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Susceptibility to a parkinsonian toxin varies during primate development

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Abstract

Symptoms of Parkinson's disease typically emerge later in life when loss of nigrostriatal dopamine neuron function exceeds the threshold of compensatory mechanisms in the basal ganglia. Although nigrostriatal dopamine neurons are lost during aging, in Parkinson's disease other detrimental factors must play a role to produce greater than normal loss of these neurons. Early development has been hypothesized to be a potentially vulnerable period when environmental or genetic abnormalities may compromise central dopamine neurons. This study uses a specific parkinsonian neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to probe the relative vulnerability of nigrostriatal dopamine neurons at different stages of primate development. Measures of dopamine, homovanillic acid, 1-methyl-pyridinium concentrations and tyrosine hydroxylase immunoreactive neurons indicated that at mid-gestation dopamine neurons are relatively vulnerable to MPTP, whereas later in development or in the young primate these neurons are resistant to the neurotoxin. These studies highlight a potentially greater risk to the fetus of exposure during mid-gestation to environmental agents that cause oxidative stress. In addition, the data suggest that uncoupling protein-2 may be a target for retarding the progressive loss of nigrostriatal dopamine neurons that occurs in Parkinson's disease and aging.

Keywords

development; dopamine; MPTP; Parkinson's disease; uncoupling protein

Introduction

It is known that the gestational environment can alter neurodevelopment, and furthermore, there is evidence that certain perturbations during the prenatal period can render an individual susceptible to neurodegenerative or neuropsychiatric disorders later in life, with the risk being further modified by the adulthood environment and genetics (Barlow, et al., 2007, Brown, 2011, Johnston, 1995, Thompson and Stanwood, 2009).

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The onset of symptoms in Parkinson's disease typically occurs after 50 years of age when the normal age-related loss of nigrostriatal dopamine (DA) neurons combine with another factor(s) detrimental to DA neurons to result in inadequate striatal DA neurotransmission and the emergence of parkinsonian symptoms (Calne and Langston, 1983). A link between developmental exposure to harmful agents (e.g., drugs, pesticides and bacterial toxins) and loss of DA neurons has been implicated by many researchers investigating the etiology of idiopathic Parkinson's disease (Barlow, et al., 2007, Callaghan, et al., 2010, Carvey, et al., 2006, Di Monte, 2003, Gao and Hong, 2011, Lloyd, et al., 2006, Sulzer, 2007, Tanner, 2010, Weidong, et al., 2009). It is not known, however, whether there are periods during development when nigrostriatal DA neurons are more or less susceptible to such agents. This issue is addressed in the current study. We have previously shown that primate nigrostriatal DA neurons undergo apoptotic natural cell death at mid-gestation, and we hypothesized that this may be a particularly vulnerable period for this neuronal population to be exposed to an insult that compromises DA neuron integrity (Morrow, et al., 2007).

There is strong evidence that nigrostriatal DA neurons in Parkinson's disease have been compromised by oxidative damage, which may arise from either genetic and/or environmental abnormalities (Horowitz and Greenamyre, 2010, Migliore and Coppede, 2009, Schapira and Jenner, 2011, Vance, et al., 2010, Zhou, et al., 2008). Exposure of experimental animals to chemicals that induce oxidative stress, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), methamphetamine, paraquat or rotenone, has produced animals with loss of nigrostriatal DA neurons and signs of Parkinson's disease. Of these agents, MPTP administration induces the most specific loss of nigrostriatal DA neurons and produces the most faithful model of Parkinson's disease (Betarbet, et al., 2002, Bezard and Przedborski, 2011, Duty and Jenner, 2011). Thus, we examined the relative susceptibility of DA neurons to MPTP in the young and adult monkey, in addition to two stages during prenatal development. These studies are not built on the premise that MPTP itself is an environmental toxin; instead, MPTP is being used here as a specific probe for the age-dependent vulnerability of nigrostriatal DA neurons to a parkinsonian toxin. A greater understanding of the impact of a DA neurotoxin during development may provide insights for risk assessment and the reduction of adverse postnatal consequences (Wells, et al., 2009). Furthermore, understanding which factors are responsible for the changing sensitivity of developing DA neurons to toxicity may identify strategies and targets for treating DA dysfunction in adults. Our previous work has highlighted a possible protective role of uncoupling protein 2 (UCP2) against oxidative stress by reducing the impact of reactive oxygen species (ROS) generated in substantia nigra (SN) DA neurons (Andrews, et al., 2005, Horvath, et al., 2003). This protein is located in the inner mitochondrial membrane and dissipates the proton gradient and membrane potential between the intermembrane space and the mitochondrial matrix to uncouple electron transport from ATP synthesis and so reduce ROS production. Thus, in this study we also examined the relative expression of UCP2 in age groups with differing susceptibility to MPTP.

