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13. ABSTRACT ***(Maximum 200 Words)***

The goal in this proposal is to understand mechanisms by which neurotoxicity destroys cells in the substantia nigra. Our hypothesis is that c-JUN kinases (JNK), which is a set of enzymes known to participate in death of neurons, mediates neurodegeneration in the substantia nigra after exposure to MPTP or glutamate excitotoxicity. Spurred by the DOD meeting in Potomac, 2001, I have extended our research in Parkinson's disease to include new stategies to improve survival of stem cells in mammalian brain. We also modified our protocol for MPTP neurotoxicity after *ad hoc* reviews of our data at the DOD meeting. Results in year 3 of our proposal indicated that mice lacking JNK 1 or JNK 3 lack neuroprotection against MPTP neurotoxicity, as measured by loss of tyrosine hydroxylase labeled neurons. The changes in protocol made our results more robust and reproducible, compared to the former protocol. Results favored an apoptotic pathway where JNK activity is proximal to mitochondrial initiation of apoptosis. Our group reported a role for p38 and not JNK as a target of MAP kinase apoptosis after nerve growth factor withdrawal. Mutant huntingtin gene causes striatal neurons to have increased responsiveness to NMDA receptor activation. Caspase 3, a key enzyme for transducing apoptotic signals, cleaves mutant huntingtin in cells transfected with huntingtin cDNA and in Huntington's disease brain. Pilot results show that intravenously injected, bone-marrow derived stem cells form neuron-like cells in mice genetically engineered to accept transplanted tissues. In year 4, we will submit manuscripts on neuroprotection of JNK knockout mice for MPTP, the role of AP-1 transcription in this paradigm, and neurotoxicity of quinolinic acid in JNK knockout and Huntington's disease mouse models. We will initiate studies to improve stem cell transplantation in mouse brains after neurotoxic lesions. Our current studies support a selective role for JNK in neuronal apoptosis, with emphasis on a proximal modulation of mitochondrially initiated apoptotic cascade.

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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, causing a rapidly progressive and severe form of Parkinson's disease (1,2). This laboratory is involved in two areas of research in neurotoxicity models of Parkinson's disease: mechanisms of MPTP-dependent apoptotic cell death and, initiated this year, new strategies to treat neurotoxic cell death with bone-marrow derived stem cells.

Apoptosis is strongly implicated in neurodegeneration in Parkinson's disease and other neurological 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 brain from non-specific spillage of cell contents.

Scope. The initiation and internal cell signaling is complex and still evolving. Contributing to apoptosis are extracellular cues, such as the binding of the protein FAS ligand to some cells, and intracellular signals, including the release of cytochrome C from mitochondria. c-JUN kinase (JNK) mediates or influences several of the intracellular pathways in apoptosis (7,8). Of the three main forms of JNK, JNK 3 is thought to be most closely involved with neurotoxin related apoptosis. 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). Surprisingly, 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.

We originally hypothesized that the JNK 3 isoform predominantly contributes to apoptosis, rather than JNK 1 or 2, and that JNK can mediate mitochondrially dependent apoptosis. In the past year, alternative theories have evolved for the JNK and mitochondria relationship. JNK is speculated to modify mitochondrial release of cytochrome C, rather than cytochrome C activating JNK (15). Therefore, our studies should clarify whether JNK mediates mitochondrially initiated apoptosis.

The Workshop on DoD Sponsored Parkinson's Related Research (Potomac, MD, 22-24 March 2001) had an important impact on our research direction in Neurotoxicity and Parkinson's disease. Although there is general agreement that signaling mechanisms mediating neurotoxin effects need to be clarified, the field has rapidly changed. Neuronal replacement of damaged neurons (embryonic or stem cell transplanation) or gene therapy to prevent or ameliorate cell death are poised to offer viable therapies. We therefore formed a team to develop a new strategy to improve survival of bone-marrow derived stem cells. The team includes the Neuroendocrine laboratory under my direction, Dale Greiner, Ph.D, and Leonard Schulz, Ph.D. Dr. Greiner is a transplantation immunologist in the Department of Medicine at the University of Massachusetts Medical School. Dr. Schulz is expert in mouse genetics and mouse modeling of disease; he is located at the Jackson Laboratories, Bar Harbor, Maine. Dr. Schulz also has a joint appointment in the Department of Medicine, UMMS.

