| PI: **Chaudhury, ARUN** | Title: Role of shank in pathophysiology of gastrointestinal motility disorders in autism | |
|---|---|---|
| Received: 02/14/2014 | FOA: PA13-303 | Council: 10/2014 |
| Competition ID: FORMS-C | FOA Title: NIH Exploratory/Developmental Research Grant Program (Parent R21) | |
| **1 R21 HD082747-01** | Dual: | Accession Number: 3669108 |
| IPF: 3212902 | Organization: HARVARD UNIVERSITY (MEDICAL SCHOOL) | |
| Former Number: | Department: VA West Rox | |
| IRG/SRG: ZRG1 MDCN-P (57)S | AIDS: N | Expedited: N |
| Subtotal Direct Costs (excludes consortium F&A)<br>Year 1: 150,000<br>Year 2: 125,000 | Animals: Y<br>Humans: N<br>Clinical Trial: N<br>Current HS Code: 10<br>HESC: N | New Investigator: N<br>Early Stage Investigator: N |
| | | |
| *Senior/Key Personnel:* | *Organization:* | *Role Category:* |
| Arun Chaudhury | President and Fellows of Harvard College | PD/PI |

OMB Number: 4040-0001
Expiration Date: 06/30/2016

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**
2014-02-14

**Application Identifier**
6696909-01-5681734

**c. Previous Grants.gov Tracking Number**

**5. APPLICANT INFORMATION** **Organizational DUNS*:** 047006379

Legal Name*: President and Fellows of Harvard College
Department: Sponsored Programs Admin
Division: Harvard Medical School
Street1*: 25 Shattuck St
Street2:
City*: Boston
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02115-6027

Person to be contacted on matters involving this application
Prefix: First Name*: Rachel Middle Name: M. Last Name*: Cahoon Suffix:
Position/Title: Director, Sponsored Programs Administration
Street1*: Harvard Medical School
Street2: SPA, Gordon Hall 509
City*: Boston
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02115-0000
Phone Number*: 617-432-1596 Fax Number: 617-432-2651 Email: spa_award@hms.harvard.edu

**6. EMPLOYER IDENTIFICATION NUMBER** *(EIN) or (TIN)** 1042103580C5

**7. TYPE OF APPLICANT*** O: Private Institution of Higher Education

Other (Specify):
**Small Business Organization Type** ❍ Women Owned ❍ Socially and Economically Disadvantaged

**8. TYPE OF APPLICATION***
● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

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

**9. NAME OF FEDERAL AGENCY***
NIH

**10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER**
TITLE:

**11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT***
Role of shank in pathophysiology of gastrointestinal motility disorders in autism

**12. PROPOSED PROJECT**
Start Date* Ending Date*
12/01/2014 11/30/2016

**13. CONGRESSIONAL DISTRICTS OF APPLICANT**
MA-007

# SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE — Page 2

**14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION**

Prefix: Dr. First Name*: Arun Middle Name: Last Name*: Chaudhury Suffix:
Position/Title: Instructor in Surgery
Organization Name*: President and Fellows of Harvard College
Department: VA West Rox
Division: Harvard Medical School
Street1*: Harvard Medical School at VA Boston Healthcare System
Street2: 1400 VFW Parkway
City*: West Roxbury
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02132-4927
Phone Number*: 857-203-6044 Fax Number: 857-203-5592 Email*: arun_chaudhury@hms.harvard.edu

**15. ESTIMATED PROJECT FUNDING**

| | |
|---|---|
| a. Total Federal Funds Requested* | $353,925.00 |
| b. Total Non-Federal Funds* | $0.00 |
| c. Total Federal & Non-Federal Funds* | $353,925.00 |
| 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*: Kelly Middle Name: A. Last Name*: Evans Suffix:
Position/Title*: SPA Team IV Manager
Organization Name*: President and Fellows of Harvard College
Department: Sponsored Programs Admin
Division: Harvard Medical School
Street1*: Harvard Medical School
Street2: Gordon Hall Suite 509
City*: Boston
County:
State*: MA: Massachusetts
Province:
Country*: USA: UNITED STATES
ZIP / Postal Code*: 02115-0000
Phone Number*: 617-432-1596 Fax Number: 617-432-2651 Email*: spa_award@hms.harvard.edu

| Signature of Authorized Representative* | Date Signed* |
|---|---|
| Evans, Kelly A. | 02/14/2014 |

**20. PRE-APPLICATION** File Name:

**21. COVER LETTER ATTACHMENT** File Name:Chaudhury_R21_Cover_Letter.pdf

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


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OMB Number: 4040-0010
Expiration Date: 06/30/2016

# 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: | President and Fellows of Harvard College |
| Duns Number: | 0470063790000 |
| Street1*: | Harvard Medical School at VABHS |
| Street2: | 1400 VFW Parkway |
| City*: | West Roxbury |
| County: | |
| State*: | MA: Massachusetts |
| Province: | |
| Country*: | USA: UNITED STATES |
| Zip / Postal Code*: | 02132-4927 |
| Project/Performance Site Congressional District*: | MA-008 |

File Name

**Additional Location(s)**



Tracking Number: GRANT11583047

Funding Opportunity Number: PA-13-303. Received Date: 2014-02-14T16:40:33.000-05:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

# 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 A3431-01

**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*** | Chaudhury_R21_Abstract.pdf |
| **8. Project Narrative*** | Chaudhury_R21_Project_Narrative.pdf |
| **9. Bibliography & References Cited** | BIBLIOGRAPHY_final.pdf |
| **10.Facilities & Other Resources** | Chaudhury_R21_Facilities_Resources_2.pdf |
| **11.Equipment** | |



Tracking Number: GRANT11583047

Funding Opportunity Number: PA-13-303. Received Date: 2014-02-14T16:40:33.000-05:00

