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AWARD NUMBER: W81XWH-04-1-0802

TITLE: Bioenergetic Approaches and Inflammation of MPTP Toxicity

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REPORT DATE: September 20FF

TYPE OF REPORT: ~~Other~~

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

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1. REPORT DATE September 2011		2. REPORT TYPE Final		3. DATES COVERED 1 September 2004 – 31 August 2011	
4. TITLE AND SUBTITLE Bioenergetic Approaches and Inflammation of MPTP Toxicity				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) M. Flint Beal, M.D. E-Mail: fbeal@med.cornell.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cornell University New York, NY 10065				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. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This project was to develop novel therapies for Parkinson' Disease (PD), to test the effects of human dopaminergic stem cells, and to utilize metabolomic profiling to develop biomarkers for PD. We showed that treatment with rolipram, mitochondrial antioxidant peptides SS22 and SS31, celastrol, promethazine, reduced CoQ and triterpenoids, which activate Nrf2/ARE, were all neuroprotective against MPTP. We developed a new transgenic mouse model of PD by expressing the LRRK2 mutation, R1441G, in the full-length human LRRK2 protein utilizing a bacterial artificial chromosome (BAC). These mice develop a profound parkinsonian phenotype in which they become markedly slowed with a flexed posture. They have impaired release of dopamine as assessed using microdialysis, a normal complement of dopaminergic neurons, but axonal pathology in which there was phosphorylated tau, which formed spheroids. We carried out studies of PINK1 in cultured neurons and demonstrated that PINK 1 deficiency results in mitochondrial impairment. We also made a knockout PINK1 mouse. We utilized HPLC coupled to coularray electrochemical detection to perform metabolomic profiling. We showed that we could separate the patients with LRRK2 mutations from idiopathic PD and controls. Similarly, we could separate gene positive at risk from both controls and LRRK2 mutation negative subjects. We showed that dopaminergic stem cells were effective in a 6-OH-dopamine treated mice, and that dopaminergic neurons from human IPS cells were effective in primates. These studies have been highly successful in meeting the research goals outlined in our tasks.					
15. SUBJECT TERMS None provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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4. INTRODUCTION

This is a project, which has been ongoing since September of 2004. The grant initially was designed to study neuroprotective agents in an MPTP model of Parkinson's Disease (PD), as well as the pathophysiology of mitochondrial dysfunction in PD. We modified the proposal to characterize a new animal model of PD made by knocking out PINK1, a nuclear encoded kinase localized to mitochondria, and to study the effects of human dopaminergic stem cells in a 6-hydroxydopamine (6-OHDA) model of PD. We also developed a new model of LRRK1 induced PD in a transgenic mouse using the R1441G mutations in a BAC with full-length LRRK2. In recent studies, we studied the ability of a new technique to produce dopaminergic neurons from human IPS stem cells. More recently, we revised our efforts to focus on the development of metabolomic profiling to identify biomarkers for PD. These studies have made considerable progress and we have accomplished our original goals.

Outline of Research Goals:

Task 1: To determine the ability of pharmacologic agents to prevent MPTP neurotoxicity.

Task 2: To develop a new transgenic mouse model of PD by knocking out PINK1, a protein in which mutations can cause autosomal recessive PD.

Task 3: To utilize metabolomic profiling to develop biomarkers for PD.

Task 4: To determine the efficacy of human dopaminergic stem cells in the 6-hydroxydopamine model of PD.

5. BODY

Task 1: To determine the ability of pharmacologic agents to prevent MPTP neurotoxicity. These studies have been largely completed.

We did studies using the phosphodiesterase inhibitor rolipram. This leads to an increase in cAMP and phosphoCREB, which is neuroprotective. We saw protective effects with either 1.2 or 2.5 mg/kg. We showed significant protection against loss of dopamine and depletion of tyrosine hydroxylase neurons. These results were published in *Experimental Neurology*.

