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Profiling genes related to mitochondrial function in mice treated with *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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Abstract

Since mitochondrial dysfunction plays an important role in the pathogenesis of dopaminergic neurodegeneration in Parkinson's disease, we determined the expression of genes related to mitochondrial function in the substantia nigra of mice treated with *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) using a cDNA array. MPTP treatment significantly depleted striatal dopamine, but did not result in apparent neuronal loss in the substantia nigra at 3 and 18 days post-treatment. We also examined changes in genes in the hypothalamus, a region containing dopaminergic neurons that are relatively resistant to MPTP. Finally, we confirmed those genes identified by microarrays as differentially expressed in the substantia nigra but not in the hypothalamus using in situ hybridization. Our results demonstrated that MPTP significantly changed the expressions of six genes in nigral neurons, four of which were related to the mitochondrial electron transport chain: the NADH–ubiquinone oxidoreductase 13 kDa B subunit, the NADH–ubiquinone oxidoreductase MNLL subunit, cytochrome *c*, and the cytochrome *c* oxidase Va subunit. Two other differentially expressed genes were the dihydropyridine-sensitive L-type calcium channel α-2 subunit precursor and type III α-1 procollagen. None of these six genes are encoded by mitochondrial DNA. The potential significance of these gene alterations in the context of Parkinson's disease is discussed.

Keywords: Microarray; Parkinsonism; Movement disorders; Mitochondria; Free radicals; Mice; Aging

Parkinson's disease (PD) is characterized pathologically by a loss of dopaminergic neurons in the substantia nigra (SN) and the resultant depletion of striatal dopamine (DA) [1]. The etiology of PD likely involves intertwined processes of aging, genetic susceptibility, and environmental exposures. A prevailing hypothesis is that mitochondria dysfunction plays a fundamentally important role in dopaminergic neurodegeneration [2–6]. This concept has been reinforced repeatedly by experiments showing that environmental toxicants that inhibit mitochondrial function also cause selective nigrostriatal dopaminergic neurodegeneration in animals [7–9].

Mitochondrial oxidative phosphorylation along the respiratory chain is carried out by five enzyme complexes that are located in the mitochondrial inner membrane [10] and designated as complex I (NADH:ubiquinone oxidoreductase), II (succinate:ubiquinone oxidoreductase), III (ubiquinol:ferrocytochrome *c* oxidoreductase), IV (ferrocytochrome *c*:oxygen oxidoreductase or cytochrome *c* oxidase), and V (ATP synthase). Several studies have demonstrated dysfunction of complex I in PD patients [6,11,12] and other mitochondrial complexes may also be affected [13,14]. In support of the latter argument, we recently found that mitochondrial DNA (mtDNA)

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damage in PD is widespread without restriction to any given regions of the mtDNA or to the region of the SN [15,16]. Furthermore, there are hundreds of protein peptides in mitochondria [17], only 13 of which are encoded by mtDNA [18]. Consequently, a defect in any components of mitochondrial respiratory chain or in other genes products involved in normal mitochondrial function may compromise mitochondrial function and potentially culminate in dopaminergic neurodegeneration in PD.

cDNA microarrays offer a powerful tool for identifying targets or pathways involved in mitochondrial dysfunction in PD [19], since gene transcripts related to mitochondria, whether encoded by mtDNA or not, may be altered when mitochondrial dysfunction is involved. Intake of *N*-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in parkinsonism in humans and in other susceptible species, including mice [9,12,20,21]. MPTP is metabolized to an active toxin, 1-methyl-4-phenylpyridinium (MPP^+), which inhibits complex I of mitochondrial electron transport chain [11,22,23]. In several recent studies, Youdim and colleagues [24,25] have demonstrated MPTP-induced changes in global gene expression using a cDNA expression array membrane, reporting that MPTP altered the expression of 51 genes (out of 1200 genes) involved in oxidative stress, inflammation, excitotoxicity, and factors regulating cell cycle. However, these studies did not use the SN as the sole source of RNA to probe with, instead they used a block of tissue encompassing the mes/diencephalons with some additional telencephalic tissues. In an attempt to identify those genes that are more directly relevant to mitochondrial dysfunction in PD, in current study we assessed the expression of genes related to mitochondrial function with the SN of control and MPTP-treated mice as the sole source of RNA, and also compared differences in gene expression between the SN and the hypothalamus, a region with several different dopaminergic cell groups that are thought to be relatively resistant to MPTP-induced neurotoxicity [26–28]. Furthermore, to avoid nonspecific effects of MPTP at higher doses, we used an MPTP protocol that produces minimal loss of dopaminergic perikarya when examined 2–3 weeks after treatment, yet results in an extensive loss of striatal dopaminergic stores [29]. This study provides a temporally and spatially relevant model of gene expression changes in PD that is directed towards genes thought to be pertinent to PD pathology.

