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Award Number: W81XWH-04-1-0001

TITLE: Signaling Pathways that Mediate Neurotoxin-Induced Death of Dopamine Neurons

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REPORT DATE: November 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2004	Annual (1 Nov 2003 - 31 Oct 2004)

4. TITLE AND SUBTITLE
Signaling Pathways that Mediate Neurotoxin-Induced Death of Dopamine Neurons

5. FUNDING NUMBERS
W81XWH-04-1-0001

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Parkinson's disease (PD) is characterized by progressive loss of dopaminergic neurons in the nigrostriatal pathway resulting in significant motor dysfunction. The pathology of PD is mimicked by exposure to 1-methyl-4-phenyl-1,2,3,tetrahydropyridine (MPTP) or the pesticide rotenone. These neurotoxins inhibit complex I of the mitochondrial respiratory chain resulting in the production of reactive oxygen species (ROS) and increased cytosolic calcium. We hypothesize that ROS promotes opening of the mitochondrial permeability transition pore which triggers the death pathway. In parallel, increases in cytosolic calcium leads to oxidative stress and activation of c-Jun-NH_2-terminal kinase (JNK). JNK/c-Jun signaling augments activation of the mitochondrial apoptotic cascade by suppressing Bcl-2 pro-survival signals via phosphorylation of Bcl-2 or transcription of the BH3-only, Bcl-2 antagonist Bim. The interactions between the oxidative stress pathway, the JNK/c-Jun signaling cascade, and the mitochondrial apoptotic machinery ultimately determine the fate of dopamine neurons. We will utilize primary ventral mesencephalic cultures obtained from E15 embryonic rats to investigate our hypothesis. The data obtained should lead to the identification of promising therapeutic strategies to slow or halt the dopaminergic neurodegeneration that occurs during progression of PD.

14. SUBJECT TERMS
Neurodegeneration, apoptosis, dopamine neurons, MPTP, rotenone, intrinsic death pathway

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Annual Progress Report- #W81XWH-04-1-0001, "Signaling Pathways that Mediate Neurotoxin-induced Death of Dopamine Neurons". November 1, 2003-October 31, 2004 (1st year of funding).

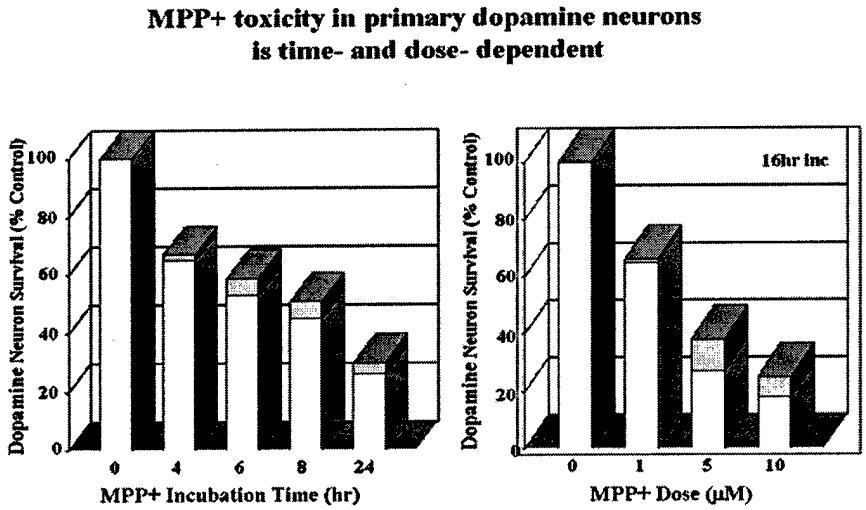
We hypothesize that the Parkinsonian neurotoxins, MPP+ and rotenone, share a common mechanism of action to induce death in dopaminergic neurons. By inhibiting complex I of the mitochondrial respiratory chain, these toxins result in the production of reactive oxygen species (ROS) that leads to opening of the mitochondrial permeability transition pore (mitoPTP) and activation of a JNK/c-Jun signaling cascade. Opening of the mitoPTP induces Bax translocation to mitochondria and Bax-dependent cytochrome C release that initiates the intrinsic apoptotic cascade. Activation of the intrinsic death pathway is augmented by JNK/c-Jun-dependent inhibition of Bcl-2 pro-survival signals. We have utilized primary ventral mesencephalic cultures obtained from fetal rats as a neuronal cell model to investigate our hypothesis.

Specific Aim 1. To investigate the role of the mitochondrial (intrinsic) death pathway in the apoptosis of primary dopamine neurons induced by the neurotoxins MPP+ and rotenone.

