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| <b>13. ABSTRACT (Maximum 200 Words)</b><br>The selective neurotoxins 1-methyl-4-phenylpyridinium (MPP <sup>+</sup> ) and 6-hydroxydopamine (6-OHDA) have been widely used to generate animal models of Parkinson's disease (PD). To understand the genetic events associated with these neurotoxins, microarray technology served to monitor differences in gene expression patterns in normal versus pathological conditions. Microarray analysis of RNA isolated from toxin treated samples revealed that the stress induced transcription factor CHOP was dramatically up regulated by both toxins. 6-OHDA also induced a large number of genes involved in endoplasmic reticulum (ER) stress and unfolded protein response (UPR) such as ER chaperones and elements of the ubiquitin-proteasome system. RT-PCR, Western blotting, and immunocytochemical approaches were used to quantify and temporarily order the UPR pathways involved in neurotoxin-induced cell death. 6-OHDA, but not MPP <sup>+</sup> , significantly increased hallmarks of UPR such as BiP, c-jun, and processed Xbp1 mRNA. Both toxins increased the phosphorylation of UPR proteins, PERK and eIF2 $\alpha$ , but only 6-OHDA increased phosphorylation of c-jun. Thus, 6-OHDA triggers multiple pathways associated with UPR, whereas MPP <sup>+</sup> exhibits a more restricted response. 6-OHDA induced similar responses in primary dopaminergic neurons. These experiments will help clarify the molecular mechanisms associated with 6-OHDA and MPP <sup>+</sup> toxicity and might aid in developing novel therapeutic avenues relevant to PD. |   |  |  |  |
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## Introduction

Accumulating evidence suggests that Endoplasmic Reticulum (ER) stress/Unfolded Protein Response (UPR)-mediated cell death plays a role in Parkinson's disease (PD) based on genetic, pharmacological and environmental factors. For example,  $\alpha$ -synuclein, the major component of Lewy bodies (Spillantini et al., 1997), is associated with protein aggregation and proteosomal dysfunction (Betarbet et al., 2002). Additionally, Parkin, the protein associated with autosomal recessive juvenile Parkinsonism (AR-JP; Kitada et al., 1998) has been shown to be an E3 ubiquitin-protein ligase (Shimura et al., 2000). Reports that Parkin substrates misfold, aggregate, and trigger ER stress/UPR suggest that Parkin activity prevents the accumulation of misfolded proteins (Imai et al., 2001; Tsai et al., 2003). The role of proteosomal impairment has been further emphasized by recent findings that pharmacological inhibition of proteasome function leads to selective degeneration of dopaminergic neurons in culture (McNaught et al., 2002a) as well as in vivo (McNaught et al., 2002b). Finally, recent studies from this lab (Holtz and O'Malley, 2003) as well as others (Ryu et al., 2002) have linked oxidative stress, a well-documented factor in PD, with ER stress/UPR as well.

Beginning with a functional genomics approach to identify transcriptional alterations in a well-characterized model of 6-OHDA and MPP<sup>+</sup> toxicity, studies conducted under the auspices of this grant identified numerous changes in genes associated with ER stress/UPR (Holtz and O'Malley, 2003). Reverse transcription/PCR amplification, western blots and immunocytochemistry were used to verify changes in selected subsets of differentially regulated transcripts. Selected transcripts were also tested for toxin-induced changes in primary cultured dopaminergic neurons. Just as studies in other model systems have uncovered novel signaling pathways, these experiments are also revealing unanticipated pathways that contribute to MPP<sup>+</sup> and 6-OHDA neurotoxicity. Taken together, these and other findings support the theory that proteosomal dysfunction with ensuing ER stress/UPR contribute to PD.

## Body

### A. Does the neurotoxin MPP<sup>+</sup> differentially regulate sets of genes?

To test the hypothesis that MPP<sup>+</sup> alters gene transcription as part of its neurotoxic program, a time course study using cycloheximide to block MPP<sup>+</sup> toxicity, was performed as previously described. Briefly, cells were treated with 50  $\mu$ M MPP<sup>+</sup> with 10  $\mu$ M cycloheximide being added for varying periods of time. The point at which about 50 % of the cells were rescued by blocking protein synthesis, 9 hours following MPP<sup>+</sup> treatment, was chosen as the best time point at which to harvest RNA.

In consultation with experts from our onsite Affymetrix gene chip core facility, we subsequently designed our experiments such that a minimum of 3 separate experiments were performed in which cells were treated with MPP<sup>+</sup> for 9 hours and then harvested for RNA preparation at that time point. Cell death was verified in each case by independent experiments done on sibling cultures. RNAs from all three experiments were pooled to form an RNA resource that would minimize experimental variation.

RNA sample preparation was done according to protocols devised by Affymetrix to achieve the best results, particularly for mammalian cells. Specific details of preparation and hybridization

were described previously and are detailed in the attached manuscript (Holtz and O'Malley, 2003).

The data obtained for MPP<sup>+</sup> are compiled values from three separate experiments done in triplicate as described above. The expression level of each probe set was plotted to determine the reproducibility of the array-based hybridization signals and to compare gene expression levels by MN9D cells treated with and without MPP<sup>+</sup>. The ratio of gene intensity in toxin-treated cells to that in control samples was used to represent the toxin-mediated induction. The reciprocal ratio represented repression. Genes were considered up or down-regulated if the fold change was at least 2.0 in individual experiments as well as in averaged, triplicated experiments. These limits are in general agreement with most gene chip experiments.

Out of the approximately 12,000 genes and ESTs represented on the MG-U74Av2 GeneChip, 4,304 (~35% of total) were defined as "present" by the microarray analysis software for MPP<sup>+</sup>-treated samples. Transcripts were subsequently grouped by individual toxin treatment or by both 6-OHDA and MPP<sup>+</sup>. As indicated in Table 1, only 59 transcripts increased in response to MPP<sup>+</sup>. Results for decreasing transcripts were somewhat less (Table 2). Both neurotoxins induced a number of the same transcripts, with 43 of the 59 transcripts induced by MPP<sup>+</sup> also induced by 6-OHDA (Table 3). These included genes involved in cell cycle and/or differentiation, signaling, stress, and transcription factors, indicating possible common cell death mechanisms. The most highly induced transcript in response to either treatment was that to the stress protein CHOP/Gadd153. These results support previous findings showing that MPP<sup>+</sup> and 6-OHDA promote distinct yet overlapping programs of cell death.

As described in more detail below (C; Holtz and O'Malley, 2003), MPP<sup>+</sup> appears to specifically induce one arm of the ER stress/Unfolded Protein Response (UPR)-mediated cell death pathway. Interestingly, time course data generated for pathway constituents indicated that between 1-3 hours something occurs in MPP<sup>+</sup> treated cells that leads to a decline in the ER stress/UPR markers (see Figs 4-5, attached publication Holtz and O'Malley, 2003). As part of the original research proposal and in order to determine whether transcriptional changes are mediating this effect, we have now prepared MPP<sup>+</sup>-treated RNA at 1 and 6 hours exactly as previously described. Aliquots of the pooled RNA from these experiments are queued at the Washington University School of Medicine DNA array facility. We should receive these new data in the next few weeks. The additional information will allow us to analyze MPP<sup>+</sup>-mediated transcriptional changes in significantly more depth than our original single time point has allowed.

## **B. Does the neurotoxin 6-OHDA differentially regulate sets of genes?**

To test the hypothesis that 6-OHDA neurotoxicity alters fundamental patterns of gene expression, experiments were conducted exactly as described above for MPP<sup>+</sup>. Out of the approximately 12,000 genes and ESTs represented on the GeneChip, 4,580 (~37% of total) were defined as present for 6-OHDA-treated samples. Notably, 6-OHDA treatment affected almost three times as many transcripts as MPP<sup>+</sup>. Specifically, 157 transcripts increased in response to 6-OHDA (Table 4) and 41 decreased (Table 5). As described, the most highly induced transcript in response to either treatment was that to CHOP. 6-OHDA also induced a large number of transcripts that were unchanged by MPP<sup>+</sup> treatment, including molecular chaperones and other genes involved in protein folding, trafficking, and the ubiquitin-proteasome pathway (Table 3).

Because 6-OHDA appeared to induce apoptosis in this model system as well as in primary cultured neurons (Oh et al., 1995; Lotharius et al., 1999; Choi et al., 1999), we anticipated the identification of functional clusters of neurotoxin-responsive genes that would overlap with apoptotic patterns observed in other models. Surprisingly, however, many of the genes that were up regulated were again members of the ER stress/UPR cell death pathway. Indeed, Chop induction was even more pronounced in 6-OHDA treated cells than in MPP<sup>+</sup> (Table 3).

Currently, we have also prepared RNA from MN9D cells treated with 6-OHDA for 1 and 6 hours. These samples, like those for the MPP<sup>+</sup>-treated RNA pools are awaiting processing at our DNA array facility. The wealth of new information being generated with further our attempts to order and delineate 6-OHDA mediated cell death pathways.

### C. Verification in MN9D Cells

To verify induction or repression by an independent method, a subset of the most interesting differentially regulated genes were examined by RT/PCR, Western blotting and immunocytochemical approaches. These methodologies allowed us to quantitate and temporally order the ER stress/UPR pathways involved in neurotoxin-induced cell death. As detailed in the attached publication (Holtz and O'Malley, 2003), 6-OHDA, but not MPP<sup>+</sup>, significantly increased hallmarks of UPR such as BiP, c-jun, and processed Xbp1 mRNA. Both toxins increased the phosphorylation of UPR proteins, PERK and eIF2 $\alpha$ , but only 6-OHDA increased phosphorylation of c-jun. Thus, 6-OHDA is capable of triggering multiple pathways associated with UPR, whereas MPP<sup>+</sup> exhibits a more restricted response. These results allowed us to derive a working model (Fig. 1) from which we can test further hypotheses.

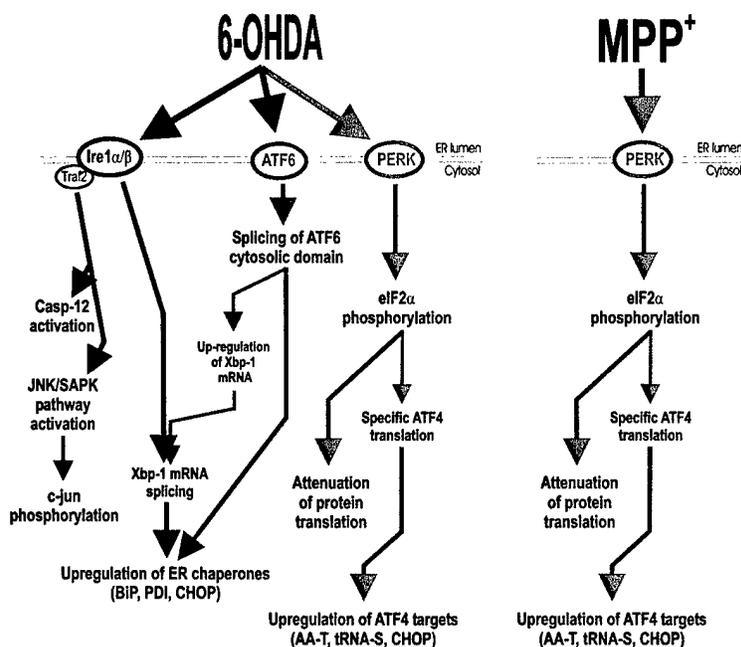


Fig. 1. 6-OHDA induces multiple targets of UPR, while MPP<sup>+</sup> is restricted to the PERK pathway. The mammalian UPR consists of three ER membrane resident proteins (Ire1 $\alpha/\beta$ , ATF6, and PERK) that sense ER stress and activate the UPR pathway resulting in transcriptional changes and attenuation of protein translation. The current studies demonstrate that 6-OHDA induces all three arms of the UPR leading ultimately to the transcriptional changes first identified by microarray analysis. In contrast, MPP<sup>+</sup> is restricted to phosphorylation of PERK and eIF2 $\alpha$ , resulting in up-regulation of a subset of genes induced by 6-OHDA (Holtz and O'Malley, 2003).

To aid in analyzing additional transcriptional changes generated from the 9-hour time point and our anticipated new data from the one and 6-hour points, we have developed protocols to analyze transcript levels using real time PCR. As an example, new primers for CHOP cDNA were prepared and used to reverse-transcribe total RNA from MN9D cells exposed to 6-OHDA or MPP<sup>+</sup> for 0, 1, 3, 6, 9, or 12 hours. This cDNA was then analyzed using real time PCR. cDNA from the constitutively transcribed GAPDH gene was also analyzed to normalize the

CHOP values. The resulting data were used to determine the relative-fold induction of CHOP as a function of time exposed to 6-OHDA or MPP<sup>+</sup>. As shown in Fig. 2, the real time PCR results verified previous data indicating that CHOP is induced up to 6 and 8 fold by MPP<sup>+</sup> and 6-OHDA, respectively.

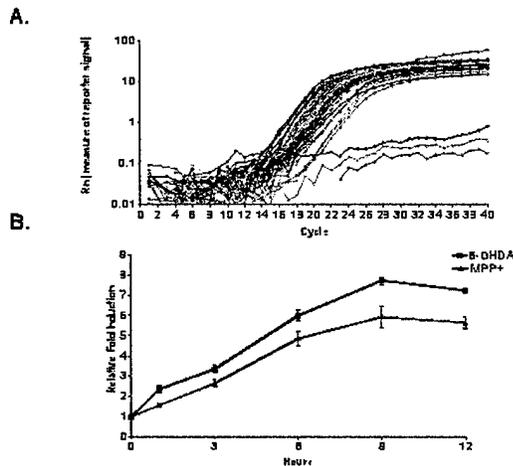


Fig. 2. Real time PCR confirms microarray results: COP is upregulated by MPP<sup>+</sup> and 6-OHDA.

elf2 $\alpha$  and c-jun. In contrast, none of the markers seen in the dopaminergic cell line were up regulated in mesencephalic cultures treated with MPP<sup>+</sup>.

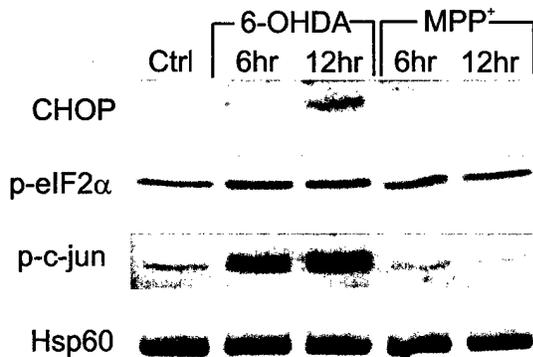


Fig. 3. 6-OHDA up-regulates CHOP in primary mesencephalic neurons. Protein lysates were prepared from primary mesencephalic cultures treated with 6-OHDA and MPP<sup>+</sup>. Western blot analysis of primary lysates was done using antibodies against CHOP, phosphorylated eIF2 $\alpha$  (p-eIF2 $\alpha$ ), phosphorylated c-jun (p-c-jun), and Hsp60 as a protein loading control.

#### D. Are neurotoxin-mediated changes in gene expression recapitulated in cultured dopaminergic neurons?

To confirm and extend results obtained using the dopaminergic cell line model, we are using primary cultures of dopaminergic neurons. The advantages of using this paradigm include the ease of preparation and culture manipulation and the well-documented similarity in responses (Oh et al., 1995; Lotharius et al., 1999; Holtz and O'Malley, 2003). To determine whether UPR induction could be observed in mesencephalic cultures following neurotoxin treatment, Western blot analysis and immunocytochemistry were performed. Similar to results from the dopaminergic MN9D cells, 6-OHDA increased levels of CHOP protein at 6 and 12 hours (Fig. 3). 6-OHDA also increased phosphorylation of

Immunostaining of primary cultures with CHOP and phospho-c-jun antibodies allowed individual dopaminergic neurons to be examined via co-staining with TH. 6-OHDA treated cultures displayed intense nuclear staining of CHOP in both dopaminergic neurons as well as in many other cell types (Fig. 4). Cultures treated with MPP<sup>+</sup> did not appear different from controls in overall expression of CHOP, nor was CHOP induction detected in dopaminergic neurons over a 24-hour period. Similarly, increased expression of phospho-c-jun was widespread with 6-OHDA treatment in both dopaminergic and non-dopaminergic neurons, whereas there was no obvious change in phosphorylation of c-jun following MPP<sup>+</sup> administration. Taken together, these results suggest that MPP<sup>+</sup> can induce a partial UPR response in the MN9D cell line but not in cultured dopaminergic neurons. In contrast, 6-OHDA induces a broad spectrum of UPR

responses in both MN9D cells as well as in dissociated dopaminergic neurons. Thus, these cells will serve as a useful model in determining the temporal and molecular events associated with 6-OHDA neurotoxicity. As new data become available from our additional microarray

experiments, we will continue screening both the MN9D cells as well as our primary culture model to confirm and extend these results.