The rationale underlying our approach is that bone marrow derived stem cells are currently an acceptable source of stem cells for transplantation. These stem cells have been shown to replace injured cells. Two problems limit the survival of stem cells in brain. First, intravenously administered stem cells are removed from the circulation by standard immune responses, thereby reducing access of the stem cells to the brain. Second, brain responses to the introduction of donor cells can deter their survival. Although incompletely understood, these brain responses can include removal of donor cells by macrophages, which require antibody recognition of the donor cell and often complement activation. We speculated that changes in immune recognition could improve stem cell transplantation. Our initial studies used the *rag* (-/-) mouse. RAG, or recombinase activation gene, encodes a protein that is critical for the generation of epitope specific antibodies. Mice deficient in *rag* accept foreign donor tissues more readily than do wild-type mice. Thus, *rag* deficient mice should permit better survival of intravenously administered stem cells and allow better access of stem cells to enter the brain. These knockout mice might also improve survival of stem cells directly injected into the brain. We will show preliminary data on this model system in the **Results**.

Another neurotoxin damaging mitochondria, 3-nitroproprionate (3-NP) was examined in striatal cultures. Donor mice were JNK replete or lacked either JNK 1 or JNK 3, as in the MPTP studies. In continuing studies, we found that a marker for mitochondrial integrity (MTT) was less reduced in neurons lacking JNK1 and JNK3 than in wild-type mice. The caveat is that MTT might also be found in non-mitochondrial compartments. We are comparing neuronal survival in these experiments. In addition, we undertook studies on NMDA receptor activation in a newly generated transgenic model of Huntington's disease and roles for caspase 3 in cleavage of mutant huntingtin.

Background summary. Several lines of evidence converge to support investigation of JNK in neurotoxin dependent Parkinsonism. 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. Our hypothesis was that blocking the activity of selective JNK isoforms would protect against neurotoxin induced apoptosis. Recent data, however, indicates that the role of JNK might be more complicated than at first perceived. JNK appears to be more likely to mediate external stress signals (e.g., cell membrane receptor activation) than in direct mitochondrial perturbation (15). Thus, our studies should impart an understanding of these possible JNK roles in apoptosis.

BODY

Experiments were performed to study the effects of MPTP on neuronal survival in the substantia nigra in JNK knockout and JNK replete mice. Experimental designs were modified based on information imparted at the DOD conference. Comparative studies on another mitochondrial toxin, 3-NP, were undertaken in striatal neurons with or without JNK1 or JNK3. Additional studies on caspase 3 (key enzyme in apoptosis) and NMDA receptor activation in HD were completed. New studies to improve stem cell survival in brain were started.

Experimental methods.

Animals. We used JNK1, JNK2 and JNK3 knockout mice for the MPTP study. Control mice were siblings with both alleles for each JNK isoform, as determined by PCR. Mice were 8-12 weeks old in these studies, a change from prior experiments (where mice were older than 6 months). Dr. Serge

Przedborski recommended the change at the DOD conference and in private communication, in order to improve animal survival after MPTP and obtain more consistent neurotoxic effects. 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. For primary neuronal cultures, JNK1 and JNK 3 knockout mice were super-ovulated and day 15 fetal gestation fetuses were harvested to make primary striatal neuronal cultures, to be tested for 3-NP toxicity. Transgenic huntingtin disease murine models were generated in SJL/C57bl/6 mice (see manuscript). Irradiated rag(-/-) mice were used for stem cell survival studies.

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

Immunohistochemistry. Animals were anesthetized with Avertin (0.23 ml/10g 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 product. The full substantia nigra was sectioned, with every fourth section used for counting.

Cell analysis. As reported here, cell counting was performed on every fourth section. The observer was blinded to the animal. Stereological counting will be used. Dr. DiFiglia will examine the morphology of the neurons (soma size and shape, dendritic size and shape, vacuoles, nuclear shrinkage and overal neuropil labeling) and the intensity of tyrosine hydroxylase immunoreactivity (Sigma scan densitometry).

Primary neuronal cultures. Dr. Genevieve Laforet in the laboratory developed a method of culturing primary striatal neurons. She dissects the primordial striatal buds, lightly triterates the tissue and plates the neurons. After 10 days, the cultures are studied for 3-NP neurotoxicity. 3-NP is added to the culture and the neurons are examined for cell survival at baseline, 24, 48 and 72 hours. Images of surviving neurons are captured on a digital microscope. In separate tests, MTT (mitotracker) is used to measure the integrity of the mitochondria. The surviving cells are counted by four impartial observers.

