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TITLE: Protective Mechanisms Against Apoptic Neurodegeneration in the Substantia Nigra

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The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
The goal in this proposal is to understand mechanisms by which neurotoxicity destroys cells in the substantia nigra. Our hypothesis is that c-JUN kinase (JNK) mediates neurodegeneration in the substantia nigra after exposure to MPTP or glutamate excitotoxicity. In the first year, we examined mice for susceptibility to MPTP induced neurodegeneration. Our results indicated that JNK knockout mice (lacking isoform 1) were resistant to the toxic effects of MPTP, compared to wild-type (WT) mice. Our objective in year 2 was to confirm these results with WT of the same strain background as the JNK knockout mice. Neuroprotection is still evident with a larger group of JNK 1 animals. We also extended the results to JNK 2 knockout and JNK 3 knockout mice. We completed the inbreeding of the 129/B6 mice to form a B6 background (10 generations). Furthermore, we initially proposed to find whether JNK activation of AP-1 regulated gene transcription (through c-JUN phosphorylation) was necessary for JNK mediated cell death. In year 2 we developed a rapid PCR method to detect transcription of luciferase in mice containing an AP-1 dependent luciferase transgene. In year 3, we will complete the studies on the role of JNK isoforms in MPTP induced neurotoxicity in the original 129/B6 and the newly derived B6 strains of mice. We will crossbreed the AP-1 luciferase transgenics with the JNK knockout and then investigate the molecular mechanism of possible protection in the JNK knockout mice.
INTRODUCTION

Subject and purpose. Cell death in the substantia nigra causes Parkinson’s disease. Ingestion of the neurotoxin MPTP destroys neurons in the substantia nigra and leads to a rapid and severe form of Parkinson’s disease (1,2). Studies on neurotoxins, including MPTP, can provide information on mechanisms in the pathogenesis of Parkinson’s disease and potentially other neurodegenerative diseases.

There is compelling evidence that neurons die an apoptotic death in Parkinson’s disease and several other neurodegenerative diseases, such as Huntington’s disease (3-6). In apoptosis, neurons recruit intracellular signals that ultimately lead to DNA cleavage, nuclear shrinkage and rapid engulfment of the dying cells by glia. The rapid removal of cells protects the surrounding tissue from non-specific spillage of cell contents.

Scope. The initiation and internal cell signaling of apoptosis is complex. Extracellular cues (binding of FAS ligand to FAS) and intracellular signals (release of cytochrome C from the mitochondria) can initiate or mediate apoptosis. c-JUN kinase (JNK) mediates several of the intracellular pathways in apoptosis (7,8). One of the isoforms of JNK (JNK 3) participates in apoptosis in the hippocampus after treatment with the neurotoxin, kainate. The absence of JNK 3 (in knockout mice) protects the hippocampus from apoptosis.

Activation of JNK leads to phosphorylation of the transcription factor, c-JUN. Phospho-c-JUN, in combination with other transcription factors binds to regulatory domains of gene promoters and (usually) increases gene transcription in many genes (11-14). A direct role for JNK dependent, c-JUN phosphorylation in apoptosis has not been demonstrated. This JNK activity would be clinically important. If the two events can be dissociated (AP-1 regulation of gene transcription and apoptosis), then pharmaceutical blockage of JNK to protect against neurodegeneration would not also repress essential transcription of genes.

Our original hypothesis is that JNK, especially JNK 3 isoform, mediates degeneration of substantia nigra neurons after exposure to the neurotoxin MPTP or excitotoxins. Our first approach has been to examine the effects of MPTP in knockout mice for JNK 1, 2 or 3 on survival of dopamine containing neurons in the substantia nigra. We have developed a PCR method to resolve whether MPTP dependent cell death involves AP-1 regulated gene transcription. We use transgenic mice containing a gene with an AP-1 promoter and a luciferase reporter.