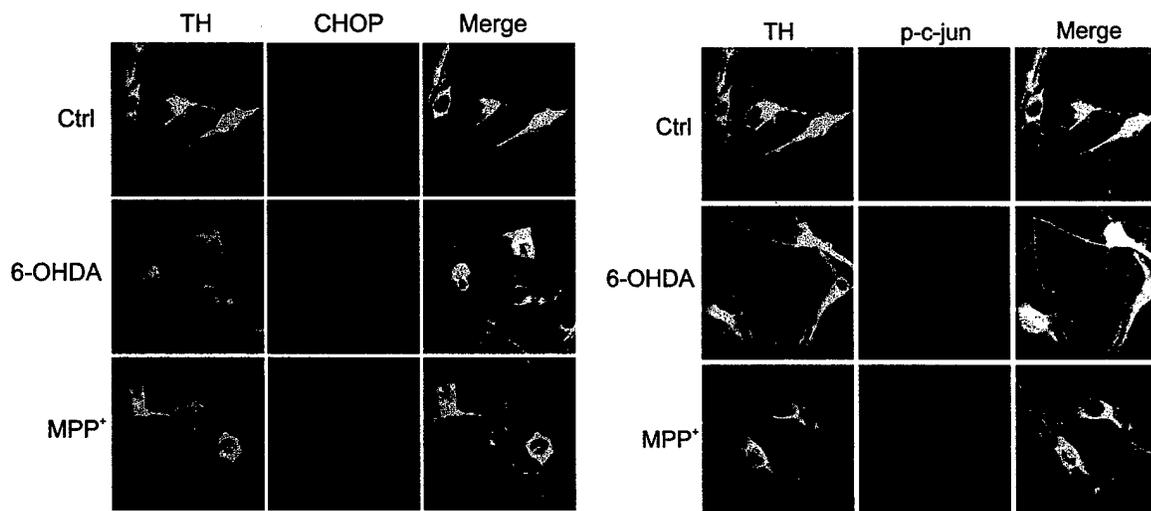


Fig. 4. 6-OHDA up-regulates CHOP and p-c-jun in dissociated dopaminergic neurons. Primary cultures treated for 18 hours were fixed and stained for CHOP and TH. Primary cultures treated for 12 hours were fixed and stained for phospho-c-jun and TH.

What are the signaling pathways involved in this response? In contrast to the well-delineated mitochondrial-mediated cell death pathways, ER stress/UPR signaling cascades are still unclear. Because identification of the initiators of this process will allow new therapeutic avenues to be pursued, we have used our microarray data to identify molecules that might be involved in this response. Potential candidates include the cysteinyl aspartate proteinases (caspases) that mediate programmed cell death and/or the so-called BH3-only proteins that affect many cellular processes to trigger cell death responses.

Inasmuch as caspase 12 specifically localizes to ER membranes and has been shown to be cleaved in the course of ER stress/UPR mediated cell death (Nakagawa et al., 2000), caspase 12 is a prime candidate for being the "ER stress mediator". To test this hypothesis we looked for evidence of caspase 12 involvement in our model. Surprisingly, antibodies that easily recognized caspase 12 activation in control cells were unable to detect similarly sized proteins in either our dopaminergic cell line or primary culture model. Moreover, our microarray results suggested that caspase 12 transcripts were not present nor could we directly amplify caspase 12 fragments using RT/PCR. Finally pre-treatment with inhibitors of the caspase 12 activators, calpains (I, II) failed to prevent CHOP or caspase 3 activation. Therefore, it would not appear that caspase 12 is triggering neurotoxin-mediated dopaminergic cell death.

As indicated in Table 6, only caspase 2,3,7, and 9 are expressed in the MN9D cells. Because very recent studies have shown that caspase 2 may serve as an initiating caspase particularly in models that also involve ER stress/UPR (e.g.  $\beta$ -amyloid toxicity; Troy and Shelanski, 2003), we screened our dopaminergic model for the presence and activation of caspase 2. Briefly, no evidence of caspase 2 activation was observed using real-time PCR, Western blotting techniques, activity assays, and/or caspase 2 inhibitors. Thus it would appear that caspase 2 does not initiate cell death in this system. Previously we've shown caspase 3 activation beginning around 6 hours after 6-OHDA treatment (Jensen et al., 2003). Caspase 7 and 9 are also activated within that time frame (not shown).

The results described above have re-focused our attention on the BH3-only proteins. Previously, we have determined that pro-apoptotic Bax is not involved since Bax deficient animals do not rescue primary dopaminergic neurons from 6-OHDA or MPP<sup>+</sup>-mediated cell death (O'Malley et al., 2003). Similarly we've ruled out Bad and Bak (not published). However, this is a large family of proteins and new ones are discovered with regularity. We are in the process of systematically testing every known BH3-only protein as a possible mediator of this response. For example, BH3-only proteins, Bim and Bid are present in MN9D cells although their transcript levels do not change following 6-OHDA or MPP<sup>+</sup> treatment (Table 6), nor do their protein levels (not shown). Another BH3-only protein recently shown to mediate ER stress/UPR cell death is Bbc3/PUMA (Reimertz et al., 2003). Because this gene is not on the microarray that we originally screened, we're using PCR based methodologies to determine whether it is present in MN9D cells and/or primary mesencephalic cultures and whether it is induced in response to ER stress/UPR.

## **Key Research Accomplishments**

Analyzed hybridization patterns of normal and 9-hour toxin-treated cRNAs using in-house GeneChip Facility and Affymetrix 12,000 gene chip set.

Verified differential regulation of particular gene subsets using RNA, Western blot, and immunocytochemical analysis in MN9D cells and cultured dopaminergic neurons.

**Discovered that both MPP<sup>+</sup> and 6-OHDA induce markers of ER stress.**

Prepared mRNA from normal, 6-OHDA and MPP<sup>+</sup>-treated dopaminergic cells at 1 and 6 hours post treatment.

Established real time PCR techniques to evaluate microarray data.

Initiated delineation of signaling pathways mediating neurotoxin responses.

## **Reportable Outcomes**

A poster describing our initial studies was presented at the Society for Neuroscience Annual Meeting, 2002.

A slide presentation describing our current studies will be presented at the Society for Neuroscience Annual Meeting, 2003.

Initial studies were published as: Holtz WA and O'Malley KL (2003) Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem.* 278:19367-77. Epub 2003 Feb 21.

## **Conclusions**

The central hypothesis of these studies is that changes in gene expression underlie much of the damage that ultimately leads to the death of dopaminergic neurons after treatment with 6-OHDA

or MPP<sup>+</sup>. Using DNA microarray technology we determined that both of these neurotoxins induce ER stress although not to the same degree. Identification of key genetic components of this response may suggest new points of intervention. Taken together, these experiments will help clarify the molecular mechanisms associated with 6-OHDA and MPP<sup>+</sup> toxicity and might aid in developing novel therapeutic avenues to pursue relevant to PD.

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Table 1. Transcripts increased by MPP\*.

| GenBankID | GeneSymbol    | GeneName   | fold change |        | Signal log ratio |        |
|-----------|---------------|--|-------------|--------|------------------|--------|
|           |               |  | MPP*        | 6-OHDA | MPP*             | 6-OHDA |
| X67083    | Ddit3         | DNA-damage inducible transcript 3  | 9.2         | 26.0   | 3.2              | 4.7    |
| AA770736  | Ifid2         | induced in fatty liver dystrophy 2   | 4.4         | 4.0    | 2.2              | 2.0    |
| M61007    | Cebpβ         | CCAAT/enhancer binding protein C/EBP beta  | 4.0         | 4.6    | 2.0              | 2.2    |
| A1852641  | P8-pending    | p8 protein   | 3.9         | 5.3    | 2.0              | 2.4    |
| AW124369  | Gtbbp2        | GTP binding protein 2  | 3.2         | 2.9    | 1.7              | 1.6    |
| A1849556  |               | ESTs   | 3.1         | 2.3    | 1.7              | 1.2    |
| A1839690  | 1500005G05Rik | RIKEN cDNA 1500005G05 gene   | 3.1         | 3.9    | 1.7              | 2.0    |
| U19118    | Atf3          | activating transcription factor 3  | 2.6         | 9.8    | 1.4              | 3.3    |
| A1849939  | 5830413E08Rik | RIKEN cDNA 5830413E08 gene   | 2.5         | 1.7    | 1.4              | 0.8    |
| A1854851  |               | ESTs   | 2.5         | 1.1    | 1.3              | 0.2    |
| U75215    | Sic1a4        | neutral amino acid transporter   | 2.4         | 1.7    | 1.3              | 0.8    |
| X51829    | Myd116        | myeloid differentiation primary response gene 116  | 2.3         | 4.3    | 1.2              | 2.1    |
| A1848732  | Cars          | cysteiny/ t-RNA synthetase   | 2.1         | 2.2    | 1.1              | 1.2    |
| X95761    | Lbcd1         | lymphoid blast crisis-like 1   | 2.1         | 2.5    | 1.1              | 1.4    |
| A1838015  | 1200017E13Rik | RIKEN cDNA 1200017E13 gene   | 2.1         | 2.8    | 1.1              | 1.5    |
| A1846545  |               | Mus musculus Similar to phosphoserine phosphatase clone MGC 7574 mRNA complete cds         | 2.0         | 1.7    | 1.0              | 0.8    |
| U28656    | Elf4ebp1      | eukaryotic translation initiation factor 4E binding protein 1                              | 2.0         | 1.6    | 1.0              | 0.7    |
| U63387    | Cbx4          | chromobox homolog 4 Drosophila Pc class  | 2.0         | 1.3    | 1.0              | 0.4    |
| M94087    | Atf4          | activating transcription factor 4  | 1.9         | 2.0    | 1.0              | 1.0    |
| AB017189  | Sic7a5        | solute carrier family 7 cationic amino acid transporter y system member 5                  | 1.9         | 1.5    | 1.0              | 0.6    |
| X78709    | Nfe211        | nuclear factor erythroid derived 2 like 1  | 1.9         | 1.6    | 1.0              | 0.7    |
| U40930    | Sqstm1        | sequestosome 1   | 1.9         | 7.0    | 0.9              | 2.8    |
| A1845237  | Clic4         | chloride intracellular channel 4 mitochondrial   | 1.8         | 1.5    | 0.9              | 0.6    |
| AW122372  | D10Bwg0791e   | DNA segment Chr 10 Brigham Women s Genetics 0791 expressed                                 | 1.8         | 0.5    | 0.9              | -1.2   |
| A1849533  | Clic4         | chloride intracellular channel 4 mitochondrial   | 1.7         | 1.5    | 0.8              | 0.6    |
| AA684508  |               | ESTs   | 1.7         | 4.0    | 0.8              | 2.0    |
| A1849620  |               | Mus musculus 10 days embryo cDNA RIKEN full-length enriched library clone 2600002G09       | 1.7         | 1.9    | 0.8              | 1.0    |
| AW125480  |               | ESTs Weakly similar to 16.7Kd protein [H.sapiens]  | 1.7         | 1.4    | 0.8              | 0.5    |
| A1849615  | Gas5          | growth arrest specific 5   | 1.7         | 2.1    | 0.8              | 1.1    |
| A1854884  | 2610007K22Rik | RIKEN cDNA 2610007K22 gene   | 1.7         | 0.8    | 0.8              | -0.4   |
| A1844089  |               | ESTs Highly similar to LEUCYL-tRNA SYNTHETASE CYTOPLASMIC [Saccharomyces cerevisiae]       | 1.7         | 1.8    | 0.8              | 0.9    |
| X67056    | Glyt1         | glycine transporter 1  | 1.7         | 1.6    | 0.8              | 0.7    |
| A1839392  |               | ESTs Highly similar to ALANYL-tRNA SYNTHETASE [Homo sapiens]                               | 1.7         | 1.9    | 0.8              | 0.9    |
| AF023482  | Hax1-pending  | HS1 binding protein  | 1.7         | 1.8    | 0.8              | 0.9    |
| A1839918  |               | Mus musculus 10 11 days embryo cDNA RIKEN full-length enriched library clone 2810017N01    | 1.6         | 1.5    | 0.7              | 0.6    |
| X54327    |               | ESTs Weakly similar to CG11414 gene product [D.melanogaster]                               | 1.6         | 1.5    | 0.7              | 0.6    |
| X76505    | Ddr2          | discoitin domain receptor family member 2  | 1.6         | 1.4    | 0.7              | 0.5    |
| X14309    | Mdu1          | antigen identified by monoclonal antibodies 4F2  | 1.6         | 1.6    | 0.7              | 0.7    |
| AW125874  | 3010001M15Rik | RIKEN cDNA 3010001M15 gene   | 1.6         | 2.2    | 0.7              | 1.2    |
| A1852087  | 1200017E04Rik | RIKEN cDNA 1200017E04 gene   | 1.6         | 1.7    | 0.7              | 0.8    |
| A1851163  | Wars          | tryptophanyl-tRNA synthetase   | 1.6         | 1.6    | 0.7              | -0.3   |
| J04627    | Mthfd2        | methyltetrahydrofolate dehydrogenase NAD dependent methenyltetrahydrofolate cyclohydrolase | 1.6         | 1.3    | 0.7              | 0.4    |
| D17666    |               | Mus musculus clone IMAGE 3491909 mRNA partial cds  | 1.5         | 1.6    | 0.6              | 0.7    |
| A1848393  |               | RIKEN cDNA 1200003J13 gene   | 1.5         | 1.4    | 0.6              | 0.5    |
| AW060270  | 1200003J13Rik |  | 1.5         | 1.1    | 0.6              | 0.2    |

Table 1. Transcripts increased by MPP\*.

| GenBankID | GeneSymbol    | GeneName   | fold change |        | Signal log ratio |        |
|-----------|---------------|--|-------------|--------|------------------|--------|
|           |               |  | MPP*        | 6-OHDA | MPP*             | 6-OHDA |
| AA798624  | Ero1l-pending | ERO1-like <i>S. cerevisiae</i>   | 1.5         | 1.5    | 0.6              | 0.6    |
| A1929971  | 1010001P14Rik | RIKEN cDNA 1010001P14 gene   | 1.5         | 1.7    | 0.6              | 0.8    |
| A1841996  | D12Bwg0579e   | DNA segment Chr 12 Brigham Women s Genetics 0579 expressed               | 1.5         | 1.3    | 0.6              | 0.4    |
| AF004294  | Myt1          | myelin transcription factor 1  | 1.5         | 1.2    | 0.6              | 0.3    |
| A1845237  | Clic4         | chloride intracellular channel 4 mitochondrial                           | 1.5         | 1.2    | 0.6              | 0.3    |
| AW120614  | Ero1l-pending | ERO1-like <i>S. cerevisiae</i>   | 1.5         | 1.7    | 0.6              | 0.8    |
| A1849432  | Cicn3         | chloride channel 3   | 1.5         | 1.8    | 0.6              | 0.9    |
| AF103809  | Ap3b1         | adaptor-related protein complex AP-3 beta 1 subunit                      | 1.5         | 1.2    | 0.6              | 0.3    |
| A1853918  |               | ESTs Weakly similar to PRP3 MOUSE PROLINE-RICH PROTEIN MP-3 [M.musculus] | 1.5         | 0.8    | 0.6              | -0.3   |

Table 2. Transcripts decreased by MPP\*.

| GenBankID | GeneSymbol    | GeneName   | fold change |        | Signal log ratio |        |
|-----------|---------------|--|-------------|--------|------------------|--------|
|           |               |  | MPP*        | 6-OHDA | MPP*             | 6-OHDA |
| AJ002387  | Hspa5         | heat shock 70kD protein 5 glucose-regulated protein 78kD | 0.5         | 3.0    | -1.2             | 1.6    |
| AW106745  | Nsdh1         | NAD P dependent steroid dehydrogenase-like               | 0.5         | 0.5    | -1.1             | -1.0   |
| M14223    | Rrm2          | ribonucleotide reductase M2                              | 0.5         | 0.5    | -1.1             | -1.2   |
| X13135    | Fasn          | fatty acid synthase                                      | 0.5         | 0.5    | -1.0             | -1.0   |
| X54401    |               |  | 0.6         | 1.2    | -0.8             | 0.3    |
| Z19521    | Ldlr          | low density lipoprotein receptor                         | 0.6         | 0.6    | -0.8             | -0.8   |
| D42048    | Sqle          | squalene epoxidase                                       | 0.6         | 0.6    | -0.7             | -0.7   |
| M21285    |               |  | 0.6         | 0.5    | -0.7             | -0.9   |
| AF020185  | Dndc1         | dynein cytoplasmic light chain 1                         | 0.7         | 1.0    | -0.6             | -0.1   |
| A1848479  |               | ESTs Weakly similar to open reading frame [M.musculus]   | 0.7         | 0.6    | -0.6             | -0.8   |
| AW045533  |               | ESTs Highly similar to FARNESYL PYROPHOSPHATE SYNTHETASE | 0.7         | 0.9    | -0.6             | -0.1   |
| X62154    | Mcmd          | mini chromosome maintenance deficient S. cerevisiae      | 0.7         | 0.7    | -0.6             | -0.5   |
| AW227650  | 0610038P07Rik | RIKEN cDNA 0610038P07 gene                               | 0.7         | 1.3    | -0.6             | 0.4    |
| A1846851  |               | ESTs Highly similar to FARNESYL PYROPHOSPHATE SYNTHETASE | 0.7         | 1.0    | -0.6             | 0.1    |
| AA529583  | Mrgx-pending  | MORF-related gene X                                      | 0.7         | 1.2    | -0.5             | 0.3    |