Materials and methods

Subjects. Male C57-Bl/6 mice (Harlan, Birmingham, AL), 20–30 g (8–12 weeks), were individually housed in an AALAC-approved vivarium under a 12-h light/dark cycle (lights on at 6:00 A.M.) with food and water available ad libitum. All experiments were performed in accord with the Guide for the Care and Use of Laboratory Animals as promulgated by the National Institutes of Health. Mice were handled daily and allowed at least 3 days to acclimate after arrival to the vivarium before any treatment.

Drugs and sample collections. Treatment of mice ($n = 6$ per group) with MPTP was performed as described previously [29]. Briefly, mice were injected with either vehicle (0.9% normal saline) or 26.5 mg/kg (i.p.) MPTP (Sigma, St. Louis, MO) twice, with the interval between each dose being 12 h. Thirty minutes before each MPTP injection, mice were treated with diethyldithiocarbamate (DDC, 400 mg/kg, i.p.; Sigma). This protocol, chosen deliberately to avoid nonspecific effects of MPTP at higher doses, results in significant striatal DA deletion without apparent nigral neuronal loss at 2–3 weeks post-treatment. In agreement with previous reports [30,31], we found that DDC administration alone did not alter striatal DA or DOPAC concentrations, and consequently we did not include a DDC control in this study. Animals were euthanized with an excess dosage of isoflurane 3 or 18 days after MPTP treatment. The brains were rapidly removed and cut into 1-mm thick coronal slices using a chilled aluminum brain block (Harvard Instruments, Holliston, MA). A 13-g puncher was used to obtain a sample from the dorsolateral striatum for measurement of DA. The SN and ventral tegmental area (VTA) were dissected from a 1-mm slice as described previously by Deutch et al. [32]. The hypothalamus was dissected from the intervening brain tissue using a method described by Glowinski and Iversen [33]. Tissue samples were placed in microfuge tubes and stored at −70 °C until assayed.

Measurement of striatal dopamine. Striatal DA concentrations were measured as described previously [34] with minor modifications. Briefly, the tissue samples were sonicated on ice in 450 μl buffer solution containing 125 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM Hepes, and 100 μM pargyline. Brain homogenates were mixed with 2.0 M perchloric acid/2.5% EDTA (v/v 20% final), centrifuged at 8000*g*, and supernatant collected and filtered through a 0.22-μm centrifuge tube filter before injection onto an HPLC equipped with a Coulochem electrochemical detector (EC) (ESA Coulochem II 5020, Coulochem, Chelmsford, MA). The HPLC-EC employed a Shimadzu LC-10ADVP pump, Shimadzu SIL-10ADVP autoinjector (Columbia, MD) with a 50-μl sample loop, and an ESA Coulochem II detector with guard cell set at 350 mV, analytical cell set at 220 mV, and detector sensitivity of 500 nA. A reversed phase C-18 column (Waters Nucleosil, 5 μm, 4.6 × 250 mm, Milford, MA) was used with a guard column (Metaguard ODS-2 Inertsil, 5 μm, 4.6 mm; Metachem, Torrance, CA). The mobile phase consisted of 75 mM monobasic sodium phosphate, 1.7 mM 1-octanesulfonic acid, 720 μM triethylamine, and 25 μM EDTA in 7.5% acetonitrile/deionized water at pH 3.0. Quantification was accomplished by comparison of peak areas with a standard curve generated with authentic standards during each series of analysis. Protein concentration was determined using a Bio-Rad Kit (Cambridge, MA) protein assay with bovine serum albumin as the standard.

Immunohistochemistry and histochemistry. In separate experiments, control and experimental mice ($n = 3$/group) were treated with the MPTP protocol as described earlier. Frozen sections through the SN of mice, whose striatal DA concentrations were depleted to less than 30% of controls, were cut at 10 μm and mounted onto poly-L-lysine coated glass slides for histological and in situ hybridization studies.

In order to assess the extent of dopaminergic neuronal loss in the SN, sections were processed to reveal tyrosine hydroxylase (TH)-like immunoreactivity. Frozen sections were treated with 3.0% H_2O_2 in Tris–HCl buffer at room temperature for 30 min to remove endogenous peroxidase activity and were then incubated with a mouse monoclonal anti-TH (1:1000; Sigma) for 2 h at room temperature, followed by 1-h incubation with biotinylated secondary antibody. TH-like immunoreactivity was revealed by incubating the antibody complex with 3,3′-diaminobenzidine tetrachloride in the presence of H_2O_2. Adjacent sections were subjected to a Nissl stain with cresyl violet.

