PI: **Guilarte, Tomas R**

Title: Cholinergic Neuron Degeneration in Mn Neurotoxicity

Received: 07/10/2017

FOA: PA16-160

Council: 01/2018

Competition ID: FORMS-D

FOA Title: NIH Research Project Grant (Parent R01)

1 R01 ES029344-01

Dual:

Accession Number: 4073976

IPF: 513809

Organization: FLORIDA INTERNATIONAL UNIVERSITY

Former Number:

Department: Envir & Occupational Health

IRG/SRG: NAL

AIDS: N

Expedited: N

Subtotal Direct Costs
(excludes consortium F&A)
Year 1: 250,000
Year 2: 250,000
Year 3: 250,000

Animals: N
Humans: N
Clinical Trial: N
Current HS Code:
HESC: N

New Investigator: N
Early Stage Investigator: N

Senior/Key Personnel:	*Organization:*	*Role Category:*
Tomas Guilarte Ph.D	Florida International University	PD/PI
Changwon Yoo	FLORIDA INTERNATIONAL UNIVERSITY	Other (Specify)-Statistician

OMB Number: 4040-0001
Expiration Date: 10/31/2019

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE **State Application Identifier**

1. TYPE OF SUBMISSION*

● Pre-application ● Application ● Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

2. DATE SUBMITTED **Application Identifier**

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION **Organizational DUNS*:**

Legal Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:

Person to be contacted on matters involving this application

Prefix: First Name*: Middle Name: Last Name*: Suffix:

Position/Title:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email:

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)**

7. TYPE OF APPLICANT*

Other (Specify):

Small Business Organization Type ● Women Owned ● Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

If Revision, mark appropriate box(es).

● New ● Resubmission ● A. Increase Award ● B. Decrease Award ● C. Increase Duration

● Renewal ● Continuation ● Revision ● D. Decrease Duration ● E. Other *(specify)*

Is this application being submitted to other agencies?* ●Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*

12. PROPOSED PROJECT
Start Date* Ending Date*

13. CONGRESSIONAL DISTRICTS OF APPLICANT

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Middle Name: Last Name*: Suffix:
Position/Title:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email*:

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$1,098,750.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$1,098,750.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ● THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

● PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: Middle Name: Last Name*: Suffix:
Position/Title*:
Organization Name*:
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email*:

Signature of Authorized Representative* **Date Signed***

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040010
Expiration Date: 10/31/2019

Project/Performance Site Location(s)

Project/Performance Site Primary Location

● I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: **FLORIDA INTERNATIONAL UNIVERSITY**
Duns Number: **0712988140000**
Street1*: **11200 SW 8th ST**
Street2: **AHC4 340**
City*: **MIAMI**
County:
State*: **FL: Florida**
Province:
Country*: **USA: UNITED STATES**
Zip / Postal Code*: **331990001**
Project/Performance Site Congressional District*: **FL-026**

Additional Location(s)

File Name:

Funding Opportunity Number: PA-16-160. Received Date: 2017-07-10T16:00:57.000-04:00

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ● **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ● Yes ● No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6

If NO, is the IRB review Pending? ● **Yes** ● **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ● **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ● No

IACUC Approval Date:

Animal Welfare Assurance Number

3. Is proprietary/privileged information included in the application?* ● **Yes** ● **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ● **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ● Yes ● No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ● **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ● **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	**Guilarte_MnGrant_Project_Summary_2017_FINAL.pdf**
8. Project Narrative*	**Guilarte_MnGrant_Proj_Narrative_2017_FINAL.pdf**
9. Bibliography & References Cited	**Guilarte_MnGrantREFERENCES_2017_FINAL.pdf**
10.Facilities & Other Resources	**Guilarte_FIU_resources_2017.pdf**
11.Equipment	**Guilarte_FIU_Major_Equipment_2017.pdf**

4.2. Project Summary (Abstract): The long-term goal of the proposed research is to understand the role of the cholinergic system in manganese (Mn)-induced neurological dysfunction. Today, millions of welders, smelters, and miners in the United States (US) and throughout the world are chronically exposed to Mn-containing fumes, aerosols, and particles on a regular basis. Furthermore, drinking water with naturally high Mn concentrations is now recognized as an important source of chronic Mn exposure to large segments of the population in the US and globally. Therefore, the number of humans that are potentially exposed to neurotoxic levels of Mn worldwide are much larger than previously recognized, making it a public health problem of global proportion. Exposure to contemporary levels of Mn results in impairments in working memory and executive function and produces deficits in fine motor control and postural stability. These neurological effects of chronic Mn exposure are likely to have a pathophysiology that involves multiple neuronal systems. Previous studies from our laboratory have shown that chronic exposure to moderate levels of Mn in non-human primates produces dysfunction of nigrostriatal dopaminergic (DAergic) neurons by inhibiting striatal dopamine release. We now find a marked loss of striatal cholinergic interneurons (ChI) and these findings challenge the current dogma of Mn-induced pathophysiology from a solely DAergic perspective to one in which there is disruption of the DAergic-Cholinergic balance in the basal ganglia.

Cholinergic neurons are important in the physiology of cognition, emotion, compulsive behavior, locomotion, and gait, domains that are affected in Mn-induced neurological dysfunction. Here, we also provide initial evidence that chronic Mn exposure in non-human primates results in an apparent basal forebrain cholinergic neuron loss or injury similar to what is found in Alzheimer's disease and other neurodegenerative disorders. Thus, we propose to rigorously characterize the effect of chronic Mn exposure on choline acetyltransferase (ChAT)-positive cholinergic neurons in the caudate/putamen/nucleus accumbens as well as in the basal forebrain and pedunculopontine nucleus in the non-human primate brain (specific aim 1). These studies will use rigorous unbiased stereological cell counting and soma size determination methods. We will also determine the effect of chronic Mn exposure on vesicular acetylcholine transporter (vAChT) in cholinergic axon terminals and varicosities (specific aim 2) to assess if chronic Mn exposure produces cholinergic neuron axonopathy. Finally, we will examine the role of neurotrophic factors on the Mn-induced loss of cholinergic neurons (specific aim 3) by measuring concentrations of Brain-Derived Neurotrophic Factor and Nerve Growth Factor in relevant brain regions. The proposed studies will provide a more precise mechanistic understanding of Mn-induced pathophysiology that can lead to the development of cholinergic- and/or neurotrophic factor-based therapies for the treatment of Mn-induced neurological dysfunction.

Project Narrative: Humans are exposed to neurotoxic levels of manganese (Mn) from a variety of sources resulting in neurological disease. The proposed research will examine the effect of chronic Mn exposure on the cholinergic system and preliminary findings indicate the loss or injury of cholinergic neurons in brain regions that are relevant to Mn-induced parkinsonism, Alzheimer's disease and other neurodegenerative disorders.

RESOURCES

Laboratory:

Dr. Guilarte has over 1600 square feet of designated laboratory space in the newly built Academic Health Center 4 (AHC4) building at Florida International University, which will be used for the proposed research. All laboratories are part of the Robert Stempel College of Public Health & Social Work, Department of Environmental and Occupational Health and located on the 3rd floor of the AHC4 building. The main lab is equipped with 2 chemical fume hoods, 3 refrigerators (including one for flammable storage), a chromatography refrigerator, 3 -20˚C freezers, 2 cryostats, 2 microtomes, and a high speed centrifuge. This is where much of the routine neurochemistry is performed, as well as gel electrophoresis and western blotting, including a LiCor Odyssey system for quantification. In addition, there is a new Applied Biosystems QuantStudio 6 Real-Time PCR system and a thermocycler for gene expression analysis. There are also 2 new Dionex Ultimate 3000 HPLC systems sharing an autosampler: one with both a Fluorescence and a UV/VIS Diode Array detectors, and a second with an Electrochemical detector for catecholamine and acetylcholine analysis. A separate radioactive area is equipped with a Brandell filtration system and scintillation counter. This is where *in situ* hybridization studies, autoradiography and radioligand binding assays are performed. There is a separate tissue culture room with 2 biosafety cabinets, 3 incubators, an inverted microscope with camera, and an incubated shaker. In addition, a separate microscope/imaging room is home to a MCID image analysis system, 2 Olympus microscopes with stereology capabilities, and an Olympus FluoView self-contained confocal microscope. All microscopes in this room are housed on anti-vibration tables. On each floor there is also a shared 4˚C cold room, common room with a dishwasher and autorclave, and allocated equipment rooms for 4 -80˚C freezers and 3 additional -20˚C freezers.

Animal:

Dr. Guilarte has animal holding rooms within the animal facilities on the 5th floor of the AHC4 Building for breeding and housing animals. He also has access to a shared surgical suite and procedure rooms within these facilities.

Neurobehavioral Core Facility: In addition to the space in AHC4, there is dedicated space in the Owa Ehan (OE) building exclusively for a Neurobehavioral Core where Dr. Guilarte's staff perform all animal behavioral experiments. The space is equipped with holding rooms for mice and rats, a cage wash and autoclave, as well as multiple rooms to house separate behavioral tasks. The facility is fully equipped to perform Morris water maze studies on adult and young rats, including Videomax equipment with a camera from Columbus Instruments, along with a large and a small water tank. A 16 arm Radial Arm maze from Noldus, two Omnitech activity cages, 2 Contextual Fear Conditioning boxes and equipment from Coulbourn Instruments, 2 Condition Place Preference boxes, 4 SR-Lab chambers from San Diego Instruments for Startle Reflex and Pre-Pulse Inhibition studies, a rotorod and an elevated plus maze are also located in this room, in addition to enrichment cages and voluntary exercise running wheels.

Computers:

12 PC computers and MacBook Pro are linked to a color laser printer for use in preparation of manuscripts, posters, reports, figures, etc. Dr. Guilarte also has an iMac linked to a color laser printer in his office.

Office:

In the Robert Stempel College of Public Health and Social Work (AHC5 Building), Dr. Guilarte has an office suite for himself, and there is designated office space in the AHC4 Building for his staff and post docs, as well as desk space in the labs.

MAJOR EQUIPMENT:

Olympus FluoView self-contained confocal microscope (confocal-in-a-box); Beckman-Coulter LS6500 Liquid Scintillation counter; 4 x Forma -80ºC freezers; BioRad benchtop spectrophotometer; Leica microtome; American Optical Co microtome; BioRad protein electrophoresis system for large and small gels; Sorvall RC 6 plus superspeed centrifuge; 2 Leica freezing cryostats; Brandell filtration system; Nikon Inverted Phase-contrast microscope with a Nikon digital camera; 3 x Forma Scientific CO_2 incubators; 2 Dionex Ultimate 3000 HPLC systems sharing an autosampler: one with both a Fluorescence and a UV/VIS Diode Array detector, and a second with an Electrochemical detector; Applied Biosystems QuantStudio 6 Real-Time PCR system; thermocycler; 6 x -20°C freezers; 1 x Refrigerator/Freezer for flammables storage; 2 chemical fume hoods with storage; 2 biosafety cabinets; MCID Image Analysis System with Northern Lights Light Box; 2 x Olympus microscopes with cameras, motorized stages, computers, and stereology capabilities; Li Cor Odyssey CLx NIR system for fluorescent detection and quantification of western blots; fluorescent plate reader; Millipore system; a variety of refrigerators, pH meters, balances, waterbaths, vortexes, polytrons, McIlwain tissue slicer. Neurobehavioral Core Equipment: 16 arm Radial Arm Maze – Noldus; Morris water maze set-up for adult and young rats with computer, Videomex - Columbus Instruments and large and small water tanks; 2 Omnitech activity cages; 2 Fear Conditioning chambers and software – Coulboum Instruments; Rota-rod – Columbus Instruments; 4 SR-Lab ABS Chambers for Startle Reflex and Pre-pulse Inhibition measurements – San Diego Instruments; 2 Conditioned Place Preference chambers – Any Maze; Elevated Plus Maze; 8 Environmental Enrichment Cages; 20 rat cages with running wheels and counters for voluntary exercise measurements - Muromachi Microwave Fixation System - 5 kW for phosphorylation work

OMB Number: 4040001
Expiration Date: 10/31/2019

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. First Name*: Tomas Middle Name R Last Name*: Guilarte Suffix: Ph.D

Position/Title*: Professor & Dean
Organization Name*: Florida International University
Department: Envir & Occupational Health
Division: Robert Stempel CPHSW
Street1*: 11200 SW 8th Street, AHC5-507
Street2:
City*: Miami
County:
State*: FL: Florida
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 331990000

Phone Number*: 305-348-5344 Fax Number:

E-Mail*: tguilart@fiu.edu

Credential, e.g., agency login: [eRA Commons User Name]

Project Role*: PD/PI Other Project Role Category:

Degree Type: PHD,MS,BS Degree Year:

Attach Biographical Sketch*: File Name: Guilarte_MnGrant_GuilarteBioSketch_2017_FINAL.pdf

Attach Current & Pending Support: File Name:

Funding Opportunity Number: PA-16-160 . Received Date: 2017-07-10T16:00:57.000-04:00

PROFILE - Senior/Key Person

Prefix: First Name*: Changwon Middle Name Last Name*: Yoo Suffix:

Position/Title*: Assistant Professor
Organization Name*: FLORIDA INTERNATIONAL UNIVERSITY
Department:
Division:
Street1*: 11200 S.W. 8th St, AHC2 580
Street2:
City*: Miami
County:
State*: FL: Florida
Province:
Country*: USA: UNITED STATES
Zip / Postal Code*: 331990000

Phone Number*: 305-348-7779 Fax Number:

E-Mail*: cyoo@fiu.edu

Credential, e.g., agency login: [eRA Commons User Name]

Project Role*: Other (Specify) Other Project Role Category: Statistician

Degree Type: PHD,MS,BS Degree Year:

Attach Biographical Sketch*: File Name: Guilarte_MnGrant_YooBioSketch_2017_FINAL.pdf

Attach Current & Pending Support: File Name:

OMB No. 0925-0001 and 0925-0002 (Rev. 11/16 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: **Guilarte, Tomás** R.

eRA COMMONS USER NAME (credential, e.g., agency login): [eRA Commons User Name]

POSITION TITLE: Professor & Dean, Robert Stempel College of Public Health & Social Work

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Florida, Gainesville, FL	BS	05/1974	Zoology
University of Florida, Gainesville, FL	MS	05/1976	Medical Physics
Johns Hopkins University School of Hygiene & Public Health (Bloomberg School of Public Health), Baltimore, MD	PhD	03/1980	Environmental Health Sciences

A. Personal Statement.

I am an established investigator with experience and expertise in neurotoxicology and neuroscience studying the effects of environmental toxins on the brain. In all of my studies, I use a comprehensive approach from behavioral, systems, cellular, and molecular endpoints. For some studies, I also use primary culture of brain cells (neuronal cultures and culture of microglia and astrocytes) as well as different neuroimaging platforms including Positron Emission Tomography, Single Photon Emission Computed Tomography, Magnetic Resonance Imaging and Spectroscopy, and Diffusion Tensor Imaging. My studies go beyond understanding mechanism(s) by which environmental toxins induce neurological and neurodegenerative disease. That is, once I understand mechanisms, I also investigate potential therapeutic strategies to mitigate environmentally-induced neurological disease. For example, my laboratory was the first to demonstrate that environmental enrichment reverses the adverse effects of early-life lead exposure on brain neurochemistry and cognitive function.

I am also interested in neuroinflammation and its role in neurodegenerative disease. My laboratory has pioneered the validation and application of a biomarker of brain injury and inflammation called Translocator Protein 18 kDa (TSPO) that is now widely used in clinical and preclinical studies. I am currently doing studies to understand the function of this biomarker in glial cells. We also study manganese neurotoxicity and have made many novel findings on the neurotoxicology of manganese using multidisciplinary collaborations. I have extensive experience in developing multidisciplinary research teams that involve multiple research groups and universities.