Table 3. Transcripts increased by both 6-OHDA and MPP\*.

| GenBankID | GeneSymbol    | GeneName  | fold change |      | Signal log ratio |      |
|-----------|---------------|---|-------------|------|------------------|------|
|           |               |   | 6-OHDA      | MPP* | 6-OHDA           | MPP* |
| X67083    | Ddit3         | DNA-damage inducible transcript 3   | 26.0        | 9.2  | 4.7              | 3.2  |
| U19118    | Atf3          | activating transcription factor 3   | 9.8         | 2.6  | 3.3              | 1.4  |
| U40930    | Sqstm1        | sequestosome 1  | 7.0         | 1.9  | 2.8              | 0.9  |
| AI852641  | P8-pending    | p8 protein  | 5.3         | 3.9  | 2.4              | 2.0  |
| M61007    | Cebpb         | CCAAT/enhancer binding protein C/EBP beta   | 4.6         | 4.0  | 2.2              | 2.0  |
| X51829    | Myd116        | myeloid differentiation primary response gene 116                                       | 4.3         | 2.3  | 2.1              | 1.2  |
| AA684508  |               | ESTs  | 4.0         | 1.7  | 2.0              | 0.8  |
| AA770736  | Ifid2         | induced in fatty liver dystrophy 2  | 4.0         | 4.4  | 2.0              | 2.2  |
| AI839690  | 1500005G05Rik | RIKEN cDNA 1500005G05 gene  | 3.9         | 3.1  | 2.0              | 1.7  |
| AW124369  | Gtpbp2        | GTP binding protein 2   | 2.9         | 3.2  | 1.6              | 1.7  |
| AI838015  | 1200017E13Rik | RIKEN cDNA 1200017E13 gene  | 2.8         | 2.1  | 1.5              | 1.1  |
| X95761    | Lbcl1         | lymphoid blast crisis-like 1  | 2.5         | 2.1  | 1.4              | 1.1  |
| X14309    | Mdu1          | antigen identified by monoclonal antibodies 4F2   | 2.2         | 1.6  | 1.2              | 0.7  |
| AI848732  | Cars          | cysteiny/1-RNA synthetase   | 2.2         | 2.1  | 1.2              | 1.1  |
| AI849615  | Gas5          | growth arrest specific 5  | 2.1         | 1.7  | 1.1              | 0.8  |
| M94087    | Atf4          | activating transcription factor 4   | 2.0         | 1.9  | 1.0              | 1.0  |
| AI849620  |               | Mus musculus 10 days embryo cDNA RIKEN full-length enriched library clone 2600002G09    | 1.9         | 1.7  | 1.0              | 0.8  |
| AI839392  |               | ESTs Highly similar to ALANYL-TRNA SYNTHETASE [Homo sapiens]                            | 1.9         | 1.7  | 0.9              | 0.8  |
| AF023482  | Hax1-pending  | HS1 binding protein   | 1.8         | 1.7  | 0.9              | 0.8  |
| AI849432  | Clcn3         | chloride channel 3  | 1.8         | 1.5  | 0.9              | 0.6  |
| AI844089  |               | ESTs Highly similar to LEUCYL-TRNA SYNTHETASE CYTOPLASMIC [Saccharomyces cerevisiae]    | 1.8         | 1.7  | 0.9              | 0.8  |
| AI846545  |               | Mus musculus Similar to phosphoserine phosphatase clone MGC 7574 mRNA complete cds      | 1.7         | 2.0  | 0.8              | 1.0  |
| AW125874  | 3010001M15Rik | RIKEN cDNA 3010001M15 gene  | 1.7         | 1.6  | 0.8              | 0.7  |
| U75215    | Slc1a4        | neutral amino acid transporter  | 1.7         | 2.4  | 0.8              | 1.3  |
| AW120614  | Ero1l-pending | ERO1-like S. cerevisiae   | 1.7         | 1.5  | 0.8              | 0.6  |
| AI929971  | 1010001P14Rik | RIKEN cDNA 1010001P14 gene  | 1.7         | 1.5  | 0.8              | 0.6  |
| AI849939  | 5830413E08Rik | RIKEN cDNA 5830413E08 gene  | 1.7         | 2.5  | 0.8              | 1.4  |
| AI851163  | Wars          | tryptophanyl-tRNA synthetase  | 1.6         | 1.6  | 0.7              | 0.7  |
| U28656    | Eif4ebp1      | eukaryotic translation initiation factor 4E binding protein 1                           | 1.6         | 2.0  | 0.7              | 1.0  |
| X67056    | Glyt1         | glycine transporter 1   | 1.6         | 1.7  | 0.7              | 0.8  |
| X76505    | Ddr2          | discoidin domain receptor family member 2   | 1.6         | 1.6  | 0.7              | 0.7  |
| D17666    |               |   | 1.6         | 1.5  | 0.7              | 0.6  |
| X78709    | Nfe2l1        | nuclear factor erythroid derived 2 like 1   | 1.6         | 1.9  | 0.7              | 1.0  |
| AI849533  | Clfc4         | chloride intracellular channel 4 mitochondrial  | 1.5         | 1.7  | 0.6              | 0.8  |
| X54327    |               | M. musculus mRNA for glutamyl-tRNA synthetase   | 1.5         | 1.6  | 0.6              | 0.7  |
| AA798624  | Ero1l-pending | ERO1-like S. cerevisiae   | 1.5         | 1.5  | 0.6              | 0.6  |
| AI839918  |               | Mus musculus 10 11 days embryo cDNA RIKEN full-length enriched library clone 2810017N01 | 1.5         | 1.6  | 0.6              | 0.7  |
| AI845237  | Clfc4         | chloride intracellular channel 4 mitochondrial  | 1.5         | 1.8  | 0.6              | 0.9  |
| AB0117189 | Slc7a5        | solute carrier family 7 cationic amino acid transporter y system member 5               | 1.5         | 1.9  | 0.6              | 1.0  |

Table 4. Transcripts increased by 6-OHDA

| GenBankID | GeneSymbol    | GeneName   | fold change |      |             | Signal log ratio |
|-----------|---------------|--|-------------|------|-------------|------------------|
|           |               |  | 6-OHDA      | MPP* | 6-OHDA MPP* |                  |
| X67083    | Ddit3         | DNA-damage inducible transcript 3  | 26.0        | 9.2  | 4.7         | 3.2              |
| U19118    | Atf3          | activating transcription factor 3  | 9.8         | 2.6  | 3.3         | 1.4              |
| AW120711  | Dnajb9        | DNA J protein b9   | 7.0         | 0.7  | 2.8         | -0.6             |
| U40930    | Sqstm1        | sequestosome 1   | 7.0         | 1.9  | 2.8         | 0.9              |
| A1846938  | Herpud1       | homocysteine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain member 1 | 6.5         | 1.3  | 2.7         | 0.4              |
| AF105222  | Lagf1-pending | leukemia-associated gene-like  | 6.1         | 1.3  | 2.6         | 0.4              |
| X56824    | Hmox1         | heme oxygenase decycling 1   | 5.7         | 1.5  | 2.5         | 0.6              |
| A1852641  | P8-pending    | p8 protein   | 5.3         | 3.9  | 2.4         | 2.0              |
| M61007    | Cebpb         | CCAAT/enhancer binding protein C/EBP beta  | 4.6         | 4.0  | 2.2         | 2.0              |
| AJ011967  | Gdf15         | growth differentiation factor 15   | 4.4         | 3.0  | 2.2         | 1.6              |
| X51829    | Myd116        | myeloid differentiation primary response gene 116  | 4.3         | 2.3  | 2.1         | 1.2              |
| AA684508  |               | ESTs   | 4.0         | 1.7  | 2.0         | 0.8              |
| AA770736  | Ifid2         | induced in fatty liver dystrophy 2   | 4.0         | 4.4  | 2.0         | 2.2              |
| A1839690  | 1500005G05Rik | RIKEN cDNA 1500005G05 gene   | 3.9         | 3.1  | 2.0         | 1.7              |
| AW123904  | 3110025G09Rik | RIKEN cDNA 3110025G09 gene   | 3.7         | 1.2  | 1.9         | 0.3              |
| A1845538  | Etv6          | ets variant gene 6 TEL oncogene  | 3.6         | 0.6  | 1.9         | -0.8             |
| AW121716  | 0610031F24Rik | RIKEN cDNA 0610031F24 gene   | 3.0         | 1.0  | 1.6         | -0.1             |
| AW122364  | 3230402M22Rik | RIKEN cDNA 3230402M22 gene   | 3.0         | 0.4  | 1.6         | -1.5             |
| AJ002387  | Hspa5         | heat shock 70kD protein 5 glucose-regulated protein 78kD                                     | 3.0         | 0.5  | 1.6         | -1.2             |
| AW124369  | Gtpbp2        | GTP binding protein 2  | 2.9         | 3.2  | 1.6         | 1.7              |
| X61940    | Ptpn16        | protein tyrosine phosphatase non-receptor type 16  | 2.9         | 1.1  | 1.6         | 0.2              |
| X67644    | Ier3          | immediate early response 3   | 2.8         | 1.1  | 1.5         | 0.2              |
| A1838015  | 1200017E13Rik | RIKEN cDNA 1200017E13 gene   | 2.8         | 2.1  | 1.5         | 1.1              |
| AV305832  | Ubc           | ubiquitin C  | 2.8         | 0.8  | 1.5         | -0.3             |
| AW122851  | 1110002O23Rik | RIKEN cDNA 1110002O23 gene   | 2.7         | 0.7  | 1.5         | -0.5             |
| A1843420  | Bag3          | Bcl2-associated athanogene 3   | 2.6         | 1.0  | 1.4         | -0.1             |
| AW045202  | 1700015E05Rik | RIKEN cDNA 1700015E05 gene   | 2.5         | 0.5  | 1.4         | -0.9             |
| A1839280  | 1810045K07Rik | RIKEN cDNA 1810045K07 gene   | 2.5         | 1.4  | 1.4         | 0.5              |
| X95761    | Lbcl1         | lymphoid blast crisis-like 1   | 2.5         | 2.1  | 1.4         | 1.1              |
| U95053    | Gclm          | glutamate-cysteine ligase modifier subunit   | 2.5         | 1.0  | 1.4         | -0.1             |
| J03297    | Tra1          | tumor rejection antigen gp96   | 2.3         | 0.8  | 1.2         | -0.4             |
| AW045202  | 1700015E05Rik | RIKEN cDNA 1700015E05 gene   | 2.3         | 0.5  | 1.2         | -1.0             |
| AW122690  |               | ESTs   | 2.3         | 0.7  | 1.2         | -0.5             |
| X14309    | Mdu1          | antigen identified by monoclonal antibodies 4F2  | 2.2         | 1.6  | 1.2         | 0.7              |
| A1848732  | Cars          | cysteinyl-tRNA synthetase  | 2.2         | 2.1  | 1.2         | 1.1              |
| A1837625  | Csrp          | cysteine rich protein  | 2.2         | 1.1  | 1.2         | 0.2              |
| D88793    | Csrp          | cysteine rich protein  | 2.1         | 1.0  | 1.1         | 0.0              |
| A1848343  | Sec23b        | SEC23B S. cerevisiae   | 2.1         | 1.0  | 1.1         | 0.0              |
| A1849615  | Gas5          | growth arrest specific 5   | 2.1         | 1.7  | 1.1         | 0.8              |
| A1845293  | 1500032E05Rik | RIKEN cDNA 1500032E05 gene   | 2.1         | 1.0  | 1.1         | -0.1             |
| AF100956  | Sacm2l        | SAC2 suppressor of actin mutations 2 homolog like S. cerevisiae                              | 2.1         | 1.0  | 1.1         | 0.1              |
| D17571    | Por           | P450 cytochrome oxidoreductase   | 2.1         | 1.0  | 1.1         | 0.1              |
| AW122075  | 251000110Rik  | RIKEN cDNA 251000110 gene  | 2.1         | 0.9  | 1.1         | -0.1             |
| A1837395  |               | ESTs Highly similar to SERYL-TRNA SYNTHETASE [Cricetulus griseus]                            | 2.0         | 1.4  | 1.0         | 0.5              |
| M94087    | Atf4          | activating transcription factor 4  | 2.0         | 1.9  | 1.0         | 1.0              |
| D50527    | Ubc           | ubiquitin C  | 2.0         | 1.0  | 1.0         | -0.1             |