## Abstract

PSD proteins in postsynaptic densities of excitatory synapses are relatively immobile components and there is a structured organization of mobile scaffolding proteins lying beneath the PSDs. For example, shank proteins are located further away from the membrane in the cytosolic faces of the PSDs, facing the actin cytoskeleton. The rationale of this organization may be related to important roles of these proteins as “exchange hubs” for the signaling proteins for their migration from the subcortical cytosol to the membrane. Notably, PSD95 have also been demonstrated in prejunctional nerve terminals of the nitrergic neuronal processes traversing the gastrointestinal smooth muscle bundles. It has been recently reported that motor proteins like myosin Va play important role in transcytosis of nNOS. In this proposal, we hypothesize that nNOS requires important interactions with scaffolding proteins in the cortical cytoskeleton of the nerve terminal prior to docking at the membrane. In this context, we propose to examine the role of “shank”, named for SRC homology (SH3) and multiple ankyrin repeat domains, in nitric oxide synthesis. We hypothesize that dynein light chain LC8-nNOS from acto-myosin Va is exchanged with shank, which thereafter facilitates transposition of nNOS for binding with palmitoyl-PSD95 at the nerve terminal membrane. We plan to examine these protein interactions in enteric nerve terminals by the powerful imaging technique of proximity ligation assay. We will also examine in vitro nitric oxide production of electrically stimulated enteric nerve terminals. We plan to compare nitric oxide production in wild type mice with shank3 knockout mice with an aim to directly establish the role of shank in nitric oxide production. We anticipate significant reduction of nitric oxide production in shank3 knockout mice. In a separate set of experiments, we propose to inject poly-I:C to pregnant female wild type mice to mimic viral infection. Pups born to these mice are known animal models of autism spectrum disorders and display behavioral abnormalities that recapitulate features of human patients with these pervasive neurodevelopmental disorders. Deletion of shank3 in humans is a monogenic cause of autism called Phelan-Mcdermid syndrome. Numerous gastrointestinal motility disorders are identified in these patients, including chronic constipation and cyclical vomiting disorder. The current proposal is significant and novel from the perspective of the first-level investigations into these complex GI motility problems that may result from defective nitric oxide synthesis in the enteric nerve-smooth muscle junctions and lays groundwork for development of rational pharmacotherapy for functional bowel disorders in autism.

## Project Narrative

Complex protein organization in nerve terminals controls precise release of gaseous neurotransmitters like nitric oxide to facilitate passage of food through the intestines. Based on previous studies, rational hypothesis is forwarded to perform rigorous and systematic animal studies to examine the role of shank protein in nitric oxide synthesis. Shank protein is deleted in Phelan-McDermid syndrome and may be the basis for cyclical vomiting and chronic constipation seen in these patients and others with autism spectrum disorders.

## Facilities and Equipment to conduct proposed research

The research involves examining the role of shank proteins in nitric oxide synthesis in enteric nerve terminals. Research will be conducted either in isolated nerve terminals (varicosities) or whole mounts. Western blotting and optical imaging are the mainstay of experiments. The investigator has several years of experience of performing these techniques (Chaudhury et al 2009, Chaudhury et al 2011, Chaudhury et al 2012, Chaudhury et al 2013). The details are described below.

1. Laboratory and office space including computers are available to the investigator for conducting the experiments and analyses.
2. In addition, **dedicated animal facilities** are available for maintaining animals proposed for the experiments.
3. For **optical imaging analyses**, **confocal microscopy suites** in core research facility at VA Boston HealthCare System (West Roxbury) campus will be used. Zeiss and Nikon confocal microscopes are available. The team have greater than decade long experience in cutting edge microscopy and have incorporated microscopy for novel translational investigations.
4. **Vibratomes** and **cryostat** machines are available for obtaining sections. **Stereo-microscope** are available for making whole mounts. These are all available in the Core facilities of HMS Research Programs at the VA Medical Center West Roxbury without any additional cost to the investigator.
5. **Spectrophotometers, ultracentrifuges** and **western blotting apparati, including access to cold room in core research area** are available.
6. Shank3 knockout mice will be obtained from Jackson Labs.
7. Source of chemicals, antibodies and other needed reagents
    - chemicals (poly-I:C, DAF etc) shall be obtained from Sigma-Aldrich (St. Louis, MO)
    - Western Blotting supplies (gel, loading buffer): BioRad
    - Shank antibodies: SySy (Synaptic Systems), Santa Cruz Biotech, Antibodies Inc
    - $nNOS_{k-20}$, LC8, actin antibodies: Santa Cruz Biotech (all antibodies standardized)
    - Proximity Ligation Assay Kit: Duolink (Olink Bioscience)

OMB Number: 4040-0001
Expiration Date: 06/30/2016

# RESEARCH & RELATED Senior/Key Person Profile (Expanded)

**PROFILE - Project Director/Principal Investigator**

Prefix: Dr. First Name*: Arun Middle Name Last Name*: Chaudhury Suffix:

| | |
|---|---|
| Position/Title*: | Instructor in Surgery |
| Organization Name*: | President and Fellows of Harvard College |
| Department: | VA West Rox |
| Division: | Harvard Medical School |
| Street1*: | Harvard Medical School at VA Boston Healthcare System |
| Street2: | 1400 VFW Parkway |
| City*: | West Roxbury |
| County: | |
| State*: | MA: Massachusetts |
| Province: | |
| Country*: | USA: UNITED STATES |
| Zip / Postal Code*: | 02132-4927 |

Phone Number*: 857-203-6044 Fax Number: 857-203-5592 E-Mail*: arun_chaudhury@hms.harvard.edu

Credential, e.g., agency login: ACHAUDHURY

| | |
|---|---|
| Project Role*: PD/PI | Other Project Role Category: |
| Degree Type: M.D. | Degree Year: 2004 |

| | File Name |
|---|---|
| **Attach Biographical Sketch*:** | Chaudhury_R21_Biosketch.pdf |
| **Attach Current & Pending Support:** | |



Tracking Number: GRANT11583047 Funding Opportunity Number: PA-13-303 . Received Date: 2014-02-14T16:40:33.000-05:00

## BIOGRAPHICAL SKETCH

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

| NAME<br>Chaudhury, Arun | POSITION TITLE<br>Instructor in Surgery |
|---|---|
| eRA COMMONS USER NAME (credential, e.g., agency login) ACHAUDHURY | |

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

| INSTITUTION AND LOCATION | DEGREE *(if applicable)* | MM/YY | FIELD OF STUDY |
|---|---|---|---|
| Medical College Kolkata, India | MBBS | 09/2000 | Medicine |
| All India Institute of Medical Sciences | MD (Residency) | 06/2004 | Anatomy |
| Monell Chemical Senses Center, Philadelphia | Postdoctoral | 02/2007 | Nutritional Neurosciences |
| Harvard Medical School, Boston | Postdoctoral | 06/2013 | Neurogastroenterology |

### A. Personal Statement

My investigative interests are focused on pathophysiology of gastrointestinal orders and include keen translational endeavors to address refractory problems in gastroenterology, specially focused on functional bowel disorders spanning all age groups. In this application, I extend my prior studies of nitrergic neurotransmission to propose a possible basis for chronic constipation and cyclical vomiting in a subset of patients with autism spectrum disorder and investigate these issues using appropriate mice model.