Material and methods

Animals

These studies used African green monkeys (*Chlorocebus sabaeus*) at the St. Kitts Biomedical Research Foundation. All procedures were approved by both the Yale University and the St Kitts Biomedical Research Foundation Institutional Animal Care and Use Committees. The use of monkeys provides a model especially relevant to man, as the anatomy, biochemistry and pharmacology of primate nigrostriatal DA neurons differ in several aspects from those in the rodent. Specifically, there is a distinction between primates and rodents in regulation of DA synthesis and catabolism (Elsworth and Roth, 2009), and in sensitivity to dopaminergic neurotoxins such as MPTP (Chiueh, et al., 1984). In addition,

the topographical organization and innervation pattern of midbrain DA neurons differs between primates and rodents (Berger, et al., 1992, Levitt and Rakic, 1982, Lewis and Sesack, 1997, Williams and Goldman-Rakic, 1998). Importantly, primates and rodents have distinctively differing rates of brain development with respect to individual brain regions and their maturity at the time of birth (Bayer, et al., 1993, Burke, 2004, Clancy, et al., 2001, Morrow, et al., 2007, Wood, et al., 2003). Furthermore, there is firm evidence for differences in the ontogeny of postnatal DA-dependent behaviors, such as motor and cognitive functions (Wood, et al., 2003).

Age-dependent effect of MPTP on tyrosine hydroxylase expression

Nigrostriatal tyrosine hydroxylase (TH) expression was studied after MPTP administration in 3 age groups: mid-gestation (target age embryonic day (E) 80), late-gestation (target age E140), and young animals (target age postnatal days (P)<30). The gestational age was estimated by ultrasound and/or direct measurement of fetal femur length (see Morrow, et al., 2005); mean gestation in this species is 165 days. Each animal in the MPTP-treated groups was given a total of 1.2 mg/kg MPTP HCl (3 consecutive daily injections of 0.4 mg/kg, i.m.), half the dose used in studies of moderate to severe Parkinsonism in adults. Fetal exposure to MPTP was achieved by treatment of the pregnant female. MPTP treatment of these groups was started near the target ages, with euthanasia at a time when the impact on DA neurons should be maximal, at 1–3 weeks after injection (Brooks, et al., 1987, Burns, et al., 1983, Elsworth, et al., 1987). Actual time (mean $\pm$ standard deviation) between MPTP treatment and euthanasia for the different groups was as follows: mid-gestational fetuses: 14 ± 3 days, late-gestational fetuses: 20 ± 3 days, and young animals: 16 ± 0 days. The mean age ($\pm$ standard deviation) at time of euthanasia for the groups was as follows: MPTP-exposed animals (mid-gestation $n=3$, $E98 \pm 8$; late gestation $n=3$, $E148 \pm 5$; young animals $n=3$ (1 female, 2 male) $P22 \pm 2$), and for age-matched controls (mid-gestation $n=3$, $E99 \pm 11$; late gestation $n=3$, $E133 \pm 16$; young animals $n=3$ (1 female, 2 male) $P20 \pm 6$). There was no significant difference in the interval between MPTP treatment and euthanasia for any of the age groups, and no significant difference in the age at euthanasia between treated subjects and controls for any of the age groups. Animals were euthanized under deep pentobarbital anesthesia. Brains were fixed by perfusion with heparin-containing saline followed either by perfusion with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.3) or in the case of some postnatal brains, by post-fixation in this solution. Past experience has shown that these 2 fixation variants produced no difference in immunostaining for TH (Morrow, et al., 2011, Sladek, et al., 1995). Brains were kept in 4% paraformaldehyde in 0.1M phosphate buffer at 4 °C either overnight (postnatal brains) or for 48 hours (fetal brains) and then stored in 0.1M phosphate buffer containing 0.1% sodium azide until preparation for immunohistochemistry.

The midbrain region was cut into 40 micron sections and stored as 10 sets of tissue, each set having tissue sections 400 microns apart and so represented a uniform sampling of the midbrain starting at a random point anterior to the SN and ventral tegmental area (VTA). A set of tissue from each fetal brain was immunostained for TH-immunoreactivity (ir) using published methods (Morrow et al., 2002). Briefly, TH-ir was identified by incubation of tissue with a primary antibody (1:1000 dilution, MAB-318, Chemicon, Temecula, CA), followed by the ABC reaction (Vectastain Elite kit, Vector Laboratories, Burlingame, CA), with visualization by 0.05% 3,3'-diaminobenzidine with 0.002% hydrogen peroxide. Sections were mounted onto glass slides and lightly counterstained for Nissl substance using thionin. This method has been demonstrated to result in reliable detection of TH-ir in the middle of a 40 micron thick section (Morrow, et al., 2005).

The number of TH-ir cells in the SN (A9) and VTA (A10) were determined using methods previously described (Morrow, et al., 2005). The investigator who counted the stained cells

was blind to the ages and treatments of the subjects; however, masking with respect to age could not be considered complete because of the obvious differences in brain appearance at the different developmental stages. Briefly, the number of cells was estimated from counted nuclei, using a modified single section disector method on an immunostained set of serial sections (Howard and Reed, 1998, Moller, et al., 1990) with an unbiased counting frame (0.0128 mm^2) using a systematic random sampling technique (50x objective with oil, Olympus BH-2 with a Prior Optiscan XYZ motorized stage). A9/A10 cells were assessed on one randomly selected side of the brain only for each subject. If the chosen side of a tissue section contained a fold or a tear that impacted counting, then the other side of that tissue section was used instead. A8 neurons, representing only 5–15% of the total number of midbrain DA neurons in the adult monkey (German, et al., 1988) were not counted. Assessment started on tissue sections anterior to the appearance of the TH-ir cells and continued in a posterior direction through and past these neurons.