Table 4. Transcripts increased by 6-OHDA

| GenBankID | GeneSymbol    | GeneName  | 6-OHDA | MPP* | 6-OHDA | MPP* | Signal log ratio |
|-----------|---------------|---|--------|------|--------|------|------------------|
| AA866971  |               | ESTs Moderately similar to hypothetical protein [H.sapiens]   | 1.7    | 1.3  | 0.8    | 0.4  | 0.4              |
| Y18505    | D0HXS9928E    | DNA segment human DXS9928E  | 1.7    | 1.2  | 0.8    | 0.3  | 0.3              |
| Z84471    | G6pd2         | glucose-6-phosphate dehydrogenase 2   | 1.7    | 0.9  | 0.8    | -0.2 | -0.2             |
| AF062071  | Zfp216        | zinc finger protein 216   | 1.7    | 1.0  | 0.8    | -0.1 | -0.1             |
| AW121539  | 1110014C03Rik | RIKEN cDNA 1110014C03 gene  | 1.6    | 0.8  | 0.7    | -0.3 | -0.3             |
| AW125736  | 1110003F06Rik | RIKEN cDNA 1110003F06 gene  | 1.6    | 0.8  | 0.7    | -0.3 | -0.3             |
| AW047320  | 2600006O07Rik | RIKEN cDNA 2600006O07 gene  | 1.6    | 1.2  | 0.7    | 0.3  | 0.3              |
| AI841920  | D13Wsu115e    | DNA segment Chr 13 Wayne State University 115 expressed   | 1.6    | 0.9  | 0.7    | -0.1 | -0.1             |
| AJ010391  |               | tryptophanyl-tRNA synthetase  | 1.6    | 1.1  | 0.7    | 0.1  | 0.1              |
| AI851163  | Wars          | tryptophanyl-tRNA synthetase  | 1.6    | 1.6  | 0.7    | 0.7  | 0.7              |
| M14044    | Anxa2         | annexin A2  | 1.6    | 1.1  | 0.7    | 0.1  | 0.1              |
| AI843665  | Sec23a        | SEC23A S. cerevisiae  | 1.6    | 1.0  | 0.7    | 0.1  | 0.1              |
| Z11911    | G6pdx         | glucose-6-phosphate dehydrogenase X-linked  | 1.6    | 1.0  | 0.7    | 0.1  | 0.1              |
| AW046691  | 593042611Rik  | RIKEN cDNA 593042611 gene   | 1.6    | 1.0  | 0.7    | 0.1  | 0.1              |
| AA683712  | 3110079L04Rik | RIKEN cDNA 3110079L04 gene  | 1.6    | 0.9  | 0.7    | -0.2 | -0.2             |
| AJ005253  | Cipp          | caseinolytic protease ATP-dependent E. coli proteolytic subunit homolog                               | 1.6    | 1.1  | 0.7    | 0.1  | 0.1              |
| AI848699  | 0610010112Rik | RIKEN cDNA 0610010112 gene  | 1.6    | 1.1  | 0.7    | 0.2  | 0.2              |
| U28656    | Ei4ebp1       | eukaryotic translation initiation factor 4E binding protein 1   | 1.6    | 1.3  | 0.7    | 0.4  | 0.4              |
| X67056    | Glyt1         | glycine transporter 1   | 1.6    | 2.0  | 0.7    | 1.0  | 1.0              |
| X76505    | Ddr2          | discoidin domain receptor family member 2   | 1.6    | 1.7  | 0.7    | 0.8  | 0.8              |
| X58990    | Ppib          | peptidylprolyl isomerase B  | 1.6    | 1.6  | 0.7    | 0.7  | 0.7              |
| AI845953  |               | ESTs Highly similar to arsenate resistance protein ARS2 [H.sapiens]                                   | 1.6    | 1.0  | 0.7    | -0.1 | -0.1             |
| D17666    |               | ESTs  | 1.6    | 0.8  | 0.7    | -0.4 | -0.4             |
| AI846118  |               | Mus musculus stress-associated endoplasmic reticulum protein 1 ribosome associated membrane protein 4 | 1.6    | 0.8  | 0.7    | -0.4 | -0.4             |
| AI843466  |               | RIKEN cDNA 5730406115 gene  | 1.6    | 0.8  | 0.7    | -0.3 | -0.3             |
| AI839946  | 5730406115Rik | RIKEN cDNA 5730406115 gene  | 1.6    | 0.7  | 0.7    | -0.5 | -0.5             |
| M73329    | Grp58         | glucose regulated protein 58 kDa  | 1.6    | 1.0  | 0.7    | 0.1  | 0.1              |
| K02236    | Mt2           | metallothionein 2   | 1.6    | 1.2  | 0.7    | 0.3  | 0.3              |
| J04509    | Jun1          | Jun proto-oncogene related gene d1  | 1.6    | 1.2  | 0.7    | 0.3  | 0.3              |
| AI838735  | 1810024J13Rik | RIKEN cDNA 1810024J13 gene  | 1.6    | 1.3  | 0.7    | 0.4  | 0.4              |
| AF033620  | Cd151         | CD151 antigen   | 1.6    | 1.1  | 0.7    | 0.1  | 0.1              |
| X78709    | Nfe211        | nuclear factor erythroid derived 2 like 1   | 1.6    | 1.9  | 0.7    | 1.0  | 1.0              |
| AI849533  | Clic4         | chloride intracellular channel 4 mitochondrial  | 1.5    | 1.7  | 0.6    | 0.8  | 0.8              |
| AI836446  | 5730465C04Rik | RIKEN cDNA 5730465C04 gene  | 1.5    | 1.0  | 0.6    | -0.1 | -0.1             |
| AF013099  | Psm4          | proteasome prosome macropain 26S subunit non-ATPase 4   | 1.5    | 1.1  | 0.6    | 0.2  | 0.2              |
| AW125336  | 2610103L06Rik | RIKEN cDNA 2610103L06 gene  | 1.5    | 1.1  | 0.6    | 0.1  | 0.1              |
| U67328    | Ei13s8        | eukaryotic translation initiation factor 3 subunit 8 110 kDa  | 1.5    | 1.4  | 0.6    | 0.5  | 0.5              |
| X54327    |               | M.musculus mRNA for glutamyl-tRNA synthetase  | 1.5    | 1.6  | 0.6    | 0.7  | 0.7              |
| AA798624  | Ero11-pending | ERO11-like S. cerevisiae  | 1.5    | 1.5  | 0.6    | 0.6  | 0.6              |
| U59807    | Cstb          | cystatin B  | 1.5    | 1.1  | 0.6    | 0.1  | 0.1              |
| AW046847  |               | ESTs Highly similar to PRE-MRNA SPLICING FACTOR PRP6 [Saccharomyces cerevisiae]                       | 1.5    | 1.1  | 0.6    | 0.1  | 0.1              |
| AA388099  | D7Wsu105e     | DNA segment Chr 7 Wayne State University 105 expressed  | 1.5    | 0.7  | 0.6    | -0.6 | -0.6             |
| U42443    | Bcat1         | branched chain aminotransferase 1 cytosolic   | 1.5    | 1.4  | 0.6    | 0.5  | 0.5              |
| M16465    | S100a10       | calcium binding protein A11 calgizzarin   | 1.5    | 0.9  | 0.6    | -0.1 | -0.1             |
| U11027    | Sec61g        | SEC61 gamma subunit S. cerevisiae   | 1.5    | 0.9  | 0.6    | -0.2 | -0.2             |
| Z50159    | Sui1-rs1      | suppressor of initiator codon mutations related sequence 1 S. cerevisiae                              | 1.5    | 1.2  | 0.6    | 0.3  | 0.3              |

Table 4. Transcripts increased by 6-OHDA

| GenBankID | GeneSymbol    | GeneName   | fold change |                  |                         | Signal log ratio |
|-----------|---------------|--|-------------|------------------|-------------------------|------------------|
|           |               |  | 6-OHDA      | MPP <sup>+</sup> | 6-OHDA MPP <sup>+</sup> |                  |
| AF069954  | Gng3lg        | G protein gamma 3 linked gene  | 1.5         | 1.1              | 0.6                     | 0.2              |
| A1839918  |               | Mus musculus 10 11 days embryo cDNA RIKEN full-length enriched library clone 2810017N01 full insert sequence | 1.5         | 1.6              | 0.6                     | 0.7              |
| A1845237  | Clic4         | chloride intracellular channel 4 mitochondrial   | 1.5         | 1.8              | 0.6                     | 0.9              |
| AB025313  | Uchl1         | ubiquitin carboxy-terminal hydrolase L1  | 1.5         | 1.1              | 0.6                     | 0.1              |
| A1836034  | 1110003B01Rik | RIKEN cDNA 1110003B01 gene   | 1.5         | 1.0              | 0.6                     | 0.1              |
| AW122255  |               | ESTs Moderately similar to T00076 hypothetical protein KIAA0462 [H.sapiens]                                  | 1.5         | 1.3              | 0.6                     | 0.4              |
| AW122052  |               | Mus musculus mRNA for N-acetylneuraminic acid 9-phosphate synthetase complete cds                            | 1.5         | 0.8              | 0.6                     | -0.4             |
| A1153421  |               | Mus musculus mRNA for erythroid differentiation regulator partial  | 1.5         | 1.1              | 0.6                     | 0.2              |
| X57349    | Trfr          | transferrin receptor   | 1.5         | 0.9              | 0.6                     | -0.1             |
| AB017189  | Slc7a5        | solute carrier family 7 cationic amino acid transporter y system member 5                                    | 1.5         | 1.9              | 0.6                     | 1.0              |
| U11812    | Ptprm         | protein tyrosine phosphatase receptor-type N   | 1.5         | 1.1              | 0.6                     | 0.2              |
| L39879    | Ftl1          | ferritin light chain 1   | 1.5         | 1.1              | 0.6                     | 0.2              |
| X51703    | Ubb           | ubiquitin B  | 1.5         | 1.0              | 0.6                     | 0.1              |
| AU020229  | Fzd3          | frizzled homolog 3 Drosophila  | 1.5         | 0.9              | 0.6                     | -0.1             |
| D87691    | D6Erttd109e   | DNA segment Chr 6 ERATO Doi 109 expressed  | 1.5         | 1.0              | 0.6                     | 0.0              |

Table 5. Transcripts decreasing with 6-OHDA.

| GenBankID | GeneSymbol    | GeneName  | fold change |                  | Signal log ratio |                  |
|-----------|---------------|---|-------------|------------------|------------------|------------------|
|           |               |   | 6-OHDA      | MPP <sup>+</sup> | 6-OHDA           | MPP <sup>+</sup> |
| M29260    | Fasn          | fatty acid synthase   | 0.2         | 1.0              | -2.5             | 0.0              |
| X13135    | Trmpo         | thymopoletin  | 0.5         | 0.5              | -1.0             | -1.0             |
| AW046443  | B2-pending    | immunosuperfamily protein B2                                  | 0.5         | 1.0              | -1.0             | 0.0              |
| AF061260  | Sox11         | SRY-box containing gene 11                                    | 0.5         | 1.1              | -1.0             | 0.1              |
| AF009414  | Cbfb          | core binding factor beta                                      | 0.5         | 1.1              | -1.0             | 0.1              |
| D14572    | 1010001C05Rik | RIKEN cDNA 1010001C05 gene                                    | 0.5         | 0.7              | -0.9             | -0.2             |
| A1837302  | Hmgb3         | high mobility group box 3                                     | 0.5         | 0.6              | -0.9             | -0.7             |
| M21285    |               | ESTs Moderately similar to JC4928 histone H1x [H.sapiens]     | 0.6         | 0.8              | -0.9             | -0.3             |
| AF022465  | Etoh16        | ethanol induced 6   | 0.6         | 0.9              | -0.9             | -0.2             |
| A1851599  | Cbfb          | core binding factor beta                                      | 0.6         | 1.2              | -0.8             | 0.3              |
| A1843895  | Ccnb2         | cyclin B2   | 0.6         | 0.9              | -0.8             | -0.1             |
| X66032    | 1810037117Rik | RIKEN cDNA 1810037117 gene                                    | 0.6         | 1.1              | -0.8             | 0.2              |
| AW125347  | 1110038L14Rik | RIKEN cDNA 1110038L14 gene                                    | 0.6         | 0.8              | -0.8             | -0.3             |
| AA681998  | ESTs          | mini chromosome maintenance deficient 2 S. cerevisiae         | 0.6         | 0.8              | -0.8             | -0.3             |
| AW120755  | Mcmd2         | mini chromosome maintenance deficient 2 S. cerevisiae         | 0.6         | 0.9              | -0.8             | -0.1             |
| D86725    | Ccna2         | cyclin A2   | 0.6         | 0.8              | -0.7             | -0.4             |
| X75483    | Gata2         | GATA-binding protein 2  | 0.6         | 1.0              | -0.7             | -0.1             |
| AB000096  | Sqle          | squalene epoxidase  | 0.6         | 0.9              | -0.7             | -0.2             |
| D42048    | 9-Sep         | septin 9  | 0.6         | 0.6              | -0.7             | -0.7             |
| AJ250723  | Nifh          | neurofilament heavy polypeptide                               | 0.6         | 0.9              | -0.7             | -0.2             |
| M35131    | Pcnt          | pericentrin   | 0.7         | 0.8              | -0.7             | -0.3             |
| AW047671  | Nek2          | NIMA never in mitosis gene a related expressed kinase 2       | 0.7         | 0.8              | -0.6             | -0.4             |
| A1194767  | 1810012N18Rik | RIKEN cDNA 1810012N18 gene                                    | 0.7         | 0.9              | -0.6             | -0.1             |
| AF013166  | Hmgb1         | high mobility group box 1                                     | 0.7         | 1.0              | -0.6             | -0.1             |
| A1839212  | Mlp           | MARCKS-like protein   | 0.7         | 0.8              | -0.6             | -0.4             |
| U00431    | Bmi1          | B lymphoma Mo-MLV insertion region 1                          | 0.7         | 0.8              | -0.6             | -0.3             |
| X61399    | 3110023F10Rik | RIKEN cDNA 3110023F10 gene                                    | 0.7         | 1.0              | -0.6             | 0.0              |
| M64068    | Mcmd4         | mini chromosome maintenance deficient 4 homolog S. cerevisiae | 0.7         | 0.9              | -0.6             | -0.2             |
| A1195392  | Impnb         | importin beta   | 0.7         | 1.3              | -0.5             | 0.4              |
| X58196    | Impnb         | importin beta   | 0.7         | 0.7              | -0.5             | -0.6             |
| D26089    | Hnrpab        | Mus musculus Pumilio 2 Pum2 mRNA complete cds                 | 0.7         | 1.0              | -0.5             | 0.1              |
| D45836    | Cd24a         | heterogeneous nuclear ribonucleoprotein A/B                   | 0.7         | 1.1              | -0.5             | 0.1              |
| A1837010  | ESTs          | CD24a antigen   | 0.7         | 0.9              | -0.5             | -0.2             |
| D90151    |               | ESTs  | 0.7         | 0.9              | -0.5             | -0.2             |
| M58661    |               | ESTs  | 0.7         | 1.0              | -0.5             | -0.2             |
| A1848984  |               | ESTs  | 0.7         | 1.0              | -0.5             | -0.1             |

Table 6. Transcripts Related to Programmed Cell Death

| Gene         | Control | MPP+      | 6-OHDA    |
|--------------|---------|-----------|-----------|
| caspase 1    | absent  | absent    | absent    |
| caspase 2    | present | no change | no change |
| caspase 3    | present | no change | no change |
| caspase 6    | absent  | absent    | absent    |
| caspase 7    | present | no change | no change |
| caspase 8    | absent  | absent    | absent    |
| caspase 9    | present | no change | no change |
| caspase 11/4 | absent  | absent    | absent    |
| caspase 12   | absent  | absent    | absent    |
| caspase 14   | absent  | absent    | absent    |
| apaf1        | present | no change | no change |
| bax          | present | no change | no change |
| bak          | present | no change | no change |
| bad          | present | no change | no change |
| boo/diva     | absent  | absent    | absent    |
| bim/bod      | present | no change | no change |
| dp5/hrk      | absent  | absent    | absent    |
| bok          | present | decreased | decreased |
| bid          | present | no change | no change |
| bag1         | present | no change | no change |
| bag2         | present | no change | no change |
| bag3         | present | no change | no change |
| bcl-2        | absent  | absent    | absent    |
| bcl-x        | absent  | absent    | absent    |
| bcl-w        | absent  | absent    | absent    |
| bcl-rambo    | present | no change | no change |

## Parkinsonian Mimetics Induce Aspects of Unfolded Protein Response in Death of Dopaminergic Neurons\*

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Genes associated with Parkinson's disease (PD) have suggested a role for ubiquitin-proteasome dysfunction and aberrant protein degradation in this disorder. Inasmuch as oxidative stress has also been implicated in PD, the present study examined transcriptional changes mediated by the Parkinsonism-inducing neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) in a dopaminergic cell line. Microarray analysis of RNA isolated from toxin treated samples revealed that the stress-induced transcription factor CHOP/Gadd153 was dramatically up-regulated by both 6-OHDA and MPP<sup>+</sup>. Treatment with 6-OHDA also induced a large number of genes involved in endoplasmic reticulum stress and unfolded protein response (UPR) such as ER chaperones and elements of the ubiquitin-proteasome system. Reverse transcription-PCR, Western blotting, and immunocytochemical approaches were used to quantify and temporally order the UPR pathways involved in neurotoxin-induced cell death. 6-OHDA, but not MPP<sup>+</sup>, significantly increased hallmarks of UPR such as BiP, c-Jun, and processed Xbp1 mRNA. Both toxins increased the phosphorylation of UPR proteins, PERK and eIF2 $\alpha$ , but only 6-OHDA increased phosphorylation of c-Jun. Thus, 6-OHDA is capable of triggering multiple pathways associated with UPR, whereas MPP<sup>+</sup> exhibits a more restricted response. The involvement of UPR in these widely used neurotoxin models supports the role of ubiquitin-proteasome pathway dysfunction in PD.

Parkinson's disease (PD)<sup>1</sup> involves an irreversible degeneration of the dopaminergic nigrostriatal pathway, resulting in marked impairments of motor control. Although the etiology of PD remains unknown, both genetic and environmental factors appear to play a role. For example, three genes and several putative loci have been identified (1), including two autosomal dominant mutations of the  $\alpha$ -synuclein gene, that were linked to rare familial early-onset PD (2, 3).  $\alpha$ -Synuclein was subse-

quently shown to be the major component of Lewy bodies, the hallmark inclusion of PD (4). Parkin, a second gene with mutations associated with PD (5), has been shown to be an ubiquitin-protein isopeptide ligase (6). Loss of Parkin activity is linked to endoplasmic reticulum (ER) stress and unfolded protein response (UPR; Refs. 7 and 8). Finally, a missense mutation in the gene encoding ubiquitin C-terminal hydrolase L1 is also associated with rare cases of PD (9). Thus, aggregation of  $\alpha$ -synuclein together with defects in the ubiquitin pathway support the notion that a dysfunctional ubiquitin-proteasome system in which aberrant proteins are not cleared may play a major role in PD. The role of proteasomal impairment has been further emphasized by recent reports that pharmacological inhibition of proteasome function leads to selective degeneration of dopaminergic neurons in culture (10) as well as *in vivo* (11). In particular, cell death was associated with increased cytoplasmic levels of  $\alpha$ -synuclein and ubiquitin, as well as the formation of inclusion bodies (10, 11). Taken together, accumulating genetic and molecular evidence suggests that defects in ER and ubiquitin-proteasomal processing contribute to the pathogenesis of PD.