I am currently Professor & Dean of the Robert Stempel College of Public Health & Social Work at Florida International University (FIU). My research [EFFORT] as I continue to guide my laboratory. This effort is consistent with an agreement with the Provost [EFFORT] for research-see letter in Letters of Support section) and has resulted in a fruitful, collaborative, and rich research environment. I arrived at FIU on January 2016 and my lab came to FIU from Columbia University in May 2016. Although we were not operational for several months, we now occupy brand new laboratory space and my lab is fully functional with post-docs, doctoral and undergraduate students, lab techs, and a highly skilled and knowledgeable senior research scientist that oversees the daily operations of my lab and has been with me for over 20 years. I direct weekly lab meetings and I actively communicate with my postdocs, graduate students, and lab personnel on a daily basis. I also have established a more streamlined and responsive Office of the Dean with support personnel as Associate Deans and an Executive Director of Operations that maintains a high level of performance and function at the College level. Therefore, my role as Dean, does not in any way interfere with my official role as Professor and researcher consistent with my agreement with the Provost.

B. Positions and Honors

Positions and Employment

1980-1981 Research Associate, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland

1981-1987	Assistant Professor, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland
1987-1994	Associate Professor, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland
1990-2010	Joint appointment, Division of Human Nutrition, Department of International Health, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland
1990-1993	Associate Director, Neurotoxicology Program, Environmental Health Sciences Center, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland
1994-2010	Professor, Department of Environmental Health Sciences, Johns Hopkins University Bloomberg School of Public Health. Baltimore, Maryland.
1993-1996	Director, Neurotoxicology Program, Environmental Health Sciences Center, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland.
2004-2009	Director, Program in Molecular Imaging, Department of Environmental Health Sciences, Johns Hopkins University School Bloomberg School of Public Health, Baltimore, Maryland.
2010-2015	Leon Hess Professor (Inaugural) & Chairman, Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY.
2011-2015	Training Grant Director, NIEHS training entitled: Interdisciplinary Training in Environmental Health Sciences, Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY.
2011-2015	Deputy Director, NIEHS Center for Environmental Health in Northern Manhattan, Dept. of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY.
2010-present	Adjunct Professor, Department of Radiology, Johns Hopkins Hospital, Baltimore, Maryland
2016-present	Adjunct Professor, Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University. New York, NY.
2016-present	Dean, Robert Stempel College of Public Health & Social Work, Florida International University, Miami, Florida.
2016-present	Professor, Department of Environmental & Occupational Health, Robert Stempel College of Public Health & Social Work, Florida International University, Miami, Florida.
2016-present	Professor, Cognitive Neuroscience & Imaging, Department of Physics, Florida International University, Miami, Florida.
2016-present	Member, Biomolecular Sciences Institute, Florida International University. Miami, Florida.

Other Experience and Professional Memberships

1992	Lead Advisory Panel, NIEHS
1993	Ad Hoc reviewer, NIH-Toxicology SS2
1995-1999	Member, NIH-ALTX-3 Study Section
1995-1999	Member, Science Advisory Board-National Center for Toxicological Research, FDA
1999-2002	Environmental Health Review Committee, NIEHS
2002-2012	Ad hoc Reviewer on Multiple Study Sections, National Institutes of Health
2006-2012	Member, Steering Committee, Manganese Health Research Program
2008-present	Member, Institute for NanoBiotechnology at Johns Hopkins University
2013-2017	Member, Advisory Board of the National Institute of Environmental Health Sciences
2016-present	Miami-Dade County Mayor's HIV/AIDS Task Force
2017-pesent	Miami-Dade County Mayor's Opioid Task Force

Professional societies

1980-present	Delta Omega National Public Health Honorary Society
1989-1998	American Association for the Advancement of Science
1991-present	Society for Neuroscience
1995-present	Society of Toxicology
1997	Vice-President elect, Neurotoxicology Specialty Section of the Society of Toxicology
1998	Vice-President, Neurotoxicology Specialty Section of the Society of Toxicology
1999	President, Neurotoxicology Specialty Section of the Society of Toxicology
2000	Chair, Student Competition Awards, Neurotoxicology Specialty Section, Society of Toxicology
2003-2010	International Behavioral Neuroscience Society
2004-present	International Neurotoxicology Association

2005-2010 Society for Molecular Imaging

Honors:

1978 Recipient of 1st USDA Student Fellowship Grant Awarded to The Johns Hopkins University of Hygiene and Public Health, Baltimore, MD

1980 Winning Presentation; First Annual Student Research Day, The Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD

1981 Outstanding Young Men of America for 1981

1982 First Prize; Scientific Papers Section, Mid-Eastern Meeting of the Society of Nuclear Medicine

1989 American Men and Women of Science

Mentoring: Awards to Doctoral Students, Post-doctoral fellows, and Junior Faculty

1996-2012 Society of Toxicology Annual Meeting, Neurotoxicology Specialty Section Student Poster Competition Awards:

1996: Pre-doctoral- Anthony C. Kuhlmann (2nd); Post-doctoral- David A Jett Ph.D. (1st)
1997: Pre-doctoral- Anthony C. Kuhlmann (2nd)
1998: Pre-doctoral- Anthony C. Kuhlmann (2nd)
1999: Post-doctoral- Michelle K. Nihei Ph.D. (1st)
2000: Pre-doctoral- Angela S. Howard (2nd); Post-doctoral- Michelle K. Nihei Ph.D. (2nd)
2001: Pre-doctoral- Christopher D. Toscano (3rd)
2002: Pre-doctoral- Christopher D. Toscano (1st) and Ming-Kai Chen (2nd)
2006: Pre-doctoral- Neal C. Burton (1st)
2007: Pre-doctoral- Ming-Kai Chen (1st) and April Neal (3rd)
2009: Pre-doctoral- April Neal (1st) and Judy Choi (2nd)
2010: Pre-doctoral- Judy Choi (3rd)
2011: Post-doctoral- Judy Choi (1st)
2012: Post-doctoral- Kirstie Stansfield (2nd)

2008 25th Anniversary of the International Neurotoxicology Conference, Pre-doctoral student award- April Neal (2nd)

2014 TSPO Structure-Function International Meeting, Royal Veterinary College, London England- Award to Meredith K. Loth (1st)

2014 NIEHS Center for Environmental Health in Northern Manhattan Career Development Award- Kirstie H. Stansfield PhD, Associate Research Scientist

2016 Travel Award (competitive) Hispanic Organization of Toxicologists (HOT) Travel Award, Society of Toxicology- Kalynda Gonzales, PhD

2016 28th International Neurotoxicology Conference, Postdoctoral fellow poster competition- Kalynda Gonzales (1st Place)

C. **Contribution to Science (selected publications): Google Scholar H-Index= 52; i10-index= 111**

1. NMDA Receptor Function in Lead Neurotoxicity-Effects on cognitive function and molecular mechanisms: My laboratory was one of two research labs that initially showed that lead is a potent inhibitor of the NMDA receptor. This initial finding was followed by a series of studies showing that early life lead exposure disrupts the normal ontogeny of NMDA receptor subunits in the hippocampus leading to impairments of synaptic plasticity in the form of long-term potentiation and deficits in spatial learning. This work also showed that early life lead exposure impairs adult neurogenesis in the hippocampus. My laboratory was the first to show that environmental enrichment is able to reverse the lead-induced deficits in spatial learning and normalize the deficits in NMDA receptor subunits. This worked formed the basis for my lab developing a working model of the molecular mechanisms by which lead exposure alters synapse development and function. During the last five years, my work has shown that early life lead exposure may be a risk factor for mental disorders, specifically schizophrenia.

a. **Guilarte TR**, Toscano CD, McGlothan JL, Weaver SA (2003) Environmental enrichment reverses cognitive and molecular deficits induced by developmental lead exposure. Annals of Neurology 53: 50-56. PMID: 12509847. [Impact factor=9.9]

b. Abazyan B, Dziedzic J, Hua K, Abazyan S, Yang C, Mori S, Pletnikov MV, **Guilarte TR** (2014) Chronic exposure of mutant DISC1 mice to lead produces sex-dependent abnormalities consistent with schizophrenia and related mental disorders: a gene-environment interaction study. Schizophrenia Bulletin 40: 575-84. PMCID: PMC3984515. [Impact factor=8.8]

c. Stansfield KH, Ruby KN, Soares BD, McGlothan JL, Liu X, **Guilarte TR** (2015) Early life lead exposure recapitulates the selective loss of parvalbumin-positive GABAergic interneurons and subcortical dopamine

system hyperactivity present in schizophrenia. Translational Psychiatry 5e522. PMCID: PMC4354343. [Impact factor=5.6]

d. Wagner PJ, Park HR, Wang Z, Kirchner R, Wei Y, Su L, Stansfield K, **Guilarte TR**, Wright RO, Christiani DC, Lu Q (2017) In vitro effects of lead on gene expression in neural stem cells and associations between up-regulated genes and cognitive scores in children. Environmental Health Perspectives 125: 721-729. PMCID: PMC5381979. [Impact factor=8.4]

2. Translocator Protein 18 kDa (TSPO): A Molecular Biomarker of Brain Injury & Inflammation: My laboratory has performed pioneering work in the validation and application of Translocator Protein 18 kDa (TSPO) as a biomarker of brain injury and inflammation. TSPO is currently being used in clinical and preclinical neuroimaging studies in major research institutions around the world. In the normal brain neuropil, TSPO levels are almost non-detectable but following brain injury, TSPO levels increase markedly in microglia and astrocytes and this effect depends on the type and severity of the injury. My most recent work relates to the function of TSPO in glial cells, specifically microglia and astrocytes. My work has recently shown that TSPO ligands induce reactive oxygen species production in microglia and this effect may be associated with the activation of NADPH oxidase. Further, my work has shown the utility of TSPO to detect inflammation in other organs systems besides the brain, for example myocardial inflammation.

a. Chen MK, **Guilarte TR** (2008) Translocator protein 18 kDa (TSPO): molecular sensor of brain injury and repair. Pharmacology & Therapeutics 118:1-17. PMCID: PMC2453598. [Impact factor=11.0]

b. Choi J, Ifuku M, Noda M, **Guilarte TR** (2011) Translocator protein (18 kDa)/peripheral benzodiazepine receptor specific ligands induce microglia functions consistent with an activated state. GLIA 59: 219-230. PMCID: PMC4425360. [Impact factor=5.1]

c. **Guilarte TR**, Loth MK, Guariglia SR (2016) TSPO finds NOX2 in microglia for redox homeostasis. Trends in Pharmacological Sciences 37: 334-343, 2016. PMID: 27113160. [Impact factor=8.4]

d. Coughlin JM, Wang Y, Minn I, Bienko N, Ambinder EB, Xu X, Peters ME, Dougherty JW, Vranesic M, Lee M, Cottrell C, Sair HI, Sawa A, Munro CA, Nowinski CJ, Dannals RF, Lyketsos CG, Kassiou M, **Guilarte TR**, Pomper MG (2017) Imaging of glial cell activation and white matter integrity in brains of active and recently retired National Football League players. JAMA-Neurology 74: 67-74. PMID: 27893897 DOI: 10.1001/jamaneurol.2016.3764. [Impact factor=10.0]

3. Behavioral and Molecular Effects of Chronic Manganese Exposure in Non-Human Primates: During the last 12 years, my laboratory has been studying the effects of chronic manganese exposure on neurological health in non-human primates. This work, in collaboration with Dr. Jay Schneider (Thomas Jefferson) and Dr. Dean Wong (Johns Hopkins) utilizes behavioral, neuroimaging and neuropathological endpoints to determine the mechanisms of manganese-induced parkinsonism. This research was the first to show that manganese-induced parkinsonism differs from idiopathic Parkinson's disease in that manganese-induced parkinsonism does not involve degeneration of dopamine neuron cell bodies in the substantia nigra pars compacta but in fact, it is related to a marked inhibition of dopamine release in the dorsal striatum. We propose that the marked inhibition of striatal dopamine release by manganese in conjunction with degeneration of cells intrinsic to the basal ganglia (Cholinergic intemeurons) will form the basis for the movement abnormalities documented in humans and experimental animals. Using this same non-human primate model, my work was the first to show that chronic manganese exposure results in beta-amyloid aggregation and neurodegeneration in the frontal cortex. This effect was associated with impairment of working memory and paired-associative learning in the same animals.

We have 3 publications for submission on our Mn work that have been delayed due to the lab move.

a. **Guilarte TR**, Burton NC, McGlothan JL, Verina T, Zhou Y, Alexander M, Pham L, Griswold M, Wong DF, Syversen T, Schneider JS (2008) Impairment of nigrostriatal dopamine neurotransmission by manganese is mediated by presynaptic mechanism(s): implications to manganese-induced parkinsonism. Journal of Neurochemistry 107: 1236-1247. PMCID: PMC3675778. [Impact factor=4.2]

b. **Guilarte TR**, Burton NC, Verina T, Prabhu VV, Becker KG, Syversen T, Schneider JS (2008) Increased APLP1 expression and neurodegeneration in the frontal cortex of manganese-exposed non-human primates. Journal of Neurochemistry 105: 1948-1959. PMCID: PMC4335763. [Impact factor=4.2]

c. Schneider JS, Williams C, Ault M, **Guilarte TR** (2015) Effects of chronic manganese exposure on attention and working memory in non-human primates. Neurotoxicology 48: 217-222. PMCID: PMC4442708. [Impact factor=2.7]

d. **Guilarte TR**, Gonzales KK (2015) Manganese-induced parkinsonism is not idiopathic Parkinson's disease: Environmental and Genetic evidence. Toxicological Sciences 146: 204-212. PMCID: PMC4607750. [Impact factor=3.9]

4. Presynaptic mechanisms of lead neurotoxicity**:** My laboratory has played an important role in understanding the molecular mechanism(s) by which chronic early life lead exposure inhibits presynaptic vesicular release. This work has shown for the first time that inhibition of vesicular release, both in vitro and in vivo, is the result of changes in key vesicular proteins. This effect of lead on vesicular release was also associated with ultrastructural changes in vesicle number in the readily releasable/docked vesicle pool, disperse vesicle clustering in the resting pool, and a reduced number of presynaptic terminals with multiple mitochondria. Based on these findings, we are currently testing a therapeutic strategy that may mitigate these lead effects on vesicular release.

a. Neal AP, Stansfield KH, Worley PF, Thompson RE, **Guilarte TR** (2010) Lead exposure during synaptogenesis alters vesicular proteins and impairs vesicular release: potential role of NMDA receptor-dependent BDNF signaling. Toxicological Sciences 116: 249-263. PMCID: PMC2920085. [Impact factor=3.9]

b. Stansfield KH, Pilsner JR, Lu Q, Wright RO, **Guilarte TR** (2012) Dysregulation of BDNF-TrkB signaling in developing hippocampal neurons by lead: implications for an environmental basis of neurodevelopmental disorders. Toxicological Sciences 127: 277-295. PMCID: PMC3327871. [Impact factor=3.9]

c. Zhang X-L, Guariglia SR, McGlothan JL, Stansfield KH, Stanton PK, **Guilarte TR** (2015) Presynaptic mechanisms of lead neurotoxicity: effects on vesicular release, vesicle clustering and mitochondria number. PLoS One 10(5): e0127461. PMCID: PMC4444102. [Impact factor=4.4]

d. Guariglia SR, Stansfield KH, McGlothan J, **Guilarte TR** (2016) Chronic early life Pb^{2+} exposure alters presynaptic vesicle pools in hippocampal synapses. BMC Pharmacology & Toxicology 17: 56. PMCID: PMC5090882. [Impact factor=2.3 (new journal)]

Complete List of Published Work in MyBibliography:

http://www.ncbi.nlm.nih.gov/sites/myncbi/tomas.guilarte.1/bibliograpahy/41163350/public/?sort=date&direction=descending

D. **Additional Information: Research Support and/or Scholastic Performance:**

Ongoing Research Support

1. **2RO1 ES006189-22A1 (P.I.: Guilarte, TR), NIH/NIEHS**
   Title: NMDA receptor function in Lead Neurotoxicity
   Project Period: 12/01/92 – 06/30/20
   Budget period: 07/01/15 – 06/30/20 (years 21-25)
   The major goal of the competitive renewal is to investigate the role of the NMDA receptor complex in lead-induced neuropathology that is relevant to schizophrenia.
2. **2RO1 ES007062-19 (P.I.: Guilarte, TR), NIH/NIEHS**
   Title: Peripheral BDZ receptor: A Biomarker of Neurotoxicity
   Project Period: 09/01/95 – 05/31/18
   Budget period: 09/01/13 – 05/31/18 (years 15-19)
   The major goal of this project is to validate the use of the peripheral benzodiazepine receptor, recently named Translocator protein 18 kDa (TSPO) as a quantitative biomarker of chemical-induced neurotoxicity using *in vitro* autoradiography and *in vivo* Positron Emission Tomography (PET) techniques. We are currently assessing studying the functional role of TSPO in primary microglia.