### B. Positions and Honors

**Positions and Employment**

1999-2000 Internship, Dept of Medicine, Surgery, OBGYN, Medical College Hospitals, Kolkata
2000-2004 Junior & Senior Resident, Department of Anatomy, All India Institute of Medical Sciences (AIIMS), New Delhi
2005-2007 Postdoctoral Fellow in Nutritional Neurosciences, Monell Chemical Senses Center, University of Pennsylvania, Philadelphia
2007-2013 Research Fellow in Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School and Center for Swallowing & Motility Disorders, VA Medical Center, Boston
2007-present Member, The Harvard Clinical and Translational Science Center (Harvard CATALYST)
2013-present Instructor, Department of Surgery, Brigham and Women’s Hospital

**Honors**

1994 Ranked 5$^{th}$ in Medical Entrance Exam (West Bengal Joint Entrance Examination)
1998 Govt of West Bengal Merit Award for Medical Class Topper (full tuition waived)
1999 Certificate of Honors, Ophthalmology, RIO, MCH, India
2003 Commonwealth Science Council Travel Award to MBL, Woods Hole
2003 Department of Science and Technology (DST, Govt. of India) Travel Award to MBL, Woods Hole

| | |
|---|---|
| 2003 | International Brain Research Organization (IBRO) (Asia-Pacific) Travel Fellowship for Analytical and Quantitative Light Microscopy (AQLM) Course at Marine Biological Lab (MBL), Woods Hole |
| 2003 | IBRO Fellowship for FENS (Federation of European Neurosciences) Summer School on Peripheral Nervous System, Ofir |
| 2003 | CSIR (Council for Scientific & Industrial Research, Govt. of India) Fellowship for Enteric Nervous System (ENS) Conference, Banff |
| 2003 | European Union Travel Fellowship, Baltic Summer School on Neurodegenerative Diseases, Kiel |
| 2004 | 16th IFAA (International Federation of Association of Anatomists) Young Investigator Award, Kyoto |
| 2004 | WERC/IBRO (West European Regional Council/International Brain Research Organization) PhD fellowship (one out of only 3 candidates chosen from a global pool of applicants) |
| 2006 | American Motility Society (AMS) Young Investigator Award |
| 2010 | Extraordinary Scientific Investigator, Department of State, Govt. of USA |
| 2011 | Best Scientific Poster, 16th American Motility Society Meeting, St. Louis |
| 2013 | US Permanent Residency, Extraordinary Investigator Category |

**C. Selected Peer-reviewed Publications**

**Most relevant to the current application**

1. Chaudhury A, Shariff A, Srinivas M, Sabherwal U. Changes in nitrergic innervation of defunctionalized rat colon after diversion colostomy. *Neurogastroenterol Motil.* 2004 Aug;16(4):475-87. PMID: 15306003
2. Horn CC, Ciucci M, Chaudhury A. Brain Fos expression during 48 h after cisplatin treatment: neural pathways for acute and delayed visceral sickness. *Auton Neurosci*. 2007 Mar 30;132(1-2):44-51. PMID 17092780
3. Rao YM, Chaudhury A, Goyal RK. Active and inactive pools of nNOS in the nerve terminals in mouse gut: implications for nitrergic neurotransmission. *Am J Physiol Gastrointest Liver Physiol*. 2008 Mar;294(3):G627-34. PMID: 18096606
4. Goyal RK, Chaudhury A. Physiology of normal esophageal motility. *J Clin Gastroenterol*. 2008 May-Jun;42(5):610-9. PMID: 18364578
5. Chaudhury A, Rao YM, Goyal RK. PIN/LC8 is associated with cytosolic but not membrane-bound nNOS in the nitrergic varicosities of mice gut: implications for nitrergic neurotransmission. *Am J Physiol Gastrointest Liver Physiol*. 2008 Sep;295(3):G442-51. PMID: 18635601
6. Chaudhury A, He XD, Goyal RK. Role of PSD95 in membrane association and catalytic activity of nNOSalpha in nitrergic varicosities in mice gut. *Am J Physiol Gastrointest Liver Physiol*. 2009 Oct;297(4):G806-13. Erratum in: *Am J Physiol Gastrointest Liver Physiol*. 2010 Oct;299(4):G100-2. PMID: 19679819
7. Goyal RK, Chaudhury A. Mounting evidence against the role of ICC in neurotransmission to smooth muscle in the gut. *Am J Physiol Gastrointest Liver Physiol*. 2010 Jan;298(1):G10-3. PMID:19892937
8. Goyal RK, Chaudhury A. Pathogenesis of achalasia: lessons from mutant mice. *Gastroenterology*. 2010 Oct;139(4):1086-90. PMID: 20800108
9. Chaudhury A, He XD, Goyal RK. Myosin Va plays a key role in nitrergic neurotransmission by transporting nNOSα to enteric varicosity membrane. *Am J Physiol Gastrointest Liver Physiol*. 2011 Sep;301 (3):G498-507. PMID: 21680773
10. Chaudhury A, He, XD, Goyal RK. Role of myosin Va in purinergic vesicular neurotransmission in the gut. *Am J Physiol Gastrointest Liver Physiol*. 2012 Mar;302 (6):G598-607. PMID: 22207579
11. Chaudhury A, Goyal RK. Myosin activators in gaseous neurotransmission. Patent application pending, USPTO/Harvard University, November 2012.
12. Goyal RK, Sullivan MR, Chaudhury A. Progress in understanding of inhibitory purinergic neuromuscular transmission in the gut. *Neurogastroenterology Motility*, 2013 Mar;25(3):203-7. PMID: 23414428
13. Goyal RK, Chaudhury A. Structure activity relationship of synaptic and junctional neurotransmission. *Autonomic Neurosciences: Basic and Clinical*, 2013 Jun;176(1-2):11-31. PMID: 23535140
14. Chaudhury A. Evidence for Dual Pathway for Nitrergic Neuromuscular Transmission in Doubt: Evidence Favors Lack of Role of ICC. Gastroenterology. 2013 Nov;145(5):1160-1. PMID: 24070723
15. Chaudhury A, Cristofaro V, Carew J, Goyal RK, Sullivan MP. Myosin Va plays a role in nitrergic smooth muscle relaxation in gastric fundus and corpora cavernosa of penis. Accepted, PloS One, December 18th 2013.

