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PRINCIPAL INVESTIGATOR: Eric Richfield, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14627-0140

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13. ABSTRACT (Maximum 200 Words) The purpose of this project is to study environmental and genetic interactions in the etiology of Parkinson's disease. The scope of the project is to use transgenic mice and tissue culture methods to determine the role of alpha-synuclein, MPTP, and paraquat in damaging dopaminergic neurons. The major findings to date include the generation and characterization of transgenic lines of mice expressing alpha synuclein in catecholaminergic cell groups, their increased vulnerability to MPTP and their vulnerability to combined maneb and paraquat. A construct to generate somatic mosaic animals has been created and tested. It will be used to create a second model of alpha synuclein over expression. Viral vectors overexpressing alpha synuclein have been created, tested and result in synuclein aggregates in neurons. The first year has been devoted to the generation of transgenic mice and the creation of cloning molecules and vectors. Significant development of resources and techniques was expected and accomplished in this year. Later years will result in production of data to address specific hypotheses.				
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FOREWORD

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X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

E. R. [Signature] *2/10/00*

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INTRODUCTION

The subject of this research is to understand the role of genes, aging, and neurotoxicants on the etiology of Parkinson's disease (PD). One specific gene, the human α -synuclein gene, was selected for study because of its role in a familial form of Parkinson's disease. The neurotoxicants to be examined include MPTP and paraquat as suspected causes of PD in humans. The purpose of this research is to develop appropriate models using *in vitro* and *in vivo* techniques to understand mechanisms of neuronal dysfunction and death and determine ways to prevent the disease. The scope of the research involves developing a variety of molecular tools, ranging from viral vectors to transgenic mice, based on the known mutations in the human α -synuclein gene and understand their adverse impact on neuronal functioning in the presence and absence of neurotoxicants and in relationship to aging.

BODY

Our application stated that environmental toxins or genetic mutations in the human α -synuclein gene produce forms of Parkinson's disease (PD). We hypothesized that sporadic cases of PD were the result of an interaction of three factors including genetic predisposition, life-long acute or intermittent exposure to neurotoxins, and aging. We hypothesized that: 1) a mutated form of the human α -synuclein gene would be toxic to substantia nigra neurons by a toxic gain of function when focally over-expressed in the mouse and 2) that expression of the human mutated α -synuclein gene would confer additional vulnerability to the environmental toxins MPTP and paraquat in mouse substantia neurons. The adverse effect of the human mutated α -synuclein gene would also be amplified by aging.

We have focused our resources during the first year of the project toward generating a variety of tools which will help us test those hypotheses. We have modified our **Statement of Work** (see Appendix) to reflect these changes for years 1 and 2. Of note, we have only **added** additional work and not removed any plans for our somatic mosaic mice. We have initial data from our first group of transgenic mice supporting our initial hypothesis that overexpression of the human α -synuclein gene in mouse substantia neurons increases their vulnerability to the neurotoxicant MPTP.

In addition to the somatic mosaic mice (SYN XATs) proposed in our approved application (Fig 1C, see end of report), we have embarked upon making and testing an additional transgenic mouse model using the human wild type (hwSYN) or mutated (hm²SYN) α -synuclein gene (Fig 1A and B, at end of report). These transgenic mice express the human wild-type (hwSYN) and a doubly mutated (hm²SYN) form of α -synuclein under the control of the full length (9 kb) tyrosine hydroxylase promoter. The hwSYN and hm²SYN mice share many characteristics of our proposed somatic mosaic mice (SYN XATs). Both models use the same promoter (9 kb tyrosine hydroxylase promoter) as specified in our original application. They also use the same wild-type and mutated forms of the human α -synuclein gene (hSYN). However, the mutated human α -synuclein lines of mice express a mutated form of α -synuclein which has been mutated at two sites, G88C and G209A, rather than the site (G209A) as originally specified in our proposal. Mutations were created by PCR directed mutagenesis. All sequences and mutations were verified by sequencing and restriction digests. Both of these mutations have been independently associated with a familial form of PD. We elected to use a doubly mutated form of α -synuclein for the following reason. If each mutation by itself results in a toxic gain of function then the double mutation *may* be more likely to result in toxicity or greater toxicity compared to each mutation alone. Also, it is unclear if either mutation will result in toxicity in mice and this allows us to test the potential effect of each together. Should toxicity result from the double mutated form of hSYN, we would be in a position to study each mutation individually, using the exact same paradigms and reagents after creating an additional transgenic line. In addition, the mouse sequence for α -synuclein already contains one of these changes in its homologous sequence (Thr53) which may prevent that change in the human sequence from being toxic in mice. If both mutations are toxic, it will be imperative to test each separately to see if they act via the same mechanism.