Total RNA extraction and mRNA amplification. The nigral and hypothalamic samples ($n = 6$ from control and experimental groups) were pooled. Pooling of samples was required because of the small

amount of RNA isolated from a single mouse SN (0.5–1 μg). In addition, pooling of tissue minimized interanimal variability. A disadvantage of this approach is that we will not be able to discern if the differences we find are due to a gene highly abundant in only a single mouse (i.e., in 1 of 6) or due to abundance in many of them (2 or more of 6). This issue will be addressed by in situ hybridization study (see below). A second risk is that some valuable gene could be identifiable in a majority of mice, but by sampling only six we could miss it. This scenario is highly unlikely. Specifically, if a fraction p of mice has an increased or decreased gene, the probability of selecting at least one mouse in six mice is: selection probability $= 1 - (1 - p)^6$. With $p = 0.9$ and 0.3 the selection probability is 0.99 and 0.88, respectively, i.e., we will be able to identify over 88% of genes that are present in only 30% of mice.

Total RNA was isolated from pooled nigral or hypothalamic samples with Trizol reagent (Invitrogen, Frederick, MD), and since total RNA amounts were not adequate even with pooling of samples for microarray experiments, mRNAs were amplified from pooled total RNA (ranging from 2 to 6 μg). We choose a procedure that optimizes amplification of low-abundance mRNA samples [35], which yields comparable expression profiles to those observed with conventional poly(A) RNA- or total-RNA probe labeling. The first-strand cDNA was prepared from 1 μg total RNA in 9 μl H_2O containing 1 μl (0.5 μg/μl) oligo(dT) (15)-T7 primer (5′-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T(15)-3′). Total RNA was denatured at 70 °C for 3 min, followed by the addition of 4 μl of 5× first-strand reaction buffer, 2 μl of 0.1 M dithiothreitol (DTT; Gibco-BRL), 2 μl of 10 mM dNTP, 1 μl (1 μg/μl) template switch primer (5′-AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG-3′) (Clontech, Palo Alto, CA), and 2 μl Superscript II (SSII) reverse transcriptase (RT) (Gibco-BRL, Shelton, CT). cDNA synthesis was completed at 42 °C for 1.5 h. Second strand (full-length double-stranded (ds) cDNA) was completed by adding 106 μl of DNase-free water, 15 μl Advantage PCR buffer (Clontech, Palo Alto, CA), 3 μl of 10 mM dNTP, 1 μl RNase-H (2 U/μl Promega), and 3 μl Advantage cDNA polymerase (Clontech). The following temperature cycle was used: 5 min at 37 °C for RNA digestion, 2 min at 94 °C for denaturation, 1 min at 65 °C for annealing, and 30 min at 75 °C for extension. Reactions were terminated by incubation in 7.5 μl of 1 M NaOH and 2 mM EDTA at 65 °C for 10 min. cDNA was extracted with phenol–chloroform–isoamyl (25:24:1) and precipitated in the presence of 0.1 μg linear acrylamide (0.1 μg/μl, Ambion, Austin, TX). cDNA was passed through a Bio-6 chromatography column (Bio-Rad), pre-washed with DEPC-treated H_2O, lyophilized, and re-suspended in 8 μl water. In vitro transcription was conducted at 37 °C for 5 h in a mixture containing 8 μl of purified full-length ds-cDNA, 2 μl of each 75 mM NTP (ATP, GTP, CTP, and UTP), 2 μl of 10× reaction buffer, and 2 μl of transcription enzyme mixture (T7 Megascript Kit 1334, Ambion). RNA recovery and removal of template DNA was achieved by Trizol purification. For the second round amplification, aliquots of 1 μg amplified RNA (aRNA) were reverse-transcribed into cDNA using 2 μg of random hexamer first heated to 70 °C for 3 min and then cooled to room temperature. To the sample, we added 4 μl of 5× first-strand buffer, 1 μg oligo(dT)-T7 primer, 2 μl of 0.1 M DTT, 2 μl of 10 mM dNTP, and 2 μl of SSII (to 22 μl with DEPC H_2O). Then, synthesis was continued at 42 °C for 1.5 h. The rest of the second round of RNA amplification was continued as above. The yield of amplification was about 80 μg for all the samples.

Microarray. We labeled aRNAs with either Cy3 or Cy5 for both control and experimental samples in an RT reaction employing 8 μg of random hexamer primer in the presence of Cy3- or Cy5-labeled dCTP (Amersham, Piscataway, NJ) and SSII (Gibco-BRL). Reaction products were purified with a Bio-6 chromatography column followed by Microcon concentration (purified and labeled cDNA target in 20 μl solution containing 2.6 μl of 20 SSC, 8 μg of poly(dA), 4 μg of yeast tRNA, and 10 μg of human Cot I DNA (Gibco-BRL)). Before hybridization, the mixture was heated to 99 °C for 2 min and then cooled to room temperature. At that point, 0.46 μl of 10% SDS was added.

