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PRINCIPAL INVESTIGATOR: Jeffrey H. Kordower, PhD

CONTRACTING ORGANIZATION: Rush University Medical Center
Chicago, IL 60612

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Jeffrey H. Kordower, Ph.D.

E-Mail: jkordowe@rush.edu

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The discovery that mutations in the gene for alphasynuclein could cause Parkinson□s disease (PD) was the first demonstration that PD can have a genetic etiology. Since this initial discovery it has become apparent that alphasynuclein may also be a central feature in the pathogenesis of PD. We have recently demonstrated that alphasynuclein is central to early pathogenic events within the nigra of PD as only nigral neurons that display alphasynuclein positive aggregates display decreases in the dopaminergic markers tyrosine hydroxylase and Nurr1. Adjacent nigral PD neurons without aggregates display normal levels of these markers. Additionally, we have found that monkeys and humans have age-related increases in cytoplasmic alphasynuclein in nigral neurons as leading to our hypothesis that preventing age-related upregulation of alphasynuclein, rather than attempting to disaggregate already aggregated alphasynuclein might be a simpler therapeutic target. Based upon this line of thought, we propose in this application to determine to following: We will use single cell gene arrays to test the hypothesis that the molecular signature of cells under attack in PD (those expressing alphasynuclein aggregates) is different than those that normal (those without alphasynuclein aggregates). We will also test the hypothesis that early onset PD engendered via viral delivery of alpha synuclein to young monkeys will display a slower course of progression, more drug-induced complications, and attenuated nigrostriatal dysfunction relative to late onset PD engendered via viral vector administration of alpha synuclein to aged monkeys. Finally, we will test the hypothesis that decreasing cytoplasmic expression of alpha synuclein using siRNA directed against alphasynuclein will the increase phenotypic dopaminergic markers in an aged monkey model of PD. Taken together, these studies will aid in our understanding of the role of alpha synuclein and other gene markers in the pathogenesis of PD, the role of age related accumulation of these proteins in the manifestation of PD-related changes and determine whether altering the age-related overexpression of alphasynuclein will be a useful therapeutic strategy for patients with PD.

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1. **INTRODUCTION:**
   Alpha synuclein is a key target in the pathogenesis of Parkinson's disease. The present application studies the effects of alpha synuclein positive versus alpha synuclein negative nigral neurons in patients with Parkinson's disease. We also studied the effects of alpha synuclein viral overexpression in young versus aged monkeys. The overarching purpose of these studies is to have a better understanding of PD pathogenesis and provide a target for therapy.

2. **KEYWORDS:** Alpha synuclein; monkeys, AAV, aging

3. **OVERALL PROJECT SUMMARY:** Summarize the progress during appropriate reporting period (single annual or comprehensive final). This section of the report shall be in direct alignment with respect to each task outlined in the approved SOW in a summary of Current Objectives, and a summary of Results, Progress and Accomplishments with Discussion. Key methodology used during the reporting period, including a description of any changes to originally proposed methods, shall be summarized. Data supporting research conclusions, in the form of figures and/or tables, shall be embedded in the text, appended, or referenced to appended manuscripts. Actual or anticipated problems or delays and actions or plans to resolve them shall be included. Additionally, any changes in approach and reasons for these changes shall be reported. **Any change that is substantially different from the original approved SOW (e.g., new or modified tasks, objectives, experiments, etc.) requires review by the Grants Officer's Representative and final approval by USAMRAA Grants Officer through an award modification prior to initiating any changes.**