We decided to proceed with a conventional transgenic model (hwSYN and hm²SYN) during our first year for the following reasons. First, it was and remains unproven if human α -synuclein (wild-type or mutated) will be toxic to substantia nigra neurons in mice. The time to build and test the SYN XAT line will be lengthy and studying its toxicity time consuming. If we are able to document toxicity using a conventional model first, it will make the somatic mosaic model more likely to succeed and would assist us greatly in assessing the outcomes. Second, the reagents we develop using the traditional approach will all be useful in the somatic mosaic approach. Some of these reagents are easier to characterize in the constitutively overexpressing mice since the mRNA and protein is present in all TH expressing cells without the need for viral activation using Cre recombinase. These reagents include primers for RT-PCR, oligonucleotides for *in situ* hybridization

histochemistry, and antibodies for immunohistochemistry. Some techniques such as stereology, behavioral assessments, and others once perfected in these conventional transgenic mice will be applicable to the somatic mosaic mouse model. The preliminary data we will present will support our intentions and decision (see below).

In regards to this transgenic mouse model (hwSYN and hm²SYN), we have been successful in generating several lines of both constructs (Table 1). One contains the human wild-type α -synuclein (hwSYN) gene and the other contains the human double mutated α -synuclein gene (hm²SYN). Several founders did not pass on the gene to offspring and have not been further characterized.

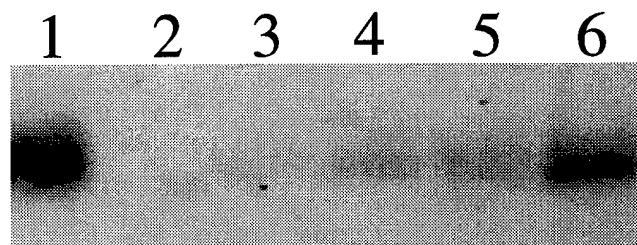
Table 1

Line	DNA Copy #	mRNA	Protein	Organs Expressing mRNA	Brain Regions Expressing mRNA	Brain Regions Expressing protein
hw5	high	+++	+++	Brain, eye, adrenal	SN, LC, CC, HpC	SN, LC, CC, HpC, AM
hw6	low	+	+	n.t.	n.t.	SN, LC, CC, HpC, AM
hw68	low	+	+	n.t.	n.t.	SN, LC, CC, HpC, AM
hw88	high	++	+++	n.t.	n.t.	SN, LC, CC, HpC, AM
hm ² 10	low	+	+	Brain, eye, adrenal	SN, LC	SN, LC, CC, HpC, AM
hm ² 27	low	+	+	Brain, eye, adrenal	SN, LC	SN, LC, CC, HpC, AM
hm ² 39	high	+++	+++	Brain, eye, adrenal	SN, LC, CC, HpC	SN, LC, CC, HpC, AM

Abbreviations, + - minimal, ++ - moderate, +++ - heavy, SN - substantia nigra, LC - locus ceruleus, CC - cerebral cortex, HpC - hippocampus, AM - amygdala.

These lines have been examined on a DNA (Fig 2), mRNA (Fig 3, see end of report), and protein level (Fig 3) and have found to vary in their number of copies, regional expression of mRNA and protein and in the quantity of mRNA and protein (Table 1).

Fig 2. Southern blot from tail DNA of transgenic and control C57BL/6 mice. Lane 1 - plasmid control, Lane 2 MW markers, Lane 3 - C57BL/6, Lane 4 - hm²-10, hm²-27, hm²-39. A specific restriction fragment is identified only in the transgenic mice and the density is proportional to the number of copies.



Other organs have also been examined and were found to express the mRNA in regions where it would be expected including the adrenal glands and eyes in several of these lines (Table 1). We have selected two specific lines, hw5 and hm²39, to focus on for behavioral, aging, and neurotoxicant actions. Both of these lines constitutively express high levels of transgene in the substantia nigra as identified by *in situ* hybridization (ISHH, Fig. 3) and express high levels of protein in the striatum and other dopaminergic projection regions of identified by immunohistochemistry (IHC, Fig. 3). All neurons of the substantia nigra and ventral tegmental area express the transgene as identified by *in situ* hybridization histochemistry and IHC. The antibody to human α -synuclein is specific for the human form of the protein and fails to detect mouse α -synuclein (Fig. 3). As such, we

are able to detect the human protein with sensitivity and specificity and are able to map the cellular and terminal location of the protein. Cell bodies identified in these two lines expressing the protein include the substantia nigra pars compacta and ventral tegmental area, locus ceruleus and neurons in the olfactory bulb and cerebral cortex. Anomalous expression occurs in cells in other regions including the hippocampus and cerebellum. Strong terminal expression of the protein, as expected, was found in projection regions of catecholaminergic nuclei including the striatum, olfactory bulb, cerebral cortex, basolateral amygdala, hippocampus, and in other regions. Our ability to do them in these mice will be useful when the SYN XAT mice become available and support our use of the hwSYN and hm²SYN lines for toxicity studies discussed below.