The microarrays included 493 mouse cDNAs and 13 control cDNAs spotted on poly-L-lysine glass slides (CEL Associates, Perland, TX) using a custom-modified Hitachi Genetic Systems microarray printing robot. The complete list of these genes will be published in a separate manuscript [36]. The genes on this list are differentially expressed either by patients with mitochondrial disease [37] or by mice lacking the heart/muscle isoform of the adenine nucleotide translocator [38], i.e., all the genes included in the arrays are somehow related to mitochondrial dysfunction. The arrays were printed using a 32-pin print head with a spot size of 200 μm (approximately 33 pL volume per spot) and a center-to-center spot spacing of 260 μm. A humidity level of 80% was maintained during the printing of the arrays by a custom humidification system. After printing, the arrays were heated to 140 °C and immediately cross-linked with 80 mJ of ultraviolet energy (Stratalinker, Stratagene, LaJolla, CA). The arrays were then baked for 2 h at 70 °C. To block non-specific interactions on the arrays during hybridization, the slides were prehybridized for 45 min with 5× SSC, 1% bovine serum albumin, and 1% SDS. Following prehybridization, the slides were rinsed with water followed by isopropanol and dried at room temperature. After drying, the microarrays were immediately hybridized with aRNAs labeled either with Cy3 or Cy5. Hybridization was carried out at 65 °C for 12–18 h in a waterbath. Before scanning, slides were washed to a final stringency of 0.05× SSC at room temperature.

All scanned arrays were saved as 16-bit TIFF files and analyzed using GenePix Pro software (Axon Instrument, Union City, CA). Local background was calculated for each individual spot and any spot with signal intensity less than two times over background or that had poor morphology was excluded from the data analysis. In addition, as a further quality control, each experimental sample was labeled with Cy3 and Cy5, and compared with control samples labeled with Cy5 and Cy3, respectively. A change (up or down) in gene expression was not accepted as significant unless two pairs of comparison gave consistent results. In other words, in the duplicates, a 40% increase in experimental-Cy3/control-Cy5 ratio should correspond to a 40% decrease in control-Cy3/experimental-Cy5 ratio.

In situ hybridization. In order to confirm the results obtained from microarray experiments, in situ hybridization was performed using a method described previously in a similar microarray study [25] to determine relative abundance of those genes that were categorized as differentially expressed in the SN but not in the hypothalamus. All clones were confirmed by sequence analysis using specific primers (Table 1). The specific PCR products were cloned into pSPT18 (Roche, Nutley, NJ) except for clone 1431493 (the glutamate receptor GluR2 subunit), which was cloned into pSPT18 directly after confirmation of the gene sequence. After linearization, antisense RNA was transcribed with a digoxygenin (DIG)-RNA Labeling Kit (SP6/T7, Roche) using the protocol included.

Tissue sections were treated first with 0.2 M HCl to neutralize endogenous alkaline phosphatase, and then with 2 μg/ml proteinase K for 15 min, rinsed with 2 mg/ml glycine, and acetylated in 0.5 ml/L acetic anhydride in 0.1 M Tris buffer (pH 8.0). Finally, sections were post fixed in 4% paraformaldehyde in PBS and prehybridized for 10 min in 1× SSC followed by 1 h in hybridization buffer containing 4× SSC, 50% formamide, 1× Denhardt's solution, and 0.5 ng/ml salmon sperm DNA. Hybridization was carried out overnight at 42 °C in maximal humidity with 5 ng/μl DIG-labeled antisense probes. The next day, slides were washed to a final stringency of 0.1× SSC at 52 °C and then with 0.1 M Tris–HCl and 0.15 M NaCl (pH 7.5). Hybrids were detected using anti-DIG antibodies (Roche) conjugated with alkaline phosphatase and NBT-BCIP as substrate. As a control, sections were hybridized with sense probes. To minimize variations among different runs of in situ hybridization, each probe was hybridized in one single experiment including every control and experimental cases.

Table 1
Genes regulated by MPTP treatment only in substantia nigra and primers used for gene characterization