We also examined neuroprotective effects of mitochondrial-targeted antioxidants (SS22 and SS31), which inhibit the mitochondrial permeability transition (MPT). We made substantial progress in these studies. We found that both of the compounds which are highly concentrated to mitochondria have ROS scavenging properties and were able to dose-dependently inhibit lipid peroxidation as measured by chemoluminescence. We also found that these compounds protect against MPP+ toxicity *in vitro*. We carried out studies against MPTP toxicity in mice. We found that both compounds were able to significantly protect against MPTP induced loss of dopamine, as well as tyrosine hydroxylase neurons. Dopamine metabolites DOPAC and HVA showed similar effects. This work has been recently published in a manuscript in *Antioxid Redox Signal*.

We examined neuroprotective effects of celastrol and promethazine on MPTP. These studies were completed and they showed marked neuroprotective effects. We also worked on the mechanism of these agents. Celastrol upregulates HSP70 and promethazine blocks the MPT. These results were published in the *Journal of Neurochemistry* and in *Neurobiology of Disease* respectively. We also examined novel forms of Coenzyme Q10 (CoQ10) in the MPTP model.

We carried out a large number of studies, which showed that there were indeed significant protective effects. In particular, we were able to show that CoQ administered in a chronic model of MPTP toxicity not only protected against loss of tyrosine hydroxylase neurons, but it also protected against the development of alpha-synuclein aggregates. This was a model in which MPTP was administered over one month by Alzet pump. These results we believe are particularly relevant to PD itself. These results are now published in the *Journal of Neurochemistry*.

We also examined the role of caspase 3 activation and activation of microglia and MPTP toxicity. We studied the effects of matrix metalloproteinase 3 (MMP3) and its role as a novel signaling proteinase from apoptotic neuronal cell death, which results in activated microglia. We found that MMP3 knockout mice were protected against MPTP toxicity. We also found that MMP3 was important in activating NADPH oxidase to generate superoxide and that this plays a direct role in dopamine cell death. These results were published in the *FASEB Journal*.

We also utilized a novel therapeutic approach using triterpenoids, which are agents, which target the Nrf2/ARE pathway. We demonstrated that these compounds can induce antioxidant enzymes in normal fibroblasts, however, the ability to induce the

enzymes glutathione-c transferase, NADPH quinone oxidoreductase and heme-oxygenase was blocked in fibroblasts knocked out for Nrf2/ARE. We studied the administration of a methylamide derivatized triterpenoid, designated TP224. We found that this compound produced excellent levels in the brain tissue following oral administration, and that these levels were within the range, which produces therapeutic effects *in vitro*. They were able to produce levels as high as 90 nanomolar whereas one nanomolar concentrations are effective *in vitro* in inhibiting iNOS. Following administration of this compound to mice for one week, we administered MPTP on an acute dosing regimen in which the compound was given every two hours for 4 doses. This produced a 50-60% depletion of dopamine and its metabolites as well as marked reduction in dopaminergic neurons assessed using tyrosine hydroxylase immunocytochemistry. We showed that administration of TP224 was highly effective in significantly reducing the depletion of dopamine and its metabolites as well as the loss of tyrosine hydroxylase neurons. We also examined the compound in a chronic dosing regimen of MPTP toxicity in which it is administered over one month using an Alzet pump. We administered MPTP at a dose of 30mg/kg/per day. This produced a 50% depletion of dopamine within the striatum as well as a significant reduction in TH immunoreactive neurons. We were able to show that we could significantly protect against the loss of both dopamine in the striatum as well as dopaminergic neurons within the substantia nigra. Furthermore, we demonstrated that there was a production of alpha-synuclein aggregates within the cytoplasm of the dopaminergic neurons treated chronically with MPTP, following administration of the triterpenoid, the development of these aggregates was completely blocked. We also showed that we could attenuate increases in malondialdehyde, 8-hydroxy-2-deoxyguanosine and 3-nitrotyrosine as assessed using immunohistochemistry. These results were published in *Neurobiology of Disease*.

Task 2: To develop a new transgenic mouse model of PD by knocking out PINK1, a protein in which mutations can cause autosomal recessive PD.