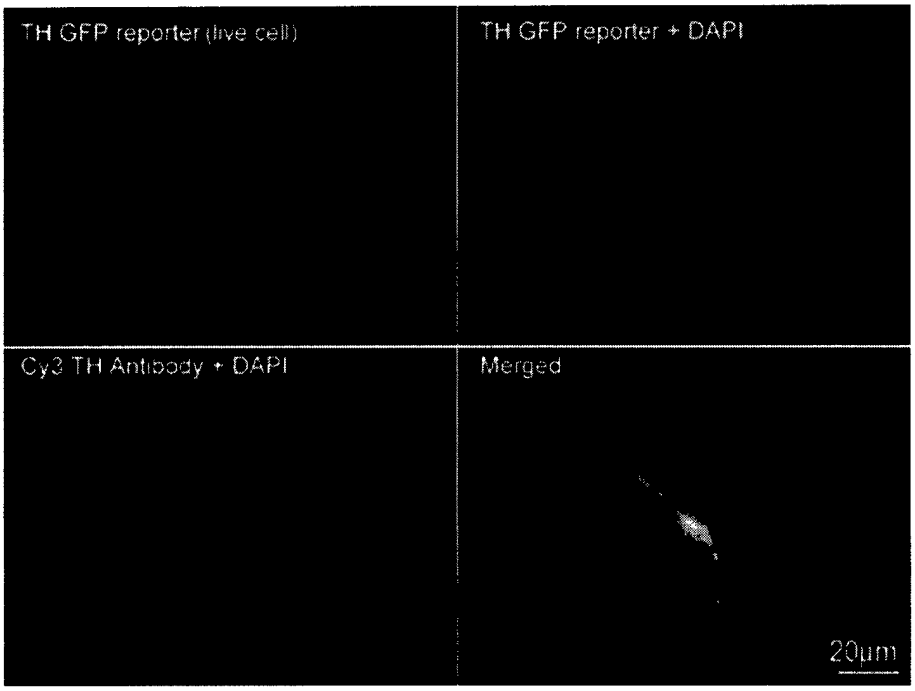
To date, most of our studies have focused on the neurotoxic effects of MPP+. Primary cultures derived from E15 rat ventral mesencephalon were prepared in Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution by mechanically dispersing tissue pieces with a 1 ml pipet. Subsequently, cells were centrifuged at 200x g for 4 min and resuspended in F12 medium containing 5% heat-inactivated human placental serum, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin and 2.2 μg/ml ascorbic acid. Cells were seeded on polyethylenimine-coated 24-well plates in 0.5 ml of medium at $6x10^4$ viable cells/cm^2. Culture medium was changed every two days. In the ventral mesencephalic cultures, approximately 2-5% of the cells are tyrosine hydroxylase (TH)-positive dopaminergic neurons.

On day 5, cultures were treated with MPP+ (time course and dose response experiments were performed). After treatment, cells were fixed with 4% paraformaldehyde and then permeabilized and blocked in PBS containing 0.2% Triton X-100 and 5% bovine serum albumin. Cells were then incubated overnight at 4 °C with a monoclonal antibody to TH (1:500). The primary antibody was aspirated, and cells were washed five times with PBS. Cells were then incubated with a donkey anti-mouse Cy3-conjugated secondary antibody (1:500) and DAPI for 2 h at room temperature. The cells were then washed 5 times with PBS, and coverslips were adhered to glass slides with mounting medium. Fluorescent images were captured on a Zeiss Axioplan-2 microscope equipped with a Cooke Sensicam deep-cooled CCD camera. All imaging data collection and analysis were carried out using the Intelligent Imaging Innovations Slide book software program. Data were obtained from at least five coverslips from five different cell preparations. Results showed that the MPP+ treatment resulted in a time- and dose-dependent loss of TH+ neurons (Fig. 1).