Genes	Microarray changes	Primer sequences
NADH–ubiquinone oxidoreductase 13 kDa B subunit	⇓ Day 3	F TAATACGACTCACTATAGGG (T7) R TGAAGCTTCCATCTGACACTGAGGTCA
Cytochrome *c*	⇓ Day 3	F TAATACGACTCACTATAGGG (T7) R ACAAGCTTCTCCTGGTGGTTAGCCATGA
Dihydropyridine-sensitive L-type calcium channel α-2 subunit precursor	⇓ Day 3	F TAATACGACTCACTATAGGG (T7) R TTAAGCTTCTGACATGCAGCCAGCGATG
Type III α-1 procollagen	⇓ Day 18	F TAATACGACTCACTATAGGG (T7) R CGAAGCTTAGCACCATTGAGACATTTTG
Cytochrome *c* oxidase Va subunit	⇑ Day 3	F CTTCTGCTCTAAAAGCTGCG(5′) R ATAAGCTTTTGTCAAGGCCCAGCTCCTC
NADH–ubiquinone oxidoreductase MNLL subunit	⇑ Day 3	F TAATACGACTCACTATAGGG (T7) R CCAAGCTTATTCCTGAAGGCAGTTAGC
Glutamate receptor GluR2 subunit	⇑ Day 18	F TAATACGACTCACTATAGGG (T7)

Cloning sites for all genes were *Eco*RI and *Hin*dIII. A ratio of signal intensities of treatment vs. respective control that is greater than 1.4 or less than 0.7 is considered as significant.

Quantification of in situ hybridization. In order to quantify expression of transcripts as revealed by in situ hybridization, the somata of all neurons in the pars compacta were outlined manually, using Scion Image (Frederick, MD). Next, optical density was obtained for each cell and the average of the mean densities (AMD) was calculated for the SN pars compacta of each mouse. Of note, only cells with grain density greater than two times of background at high magnification were included, and all values were corrected by subtracting the level of background staining determined as the average of three fields taken from the neuropil in or adjacent to the pars compacta.

Statistics. All data were analyzed with commercially available software, GraphPad (GraphPad, San Diego, CA). Student's *t* test was used to analyze DA concentrations in the striatum of mice, whereas one-way ANOVA with Bonferroni's correction test was used for comparison of the hybridization signals of each probe, i.e., AMD, in the SN between control and treated animals.

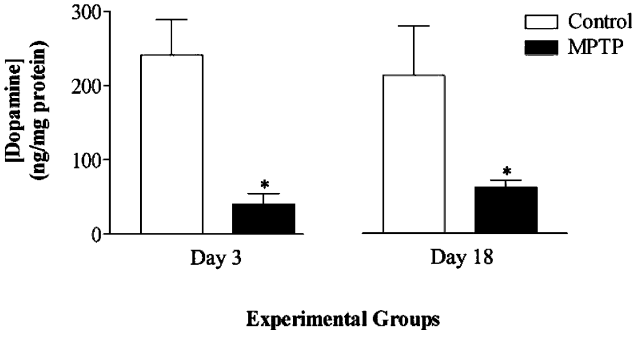

Fig. 1. Effects of MPTP on striatal DA concentrations. Striatal DA concentrations were significantly depleted at both day 3 and day 18 after two i.p. treatments with 26.5 mg/kg of MPTP. $n = 6$ for each group. $^*P < 0.01$.

Results

Effects of MPTP on striatal DA concentrations and nigral neurons

MPTP-treatment significantly decreased striatal DA concentrations at both three days (17% of control) and 18 days (29% of control) after treatment (Fig. 1). There was no statistical difference in striatal DA concentrations of mice treated with vehicles between 3 days and 18 days post-treatment. There results were very similar to those that we have published previously [29].

Despite the large decrease in striatal DA in MPTP-treated mice, there did not appear to be any gross decrease in the number of dopaminergic perikarya in the SN, as revealed by Nissl and TH staining, although the intensity of TH staining appeared somewhat decreased in the dendrites of the dopaminergic neurons (Fig. 2). We did not count cells in the SN with stereologic technique in this study; however, the impression that there was no decrease in the numbers of dopaminergic neurons was confirmed by counting the numbers of mRNA-expressing cells in the SN pars compacta.

Changes in gene expression induced by MPTP

A set of 493 mouse cDNAs related to mitochondrial function was used for hybridization. Alterations in mRNA expression in response to MPTP treatment at 3 or 18 days for each gene were determined by the ratio of signal intensities of treatment vs. respective control. We did not observe any increase or decrease in the level of gene expression that was greater than 2-fold over controls, i.e., a ratio of 2 or 0.5, at either 3 or 18 days after