Statistical analysis. Dr. Robert Lew provided the statistical analysis for the transgenic HD murine model. Several parametric and non-parametric tests were used, including Hazard scores, chi-square and ANOVA.

Results.

Positive findings. **JNK neuroprotection against MPTP administration.** In the year 2 summary, our results indicated a trend that JNK1 knockout mice offered protection against MPTP, but JNK3 knockouts did not. We noted a wide range of results, however, and were puzzled by the inconsistently. We therefore sought advice about our experimental protocol, although we used a standard protocol. We were advised (Dr. Przedborski) to use younger mice. This change resulted in improved mouse survival after MPTP injection and more reproducible results. Results indicated that neither JNK1 nor JNK3 knockouts offered neuroprotection against MPTP, with at least 3 mice per group (Fig. 1). We are increasing the numbers of mice in each group for statistical analysis. Our original hypothesis was that JNK knockouts (especially JNK3) should protect against mitochondrial dependent apoptosis. Our current

results support a role for JNK proximal to mitochondria, in keeping with recent ideas of apoptotic pathways (15).

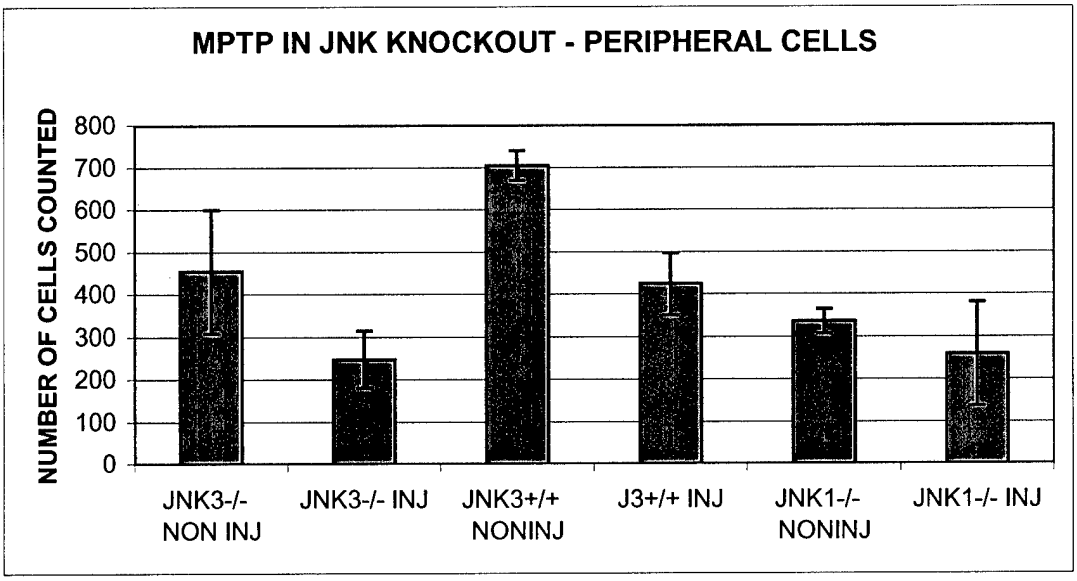
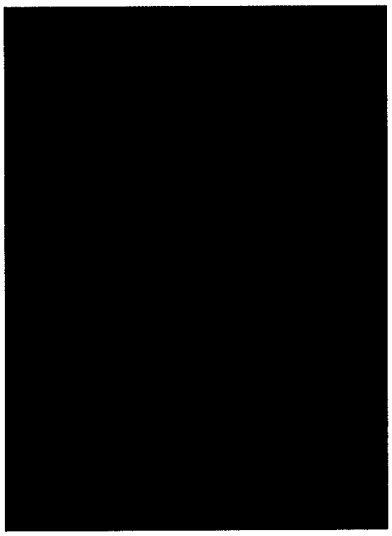


Figure 1. Neuronal loss in the substantia nigra after MPTP administration in JNK replete and JNK knockout mice. JNK replete = +/+, JNK knockout = -/-. Number of mice = 8 for JNK3 -/- non-injected; n=4 for JNK3 -/- injected; n=3 for remaining groups.