Age-dependent effect of MPTP on DA and homovanillic acid

Fresh tissue samples were taken from postmortem brains of young and adult MPTP-treated ($n=3$) and control subjects ($n=4$ or 5), as before (Morrow, et al., 2011), for measurement of DA and homovanillic acid (HVA) concentrations. Specifically, 4 young monkeys were treated with MPTP as described above and fresh nigrostriatal samples collected (below) 16 days later for DA and HVA assays when their mean age ($\pm$ standard deviation) was 21 ± 2 days, while the 5 controls were aged 19 ± 5 days. Thus, there was no significant difference in age of the young MPTP-treated subjects and their controls. MPTP-treated female adults ($n=4$, > 5 years old, but showing no signs of old age) were treated with MPTP as described above and fresh nigrostriatal samples obtained from them and 4 age- and sex- matched controls after a mean ($\pm$ S.D.) interval of 49 ± 4 days. Although there was a greater delay between MPTP treatment and euthanasia for adults than young animals, it is known that MPTP-induced DA losses in adults peak at 1–3 weeks after treatment, and then remain stable, or in some monkeys, lessen slightly over time. Thus, the longer interval in adult monkeys compared with young monkeys would if anything, bias the outcome towards lessening the apparent impact of MPTP in adults relative to young monkeys.

Punches of tissue from striatum were taken using a 1.4 mm diameter punch from a chilled 4 mm thick coronal slice, and then frozen in cryotubes by immersion in liquid nitrogen, as described before (Elsworth, et al., 2000, Morrow, et al., 2011). Dorsal and ventral subregions of both caudate nucleus and putamen were taken at the mid-coronal level, which corresponded at the caudal extreme, to the crossing of the anterior commissure. DA and its main metabolite in primate brain, HVA (Bacopoulos, et al., 1978), were extracted from striatal samples and quantified after reverse-phase HPLC separation, with electrochemical detection (Elsworth, et al., 2000, Morrow, et al., 2011). Quantification was achieved by reference to internal and external standards.

Age-dependent effect of MPTP on expression of mRNA for UCP2

Fresh tissue samples of substantia nigra were collected from control and MPTP-treated postmortem brains of the young and adult subjects described above. Total RNA was extracted using Trizol solution (Invitrogen, Carlsbad, CA). UCP2 mRNA levels were measured by Real Time PCR. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA) was used for the reverse transcription. Real Time PCR (LightCycler 480, Roche, Indianapolis, IN) was performed with diluted cDNAs in a $20 \mu\text{l}$ reaction volume in triplicates. Primers used for this study were as follow: Cat no. HS01075225_m1 for human UCP2 and Cat no. Hs02758991_g1 for glyceraldehyde-3-phosphate dehydrogenase (GADPH) rRNA (Applied Biosystems). The calculations of average crossing point (Cp) values, standard deviations, and resulting expression ratios for

each target gene were based on the Roche LightCycler 480 software. Precautions were taken to assure validity of the comparison of expression of mRNA for UCP2 in the 2 groups, including using the equivalent sizes of dissected tissue, and processing equivalent quantities of mRNA. The data are expressed relative to a housekeeping gene (GADPH) that appears to have stable expression in the midbrain from the early postnatal period to adulthood in the mouse (Boda, et al., 2009).

Comparison of 1-methyl-4-phenylpyridinium levels following MPTP treatment

As MPTP is rapidly metabolized in vivo, it has only been possible to measure circulating or tissue levels of MPTP within a few minutes of injecting a high dose of the drug in rodents (Shinka, et al., 1987). Thus, it was not feasible to measure MPTP levels following a single low dose of MPTP in the present study. However, 1-methyl-4-phenylpyridinium (MPP⁺) is the actual toxic metabolite of MPTP, and concentrations of MPP⁺ were measured in 3 adult and 3 young monkeys following a single i.m. injection of 0.4 mg/kg MPTP. Samples of venous blood were drawn 20 minutes after MPTP injection, immediately placed on ice, then centrifuged, and the resulting plasma samples were frozen in liquid nitrogen. Cisternal cerebrospinal fluid (CSF) samples were collected at 24 hours after MPTP injection and then frozen in liquid nitrogen. Postmortem brains samples were collected 24 hours after MPTP injection, as described before (Morrow, et al., 2011).

Plasma or CSF samples (0.5 ml) were mixed with an equal volume of 0.8M perchloric acid to precipitate proteins. After centrifugation, the supernatant was treated with 0.1 ml 5M sodium hydroxide to raise the pH above 10. This solution was saturated with potassium iodide to permit the extraction of MPP⁺ into 5 volumes of dichloromethane as a stable ion pair (Langston, et al., 1984). Following centrifugation to separate layers, the tube was frozen on dry ice, and the upper organic layer was poured off and dried down under a stream of helium. After evaporation, the residue was reconstituted in 150 μ l 0.1M perchloric acid, and a 50 μ l aliquot was separated by HPLC. Recovery of MPP⁺ from plasma and CSF was 80%. Brain samples (0.3 to 1 mg protein) were sonicated in 200 μ l 0.1 M perchloric acid containing 0.4 mM sodium metabisulfite and 0.1 mM EDTA disodium dihydrate (Naoi et al., 1987), and following centrifugation, a 50 μ l aliquot was separated by HPLC. The HPLC system was adapted from previous methods (Naoi, et al., 1987, Russo, et al., 1994) and comprised a 4.6 mm x 250 mm Cosmosil 5C18-MS-II column (Nacalai USA Inc., San Diego, CA), using a pH 7.75 mobile phase (20 mM boric acid, 3 mM tetrabutylammonium sulfate, 0.25 mM hexane sulfonic acid and 7.5% isopropanol) pumped at 0.8 ml/min (Shimadzu LC-10ADVP, Columbia, MD) with native fluorescence detection of MPP⁺ achieved at excitation/emission wavelengths of 295 and 375 nm (Shimadzu RF-10AXL). Less tailing of the MPP⁺ peak was obtained with this column compared to other tested reverse phase columns. MPP⁺ has a propensity to bind to metal surfaces, and it was important to clean the metal parts of the injection syringe between injections in order to avoid “ghost” peaks. MPP⁺ levels were calculated from an external standard curve (which was linear with intercept at the origin) and brain levels were corrected for the protein content of the precipitated pellet, which was determined by the Lowry method.