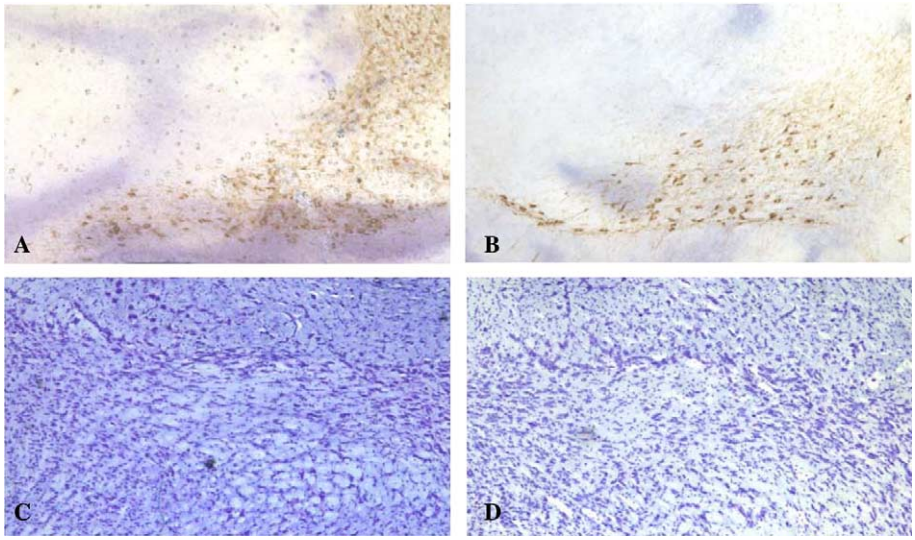

Fig. 2. Photomicrograph of immunohistochemical studies. TH-immunohistochemistry cell bodies and fibers within the substantia nigra of a control mouse (A) and an MPTP-treated mouse 18 days post-treatment (B). Nissl stains of the substantia nigra of a control mouse (C) and an MPTP-treated mouse 18 days post-treatment (D).

MPTP treatment. Accepting the fact that there is no magic absolute cut-off threshold for a meaningful interpretation of low signals in microarray expression profiling, we adopted an arbitrary value used by others [25,39,40] to consider genes with more than 40% changes over controls, i.e., a ratio of 1.4 or 0.7, as significantly differentially expressed as the first step of analysis. With this criterion, MPTP treatment resulted in changes in seven genes in the SN but not in the hypothalamus (Table 1), 11 genes in the hypothalamus but not in the SN (Table 2), and two genes in both hypothalamus and SN (Table 3) at respective time points.

In theory, the genes of interest identified by the microarray method need to be verified with an independent method, regardless of how many independent samples are studied. But practically, investigators usually only confirm a few genes of their choice, depending on the research interest. Similarly, rather than doing this for all 20 genes, we focused on the seven genes that had altered expression in the SN and not hypothalamus by MPTP, given that nigral neurons are selectively vulnerable to MPTP-induced toxicity. Among these seven genes, four were decreased by MPTP treatment: the NADH–ubiquinone oxidoreductase 13 kDa B subunit, cytochrome *c* and the dihydropyridine-sensitive L-type calcium channel α-2 subunit precursor at day 3 post-treatment, and type III α-1 procollagen at day 18 post-treatment. The other three genes, the cytochrome oxidase Va subunit and the NADH–ubiquinone oxidoreductase MNLL subunit at day 3 post-treatment and the glutamate receptor GluR2 subunit at day 18 post-treatment, were increased by MPTP treatment.

Table 2
Genes regulated by MPTP treatment only in hypothalamus

Genes	Microarray changes
Glucocorticoid-induced leucine zipper	⇑ Day 3
Procollagen type 1	⇑ Day 18
DD 48*	⇑ Day 18
Mouse creatine kinase	⇑ Day 18
mtDNA-NADH 4L	⇑ Day 18
mtDNA-NADH 5	⇑ Day 18
CCAAT/enhancer binding protein (C/EBP)	⇑ Day 18
Alkaline phosphatase	⇑ Day 18
NADH–ubiquinone oxidoreductase MNLL subunit	⇑ Day 18
mtDNA-ATPase 8	⇓ Day 3
Ubiquitin	⇓ Day 18

* Differential display (DD) results from a previous study [38]. A ratio of signal intensities of treatment vs. respective control that is greater than 1.4 or less than 0.7 is considered as significant.

Table 3
Genes regulated by MPTP treatment in both hypothalamus and substantia nigra at the same time

Genes	Microarray changes
Glucocorticoid-induced leucine	⇑ Day 3 and day 18
Procollagen type I	⇑ Day 3

A ratio of signal intensities of treatment vs. respective control that is greater than 1.4 or less than 0.7 is considered as significant.

In situ hybridization analysis

In situ hybridization histochemistry was performed with specific antisense probes and sense probes were used as negative controls. The results demonstrated that all seven genes showed diffused somal expression without restriction to any brain regions, i.e., positive signals were mainly located in the cytoplasm of neurons whether examined in the region of cortex, hippocampus, or