We generated the PINK1 knockout mice. Correctly targeted ES cells were used to inject and generate PINK1 knockout mouse founders. We are now continuing to expand the colony. We found mild defects in motor behavior and we also found impaired dopamine release using microdialysis. We also observed a number of different phosphorylated proteins on two-dimensional gels. Of particular interest, the protein OMI was phosphorylated by PINK1 and this has been confirmed by other authors. Further characterization of these mice is continuing. We published a manuscript describing the effects of PINK1 in mitochondrial dysfunction, proteasomal deficits and synuclein aggregation in *PLoS One* in 2009.

We also utilized BAC transgenesis to develop a novel model of PD based on mutations in LRRK2. The BAC clone was correctly isolated and then underwent site-directed mutagenesis. We verified the construct using sequencing. The construct was then injected into founder mice and we were able to identify viable offspring. These mice developed normally. They however, by 10 months of age developed a profound parkinsonian phenotype. We could first detect abnormalities of movement as early as 6 months of age. By 10 months of age, the animals were largely immobile and showed a marked flex posture with slowed movements. They were impaired on rearing and open field activity. They were also impaired on the pole test and rotarod testing. We used

microdialysis to demonstrate that at baseline the production of dopamine after administration of nomphensine was impaired in the LRRK2 R1441G mice. We made counts of dopaminergic neurons, which showed that they were unaltered. The neurons however, did show some very mild shrinkage. In addition, there was a loss of dopaminergic axons in the substantia nigra reticulata. We found that the dopaminergic axons in the striatum showed marked abnormalities with axonal spheroids. Using immunostaining, they showed phosphorylated tau as detected with the AT8 antibody. This result is consistent with the observations made by Matt Farrer and colleagues at the Mayo Clinic Jacksonville. We are continuing to characterize these mice and we have commenced doing therapeutic studies to determine whether they will prove to be a useful model for developing new treatments for PD. This work was published in *Nature Neuroscience*.

Task 3: To utilize metabolomic profiling to develop biomarkers for PD.

The primary aim of our ongoing studies and existing protocol is to determine whether neurochemical markers in blood or spinal fluid can be used to make early diagnoses or to follow the progression of PD. The underlying hypothesis of the existing protocol is that disordered energy metabolism may contribute to the pathogenesis of neurodegenerative diseases, and in particular, PD. We, therefore, studied both cell lines as well as body fluids from patients with PD as well as normal controls. We carried out initial studies in 25 controls and 66 PD subjects. We utilized metabolomic profiling with high performance liquid chromatography, coupled with electrochemical coulometric array detection. In these studies, we were able to completely separate unmedicated PD subjects from controls. This work was published in *Brain*. We carried out further studies of patients and gene carriers with LRRK2 mutations, which is responsible for autosomal dominant inherited PD. This enabled us to identify the variables, which were responsible for the separations in the LRRK2 PD subjects and family members who are carriers of this genetic defect, as well as patients who have manifest PD. We can clearly separate PD patients with LRRK2 mutations from idiopathic PD and controls. This work was published in *PLoS One* in 2010. Similarly, we can separate gene positive patients from both controls and LRRK2 mutation negative patients. We are now working on structural elucidation of the remaining analytes separating PD patients from controls using mass spectroscopy. We are also using an integrated parallel LCECA/LCMS device with post-column splitting between electrochemical and mass spectrometric detectors. We intend to expand these studies into a much larger cohort of patients to determine the sensitivity and specificity of these analyses as biomarkers for PD diagnosis and assessment of therapies.

We were able to identify a number of variables, which differentiated LRRK2 PD subjects from idiopathic PD subjects. We were also able to separate LRRK2 PD subjects from family members who were carriers of the genetic defect as well as patients who are controls. Our results show that we can clearly separate PD patients with LRRK2 mutations from idiopathic PD and controls. We also carried out studies of predictive testing and showed that we were able to separate gene positive patients from LRRK2 carriers as well as LRRK2 negative patients within the same families. We are now working on structural elucidation of the remaining analytes. We have developed a technique to utilize mass spectroscopy in combination with coulometric array

electrochemical detection. This involves injecting a sample, which goes through a reverse phase column and is then split post column to be analyzed both by the electrochemical and mass spectrometric detectors in parallel. We published the work on LRRK2 in *PLoS One* in 2009. Work on urate was published in *Arch Neurology* in 2009.

Task 4: To determine the efficacy of human dopaminergic stem cells in the 6-hydroxydopamine model of PD.