We have initiated toxicity studies with these animals and our first experiment involved using 12 each of C57BL/6, hw5, and hm²39 male mice (**Fig 4 below**). We treated them with a low dose of MPTP (15 mg/kg) and measured locomotor activity before and after two treatments. Both the hw5 and hm²39 groups demonstrated increased sensitivity to MPTP as defined by a greater reduction in locomotor activity after treatment and a slower recovery to baseline. These effects were seen following both doses of MPTP. Treatments were stopped after the second treatment. The animals were sacrificed, and the tissue is awaiting neurochemical outcome measures. These animals will have the density of the dopamine transporter measured in the striatum using quantitative autoradiography (**Fig. 3**) and will have the number of dopaminergic neurons in the substantia nigra and ventral tegmental area counted using unbiased stereology (**Fig. 3**).

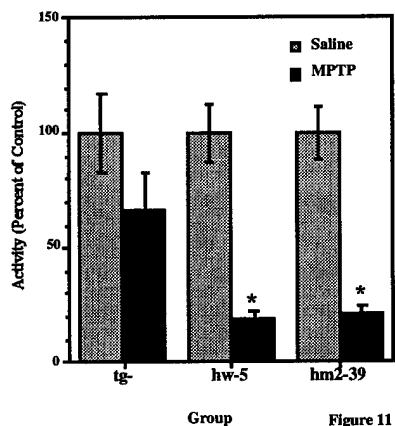


Fig 4. Three groups of mice (n=12 per group) including C57BL/6, hw5, and hm²39 were given two treatments of MPTP (15 mg/kg). Locomotor activity was measured 24 hrs before, 1 hour after each treatment and 72 hours after each treatment. Both transgenic lines (hw5, and hm²39) were significantly more affected by MPTP (*) as demonstrated by reduced locomotion and had slower recovery (data not shown).

The data obtained from these studies suggests that overexpression of either the human wild-type α -synuclein gene or a doubly mutated form may exacerbate MPTP toxicity. Toxicity due to the transgenes alone awaits further aging of our mice. These results suggest that we will be able to carry out these studies in the SYN XAT lines when they become available. They also suggest we will be able to study the effect of the human α -synuclein gene on toxicity of substantia nigra neurons as a function of aging and neurotoxicants. The data from these mice completes our efforts for Year 1 in the **modified Statement of Work (Parts Ia, b, c)**.

As proposed in our original application, we are continuing our efforts to develop a somatic mosaic transgenic mouse model in which the tyrosine hydroxylase promoter will be used to drive the expression of wild-type and mutated human α -synuclein in a temporal and regional selective manner. Initial efforts have been in developing a universal somatic mosaic construct vector (pUSMTV) which can be used to produce a variety of somatic mosaics (**Fig. 1C**). The initial cloning vector has been completed. We have tested this construct by inserting the CMV IE promoter into our "promoter insertion site" and the gene for enhanced green fluorescence protein (eGFP) into our "gene of interest site" (**Fig. 1D**). We have used this test construct to make stably transfected cells and have been able to test all functional components. The construct is transcriptionally silent under normal conditions within the cell following stable integration. Upon exposure to Cre recombinase using a viral vector (HSVCre) we have demonstrated excision of our sequences between the loxP sites with subsequent activation of expression of the eGFP gene. We have also demonstrated that the

IRES and the LacZ gene are functional. Thus, every functional component has been tested *in vitro* and works correctly. We have found the pUSMTV vector to be unstable during cloning making it difficult to clone with ease. We are making a variety of changes to make this a more stable cloning vector, including removing a large unnecessary homologous stretch to the LacZ gene present in the pBS vector, by transferring the essential components to a low copy number plasmid background (pBR322), and using a bacterial strain mutated to reduce DNA repair and the production of recombinants. With these changes we hope that cloning the large tyrosine hydroxylase promoter (9 kb) will be an easier task. After these changes, the TH promoter and the wild-type and doubly mutated human α -synuclein genes will be cloned in and the constructs (hwSYN XAT and hm²SYN XAT) sent to our transgenic facility for production. The steps following production of founders will be quite short as all of the needed reagents for testing and characterization of the lines are already available and verified using our current transgenic lines. These data complete the revised **Statement of Work for Year 1, part Id**