Because PD is largely restricted to dopaminergic neurons and because dopamine is easily oxidized *in vitro* and *in vivo* to a variety of neurotoxic metabolites, dopamine itself is considered a major factor in this disorder. For example, dopamine is readily oxidized to highly cytotoxic quinone molecules via at least three different enzymatic pathways (for review see Ref. 12). Moreover, in the presence of transition metals and hydrogen peroxide, dopamine can be converted to 6-OHDA (for review see Ref. 13), a highly potent endogenous neurotoxin widely used to create animal models of PD (13). Both 6-OHDA and other dopamine quinone derivatives have been found in post-mortem Parkinsonian brains (14–16), a finding that, together with the extensive studies documenting 6-OHDA-induced nigral degeneration, underscores the role dopamine plays in its own demise.

Similarly, another PD mimetic, *N*-methyl-4-phenyl-1,2,3,6-tetrahydroindole (MPTP) or its active derivative, MPP<sup>+</sup>, is also thought to induce oxidative stress and impair energy metabolism (for review see Ref. 17). The original finding that human exposure to MPTP results in PD (18) has been replicated in various animal models including non-human primates (for review see Ref. 17). Thus, both 6-OHDA and MPP<sup>+</sup> have been shown to produce reactive oxygen species and to inhibit mitochondrial complex I, as well as to mimic many behavioral, pharmacological, and pathological symptoms of this disorder (for review see Refs. 13, 17, and 19). Despite these parallels, the molecular mechanisms by which these neurotoxins kill cells remain unclear. Further, their relevance to emerging genetic and pharmacological models investigating ubiquitin-proteasome pathway dysfunction and protein aggregation has yet to be studied.

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<sup>1</sup> The abbreviations used are: PD, Parkinson's disease; 6-OHDA, 6-hydroxydopamine; MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium; UPR, unfolded protein response; RT, reverse transcription; ER, endoplasmic reticulum; MPTP, *N*-methyl-4-phenyl-1,2,3,6-tetrahydroindole; PBS, phosphate-buffered saline; PERK, PKR-like ER kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; ANOVA, analysis of variance; TH, tyrosine hydroxylase; eIF, eukaryotic initiation factor.

Previous results from this laboratory and others have demonstrated that 6-OHDA and MPP<sup>+</sup> trigger morphologically distinct forms of cell death in the dopaminergic cell line MN9D and mouse primary mesencephalic cultures (13, 20, 21). Markers of apoptosis such as chromatin condensation and caspase-3 cleavage are widespread in cells treated with 6-OHDA, but not with MPP<sup>+</sup>. Despite the different forms of cell death induced by either toxin, both types of cell death seem to be dependent on *de novo* protein synthesis (22, 23). However, few studies of gene expression in 6-OHDA or MPP<sup>+</sup>-induced dopaminergic cell death models have been done. Presumably, this is a result of the scarcity and heterogeneity of the tissue involved as well as the technical limitation in analyzing a few genes at a time. Thus, at present, there is no information about the coordinated patterns of gene expression involved in 6-OHDA or MPP<sup>+</sup> toxicity.

To unravel biological processes occurring in response to 6-OHDA and MPP<sup>+</sup>, we used microarray analysis of RNA isolated from the dopaminergic cell line MN9D (24) as a starting point to identify possible pathways induced by these Parkinsonian mimetics. These cells have been shown to mimic many aspects of the dopaminergic cell type from which they were immortalized (20–25). Capitalizing on the homogeneity and similarity in response of MN9D cells, the present study used microarray results, in addition to RT-PCR, Western blotting, and immunocytochemical approaches, to reveal that 6-OHDA triggers three separate signaling pathways associated with ER stress and UPR, whereas MPP<sup>+</sup> seems to only involve one such signaling pathway. The unexpected identification of UPR induction in these models of dopaminergic cell death increases our understanding of how they may function to mimic the disease state and supports the theory that aberrations in the ubiquitin-proteasome pathway play an important role in PD.

#### MATERIALS AND METHODS

**Cell Cultures**—For primary cultures, the ventral mesencephalon was removed from embryonic day 14 CF1 murine embryos (Charles River Laboratories, Wilmington, MA) as described previously (21). Briefly, tissues were mechanically dissociated, incubated with 0.25% trypsin and 0.05% DNase in PBS for 20 min at 37 °C, and further triturated using a constricted Pasteur pipette. All plates were pre-coated overnight at room temperature with 0.5 mg/ml poly-D-lysine (Sigma) followed by 2.5 µg/ml laminin (BD Biosciences, San Jose, CA) for 2 h at 37 °C. Cells were maintained in serum-free Neurobasal medium (Invitrogen) supplemented with 1× B27 supplement (Invitrogen), 0.5 mM L-glutamine (Sigma), and 0.01 µg/ml streptomycin plus 100 units of penicillin. Half of the culture medium was replaced with fresh Neurobasal medium on the third and fifth day following plating. All experiments were conducted after 6 days *in vitro*.

MN9D cells were plated on dishes coated with 0.5 mg/ml poly-D-lysine for 1 h at 37 °C and then rinsed with sterile H<sub>2</sub>O. Cells were maintained in Iscove's Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an incubator with 10% CO<sub>2</sub> at 37 °C. Cells were switched to serum-free Iscove's Dulbecco's modified Eagle's medium/F-12 supplemented with 1× B27 prior to addition of experimental agents.

**Cycloheximide Treatment and Determination of Cell Viability**—MN9D cells were plated at a density of 40,000 cells/well in 24-well plates and treated after 3 days. One µg/ml cycloheximide (Calbiochem, La Jolla, CA) was added either immediately prior to, or at times following, addition of 100 µM 6-OHDA with ascorbic acid (dissolved in boiled water; Sigma) or 75 µM MPP<sup>+</sup> (Sigma). After 48 h, cell survival was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as previously described (22).

**Microarray Analysis**—MN9D cells were plated at a density of 200,000 cells/well in six-well plates. After 3 days, cells were treated with 75 µM 6-OHDA or 75 µM MPP<sup>+</sup>, or left untreated for control comparisons. Total RNA was isolated after 9 h of neurotoxin treatment using an RNeasy kit (Qiagen, Valencia, CA) according to the protocol from the manufacturer. Equal amounts of total RNA from three independent neurotoxin treatments were pooled together for each GeneChip hybridization experiment. Two separate GeneChip hybridizations of

pooled, treated, and control RNA were performed, representing six independent experiments. A minimum of 20 µg/sample of total RNA was sent to the Alvin J. Siteman Cancer Center GeneChip Core Facility (Washington University, St. Louis, MO) for generation of labeled cRNA target and hybridization against Affymetrix Murine Genome U74Av2 GeneChip arrays (Santa Clara, CA) using standard protocols (pathbox.wustl.edu/~mgacore). Data were analyzed by Affymetrix Microarray Suite version 5.0, as well as Spotfire Decision Site for Functional Genomics (Somerville, MA). For those transcripts designated both "present" and "increasing" in each replicate by the software, a threshold of an average signal log ratio greater than 0.5 (~1.5-fold change) was set. Transcripts for which signal was less than 3% of the maximum signal were filtered out.

**Reverse Transcription-PCR**—MN9D cells were plated and treated exactly as described for microarray experiments. Total RNA was extracted after 1, 3, 6, 9, and 12 h. Primers to 18 S ribosomal RNA (26) were used to standardize amounts of RNA in each sample. RNA was reverse transcribed using gene-specific reverse primers, and resulting cDNAs were PCR-amplified. PCR primer sequences used were: CHOP (+) and CHOP (−) described in Ref. 27, BiPFwd (TGACTGGAATTC-CTCCTGCT) and BiPRev (AGTCTTCAATGTCCGATCC), c-junFwd (GCTGAAGTGCATAGCCAGAA) and c-junRev (CTTGATCCGCTCCT-GAGACT), and Xbp1Fwd (TAGAAAGAAAGCCCGGA TGA) and Xbp1Rev (CTCTGGGAAGGACATTTGA). PCR products were resolved on a 4% PAGE gel and analyzed with Vistra Green (Amersham Biosciences) detection and quantitative fluorimaging.

**Western Blot Analysis**—For MN9D Western blots, cells were plated and treated exactly as described for microarray experiments. For primary culture Western blots, 600,000 cells/well were plated in six-well plates and treated on the 6th day *in vitro* with 40 µM 6-OHDA or 1 µM MPP<sup>+</sup> (21). MN9D lysates were taken at 1, 3, 6, 9, and 12 h, and primary lysates were taken at 6 and 12 h. Cells were washed once with PBS and harvested in ice-cold radioimmune precipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% NaDoc, 0.1% SDS, 50 mM Tris, pH 8.0) with protease inhibitor mixture (Roche, Mannheim, Germany) and placed on ice for 30 min. Insoluble cell debris was removed by centrifugation, and the protein concentration of cell lysates was determined by the Bio-Rad protein assay. Equal amounts of protein were run on SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Bio-Rad). Mouse monoclonal antibody against CHOP/Gadd153 (1:100) and goat polyclonal antibodies against Hsp60 (1:500) and BiP/Grp78 (1:125) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit polyclonal antibodies against cleaved caspase-3, phospho-c-Jun, phospho-eIF2 $\alpha$ , and phospho-PERK (all 1:1,000) were purchased from Cell Signaling Technologies (Beverly, MA). After incubation with appropriate primary and horseradish peroxidase-conjugated secondary antibodies (anti-mouse 1:5000, Sigma; anti-goat 1:5000, Jackson Immunoresearch, West Grove, PA; or anti-rabbit 1:2000, Cell Signaling Technologies), specific protein bands were detected and analyzed by enhanced chemiluminescence substrate detection (ECL Plus; Amersham Biosciences) and quantitative fluorimaging.

**Immunocytochemistry**—MN9D cells were plated at a density of 300,000 cells/well on a four-well chamber slide. Twelve hours after plating, cells were treated with 75 µM 6-OHDA or 75 µM MPP<sup>+</sup> and fixed 12 h later with 4% paraformaldehyde in PBS. Primary culture cells were plated at a density of 100,000 cells/35-mm microwell plate (1.25 × 10<sup>5</sup> cells/mm<sup>2</sup>; MatTek Corp., Ashland, MA). On day 6 *in vitro*, cells were treated with 40 µM 6-OHDA or 1 µM MPP<sup>+</sup>, and fixed after 12, 18, or 24 h with 4% paraformaldehyde in PBS. Cultures were double-stained with either mouse monoclonal anti-CHOP (1:300) or rabbit polyclonal anti-phospho-c-Jun (1:500), together with rabbit polyclonal (1:500; Pel-Freez, Rogers, AR) or mouse monoclonal (1:2,500; Immunostar, Hudson, WI) antibodies against the dopaminergic neuron marker TH, respectively. Secondary antibodies conjugated with Cy3 (anti-mouse and anti-rabbit 1:300) and Alexa488 (anti-mouse 1:500; anti-rabbit 1:2000) were used. Cells were imaged using an Olympus Fluoview confocal microscope.

**Statistics**—GraphPad Prism software (San Diego, CA) was used for statistical analysis. The significance of effects between control and drug conditions was determined by one-way ANOVA as indicated and *post hoc* Dunnett's multiple comparison tests (GraphPad Prism software).

#### RESULTS

**Cell Death Induced by 6-OHDA and MPP<sup>+</sup> Is Blocked by Inhibition of Macromolecular Synthesis**—Previous studies have characterized 6-OHDA-induced cell death as a caspase-

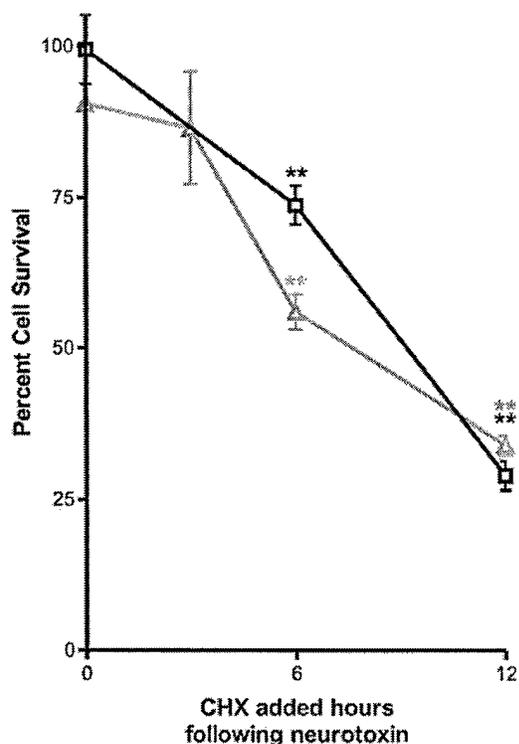


FIG. 1. New protein synthesis is required for MPP<sup>+</sup>- and 6-OHDA-induced cell death. MN9D cells were treated with 100  $\mu$ M 6-OHDA (squares) or 75  $\mu$ M MPP<sup>+</sup> (triangles). One  $\mu$ g/ml cycloheximide (CHX) was added either immediately prior to or at various times following neurotoxin addition. Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and expressed as a percentage of survival compared with control cultures treated with cycloheximide alone. Values represent mean  $\pm$  S.E.,  $n = 4$ . \*\*,  $p < 0.01$  compared with control (one-way ANOVA with post-hoc Dunnett's multiple comparison test). Error bars of less than 2% are buried in the symbol.

dependent, apoptotic process, whereas MPP<sup>+</sup>-induced cell death can occur independent of caspase activation, and without canonical markers of apoptosis (13, 20, 21, 28). Some forms of apoptotic and non-apoptotic cell death require *de novo* synthesis of cell death proteins (29, 30), whereas others do not (31, 32). To determine whether 6-OHDA- or MPP<sup>+</sup>-induced cell death require *de novo* macromolecular synthesis, cultures were treated with the protein synthesis inhibitor cycloheximide. Addition of 1.0  $\mu$ g/ml cycloheximide together with 100  $\mu$ M 6-OHDA or 75  $\mu$ M MPP<sup>+</sup> provided significant protection. In contrast, delaying addition of cycloheximide following neurotoxin treatment resulted in increasing cell death in a time-dependent manner (Fig. 1). These data indicate that, although 6-OHDA induces an apoptotic form of cell death and MPP<sup>+</sup> does not, both types of cell death require *de novo* protein synthesis. Therefore, it may be possible to identify changes in gene expression associated with the cell death process.

**Microarray Analysis Identifies Distinct Changes in Gene Expression following 6-OHDA and MPP<sup>+</sup> Treatment**—Microarray analysis was used to examine the expression profile of a large number of transcripts. Out of the ~12,000 genes and expressed sequence tags represented on the MG-U74Av2 GeneChip, 4,304 (~35% of total) were defined as "present" by the microarray analysis software for MPP<sup>+</sup>-treated samples. Similarly, 4,580 (~37% of total) were defined as present for 6-OHDA-treated samples. Transcripts were subsequently grouped by individual toxin treatment, or by both 6-OHDA and MPP<sup>+</sup> (Fig. 2). Notably, 6-OHDA treatment affected almost three times as many transcripts as MPP<sup>+</sup>. Specifically, 153 transcripts in-

creased in response to 6-OHDA, whereas only 55 transcripts increased in response to MPP<sup>+</sup>. Results for decreasing transcripts were similar (data not shown). Both neurotoxins induced a number of the same transcripts, with 39 of the 55 transcripts induced by MPP<sup>+</sup> also induced by 6-OHDA (Table I). These included genes involved in cell cycle and/or differentiation, signaling, stress, and transcription factors, indicating possible common cell death mechanisms. The most highly induced transcript in response to either treatment was that to the stress protein CHOP/Gadd153. 6-OHDA also induced a large number of transcripts that were unchanged by MPP<sup>+</sup> treatment, including molecular chaperones and other genes involved in protein folding, trafficking, and the ubiquitin-proteasome pathway (Table II). These results support previous findings showing that MPP<sup>+</sup> and 6-OHDA promote distinct yet overlapping programs of cell death.

**CHOP Is Induced in Response to 6-OHDA and MPP<sup>+</sup>**—To confirm the microarray findings that CHOP mRNA was up-regulated by 6-OHDA and MPP<sup>+</sup> in MN9D cells, RT-PCR was performed (Fig. 3A). 6-OHDA induced a large and rapid induction of CHOP mRNA that peaked between 6 and 9 h. MPP<sup>+</sup> induction of CHOP mRNA lagged behind that of 6-OHDA, but continued to increase for at least 12 h (Fig. 3, A and C). These data are consistent with the GeneChip results from a 9-h time point showing greater induction with 6-OHDA than with MPP<sup>+</sup> (Fig. 2 and Table I). Western blotting of MN9D total cell lysates confirmed that levels of CHOP protein were also increasing (Fig. 3, B and C). Again, 6-OHDA induced a larger and more rapid increase in protein expression than did MPP<sup>+</sup> (Fig. 3C). To visualize CHOP induction *in situ* (Fig. 3D), treated cells were fixed, stained, and imaged using confocal microscopy. Control cultures had dim, diffuse staining, whereas both 6-OHDA and MPP<sup>+</sup> treated cells showed intense nuclear staining. This localization is consistent with the role of CHOP as a transcription factor. Together, these results confirm and extend the GeneChip findings that toxin treatment of dopaminergic cells leads to an up-regulation of CHOP mRNA and protein levels.