Data analysis

Data were analyzed using either unpaired 2-tailed t-test, or a 1- way or 2-way or repeated measures ANOVA as appropriate (SuperAnova, Abacus Concepts, Berkeley, CA), considering $p < 0.05$ as significant for all analyses. Data are presented as group means $\pm$ standard error of the mean, except where noted.

Results

Age dependent susceptibility of TH-ir to MPTP treatment

Two-way ANOVA identified a significant effect of treatment [$F(1,12) = 5.7$, $p < 0.05$] and age [$F(2,12) = 8.9$, $p < 0.005$] on the number of TH-ir neurons, and an interaction between age and treatment [$F(2,12) = 8.9$, $p < 0.005$], with the effect of MPTP being significant for only the mid-gestational group. Exposure to MPTP reduced the number of A9/A10 neurons in the mid-gestational fetal samples by greater than 90% (Figs. 1, 2A and 2B). In contrast, in the late-gestational fetuses and young monkeys, MPTP exposure did not induce a significant change in the number of A9/A10 neurons compared with samples from controls of the same age (Figs. 1 and 2C). These data demonstrate that midbrain DA neurons are more susceptible to MPTP at the mid-gestation compared with late gestation stage of development and in the young monkey.

When viewing the slides of the pars compacta subregion of SN (SNpc) region of MPTP-treated mid-gestation fetuses, in addition to a decrease in number of TH-ir neurons, we noted an apparent increase in the number of Nissl-stained neurons without TH-ir, recognized by the typical morphology of SNpc DA neurons (Fig. 2A). Thus, we quantified SNpc neurons lacking TH-ir at the mid-gestational age and compared their frequency in another developmental group in which there was no loss of TH-ir neurons, namely the young animals (Figs. 2C and 3). As expected based on the data from the combined SN and VTA regions (above), MPTP treatment reduced the number of TH-ir neurons in the SNpc region of mid-gestational fetuses, but not young animals [treatment $F(1,8) = 7.6$, $p < 0.05$; age $F(1,8) = 6.5$, $p < 0.05$; interaction $F(1,8) = 6.5$, $p < 0.05$]. The numbers of Nissl-stained neurons without TH-ir in the SNpc regions of controls of both ages were relatively small, representing less than 10% of the SNpc population. As there is a substantial difference in the number of TH-ir neurons at these 2 ages (Morrow, et al., 2005), the number of non-TH-ir neurons was expressed as percent of the control number of TH-ir cells at that age to facilitate comparison. The number of Nissl-stained, non-TH-ir neurons was significantly increased by MPTP treatment in the mid-gestation fetuses but not in the young animals. Thus, 2-way ANOVA identified a significant effect of treatment [$F(1,8) = 72.9$, $p < 0.0001$], age [$F(1,8) = 39.8$, $p < 0.0005$] and interaction between the factors [$F(1,8) = 39.6$, $p < 0.0005$], with post-hoc tests showing a significant increase only in the mid-gestational samples. When the size of the 2 populations of cells were added together, the mean number ($\pm$ S.E.M.) of TH-ir neurons plus Nissl-stained/non-TH-ir neurons in the A9pc was not significantly different between the samples of mid-gestation tissue (control, 28,271+8324; MPTP, 30,931+624) and between samples from young animals (control: 63,261+4727, MPTP: 69,966+4893). These data indicate that the loss of TH-ir SNpc neurons induced by MPTP at the mid-gestation stage of development could be accounted for by an increase in Nissl-stained/non-TH-ir neurons.

Age dependent susceptibility of DA and HVA levels to MPTP treatment

MPTP treatment induced the well-documented marked losses of DA and HVA concentrations in the striatum of adult monkeys (Fig. 4). Statistical analysis by 2-way ANOVA identified a significant effect of treatment [$F(1,24) = 776.0$, $p < 0.0001$] and striatal subregion [$F(3,24) = 16.2$, $p < 0.0001$] on DA concentration. In MPTP-treated adults, the decrease in DA concentration in ranged from 94 to > 99%, depending on striatal subregion. Similarly, for HVA there was a significant effect of treatment [$F(1,24) = 155.3$, $p < 0.0001$] and region [$F(3,24) = 10.4$, $p < 0.0001$], and the decrease in HVA concentration ranged from 71 to 83% depending on striatal subregion (Fig. 4).