Background summary. Several lines of studies converge to support investigation of a role of JNK in neurotoxin dependent Parkinsonism. First, there is ample evidence that neurotoxins can kill neurons via apoptosis (5). JNK proteins have important roles in apoptosis; absence of JNK 3 prevents apoptosis in a well-recognized animal model of excitotoxin induced apoptosis in the brain (9). The substantia nigra in patients with Parkinson’s disease can exhibit apoptotic changes. Altogether, these findings support a possible role for JNK in neurotoxin induced degeneration of substantia nigra neurons.
PROTECTIVE MECHANISMS AGAINST APOPTOTIC NEURODEGENERATION IN THE SUBSTANTIA NIGRA

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Thus, if we can substantiate our hypothesis, blocking the activity of a selective JNK isoform would serve to protect against neurotoxin induced apoptosis.

**BODY**

Experiments were performed to study the effects of MPTP on neuronal morphology and survival in the substantia nigra in JNK knockout and JNK replete mice.

*Experimental methods.*

*Animals.* We used JNK 1, JNK 2 and JNK 3 knockout mice. Control mice were sibling mice with both alleles for each JNK isoform. Thus, the strain was 129/B6 for each experimental animal. (In year 1, non-129/B6 strains were used as controls.) Mice were at least 6 months old, because MPTP neurotoxicity is age dependent and most apparent in older mice. Animals were injected with MPTP or vehicle in a glove box in a P3 facility and were kept alive for 7 days before immunohistochemical analysis.

*MPTP use.* MPTP was injected intraperitoneally. From experiments in year 1, we standardized the dose of MPTP to 20 mg/kg ip x 5 days. Animals were allowed to survive 2 additional days.

*Immunohistochemistry.* Animals were anesthetized with Avertin (0.23 ml/10 g bw ip), perfused with 4% paraformaldehyde and post-fixed for 2 hours. Brain sections (50 μm) were cut on a Vibratome, blocked with horse serum and treated with anti-tyrosine monoclonal antibody (Sigma) at a dilution of 1:100. Vectorstain anti-mouse ABC kit was used to detect the diaminobenzidine reaction product. Five or six sections through the substantia nigra were processed.

*Cell analysis.* Three MPTP dependent outcomes were measured. We counted tyrosine hydroxylase (TH) labeled cells in the substantia nigra, excluding the ventral tegmental area. Three investigators counted TH labeled neurons in two sections in each mouse. Sections that had the fullest extent of the substantia nigra were selected. The investigators had no prior knowledge of the treatment or JNK status of the mice. The cell counts were then averaged for each mouse.

Dr. Marian DiFiglia evaluated the morphology of each experimental animal. Morphological characteristics included soma size and shape, dendritic size and shape, presence of vacuoles, nuclear shrinkage, and overall neuropil immunohistochemical labeling.

We used computer based image analysis (Sigma scan densitometry) to measure the intensity of tyrosine hydroxylase immunoreactivity in the neuropil of the lateral substantia nigra. The neuropil labeling correlates mainly with dendritic arborization. The lateral substantia nigra, in our observations, is affected foremost after MPTP injection. We will undertake this analysis for all of our mice. Information on 23 mice is provided below.
Statistical analysis. Measurements will be provided to the medical school statistician, Dr. Stephen Baker. We found an effect of JNK knockout on cell survival in year 1 and used a Tukey's test. Most of those animals were JNK 1 knockout. We have extensively increased our populations and will require at least 6 mice per group (anticipated ≥8), to be accomplished this year. We will carry out an ANOVA with Bonferroni corrections once we have sufficient animals in each group.

Results.

Positive findings. In year 1, we started colonies of the three JNK knockout lineages (JNK1, JNK2 and JNK3). Our initial results included 7 vehicle (3 WT and 4 JNK knockout) and 14 MPTP treated (4 WT and 10 JNK). To date, we now have used over 100 mice. Each is genotyped to ensure the appropriate JNK knockout background.

Based on cell counts alone (with the caveat that we need to study more mice), the JNK 1 knockout is most protective against MPTP neurotoxicity (Figure 1). We did not undertake statistical analysis, because we need to increase the number of JNK 1 (+/+) sibling controls. The number of TH labeled neurons in the substantia nigra in JNK 1 knockout mice was approximately twice the number of TH neurons in WT sibling mice that had the normal complement of JNK 1 alleles. Furthermore, the JNK 1 knockout mice treated with MPTP had a similar number of TH neurons as did untreated JNK 1 knockout and WT controls. We need to increase the number of WT animals; these mice are nearing the appropriate age (>6 months) for experimental use.