**RT-PCR Reveals Markers of Unfolded Protein Response Are Up-regulated by 6-OHDA and MPP<sup>+</sup> Treatment**—CHOP is up-regulated by a variety of cellular stresses including ER stress (27, 33–35). Following confirmation of CHOP induction, further analysis of GeneChip results revealed a pattern of induction of other stress-induced genes including many involved in UPR (Fig. 2, Tables I and II). These included molecular chaperones such as BiP/Grp78 and UPR-induced transcription factors other than CHOP (Atf4 and Xbp1). To examine the role that UPR may play in 6-OHDA and MPP<sup>+</sup> toxicity, induction of these transcripts was verified by RT-PCR (Fig. 4, A and B). BiP is an ER-resident chaperone protein central to UPR (36). Levels of BiP mRNA were increased greater than 2-fold over control from 6 to 12 h following 6-OHDA exposure. BiP expression, however, decreased slightly in response to MPP<sup>+</sup> exposure over 12 h. These results were consistent with GeneChip results at 9 h for both 6-OHDA and MPP<sup>+</sup> (Table II). Although not specific to ER stress, activation of the c-Jun N-terminal kinase/stress-activated protein kinase pathway (JNK/SAPK) occurs during UPR (37, 38). Expression of c-Jun mRNA was increased rapidly by 6-OHDA and then maintained at levels 5–6-fold that of control from 3 to 12 h following exposure. MPP<sup>+</sup> treatment resulted in a rapid induction of c-Jun mRNA to 3-fold that of control at 1 h, identical to exposure to 6-OHDA. However, MPP<sup>+</sup> induction of c-Jun mRNA was not sustained and returned to control levels by 9 h.

Another feature of the UPR pathway is the non-conventional removal of 26 base pairs of Xbp1 mRNA by the ER membrane

**FIG. 2. Microarray analysis reveals both common and distinct transcriptional changes induced by 6-OHDA and MPP<sup>+</sup>.** Total RNA from MN9D cells treated with 6-OHDA or MPP<sup>+</sup> in addition to untreated control was used for Affymetrix MG-U74Av2 GeneChip array probe hybridization. Data were analyzed by Affymetrix Microarray Suite version 5 as well as Spotfire Decision Site for Functional Genomics. Transcriptional changes were defined as described in the text. Large plot shows known genes induced by 6-OHDA or MPP<sup>+</sup> treatment plotted as average-fold induction on the x axis and y axis, respectively, with a scale of log<sub>2</sub>. Several genes of interest have been labeled (see Tables I and II for abbreviations used). Independent of their position on the plot, genes were grouped according to those induced by 6-OHDA but not induced by MPP<sup>+</sup> (green squares), those induced by MPP<sup>+</sup> but not induced by 6-OHDA (blue circles), or those induced by both 6-OHDA and MPP<sup>+</sup> (red points). Inset shows all ~12,000 genes represented on the Affymetrix MG-U74Av2 GeneChip. Red points represent the 169 increasing genes identified as described in text.

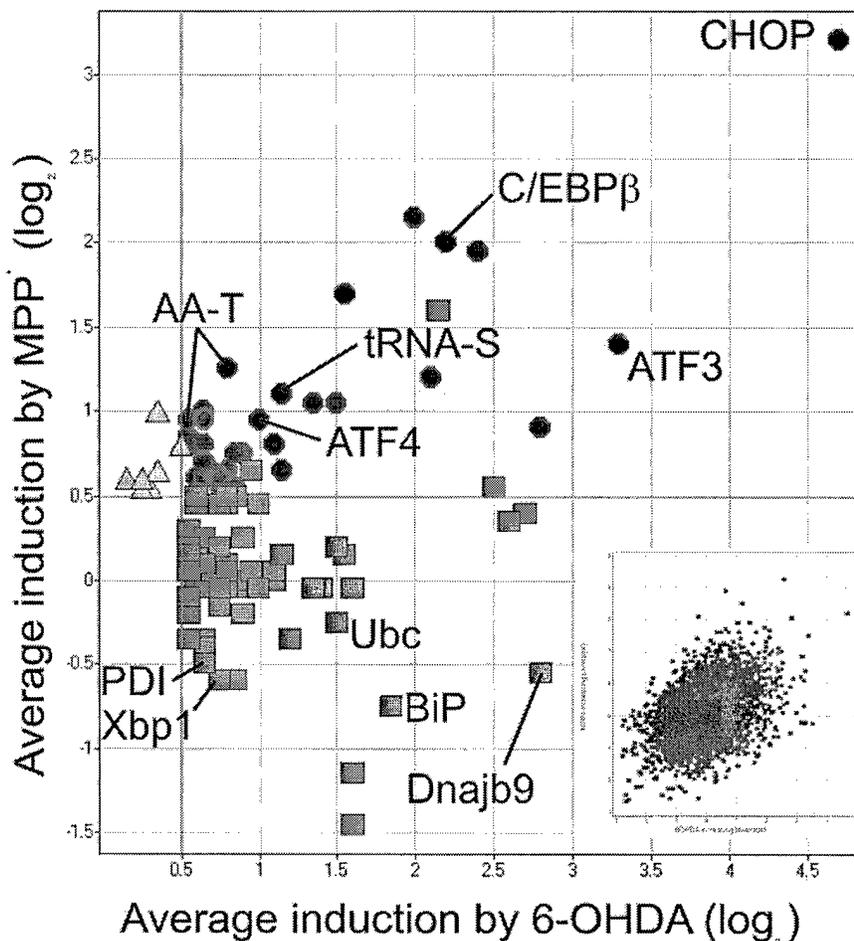


TABLE I  
Genes increased by both 6-OHDA and MPP<sup>+</sup>

Table lists 18 of 39 transcripts increased by both 6-OHDA and MPP<sup>+</sup>.

| Gene symbol     | Gene name   | Change      |                  |
|-----------------|---|-------------|------------------|
|                 |   | 6-OHDA      | MPP <sup>+</sup> |
|                 |   | <i>fold</i> |                  |
| <i>Gadd153</i>  | CHOP/Gadd153  | 26.0        | 9.2              |
| <i>Atf3</i>     | Activating transcription factor 3                             | 9.8         | 2.6              |
| <i>Cebpb</i>    | CCAAT/enhancer-binding protein C/EBP-β                        | 4.6         | 4.0              |
| <i>Sqstm1</i>   | Sequestosome 1  | 7.0         | 1.9              |
| <i>Myd116</i>   | Myeloid differentiation primary response gene 116             | 4.3         | 2.3              |
| <i>Gtpbp2</i>   | GTP-binding protein 2   | 2.9         | 3.2              |
| <i>Lbcl1</i>    | Lymphoid blast crisis-like 1 (cell growth and maintenance)    | 2.5         | 2.1              |
| <i>Cars</i>     | CysteinyI-tRNA synthetase                                     | 2.2         | 2.1              |
| <i>Slc1a4</i>   | Neutral amino acid transporter                                | 1.7         | 2.4              |
| <i>Atf4</i>     | Activating transcription factor 4                             | 2.0         | 1.9              |
| <i>Gas5</i>     | Growth arrest-specific 5                                      | 2.1         | 1.7              |
| <i>Eif4ebp1</i> | Eukaryotic translation initiation factor 4E binding protein 1 | 1.6         | 2.0              |
| <i>Slc7a5</i>   | Solute carrier family 7 cationic amino acid transporter       | 1.5         | 1.9              |
| <i>Glyt1</i>    | Glycine transporter 1   | 1.6         | 1.7              |
| <i>Clic4</i>    | Chloride intracellular channel 4 mitochondrial                | 1.5         | 1.8              |
| <i>Cln3</i>     | Chloride channel 3  | 1.8         | 1.5              |
| <i>Wars</i>     | Tryptophanyl-tRNA synthetase                                  | 1.6         | 1.6              |
| <i>Hspa9a</i>   | Heat shock protein cognate 74 (mitochondrion)                 | 1.6         | 1.5              |

resident protein, Ire1 $\alpha/\beta$ , under conditions of ER stress (39, 40). Moreover, levels of unprocessed Xbp1 mRNA are also increased by ER stress. In response to 6-OHDA but not MPP<sup>+</sup>, Xbp1 was induced almost 2-fold according to the GeneChip analysis (Fig. 2, Table II). To determine whether Xbp1 mRNA was processed, primers flanking the excised portion of Xbp1 mRNA were used to reveal a shift in size of the RT-PCR product

(Fig. 4A). As indicated in Fig. 4B, 6-OHDA produced a large, transient induction of processed Xbp1 mRNA peaking at 3–6 h and returning to near control levels after 12 h. In contrast, MPP<sup>+</sup> treatment resulted in a sustained inhibition of Xbp1 mRNA processing from 3 to 12 h.

*Western Blotting Reveals Markers of Unfolded Protein Response Are Up-regulated by 6-OHDA and MPP<sup>+</sup> Treatment—*

TABLE II  
Genes increased by 6-OHDA onlyTable lists 21 of 114 transcripts increased by 6-OHDA, but not by MPP<sup>+</sup>.

| Gene symbol    | Gene name   | Change      |                  |
|----------------|---|-------------|------------------|
|                |   | 6-OHDA      | MPP <sup>+</sup> |
|                |   | <i>fold</i> |                  |
| <i>Dnajb9</i>  | DNA j protein b9  | 7.0         | 0.7              |
| <i>Herpud1</i> | ER stress-inducible ubiquitin-like domain member 1                            | 6.5         | 1.3              |
| <i>Hmox1</i>   | Heme oxygenase decycling 1  | 5.7         | 1.5              |
| <i>Hspa5</i>   | Bip/Grp78   | 3.0         | 0.5              |
| <i>Ubc</i>     | ubiquitin C   | 2.8         | 0.8              |
| <i>Bag3</i>    | Bcl2-associated athanogene 3 (cytosol anti-apoptotic)                         | 2.6         | 1.0              |
| <i>Tra1</i>    | Tumor rejection antigen gp96 (chaperone calcium binding)                      | 2.3         | 0.8              |
| <i>Sec23b</i>  | SEC23B <i>Saccharomyces cerevisiae</i> (ER intracellular protein trafficking) | 2.1         | 1.0              |
| <i>Por</i>     | P450 cytochrome oxidoreductase  | 2.1         | 1.0              |
| <i>Txnrd1</i>  | Thioredoxin reductase 1   | 1.9         | 1.0              |
| <i>Spc18</i>   | Signal peptidase complex 18-kDa   | 1.9         | 0.9              |
| <i>Arhb</i>    | Aplysia Ras-related homolog B RhoB  | 1.8         | 1.0              |
| <i>Xbp1</i>    | X-box-binding protein 1   | 1.7         | 0.7              |
| <i>Hsc70t</i>  | Heat shock protein cognate 70 testis  | 1.7         | 0.9              |
| <i>Sec23a</i>  | SEC23A <i>S. cerevisiae</i> (ER intracellular protein trafficking)            | 1.6         | 1.0              |
| <i>Ppib</i>    | Peptidylprolyl isomerase B  | 1.6         | 1.0              |
| <i>Grp58</i>   | grp58 kDa (protein-disulfide isomerase)                                       | 1.6         | 0.7              |
| <i>Psmc4</i>   | Proteasome 26 S subunit, non-ATPase, 4  | 1.5         | 1.1              |
| <i>S100a10</i> | Calcium-binding protein A11 calgizzarin                                       | 1.5         | 0.9              |
| <i>Sec61g</i>  | SEC61 gamma subunit <i>S. cerevisiae</i>                                      | 1.5         | 0.9              |
| <i>Uch11</i>   | Ubiquitin C-terminal hydrolase L1   | 1.5         | 1.1              |

Induction of the UPR pathway triggers not only transcriptional changes, but also involvement of protein kinase signaling pathways. One such pathway is that of JNK/SAPK, activation of which leads to phosphorylation of c-Jun (37, 38). In addition to changes in c-Jun mRNA expression (Fig. 4, A and B), Western blot analysis using antibodies against phospho-c-Jun indicated that 6-OHDA administration increased phosphorylation of c-Jun ~6-fold over control levels at 9–12 h (Fig. 5, A and B). In contrast, treatment with MPP<sup>+</sup> induced a transient increase of phosphorylated c-Jun at 3 h, returning to control levels by 6–9 h. These data are consistent with the RT-PCR results indicating a slight, early MPP<sup>+</sup> mediated increase in c-Jun mRNA that was not sustained (Fig. 4, A and B). Taken together these results indicate that cellular responses to 6-OHDA led to the activation of the JNK/SAPK pathway.

Another consequence of UPR is translational attenuation caused by phosphorylation of eIF2 $\alpha$  by the ER membrane resident kinase PERK. Western blotting using antibodies against phospho-eIF2 $\alpha$  revealed that both 6-OHDA- and MPP<sup>+</sup>-mediated toxicity resulted in eIF2 $\alpha$  phosphorylation (Fig. 5, A and B). Specifically, MPP<sup>+</sup> exposure induced a rapid, transient response, whereas 6-OHDA exposure resulted in sustained phosphorylation of eIF2 $\alpha$  from 3 to 12 h. The eIF2 $\alpha$  kinase PERK is itself activated by phosphorylation, and Western results indicated that MPP<sup>+</sup> induced PERK phosphorylation in a profile almost identical to eIF2 $\alpha$  phosphorylation. In contrast, PERK phosphorylation induced by 6-OHDA exhibited delayed kinetics, staying at baseline levels for 3 h following treatment, and then rising 3-fold over the next 9 h. BiP protein levels showed a slight increase over 12 h with 6-OHDA treatment, but not with MPP<sup>+</sup> (Fig. 5A), again consistent with both GeneChip and RT-PCR data. In accordance with previous reports that 6-OHDA induced apoptosis (20, 21), but MPP<sup>+</sup> does not, activated caspase-3 was detected only in 6-OHDA-treated cultures (Fig. 5A). Collectively, these data reveal that many components of UPR, including multiple signaling pathways, were up-regulated in response to 6-OHDA toxicity. In contrast, treatment with MPP<sup>+</sup> led to the up-regulation of some, but not all, markers of UPR. Thus, MPP<sup>+</sup> may ultimately lead to dopaminergic cell death by a pathway that is at least partially independent of UPR.