In contrast, in young monkeys there was no significant MPTP-induced loss of striatal DA levels (Fig 5). However, a reduction in HVA concentration was noted in the young MPTP-treated monkeys, with ANOVA indicating a significant effect of treatment [$F(1,28) = 50.3$, $p < 0.0001$] and region [$F(3,28) = 25.4$, $p < 0.0001$] (Fig 5), which ranged from 45 to 51% in different striatal subregions.

When changes in DA and HVA concentrations were expressed as a ratio, MPTP treatment was observed to increase HVA/DA in adults (up to 40-fold), with a 2-way ANOVA indicating a significant effect of treatment [$F(1,24) = 25.8$, $p < 0.0001$] and brain region [$F(3,24) = 3.6$, $p < 0.05$], and the increase in ratio varied from 9- to 45-fold, depending on striatal subregion (Table 1). However, the HVA/DA ratio modestly *decreased* (up to 2-fold) in the young MPTP-treated monkeys (Table 1), with a 2-way ANOVA indicating a significant effect of treatment [$F(1,28) = 24.0$, $p < 0.0001$] and brain region [$F(3,28) = 6.8$, $p < 0.005$], with the decrease in ratio varying from 1.4- to 2.3-fold, depending on region (Table 1).

These data show that in adult monkeys, MPTP exposure induced a greater loss of DA than HVA in striatum, leading to an increase HVA/DA ratio compared with controls. In young animals, MPTP exposure did not result in a loss of DA, although a decrease in HVA was found, resulting in a lower HVA/DA ratio compared with controls at this age.

MPP+ levels in MPTP-treated young and adult monkeys

No difference in plasma or CSF levels of MPP+, measured 20 mins or 24 hours after MPTP injection respectively, was detected (2-tailed unpaired t-test) between young and adult monkeys (Table 2). Repeated measures ANOVA using a 1-between (age) and 1-within (region) design was calculated to analyze MPP+ levels in 6 brain regions from young and adult monkeys at 24 hours after a single injection of MPTP. This revealed a significant effect of age [$F(1,20) = 167.6$, $p < 0.0002$], region [$F(5,20) = 136.2$, $p < 0.0001$], and an interaction between these factors [$F(5,20) = 41.0$, $p < 0.0001$] (Fig. 6). Further analysis of the effect of region on MPP+ levels in each age group (1-way ANOVA, followed by Student-Newman-Keuls test) revealed significant differences in accumulation of MPP+ in different brain regions. Ventral putamen contained the highest MPP+ concentration, and substantia nigra and occipital cortex the lowest concentration in both age groups. Ventral putamen MPP+ concentration was higher than dorsal caudate nucleus in both young and adult monkeys. The level of MPP+ was also compared between young and adult monkeys in each brain region (2-tailed unpaired t-test). This analysis indicated that adult brain contained more MPP+ than the young brain in the following regions: ventral putamen [$t(4) = 12.5$, $p < 0.0002$], dorsal caudate [$t(4) = 10.1$, $p < 0.0005$], and cingulate cortex [$t(4) = 5.1$, $p < 0.01$].

These data revealed that there was no difference detected in plasma or CSF levels of MPP+ between young and adult monkeys following an acute MPTP injection. However, several brain regions of adult monkeys contained higher MPP+ concentrations than in young monkeys following acute MPTP administration.

Age-dependent expression of UCP2

Substantia nigra from untreated young monkeys had significantly higher levels of mRNA for UCP-2 (> 2-fold), relative to GADPH, than untreated adults (2-tailed unpaired t-test, $t(6) = 2.8$, $p < 0.05$) (Fig 7). These data indicate that there is a higher expression of UCP2 in the substantia nigra of young monkeys compared with adult monkeys.

Discussion

As hypothesized, the present data indicate that DA neurons at the time of natural cell death are especially vulnerable to the dopaminergic neurotoxin, MPTP. However, when MPTP is given later in gestation, or even directly to the young monkey, developing DA neurons appear remarkably resistant to the toxic effects, compared with the substantial damage to DA neurons elicited by MPTP in either E80 fetuses or young adult monkeys. These conclusions are based on the loss of TH-ir neurons and/or striatal DA concentration following MPTP administration. The data demonstrate the vulnerability of developing DA neurons to MPTP treatment at mid-gestation compared with later in development, and closely resemble the results from our recent study of the relative impact of methamphetamine at different stages of primate development (Morrow, et al., 2011). Together these data imply that mid-gestation is a period of increased susceptibility to compounds that induce oxidative stress in DA neurons. It is not known yet to what extent DA neurons impacted at mid-gestation can recover from such an insult, but it is possible that lasting detrimental effects ensue. If a permanent loss of a portion of the nigrostriatal DA population were to occur during human fetal development, it would presumably increase the risk for Parkinson's disease later in life, when the natural age-related loss of DA neuron function (Chu, et al., 2002, Collier, et al., 2011, Kish, et al., 1992) adds to the loss incurred during development, and exceeds the threshold of compensations that are invoked when the nigrostriatal DA population is compromised. Furthermore, even in the case of full recovery of DA neurons that are impacted during mid-gestation, a temporary loss of DA signalling during brain growth might permanently affect the development and function of other neuronal systems in the brain, as DA is a recognized morphogen, affecting for example, growth cone steering in target and non-target cells (Herlenius and Lagercrantz, 2001, Ruediger and Bolz, 2007, van Kesteren and Spencer, 2003).