Recently Completed Research Support:

1. **2RO1 ES010975-10 (P.I.: Guilarte, TR), NIH/NIEHS**
   Title: Molecular & Behavioral effects of low-level Mn exposure
   Project Period: 04/01/01 – 04/30/16
   Budget period: 05/01/09 – 05/31/18 (years 8-10)
   The goal of this project is to determine the behavioral and brain anatomical and biochemical changes using associated with chronic low-level manganese exposure in non-human primates.
2. **1RO1ES020465-05 (P.I.: Guilarte, TR) [this grant will end is 6months and will not be renewed]**
   Presynaptic Mechanisms of Lead Neurotoxicity.
   Project Period: 4/1/12-1/31/18
   Budget Period: 4/1/12-1/31/18 (years 1-5; currently on a no cost extension)
   The goal of this proposal is to examine a novel mechanism by which lead exposure alters neurotransmitter release and presynaptic plasticity via modification of NMDA receptor-dependent BDNF-TrkB trans-synaptic signaling.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Changwon Yoo

eRA COMMONS USER NAME (credential, e.g., agency login): [eRA Commons User Name]

POSITION TITLE: Associate Professor of Biostatistics

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
KyungHee University, Seoul, Korea	B.S.	02/1991	Mathematics
Harvard University, Cambridge, MA	S.M.	05/1997	Computer Science
University of Pittsburgh, Pittsburgh, PA	Ph.D.	05/2003	Biomedical Informatics
Virginia Bioinformatics Institute, Virginia Tech, VA	Postdoctoral	07/2004	Bioinformatics/ Biostatistics

A. Personal Statement

I am highly qualified to perform the biostatistical analyses needed for this study. My area of expertise is Causal Bayesian Networks (CBN), and I have performed multiple analyses of large and complex datasets. These analyses were for research projects ranging from discovering gene networks from microarray experiments to analyzing clinical and/or public health population data. More specifically, I have been working on analyses of large genome-wide datasets to discover gene networks for chronical diseases, such as brain tumor, Alzheimer, mesothelioma, using causal Bayesian networks and handling patient datasets to build computational causal models to better understand how such diseases develop. I have also developed an informatics database core — to help translational research and integrate other sources of data — that was supported by NIH Centers of Biomedical Research Excellence and NSF Experimental Program to Stimulate Competitive Research (EPSCoR) grants.

CBN is a knowledge representation formalism that has been valuable in translational biomedical research for constructing statistical graphical models, e.g., Structured Equation Models, and for generating causal hypotheses from data. Given the emergence of datasets in medicine, epidemiology, and biology with large number of variables, I have been developing efficient algorithms that scale up to datasets involving thousands of variables in learning high quality CBNs from data especially with interventions. For example, one of the algorithms that I have developed (called Implicit Latent Variable Scoring method) could identify latent variable models a hundred times faster than well-known methods (such as Gibbs Sampling and Importance Sampling) with comparable or better identification ability. I have also extended the method to incorporate Markov Chain Monte Carlo search and showed that it can better predict causal relationships.

Along with my statistical methodological development, I have been collaborating with researchers in public health, behavioral, clinical, and genomic fields that requires multivariate statistical analyses of biomedical datasets (including longitudinal data, data from different sources, and data with missing values) to better understand clinical, physiological, and psychological observations.

B. Positions and Honors

Positions and Employment

1991-1992 Programmer and lecturer, Army Admission School, Seoul, Korea
1993-1996 Lecturer, Ssangyong Information and Communications Corp. Education Center, Seoul, Korea

1992-1996 Software Engineer, Ssangyong Information and Communications Corp., Geographic Information Systems, Seoul, Korea
1996-1998 Graduate Research Associate, Electric Engineering and Computer Science, Massachusetts Institute of Technology, Cambridge, MA
1998-2002 Research Fellow / Graduate Research Associate, Center for Biomedical Informatics, University of Pittsburgh, Pittsburgh, PA
2002-2004 Senior Research Associate, Virginia Bioinformatics Institute, Virginia Polytechnic Institute and State University, Blacksburg, VA
2004-2009 Assistant Professor, Computer Science and Center for Environmental Health Science, University of Montana, Missoula, MT
2009-2015 Assistant Professor, Department of Biostatistics, Florida International University, Miami, FL
2015-present Associate Professor, Department of Biostatistics, Florida International University, Miami, FL
2016-present Interim Chair, Department of Biostatistics, Florida International University, Miami, FL

Synergistic Activities

1999-2003 Collaborative research in Systems Biology Group that includes biologists, physicians, and computer scientist at University of Pittsburgh, Carnegie Mellon University, and Institute of Systems Biology
2002-2003 Collaborative research in Virginia Bioinformatics Institute at Virginia Tech
2004-2006 Developed and taught a Bioinformatics course that is for students in Biological Science, Spring
2004-2006 Instructor of Bioinformatics course that covers issues in chemistry, biology, and computer science, Spring
2003-2009 Collaborative research in NIH Center for Environmental and Health Sciences and Division of Biological Studies at the University of Montana
2009-present Collaborative research in Statistical Bioinformatics models of Brain Cancer key genes at Florida International University
2009-present Collaborative research in Center for Research on US Latino HIV/AIDS and Drug Abuse at Florida International University
2011-present Collaborative research in developing Markov Chain Monte Carlo method in HIV presentation at Florida International University

Honors and Awards

2009-2011 FIU Faculty research award
2011 Department travel award

C. Contribution to Science

1. My early publications represent novel statistical causal discovery methods that I have developed. At that time most of the gene-gene interactions were modeled with correlation not causation. In the new systems biology era, my causal model has been praised by many eminent scholars, e.g., Kitano (in his 2002 Science magazine article)* references my causal analysis paper and states that the causal modeling that I am using is the future direction that systems biology researchers should follow. Indeed, now the causal modeling has become more and more important in systems biology research.
    a. **C. Yoo**, V. Thorsson, G.F. Cooper. Discovery of causal relationships in a gene-regulation pathway from a mixture of experimental and observational DNA microarray data, Pacific Symposium On Biocomputing, p498-509, 2002
    b. **C. Yoo**, E. Blitz. Local Causal Discovery Algorithm using Causal Bayesian networks, *Annals of the NY Academy of Science*, 1158, p93-101, 2009
    c. **C. Yoo**. Bayesian Method for Causal Discovery of Latent-Variable Models from a Mixture of Experimental and Observational Data, *Computational Statistics and Data Analysis*, 56, p2183-2205, 2012
    d. **C. Yoo**, E. Brilz. Efficient and Scalable Bayesian Statistical Method for Identifying Causal Relationships from Intervention Studies, *Advances and Applications in Statistics*, 37(2), p95-122, 2013

    * Kitano, H., Systems Biology: a brief overview, *Science*, 2002 Mar 1;295(5560):1662-4.

2. In addition to the contributions described above, I have also applied the value of information so the researchers can design better experiments for discovering more significant causal relationships from statistical analysis. Statistical methods that I have developed show that they can identify causal relationships more effectively than the traditional analysis methods.
    a. **C. Yoo**, G. Cooper. A Computer-Based Experiment Design Recommendation System for Gene-Regulation Pathways Discovery, Proceedings of AMIA Symposium, p733-737, 2003
    b. **C. Yoo**, G. Cooper, M. Schmidt. A Control Study to Evaluate a Computer-Based Microarray Experiment Design Recommendation System for Gene-Regulation Pathways Discovery. *Journal of Biomedical Informatics*, 39, 126-146, 2006
    c. **C. Yoo**. Statistical Methods in Experimentation Recommendation Models for Discovering Gene Regulation Pathways, *Wiley WIREs Computational Statistics*, 5(2) p121-134, 2013

3. Extending the statistical models that were described above and working with various collaborators, I have shed a light on how chronic diseases, such as a brain tumor, progress especially in genomic level. This served the basis of my NIH grant.
    a. **C. Yoo**, E. Blitz, M. Wilcox, M. Pershouse, E. Putnam. Gene Pathways Discovery in Asbestos-Related Diseases using Local Causal Discovery Algorithm, *Communications in Statistics - Simulation and Computation*, 41(10), p1840-1859, 2012
    b. B. Kunkle, **C. Yoo**, D. Roy. Discovering gene-environment interactions in Glioblastoma through a comprehensive data integration bioinformatics method, *NeuroToxicology*, 35, p1-14 2013
    c. B. Kunkle, **C. Yoo**, D. Roy. Reverse Engineering of Modified Genes by Bayesian Network Analysis Defines Molecular Determinants, *PLOS One*, 8(5): e64140. doi:10.1371/journal.pone.0064140, 2013
    d. B. Luna, S. Bhatia, **C. Yoo**, Q. Felty, D.I. Sandberg, M. Duchowny, Z. Khatib, I. Miller, J. Ragheb, J. Prasanna, D. Roy, Proteomic and mitochondrial genomic analyses of pediatric brain tumors, *Molecular Neurobiology*, DOI:10.1007/s12035-014-8930-3, 2014

4. With various collaborators, I have also helped physicians make better diagnosis and treatment plans by understanding relationships among various clinical variables. In one instance, we have used my statistical method to show that non-invasive testing performs as well as the traditional invasive testing for Benign Prostatic Hyperplasia.
    a. M. Agudelo, **C. Yoo**, M.P. Nair, Alcohol-induced serotonergic modulation: The role of histone deacetylases, *Alcohol*, 46(12), p635-642, 2012
    b. T. Samikkannu, K.V.K. Rao, A.Y. Arias, A. Kalaichezian, V. Sagar, **C. Yoo**, M.P.N. Nair, HIV infection and drugs of abuse: role of acute phase proteins, *Journal of Neuroinflammation*, **10**:113 doi:10.1186/1742-2094-10-113, 2013
    c. M. Kim, A. Cheeti, **C. Yoo**, M. Choo, SJ Oh. Non-invasive parameters for the prediction of urodynamic bladder outlet obstruction: analysis using causal Bayesian networks, *PLOS One,* DOI: 10.1371/journal.pone.0113131, 2014
    d. M. Kim, L Ramirez, **C. Yoo**, MS Choo, JS Paick, SJ Oh, Factors Influencing Nonabsolute Indications for Surgery in Patients With Lower Urinary Tract Symptoms Suggestive of Benign Prostatic Hyperplasia: Analysis Using Causal Bayesian Networks, *International Neurourology Journal*, 18(4):198-205. DOI: 10.5213/inj.2014.18.4.198, 12/2014

5. I also showed how to apply Bayesian statistical analyses in questionnaire data with many variables and longitudinal data with missing data in understanding psychological measurements. In one longitudinal dataset with substantial missing data, we have shown the Bayesian method better imputes the missing data.
    a. **C. Yoo**, A. Saxena, K. Krupp, K. Winter, J.D. Klausner, V. Kulkarni, J. Devieux, P. Madhivanan, Using Integrated Logistic Regression and Bayesian Statistical Modeling to analyze Acceptance of Male Circumcision in Pune, MODSIM 2013, Proceeding of 20th International Congress on Modeling and Simulation, Adelaide, Australia, p2023-2028, 2013
    b. **C. Yoo**, A. Cheeti, D. Brooten, J. Youngblut, Modified Second Degree Markov Model in Missing Data Analysis in Child and Family Functioning after Pediatric Head Trauma, MODSIM 2013, 20th

International Congress on Modelling and Simulation, Modelling and Simulation Society of Australia, p2018-2022, 2013

c. M. Agudelo, P. Khatavkar, A. Yndart, **C. Yoo**, R. Rosenberg, J.G. Dévieux, R.M. Malow, M. Nair, Alcohol Abuse and HIV infection: Role of DRD2, *Current HIV research*, PMID:25053368, July 2014

d. JM Youngblut, D. Brooten, K. Blais, C. Kilgore, **C. Yoo**, Health and Functioning in Grandparents After a Young Grandchild's Death, *Journal of Community Health*, DOI: 10.1007/s10900-015-0018-0, 2015

Complete List of Published Work in MyBibliography:

http://www.ncbi.nlm.nih.gov/sites/myncbi/1hA3Qwv_hkEA1/bibliography/46169487/public/?sort=date&direction=descending

D. **Research Support**

Ongoing Research Support

- 1 SC3 GM096948-01A1 Identifying Key Genes that cause Aggressive Brain Cancer
  Funded by NIH's National Institute of General Medical Sciences.
  (PI; 09/01/12-07/31/17)
  The major goal of this project is to develop a statistical model to identify key genes that cause aggressive brain cancer and further model the key genes causal interactions.

- NIH RISE Undergraduate Research 08/16-07/17 Role: Student Research Advisor

Completed Research Support (selected from completed projects)

- [Private Source]

  Benign Prostatic Hyperplasia Diagnosis and Surgery Decision Support Smart Phone App Development
  We propose to develop an app in smart phone to help physician make decision and students leanrn how to make decision in diagnosing and surgery of Benign Prostatic Hyperplasia.

- R01-NR012675 Children's Reactions to the Death of a Sibling in the PICU/NICU in 3 Racial/Ethnic Groups. Funded by the NIH's National Institute of Nursing Research. 9/30/10 – 6/30/16. Youngblut & Brooten, Principal Investigators. Silverman, Yoo Co-Investigators.

  The purpose of this longitudinal study is to examine children's mental health, physical health, personal growth and functioning from 2 to 13 months after the death of a brother/sister in the NICU/PICU and to identify factors related to these outcomes, using both qualitative and quantitative methods, in three racial/ethnic groups.

- R01-NR04430 National Institute for Nursing Research, NIH. Child & Family Functioning after Pediatric Head Trauma. Youngblut J, PI. Brooten D, Co-PI. 1997-2004. Yoo C, Statistician. 2012-2015.

  The purpose of this longitudinal study was to describe the impact of traumatic brain injury (TBI) severity, resistance resources, parental appraisal on the adaptation of preschool children with TBI and their parent(s), and the quality of parent-child and family relationships in the first year post-discharge.