These studies were completed. We showed that the stem cells survived readily. The acquisition of highly enriched dopaminergic populations is an important prerequisite to human embryonic stem cell (HES)-derived dopaminergic neurons for cell-based therapy. We utilized a new strategy improving the efficiency of dopaminergic neurogenesis from human ES cells. This involved co-culture with telomerase immortalized human mesencephalic astrocytes during induction of a dopaminergic phenotype using sonic hedgehog and FGF8. Utilizing these enriched dopaminergic neurons, we were able to achieve a substantial and long-lasting restitution of motor function in 6-OHDA-lesioned adult rats. We also showed effective generation of TH positive neurons in all six animals studied. We examined BDRU incorporation, which showed that there was a small percentage of neurons, which showed BDRU incorporation consistent with evidence of mitosis. These extremely promising results are important in setting the stage for further work on human transplantation.

Together, these results suggest the utility of mesencephalic astroglial coculture in driving dopaminergic neurogenesis from human ES cells, while demonstrating the potential of the result in grafts in mediating behavior recovery in a model of nigrostriatal loss. The findings, however, suggest a need for caution in the clinical application of human ES derived grafts given their potential for phenotypic instability and undifferentiated expansion. These late complications of otherwise successful HES-derived dopaminergic neuronal grafts suggest that strategies not only for high efficiency of dopaminergic neurogenesis but also methods for ridding the preparations of undifferentiated cells need to be developed. More recently, in collaboration with Dr. Lorenz Studer we carried out further studies of dopaminergic neurons generated from human IPS cells. These studies were published in *Nature Medicine*.

Human pluripotent stem cells (PSCs) are a promising source of cells for applications in regenerative medicine. Directed differentiation of PSCs into specialized cells such as spinal motoneurons or midbrain dopamine (DA) neurons has been achieved. However, the effective use of PSCs for cell therapy has lagged behind. Whereas mouse PSC-derived DA neurons have shown efficacy in models of Parkinson's disease, DA neurons from human PSCs generally show poor in vivo performance. There are also considerable safety concerns for PSCs related to their potential for teratoma formation or neural overgrowth. Here we present a novel floor-plate-based strategy for the derivation of human DA neurons that efficiently engraft in vivo, suggesting that past failures were due to incomplete specification rather than a specific vulnerability of the cells. Midbrain floor-plate precursors are derived from PSCs 11 days after exposure to small molecule activators of sonic hedgehog (SHH) and canonical WNT signalling. Engraftable midbrain DA neurons are obtained by day 25 and can be maintained in vitro for several months. Extensive molecular profiling, biochemical and electrophysiological data define developmental progression and confirm identity of PSC-derived midbrain DA neurons. In

vivo survival and function is demonstrated in Parkinson's disease models using three host species. Long-term engraftment in 6-hydroxy-dopamine-lesioned mice and rats demonstrates robust survival of midbrain DA neurons derived from human embryonic stem (ES) cells, complete restoration of amphetamine-induced rotation behaviour and improvements in tests of forelimb use and akinesia. Finally, scalability is demonstrated by transplantation into parkinsonian monkeys. Excellent DA neuron survival, function and lack of neural overgrowth in the three animal models indicate promise for the development of cell-based therapies in Parkinson's disease. This work was published in *Nature*.

6. KEY RESEARCH ACCOMPLISHMENTS

A. The finding that mitochondrial targeted antioxidants SS02 and SS31 which inhibit the MPT are neuroprotective against MPTP toxicity in mice, as well as against MPP+ toxicity in cell culture.

B. The finding that a reduced novel form of CoQ10 is effective in the MPTP model of PD. We have also demonstrated that there is dose-responsive protection with other formulations of CoQ10 against MPTP toxicity in young mice. We have evaluated the role of MMP3 activation by microglia in MPTP toxicity. We found that MPTP toxicity was significantly attenuated in MMP3 deficient mice. Furthermore, that an activated isoform of MMP was released from injured dopaminergic neurons, which led to the subsequent activation of microglia. This was accompanied by an increase in superoxide generation, which was mediated by NADPH oxidase.