We have added an additional area of work related to our current project using HSV viral vectors. We have created a replication defective HSV amplicon in which the human wild-type (pHSV/eGFP/hwSYN) and doubly-mutated α -synuclein (pHSV/eGFP/hm²SYN) are under control of the Herpes virus IE/45 promoter and a reporter (eGFP) is under control of a CMV IE promoter. This construct has been packaged into virus and used to infect primary mouse cortical neurons *in vitro* (Fig. 5, see end of report). As can be seen, this virus is functional and expresses both α -synuclein and eGFP protein. The eGFP protein can be monitored using epifluorescence and is green. The α -synuclein protein can be identified using fluorescent immunocytochemistry and using the appropriate filters and imaging can be visualized in the same cell expressing eGFP. We have demonstrated that the α -synuclein protein appears to spontaneously aggregate in neurons and may be toxic to cells. Further work is planned using this virus both *in vitro* and *in vivo* in mouse brain to demonstrate toxicity. The studies will be useful to identify whether acute overexpression of wild-type or mutated α -synuclein is directly toxic to neurons, what type of neurons might be involved and whether this toxicity can be combined with environmental neurotoxicants to increase toxicity. These data complete our Year 1, part Ie in our revised **Statement of Work**.

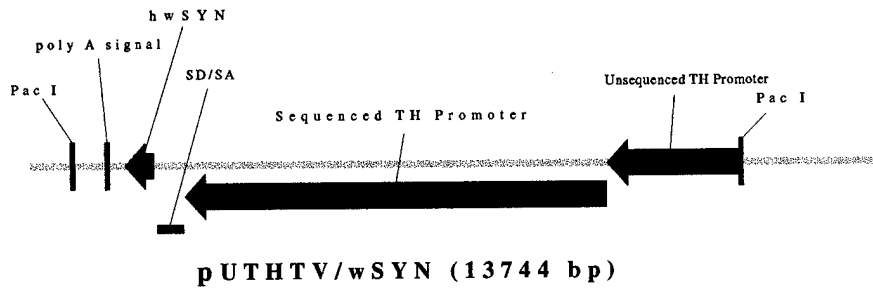
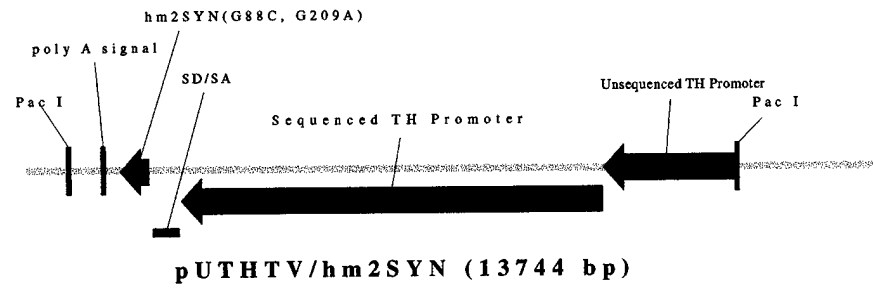
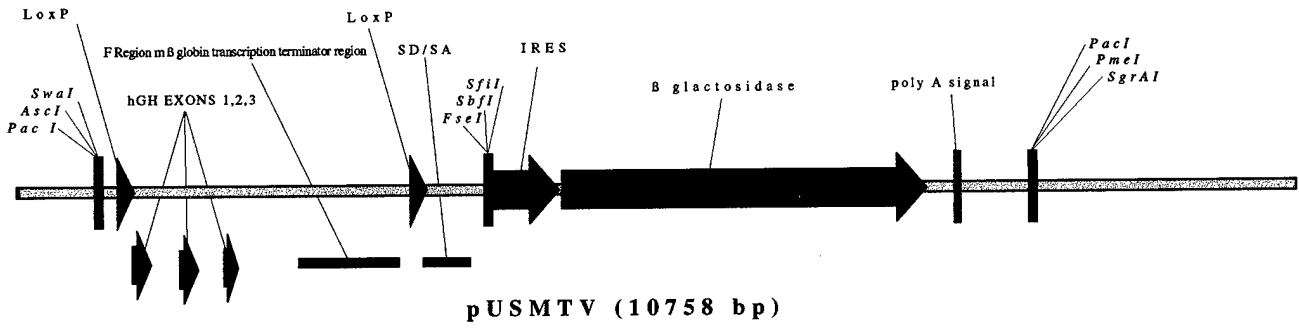
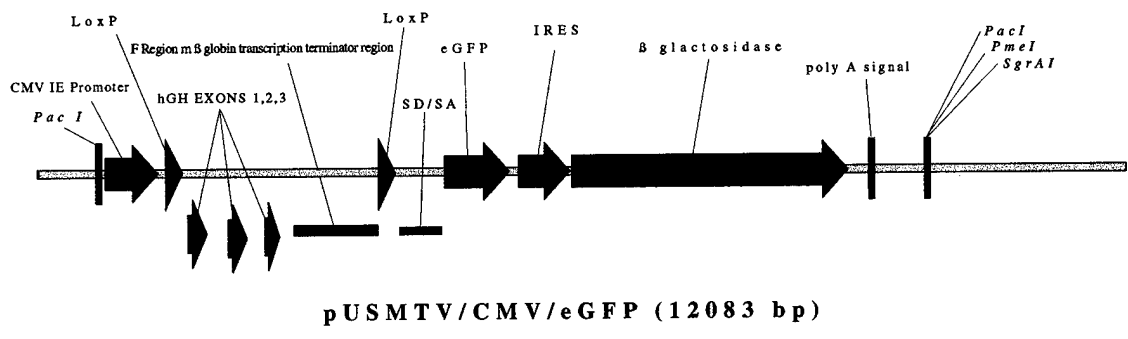
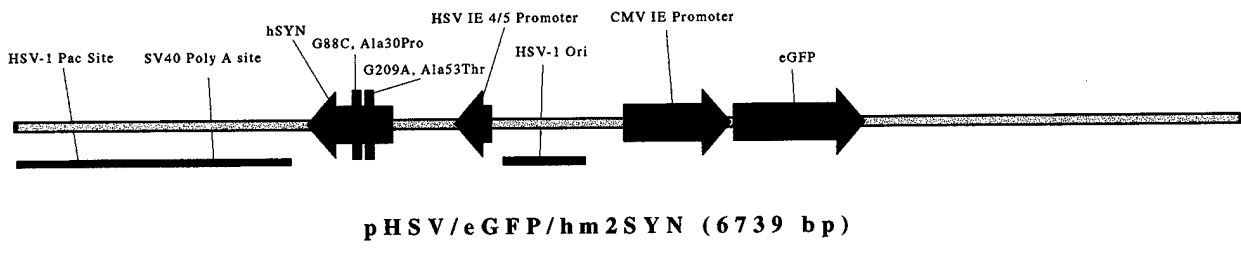
A**B****C****D****E**