- [Private Source]

  Analysis of Clinical Parameters of Benign Prostatic Hyperplasia using Causal Bayesian Network
  We propose to learn interactions among the genes and environment factors. We will test the model using simulated data. Based on the clinical electronic database of Benign Prostatic Hyperplasia patients, we will develop a statistical model that provides better ways to measure and understanding of Bladder Outlet Obstruction.

- **P20MD002288** NIH **Center for Substance Use and Aids Research on Latinos in the US (CoPI; 01/15/10-08/14/12)**
  HIV Risk Behavior and Substance Abuse among Recent Latino Immigrants

  The central hypothesis of the study is that pre-immigration assets, mediated by acculturation related stress and substance use, influence the HIV risk behavior of Latino immigrants who have lived in the U.S. for less than one year, while controlling for pre-immigration substance use and HIV risk behavior.

- **NSF EPSCoR and NIH COBRE grant (PI; $20,000; 2006 – 2007) to develop an infonmatics core**

- **NSF EPSCoR Undergraduate Research grant (2007-2009) to mentor two undergraduate students from University of Montana**

- **P20RR017670 NIH Center of Biomedical Research Excellence (COBRE) grant (CoPI; $107,500; 2003-2006) to develop a causal Bayesian networks for asbestos related disease gene regulation pathways**

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ● Yes ● No

*Agency-Defined Phase III Clinical Trial? ● Yes ● No

2. Vertebrate Animals Section

Are vertebrate animals euthanized? ● Yes ● No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes ● No

If "No" to AVMA guidelines, describe method and proved scientific justification

3. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

● Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period *Anticipated Amount ($) *Source(s)

PHS 398 Cover Page Supplement

4. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ● Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

5. Inventions and Patents Section (RENEWAL)

*Inventions and Patents: ● Yes ● No

If the answer is "Yes" then please answer the following:

*Previously Reported: ● Yes ● No

6. Change of Investigator / Change of Institution Section

❑ Change of Project Director / Principal Investigator

Name of former Project Director / Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❑ Change of Grantee Institution

*Name of former institution:

PHS 398 Modular Budget

OMB Number: 0925-0001
Expiration Date: 10/31/2018

Budget Period: 1

Start Date: 04/01/2018 End Date: 03/31/2019

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[Indirect Costs]
Consortium Indirect (F&A)	
Total Direct Costs*	

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC			[Indirect Costs]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Theodore Foster, 214-767-3261

Indirect (F&A) Rate Agreement Date: 04/28/2016

Total Indirect (F&A) Costs: [Indirect Costs]

C. Total Direct and Indirect (F&A) Costs (A + B) Funds Requested ($): [Indirect Costs]

PHS 398 Modular Budget

Budget Period: 2

Start Date: 04/01/2019 End Date: 03/31/2020

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[Indirect Costs]
Consortium Indirect (F&A)	
Total Direct Costs*	

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC		[Indirect Costs]	
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Theodore Foster, 214-767-3261

Indirect (F&A) Rate Agreement Date: 04/28/2016

Total Indirect (F&A) Costs: [Indirect Costs]

C. Total Direct and Indirect (F&A) Costs (A + B) Funds Requested ($): [Indirect Costs]

Tracking Number: GRANT12449073

Funding Opportunity Number: PA-16-160 Received Date: 2017-07-10T16:00:57.000-04:00

PHS 398 Modular Budget

Budget Period: 3

Start Date: 04/01/2020 End Date: 03/31/2021

A. Direct Costs

	Funds Requested ($)
Direct Cost less Consortium Indirect (F&A)*	[Indirect Costs]
Consortium Indirect (F&A)	
Total Direct Costs*	

B. Indirect (F&A) Costs

	Indirect (F&A) Type	Indirect (F&A) Rate (%)	Indirect (F&A) Base ($)	Funds Requested ($)
1.	MTDC			[Indirect Costs]
2.				
3.				
4.				

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS, Theodore Foster, 214-767-3261

Indirect (F&A) Rate Agreement Date: 04/28/2016

Total Indirect (F&A) Costs: [Indirect Costs]

C. Total Direct and Indirect (F&A) Costs (A + B) Funds Requested ($): [Indirect Costs]

Tracking Number: GRANT12449073

Funding Opportunity Number: PA-16-160 Received Date: 2017-07-10T16:00:57.000-04:00

PHS 398 Modular Budget

Cumulative Budget Information	
1. Total Costs, Entire Project Period	
Section A, Total Direct Cost less Consortium Indirect (F&A) for Entire Project Period ($)	[Indirect Costs]
Section A, Total Consortium Indirect (F&A) for Entire Project Period ($)	
Section A, Total Direct Costs for Entire Project Period ($)	
Section B, Total Indirect (F&A) Costs for Entire Project Period ($)	
Section C, Total Direct and Indirect (F&A) Costs (A+B) for Entire Project Period ($)	
2. Budget Justifications	
Personnel Justification	Guilarte_Mn_Grant_Personnel_Justification_June2017.pdf
Consortium Justification	
Additional Narrative Justification	

Personnel Justification

A. Senior Personnel:

Tomás R. Guilarte, PhD (effort = [EFFORT]): Dr. Guilarte is the principal investigator and is responsible for carrying out all aspects of the work performed under the auspices of the grant. He is responsible for the design and the implementation of all experiments, data analysis and interpretation. He is also responsible for writing progress reports and publication of original findings.

B. Other Personnel:

Changwon Yoo, PhD (effort = [EFFORT]): Dr. Yoo is an outstanding biostatistician and will be responsible for statistical design and statistical analysis of data as it is generated.

Kalynda Gonzales, PhD (effort = [EFFORT]): Dr. Gonzales is a postdoctoral fellow that has been in Dr. Guilarte's laboratory for 4 years. Dr. Gonzales will be responsible for all of the immunohistochemistry and stereological and pathological analysis of the tissues. She will also assist with preparation of publications.

Jennifer Dziedzic. MS (effort = [EFFORT]): Mrs. Dziedzic is a Research Associate that has been in Dr. Guilarte's laboratory for over 20 years. Ms. Dziedzic is a superb researcher and supervises the day-to-day operation of Dr. Guilarte's labs. She will also be responsible for performing and analyzing the autoradiography assay for ^{3}H-Vesamicol and ELISA assays for BDNF and NGF. She will also assist with preparation of publications.

Juan Perez, BS (effort = [EFFORT]): Mr. Perez is a Laboratory Technician that has been in Dr. Guilarte's laboratory for 1 year. Mr. Perez will be responsible for slicing all of the tissue for autoradiography and immunohistochemistry assays. He will also assist with the autoradiography assay and immunohistochemistry staining.

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 10/31/2018

Introduction	
1. Introduction to Application (Resubmission and Revision)	
Research Plan Section	
2. Specific Aims	Guilarte_MnGrant_Spec._Aims_2017_FINAL.pdf
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5.5.2. Specific Aims:

Conceptual framework and hypothesis: Chronic exposure to manganese (Mn) from environmental and occupational sources damages cholinergic neurons and results in cellular and circuit level effects that can explain Mn-induced neurological dysfunction. Exposure to contemporary sources of Mn impairs working memory/executive function, and produces deficits in fine motor control and postural stability. The goal of this research is to understand the pathophysiology of chronic Mn exposure on the cholinergic system. Our proposed studies challenge the current view of Mn-induced neurological disease from a dopamine-centric perspective to one in which there is disruption of the cholinergic-dopaminergic balance in the basal ganglia and cholinergic changes in other brain structures. A precise understanding of molecular, cellular, and circuit level effects of chronic Mn exposure on the cholinergic system and interactions with dopamine neurons will provide a more precise mechanistic understanding and advance the development of effective therapeutic strategies.

Experience and expertise: We have studied the neurotoxicity of chronic Mn exposure in non-human primates using rigorous behavioral, neuroimaging, and neuropathological methods for over 10 years. These studies have provided novel findings related to: 1) inhibition of striatal dopamine (DA) release (DAR) in the absence of dopamine terminal degeneration and deficits in fine motor control and activity [1-4], 2) Mn-induced impairments of working memory, attention and paired associative learning [3,5-9], and 3) have advanced the understanding of Mn neuropathology [10-17] including β-amyloid and α-synuclein aggregation in the frontal cortex [5,9,14]. The current proposal is a natural extension of our work and builds upon exciting preliminary findings. We provide evidence that chronic Mn exposure results in the loss of striatal cholinergic interneurons (ChI). This finding is impactful because striatal ChI modulate striatal DAR and we have shown *in vivo* inhibition of striatal DAR by Mn [1,4]. Therefore, Mn-induced cholinergic neuron loss or dysfunction may mediate the inhibition of DAR providing a mechanistic understanding of Mn-induced inhibition of DAR and neurological dysfunction. Cholinergic neurons are important in the physiology of cognition, compulsive behavior, locomotion, and gait; domains that are associated with the clinical expression of Mn neurotoxicity [18]. Thus, the specific aims are:

Specific Aim 1 (SA1): The goal of SA1 is to determine the number and soma size of choline acetyltransferase (ChAT)-positive cholinergic neurons in the caudate/putamen/nucleus accumbens, basal forebrain (BF), and the pedunculopontine nucleus (PPN) of Mn-exposed and non-exposed *Cynomolgus macaques*. These studies will use unbiased stereological cell counting and soma size determinations, rigorous methods that are well-established in our laboratory. Our preliminary findings provide strong support for SA1 in that there is an apparent loss of striatal ChIs in the limited number of Mn-exposed animals that we have analyzed relative to controls. We propose to assess whether this putative loss of ChAT-positive ChI is confirmed in the larger cohort of animals and whether it occurs in other cholinergic brain nuclei such as the BF and PPN. We also propose to perform unbiased stereological cell counting of Nissl and NeuN stain sections as an alternative method to confirm cholinergic neuron degeneration.

Specific Aim 2 (SA2): The goal of SA2 is to determine the effect of chronic Mn exposure on ChI axon terminals and varicosities in the striatum, and efferents from the BF and PPN cholinergic neurons that extensively innervate the cerebral cortex, hippocampus, amygdala, and thalamus. This will be accomplished by visualizing vesicular acetylcholine transporters (vAChT) in cholinergic terminals and varicosities using: 1) [^{3}H]-vesamicol quantitative autoradiography. Vesamicol is a selective ligand for vAChT and autoradiography is quantitative and has excellent spatial resolution, and 2) vAChT immunohistochemistry as a second method to visualize and assess the Mn-induced effect on vAChT protein in cholinergic terminals in order to provide scientific rigor to our work. The work proposed in SA2 is important because it is possible that in some brain regions there is little or no loss of ChAT-positive cholinergic soma, but Mn exposure could produce axonal degeneration (axonopathy).

Specific Aim 3 (SA3): The goal of SA3 is to determine the putative role of neurotrophic factors in Mn-induced degeneration or injury of cholinergic neurons. It is known that neurotrophic factors are needed for cholinergic neuron survival. Thus, we will determine if Brain-Derived Neurotrophic Factor (BDNF) and Nerve Growth Factor (NGF) are decreased in brain regions where there is a Mn-induced effect on ChAT-positive neurons and/or their vAChT-positive axon terminals. The findings made under SA3 will provide valuable insights on mechanism(s) by which chronic Mn exposure results in degeneration or injury of cholinergic neurons.

Impact of proposed research: The proposed studies are novel, translational, and point to a role of the cholinergic system in Mn-induced neurological dysfunction. Since there is no current therapy for Mn-induced neurological disease, the results of the proposed research could be impactful in the future development of cholinergic- and/or neurotrophic factor-based therapies, since Mn-induced neurological disease is refractory to dopaminergic drug therapy.

5.5.3. Research Strategy:

(A) Significance: A.1.: Biometals and neurodegenerative disease: Biologically essential metals, such as copper, zinc, iron, and manganese have many physiological functions in the brain and an increasing body of scientific evidence during the last decades supports the hypothesis that altered metal homeostasis plays an important role in brain disorders. There is substantial evidence that dysregulation of metal homeostasis is associated with neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD) amongst others [19-21]. Studies have also shown that while there is a genetic component to these neurodegenerative disorders, genetics does not account for the total number of sporadic cases and gene-environment interactions are likely to play a significant role in disease onset and/or progression [22,23]. Therefore, it has become clear that environmental factors, including, heavy metals, pesticides, insecticides, and other industrial pollutants are also likely to play a significant role in the etiology of neurodegenerative disorders [24-26].

A.2.: Manganese and neurodegenerative disease (scientific premise and need for studying manganese-induced neurodegeneration): Manganese (Mn) is an essential trace element that is required as a cofactor for many enzymes critical for human health [27]. Multiple lines of evidence indicate that when brain Mn concentrations accumulate above physiological levels, Mn is neurotoxic and it results in neurological disease characterized by early neuropsychiatric symptoms with cognitive deficits followed by an atypical form of parkinsonism that is refractory to L-dopa therapy [12,15,17]. Currently, humans are exposed to Mn from a variety of sources including: 1) occupations in which there are increased concentrations of Mn in the air from welding, smelting, mining, and steel production, 2) from environmental contamination when segments of the population live near smelters or other industries in which Mn is emitted into the air or released as a byproduct into the water table or wells; 3) in countries where gasoline containing the Mn additive methyl cyclopentadienyl manganese tricarbonyl (MMT) is used and Mn is released into the environment as an automobile combustion product; and importantly, 4) in parts of the world including the US where large segments of the population drink water with naturally high Mn concentrations. Historically, chronic Mn exposure and Mn-induced neurological disease, have been most widely studied in occupational settings. However, emerging evidence indicates that drinking water with naturally high Mn concentrations is a significant environmental source of exposure to large population segments [28]. For example, there is evidence that potentially toxic concentrations of Mn in drinking water exist in over 50 countries including in the US [28]. Therefore, chronic exposure of human populations to Mn has been significantly underestimated and high Mn concentrations in drinking water is a significant source of exposure with untold consequences to neurological health. This is supported by recent studies from different parts of the world describing an association between Mn in drinking water and deficits in children's intellectual functioning, cognitive abilities, and academic achievement, motor function and gait abnormalities, and delayed fetal growth [29-37]. These studies have brought to light an important association between chronic exposure to drinking water with naturally high Mn concentrations and deleterious effects on neurological health. Additional evidence of the neurological consequences of elevated brain Mn concentrations has also been recently described in: 1) young drug users injecting high Mn concentrations as a byproduct of the synthesis of the illicit psychostimulant drug, ephedron [38-51]; and 2) in individuals with inherited recessive mutations of the Mn transporter genes *SLC30A10* [52-59] and *SLC39A14* [60]. These clinical populations have significantly advance our understanding of Mn-induced neurological disease. They have been an important source of knowledge and have provided confirmation to many of the novel findings that we have made in our Mn studies using non-human primates.

A.3.: Rationale and scientific premise for the proposed studies: expertise and experience of the multidisciplinary approach of the Guilarte (PI) lab in studying Mn-induced neurological dysfunction:

Behavior: Our laboratory in collaboration with Dr. Jay Schneider at Thomas Jefferson University has been studying the neurological consequences of chronic Mn exposure in non-human primates. Our work has shown that non-human primates chronically exposed to levels of Mn resulting in blood Mn concentrations in the upper range of those documented in Mn-exposed human populations exhibit: 1) deficits in spatial working memory and modest decreases in spontaneous activity and fine motor control [3]. We also found stereotypic and compulsive-like behaviors such as compulsive grooming and licking [3]. 2) Another study using different behavioral paradigms showed that Mn-exposed non-human primates develop deficits in spatial working memory, greater deficits in non-spatial working memory, and no effect in reference memory [6]. 3) We found that the earliest effect of chronic Mn exposure was impairment of paired associative learning [7], a task that has been shown to be affected in early Alzheimer's disease. This finding was important because we have also described significant neuropathology in the frontal cortex of these Mn-exposed monkeys including diffuse β-amyloid plaques and α-synuclein aggregation [5,14]. Parenthetically, the monkeys used in all of our studies are research naïve young adults and based on their age [less than 12 years of age], they should not have any age-associated diffuse β-

amyloid plaques nor α-synuclein aggregation; 4) lastly, using behavioral tasks specifically designed to preferentially assess attention, working memory, and executive function, our work also showed that besides deficits in a paired associative learning task; attention and cognitive processing speed were impaired in Mn-exposed non-human primates [8]. These Mn-induced behavioral deficits have been described in humans chronically exposed to Mn [12,15,17,61,62]. Therefore, our non-human primate animal model recapitulates many of the behavioral deficits observed in humans chronically exposed to contemporary levels of Mn.