Stem cell survival in brain: adminstration of bone-marrow derived stem cells in peripheral circulation. We undertook new studies on stem cell survival. At the DOD conference, neuronal transplanation and stem cell implants seemed to be viable options for treating neurodegenerative diseases. However, compelling evidence was presented that survival of the donor cells was suboptimal. One area for study that received little attention was a change in host defenses against donor cells. Although the brain is somewhat protected against transplantation rejection, there remain immune processes in brain capable of impeding donor cell survival. We therefore formed a new team to examine these issues. Dr. Dale Greiner is an immunologist at U Mass, with focus on transplantation immunology. Dr. Leonard Schulz is a mouse geneticist, also with focus on immunology. In a preliminary study, we introduced bone-marrow derived stem cells (from Dr. Greiner) into mice modified genetically to become immune incompetent against transplanted cells (rag -/-) and previously irradiated. The stem cells were given in the peripheral circulation. Although most of the cells were taken up in the spleen (Fig. 2.b), numerous examples were found where cells resided in the brain and developed neuronal morphological features (Fig. 2a). We repeated these studies by directly injected the stem cells into the substantia nigra of MPTP treated rag (-/-), irradiated mice.



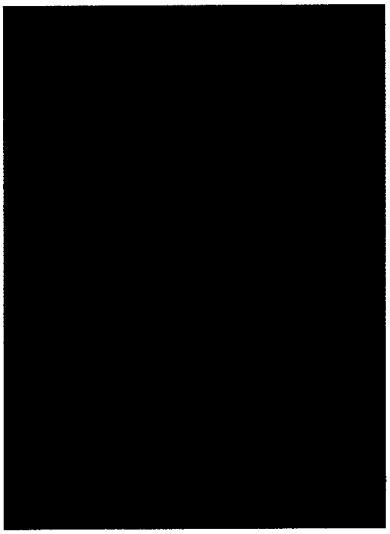


Figure 2. Bone-marrow derived stem cells into irradiated, rag-/- mice: 2 weeks post iv injection. Left: neuron like cell in striatum. Right: spleen. The donor cells were derived from GFP positive mice, to facilitate identifying donor cells in the prepared recipient mice.

Completed studies on the role of p38 and JNK in apoptosis in PC12 cells after withdrawal of nerve growth factor (17), role of caspase 3 in huntingtin processing (19), and mutant huntingtin effects on NMDA receptor activation (18) are published or in press. This grant is sited in each acknowledgment.

Negative findings. We believe that we will disprove the hypothesis on the interaction between mitochondrial damage by MPTP and JNK mediation of apoptosis. In the scientific process, this finding allows us to reformulate our hypothesis. An increasingly more important study will be a comparison of MPTP neurotoxicity with that of quinolinic acid or kainic acid, which are glutamate receptor agonists directed to different glutamate receptors. JNK 3 knockout mice show protection against kainic acid administration in hippocampal neurons.

Problems in accomplishing tasks. As noted in the year 2 summary, we were uncomfortable with the lack of cell death in some mice receiving MPTP. Also, about 50% of the older mice died after MPTP injection. We were concerned that the surviving mice might be a selected population, which would skew our results. As discussed above, we sought and received input from participants at the DOD conference. We have implemented these recommendations.

KEY RESEARCH ACCOMPLISHMENTS

- Placement of c-JNK involvement in apoptosis is proximal to mitochondria.

- p38, rather than JNK, is a mediator of MAO-A dependent apoptosis in PC12 cells

- Caspase 3 cleaves mutant huntingtin, which might increase huntingtin aggregation.

- Mutant huntingtin increases striatal neuron response to NMDA activation, with increase in Ca++ influx.

- Pilot study indicates feasibility that diminishing brain immune competency might improve survival of bone-marrow derived stem cells in brain; stem cells might take on neuronal-like morphological traits.

REPORTABLE OUTCOMES.

DeZutter GS and Davis RJ. Pro-apoptotic gene expression mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Proc Natl Acad Sci USA 98:6168-6173, 2001.

Laforet GA, Sapp E, Chase K, McIntyre C, Boyce FM, Campbell M, Cadigan BA, Warzecki L, Tagle DA, Reddy H, Cepeda C, Calvert CR, Jokel ES, Klapstein GJ, Ariano MA, Levine MS, DiFiglia M, and Aronin N. Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. J Neuroscience, in press.

Kim YJ, Yi Y, Sapp E, wang Y, Cuiffo B, Kegel KB, Qin Z-H, Aronin N, and DiFiglia M. Caspase 3-cleaved N-terminal fragments of wild-tyupe and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc Natl Acad Sci USA, in press.