C. We demonstrated that several novel pharmacologic agents exert neuroprotective effects in the MPTP model of PD. In particular, in the most recent grant, we have demonstrated that triterpenoids, which activate the Nrf2/ARE pathway are highly effective in both acute and chronic MPTP models of PD. This suggests that these agents are worthwhile for further development for treatment of PD patients.

C. We have developed a knockout model of PINK1. We have found that these mice do have behavioral alterations and show abnormalities in their phosphoprotein profiles. These mice are undergoing continuing evaluation.

D. We have continued metabolomic profiling and have now made the observation that we can separate patients with the G2019S LRRK2 mutation from both family members, as well as normal controls. We can also separate these patients from those with idiopathic PD.

E. We have developed a BAC R1441G LRRK2 transgenic mouse model for PD. These animals are undergoing further characterization in order to carry out studies of therapeutic interventions. We have initiated studies of a number of promising therapeutic compounds, which are continuing to progress.

F. We have developed a novel technique for increasing the induction of a dopaminergic phenotype in human ES derived dopaminergic neurons. We showed that transplantation of these stem cell derived neurons into the 6-hydroxydopamine level of PD was efficacious in reversing the behavioral deficits. We found that there was no frank anaplasia. However, there were continuing dividing neurons in the center of the graft raising the possibility that neoplasia could occur and, therefore, warranting caution in the use of this approach for treating human PD.

G. We have continued metabolomic profiling and now have shown that we can separate unmedicated PD patients from controls as well as medicated PD patients from controls. This work has been published. We have also showed that a number of specific metabolites are altered including 8-hydroxy-2-deoxyguanosine and reduced uric acid. We have also found abnormalities in both hypoxanthine and xanthine. We can separate idiopathic PD patients from patients with the LRRK2 mutation and in addition, we can separate LRRK2 gene positive carriers from controls and LRRK2 mutation negative patients. This provides evidence that it may be possible to screen patients early in life and determine whether they will eventually be at high risk of developing PD.

H. We have generated IPS cells and dopaminergic neurons starting with fibroblasts from patients with idiopathic PD as well as patients with defined mutations in LRRK2. We are now studying these fibroblasts induced dopaminergic neurons further to determine their phenotype with regard to dopamine, mitochondrial function and production of reactive oxygen species.

I. We generated dopamine neurons from human ES cells efficiently and used them to engraft animal models of PD, including Parkinsonian monkeys.

7. REPORTABLE OUTCOMES

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8. CONCLUSION

This project was to develop novel therapies for Parkinson's Disease (PD), to test the effects of human dopaminergic stem cells, and to utilize metabolomic profiling to develop biomarkers for PD. We also developed a new transgenic mouse model of PD by knocking out the gene PINK1. Utilizing microdialysis, we showed that PINK1 deficient mice have impaired dopamine release. We demonstrated that rolipram mitochondrial targeted antioxidant peptides, celastrol and promethazine were neuroprotective against MPTP toxicity. We showed that novel forms of Coenzyme Q exert neuroprotective effects. We demonstrated that MMP3 knockout mice were protected against MPTP toxicity. Utilizing microdialysis, we showed that PINK1 deficient mice have impaired dopamine release. We showed that human dopaminergic stem cells reverse deficits in the 6-hydroxy-dopamine models of PD. We carried out studies in collaboration with Dr. Studer using a refined technique to convert IPS stem cells into dopaminergic neurons. These dopaminergic neurons reversed motor deficits in a Parkinsonian rodent model and were sufficient to produce enough cells to graft primate models. This is a major step forward to human clinical trials. We carried out studies using metabolic profiling to develop biomarkers for PD. We utilized metabolomic profiling with high performance liquid chromatography coupled to electrochemical coulometric array detection. In these studies, we were able to completely separate unmedicated PD subject from controls. We subsequently were able to clearly separate PD patients with the LRRK2 mutations from idiopathic PD and controls. Similarly, we could separate gene positive patients from both controls and LRRK2 mutation negative patients. Lastly, we studied a number of agents, which produced significant neuroprotective effects against MPTP toxicity and which, therefore, might be useful in human clinical trials to treat PD.

9. REFERENCES

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10. APPENDICES

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