Neuroimaging & neuropathology: We have made novel findings and provided resolution to long-standing questions related to the role of the dopaminergic (DAergic) system on the pathophysiology of Mn-induced motor function deficits. Our laboratory in collaboration with Dr. Dean Wong in the department of Radiology and PET Center at the Johns Hopkins Medical Institutions (Dr. Guilarte started this work when he was at Johns Hopkins) was the first to demonstrate that monkeys chronically exposed to Mn that exhibit subtle motor function deficits and fine motor control problems, do not express degeneration of nigrostriatal DAergic terminals as measured by dopamine transporter (DAT) positron emission tomography (PET) in the striatum [1,4]. However, we made the novel discovery that chronic Mn exposure produces nigrostriatal DAergic neuron dysfunction in the form of marked inhibition of *in vivo* striatal DA release (DAR) measured by PET [1,4].

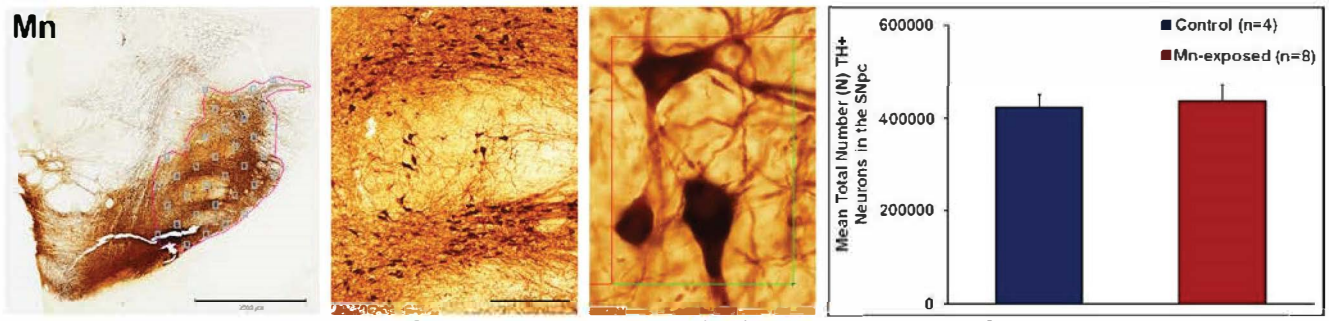


Figure 1: Representative images of tyrosine hydroxylase (TH) immunostaining of dopaminergic neurons in the substantia nigra of a Mn-exposed monkey at 4x, 10x, and 100x (from left to right panels). Unbiased stereological cell counting of TH-positive neurons (mean ± SEM) in the complete substantia nigra pars compacta (SNpc) showed no significant differences in the number of TH-positive neurons between control (n=4; 423,538 ± 16,069) and Mn-exposed animals (n=8; 436,864 ± 34,599) (p>0.05) (right-side graph). Cumulative Mn dose for the Mn-exposed animals (n=8) was 3082 ± 214 mg Mn (range: 2339 – 4293) or 401 ± 27 mg Mn/kg body weight (range= 333 – 517 mg Mn/kg body weight). Scale bars: 2500 μm for first left-side panel; 350 μm for the 2nd left-side panel. [manuscript in preparation for submission].

The finding that chronic Mn exposure does not result in striatal DA neuron terminal degeneration has been confirmed in the following ways providing support and rigor to our previous work: 1) as shown in Figure 1, we now have performed unbiased stereological cell counting of tyrosine hydroxylase (TH)-positive DA neurons in the entire volume of the substantia nigra pars compacta (SNpc) of these Mn-exposed monkeys and controls and found no significant differences in SNpc DA neuron number; 2) individuals who abuse ephedron (a Mn-containing psychostimulant drug), chronically inject large amounts of Mn and develop Mn-induced clinical parkinsonism that is refractory to L-dopa therapy and they have normal striatal DAT levels based on SPECT imaging [38,45,46] indicating that striatal DA neuron terminals do not degenerate despite exhibiting clinical parkinsonism; 3) similarly, recent cases of individuals with inherited recessive mutations of the *SLC30A10* gene (a manganese efflux transporter that becomes dysfunctional by the mutation) have highly elevated Mn concentrations in blood and brain (the later assessed by T1-weighted MRI) and exhibit Mn-induced parkinsonism with dystonia. As with the ephedron drug users, the Mn-induced parkinsonism in subjects expressing SLC30A10 mutations is refractory to L-dopa therapy and striatal DAT levels are also normal based on DAT-SPECT imaging [53,59]; 4) another study in smelter workers with Mn-induced clinical parkinsonism (also not responsive to L-dopa) has shown lack of striatal DA terminal degeneration measured by vesicular monoamine transporter type-2 (VMAT2) PET after more than 20 years from the original exposures and onset of parkinsonism [63]. 5) Lastly, recent studies in Mn-exposed mice have also shown (as in our monkeys) a Mn-induced inhibition of striatal DAR with no change in striatal DA concentrations or DA neuron number in the SNpc, although the study did not use unbiased stereological cell counting [64,65]. Thus, based on the above-mentioned human and animal literature and our own non-human primate results [1,4 and Figure 1]; Mn-induced motor function deficits are not the result of DA neuron degeneration in the SNpc. On the other hand, they are associated with DA neuron dysfunction (decreased DAR) as we have shown by PET imaging [1,4]. These findings have brought to light the possibility

that other neuronal systems intrinsic to the basal ganglia are likely to be affected by chronic Mn exposure and we have started to assess the effect of chronic Mn exposure on cholinergic interneurons (ChI) in the striatum.

A.4. Cholinergic interneurons in the basal ganglia: rationale and scientific premise for studying the cholinergic system in Mn neurotoxicity: Cholinergic neurons are crucial in the physiology of cognition, emotion, compulsive behavior, locomotion, and gait; domains that are affected by chronic Mn exposure [18]. We selected to initially examine striatal cholinergic interneurons (ChI) for the following reasons: (1) ChI strongly modulate DAR from striatal DA axon terminals [66,67], and we have previously shown that chronic Mn exposure markedly inhibits *in vivo* DAR from striatal DAergic terminals [1,4]; and (2) synchronized ChI firing drives striatal DAR via nAChR activation that is independent of DA neuron activity in the SNpc [67]. Combined, these studies indicate that the overall striatal cholinergic tone needs to be balanced for physiological DAergic activity to be maintained [68]. Thus, the scientific premise and rationale for studying the cholinergic system in our proposed studies is not only scientifically sound but it is also supported by our preliminary studies (see Figure 2 below), and by a number of recent human studies in clinical populations with Mn-induced parkinsonism with dystonia indicating that degeneration of the DAergic system does not explain the clinical presentation of Mn-induced neurological disease [38,45,46,53,59]. Therefore, studying cholinergic circuits will not only provide an improved understanding of Mn effects on the brain, but cholinergic-based therapies could offer a new line of intervention in the clinical management of Mn-induced neurological disease. This is especially important since Mn-induced neurological dysfunction is not responsive to DA-based drugs such as L-dopa [12,15,17].

(B) Innovation: Our studies are highly translational and innovative because they provide a new avenue of investigation that can provide a more precise and comprehensive understanding of mechanism(s) associated with the cognitive, attention, motor function, and gait deficits in humans chronically exposed to Mn. The proposed studies challenge the current view of the cellular and circuit level mechanisms of Mn-induced neurological disease from a primary perspective on DA neurons to one in which there is disruption of the Cholinergic-DAergic balance in the basal ganglia. A better understanding of Mn effects on cholinergic circuits can be useful in the design of new therapeutic approaches with cholinergic drugs. This is critically important because DAergic drugs are not effective in Mn-induced neurological dysfunction.

Another innovative aspect of our proposed studies is that cholinergic neuron degeneration occurs in a number of neurodegenerative disorders including Alzheimer's and Parkinson's disease with dementia but yet, there is little knowledge on etiological factors that produce this neurodegeneration. Our studies provide the first evidence that Mn, an endogenous essential nutrient for human health, may promote degeneration or injury of cholinergic neurons if brain concentrations are chronically increased above physiological levels with significant implications to the etiology of neurodegenerative disorders. Further, our work uses a non-human primate model that exhibits the same neurological symptoms as humans exposed to Mn and it is an excellent animal model to study human Mn neurotoxicity. The non-human primate is a particularly valuable model because it has major similarities with the neural organization of the human brain. Lastly, from a methodological perspective, our studies are innovative because we use rigorous unbiased stereological analysis in the context of functional brain circuits which is unique to the field of neurotoxicology.

(C) Approach: C.1. Preliminary Studies: C.1.1.: Striatal cholinergic interneurons in the basal ganglia of Mn-exposed monkeys-experimental support for proposed studies: We used choline acetyltransferase (ChAT) immunoreactivity to determine the estimated total number of striatal ChI in a limited number [control (n=3) and Mn-exposed animals (n=3)] of our larger cohort of animals [see Table 1] using the optical fractionator method [69] with a uniform random sampling scheme of the region of interest and reciprocal sampling fraction [70,71]. ChAT is the enzyme responsible for the synthesis of the neurotransmitter acetylcholine from choline and acetyl-CoA and has been widely used as a specific biomarker for the large striatal ChI in non-human primates and humans [68]. For this study, we delineated the striatal functional regions (Figure 2) based on the corticostriatal topography described in prior publications [72,73] to determine if a selective, functional pattern of ChI cell loss occurs after chronic Mn exposure. Our stereological cell counts revealed that the total number of ChAT-positive ChI was not significantly decreased in the entire caudate nucleus (26% loss; p= 0.1) but there was a highly significant loss of ChIs in the entire putamen (86% loss; p=0.00008) after chronic Mn exposure compared to normal controls (Figure 2). Within the putamen, the largest decrease (90%; p=0.00008) of ChI occurred in the post-commissural (mainly sensorimotor) region of Mn-exposed monkeys (24,860 ± 9,759) compared to control monkeys (254,993 ± 10,544)-see Figure 2. Although all regions of the putamen were dramatically affected by Mn exposure (Figure 2). Regional assessment of the caudate nucleus indicates that the post-commissural tail (mainly associative) is the only region that displayed a significant loss (59%; p= 0.008) of ChIs in the caudate of Mn-exposed (8,438 ± 2,070) compared to control monkeys (20,772 ± 1,476)-see Figure 2.

Results from these studies (Figure 2) represent the first unbiased stereological cell counts of the total number of striatal ChIs in monkeys, as well as, the first to demonstrate a pathological decrease of dorsal striatum (caudate/putamen) ChIs after chronic Mn intoxication. A Mn-induced loss of ChIs occurs throughout the entire rostrocaudal extent of the putamen, whereas this loss is more selective in the caudate nucleus, localizing only to the Post-Commissural (tail) region. These affected neurons largely reside in the "associative" and "sensorimotor" functional regions of the striatum that receive diverse inputs from circuits related to reward, working memory, and motor outcome, including the corticostriatal and nigrostriatal pathways [73,74].

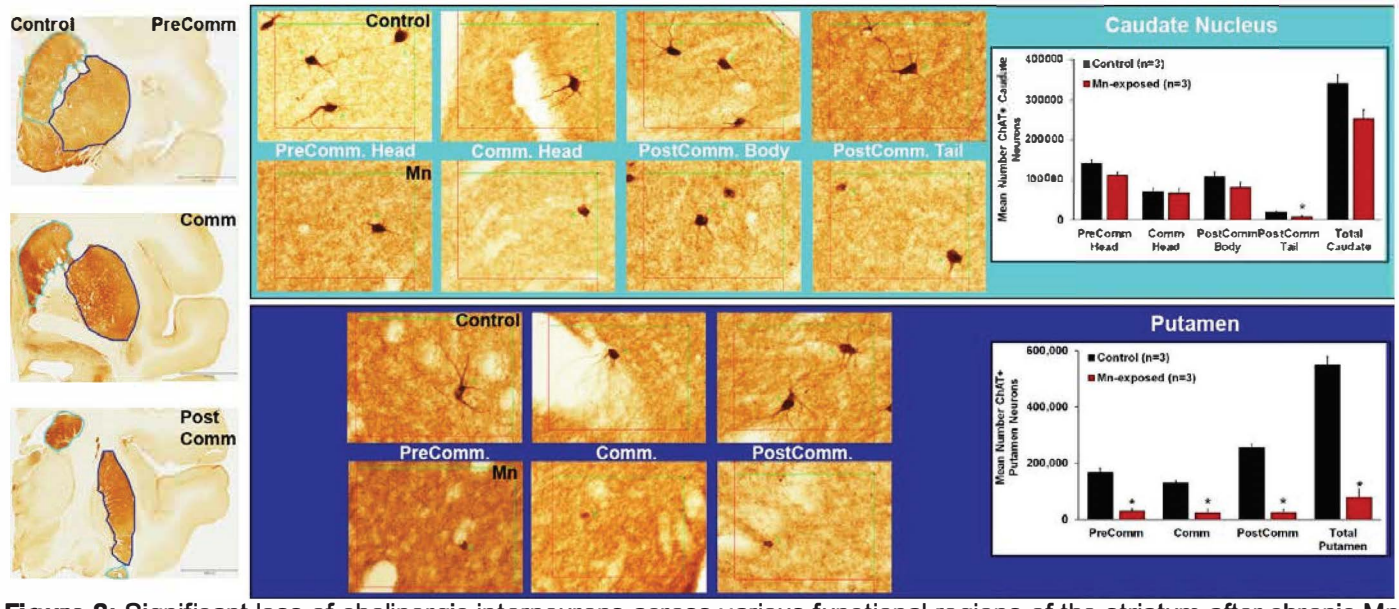


Figure 2: Significant loss of cholinergic interneurons across various functional regions of the striatum after chronic Mn exposure. Photomicrographs of three representative coronal sections (4x) immunolabeled for ChAT from the PreCommissural (PreComm), Commissural (Comm), and PostCommissural (PostComm) striatum are shown in the left panel, along with the delineation of the caudate nucleus (turquoise outline) and putamen (dark blue outline). These images represent the different functional regions of the dorsal striatum used in our stereological analyses. A representation of ChAT-immunolabeled neurons in the caudate nucleus (turquoise panel) and putamen (dark blue panel) of control and Mn-exposed monkeys is shown at the light microscopic level (40x). The histograms (mean ± SEM) show the estimated (unbiased stereological cell counts) total number of ChAT-labeled neurons across each functional region of the dorsal striatum. Scale bars: 5500 µm for the left-side panels. All immunostaining was performed at the same time and the experimenter was blinded to the animal treatment for immunostaining, cell counts, and analysis.