Figure 1. Figures of constructs being used in this project. See text for descriptions and use.

line hm²-39

C57BL/6

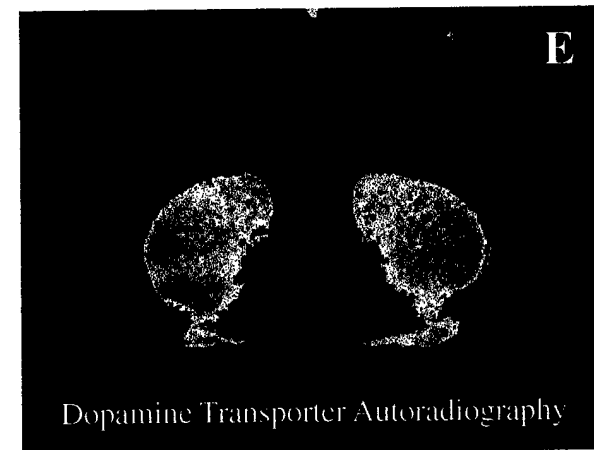
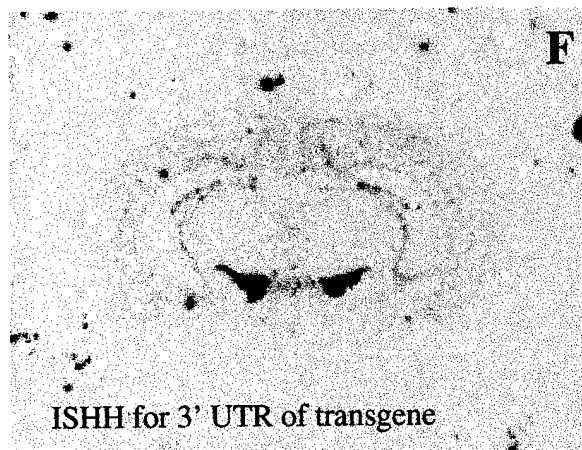
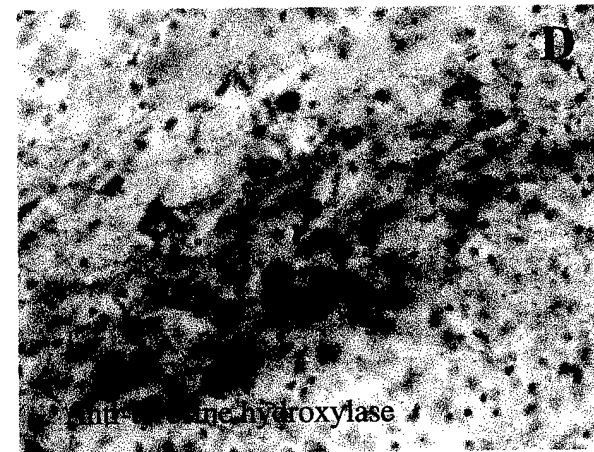
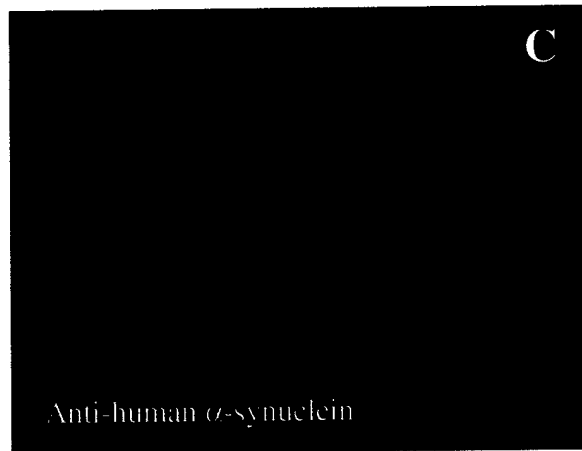
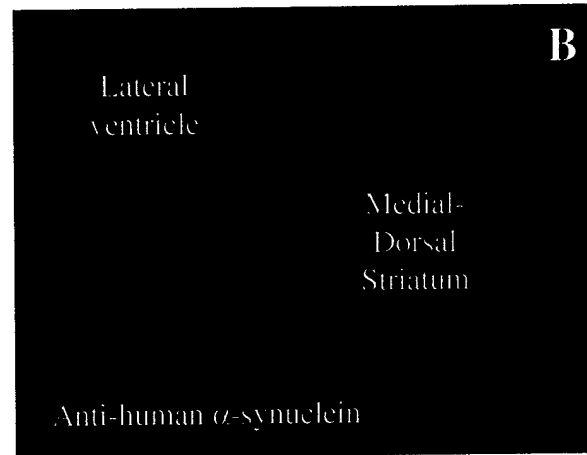
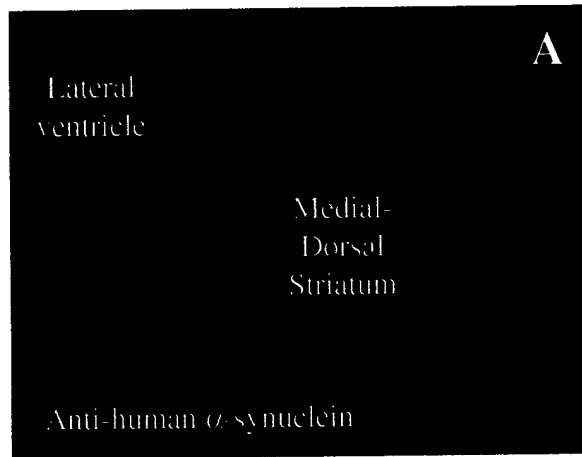


Figure 3. Selected images from transgenic mouse line hm²-39 (A, C, E) and from normal C57BL/6 mice (B, D, E). Immunochistochemistry using a human specific antibody to α -synuclein detects transgene specific protein only in the transgenic mouse lines in terminals in the striatum and cerebral cortex (A) and cell bodies in the substantia nigra (C). No labeling is seen in normal C57BL/6 mice (B). *In situ* hybridization histochemistry using a probe to the 3' UTR of the transgene demonstrates high levels of mRNA in the cell bodies of the substantia nigra and lesser levels in hippocampus and cerebral cortex (F). No hybridization is seen in control C57BL/6 mice. Chromogenic IHC combined with Nissl staining was developed for use in stereology to count substantia nigra neurons during aging and neurotoxicant treatments (D). Quantitative autoradiography for the dopamine transporter was tested in mice using techniques developed for the rat to quantitate terminal loss during aging and neurotoxicant treatment (E).