JNK 3 mice show a similar effect of MPTP in both the knockout and sibling controls. There is a wide variation in the number of TH cells (Figure 2). This variation can be explained, in part, by the persistence of morphologically altered TH neurons in the substantia nigra in the MPTP treated animals. The TH neurons remain and therefore need to be included in the counts. However, the neurons appear affected by the neurotoxin. Dr. DiFiglia has confirmed these results (see below, Figure 3).

JNK 2 knockout mice currently lack sibling littermate controls of sufficient age for comparison of the MPTP effect. Two groups of mice have been used, JNK 2 knockout treated and untreated with MPTP. We need to study more JNK 2 mice.

Neuropathologic review. To date, Dr. DiFiglia has examined 20 mice. She plans to complete her analysis in the next few months, by which time we will have sufficient numbers of mice in each category of treated vs untreated and JNK knockout vs JNK replete sibling. Her analysis has not yet been decoded.

For this progress report, we measured the neuropil intensity of TH labeling in sets of JNK 1 and JNK 3 mice (2 to 4 mice in each set). Our initial data revealed that MPTP decreased TH labeling in JNK 1 and JNK 3 sibling controls, but the absence of JNK 1 and JNK 3 protected against the MPTP neurotoxicity (Figure 3). These initial results confirm the neuroprotection offered by JNK 1 knockout as measured by TH cell counts in the substantia nigra. The results on JNK 3 knockout mice correspond to our clinical
observations that the TH neurons remaining after MPTP can be counted but are adversely affected by the MPTP. Examples of the neuropil labeling in different conditions are given in Figure 4).

**Measurement of AP-1 dependent gene transcription in situ.** One of our goals in years 3 and 4 of the project is to find if JNK activity that mediates apoptosis requires AP-1 dependent gene transcription. The best known role of JNK in cells is to phosphorylate c-JUN, to regulate gene transcription at AP-1 promoter sites. To accomplish this study, we planned to use transgenic mice that contain a transgene composed of an AP-1 promoter and a luciferase gene reporter. As a proof of principle, we showed that luciferase activity in the striatum was increased after local injection with quinolinic acid (an NMDA receptor agonist). The brain tissue was homogenized and luciferase activity was measured in a luminometer after addition of luciferin. The two to three-fold increase in luciferase activity was optimal 48 hours after injection. To get a faster measurement of the AP-1 promoter action, we devised a PCR method to estimate luciferase mRNA content in the brain tissue. Luciferase mRNA was detected by RT-PCR using primers directed against the luciferase cDNA sequence. Figure 5 shows the results from this analysis. No luciferase mRNA is detected in either injected or uninjected wild type mouse striatum, but AP-1 transgenic mice show substantial induction of luciferase mRNA 7 or 24 hours after intrastrital injection with quinolinic acid. Injection with vehicle (PBS) results in only trace induction compared to injection with quinolinic acid (data not show).

To confirm that this RT-PCR product corresponds to luciferase mRNA, we sequenced the DNA band generated by the RT-PCR. The sequence obtained corresponds exactly to bases 1523-1826 of luciferase cDNA, as we would have predicted.

Therefore, we found that activation of the AP-1 promoter using the AP-1 promoter/luciferase reporter system results in rapid induction of luciferase message (7 hours), which is followed by a slower accumulation of luciferase protein over the course of several days. Because AP-1 transgenic mice are derived in a B6 strain background and we have inbred our JNK knockout mice into this same strain, we can now mate AP-1 transgenic and JNK knockout as were originally proposed. We plan to use these combined transgenic/knockout mice to study a possible role of AP-1 dependent gene transcription in JNK mediated (or JNK knockout protected) cell death after neurotoxin treatment.

**Negative findings.** Further study of JNK 2 knockout mice did not show an extensive neuroprotection, as we had anticipated in year 1.