CONCLUSIONS

Our hypothesis has been that JNK mediates degeneration of substantia nigra neurons after treatment with MPTP. Our recent results indicate that JNK participates in apoptosis proximal to mitochondrial activation of apoptosis, based on the lack of neuroprotection against MPTP in the substantia nigra of JNK1 and JNK3 knockout mice. Use of JNK antagonists would not expected to protect against MPTP neurotoxicity or exposure. Another MAP kinase target, p38, has a prominent role in MAO-A mediated neurotoxicity in PC12 cells; again, JNK activity does not transduce this form of apoptosis. Caspase 3, a site of apoptotic integration, has additional activities that could lead to increased neuronal susceptibility to a variety of stresses. Caspase 3 cleaves mutant huntingtin, which facilitates mutant huntingtin aggregation. Mutant huntingtin can also render striatal neurons especially responsive to NMDA receptor activation. Finally, modifications in brain immunity might improve stem cell survival as neurons.

REFERENCES

1. Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, and Kopin IJ. A primate model of parkinsonism: Selective destruction of dopaminergic neurons in the part compacta of the substantia nigra by N-methyl-4-phenyl-1.2.3.6-tetrahydorpyridine. Proc Natl Acad sci USA 80:4546-4550, 1983.

2. Langston JW, Ballard P, Tetrud, and Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219:979-980, 1983.

3. Majno G and Joris I. Apoptosis, oncosis, and necrosis. Amer J Pathol 146:3-15, 1996.

4. Singer TP, Ramsey RR, Sonsalla PK, Nicklas WJ, and Heikkila RE. Biochemical mechanisms underlying MPTP-induced and idiopathic parkinsonism. Adv Neurol 60:300-305, 1993.

5. Choi DW. Glutamate neurotoxicity and diseases of the nervous system. Neuron 1:623-634, 1988.

6. DiFiglia M. Excitotoxic injury of the neostriatum: A model for Huntington's disease. Trends in Neurosciences 13:286-289, 1990.

7. Burns RS, Chiueh CC, Markey SP, Ebert MH, Jacobowitz DM, and Kopin IJ. A primate model of parkinsonism: Selective destruction of dopaminergic neurons in the part compacta of the substantia nigra by N-methyl-4-phenyl-1.2.3.6-tetrahydorpyridine. Proc Natl Acad sci USA 80:4546-4550, 1983.

8. Langston JW, Ballard P, Tetrud, and Irwin I. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. Science 219:979-980, 1983.

9. Majno G and Joris I. Apoptosis, oncosis, and necrosis. Amer J Pathol 146:3-15, 1996.

10. Singer TP, Ramsey RR, Sonsalla PK, Nicklas WJ, and Heikkila RE. Biochemical mechanisms underlying MPTP-induced and idiopathic parkinsonism. Adv Neurol 60:300-305, 1993.

11. Xia Z, Dickens M, Raingeaud J, Davis RJ, and Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270:1326-1331, 1995.

12. Dickens M, Rogers J, Cavanagh J, Raitano A, Xia Z, Halpern JR, Greenberg M, Sawyers C, and Davis RJ. A cytoplasmic inhibitor of the JNK signal transduction pathway. Science 277:693-696, 1997.

13. Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, and Flavell RA. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature 389:865-870, 1997.

14. Saporito MS, Brown EM, Miller MS, and Carswell S. CEP-1347/KT-7515, an inhibitor of c-jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydorpyridine-mediated loss of nigrostriatal dopaminergic neurons In vivo. J Pharmacol Exp Ther 288:421-427, 1999.

15. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 103: 239-252, 2000.

16. DeZutter GS and Davis RJ. Pro-apoptotic gene expression mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Proc Natl Acad Sci USA 98:6168-6173, 2001.

17. Laforet GA, Sapp E, Chase K, McIntyre C, Boyce FM, Campbell M, Cadigan BA, Warzecki L, Tagle DA, Reddy H, Cepeda C, Calvert CR, Jokel ES, Klapstein GJ, Ariano MA, Levine MS, DiFiglia M, and Aronin N. Changes in cortical and striatal neurons predict behavioral and electrophysiological abnormalities in a transgenic murine model of Huntington's disease. J Neuroscience, in press.

18. Kim YJ, Yi Y, Sapp E, wang Y, Cuiffo B, Kegel KB, Qin Z-H, Aronin N, and DiFiglia M. Caspase 3-cleaved N-terminal fragments of wild-tyupe and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. Proc Natl Acad Sci USA, in press.