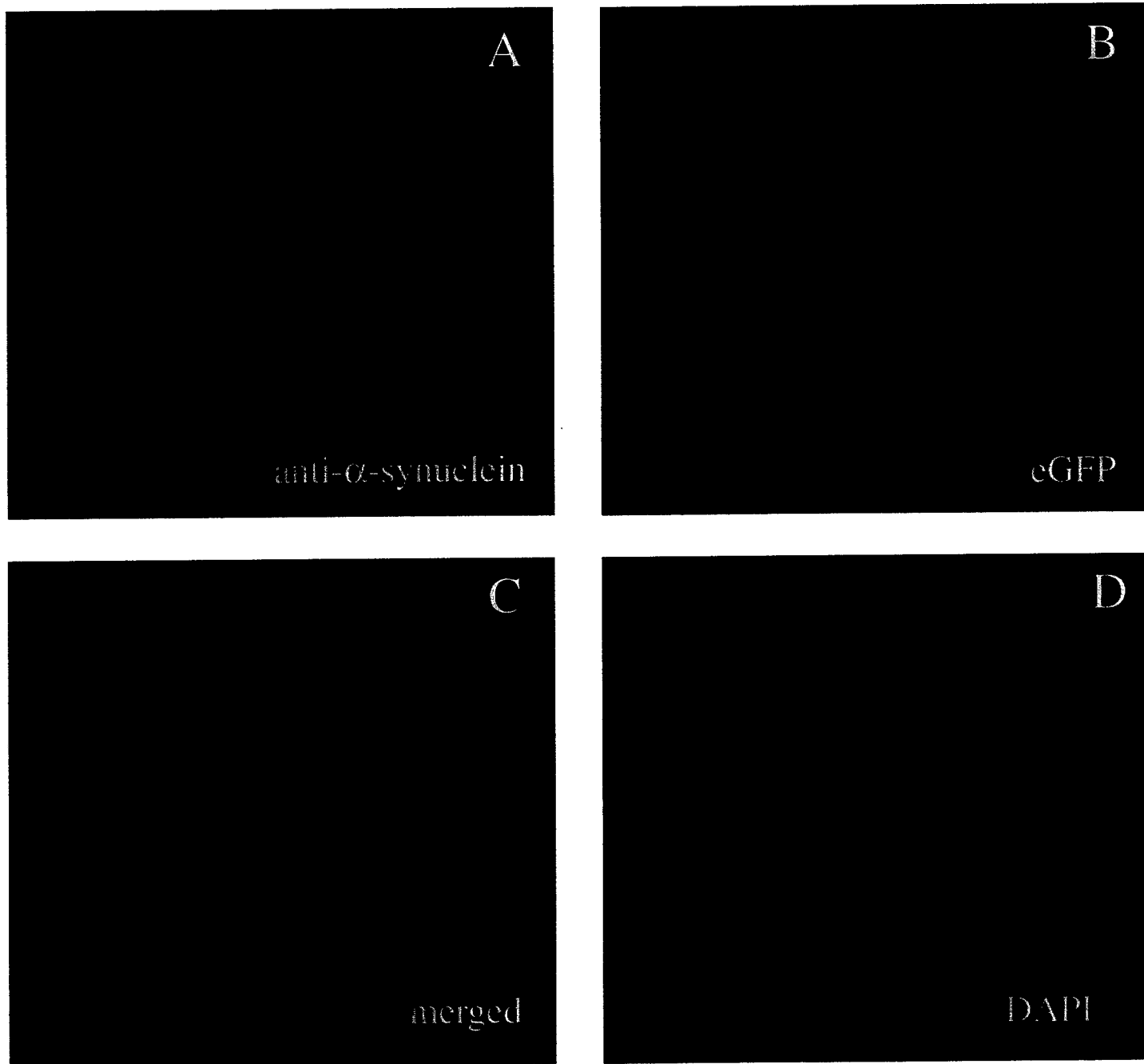


Figure 5. Primary mouse cortical neurons were infected with an HSV amplicon vector (pHSV/eGFP/hwSYN) expressing eGFP under control of the CMV promoter and wild-type human α -synuclein under control of the Herpes virus IE4/5 promoter. Wild-type human α -synuclein (A) was detected using a Rb polyclonal 1^o antibody and Alexa 590 Gt anti-Rb 2^o Ab. The same field was captured using a 100X oil immersion objective and different filters (A - TRITC, B - FITC, and D - UV). The images from A and B were merged in panel C. Wild-type α -synuclein forms aggregates only in cells infected with the amplicon vector and is otherwise undetectable in these cells.

KEY RESEARCH ACCOMPLISHMENTS

1. Generation of transgenic mice continuously overexpressing human wild-type α -synuclein (hwSYN, lines hw5, 6, 68, and 88) and human doubly-mutated α -synuclein (hm²SYN, lines hm²10, 27, and 39) in catecholaminergic neurons. These mice have been characterized and are ready for toxicity and aging studies. Generation of reagents (probes, primers, and antibodies) for use in characterizing α -synuclein XAT mice when they become available.
2. Identification of increased susceptibility of two lines of mice (hw5 and hm²39) to the environmental neurotoxicant MPTP in comparison to littermate C57BL/6 mice.
3. Construction of the pUSMTV cloning vector.
4. Verification of functionality of all DNA components pUSMTV. Generation of fragments (TH promoter, wild-type and doubly-mutated) human α -synuclein for cloning into pUSMTV to make the XAT mice.
5. Construction of viral vectors expressing human wild-type and doubly mutated α -synuclein.
6. Identification of spontaneous aggregation of α -synuclein in primary cortical neuronal cultures.