**Problems in accomplishing tasks.** We have now bred the JNK mice lineages to a B6 background through generation 10. We were able to accomplish the inbreeding by inducing ovulation in adolescent females at one month, rather than waiting 2 months. The JNK knockouts are young and will be tested in about 6 months. We decided to use 10 inbred crosses, which is a conservative estimate to create a single genetic strain. As
noted, JNK 1 and JNK 2 littermate controls need to be increased for study. We also
found that MPTP injection did not always cause loss of tyrosine hydroxylase containing
neurons. Instead, cells remained, but appeared shrunken with poor dendritic arborization.
We therefore used other morphological criteria to evaluate the MPTP effects. These
included extent of dendritic arborization, shape and size of the neurons, and neuropil
labeling for tyrosine hydroxylase immunoreactivity.

KEY RESEARCH ACCOMPLISHMENTS

• Involvement of c-JUN kinase in MPTP induced neurotoxicity in the substantia
nigra

• Knockout mice lacking c-JUN kinase, isoform 1, have neuroprotection against
MPTP induced neurotoxicity

• Neuroprotection by absence of c-JUN kinase might be graded: c-JUN kinase 1
has better overall protection than does c-JUN kinase 3

• Demonstration that NMDA agonist, quinolinic acid, can increase AP-1
promoter activity: use of AP-1 promoter/luciferase reporter and measurement of
luciferase gene transcription by RT-PCR

REPORTABLE OUTCOMES. We expect to have several manuscripts submitted and
abstracts presented this year, upon completion of the first set of experiments.

CONCLUSIONS

Our hypothesis is that JNK mediates degeneration of substantia nigra neurons
after treatment with MPTP. Our results to date indicate that JNK might mediate the
neurotoxicity of MPTP. In particular, JNK 1 knockout mice have neuroprotection as
measured by survival of neurons and neuronal elements containing tyrosine hydroxylase.
JNK 3 knockout mice offer somewhat less neuroprotection, as measured by cell count
and neuropil labeling. Separate effects of JNK 1 and JNK 3 could be clinical useful, to
permit administration of a selective JNK inhibitor. Furthermore, we are now in a position
to study a role for gene transcription in JNK mediated apoptosis; we developed an assay
for measurement of rapid gene transcription of luciferase in AP-1 luciferase transgenic
mice.

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FIGURE LEGENDS

Figure 1. Survival of tyrosine hydroxylase labeled neurons in the substantia nigra: effect of JNK 1 knockout. Mice were injected with MPTP as described in the methods. Immunohistochemistry was performed with an anti-tyrosine hydroxylase monoclonal antibody. A = JNK 1 knockout, MPTP treated (n=8), B = JNK 1 knockout, no treatment (n=3), C = JNK 1 WT, MPTP treated (n=2), D = JNK 1 WT, no treatment (n=3). TH = tyrosine hydroxylase labeled neurons.

Figure 2. Survival of tyrosine hydroxylase labeled neurons in the substantia nigra: effect of JNK 3 knockout. The same experimental paradigm as in Figure 1 was used. A = JNK3 knockout, MPTP treated (n=6), B = JNK 3 knockout, no treatment (n=5), C = JNK 3 WT, MPTP treated (n=8), D = JNK 3 WT, no treatment (n=5).

Figure 3. TH neuropil intensity in the substantia nigra after MPTP injection: effect of JNK 1 and JNK 3 knockouts. The effect of MPTP on survival of neuronal elements labeled for tyrosine hydroxylase (TH) was estimated using Sigma scan densitometry. The intensity levels are presented as relative units of intensity. J(-/-) = JNK knockout; J (+/+) = JNK replete. No inj = no injection.

Figure 4. Neuropil and neuronal TH labeling in the substantia nigra after MPTP injection: immunohistochemistry. Examples of TH immunohistochemistry are presented in JNK knockouts and controls.

Figure 5. RT-PCR for luciferase mRNA after 7 and 24 hour quinolinic acid (QA) injection. Littermate controls and AP-1 transgenics expressing the luciferase reporter gene were injected with 30 nM QA and sacrificed 7 or 24 hours after injection. RNA was extracted from brain tissues and RT-PCR was performed using primers designed to recognize the luciferase sequence. M: 100 bp DNA ladder; A: littermate control, un.injected side; B: control, injected side; C: AP-1 transgenic, 7 hour injected side; D: AP-1 transgenic, 24 hour injected animal; E: negative control, no RNA.
Figure 3
Figure 5