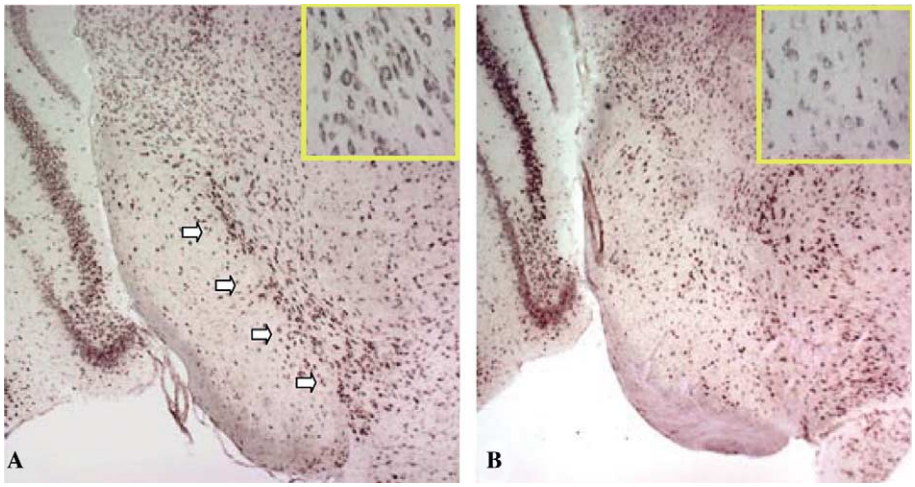

Fig. 3. In situ hybridization with the complex I 13 kDa B subunit. (A) Control, (B) mouse treated with MPTP at day 3 post-treatment. The area of substantia nigra pars compacta is indicated with arrows. Expression of the complex I 13 kDa B subunit decreased significantly (B at low magnification) even though most cells were still preserved in the SN of the mouse treated with MPTP (see the inset in B compared to A).

SN. The representative images are illustrated in Fig. 3. To quantify changes in hybridization signals, AMD of total neurons was calculated in the SN pars compacta for each mouse. The number of neurons analyzed for each probe was obtained at two comparable levels of the SN pars compacta in each mouse from both control and experimental groups. Statistical analysis indicated that an increase or decrease in AMD in the nigral neurons was entirely consistent with the results obtained with microarray studies in six out of seven genes (Table 4). Gene expression of the GluR2 subunit, which increased significantly at day 18 after MPTP treatment as determined by microarray analysis, was the only inconsistent example that did not show significant increase in hybridization signal (AMD) in the SN of treated mice compared to controls.

Discussion

The major finding emerging from this work was that six genes were altered by MPTP treatment in neurons of the SN. Four of these six genes encode components of the mitochondrial electron transport chain: the NADH–ubiquinone oxidoreductase 13 kDa B subunit, the NADH–ubiquinone oxidoreductase MNLL subunit, cytochrome *c*, and the cytochrome *c* oxidase Va subunit. The other two genes were type III α-1 procollagen and the dihydropyridine-sensitive L-type calcium channel α-2 subunit precursor. None of these genes are derived from mtDNA.

NADH:ubiquinone oxidoreductase (complex I) consists of more than 42 subunits [41]. Seven are encoded by mtDNA, whereas the remaining subunits, including the two altered by MPTP treatment in this study, are nuclear gene products imported into mitochondria from the cytoplasm [42]. The precise roles of the mitochondrial complex I MNLL subunit, one of the up-regulated genes, are not well understood. However, the down-regulated 13 kDa B subunit of complex I appears to be critical in multiple cellular functions. For instance, decreased expression of this subunit is associated with incomplete or incorrect assembly of a functional complex I, leading to a mitochondrial disease with myopathy

Table 4
Comparison between average of mean densities of antisense probes in substantia nigra and microarray changes following MPTP treatment

Genes	Control	MPTP	Microarray changes
NADH–ubiquinone oxidoreductase 13 kDa B subunit	85.01 ± 9.69 (449)	49.78 ± 10.68* (380)	⇓ Day 3
Cytochrome *c*	103.30 ± 11.94 (231)	58.26 ± 11.94* (308)	⇓ Day 3
Dihydropyridine-sensitive L-type calcium channel α-2 subunit precursor	50.69 ± 11.92 (361)	24.59 ± 9.66* (335)	⇓ Day 3
Type III α-1 procollagen	77.18 ± 14.49 (188)	30.96 ± 9.56* (273)	⇓ Day 18
Cytochrome oxidase Va subunit	46.93 ± 12.58 (334)	103.60 ± 13.28* (284)	⇑ Day 3
NADH–ubiquinone oxidoreductase MNLL subunit	28.02 ± 9.23 (304)	60.88 ± 11.03* (319)	⇑ Day 3
Glutamate receptor GluR2 subunit	44.13 ± 8.68 (136)	44.40 ± 7.68 (170)	⇑ Day 18

Parentheses indicate the number of neurons counted from comparable sections of control and experimental groups for each gene. Of note, the total number of neurons in the SN of mice treated with MPTP were not statistically different from those of controls; variations in cell counts with different probes were due to different anatomical levels of SN that were used *$P < 0.0001$ (one-way ANOVA with Bonferroni's correction).