Figure 1

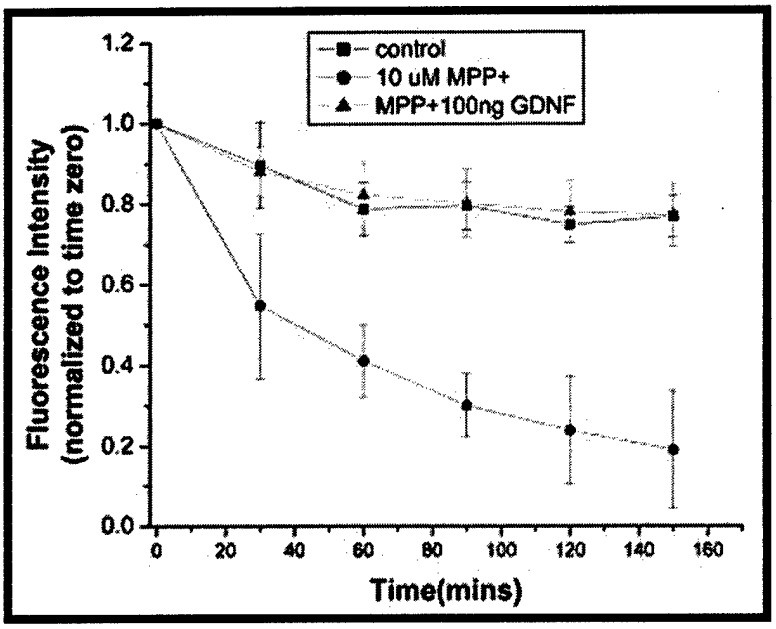


To determine the mechanism of cell death in response to MPP+, we assessed an early step in the intrinsic apoptotic cascade, mitochondrial membrane depolarization ($\Delta\psi_m$). For these experiments, dopamine neurons were identified by transfecting the cultures with a GFP-TH reporter using the Helios gene-gun. At 48 h post-transfection, approximately 10 to 50 GFP-positive neurons were apparent on each coverslip and 100% of these cells stained positively with an antibody against TH. See Figure 2 below.



To measure mitochondrial membrane potential ($\Delta\psi_m$), cells were washed with Ringer's solution containing 130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 5 mM glucose, pH 7.4. The cells were loaded with 500 nM of tetramethylrhodamine ethyl ester (TMRE) for 30 min at room temperature. TMRE fluorescence was excited at 545 nm using excitation light provided by a HBO 100 W lamp in a computer-controlled filter wheel. Emitted fluorescence light was reflected through a 590 nm long-pass filter to the camera. Changes in $\Delta\psi_m$ were monitored in single dopamine neurons over time. Results show that MPP+ induced rapid mitochondrial depolarization and that GDNF completely protected dopamine neurons from the effects of MPP+ (Fig. 3).

Figure 3



Specific Aim 4. To evaluate the effects of glial cell line-derived neurotrophic factor (GDNF) on neurotoxin-induced generation of reactive oxygen and nitrogen species, activation of JNK/c-Jun, and mitochondrial death signaling.

As mentioned above, we investigated the effects of GDNF on the loss of mitochondrial membrane potential triggered by MPP+ in primary dopamine neurons. Ventral mesencephalic cultures from E15 rats were transfected with a TH promoter-driven GFP construct using a helium-powered gene-gun. After transfection, cultures were exposed to MPP+ alone or following pre-incubation with GDNF. Live dopamine neurons were identified by GFP fluorescence and mitochondrial membrane potential was measured continuously using TMRE. Incubation with MPP+ induced a rapid (within 3 hr) collapse of mitochondrial membrane potential which was prevented by pretreatment with GDNF. The effects of GDNF were antagonized by the ERK pathway inhibitor, PD98059. Inhibitors of oxidative stress

(glutathione), the classical mitoPTP (cyclosporin A), or a Ca^{2+}-triggered permeability transition (2-APB), were each less effective than GDNF at blocking mitochondrial depolarization induced by MPP+. In contrast to MPP+, mitochondrial depolarization elicited by the Bcl-2 antagonist compound, HA14-1, was completely blocked by glutathione, partially attenuated by GDNF, but unaffected by cyclosporin A or 2-APB. Collectively, these data indicate that GDNF inhibits multiple pathways that induce mitochondrial depolarization in dopaminergic neurons. GDNF blocks both the classical, cyclosporin A-sensitive permeability transition pore and a Ca^{2+}-triggered permeability transition activated by complex I inhibition. Moreover, GDNF partially inhibits a redox-sensitive, but cyclosporin A/2-APB-insensitive, pathway that is regulated by Bcl-2. These results are the first to show that GDNF acts at the level of mitochondrial depolarization to protect dopamine neurons from MPP+-induced toxicity

Key Research Accomplishments

The key research accomplishments this year were to establish the primary ventral mesencephalic cultures and develop live-cell imaging techniques to explore death pathways in dopamine neurons. These experiments are technically difficult since such a small percentage of the cells are dopaminergic neurons. We were able to identify living dopamine neurons by transfecting a GFP-TH reporter contruct using gene-gun delivery. This allowed us to measure mitochondrial depolarization using fluorescence microscopy in real time. The experiments showed that MPP+ leads to a loss in mitochondrial membrane potential by activating several channels in the outer mitochondrial membrane that lead to the release of apoptogenic factors that cause cell death. The novel finding of our studies was that GDNF blocks activation of several of these channels, thereby blocking dopamine neuron death at a very early step in the intrinsic apoptotic cascade

Reportable Outcomes

Publications

Chen, C, DA Linseman, BD Butts, RJ Bouchard, ML McClure, **KA Heidenreich**. GDNF protects primary dopamine neurons from MPP+ induced toxicity by blocking diverse pathways to mitochondrial depolarization. Society for Neuroscience , San Diego, CA 2004.