Thus, the working memory impairment and motor deficits caused by Mn intoxication in our monkey model of Mn-induced neurological dysfunction [3,6-8] could result from abnormal nigrostriatal activity i.e., decreased striatal DAR from intact DA terminals [1,4] that is likely mediated via deficits in acetylcholine release due to striatal cholinergic neuron degeneration or dysfunction, principally in the putamen (preliminary results-Figure 2). Additionally, intra-striatal connectivity and function of other striatal neuronal populations, as well as striatal outflow to the globus pallidus and substantia nigra pars reticulata (SNpr), could be interrupted due to the strong influence of the striatal cholinergic system on normal striatal activity [68]. These findings strongly suggest that chronic exposure to levels of Mn used in our study [upper range of those documented in environmentally exposed populations] results in extensive striatal ChI degeneration raising the question whether other major cholinergic brain nuclei such as the basal forebrain (BF) and the pedunculopontine nucleus (PPN) are also affected by chronic Mn exposure. Further, based on qualitative observations during our stereological analyses, we also found a noticeable difference in the morphology of striatal ChAT-positive ChI between the two groups of monkeys. For example, striatal ChI exhibiting shrunken somata along with truncated proximal processes were present in the Mn-exposed striatum (Figure 2, bottom panels of the caudate nucleus and putamen), which may be more pronounced in the putamen than in the caudate of Mn-exposed monkeys. To quantitatively examine these Mn-induced changes, we propose to measure soma size (ChAT immunostaining) in SA1, and putative changes in axonal processes using vesicular acetylcholine transporter (vAChT) immunohistochemistry and [^{3}H]-vesamicol quantitative receptor autoradiography in SA2.

C.1.2.: Are cholinergic neurons in other brain nuclei affected by chronic Mn exposure? We examined ChAT immunohistochemistry at the level of the basal forebrain (intermediate division of Ch4 of the nucleus basalis of Meynert=NBM) [75] in a control and a Mn-exposed monkey in which we had excellent correspondence

of anatomical regions. Figure 3 shows ChAT-immunostaining in these two animals. The results strongly suggest that there is a striking loss of ChAT-positive staining in cholinergic neurons of the NBM from the Mn-exposed animal relative to the control. However, this is only one set of animals and we have not performed stereological cell counting; therefore, this is only a suggestion based on visual examination of the ChAT-immunostaining. While this observation is very preliminary, if this finding is confirmed in the larger cohort of Mn-exposed and control monkeys following unbiased stereological cell counting, it will provide the first evidence that Mn, an endogenous essential trace metal that is also an environmental/endogenous neurotoxicant could be a putative etiological factor in the degeneration of BF cholinergic neurons with significant implications to the etiology of neurodegenerative disorders.

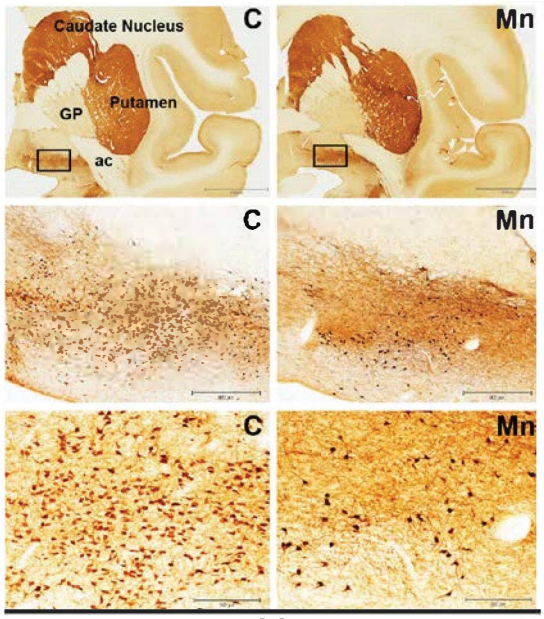


Figure 3: Example of ChAT immunostaining performed at the same time with the same antibody solution in a control (C) and a Mn-exposed monkey (Mn8 in Table 1-see cumulative Mn dose and exposure duration) at the level of the cholinergic basal forebrain (Ch4 or the Nucleus Basalis of Meynert; see rectangles in the upper panel). The middle (10x) and bottom panels (20x) represent higher magnification images of the rectangles in the upper panel. In comparison to the control animal, an apparent loss of ChAT-positive cholinergic neurons in Ch4 is apparent in the Mn-exposed animal. Scale bars: 5500 µm for the top panel, 900 µm for the middle panel, and 350 µm for the bottom panel. GP= globus pallidus; ac= anterior commissure.

C.1.3.: Is reduced neurotrophic support an important factor in the Mn-induced cholinergic interneuron loss observed in the striatum? An important question raised by the preliminary findings provided in Figure 2 is why there is an apparent preferential Mn-induced loss of ChI in the putamen relative to the caudate nucleus? It is known that neurotrophic factors such as Nerve Growth Factor (NGF) and Brain-Derived Neurotrophic Factor (BDNF) are important for the health and survival of striatal ChI [76-78] and can modulate cholinergic neuron proteins such as ChAT and vAChT [79,80]. Prior to obtaining the preliminary findings in Figure 2 of an apparent preferential Mn-induced loss of ChI in the putamen relative to the caudate; we have published that BDNF concentrations were significantly decreased in the putamen but not in the caudate nucleus of our Mn-exposed monkeys relative to controls [16]. This previous finding is consistent with our current data on the loss of ChIs in the entire putamen with no significant loss of ChIs in the entire caudate nucleus of Mn-exposed animals (Figure 2) providing support to the hypothesis articulated in SA3 that decreased growth factor concentrations may be responsible for the Mn-induced loss of ChI. Therefore, it is possible that a Mn-induced decrease in BDNF, and possibly of other neurotrophins (such as NGF) may contribute to the loss of ChIs observed in our preliminary findings. Notably, in the same study [16], we also provide evidence that exposure of primary cortical and hippocampal neuron cultures to relatively low levels of Mn alters huntingtin phosphorylation at a site that is important for the anterograde transport of BDNF. Since the only source of neurotrophic support of striatal ChI is from anterograde transport by cortical afferents to the striatum, it is possible that the transport of BDNF to the striatum is decreased by chronic Mn exposure by altering the function of huntingtin-mediated BDNF anterograde transport [16]. Thus, a detailed study of regional concentrations of NGF and BDNF in the Mn-exposed brain is needed in order to determine if the loss of neurotrophic support promotes Mn-induced loss of ChAT-positive cholinergic interneurons as observed in Figure 2 and possibly in other brain structures such as the basal forebrain (Figure 3). This point will be addressed in SA3. Directly relevant to these Mn/BDNF/ChI findings, a recent human study in a large cohort of Mn-exposed smelter workers and appropriate controls has shown that Mn exposure impairs cognitive performance in the Montreal Cognitive Assessment Test in a dose-dependent manner and this effect is associated with decreased plasma BDNF concentrations in Mn-exposed workers relative to non-exposed workers [81]. This human study lends further supports to our non-human primate findings that decreased neurotrophic support may be a putative mechanism by which chronic Mn exposure results in cholinergic neuron degeneration or injury with significant implications to neurodegenerative disorders in which there is cholinergic neuron loss.

(D) Experimental Design: D.1. Overall experimental plan: The proposed studies will use complete brains that have already been harvested from a total of 19 males and 3 female young adult *Macaca fascicularis* (cynomolgus) monkeys [see Table 1].

Animal ID	Experimental Group	Sex	Final Age	Total Exposure Duration (weeks)	Total Mn Administered (mg/kg)
C1	Naive Control	Female	8y4m	N/A	N/A
C2	Naive Control	Female	8y4m	N/A	N/A
C3	Naive Control	Female	4y	N/A	N/A
C4	Naive Control	Male	6y6m	N/A	N/A
C5	Naive Control	Male	7y	N/A	N/A
C6	Naive Control	Male	7y5m	N/A	N/A
C7	Naive Control	Male	6y9m	N/A	N/A
Mn13	Naive Control	Male	8y9m	N/A	N/A
Mn14	Naive Control	Male	8y8m	N/A	N/A
Mn2	Imaged Control	Male	8y8m	N/A	N/A
Mn5	Imaged Control	Male	9y	N/A	N/A
Mn7	Imaged Control	Male	8y11m	N/A	N/A
Mn15	Imaged Control	Male	9y4m	N/A	N/A
Mn1	Mn	Male	8y7m	80	511.9
Mn4	Mn	Male	10y3m	87	517.2
Mn8	Mn	Male	11y1m	57	333.2
Mn9	Mn	Male	10y	60	382.0
Mn10	Mn	Male	10y8m	57	333.2
Mn11	Mn	Male	9y9m	61	375.5
Mn12	Mn	Male	10y6m	57	333.2
Mn3	Mn + Reversal	Male	9y10m	80	512.0
Mn6	Mn + Reversal	Male	9y4m	71	421.0

Table 1: Information of animal tissue available for the proposed studies.

Amongst these animals there are 9 Mn-exposed and 4 imaged-control animals that have undergone behavioral training and assessment of behavioral performance as well as neuroimaging studies (PET and T1-weighted MRI for brain Mn distribution) and 2 naïve controls. "Imaged-controls" are control animals that received the same type of behavioral and imaging studies as the Mn-exposed animals but were not exposed to Mn. All of these animals (n=15) were studied in a 5-year grant that ended May 31, 2016. Parenthetically, two of the Mn exposed animals (Mn3 and Mn6) were used in a reversal study in which the Mn exposure ended and behavioral and neuroimaging data was continued in order to obtain knowledge on the reversibility of Mn effects on behavioral deficits and to obtain data on brain Mn clearance [see Figure 4 for T1-weighted MRI of one (Mn6) of these two animals describe in Table 1]. These two animals should provide important information on whether the loss of ChAT that we have identified in Mn-exposed animals can or cannot recover after 10 months of terminating the Mn exposure.

We also have PET data (*in* vivo DAR in the frontal cortex) in these imaged-controls and Mn-exposed animals that can serve as an additional resource of information to our proposed studies. During the 5-year funding cycle, we also acquired naïve control male and female monkey brains that did not go through the experimental protocol and the brain tissue is used as naïve controls [C1-C7 in Table 1].

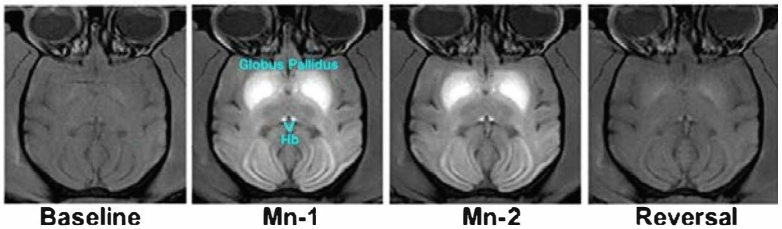


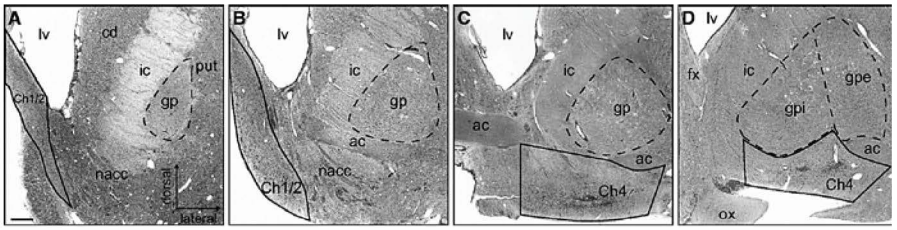
Figure 4: High resolution T1-weighted MRI in the brain of the same Mn-exposed monkey (Mn6 in Table 1) at baseline (prior to Mn administration; blood Mn = 8.9 μg/L), at Mn-1 (35 weeks after initiation of Mn exposure; blood Mn= 132 μg/L), Mn-2 (66 weeks after initiation of Mn exposure; blood Mn= 121 μg/L) and following a 10-month removal from the Mn exposure (reversal; blood Mn= 33 μg/L). T1-relaxation times for the globus pallidus were: Baseline= 826 msec; Mn-1= 469 msec; Mn-2= 476 msec; reversal= 837 msec. Since Mn is paramagnetic, it decreases the T1-relaxation time. The hyperintensive white regions of the brain images in Mn1 and Mn2 indicates increased Mn deposition relative to baseline (prior to Mn exposure) and reversal. The decrease in T1-relaxation time is depicted as a hyperintensive white signal. The brightest hyperintensive signal in the Mn-1 and Mn-2 images is from the globus pallidus. Hb= habenula.

D.1.1.: Consideration of sex as a biological variable: The Mn-exposed brain tissue to be used in the proposed studies are from young male *cynomolgus macaques*. Our studies using non-human primates were initiated prior to the new NIH guidelines in considering sex as a biological variable. As a result, we do not have brain tissue from Mn-exposed female monkeys. However, we do have brain tissue from naïve female animals to compare to naïve male brain tissue. Therefore, at a minimum, our studies will provide new data on regional brain cholinergic neuron number in naïve control male and female monkeys that will be useful information to the scientific community.

D.2.: Experimental Design for Specific Aim 1 (SA1): The goal of SA1 is to perform rigorous unbiased stereological cell count and soma size measurements of ChAT-positive cholinergic neurons in the caudate/putamen/nucleus accumbens, basal forebrain (BF) and the pedunculopontine nucleus (PPN) of Mn-exposed and control animals. The rationale for the selection of the BF and PPN as additional brain regions to analyze is as follows:

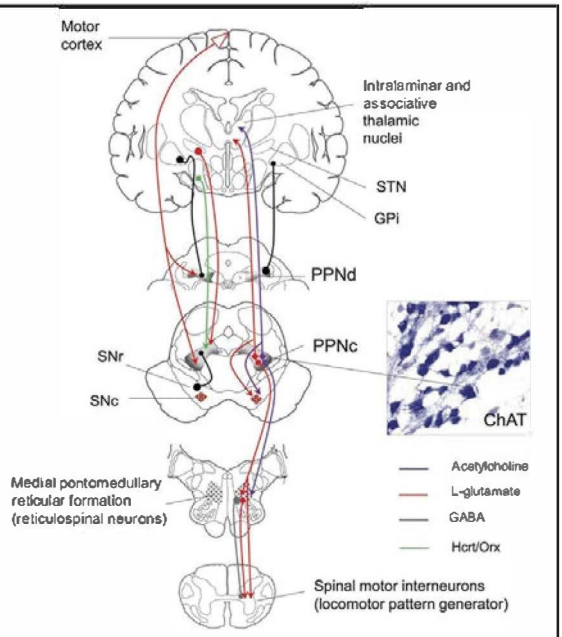
The basal forebrain (BF): The cholinergic BF structures in primates are located medial to the striatum and ventral to the globus pallidus and anterior commissure [scheme 1] and are subdivided into 4 nuclei: Ch1 group=the medial septum; Ch2 group=the vertical nucleus of the diagonal band; Ch3 group=the horizontal nucleus of the diagonal band; and Ch4=the nucleus basalis of Meynert (NBM)-scheme 1 [75]. These cholinergic cluster of cells, comprise the septal nucleus, diagonal band of Broca, and NBM that provide the major cholinergic

innervation to the cerebral cortex, hippocampus, and amygdala [83-85]. These cholinergic neurons are known to be involved in attention and in many aspects of cognitive function [86,87]. Importantly, BF cholinergic nuclei undergo a decrease in volume and neuron number and a loss in the expression of cholinergic markers in Alzheimer's and Parkinson's disease with dementia [82,83,87-94]. In our Mn-exposed monkeys, we have observed significant impairments in attention, working memory, paired associative learning and processing speed [6-8], as in Alzheimer's and Parkinson's disease [87]. Therefore, we believe it is important to examine the BF cholinergic nuclei for cholinergic neuron loss and morphological changes in our unique set of monkeys. Further, our preliminary studies point to a putative Mn-induced effect on BF cholinergic neurons (Figure 3).

