic neuronal loss in locus coeruleus of aged female dtg APP/PS1 mice. *J Chem Neuroanat*. 2007; 34:102–107. [PubMed: 17658239]
- Orth M, Tabrizi SJ. Models of Parkinson's disease. *Mov Disord*. 2003; 2018:729–37. [PubMed: 12815651]
- Pakkenberg B, Møller A, Gundersen HJG, Dam AM, Pakkenberg H. The absolute number of nerve cells in substantia nigra in normal subjects and in patients with Parkinson's disease estimated with an unbiased stereological method. *J Neurol Neurosurg Psychiatr*. 1991; 54:30–3. [PubMed: 2010756]
- Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia*. 2007; 55:453–462. [PubMed: 17203472]
- Ricaurte GA, Irwin I, Forno LS, DeLanney LE, Langston E, Langston JW. Aging and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced degeneration of dopaminergic neurons in the substantia nigra. *Brain Res*. 1987; 403:43–51. [PubMed: 3493827]
- Rudow G, O'Brien R, Savonenko AV, Resnick SM, Zonderman AB, Pletnikova O, Marsh L, Dawson TM, Crain BJ, West MJ, Troncoso JC. Morphometry of the human substantia nigra in ageing and Parkinson's disease. *Acta Neuropathol*. 2008; 115:461–70. [PubMed: 18297291]
- Sanlioglu S, Williams CM, Samavati L, Butler NS, Wang G, McCray PB Jr, Ritchie TC, Hunninghake GW, Zandi E, Engelhardt JF. Lipopolysaccharide induces Rac1-dependent reactive oxygen species

- formation and coordinates tumor necrosis factor- α secretion through IKK regulation of NF- κ B. *J Biol Chem*. 2001; 276:30188–30198. [PubMed: 11402028]
- Saitoh T, Nijima K, Mizuno Y. Long-term effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) on striatal dopamine content in young and mature mice. *J Neurol Sci*. 1987; 77:229–235. [PubMed: 3493330]
- Saper CB. Any way you cut it: a new journal policy for the use of unbiased counting methods. *J Comp Neurol*. 1996; 364:5. [PubMed: 8789271]
- Sauer H, Oertel WH. Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6-hydroxydopamine: a combined retrograde tracing and immunocytochemical study in the rat. *J Neurosci*. 1994; 59:401–415.
- Schumann RR, Leong SR, Flaggs GW, Gray PW, Wright SD, Mathison JC, Tobias PS, Ulevitch RJ. Structure and function of lipopolysaccharide binding protein. *Science*. 1990; 249:1429–1431. [PubMed: 2402637]
- Scott SA, Diaz NM, Ahmad SO. Stereologic analysis of cell number and size during postnatal development in the rat substantia nigra. *Neurosci Lett*. 2007; 419:34–37. [PubMed: 17481811]
- Shibata H, Katsuki HH, Nishiwaki M, Kume T, Kaneko S, Akaike A. Lipopolysaccharide-induced dopaminergic cell death in rat midbrain slice cultures: role of inducible nitric oxide synthase and protection by indomethacin. *J Neurochem*. 2003; 86:1201–12. [PubMed: 12911628]
- Siddiqi Z, Kemper TL, Killiany R. Age-related neuronal loss from the substantia nigra-pars compacta and ventral tegmental area of the rhesus monkey. *Neuropathol Exp Neurol*. 1999; 58:959–71.
- Singh AK, Jiang Y. How does peripheral lipopolysaccharide induce gene expression in the brain of rats? *Toxicology*. 2004; 201:197–207. [PubMed: 15297033]
- Spandidos A, Wang X, Wang H, Dragnev S, Thurber T, Seed B. A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance. *BMC Genomics*. 2008; 9:633–641. [PubMed: 19108745]
- Sulzer D. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends Neurosci*. 2007; 30:244–250. [PubMed: 17418429]
- Tatton WG, Greenwood CE, Verrier MC, Holland DP, Kwan MM, Biddle FE. Different rates of age-related loss for four murine monoaminergic neuronal populations. *Neurobiol Aging*. 1991; 12:543–56. [PubMed: 1722872]
- Thiruchelvam M, McCormack A, Richfield EK, Baggs RB, Tank AW, Di Monte DA, Cory-Slechta DA. Age-related irreversible progressive nigrostriatal dopaminergic neurotoxicity in the paraquat and maneb model of the Parkinson's disease phenotype. *Eur J Neurosci*. 2003; 18:589–600. [PubMed: 12911755]
- Wang X, Seed B. A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res*. 2003; 31:1–8. [PubMed: 12519937]
- West MJ, Slomianka L, Gundersen HJ. Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec*. 1991; 231:482–497. [PubMed: 1793176]
- Wilms H, Zecca L, Rosenstiel P, Sievers J, Deuschl G, Lucius R. Inflammation in Parkinson's diseases and other neurodegenerative diseases: cause and therapeutic implications. *Curr Pharm Des*. 2007; 13:1925–1928. [PubMed: 17584117]
- Zhang J, Stanton DM, Nguyen XV, Liu M, Zhang Z, Gash D, Bing G. Intrapallidal lipopolysaccharide injection increases iron and ferritin levels in glia of the rat substantia nigra and induces locomotor deficits. *Neuroscience*. 2005; 135:829–38. [PubMed: 16165292]