There have been few other studies on the variation in sensitivity to dopaminergic toxins during prenatal development. Even in rodents, the data are scant and apparently inconsistent (Furune, et al., 1989, Melamed, et al., 1990, Ochi, et al., 1991). There is only one report of the effects of prenatal exposure to MPTP in a primate species (Perez-Otano, et al., 1995). In this study, designed to examine the effect of MPTP in adult marmosets, MPTP was inadvertently given to 2 pregnant marmosets during most of the gestational period; the offspring had approximately 80% loss of striatal DA concentration when examined at 5 months after birth. The present data suggest that there is period during prenatal development when nigrostriatal DA neurons have increased sensitivity to MPTP, and by implication also to other drugs that compromise oxidative phosphorylation and/or induce oxidative stress. Mid-gestation (about E80) is the period when apoptotic natural cell death occurs in nigrostriatal DA neurons in primates (Morrow, et al., 2007), and the time at which MPTP injections were given in the present study. In rodents, 2 phases of apoptotic cell death have been identified; these both peak postnatally, at P2 and P14 (Oo and Burke, 1997). Our hypothesis was that during apoptotic natural cell death primate DA neurons might be more sensitive to toxicity. While the present data are consistent with this prediction, further studies are required to firmly establish this relationship and a precise mechanism between natural cell death in DA neurons and their susceptibility to toxins.

There are other mechanisms that should be considered when evaluating the difference in response to MPTP during different periods of gestation. For example, placental permeability is not constant during pregnancy, and is influenced by characteristics such as thickness, area, carrier systems, lipid and protein content, blood flow and tissue metabolic activity (Andersen, et al., 2000). Based on lipophilicity, it seems likely though that MPTP will pass easily through the placenta at all times of development. Conversely, being a charged species, it is unlikely that MPP⁺ would pass to any significant extent from the maternal to

the fetal circulation (van der Aa, et al., 1998). The blood-brain and blood-CSF barriers appear well established even at the earliest stages of development (Ek, et al., 2012, Saunders, et al., 2000). Other factors such as alterations in inward/outward transport systems or plasma protein binding during development could theoretically lead to greater MPTP-induced toxicity during mid-gestation, however to date, there are no data to support such mechanisms being relevant to MPTP or MPP+ (Ek, et al., 2012, Saunders, et al., 2000).

The only report of postnatal exposure to MPTP in any species was performed in rats (7–10 days old), which were found to be more resistant to the neurotoxic effects of MPTP than adult rats (Jarvis and Wagner, 1985). In one study (Rose, et al., 1993), juvenile marmosets required a higher cumulative dose of MPTP than young adult or aged marmosets before onset of parkinsonian signs, yet at post-mortem the juvenile brains displayed greater loss of striatal DA and cell loss in the substantia nigra. The present data clearly show that the young monkey is relatively resistant to MPTP-induced toxicity compared with the adult monkey.

The decrease of HVA concentration, but not DA concentration, in the striatum of young monkeys treated with MPTP could reflect a mild impact on DA neurons that was insufficient to cause loss of DA content, but enough to induce a down-regulation of dopaminergic activity or DA turnover. Alternatively, the release of MPP+ from storage vesicles and entry into mitochondria in DA terminals over time (see below) provides another possible explanation for the reduction in HVA following MPTP treatment. This is because MPP+ is an inhibitor of monoamine oxidase (Takamido, et al., 1987), an enzyme located on the outer mitochondrial membrane, and monoamine oxidase activity is necessary for the formation of HVA from DA. If MPP+ does produce significant inhibition of monoamine oxidase, then the resulting reduction in HVA would presumably occur in both the young and adult striatum following exposure to MPTP; this means that the loss of HVA in the adult striatum shortly after MPTP administration may be due a combination of monoamine oxidase inhibition and MPTP-induced toxicity. Thus, while the reason for the reduction in striatal HVA concentration in young animals is not clear, the lack of a significant effect on striatal DA is consistent with the absence of a loss of nigrostriatal DA neurons in young MPTP-treated animals (Zigmond, et al., 1989).

One possible explanation for the resistance of young monkeys to MPTP-induced toxicity is the finding of lower levels of MPP+ measured in the striatum (but not all brain regions) of young monkeys compared with adults. However interpretation of this result is not quite so obvious as it might appear, as the relationship between MPP+ concentration and toxicity is not straightforward. The selectivity of MPTP for DA neurons arises because the toxic metabolite, MPP+, is a substrate for the plasma membrane DA transporter. Once in the cytosol of a DA neuron, MPP+ can enter the mitochondrial matrix, where it acts as an inhibitor of Complex I of the electron transport chain, and increases production of ROS. However, MPP+ is a good substrate for the vesicular monoamine transporter (VMAT-2), located on the storage vesicles. Thus, MPP+ is sequestered in vesicles by the action of VMAT-2, and while in this compartment the neuron is protected, in the short-term, from its neurotoxic action (Miller, et al., 1999). This conclusion is reinforced by the observation that heterozygote VMAT-2 knockout mice have increased susceptibility to MPTP (Gainetdinov, et al., 1998, Takahashi, et al., 1997). The eventual release of MPP+ from vesicles may result in damage to the neuron if sufficient drug enters mitochondria. Alternatively, the sequestration of MPP+ in vesicles may allow the neuron to escape damage, if the levels released from the vesicle are low and gradual. Studies comparing the marked inter-species or inter-strain differences in sensitivity to MPTP have attempted to shed some light on the relevance of striatal MPP+ concentrations. Within strains of mice, there is a spectrum of susceptibility to MPTP, and in a study comparing the response of different strains to MPTP, a higher content of MPP+ in the striatum was associated with a greater degree of MPTP-