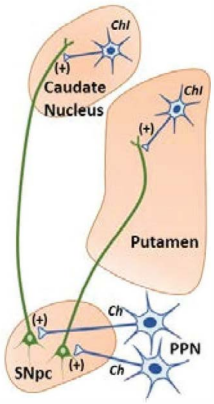


Scheme 1: Delineation of the cholinergic basal forebrain throughout its entire rostrocaudal extent via cresyl violet staining-Figure 1 from [82]. Cd=caudate nucleus; put= putamen; nacc=nucleus accumbens; ac=anterior commissure; gp=globus pallidus; gpe=external globus pallidus=gpi, internal globus pallidus; ic=internal capsule; fx=fornix; lv=lateral ventricle, and ox=optic chiasm.

The pedunculopontine nucleus (PPN): The PPN is a brain structure in the dorsal tegmentum of the midbrain and upper pons and is divided into two components, the mainly GABAergic pars dissipata (PPNd) and the cholinergic/glutamatergic pars compacta (PPNc; Ch5 group) (Scheme 2) [95]. It receives direct connections from the motor cortex and the output nuclei of the basal ganglia; i.e., the globus pallidus internus and the substantia nigra pars reticulata (SNpr), two brain regions that are known to be affected in Mn neuropathology. The PPN is reciprocally connected to the basal ganglia and provides direct input to the intralaminar thalamic nuclei, and motor regions of the brainstem and spinal cord [95]. Because of these functional connections, the PPN is involved in cortical arousal and behavioral state control and participates in locomotion, gait, and muscle tone [96-100]. Furthermore, PPN neuron loss has been shown in Alzheimer's disease and Parkinson's disease with dementia brains [100-104], and this structure currently serves as an important target for deep brain stimulation in PD patients with severe postural instability and gait abnormalities [105-107]. PPN activation is involved in higher cognitive functions such as visuospatial and attentional processes [108] that are needed for motor performance and gait control, and we have shown that these functions (i.e., visuospatial and attention) are affect by chronic Mn exposure in our non-human primate model [6-8]. Importantly, relevant to our results related to Mn-induced impairment of striatal DAR [1,4], electrical stimulation of cholinergic neurons in the PPN induces burst firing of DAergic neurons in the SNpc and induces DAR in the striatum [109-111]. Therefore, as previously noted, Mn-induced degeneration of striatal ChI could modulate striatal DAR at the level of DAergic terminals in the striatum (Figure 2). Furthermore, it is also possible that the loss or injury of cholinergic neurons in the PPN could influence the firing of DA neurons in the SNpc and reduce DAR in the striatum as we have reported in our Mn-exposed monkeys with PET imaging [1,4]. Scheme 3 depicts how Mn-induced cholinergic neuron loss or injury can result in inhibition of striatal DAR. These observations are consistent with the findings that Mn-induced motor function deficits, postural instability and gait disturbances in humans are resistant to DA-based drugs and cannot be explained by degeneration of the nigrostriatal DAergic system. The proposed studies of Mn-effects on the cholinergic system offer a fresh perspective on a circuit level understanding of Mn neurotoxicity.



Scheme 2: Efferent and afferents of the pedunculopontine nucleus (PPN) (from (Morita et al., 2014). Abs. SNr, substantia nigra; SNc, substantia nigra pars compacta; STN, subthalamic nucleus; GPi, globus pallidus internal segment; PPNc, pedunculopontine nucleus pars compacta; PPNd, pedunculopontine nucleus pars dissipatus; ChAT, choline acetyltransferase. Taken from reference [95].



Scheme 3: Cholinergic interneurons in the caudate/putamen positively modulate DAR [66,67]. Activation of cholinergic neurons in the PPN induce burst firing of DA neurons in the SNpc and DAR in the striatum [109-111]. Therefore, a Mn-induced loss or injury of striatal ChI and cholinergic neurons in the PPN would result in significant inhibition of striatal DAR as we have shown in our Mn-exposed monkeys [1,4].
ChI=cholinergic interneurons; Ch=cholinergic neurons; SNpc= substantia nigra pars compacta; PPN=pedunculopontine nucleus. (+)= acetylcholine release promotes DAR in the striatum.

D.2.1.: Methods for SA1:

D.2.1.a.: Tissue processing: After harvesting, brains were cut along the midline providing one hemisphere for analyses of fresh frozen tissue and the other hemisphere for immunohistochemistry. The side of the brain (always the same side) for immunohistochemistry was placed in a solution of 4% PFA in phosphate buffered saline (PBS; 0.01M, pH 7.4) for two weeks and then cut into 4-mm blocks in the coronal plane based on the stereotaxic coordinates in Szabo and Cowan [112]. These brain slabs underwent cryoprotection in 10% sucrose followed by 30% sucrose in PBS, until they were fully saturated with sucrose. Some of the slabs were sectioned into 50-µm-thick coronal slices using a freezing microtome and serially collected and stored in an anti-freeze solution and kept in a -20°C freezer for long-standing storage.

D.2.1.b.: Authentication and quality of primary antibody: A goat anti-ChAT antibody (1:100, ProSci, California) has been used by us to generate preliminary data [Figures 2 and 3] and will be used for our immunohistochemical studies of cholinergic neurons in the striatum, BF, and PPN. This antibody is highly specific and produce little non-specific staining [68,113]. Western blot demonstrates a single band at the known molecular weight of ChAT [114]. In addition, incubations with primary antibody omitted show a lack of immunolabeling in cholinergic nuclei [113,115].

D.2.1.c.: Experience and scientific rigor with processing for light microscopic observations, volume estimations, and stereological cell counts: Our lab has significant amount of experience (>12 years) with these methodologies and maintain technical consultation and support related to unbiased stereological cell counting, soma seize estimation, and fiber-length density with Johnnie Bremholm PhD- a world renowned expert in these methodologies and senior application scientist at Visiopharm.

D.2.1.c.1.: ChAT immunostaining of systematic uniformly random sampled sections of brain nuclei:
Scientific rigor: To ensure robust and unbiased analysis and maintain strict scientific rigor, all tissue sections are blinded to the experimenter (Dr. Kalynda Gonzales) that will perform the immunostaining, unbiased stereological cell counting, analysis of neuron number and soma seize, and reporting of results. In all of our work, including data reported in the preliminary studies, the brain tissue sections are coded with no identification of the animal number or treatment group. The code is only broken when all staining, cell counting, and analysis are finalized.
To obtain neuron number and soma size endpoints, post-fixed sections of the striatum, BF, and PPN will be processed for immunohistochemistry with immunoperoxidase localization of ChAT at the light microscopic level (for examples, see Figures 2 and 3). Systematic uniformly random samples will be collected from the entire brain structures as follows: (1) one out of every twelfth section for the caudate nucleus and putamen; (2) one out of every seventh section for the BF nuclei; and (3) one out of every third section for the PPN. The immunohistochemistry protocol will be carried out as previously described [113]. For incubation with the primary antibody, sections will be placed for 24 hours at RT in a solution containing mouse anti-ChAT, 1% normal horse serum, 0.3% Triton-X-100, and 1% BSA in PBS. The next day, sections will be incubated for 90 min at RT in the secondary antibody solution containing biotinylated horse anti-mouse IgGs (1:200 dilution; Vector Laboratories), 1% normal horse serum, 0.3% Triton-X-100, and 1% BSA. The sections will be exposed to an avidin-biotin-peroxidase complex followed by incubation with 0.025% 3,3'-diaminobenzidine (DAB), 10 mM imidazole, and 0.006% hydrogen peroxide in Tris buffer for 10 minutes at RT to reveal the immunoreaction product. Sections are mounted on slides, dehydrated with increasing ethanol dilutions followed by xylene rinses and coverslipped.

D.2.1.c.2.: Delineation of brain regions of interest: For the delineation of each brain nuclei, we will utilize two sources of information that provide a stereotaxic atlas of the basal ganglia and thalamus in the *cynomolgus* monkey [116,117], along with the standard monkey brain atlas of Paxinos. Furthermore, previous studies of ChAT immunolabeling in primates will be used to define the anatomical landmarks of interest, in particular Gonzales and Smith [68] for the striatum, Hall et al. [81] for the BF, and Mesulam et al. [118], Hepp et al. [104], and Hamani et al. [107] for the PPNc. Dr. Gonzales is an expert neuroscientist that was trained (PhD) at the Yerkes National Primate Research Center at Emory University and has extensive expertise and experience with primate tissue and her thesis was on cholinergic neurons [see reference 68].

D.2.1.c.3.: Estimation of total neuron number using the optical fractionator method: An Olympus BX51 light microscope fitted with an Olympus DP73 digital camera (0.5x), a IK220 encoder (Heidenhain, Germany), and an automated stage for imaging in the X, Y, and Z planes (ProScan II, Prior Scientific, UK) will be used to analyze ChAT-labeled tissue sections. The microscope system is calibrated using an Olympus calibration slide, and the exact alignment of the camera and stage are monitored frequently. The computer is equipped with Visiopharm stereological software (Version 6.7.0.2590, Denmark), and the entire system is mounted on a vibration isolation table (Vision IsoStation, Newport Inc., CA, USA) to allow stable high magnification imaging.
Unbiased stereological estimation of the total number (*N*) of ChAT-labeled neurons in the various brain nuclei will be quantified using the optical fractionator principle [69] with a uniform random sampling scheme of the region of interest and a reciprocal sampling fraction [70,71]. We chose the optical fractionator method that incorporates post-processing tissue shrinkage (i.e., *hsf,* the height sampling frequency) because of its insensitivity to the reference volume [71,119,120], and the entire extent of each brain nuclei is available for analysis, which is a requirement of the optical fractionator. Stereological methods are optimal because they allow for the assessment of the sources of variation within a study [119,121]. The coefficient of error (CE) will be calculated for the estimated total neuron number (*N*) of each animal, along with the mean CE, the coefficient of total variation (CV_{Total}), and the coefficient of biological variation (CV_{Bio}) for the mean total number of each group. The group standard deviation will be used to calculate the mean CV_{Total} per group; then, the CV_{Bio} will be calculated from the mean CE and CV_{Total}. These various factors will allow for the detection of the methodological precision and reproducibility of our stereological estimates [120-122]. For instance, two ratios typically used in stereological analyses will be applied to determine what proportion the method error or biological variability contribute to the total variation of our estimates for the control and Mn-exposed groups [70,123].
Data from pilot studies of neuron numbers are informative about both the degree of biological variation in experimental design and the level of sampling and counting needed to obtain an optimal precision of estimates [71,121,124]. Accordingly, we always carry out a pilot study prior to the estimation of the total number of ChAT-labeled neurons in the regions of interest. Once our optimal parameters are chosen, the number of ChAT-labeled neurons in the dorsal striatum, BF, and PPN of each animal will be assessed by a single blinded investigator (Dr. Gonzales) to ensure reliability of the analysis. Our protocol for performing the stereological cell counts will be followed in a similar manner for every section: (1) Super Image of the entire coronal section taken at 4x (Olympus, UPlanFI, NA 0.13); (2) outline of the region of interest (ROI) at 4x; (3) set the parameters for the optical dissector probe (i.e., the disector height, counting frame size, and percentage of sampling defined by our pilot studies); (4) ChAT-labeled neurons will be counted at 100x (Olympus, UPlanApo, NA 1.35, oil iris); and (5) the analyzed data will be exported into a Microsoft Excel file. Neuron cell bodies will only be included, if at the time of focus, they are within the unbiased, randomly superimposed counting frames or touched the inclusion line (green color) of the counting frames. Additionally, ChAT-labeled soma at the inclusion (green)/exclusion (red) border of the counting frame will be included in the cell counts, unlike the cells that only touched the exclusion (red) counting frame line.

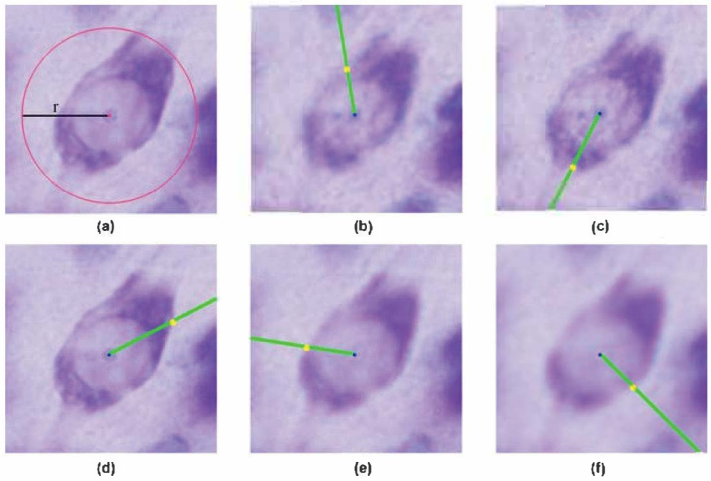


Figure 5: Example of soma volume determination using the spatial rotator approach via the Visiopharm software (Figure 11 from [124]. First, the largest radius, r, from the nucleolus to the cell boundary was determined (a) and used to identify the top and bottom of the neuron (b-f). Five test rays were used in this example to mark the intersection point with the cell boundary in a series of parallel focal planes. Thus, only one test line is needed for each focal plane, resulting in less time consumption.

Once all of the sections are counted, the Visiopharm calculator will quantify the estimated *N* in the brain nuclei of one animal. Photomicrographs of all parameters and settings and of representative cells included in the actual analysis will be collected and saved for each set of completed cell counts. The z-positions of all neurons included in this analysis will be documented. When a ChAT-labeled neuron is included in the cell counts, the local section thickness (µm) will also be measured to calculate the weighted mean section thickness for individual animals, serving as a form of quality control to ensure estimate precision [119]. As noted previously, all tissue sections will be blinded to the experimenter (Dr. Gonzales) that will perform the

immunostaining and unbiased stereological cell counting.

D.2.1.d.: Nissl and NeuN stain: We will perform Nissl and NeuN staining using standard methods and stereological cell counting of the large ChI in the striatum and cholinergic neurons in the BF and PPN to confirm the loss of these cells as suggested by the ChAT-immunostaining in Figure 2 and 3.

D.2.1.d.1: Soma size determination: To determine if chronic Mn exposure alters cholinergic neuron soma size, we will measure the volume of the ChAT-positive cell bodies in conjunction with the stereological cell counts, producing a sufficient sample size for this type of analysis. We will use a new local volume estimator, the spatial rotator [125]. This method is ideal because our coronal sections are taken from preferentially-oriented tissue in order to identify the nucleus of interest, meaning they are not isotropic or vertical uniform sections required for most local volume estimators. Thus, the spatial rotator provides a solution to this problem: the use of a virtual 3D probe to estimate the volume (see Figure 5). In addition, the spatial rotator approach is an interactive analysis (versus an automated analysis), allowing for compensation of the variability between staining of immunoreactions [125]. The distribution of these results will be produced after the analysis is completed to determine if chronic Mn exposure alters the volume of cholinergic neurons in the striatum, BF, or PPN. We will generate a soma size distribution based on the frequency of the observed number of soma seize and determine if the soma size distribution in Mn-exposed animals and controls is different for any of the brain regions.