[43]. This is not surprising, given that complex I is one of the most important components in the production of ATP. In addition, overexpression of this subunit has been hypothesized to inhibit lipid peroxidation and biological tissue damage [44]. Consistent with this argument, the expression of this gene was increased in the hypothalamus (Table 2), a region relatively resistant to MPTP-induced toxicity, 18 days post-treatment. Finally, the complex I 13 kDa B subunit may have functions in addition to oxidoreductase activity and proton translocation, as does the complex I GRIM-19 subunit, which is a cell-death regulatory gene induced by interferon-β and retinoic acid [41].

MPTP treatment also down-regulated cytochrome *c* expression at day 3 post-treatment. Cytochrome *c* plays an integral role in transfer of electrons between complex III and complex IV. Accordingly, decreased expression of this molecule may further inhibit mitochondrial function and trigger production of reactive oxygen species (ROS) [45]. However, the function of cytochrome *c* is not limited to production of ATP and ROS. For example, release of cytochrome *c* from mitochondria initiates apoptosis [46] and a recent study suggests that cytochrome *c* facilitates α-synuclein aggregation [47].

Cytochrome *c* oxidase (complex IV) is composed of three mitochondrial-encoded polypeptides and 10 nuclear-encoded polypeptides [48,49]. Some nuclear-encoded gene products such as the Va subunit are environmentally regulated, including by cellular oxygen concentrations [50]. Synthesis of ATP in higher organisms alternates between two states of energy metabolism, i.e., a relaxed state with low ROS formation and an excited state with elevated formation of ROS [51]. The regulation of these two states is accomplished by iodothyronine metabolites acting on the nuclear-encoded Va subunit. Binding of iodothyronine metabolites to the nuclear-encoded Va subunit abolishes allosteric ATP inhibition of cytochrome *c* oxidase, which typically occurs at high intramitochondrial ATP/ADP ratios, i.e., in the relaxed state [52]. Hence, MPTP-induced up-regulation of this gene may reflect a higher demand on ATP production secondary to mitochondrial inhibition by MPP^+.

The voltage-dependent calcium channel α-2 subunit (also called α2δ subunit) is part of both muscle L-type [53] and neuronal N-type Ca^{2+} channels [54]. The role of decreased expression of this gene in MPTP treated mice remains to be elucidated.

The type III procollagen α-1 subunit gene encodes the constitutive chains of type III procollagen, a major component of the blood vessels, bowel, skin, and hollow organs [55]. As a part of the extracellular matrix, procollagen has been implicated in modulating cell death, apoptosis in particular [56]. However, the type III procollagen α-1 subunit gene has received no attention in the context of the central nervous system and thus warrants further study, particularly with regard to the intriguing possibility of its functioning in repair mechanisms.

The only discrepancy between the microarray and in situ studies was with GluR2 subunit expression. The GluR2 subunit is part of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor, which is associated with glutamate-mediated excitotoxicity [57]; the functional properties of the AMPA receptor depend upon its subunit composition, since its Ca^{2+} permeability is mainly determined or even dominated by the presence of GluR2 [58,59]. The fact that increased expression of the GluR2 subunit gene was not observed with in situ studies reemphasizes the need for confirmatory studies when gene array is used as a discovery tool.

The hypothalamus contains several distinct DA cell groups (A11–A14), all of which are generally considered to be relatively resistant to MPTP-induced toxicity. Three out of 11 differentially regulated genes in this region, i.e., ATPase 8, and the NADH 4L and NADH 5 subunits are encoded by mtDNA. Expression of both the NADH 4L and NADH 5 subunits, components of complex I, went up at day 18 post-treatment, whereas expression of ATPase 8 went down at day 3 post-treatment. In light of possible involvement of mtDNA in PD [3,15,16,60], these genes warrant further studies in the future.

Finally, the microarray panel used in this study, though directly relevant to PD pathology, does not encompass all gene alterations induced by MPTP challenge. It is important to note that the genes spotted in our microarray panel only partially overlap with those spotted on mouse Atlas gene arrays used by others [24,25], and none of the genes regulated significantly in our experiment is included in their arrays (http://www.clontech.com/atlas/genelists/7741-1_Mo.pdf). On the other hand, one of the up-regulated genes in Grunblatt's study [25], BAX membrane isoform α, was included in our array panel, but it did not change significantly with MPTP treatment as compared to controls. This discrepancy may have resulted from differences in the dosage of MPTP used and/or brain regions dissected for the gene array studies.

In conclusion, our cDNA array study has discovered significant alterations in several genes that are related to mitochondrial function, which is directly related to the underlying pathology of PD. Further characterization of these gene products may lead to a better understanding of the pathology of PD and development of more specific and effective drugs to treat this disease.

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