induced striatal DA loss (Giovanni, et al., 1991), but see Vaglini et al. (1996). However, this relationship does not appear to extend between species, as the rat, which is more resistant to MPTP-induced toxicity than the mouse, actually achieves higher striatal MPP⁺ levels than the mouse after a fixed MPTP dose (Giovanni, et al., 1994a, Giovanni, et al., 1994b). In fact, mouse DA neurons are more sensitive than rat DA neurons to a direct injection of MPP⁺ into the striatum (Giovanni, et al., 1994b), so it is apparent that rat DA neurons can withstand a higher MPP⁺ exposure than mouse DA neurons without incurring damage (Giovanni, et al., 1994a, Jossan, et al., 1989). Primates are exquisitely sensitive to MPTP-induced toxicity; for example, mice given 200 times as much MPTP than monkeys (300 vs. 1.5 mg/kg cumulative dose) still maintain about 10-fold higher striatal DA concentration than monkeys (Johannessen, et al., 1985). Another striking difference of probable importance between the mouse and primate brain is the relative retention of MPP⁺ in the striatum; the primate striatum selectively retains MPP⁺ for days compared with just hours in the mouse striatum (Johannessen, et al., 1985). These early primate data were based on measurement of radioactivity following injection of labeled MPTP, and the current data are the first to our knowledge that have directly measured MPP⁺ levels in the primate striatum following MPTP injection. Our results show that despite having similar plasma levels following an MPTP injection, and similar CSF levels 24 hours later, adult monkeys achieved a higher striatal level of MPP⁺ compared with young monkeys. While this finding appears to provide a ready explanation for the resistance of young monkeys to MPTP-induced toxicity, as the discussion above indicates, accumulation of higher striatal MPP⁺ levels does not necessarily imply greater toxicity, and this is illustrated by the greater accumulation of MPP⁺ in the ventral striatum compared to dorsal striatum, yet the DA loss in dorsal striatum is greater than in ventral striatum following MPTP administration (Elsworth, et al., 2000). The determinants of MPP⁺ levels in striatum include the expression and activity of VMAT2 and the plasma membrane dopamine transporter, and the time-course of MPP⁺ accumulation and release from vesicular storage. Further work will be needed to determine the mechanism of the higher MPP⁺ levels in the adult animals treated with MPTP, and whether this is associated with the greater impact of MPTP-induced toxicity in these monkeys.

Our previous work has highlighted a possible protective role of UCP2 against MPTP-induced toxicity. This mitochondrial protein uncouples electron transport from ATP synthesis and so reduces ROS production. Interestingly, UCP2 expression and function in brain are basally increased in young rats by the fat-rich diet of maternal milk (Sullivan, et al., 2003). Similarly, we found evidence that, compared with adults, UCP2 expression is upregulated in the SN of young monkeys, which are still at the age when they feed from their mother. Our previous studies provide support for a role of UCP2 in protection from MPTP-induced toxicity in both nonhuman primates and rodents. Specifically, we have reported that administration of coenzyme Q, an obligatory cofactor for UCP (Echtay, et al., 2000), to adult monkeys induces UCP2 and protects nigrostriatal DA neurons from MPTP-induced toxicity (Horvath, et al., 2003). In addition, genetic manipulation of UCP2 in mice was found to directly affect SN DA function, with a lack of UCP2 resulting in greater MPTP-induced DA cell loss, and overexpression of UCP2 being protective against MPTP-induced toxicity (Andrews, et al., 2005). Thus, the relatively high expression of UCP2 in the young monkey SN may play a role in its protection from MPTP-induced damage to DA neurons located in that region.

These studies provide novel information on altered susceptibility of the primate nigrostriatal DA system to a parkinsonian toxin at different stages of development. In particular the results highlight the potential of greater risk to the fetus during mid-gestation of exposure to environment agents that cause oxidative stress. In addition, the current data suggest that UCP2 may be a target for amelioration of toxin-induced, and conceivably age-associated,

loss of nigrostriatal DA neurons in the adult, by essentially reinstating in the adult the protection that was afforded during early life.

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Abbreviations

CSF	cerebrospinal fluid
DA	dopamine
E	embryonic day
GADPH	glyceraldehyde-3-phosphate dehydrogenase
HVA	homovanillic acid
ir	immunoreactivity
MPP+	1-methyl-pyridinium
MPTP	1-methyl-4-phenyltetrahydropyridine
P	postnatal day
ROS	reactive oxygen species
SN	substantia nigra
SNpc	substantia nigra pars compacta
TH	tyrosine hydroxylase
UCP2	uncoupling protein 2
VTA	ventral tegmental area

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Highlights

- Impact of a parkinsonian toxin on developing primate dopamine neurons was studied
- At mid-gestation and in adults, dopamine neurons are sensitive to MPTP
- In the young monkey, dopamine neurons are resistant to MPTP
- Uncoupling protein 2 is upregulated in substantia nigra of young monkeys
- Susceptibility of dopamine neurons to oxidative stress appears to be age-dependent

Figure 1.