Alpha-Synuclein is a synaptic protein that has been directly linked to both the etiology and pathogenesis of Parkinson's disease. We have previously shown that only nigral neurons in PD expressing alpha-synuclein inclusions display a loss dopaminergic phenotype. The present study tested the hypothesis that normal aging contributes to this effect. The relative abundance of alpha-synuclein protein within individual nigral neurons was quantified in eighteen normal humans between the age of 18 and 102 and twenty four rhesus monkeys between the age of 2 and 34. Optical densitometry revealed a robust age-related increase in alpha-synuclein protein within individual nigral neurons in both species. This effect was specific for nigral alpha-synuclein as no age-related changes were found in the ventral tegmental area nor were there changes in the nigra for non-pathogenic beta-synuclein. The age-related increases in nigral alpha-synuclein were non-aggregated and strongly associated with age-related decreases in tyrosine hydroxylase (TH), the rate-limiting enzyme for dopamine production. In fact, only cells expressing alpha-synuclein displayed reductions in TH. We hypothesize that age-related increases in alpha-synuclein result in a subthreshold degeneration of nigrostriatal dopamine which, in PD, becomes symptomatic due to lysosomal failure resulting in protein misfolding and inclusion formation. We further hypothesize that preventing the age-related accumulation of non-aggregated alpha-synuclein might be a simple and potent therapeutic target for patients with PD.

We then explored the relationship between ubiquitin proteasome system (UPS) and lysosomal markers and the formation of alpha-synuclein (alpha-syn) inclusions in nigral neurons in Parkinson disease (PD). Lysosome Associated Membrane Protein 1(LAMP1), Cathepsin D

(CatD), and Heat Shock Protein73 (HSP73) immunoreactivity were significantly decreased within PD nigral neurons when compared to age-matched controls. This decrease was significantly greater in nigral neurons that contained alpha-syn inclusions. Immunoreactivity for 20S proteasome was similarly reduced in PD nigral neurons, but only in cells that contained inclusions. In aged control brains, there is staining for alpha-syn protein, but it is non-aggregated and there is no difference in LAMP1, CatD, HSP73 or 20S proteasome immunoreactivity between alpha-syn positive or negative neuromelanin-laden nigral neurons. Targeting over-expression of mutant human alpha-syn in the rat substantia nigra using viral vectors revealed that lysosomal and proteasomal markers were significantly decreased in the neurons that displayed alpha-syn-ir inclusions. These findings suggest that alpha-syn aggregation is a key feature associated with decline of proteasome and lysosome and support the hypothesis that cell degeneration in PD involves proteosomal and lysosomal dysfunction, impaired protein clearance, and protein accumulation and aggregation leading to cell death.

We then reported that myocyte enhancer factor 2D (MEF2D), a nuclear transcription factor known to promote neuronal survival, is down regulated in response α-synuclein accumulation and aggregation. Our data demonstrated that levels of cytoplasmic and nuclear MEF2D were significantly decreased in PD nigral neurons when compared to the nigra of age-matched controls and Alzheimer's disease (AD) cases. This decrease was significantly greater in the nigral neurons with α-synuclein inclusions. Viral vector-mediated overexpression of human α-synuclein in rats resulted in significantly decreased MEF2D in nigral neurons similar to what was seen in PD. The decline of MEF2D-immunoreactivity was associated with a reduction in TH-immunoreactivity. These results indicate that the neuronal survival factor MEF2D is decreased in human and experimental PD, and this decrease is specifically associated with α-synuclein accumulation and aggregation.

The progressive loss of the nigrostriatal pathway is a distinguishing feature of Parkinson's disease. As terminal field loss seems to precede cell body loss, we tested whether alterations of axonal transport motor proteins would be early features in Parkinson's disease. There was a decline in axonal transport motor proteins in sporadic Parkinson's disease that preceded other well-known nigral cell-related pathology such as phenotypic downregulation of dopamine. Reductions in conventional kinesin levels precede the alterations in dopaminergic phenotypic markers (tyrosine hydroxylase) in the early stages of Parkinson's disease. This reduction was significantly greater in nigral neurons containing α-synuclein inclusions. Unlike conventional kinesin, reductions in the levels of the cytoplasmic dynein light chain Tctex type 3 subunit were only observed at late Parkinson's disease stages. Reductions in levels of conventional kinesin and cytoplasmic dynein subunits were recapitulated in a rat genetic Parkinson's disease model based on over-expression of human mutant α-synuclein (A30P). Together, our data suggest that α-synuclein aggregation is a key feature associated with reductions of axonal transport motor proteins in Parkinson's disease and support the hypothesis that dopaminergic neurodegeneration following a 'dying-back' pattern involving axonal transport disruption.