Expected outcome and interpretation of results for SA1: We anticipate that we will discover that other brain regions such as the BF and PPN will also exhibit reductions in ChAT-positive cholinergic neurons and/or shrinkage of soma size as in the striatum. These findings would indicate a Mn-induced loss or injury of cholinergic neurons. Alternatively, another possibility is that the decrease in the ChAT immunohistochemistry signal is the result of a Mn-induced downregulation of the ChAT protein. To determine if there is frank neuronal loss and our results are not a reflection of a decrease in ChAT protein, we will also do Nissl and NeuN staining to provide a resolution to this question. Cholinergic neurons, although few in number in the striatum, have relatively large soma (on average 40 μm) and can be distinguished from other neuron types. Even if we find that the Mn effect on ChAT is the result of a downregulation in ChAT protein, this would still be a significant finding since ChAT is the enzyme responsible for the synthesis of acetylcholine.

D.3.: Experimental Design for Specific Aim 2 (SA2): The goal of SA2 is to determine the effect of chronic Mn exposure on vesicular acetylcholine transporter (vAChT) levels in order to visualize cholinergic axon terminals and varicosities using [^{3}H]-vesamicol quantitative autoradiography and vAChT immunohistochemistry. We have selected these two methods to provide scientific rigor to our work. Why use vAChT rather than ChAT for cholinergic neuron axon terminals and varicosities?

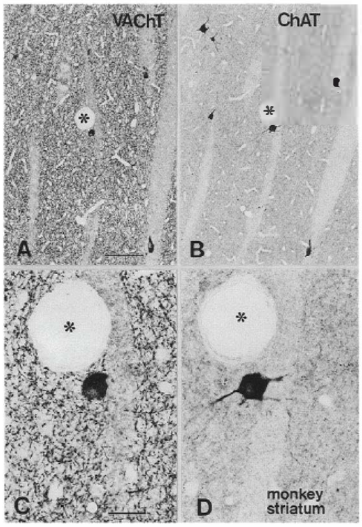


Figure 6: Immunostaining of vAChT and ChAT in cholinergic neurons from the same monkey striatum. Panels A and B depict low magnification images of adjacent sections of monkey striatum demonstrating distribution of vAChT- (panel A) and ChAT-labeled neurons (panel B). See immunoreactivities to identical neuronal cell bodies below asterisk. A dense network of vAChT-positive terminals and varicosities exhibit strong immunostaining as compared to the less intense and less abundant staining of terminals visualized with the ChAT antibody. These images indicate that vAChT is a better marker of cholinergic neuron terminals and varicosities than ChAT. Panels C and D depict high power magnification images of identical areas from (A) and (B) demonstrating coexistence of vAChT (C) and ChAT (D) immunostaining in a single neuron. (A) and (B) are at the same magnification: scale bar in (A) represents 200 μm. (C) and (D) are at the same magnification: scale bar in (C) represents 50 μm. Staining shown in these images was abolished by preincubation of vAChT antiserum with 10-20 μM human vAChT peptide. Figure and legend taken and modified from reference [126].
Asterisks in images mark the same blood vessel on the two adjacent sections.

As noted previously, it is possible that chronic Mn exposure may also affect the extensive neuronal terminals in the striatum and those from BF and PPN cholinergic neurons that innervate the cerebral cortex, hippocampus, amygdala, and thalamus. Studies that have compared both vAChT and ChAT immunostaining in the same brain areas have found that vAChT-positive varicosities exhibit a dense network of strong fiber immunostaining compared to the less intense and less abundant immunostaining of fibers with ChAT [126,127] indicating that vAChT is a better marker to visualize axonal terminals and varicosities (see Figure 6-taken from reference [126]). Therefore, we plan to use ChAT for neuron counts and soma size in SA1 and vAChT for analysis of axonal terminals and varicosities in SA2. The brain regions to be analyzed in SA2 are similar to those in SA1 with the

addition of target regions for BF and PPN neuronal projections. These include several cortical regions (frontal, occipital, parietal), hippocampus, amygdala, and thalamus.

D.3.1. Methods for specific aim 2: We will authenticate the biological integrity of the [^{3}H]-vesamicol received from the company by doing receptor binding studies in neuronal membranes prepared from monkey brain tissue prior to using the radioligand for autoradiography. We will also assess the chemical integrity and purity of the radioligand by thin layer chromatography. These are standard methods in our laboratory [for example see reference 4].

D.3.1.1.: [^{3}H]-Vesamicol autoradiography (see Figure 7 for example): Fresh-frozen brain tissue is sectioned (20 µm) in the coronal plane on a freezing cryostat, thaw-mounted onto slides (Brain Research Laboratories) and stored at –80°C until used. [^{3}H]-vesamicol autoradiography to measure vAChT levels will be performed using methods similar to those described by Altar and Marien [128]. Briefly, slides are thawed and dried at 23°C for 30 min followed by a 10-minute prewash in 50 mM Tris–HCl buffer (pH 7.6) containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ at 23°C. Sections are then incubated at 23°C for 60 min in buffer containing 40 nM [^{3}H]-vesamicol (45.2 Ci/mmol; Perkin Elmer). Non-specific binding will be assessed in adjacent tissue sections incubated in the presence of 10 µM unlabeled vesamicol. The reaction is terminated by two 30 sec washes in cold buffer (4°C) and two dips in cold dH_2O (4°C). Sections are opposed to Kodak Bio-Max MR films with tritium microscales for 4-5 weeks, and images will be acquired and quantified using the MCID software (InterFocus Imaging Ltd., Cambridge, England). Brain regional analysis of total binding is performed using the MCID software. Quantitative autoradiography is a standard method in our lab that we have used for many years.

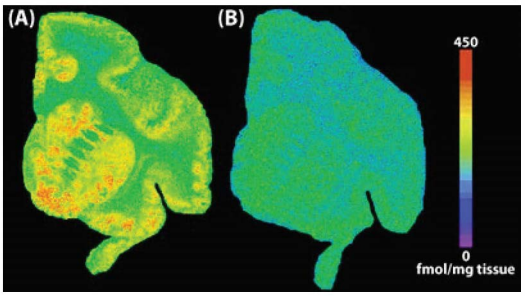


Figure 7: Pseudocolor images of [^{3}H]-vesamicol autoradiography in the pre-commissural striatum from one of our control macaque monkeys. (A) total [^{3}H]-vesamicol binding to vAChT at the level of the pre-commissural caudate/putamen. (B) non-specific binding in the adjacent tissue slide. For any one region of interest, the difference between total and non-specific binding results in specific binding. In general, there is significant [^{3}H]-vesamicol specific binding to vChAT in cortical areas as well as in the striatum. Psudocolor bar on right is indicative of the level of [^{3}H]-vesamicol binding to vChAT in fmol/mg tissue.

D.3.1.2.: vAChT immunohistochemistry: vChAT immunohistochemistry will use a validated goat anti-vAChT antibodies (1:1000, Millipore) and will be performed in a similar fashion as described above for ChAT immunostaining.

D.3.2. Estimates of vChAT immunohistochemistry-positive fiber-length density: Brain sections from the different brain regions (striatum, BF, PPN, cerebral cortex, hippocampus, etc) will be selected for analysis. Slides from control and Mn-exposed brain will be coded to blind the experimenter and a series will be selected throughout the extent of the brain structure of interest. We will determine fiber-length density of vChAT-positive fibers using a global sampling method with isotropic virtual planes generated by the CAST-GRID software. The focal plane along the section z axis will be measured. Fiber length-density will be estimated using a x100 objective lens. Sampling boxes with fixed x, y, and z parameters will be generated by the computer software in a systematic random manner 100 µm (both x and y steps) apart and are superimposed on the video images of the microscope fields that are viewed by changing focal planes throughout the thickness of the sections. Virtual planes generated by the software with a 5 µm sampling plane appear as lines moving along the z axis with the focal plane changing and have a random isotropic orientation for each counting field. The number of intersects of vChAT-labeled fibers with moving lines are counted within the limits of the counting boxes. Being proportional to the fiber length, these numbers represent a base for estimating fiber length per unit of volume (length-density). The stereological sampling is designed so that at least 150-200 intersects are counted per slide. Again, the person performing the studies will be blinded and the tissue slides will be coded until the code is broken following termination of staining, analysis, and reporting of data.

Expected outcome and interpretation of results for SA2: We do not anticipate any problems in the performance of the autoradiography or immunohistochemistry for vAChT. We hypothesize that cholinergic fibers marked by [^{3}H]-vesamicol or vAChT immunostaining will be significantly less (axonopathy) in target regions that are innervated by BF, PPN cholinergic neurons and in ChI within the striatum from Mn-exposed animals. The performance of two different methods should provide important confirmation and rigor to our results. As an alternative approach, we can also label the tissue with microtubule associated protein 2 (MAP2) as an additional method to confirm the possibility that chronic Mn exposure results in axonopathy.

D.4. Experimental design for Specific Aim 3: In SA3, we propose to measure BDNF and NGF concentrations in the striatum, BF, and PPN as well as cholinergic target areas such as the cerebral cortex, hippocampus,

amygdala, and thalamus in brain tissue (fresh frozen) from control and Mn-exposed animals. These studies will be carried out with fresh frozen (-80˚C) tissue from the opposite hemisphere to the one used for the immunohistochemistry studies. Tissue samples will be obtained by punches in the tissue slab once tissue slides have been cut for receptor autoradiography studies. The concentrations of BDNF and NGF in the tissue punches will be measured using ELISA kits per manufacturing instructions (Promega) as well as by Western blot. This approach has worked well for us using monkey brain tissue [16]. We will also obtain tissue punches adjacent to those for BDNF and NGF analysis in order to perform metals analysis using standard methods. We will analyze for Mn, Cu, Zn, and Fe concentrations using ICP-mass spectroscopy as previously performed by us [4]. Brain samples for neurotrophic factors and metals analysis will comprise brain regions such as the caudate, putamen, nucleus accumbens, globus pallidus, BF, PPN, and selected regions of the cerebral cortex (frontal, parietal, temporal, and occipital cortices), hippocampus (CA1-CA4, dentage gyrus), amygdala, and thalamus.

D.4.1.: Authentication and validation of nerve growth factors ELISA kits: we will validate the ELISA kits by spiking the tissue with known amounts of recombinant BDNF and NGF to determine percent recovery. We will use internal standards included for every assay run in order to authenticate the reproducibility of the kits.

Expected outcome and interpretation of results for SA3: We anticipate that we will find a direct relationship between BDNF and/or NGF concentrations and the Mn-induced loss of ChAT- and vAChT-positive cell number and fibers. An alternative approach is to also examine Mn-induced fiber loss by performing MAP2 immunostaining. In general, our studies will provide a more precise understanding of Mn-induced neuropathology with significant implications to behavioral deficits and the development of cholinergic and/or neurotrophic factor based pharmacotherapy. *Limitations and future goals related to SA3:* the proposed studies in our unique group of Mn-exposed non-human primates described in SA3 are highly translational but have limitations in understanding mechanism(s) by which Mn alters BDNF and NGF concentrations and the resulting loss of cholinergic neurons. However, if we confirm the Mn-induced decrease of BDNF and/or NGF concentrations in the larger cohort, then in future studies we can perform mechanistic studies using cholinergic neuron cultures in order to define putative mechanisms. We believe that at this time, it would be premature for us to propose cell-based mechanistic studies on the effects of chronic Mn exposure on BDNF and NGF and its effects on cholinergic neuron loss or injury until we confirm that an effect is consistently observed in the Mn-exposed monkey brain.

D.5. Statistical Analysis and Power Calculation (Dr. Yoo-associate professor of Biostatistics will serve as our biostatistical expert-see Biosketch:

The independent-samples t-test will be used to determine the potential differences in estimated stereological cell counting and soma size determinations of ChAT-positive cholinergic neurons in different brain regions (i.e., striatum, BF, and PPN) between control and Mn-exposed animals. Based on our previous study (Figure 2), in the entire caudate nucleus, it is reported that the mean (and standard deviation in parentheses) of ChAT-labeled cell counts in control group and Mn exposed group are 339,948 (39,170) and 251,695 (39,052) respectively. With this result, we calculate the effect size of 1.35, so at α=0.05, 9 control and 9 Mn-exposed animals would give us a power of 0.99. Thus, available samples in the proposed study (10 male controls and 9 Mn exposed from Table 1) would provide enough power for the proposed studies. We note that even if we observe 40% less in mean difference between ChAT-labeled neurons in control and Mn-exposed groups, that what we observed in the previous study (Figure 2), we would have power larger than 0.8 (with α=0.05) to detect an effect. vAChT levels in brain regions (e.g., striatum, BF, PPN, cerebral cortex, hippocampus, etc) between control and Mn-exposed animals will be analyzed by the independent-samples t-test. Also, concentrations of BDNF (or NGF) in brain regions (e.g., caudate, putamen, accumbens, globus pallidus, BF, PPN, cerebral cortex [several regions], hippocampus, and amygdala) between control and Mn-exposed animals will be analyzed by the independent-samples t-test. Multivariate regression analysis will be used to identify potential relationships among brain regions, study group (control and Mn-exposed), and concentrations of BDNF and NGF.

TIMELINE: We anticipate that the total time to perform the proposed studies is 3 years.

Specific Aim	Year 1	Year 2	Year 3
1	----------		
2 (autoradiography)	----------		
2 (immunohistochemistry)		----------	
3	----------		

5.5.5. (A) Bibliography & References Cited:

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June 1st, 2017

To Study Section Review Committee:

I express my full support for Dr. Tomas Guilarte's application entitled: Cholinergic Neuron Degeneration in Manganese Neurotoxicity. Since January 2016, Dr. Guilarte has been Dean of the Robert Stempel College of Public Health & Social Work and Professor of Environmental & Occupational Health as well as Professor of Cognitive Neuroscience & Imaging at Florida International University. As part of Dr Guilarte's hiring agreement he has protected time (40%) to continue to engage in his research, training of doctoral students and postdoctoral fellows.

To assist with the daily operations of the college, Dean Guilarte has appointed an Associate Dean for Academic Affairs, an Associate Dean for Research and a Senior Director of Administrative Services. These administrators are supported by several other staff level positions at the Dean's office. This assistance has allowed Dean Guilarte to allocate a portion of his time to the above mentioned proposed application and research activities.

He has an active laboratory with 4 post-docs, 1 doctoral student, 2 laboratory researchers and a Senior researcher that has been supervising his lab for over twenty years.

We are delighted that Dr. Guilarte is with us here at Florida International University and look forward to his continued success with research and student training.

Sincerely,

Kenneth G. Furton, Ph.D.
Provost, Executive Vice President and Chief Operating Officer

Office of the Provost

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Authentication of Key Biological and/or Chemical Resources:

The studies in this proposal will use commercially available primary antibodies and a radioligand. We will authenticate the purchased resources as described below.

Authentication of primary antibodies (ChAT and vChAT):
A goat anti-ChAT antibody (1:100, ProSci, California) has been used by us to generate preliminary data [Figures 2 and 3] and will be used for our immunohistochemical studies of cholinergic neurons in the striatum, BF, and PPN. This antibody is highly specific and produce little non-specific staining [68,113]. Western blot demonstrates a single band at the known molecular weight of ChAT [114]. In addition, incubations with primary antibody omitted show a lack of immunolabeling in cholinergic nuclei [113,115].

vChAT immunohistochemistry will use a validated goat anti-vAChT antibodies (1:1000, Millipore) and will be performed in a similar fashion as described above for ChAT immunostaining.

Authentication of [^{3}H]-vesamicol:
We will authenticate the biological integrity of the [^{3}H]-vesamicol received from the company by doing receptor binding studies in neuronal membranes prepared from monkey brain tissue prior to using the radioligand for autoradiography. We will also assess the chemical integrity and purity of the radioligand by thin layer chromatography. These are standard methods in our laboratory [for example see reference 4].