## D. Research Support

### Ongoing Research Support

SomahLution (Industry) Thatte HS (PI) 09/01/2011-08/31/2014

Title: Evaluation of Somah Technology in Preservation of Abdominal Organs. Define the role of Somah Technology in its ability to preserve structure and function in liver, pancreas and kidneys from beating and non-beating heart donors.

Role: Instructor

### Completed Research Support

None

# PHS 398 Cover Page Supplement

OMB Number: 0925-0001

## 1. Project Director / Principal Investigator (PD/PI)

Prefix: Dr.
First Name*: Arun
Middle Name:
Last Name*: Chaudhury
Suffix:

## 2. Human Subjects

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

## 3. Permission Statement*

If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?

❍ Yes ● No

## 4. Program Income*

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

## 5. Human Embryonic Stem Cells

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

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:

**Cell Line(s):** ❑ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

## 6. Inventions and Patents (For renewal applications only)

Inventions and Patents*: ❍ Yes ● No

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

Previously Reported*: ❍ Yes ❍ No

## 7. Change of Investigator / Change of Institution Questions

❑ Change of principal investigator / program director

Name of former principal investigator / program director:

Prefix:

First Name*:

Middle Name:

Last Name*:

Suffix:

❑ Change of Grantee Institution

Name of former institution*:

# PHS 398 Modular Budget

OMB Number: 0925-0001

**Budget Period: 1**

**Start Date:** 12/01/2014 **End Date:** 11/30/2015

## A. Direct Costs

| | Funds Requested ($) |
|---|---|
| Direct Cost less Consortium F&A* | 150,000.00 |
| Consortium F&A | |
| **Total Direct Costs*** | **150,000.00** |

## B. Indirect Costs

| | Indirect Cost Type | Indirect Cost Rate (%) | Indirect Cost Base ($) | Funds Requested ($) |
|---|---|---|---|---|
| 1. | MTDC Off Campus | 28.70 | 150,000.00 | 43,050.00 |
| 2. | | | | |
| 3. | | | | |
| 4. | | | | |

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS , Michael Leonard , 212.264.2069

Indirect Cost Rate Agreement Date: 05/31/2013

**Total Indirect Costs** **43,050.00**

## C. Total Direct and Indirect Costs (A + B)

**Funds Requested ($)** **193,050.00**

# PHS 398 Modular Budget

**Budget Period: 2**

**Start Date:** 12/01/2015 **End Date:** 11/30/2016

## A. Direct Costs

| | Funds Requested ($) |
|---|---|
| Direct Cost less Consortium F&A* | 125,000.00 |
| Consortium F&A | |
| **Total Direct Costs*** | **125,000.00** |

## B. Indirect Costs

| | Indirect Cost Type | Indirect Cost Rate (%) | Indirect Cost Base ($) | Funds Requested ($) |
|---|---|---|---|---|
| 1. | MTDC Off Campus | 28.70 | 125,000.00 | 35,875.00 |
| 2. | | | | |
| 3. | | | | |
| 4. | | | | |

Cognizant Agency (Agency Name, POC Name and Phone Number): DHHS , Michael Leonard , 212.264.2069

Indirect Cost Rate Agreement Date: 05/31/2013

**Total Indirect Costs** **35,875.00**

## C. Total Direct and Indirect Costs (A + B)

**Funds Requested ($)** **160,875.00**

# PHS 398 Modular Budget

## Cumulative Budget Information

### 1. Total Costs, Entire Project Period

| | |
|---|---|
| Section A, Total Direct Cost less Consortium F&A for Entire Project Period ($) | 275,000.00 |
| Section A, Total Consortium F&A for Entire Project Period ($) | |
| Section A, Total Direct Costs for Entire Project Period ($) | 275,000.00 |
| Section B, Total Indirect Costs for Entire Project Period ($) | 78,925.00 |
| Section C, Total Direct and Indirect Costs (A+B) for Entire Project Period ($) | 353,925.00 |

### 2. Budget Justifications

| | |
|---|---|
| Personnel Justification | Chaudhury_R21_Personnel_Justification2.pdf |
| Consortium Justification | |
| Additional Narrative Justification | |

## Budget Justification

### Personnel

**Dr. Arun Chaudhury, M.D., Principal Investigator**, (11.16 calendar months in Yr.1; 9.24 calendar months in Yr. 2) will supervise the overall progress of the projects, conduct all experiments and endeavor to complete the proposed specific aims. Dr. Chaudhury is an expert neuroanatomist/cell biologist and has extensive training in animal microsurgery, nutritional neurosciences and has performed systematic investigations in nitrergic neurotransmission.

Dr. Chaudhury has joint appointments with Harvard Medical School, the VA Boston Healthcare System (without compensation) and Boston VA Research Institute. The effort requested represents Dr. Chaudhury's total effort dedicated to this project. Salary requested is the university's share of Dr. Chaudhury's effort.

**TBN, Research Technician** (9.24 calendar months in Yr.1; 7.08 calendar months in Yr. 2) Will be mentored and instructed by Dr. Chaudhury on all aspects of the study.

Fringe Benefits are calculated at 26.2% for faculty and 56.5% for staff.

# PHS 398 Research Plan

Please attach applicable sections of the research plan, below.

OMB Number: 0925-0001

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Despite reports of frequent gastrointestinal motility disorders affecting both the proximal and distal portions of the gastrointestinal in children with diagnosed **pervasive neurodevelopmental disorders (PND)** (White 2003, Buie et al 2010, Verhoeven et al 2013), virtually nothing is known regarding the molecular pathogenesis of these motility disorders. These presentations include esophageal achalasia, vomiting, intestinal pseudo-obstruction and chronic constipation (Betalli et al 2013, Chaidez et al 2013, Peeters et al 2013). Currently, it is being recognized that these disorders, also called **autism spectrum disorders (ASD),** may result from distinct defects in protein components in central nervous system synapses (Sudhof 2008, Pardo and Eberhart 2007, Bourgeron 2009, Won et al 2013). Though CNS neuropathology is not well understood and mostly involve polygenic disease mechanisms, a subset of autism results from monogenic defects. For example, **Phelan-McDermid syndrome** results from deletion in chromosome 22 (Phelan and Mcdermid 2012). This deletion results in loss of the protein **shank3** (shank is abbreviated for **Src-homology domain 3 (SH3) and multiple ankyrin repeat domains**) (Phelan 2008, Phelan and McDermid 2012), which is an important structural component of post-synaptic protein clustering complex in excitatory glutamatergic synapses (Kim and Sheng 2004). Shank3 knockout mice exhibit features of autism (Yang et al 2012, Wang et al 2011, Peca et al 2011).