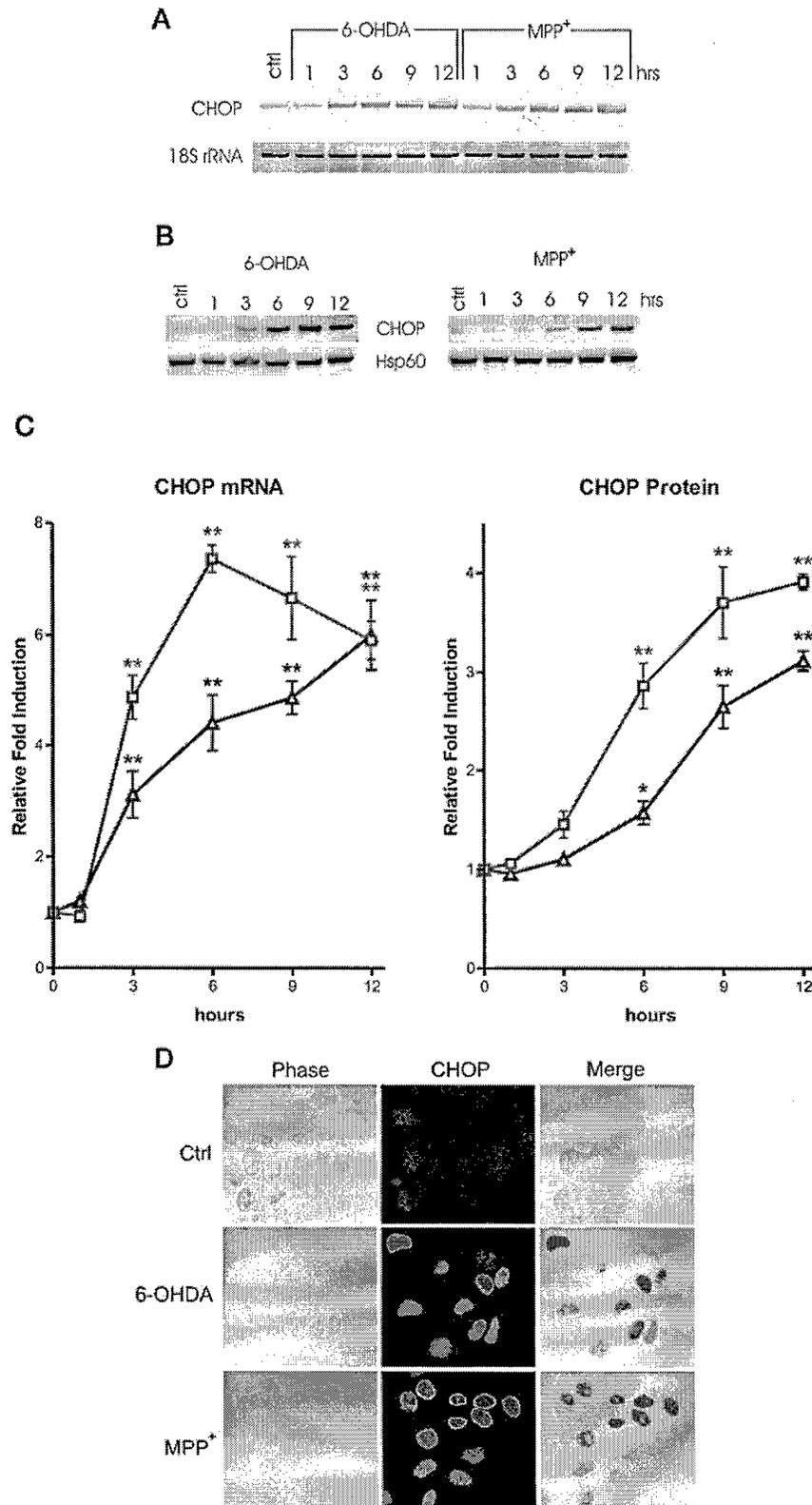
**6-OHDA, but Not MPP<sup>+</sup>, Induces Components of the UPR Pathway in Primary Mesencephalic Cultures**—To determine

whether UPR induction could be observed in primary mesencephalic cultures following neurotoxin treatment, Western blot analysis and immunocytochemistry were performed. Similar to results from the dopaminergic MN9D cells, 6-OHDA increased levels of CHOP protein at 6 and 12 h (Fig. 6A). 6-OHDA also increased phosphorylation of eIF2 $\alpha$  and c-Jun. In contrast, none of the markers seen in the dopaminergic cell line were up-regulated in mesencephalic cultures treated with MPP<sup>+</sup>. Neither 6-OHDA nor MPP<sup>+</sup> induced significant changes in levels of BiP protein over 12 h (data not shown).

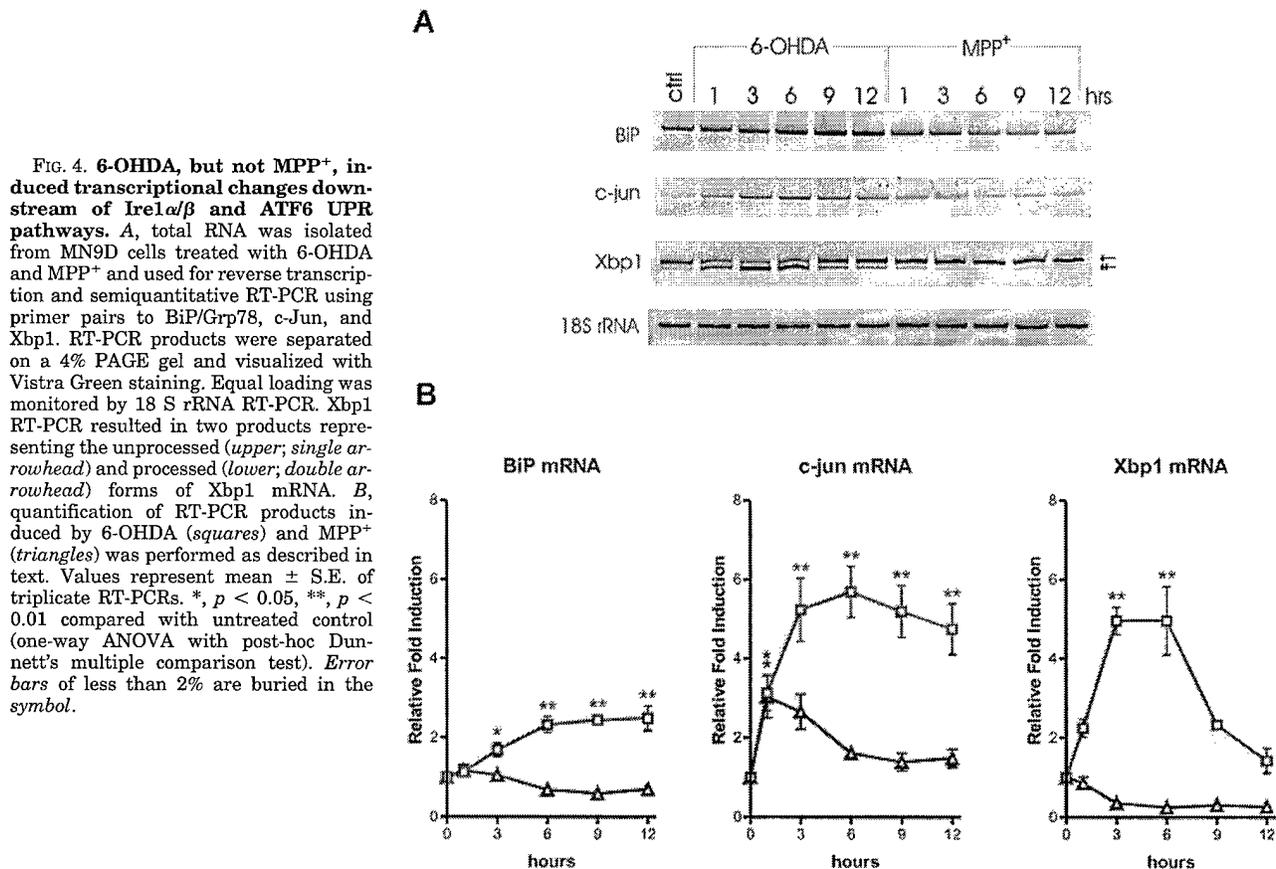
Immunostaining of primary cultures with CHOP and phospho-c-Jun antibodies allowed individual dopaminergic neurons to be examined via co-staining with TH. 6-OHDA-treated cultures displayed intense nuclear staining of CHOP in both dopaminergic neurons as well as in many other cell types. Cultures treated with MPP<sup>+</sup> did not appear different from controls in overall expression of CHOP, nor was CHOP induction detected in dopaminergic neurons over a 24-h period. Similarly, increased expression of phospho-c-Jun was widespread with 6-OHDA treatment in both dopaminergic and non-dopaminergic neurons, whereas there was no obvious change in phosphorylation of c-Jun following MPP<sup>+</sup> administration. Taken together, these results suggest that MPP<sup>+</sup> can induce a partial UPR response in the MN9D cell line but not in cultured dopaminergic neurons. In contrast, 6-OHDA induces a broad spectrum of UPR responses in both MN9D cells as well as in dissociated dopaminergic neurons. Thus, these cells will serve as a useful model in determining the temporal and molecular events associated with 6-OHDA neurotoxicity.

#### DISCUSSION

Accumulating evidence suggests that ER stress induced by aberrant protein degradation plays a role in PD. Beginning with a functional genomics approach to identify transcriptional alterations in a well characterized model of 6-OHDA and MPP<sup>+</sup> toxicity, the present study identified numerous changes in genes associated with UPR. Notably, a major target of the UPR pathway, the transcription factor CHOP, was dramatically up-regulated at both the mRNA and protein levels by either 6-OHDA or MPP<sup>+</sup>. Moreover, 6-OHDA activated numerous other markers of UPR including BiP, splicing of Xbp1 mRNA, the JNK/SAPK pathway, as well as proteins involved in the



**FIG. 3. CHOP is up-regulated following 6-OHDA and MPP<sup>+</sup> administration.** Treatment with 6-OHDA and MPP<sup>+</sup> increased levels of CHOP mRNA isolated from MN9D cells as detected by RT-PCR (A) and levels of CHOP protein isolated from MN9D cells as detected by Western blot analysis (B). Equivalent loading was monitored by 18 S rRNA and Hsp60, respectively. C, quantification of CHOP mRNA and protein induced by 6-OHDA (squares) and MPP<sup>+</sup> (triangles) was performed as described in text. Values represent mean  $\pm$  S.E. of triplicate RT-PCRs and Western blots. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  compared with untreated control (one-way ANOVA with post-hoc Dunnett's multiple comparison test). Error bars of less than 2% are buried in the symbol. D, MN9D cells were fixed after 12 h of neurotoxin treatment and stained with an antibody against CHOP. Left panels are phase bright images showing the morphology of MN9D cells. Middle panels show CHOP immunostaining. Nuclear localization of CHOP can be observed in the merged right panels.



**FIG. 4. 6-OHDA, but not MPP<sup>+</sup>, induced transcriptional changes downstream of Ire1 $\alpha$ / $\beta$  and ATF6 UPR pathways.** *A*, total RNA was isolated from MN9D cells treated with 6-OHDA and MPP<sup>+</sup> and used for reverse transcription and semiquantitative RT-PCR using primer pairs to BiP/Grp78, c-Jun, and Xbp1. RT-PCR products were separated on a 4% PAGE gel and visualized with Vistra Green staining. Equal loading was monitored by 18 S rRNA RT-PCR. Xbp1 RT-PCR resulted in two products representing the unprocessed (upper; single arrowhead) and processed (lower; double arrowhead) forms of Xbp1 mRNA. *B*, quantification of RT-PCR products induced by 6-OHDA (squares) and MPP<sup>+</sup> (triangles) was performed as described in text. Values represent mean  $\pm$  S.E. of triplicate RT-PCRs. \*,  $p < 0.05$ , \*\*,  $p < 0.01$  compared with untreated control (one-way ANOVA with post-hoc Dunnett's multiple comparison test). Error bars of less than 2% are buried in the symbol.

attenuation of translation such as PERK and eIF2 $\alpha$ . In contrast, MPP<sup>+</sup> effects appeared restricted to events associated with PERK and eIF2 $\alpha$  phosphorylation. In confirmation of these cell line results, 6-OHDA also triggered UPR responses in primary cultures of dopaminergic neurons. Collectively these data emphasize that 6-OHDA and MPP<sup>+</sup> induce distinct cell death responses. Inasmuch as 6-OHDA is widely used to create animal models of PD, the present findings further support the notion that ER stress and ubiquitin-proteasome dysfunction is associated with this disorder.

**Biological Sequelae Associated with PD Mimetics**—Oxidative stress and mitochondrial dysfunction have long been implicated in PD (41). Because of this, two neurotoxins exhibiting specificity toward dopaminergic neurons, 6-OHDA and MPP<sup>+</sup>, are commonly used to model nigral degeneration. 6-OHDA is a potent inducer of oxidative stress that can be endogenously converted from dopamine (13). Dopamine quinone derivatives including 6-OHDA have been found in post-mortem PD brains (14–16), implicating dopamine itself as a factor in this disorder. MPTP was originally identified because accidental human exposure led to PD (18, 42). MPTP, and its active metabolite MPP<sup>+</sup>, are also thought to induce oxidative stress in addition to inhibiting mitochondrial function (17). The discovery that mutations in  $\alpha$ -synuclein (2, 3), parkin, and UCH-L1 (5, 9, 43, 44) are associated with PD led to the recognition that impaired protein degradation is also an important factor in this disorder. Mechanistically, however, it is still unclear what the common thread is among these seemingly disparate cellular responses.

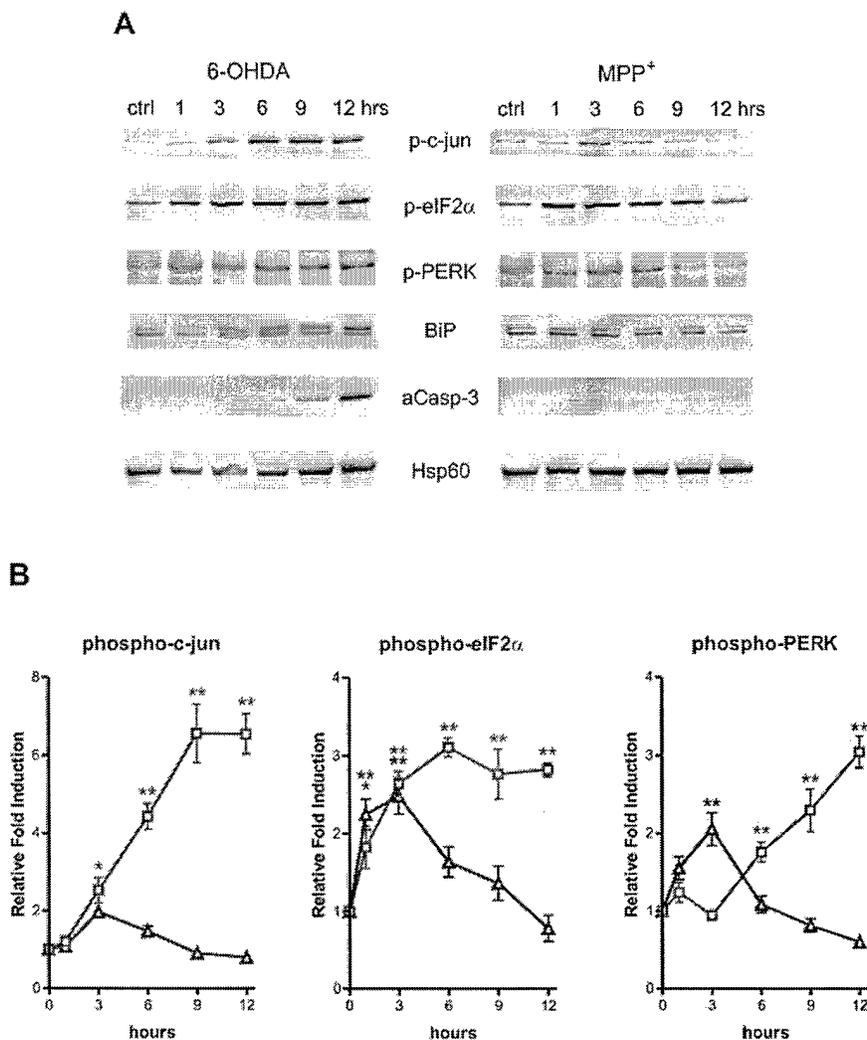
The present study utilized gene expression profiling to assess thousands of genes to obtain a more detailed understanding of the molecular programs utilized by dopaminergic cells in response to 6-OHDA and MPP<sup>+</sup>. Two important outcomes from this study include the identification of a previously unsus-

pected link between these known oxidative stress inducers and aspects of ER stress/UPR, as well as the identification of at least a subset of common transcriptional changes associated with toxin-mediated events. The latter observation emphasizes the overlapping yet divergent nature of cell death in response to 6-OHDA versus MPP<sup>+</sup>.

Commonality in response to 6-OHDA and MPP<sup>+</sup> is highlighted by the finding that the most highly induced transcript by either toxin was CHOP, a stress-induced transcription factor implicated in cell death (34, 45). The temporal and spatial up-regulation of CHOP was confirmed and extended by RT-PCR, Western blot analysis, and immunocytochemistry (Fig. 3). In support of the present findings, microarray analysis of MPP<sup>+</sup>-treated SH-SY5Y cells also resulted in an up-regulation of CHOP, albeit with a much later, more prolonged time course (46). Similarly, microarray analysis of the dopaminergic cell line, SN4741, revealed induction of stress indices following MPP<sup>+</sup> treatment (47). To date, however, this is the first report that 6-OHDA up-regulates CHOP, and that it does so to a much greater extent than MPP<sup>+</sup>.

Additional transcripts identified via microarray analysis revealed that 6-OHDA induced a large number of genes that were not positively affected by MPP<sup>+</sup>, many of which were involved in protein folding, trafficking, or degradation (Table II). In contrast, the subset of genes induced by both drugs included amino acid transporters, tRNA-synthetases, ion channels, and stress-induced transcription factors (Table I). A small number of genes was induced by MPP<sup>+</sup> but not 6-OHDA. These included Dnaja3, adaptor-related protein complex AP-3  $\beta$ 1 subunit, and myelin transcription factor 1. Currently, the significance of these changes is unclear. Overall, MPP<sup>+</sup>-induced transcripts appeared to primarily represent a subset of genes induced by 6-OHDA.