Figure 1.

Time-dependent LPS-induction of plasma TNF- α protein levels (pg/ml). Rats received LPS (1 mg/kg, i.p., n=6) and serial blood samples were collected for analysis of plasma TNF- α protein to define the time to maximal response (Tmax). Plasma TNF- α was significantly elevated at all times following LPS versus prior resting levels in the same animal ($P < 0.001$, Dunnett's t-test). Tmax was 90 to 120 min; error bars indicate SEM.

Table 1

Peripheral cytokine levels (pg/ml) following LPS injection (0.3 mg/kg, i.p.); number of mice (n) indicated in parenthesis;

		IL-1 β	IL-6	IL-10	TNF- α
YOUNG	Mean	19.66 (6)	28982.50 (6)	3320.74* (6)	2755.50 (6)
	SEM	9.13	565.96	1326.24	569.22
MIDDLE-AGED	Mean	52.60* (5)	33132.19* (6)	1637.07 (6)	3930.67 (6)
	SEM	6.16	1198.88	196.77	527.36
AGED	Mean	38.11 (3)	35334.18* (6)	1105.58 (6)	8919.67* (6)
	SEM	6.92	289.84	202.43	2490.31

* see text for details of statistical comparisons.

Levels of (A) mRNA and (B) protein for pro- and anti-inflammatory cytokines in hippocampus two hours after i.p. LPS administration. Levels of mRNA expression reported as a ratio with gene expression of the housekeeping gene tubulin;

Table 2

A. mRNA Levels					
		IL-1 β	IL-6	TNF- α	
YOUNG	Mean	1.13 (6)	0.75 (5)	0.56 (6)	
	SEM	0.37	0.06	0.20	
MIDDLE-AGED	Mean	1.93 (6)	1.59 * (6)	0.72 (6)	
	SEM	0.41	0.26	0.12	
AGED	Mean	0.23 * (6)	0.07 (5)	0.01 * (5)	
	SEM	0.11	0.07	0.01	

B. Protein Levels (pg/ml)					
		IL-1 β	IL-6	IL-10	TNF- α
YOUNG	Mean	0.93 (6)	63.06 (6)	0.28 (6)	1.52 (6)
	SEM	0.23	24.15	0.04	0.54
MIDDLE-AGED	Mean	0.57 (6)	59.44 (6)	0.46 * (6)	1.26 (6)
	SEM	0.07	7.72	0.02	0.23
AGED	Mean	0.28 (4)	23.49 (6)	0.33 (4)	0.31 * (6)
	SEM	0.09	9.27	0.06	0.11

* see text for details of statistical comparisons.

Table 3

Mean total neuron number of TH+ neurons in SN_{PC}-VTA (one-side); number of mice per group indicated in parenthesis;

		YOUNG	MIDDLE-AGED	AGED
SALINE	Mean	7092 (5)	6424 (5)	4168 * (8)
	SEM	2018	854	608
LPS	Mean	9986 (5)	6382 (5)	5841 * (8)
	SEM	1403	310	1425

*
p < 0.02 for aged versus young groups.