Importantly, protein clustering complexes are not only present in the postsynaptic compartment but have also been shown to be present in pre-synaptic structures, including nerve terminals (Chaudhury et al 2009, Aoki et al 2001). In the periphery, it has been shown that these clustering complexes are important in nitrergic neurotransmission (Chaudhury et al 2009, Chaudhury et al 2011). Nitric oxide (NO)-mediated neurotransmission is the main step of inhibitory neurotransmission that facilitate oro-aboral progression of bowel luminal contents (Burnstock 2013, Pluja et al 1999). Defective nitrergic neurotransmission may prevent adequate gastric empting or may cause constipation. Like many other systems in which there is signaling compartmentalization of nNOS (Mohamed et al 2011, ElMlili et al 2010, Villanueva and Giulvi 2010), preliminary evidence suggested that even in enteric nerve terminals, active nNOS is localized to the membrane (Chaudhury et al 2009, Chaudhury et al 2011). This membrane localization of nNOS is facilitated by specialized motor proteins like myosin Va (Chaudhury et al 2011, Chaudhury et al 2013). Through specific interaction moieties like the light chain of dynein (LC8) which acts as a protein interaction hub (Rapali et al 2011, Barbar 2008), nNOS can bind with myosin Va (Chaudhury et al 2011). **We hypothesize that LC8 may facilitate molecular exchange of nNOS from acto-myosin Va through the cortical actin cytoskeleton, which may serve as a diffusion barrier for nNOS docking to membrane, and that shank facilitates this molecular shuttling of nNOS.**

In this proposal, we plan to examine the role of shank in nitrergic neuromuscular function. Shank proteins have been localized in myenteric neurons (Raab et al 2010) and mRNA for shank-interacting proteins have been reported in mice gut muscle extracts (Daigo et al 2003), though specific location of shank in nerve varicosities have not been examined. Shank likely acts as an intermediary scaffold between nNOS delivered to the cell periphery, and its subsequent transcytosis through the actin cortical network for docking to palmitoyl-PSD, the active zone for nitric oxide synthesis at the membrane. We plan to test whole gut nerve varicosities obtained by high speed ultracentrifugation and sucrose gradient purification from wild type C57BL/6J mice and compare with **shank3$^{exon4\text{-}9}$ knockout mice** and another model of autism, the **"maternal immune activation model."** Static protein-protein interactions of shank, nNOS, LC8, actin and PSD95 will be imaged by proximity ligation assay in enteric nerve terminals. We plan to examine the role of shank in prejunctional nitric oxide synthesis by imaging KCl stimulated diaminofluorescein (DAF)-loaded varicosities. We hypothesize that nitric oxide synthesis may be defective in these autism mouse models and may result from deficiency in shank.

**Specific aim 1 To examine the role of scaffolding protein shank in prejunctional nitric oxide synthesis** We hypothesize that nNOS requires interaction with specific proteins in the cortical actin cytoskeleton that facilitates molecular exchange of cytosolic nNOS with membrane bound nNOS, and that this happens via interaction with shank proteins. Shank3 knockout mice will be utilized to evaluate the role of shank protein in nitric oxide synthesis in enteric nerve terminals.

**Specific aim 2 To examine defects in nitrergic synthesis, including shank deficiency, in maternal immune activation mice, a model of autism spectrum disorder** This model is created by mimicking viral infection during pregnancy. Pups born to mice injected with poly-inosine-cytosine are a standard model of creating mice representative of autism spectrum disorders. We will examine nitric oxide synthesis and its deficit in enteric nerve terminals in pups beyond 6 weeks of age and test shank levels and shank-nNOS binding in enteric nerve terminals.

## Background and Significance

Gastrointestinal motility disorders affecting both the proximal and distal portions of the gut affect quality of daily life in both children and adult subjects with **autism spectrum disorders (ASD)** (White 2003, Buie et al 2010, Verhoeven et al 2013). These gastrointestinal motility problems manifest as dysphagia, achalasia, refractory or cyclical vomiting, acid reflux, gastroparesis and defect in gastric emptying, intestinal stasis and pseudo-obstruction and chronic constipation (Betalli et al 2013, Chaidez et al 2013, Peeters et al 2013, Chandler et al 2013, Furuta et al 2012, Wang et al 2012, Wang et al 2011, Pang and Croaker 2011, Erickson et al 2005). Dysphagia in these patients, gastroesophageal reflux, chronic vomiting or encopresis and chronic constipation are often misconstrued in the clinical setting as behavioral issues, rather than an organic problem (Simonoff et al 2008, Dalrymple and Ruble 1992, Matson 1977). Recent studies have provided evidence that mere presence of neurotransmitters in the nerve terminals may not be adequate for inhibitory nitrergic neuromuscular transmission in the gut (Chaudhury et al 2011, Chaudhury et al 2013). Nitric oxide mediated neurotransmission, the main basis for oro-aboral movement of intestinal luminal contents, may be disrupted due to several factors, including (i) transcriptional blockade of genomic nNOS synthesis (Li et al 2013, Saur et al 2002) (ii) defect in allosteric proteins (Chaudhury et al 2009, Gangula et al 2010, Welsh et al 2013, Gorren and Mayer 2007) (iii) defects in dimerization (Rao et al 2008, Gangula et al 2007) and (iv) defective transport of nNOS within the nerve terminals that do not favor enzymatic synthesis of nitric oxide (Chaudhury et al 2011, Chaudhury et al 2013). Examining diverse organ systems reveal that nNOS remains membrane-bound during enzymatic synthesis (Van Geldre et al 2004, Meinen et al 2012, Mohamed et al 2011, Finanger Hedderick et al 2011, Cartwright et al 2009, ElMlili et al 2010, Bredt 1996, Oess et al 2006, Villanueva and Giulvi 2010, van Geldre et al 2000, Kone et al 2003). Evidence has suggested the role of motor proteins like myosin Va in transposition of nNOS within the nerve terminals to the membranes (Chaudhury et al 2011).