Quantification of MPTP-induced loss of TH-ir in A9 and A10 regions during different stages of development. An MPTP-induced loss of TH-ir neurons occurred at mid-gestation, but not later in development. Abbreviations: Gest, gestation. There were 3 samples in each control and MPTP group. * indicates a statistically significance decrease compared with appropriate control group. See text for details of statistical analyses.

Figure 2.

Images showing that the extent of MPTP-induced loss of TH-ir in SN varied during development. The tract of neurons containing the highest density TH-ir neurons is the pars compacta region of the SN. The significant impact of MPTP exposure at the mid-gestational age is shown in panel A (scale bar is 100 micrometers); panel B (scale bar is 10 micrometers) shows a higher power image of TH-ir neurons in control and MPTP-treated mid-gestational fetuses. The lack of effect of MPTP exposure on TH-ir in young monkeys is illustrated in panel C (scale bar is 100 micrometers). TH-ir produces a brown stain, thionin staining is blue.

Figure 3.

MPTP exposure induced a loss of TH-ir neurons and an increase in Nissl-stained neurons without TH-ir in the SNpc. Data derives from 3 control and 3 MPTP-exposed samples from each development stage. * indicates a significant MPTP-induced change from controls of that age. See text for details of statistical analyses.

Figure 4.

MPTP-induced loss of striatal DA and HVA concentrations in adult monkeys. DA and HVA concentrations were measured in subregions of the striatum from 4 control and 4 MPTP-treated animals. Abbreviations: dCD, dorsal caudate nucleus; dPT, dorsal putamen; vCD, ventral caudate nucleus; vPT, ventral putamen. * indicates a statistically significant difference compared to the control value for that region. See text for details of statistical analyses.

Figure 5.

Effect of MPTP on striatal DA and HVA concentrations in young monkeys. No loss of DA concentration, but a decrease in HVA concentration, was found in striatum from 4 control and 5 MPTP-treated young monkeys. Note that the Y-axes have different scale to those in Figure 4, as young monkeys have lower levels of striatal DA and HVA than adults.

Abbreviations: dCD, dorsal caudate nucleus; dPT, dorsal putamen; vCD, ventral caudate nucleus; vPT, ventral putamen. * indicates a statistically significant difference compared to the control value for that region. See text for details of statistical analyses.

Figure 6.

MPP+ accumulation in brain following MPTP injection depended on region and age. * indicates brain regions in which significantly more MPP+ was retained in the adult compared with the young monkey at 24 hours following MPTP injection. Bars with different lower case letters indicate regions in the adult brain that contained significantly different levels of MPP+. See text for details of statistical analyses. Bars with different upper case letters indicate regions in the young monkey brain that contained significantly different levels of MPP+. Data derived from 3 adult and 3 young monkeys. Abbreviations: CCx, cingulate cortex; dCD, dorsal caudate nucleus; DLPFC, dorsolateral prefrontal cortex; Nigra, substantia nigra; OccCx, occipital cortex; vPT, ventral putamen.

Figure 7.

Greater expression of mRNA UCP2 in SN of young monkeys relative to adults. Data are adjusted for expression of mRNA GAPDH, and derive from samples taken from 3 adults and 5 young monkeys. * indicates a significant different from adult levels. See text for details of statistical analyses.

Table 1

Effect of MPTP treatment on HVA/DA ratio in striatal sub-regions of young and adult monkeys ^a.

Striatal Region	Young		Adult	
	Control (n=5)	MPTP (n=4)	Control (n=4)	MPTP (n=4)
d-Caudate	1.1 ± 0.1	0.8 ± 0.1 †	0.8 ± 0.1 #	36.2 ± 11.0 *
v-Caudate	0.9 ± 0.1	0.5 ± 0.1 †	0.7 ± 0.1 #	5.7 ± 1.0 *
d-Putamen	1.5 ± 0.2	0.9 ± 0.1 †	1.0 ± 0.1 #	32.4 ± 10.6 *
v-Putamen	2.2 ± 0.3	0.9 ± 0.1 †	0.8 ± 0.1 #	10.8 ± 5.0 *

^a HVA/DA ratio was significantly increased in adults following MPTP treatment, indicated by *. In contrast, HVA/DA ratio was significantly decreased in young monkeys following MPTP treatment, indicated by †. In control monkeys, HVA/DA ratio was lower than in young monkeys, indicated by #. See text for details of statistical analyses. Abbreviations: d, dorsal; n, number of animals; v, ventral.

Table 2

Plasma and CSF levels of MPP⁺ following MPTP administration in young and adult monkeys ^b.

Age Group	Plasma (n=3, 20 mins)	Cisternal CSF (n=3, 24 hours)
Young	2.0 ± 0.4 ng/ml	0.9 ± 0.2 ng/ml
Adult	2.1 ± 0.1 ng/ml	0.8 ± 0.2 ng/ml

^b Levels of the toxic metabolite of MPTP, MPP⁺, were not significantly different in plasma or CSF between young and adult monkeys, measured 20 mins or 24 hours respectively, following an MPTP injection. Abbreviation: n, number of animals.