Grants Funded

American Parkinson Disease Association (APDA), "Neurotoxin-induced Changes in BH3-only Protein Expression Analyzed by Real-time PCR in Primary Dopaminergic Neuronal Cultures", P.I.: Daniel A. Linseman, Ph.D. (Effective: September 1, 2004 – August 31, 2005) Total direct costs - $50,000 per year

Invited talks

Kim A. Heidenreich, Plenary talk, New York Academy of Science Symposium " PD: Life Cycle of the Dopamine Neuron", Princeton, N.J. 2003.

Kim A. Heidenreich, Chairperson and Invited Speaker, Gordon Conference on Insulin-like Growth Factors, Ventura, CA. 2003.

Kim A. Heidenreich, Department of Pharmacology Retreat, Copper Mountain, CO, "Molecular Mechanisms of Neuronal Death" 2003.

Daniel A. Linseman, University of Colorado Health Sciences Center, Neuroscience Training Program Seminar, "Regulation of Bax by phosphorylation during neuronal apoptosis" 2003.

Daniel A. Linseman, Gordon Research Conference, Insulin-like Growth Factors in Physiology and Disease. "IGF-I blocks neuronal apoptosis by inhibiting Bim induction and Bax translocation to mitochondria" 2003.

Conclusions

The scope of research over the last year has focused on Specific Aims #1 and #4 our original research proposal. Our key research accomplishments are outlined above. Our findings were presented at the Society for Neurosciences in San Diego this year and a manuscript is in preparation. Both investigators have presented this work at local and national research conferences. Dr. Linseman was successful in obtaining additional research funding for a project related to the current USAMRMC funded project.

GDNF protects primary dopaminergic neurons from MPP+-induced toxicity by blocking diverse pathways to mitochondrial depolarization

Chunhe Chen, Daniel A. Linseman, Brent D. Butts, Ron J. Bouchard, Maria L. Florez-McClure, and Kim A. Heidenreich

GDNF (glial cell line-derived neurotrophic factor) protects primary dopamine neurons from the mitochondrial complex I inhibitor, 1-methyl-4-phenylpyridinium (MPP+). GDNF also inhibits MPP+-induced Parkinsonism and promotes functional recovery of dopamine neurons *in vivo*. Based on its neuroprotective properties, GDNF is currently in clinical trials of Parkinson's disease. However, the molecular mechanism underlying its neuroprotective effects is currently unknown. Here, we investigated the effects of GDNF on the loss of mitochondrial membrane potential triggered by MPP+ in primary dopamine neurons. Ventral mesencephalic cultures from E15 rats were transfected with a tyrosine hydroxylase (TH) promoter-driven GFP construct using a helium-powered gene-gun. After transfection, cultures were exposed to MPP+ alone or following pre-incubation with GDNF. Live dopamine neurons were identified by GFP fluorescence and mitochondrial membrane potential was measured continuously using TMRE. Incubation with MPP+ induced a rapid (within 3 hr) collapse of mitochondrial membrane potential which was prevented by pretreatment with GDNF. The effects of GDNF were antagonized by the ERK pathway inhibitor, PD98059. Inhibitors of oxidative stress (glutathione), the mitochondrial permeability transition pore (cyclosporin A), or a Ca^{2+}-triggered permeability transition (2-APB), were each less effective than GDNF at blocking mitochondrial depolarization. In contrast to MPP+, mitochondrial depolarization elicited by the Bcl-2 antagonist, HA14-1, was completely blocked by glutathione, partially attenuated by GDNF, but unaffected by cyclosporin A or 2-APB. Collectively, these data indicate that GDNF inhibits multiple pathways that induce mitochondrial depolarization in dopaminergic neurons. GDNF blocks both the classical, cyclosporin A-sensitive permeability transition pore and the Ca^{2+}-triggered permeability transition activated by complex I inhibition. Moreover, GDNF partially inhibits a redox-sensitive, but cyclosporin A/2-APB-insensitive, pathway that is regulated by Bcl-2. These results are the first to show that GDNF acts at the level of mitochondrial depolarization to protect dopamine neurons from MPP+-induced toxicity.