Intriguingly, myosin Va associated scaffolding proteins like **"shank"** have been reported to be depleted in monogenic conditions that result in manifestation as **pervasive neurodevelopmental disorder (PND)** (Feyder et al 2010). For example, patients with the rare condition **Phelan-McDermid syndrome** report refractory and cyclical vomiting (Phelan 2007, Phelan 2008, Grabucker et al 2014). Chromosome mutations like deletions are seen in chromosome 22 in this syndrome (del22q13.3) (Guilmatre et al 2014, Soorya et al 2013). This results in inhibition of synthesis of the protein shank3 (Phelan and Mcdermid 2012). **Shank proteins (Src-homology domain 3 (SH3) and multiple ankyrin repeat domains)**, including shank3, are known protein interaction partners of myosin Va (Yoshii et al 2013, Kreienkamp 2008, Lim et al1999, Naisbitt et al 1999, Boeckers et al 1999).

We hypothesize in this proposal that depletion of shank3 may result in defective nNOS membrane localization, resulting in defective nitric oxide synthesis. nNOS is water soluble, but a portion of nNOS remains membrane bound due to its ability to interact with palmitoyl-PSD95 (Chaudhury et al 2009, Tochio et al 2000). Membrane bound nNOS may be at an optimal cellular localization for nitric oxide synthesis due to proximity to calcium ion channels (Chaudhury et al 2011). Myosin Va facilitates cytosolic transport of nNOS to the subcortical region of the nerve terminal that is rich in actin (Chaudhury et al 2011). Actin meshwork has been reported to provide a physical barrier to vesicles involved in neurotransmission (Lemieux et al 2013, Trifaro et al 1992, Duffney et al 2013, Bleckert et al 2012, Fonseca 2012, Sankaranarayanan et al 2003, Morales et al 2000, Goyal and Chaudhury 2013, Siksou et al 2007, Phillips et al 2001). The critical role of filamentous actin in determining the extent of dynamic reorganization in postsynaptic density (PSD) molecular composition is being increasingly recognized (Kuriu et al 2006, Okabe 2012, Okabe 2013, Okabe 2007). It is not known whether actin network may provide a barrier to diffusion of nonvesicular neurotransmitter synthesizing enzymes like nNOS, but recent evidence suggests that the cytosolic streaming of nNOS is not a chaotic stochastic event (Shimmen and Yokota 1994), but rather relies on the cytoskeletal machinery like myosin Va and actin for specific domain localization (Chaudhury et al 2011, Chaudhury et al 2013, Yu et al 2010). Recently, the role of actin mutations in refractory constipation has been recognized (Rubinstein and Mayer 2012, Lehtonen et al 2012).

In excitatory synapses, the PDZ-domain-containing scaffold proteins PSD95 and the Shank family form a bilayer protein network below the postsynaptic membrane, which is bridged by guanylate kinase-associated protein (GKAP) (Kim and Sheng 2004). Shank-family scaffolds are further linked to actin filaments via cortical-actin-binding protein (cortactin) (Boeckers et al. 1999). Thus, these shank proteins form sheets that make a synaptic platform (Baron et al. 2006, Grabrucker et al. 2011). Depletion and redistribution has been shown for ProSAP2/Shank3 in PSDs of cultured neurons, an observation which was independent from protein synthesis

or degradation and could be enhanced by electrophysiological stimulation (Tsuriel et al. 2006). Whether such laminar organization occurs in enteric nitrergic nerve terminals is not known. However, preliminary evidence suggests that these scaffolding proteins like PSD95, which are normal constituents of postsynaptic compartments, may also be present in presynaptic region, including enteric nerve terminals (Chaudhury et al 2009, Aoki et al 2001).

Myosin Va has been shown to interact with nNOS via DLC8 (dynein light chain, 8kDa MW) (Chaudhury et al 2011, Chaudhury et al 2008, Lajoix et al 2004). LC8 acts as multiple cargo adapters and provides a hub for protein homo- and heterodimerization (Rapali et al 2011, Barbar 2008). LC8, also called DLC8, has been reported to bind to presynaptic components like bassoon, which form cytomatrix of the active zone (Rapali et al 2011). LC8 has been reported to associate with shank (Yoshii e al 2013). Initial evidence has shown an “active zone” for nNOS in the membrane of these nerve terminals. nNOS is tethered via the PDZ rich protein PSD95 (Chaudhury et al 2009). PSD95, apart from its PDZ domains, also have other protein domains like SH3 and guanylate kinase (Luo and Zhu 2011, Roszer 2012). In this proposal, the hypothesis is forwarded that nNOS undergoes molecular exchange in the region of the cortical cytoskeleton in which acto-myosin Va bound nNOS initiates association with shank, a cortical actin-bound scaffolding protein. nNOS may thereafter bind to PSD95 in the membrane from shank via transposition through guanylate kinase associated protein (GKAP). PDZ domain mediated transfer of these proteins or molecular exchange via LC8 may occur at the enteric nerve terminal periphery (**Figure 1**).