Both the misfolding of α-synuclein and mitochondrial dysfunction are considered two major contributors to Parkinson's disease (PD). However, the relationship between the two in normal and PD states remains unclear. Here, we report that voltage-dependent anion channel 1 (VDAC1), a major component of the outer mitochondrial membrane known to regulate mitochondrial functions, is down-regulated in response to α-synuclein accumulation and aggregation. Stereological analysis revealed that 58.33% of the neurons were VDAC1 immunoreactive in the remaining neuromelanin laden neurons in the PD group while 87.48% of the nigral neurons were VDAC1 immunoreactive in the age-matched control group. The relative

levels of VDAC1 were significantly decreased in PD nigral neurons when compared to age-matched controls. In PD, this decrease was significantly greater in nigral neurons with α-synuclein inclusions. VDAC1 was observed in fibers with granular α-synuclein but not in fibers with aggregated α-synuclein. Viral vector-mediated overexpression of mutant human α-synuclein (A30P) in rats resulted in significantly decreased VDAC1 in nigral neurons and striatal fibers. These results indicate that mitochondrial function associated with VDAC1 is decreased in sporadic and experimental PD, and this decrease is associated with α-synuclein accumulation and aggregation.

Activity-dependent neuroprotective protein (ADNP), a vasoactive intestinal peptide-regulated gene, is essential for brain development and plasticity. A synthetic peptide (NAP) derived from the ADNP sequence exhibits a neuroprotective effect in various neurodegenerative disease models, prompting the hypothesis that endogenous ADNP may play a similar role. Here we investigated ADNP expression in nigral neurons from sporadic Parkinson's disease (PD) and an experimental PD model. Compared to age-matched controls, a marked reduction in ADNP protein levels was observed in neuromelanin-containing nigral neurons of PD. Reduced ADNP levels did no relate to the progression of PD symptoms, but instead occurred at early PD stages, before reductions in tyrosine hydroxylase could be detected. Reductions in ADNP were also positively correlated with alterations in axonal transport motor protein subunits (kinesin heavy chain and dynein light chain Tctex type 3). Reductions in ADNP levels were recapitulated in a rat model of PD based on viral over-expression of human wild-type α-synuclein, suggesting that ADNP reductions in PD are a direct result of α-synuclein overexpression. These findings demonstrate that the down-regulation of protein ADNP is an early pathological alteration in dopaminergic neurodegeneration.

We injected AAV-alpha synuclein to both young and aged nonhuman primates as per the protocols described in the application. Surprisingly we found that injections into aged nonhuman primates failed to result in any lesion or behavioral impairments. In contrast, we did find significant effects when AAV-alpha synuclein was placed into young monkeys. In this study, 12 cynomolgus macaques each received monthly baseline assessments on motor activity, a functional rating scale (MPPrs) and were trained to conduct a fine motor task (mMAP test). Baseline PET scans were obtained bimonthly to visualize VMAT-2 and FDG fluorodeoxyglucose (FDG). Animals were injected with either AAV1/2 A53T aSyn or empty vector (EV) (both, 1.7 x 10exp12 gp/ml) bilaterally into the substantia nigra (SN) and killed 8 months post surgery. In an independent study (validation study), another 4 macaques were injected unilaterally with the same vector and pathology was characterized at 4 months. In the first study, postmortem analyses, showed that at 8 months, there was a 42% and a 39% reduction in putaminal DA and DAT, respectively, compared to controls (all, $P<0.05$), along with a reduction in TH positive neurons in the SN. Neurites also showed transgene expression throughout the striatum and had a dystrophic Lewy morphology. Remaining neurons showed overexpression of aSyn, however, pS129 aSyn and thioflavin-S positive inclusions were absent. By 5 months post surgery, A53T aSyn macaques showed 45% less motor activity in the 2-4 hr period of a 4 hr observation, compared to EV controls ($P<0.05$). This deficit persisted through to the final month (53%, 56% and 60%, respectively, to month 8, [all, $P<0.05$]). No significant effects were observed on mMAP performance. PET imaging showed no significant change in striatal VMAT-2 to assess dopaminergic activity or change in FDG uptake evaluated to derive the Parkinson related pattern (PrP). In the validation study, we found that, at 4 months, aSyn aggregates were positive for p129 aSyn and thioflavin-S. Relative to the contralateral side, there were reductions in the number of remaining TH positive neurons (reduced by 30%), TH optical density in the putamen and caudate nucleus (reduced by 26 and 20%, respectively), and striatal dopamine as measured by HPLC (reduced by 34%). The presence of pathological aSyn (pS129 and