**FIG. 5. 6-OHDA induced sustained phosphorylation of proteins associated with Ire1 $\alpha$ / $\beta$  and PERK UPR pathways, whereas MPP<sup>+</sup> induced only transient changes.** Protein lysates were prepared from MN9D cells treated with 6-OHDA and MPP<sup>+</sup>. **A**, antibodies against the phosphorylated forms of c-Jun (*p-c-jun*), eIF2 $\alpha$  (*p-eIF2 $\alpha$* ), and PERK (*p-PERK*) were used for Western blot analysis. Additional antibodies were used to detect BiP, activated caspase-3 (*aCasp-3*), and Hsp60 as a protein loading control. **B**, quantification of phosphorylated proteins induced by 6-OHDA (squares) and MPP<sup>+</sup> (triangles) was performed as described in text. Values represent mean  $\pm$  S.E. of triplicate Western blots. \**p* < 0.05; \*\**p* < 0.01 compared with untreated control (one-way ANOVA with post-hoc Dunnett's multiple comparison test). Error bars of less than 2% are buried in the symbol.

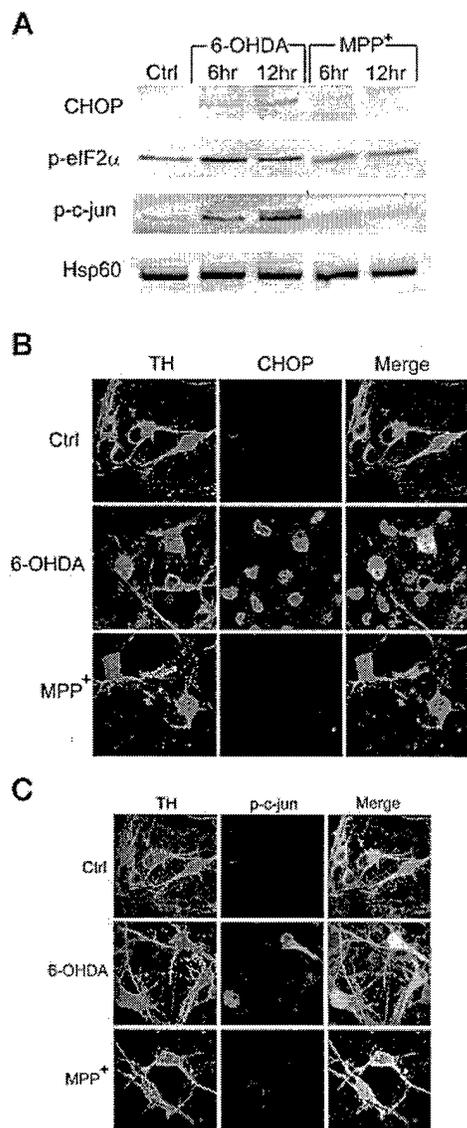


**UPR Signaling Pathways**—Three signaling pathways have been associated with UPR that are triggered by the ER proteins, Ire1 $\alpha$ / $\beta$ , ATF6, and PERK review (48). The Ire1 $\alpha$ / $\beta$  pathway is thought to activate caspase-12, the JNK/SAPK pathway, as well as Xbp1 mRNA splicing (37, 39, 40, 49). Translocation of ATF6 to the nucleus leads to the up-regulation of Xbp1 as well as various ER chaperones (48, 50). Finally, in addition to transcriptional changes, ER stress/UPR can down-regulate protein translation through phosphorylation of eIF2 $\alpha$  via PERK kinase activity (48). Of interest, there is some redundancy in these cascades. For example, CHOP can be up-regulated by both the ATF6 and PERK pathways (50, 51). CHOP, as well as many chaperone proteins, contains a binding site called the ER stress element in its promoter region. In the nucleus, ATF6 binds to ER stress element sites activating CHOP transcription. In addition, CHOP contains a second site called the amino acid response element that is bound by the transcription factors ATF4 and C/EBP $\beta$ . ATF4 is activated when eIF2 $\alpha$  is phosphorylated by PERK (48) or other eIF2 $\alpha$  kinases (52, 53). Thus, signaling through PERK also leads to the up-regulation of CHOP.

GeneChip analysis indicated that many of the genes induced by either MPP<sup>+</sup> or 6-OHDA were increased to a similar extent. A notable exception, however, was that 6-OHDA induced CHOP 26-fold compared with 9-fold with MPP<sup>+</sup> (Fig. 2, Table

I). Moreover, although both neurotoxins increased ATF4 and C/EBP $\beta$ , only 6-OHDA increased Xbp1 mRNA levels (Fig. 2). These data are consistent with the notion that 6-OHDA triggered both ATF6 and PERK pathways leading to the dual activation of the CHOP promoter. Moreover, processing of Xbp1 mRNA, indicating activation of the Ire1 $\alpha$ / $\beta$  pathway, was only observed with 6-OHDA. Although at present we have no clear evidence that caspase-12 is activated (data not shown), 6-OHDA but not MPP<sup>+</sup> also dramatically up-regulated c-Jun mRNA (Fig. 4) and markedly increased phospho-c-Jun levels (Fig. 5). Taken together, it seems reasonable to propose that 6-OHDA is activating all three branches of the UPR signaling cascade, Ire1 $\alpha$ / $\beta$ , ATF6, and PERK, whereas MPP<sup>+</sup> is only activating the PERK branch. One possible model summarizing these results is shown in Fig. 7.

Additional support for this hypothesis comes from studies showing that eIF2 $\alpha$  can also be phosphorylated by other kinases such as GCN2 in response to amino acid starvation (52) or PKR in response to viral infection (53). Thus, phosphorylation of eIF2 $\alpha$  does not require activation of the entire UPR and can lead to induction of genes downstream of ATF4, but not ATF6 (50, 51). The present findings are consistent with the model that MPP<sup>+</sup> triggers eIF2 $\alpha$  phosphorylation (Fig. 7) without involving ATF6 and Ire1 $\alpha$ / $\beta$  activation. These data are remarkably similar to a recent report showing that arsenite



**FIG. 6. 6-OHDA up-regulates CHOP in primary mesencephalic neurons.** Protein lysates were prepared from primary mesencephalic cultures treated with 6-OHDA and MPP<sup>+</sup>. **A**, Western blot analysis of primary lysates was done using antibodies against CHOP, phosphorylated eIF2 $\alpha$  (*p-eIF2 $\alpha$* ), phosphorylated c-Jun (*p-c-jun*), and Hsp60 as a protein loading control. **B**, primary cultures treated for 18 h were fixed and stained for CHOP and TH. **C**, primary cultures treated for 12 h were fixed and stained for phospho-c-Jun and TH.

exposure of primary neuronal cells led to the up-regulation of CHOP expression without a concurrent activation of UPR (54). Thus, MPP<sup>+</sup>-mediated cell death parallels that described for amino acid starvation and/or toxin treatment.

**6-OHDA- or MPP<sup>+</sup>-mediated Cell Death**—Previously we and others have shown that, although 6-OHDA and MPP<sup>+</sup> both generate oxidative stress, only 6-OHDA treatment resulted in activation of caspases and morphological changes associated with apoptosis (20, 21). Several lines of evidence from this laboratory suggest, however, that 6-OHDA does not mediate an intrinsic, mitochondrial dependent, apoptotic pathway. For example, overexpression of the anti-apoptotic protein, Bcl-2, did not attenuate 6-OHDA-induced cell death in either the MN9D cell line or in primary dopaminergic neurons (22, 25). Moreover, deletion of the pro-apoptotic Bcl-2 family member, Bax, did not rescue dopamine neurons from 6-OHDA toxicity (25),

nor was Bax protein translocated to the mitochondria in response to this toxin.<sup>2</sup> Finally, microarray analysis failed to detect up-regulation of any BH3-only family proteins thought to act upstream of the intrinsic mitochondrial pathway, even though downstream caspases were activated (Fig. 5A). Thus, these data support a model in which 6-OHDA activates apoptosis without involving the intrinsic mitochondrial pathway.

Another possibility is that 6-OHDA activates the extrinsic apoptotic pathway involving death receptors such as Fas and the induction of caspase-8. The extrinsic pathway can occur independent of *de novo* protein synthesis (32, 55, 56) as well as Bcl-2 family member expression (for review see Ref. 57). However, activation of the extrinsic pathway requires ligand-mediated death receptor multimerization, adaptor proteins such as FADD, as well as autoproteolysis of caspases-8 and -10 (for review see Ref. 58). In the case of 6-OHDA-induced apoptosis, utilization of the extrinsic pathway seems unlikely because it was dependent on new protein synthesis, known death-inducing ligands were not identified by microarray analysis, and so-called death receptors (Fas (APO-1, CD95), tumor necrosis factor receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand receptor I and II, etc.; Ref. 59) as well as Fas-associated death domain were not detected either. In contrast, a growing body of evidence indicates that ER stress can induce apoptosis independent of both extrinsic and intrinsic pathway factors requiring instead caspase-12 and caspase-9 (60, 61). Apoptosis mediated by 6-OHDA appears to have more characteristics in common with this alternative, non-mitochondrial, pathway, although the involvement of caspases-9 and -12 remains to be determined.

The present data as well as previous studies (20, 21) help to order and clarify the temporal events following neurotoxin treatment. Previous studies of primary dopaminergic neurons have shown that 6-OHDA induced an immediate increase (minutes) in reactive oxygen species (ROS) (21). The current findings suggest that following ROS generation 6-OHDA treatment quickly leads to the induction of c-Jun and processed Xbp1 mRNA (Fig. 4). These mRNAs are increased after 1 h and reach near maximal values by 3 h. Another early event is the phosphorylation of eIF2 $\alpha$ , which is also increased significantly at 1 h, peaks at 3 h, and then stays elevated for the next 9 h (Fig. 5). Presumably triggered by the aforementioned primary events, a distinct second wave of transcriptional responses occurs, exemplified by CHOP and BiP. The latter are unchanged at 1 h and then rise rapidly (Fig. 3, 4). Phosphorylation of c-Jun also occurs during this time (Fig. 5). Reflecting an earlier increase in levels of CHOP mRNA, increased CHOP protein is detected after 6 h (Fig. 3). In addition, phosphorylation of PERK is not detected until 6 h following 6-OHDA exposure (Fig. 5). The last event to occur in this study was the activation of caspase-3, which was barely detectable at 9 h and only increased significantly after 12 h (Fig. 5A). Previous studies have shown that the pan-caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone blocks 6-OHDA toxicity in MN9D cells (20) and that the pan-caspase inhibitor bocasparyl(Ome)-fluoromethylketone is similarly effective in cultured dopaminergic neurons (21). Thus, a broad, multiphasic program of transcriptional, translational, and post-translational events precedes 6-OHDA-induced dopaminergic cell death.

Following transient increases, MPP<sup>+</sup>-induced phospho-PERK, phospho-eIF2 $\alpha$ , and phospho-c-Jun levels all decreased to near control levels after 6–9 h of exposure, whereas these same proteins remained phosphorylated in response to

<sup>2</sup> W. A. Holtz and K. L. O'Malley, unpublished observation.

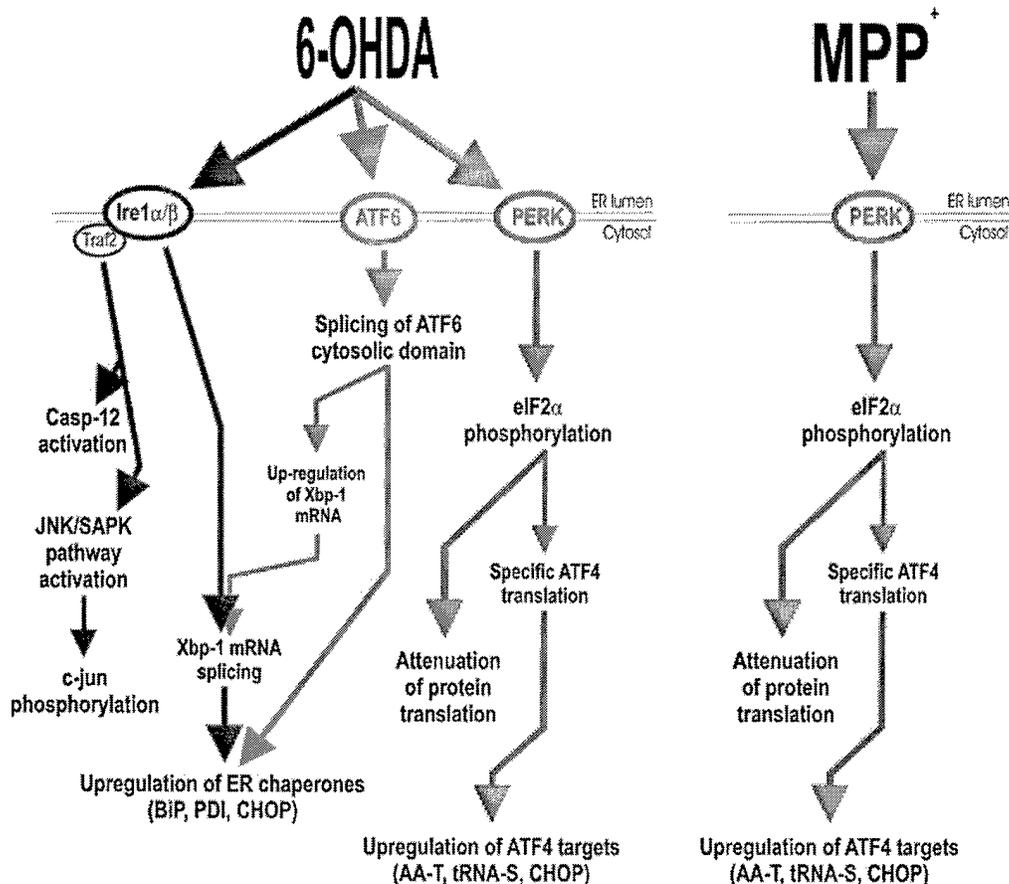


FIG. 7. 6-OHDA induces multiple targets of UPR, whereas MPP<sup>+</sup> is restricted to the PERK pathway. The mammalian UPR consists of three ER membrane resident proteins (Ire1 $\alpha/\beta$ , ATF6, and PERK) that sense ER stress and activate the UPR pathway resulting in transcriptional changes and attenuation of protein translation. The current studies demonstrate that 6-OHDA induces all three arms of the UPR leading ultimately to the transcriptional changes first identified by microarray analysis. In contrast, MPP<sup>+</sup> is restricted to phosphorylation of PERK and eIF2 $\alpha$ , resulting in up-regulation of a subset of genes induced by 6-OHDA.

6-OHDA. Why then are MPP<sup>+</sup> mediated changes transient? One possible explanation is that, although both toxins initially trigger the same response as a result of oxidative stress, this response diverges as MPP<sup>+</sup> more effectively depletes cellular energy. Conceivably, only 6-OHDA-treated cells retain sufficient energy to execute apoptosis. On the other hand, BiP and Xbp1 mRNA did not increase significantly at any time following MPP<sup>+</sup> treatment, but were induced by 6-OHDA. This might indicate that the two responses are distinct from the beginning, despite sharing common participants.

In primary cultures, the difference between 6-OHDA and MPP<sup>+</sup> appears to be even more distinct. Markers of UPR seen in 6-OHDA-treated MN9D cells were also seen in 6-OHDA-treated primary cultures (Fig. 6). In contrast, MPP<sup>+</sup> did not appear to up-regulate CHOP or to phosphorylate eIF2 $\alpha$  or c-Jun in dissociated dopaminergic neurons (Fig. 6). Further investigation will be needed to determine whether this is the result of differences between MN9D cells and primary cells, or of the manner or timing in which the cells were treated.

Unraveling the biological processes by which PD mimetics induce their neurotoxic effects is important to accurately model this disease. However, despite decades of use, the complex signaling pathways by which 6-OHDA and MPP<sup>+</sup> act remain unclear. The unsuspected finding that 6-OHDA and MPP<sup>+</sup> trigger components of the UPR pathway will lead to a better understanding of the application of these agents in models of nigral degeneration and improve the interpretation of the results. In addition, information obtained from 6-OHDA- or

MPP<sup>+</sup>-mediated cell death may also contribute toward understanding other disorders such as excitotoxicity, amyotrophic lateral sclerosis, ataxias, etc. These findings support the emerging role of ubiquitin-proteasome system dysfunction in PD, and provide a connection between oxidative stress, mitochondrial dysfunction, and impaired protein degradation.

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**Note Added in Proof**—While this manuscript was under review, Ryu *et al.* (Ryu, E. J., Harding, H. P., Angelastro, J. M., Vitolo, O. V., Ron, D., and Greene, L. A. (2002) *J. Neurosci.* **22**, 10690) demonstrated induction of the unfolded protein response in 6-OHDA-treated PC12 cells and sympathetic neurons. This supports our findings in MN9D cells and primary dopaminergic cultures that 6-OHDA is an inducer of ER stress.

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