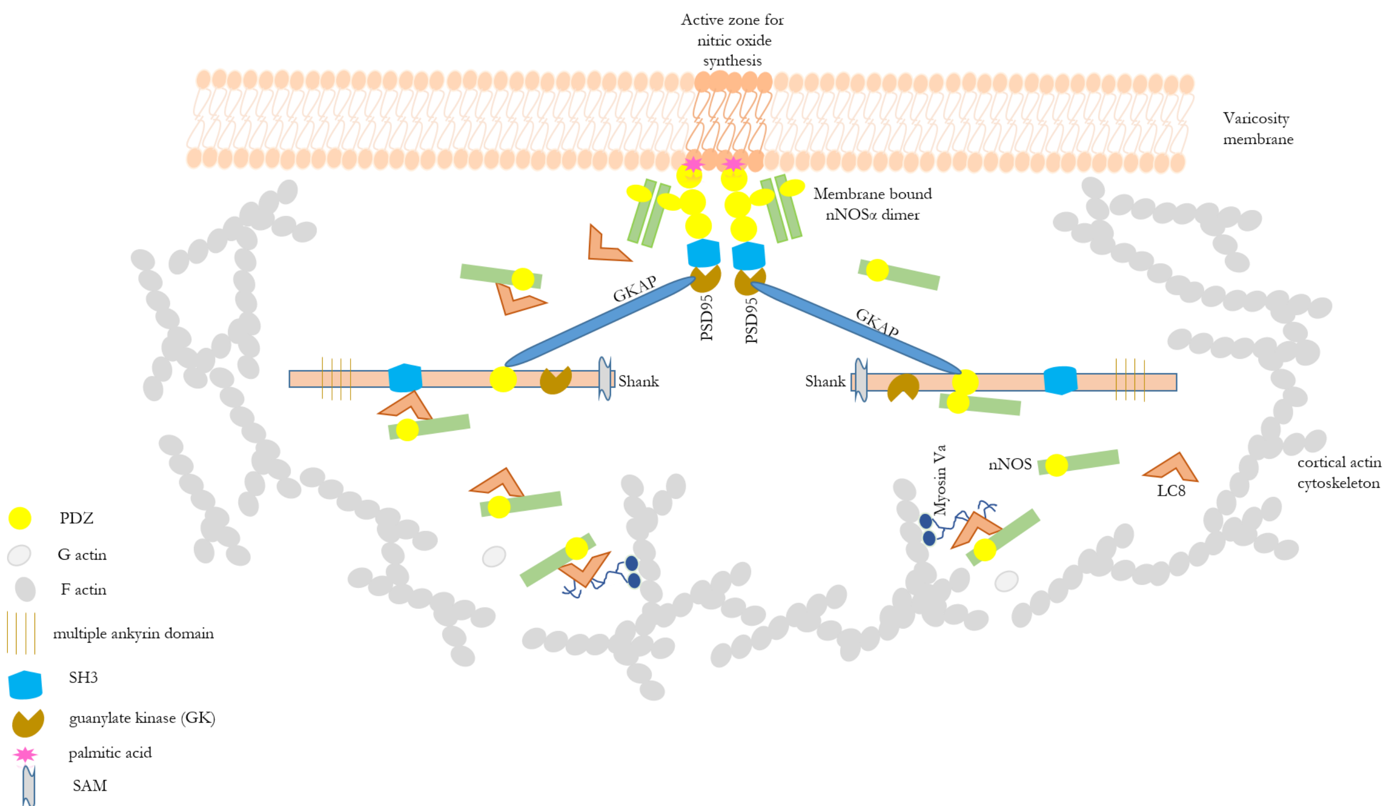


**Figure 1 Cartoon depicting possible role of shank in shuttling of cytosolic nNOS to the nitrergic nerve terminal membrane** Different scaffolding proteins are shown with the modular domains without appropriate scale to the full length of the proteins. Details of this modular domains are described in Kim and Sheng (2004). nNOS, via interaction with LC8 or PDZ domain based interaction, may be shuttled from acto-myosin Va in the cytosol through the actin cortical barrier by shank and GKAP. Shank has multiple protein interaction domains like ankyrin domains, SH3, PDZ and SAM. Static protein interactions are planned to be examined to test whether such exchanges takes place in enteric nerve terminals. By these mechanisms, shank may play a significant role in nitric oxide synthesis and nitrergic neuromuscular neurotransmission. nNOS-LC8-myosin Va and nNOS-palmitoyl-PSD95 interactions have been demonstrated in enteric nerve terminals (Chaudhury et al 2011 & Chaudhury et al 2009).

**<u>We hypothesize that shank proteins may play critical role in the subcortical cytoskeleton for transfer of cytosolic nNOS to the membrane</u>**. Though the experiments of dynamically examining these molecular exchanges by real time high resolution live imaging may not be feasible in this limited proposal, <u>protein association studies are proposed to obtain a snapshot of static interactions</u>. Importantly, the proposed studies

will examine if **shank proteins have a significant role in enteric nitrergic neurotransmission**. **Nitric oxide production will be examined in intestinal nerve terminals of shank3 knockout mice.** These knockout mice manifest behavioral patterns of autism (Yang et al 2012, Wang et al 2011, Peca et al 2011, Yoo et al 2013). This shall provide insights into the molecular pathology of refractory gastrointestinal motility disorders in patients with autism spectrum disorders. **Deficiency of inhibitory nitrergic neurotransmission, including shank defects, will be additionally examined in maternal immune activation mice pups, a model of ASD** (Naviaux et al 2013, Hsiao et al 2013, Weber and Polanco 2012, Meyer 2014). Thus, this proposal aims to provide the first comprehensive insight into the pathological basis for gastrointestinal symptoms in patients with ASD. Insights into molecular pathogenesis shall set the stage for long term investigations into designing rational pharmacological targets for addressing these conditions. The gastrointestinal symptoms may severely affect nutrition in ASD patients with already compromised social communication skills, so state-of-the-art management for gastrointestinal problems is much needed.

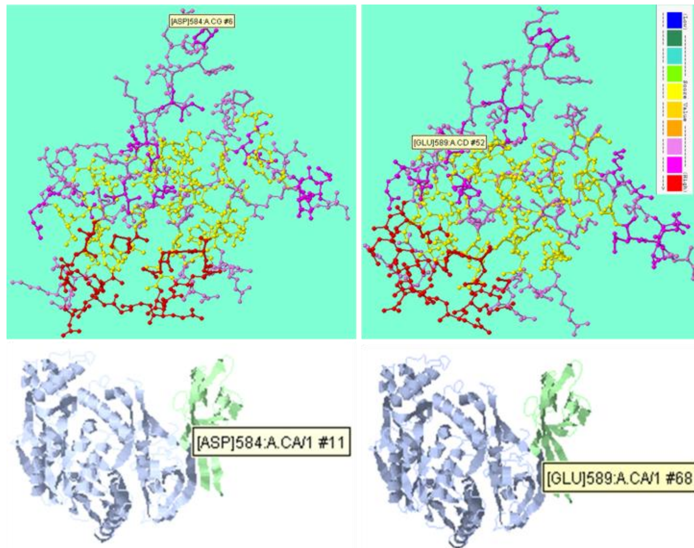


**Figure 2** ***In silico*** **prediction of possible interaction between shank and nNOS** Upper panel shows the region of shank protein that shows high probability for protein interaction. The lower panel shows predicted interaction between a shank isoform (blue) and nNOS (green). PDB files of rat shank1 and rat brain nNOS was used for this query. Best interactive sites (as suggested by lowest energy levels) were for shank: ASP584, TYR585, ILE586, ILE587, LYS588, GLU589. These corresponded to SH3 domain. SH3 domain shares 72% homology between shank1 and shank3, and it is likely this region in shank3 that mediates nNOS binding. Shank knockout mice involves deletion of the multiple ankyrin domains proximal to the SH3 domain, thus predicting the likelihood that shank may not bind to nNOS in these knockout mice. We propose to test these possibilities. In the upper panels, the purple color code shows higher probability of protein interaction. These predictions were performed with metaPPI2.0. Protein-protein interactions were examined with Patchdock and graphically represented with Firedock.