REPORTABLE OUTCOMES

No manuscripts, abstracts, or presentations have yet been made on this work.

CONCLUSIONS

We have made considerable progress in the first year of this award in constructing an animal model of α -synuclein overexpression and have demonstrated that our model likely results in enhanced sensitivity to environmental neurotoxicants. The importance of this work is that it supports our hypothesis and suggests that both normal α -synuclein and the mutated form of α -synuclein may harm cells over expressing the protein. This harm in humans may be related to an effect of aging or exposure to environmental neurotoxicants. Future studies will address the role of this overexpression in animals which are at different ages. We will continue to explore the role of wild-type and mutated human α -synuclein in neurons using transgenic mouse models and viral vectors. We hope this work will lead to an understanding of the mechanisms of toxicity and offer insights into possible therapies.

REFERENCES

None.

APPENDICES

See Revised Statement of Work (next page).

3. STATEMENT OF WORK (REVISED, 1/00)

The following goals will be performed in each year of the award. Each goal is designated according to the Technical Objectives outlined in the Body of Proposal.

Year 1

- Ia. Create transgenic mouse lines overexpressing wild-type (hwSYN) and double-mutated (hm²SYN) human α SYN under control of the TH promoter.
- Ib. Develop reagents and characterize the α SYN lines at the DNA, RNA, and protein level.
- Ic. Assess the α SYN lines for functionality and response to neurotoxins using behavioral, neurochemical and stereological measures.
- Id. Create a universal somatic mosaic cloning vector (pUSMTV) for the production of somatic mosaic mouse lines. Test all functional DNA elements.
- Ie. Create and test viral vectors expressing wild-type and double-mutated human α SYN.

Year 2

- IIa. Three α SYN XAT constructs will be made. Each of the three constructs will be made simultaneously so that all three can be carried through later steps simultaneously.
- IIb. *In vitro* testing of the three α SYN XAT constructs will be conducted using stably transfected cells to ensure the constructs are functional.
- IIc. The three α SYN XAT constructs will be used to generate lines of mice. Using methods from the previous lines of mice (hwSYN and hm²SYN), the XATs lines will be characterized on the DNA, RNA, and protein level with and without recombination, *ex vivo*. Lines will be selected for further behavioral and neurotoxic exposures.
- IIId. Viral vectors will be used *in vitro* and *in vivo* to study the toxicity and aggregation of wild-type (HSV/SYN) and double-mutated (HSV/hm²SYN) human α SYN.

Year 3

- IIa. Complete *in vivo* characterization of transgenic lines by mRNA and protein expression from adult hw α SYN and hm² α SYN XATs activated by unilateral intrastriatal delivery of HSVTH9cregfp or controls will be done. At one week, 4 weeks, 6 months, and 12 months, animals will be sacrificed and processed for ISHH and IHC using probes and antibodies specific for human or mouse α SYN will be performed.
- IIb. Determination of substantia nigra neuronal response to gene activation. Neuronal counts from adult α SYN XATs activated with HSVTH9cregfp will be compared to animals activated with HSVTH9gfp to determine if activation results in loss of SN neurons over time. Multiple stereological measures will be used to establish whether hm α SYN, hw α SYN, or m α SYN overexpression alone increase SN neuron vulnerability.
- IIc. Initiate acute treatments of MPTP and paraquat to mice. Adult α SYN XAT mice prelabeled bilaterally with intrastriatal FluoroGold (FG) will be activated unilaterally by the intrastriatal delivery of HSVTH9cregfp or control mice will receive HSVTH9gfp. When activated α SYN levels are at steady state, animals will be injected with MPTP or paraquat.

Year 4

- IIc. Complete acute treatments of MPTP and paraquat to mice. Drug administration will be completed and analysis of neuronal loss performed during this year.
- III. Initiate chronic paraquat treatment of mice. Adult α SYN XAT mice prelabeled bilaterally with intrastriatal FluoroGold (FG) will be activated unilaterally by the intrastriatal delivery of HSVTH9cregfp or control mice will receive HSVTH9gfp. Animals will be given low dose paraquat or vehicle in their diet.

Year 5

- III. Complete paraquat treatment and analysis of neuronal vulnerability.