thioflavin-S positivity) observed at 4 months and the absence of such, despite continued overexpression of the aSyn transgene, at 8 months, may be evidence of more vulnerable neurons degenerating over the period of 4-8 months. In conclusion, the macaque model of PD alpha-synucleinopathy produced here is in a position to assess therapeutics aimed at reducing or preventing aSyn accumulation and concomitant neurodegeneration in the nigrostriatal system

4. **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of key research accomplishments emanating from this research. Project milestones, such as simply completing proposed experiments, are not acceptable as key research accomplishments. Key research accomplishments are those that have contributed to the major goals and objectives and that have potential impact on the research field.
   - We established multiple neuropathologies seen in Parkinsonian brains and supported by viral over expression modeling in rodents that are associated with alphasynuclein aggregation. These include specific reductions in phenotypic markers, alterations in mitochondrial function, alterations in autophage functions, and alterations in axonal transport.
   - We established a nonhuman primate model of PD using viral over expression of alpha synuclein. This model does not apply to aged nonhuman primates

5. **CONCLUSION:** Summarize the importance and/or implications with respect to medical and /or military significance of the completed research including distinctive contributions, innovations, or changes in practice or behavior that has come about as a result of the project. A brief description of future plans to accomplish the goals and objectives shall also be included.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

   a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

      (1) Lay Press: none
      (2) Peer-Reviewed Scientific Journals:

Chu Y[1], Kordower JH. Age-associated increases of alpha-synuclein in monkeys and humans are associated with nigrostriatal dopamine depletion: Is this the target for Parkinson's disease? Neurobiol Dis. 2007 Jan;25(1):134-49.

Chu Y[1], Dodiya H, Aebischer P, Olanow CW, Kordower JH. Alterations in lysosomal and proteasomal markers in Parkinson's disease: relationship to alpha-synuclein inclusions. Neurobiol Dis. 2009 Sep;35(3):385-98. doi: 10.1016/j.nbd.2009.05.023. Epub 2009 Jun 6.

Chu Y[1], Mickiewicz AL, Kordower JH.α-synuclein aggregation reduces nigral myocyte enhancer factor-2D in idiopathic and experimental Parkinson's disease. Neurobiol Dis. 2011 Jan;41(1):71-82. doi: 10.1016/j.nbd.2010.08.022. Epub 2010 Sep 9.

Chu, Y., Morfini, GA, Kordower, Alterations in activity-dependent neuroprotective protein in sporadic and experimental Parkinson's disease. In review.

(3) Invited Articles: none
(4) Abstracts:
(5) Koprich, J., Johnston, TH., Seibyl,J., Marek, K., Ma, Y., Eidelberg, D., Zuo, C., Guan, Y , Kordower, J.H., Fox,S.H., Brotchie, J.M. A non-human primate model of Parkinson's disease based on viral vector mediated overexpression of alpha-synuclein. Soc. Neurosci. Abstr., 2015.

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

7. INVENTIONS, PATENTS AND LICENSES: None

8. REPORTABLE OUTCOMES: None

9. OTHER ACHIEVEMENTS: None

For each section, 4 through 9, if there is no reportable outcome, state "Nothing to report."

10. REFERENCES: None

11. APPENDICES: None

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