### Innovation

The proposed study is innovative from several perspectives. (i) First, this is possibly the first ever study that proposes to systematically investigate the molecular basis of gastrointestinal motility problems in patients with autism spectrum disorders. Gastrointestinal motility problems in these patients are a cause of suffering for the patients, as well as challenging issues for their caregivers including parents. Virtually nothing is known about the mechanisms underlying these disorders. (ii) This study argues based on incipient evidence from CNS neuropathology that because **synaptopathy is a major underlying pathophysiology of ASD** (Sudhof 2008, Pardo and Eberhart 2007, Bourgeron 2009, Won et al 2013), the motility problems of slowed gastrointestinal transit possibly result from defective junctional neuromuscular transmission, for example, through defects in nitric oxide mediated neuro-smooth muscle transmission. (iii) Preliminary evidence has shown that nNOS requires molecular motors for optimal conditions for enzymatic activity (Chaudhury et al 2011, Chaudhury et al 2013). Proteins partners of these molecular motors like shank (Yoshii et al 2013) have been reported to defective in animal models and human patients with ASD (Yang et al 2012, Peca et al 2011, Wang et al 2011, Jiang and Ehlers 2013, Guilmatre et al 2014, Mameza et al 2013). This novel target identification approach to understand disease pathophysiology lays the framework for developing rational pharmacotherapy.

### Approach

This proposal is based entirely on animal studies and objectively aims to examine the role of the scaffolding protein shank3 in nitrergic neurotransmission. Shank3 has been reported to be present in nNOS-positive myenteric neuronal cell body (Raab et al 2010) and protein presence described in the gut (Berglund et al 2008, Huett et al 2009, Herbert 2011), though it has not been examined specifically in the nitrergic nerve terminal. Messages for shank-interacting proteins has been described from all portions of gut muscle extracts of wild

type mice (Daigo et al 2003). In this proposal, examining the role of shank proteins in nitrergic neurotransmission is planned.

**Specific Aim 1 To examine the role of scaffolding protein shank in prejunctional nitric oxide synthesis**

**Rationale for specific aim** The enzymatic activity of nNOS requires important organizational steps, including (i) dimer formation to facilitate electron flow during NO synthesis (Welsh et al 2013, Alderton et al 2001) (ii) membrane localization to facilitate nNOS in close proximity to stimulation sources like voltage gated calcium channels (Chaudhury et al 2009, Chaudhury et al 2011) (iii) specific splice variants like nNOSα, because these splice variants have the capability (N-terminal PDZ interacting domain) to undergo lipidic modification to remain membrane-associated (Alderton et al 2001, Chaudhury et al 2009) (iv) cytoskeletal components like unconventional motor proteins like myosin Va that facilitates transcytosis of nNOSα to specific signaling domains (Chaudhury et al 2011, Chaudhury et al 2013, Su et al 2005). In this current proposal, we hypothesize that **nNOS requires interaction with specific proteins in the cortical actin cytoskeleton that facilitates molecular exchange of cytosolic nNOS with membrane bound nNOS, and that this happens via interaction with shank proteins**. If this hypothesis is correct, then nNOS should bind with shank and nitric oxide synthesis should be defective or impaired in shank knockout mice.

**Proposed experiments** Whole gastrointestinal tracts from male C57BL/6J and Shank3 knockout mice (Shank $3^{exon4-9}$, homozygotes result in nearly complete loss of shank3a&b isoforms; these mice developed on a C57BL/6J background) (Yang et al 2011) (n=30 in each group) will be utilized to obtain enteric nerve terminals by a combination of ultracentrifugation and sucrose gradient purification. nNOSα and shank will be colocalized with beta-actin and synaptophysin and imaged in both isolated nerve terminals and whole mounts prepared from stomach, small intestine and colon using confocal microscopy. nNOSα-shank3 protein interactions, as well as interactions of these proteins with LC8, actin and PSD95, will be tested by co-immunoprecipitation and visualized by proximity ligation assay as described earlier (Chaudhury et al 2011, Chaudhury et al 2012). Diaminofluorescein (DAF)-loaded nerve terminals will be stimulated by KCl to assay in vitro nitric oxide production. Tetrahydroquinoline carboxylates are potent Inhibitors of the shank PDZ domain (Saupe et al 2011). In vitro NO production will be performed by incubating nerve terminals with tetrahydroquinoline carboxylate to examine whether nNOSα binding to shank and ultimately to nerve terminal membranes is mediated by PDZ domain binding. High speed ultracentrifugation will be used to separate membranes and cytosol and membrane-bound nNOSα will be quantified by running protein extracts on a 4% SDS gel. Comparisons will be made between wild-type and shank knockout mice.

**Anticipated results** We anticipate nNOSα and shank3 co-localization in enteric nerve terminals. We expect to visualize nNOSα-shank3 protein interaction signals on proximity ligation assays in wild type nitrergic terminals. These interactions should be absent in shank knockout animals. We anticipate significant reduction in nitric oxide production during in vitro assays in shank3 knockout mice enteric nerve terminals, in comparison to wild type mice. Tetrahydroquinoline carboxylates should also impair nitric oxide production. This may result from defective localization of nNOSα to membrane, and thus we anticipate reduction or deficiency of membrane bound nNOSα in shank3 knockouts. We plan to assay these using western blots of nNOS in cold SDS PAGE, as nNOS is resistant to SDS and cold conditions are known to conserve dimeric nNOS (Rao et al 2008). Shank protein has 3 different isoforms: shanks1, 2 and 3 (Jiang and Ehlers 2013, Sheng and Kim 2001). Shank3 is the isoform most examined in relation to autism spectrum disorders (Yang et al 2012, Wang et al 2011, Peca et al 2011), though shank2 and shank1 mutations have also been reported to cause autism disorders. All these 3 isoforms have been reported to be present in the central nervous system. In the myenteric neurons, both shank2 and shank3 have been localized in myenteric neuronal cell body (Raab et al 2010). It is possible that apart from shank3, other shank isoforms may be involved in nitrergic neurotransmission. We plan to initially test the role of shank3 in nitrergic neurotransmission. Shank isoform-specific antibodies are available (SYnaptic SYstems), and if required, appropriate additional experiments will be performed.

**Feasibility, anticipated problems and troubleshooting** All the proposed experiments are feasible from a technical viewpoint. These are the major protocols that the principal investigator standardized and introduced over the last several years, including (a) extraction protocol for varicosities (ii) cold SDS PAGE and Western blots and standardization of antibodies (for example $nNOS_{K-20}$ specific antibody that detects the alpha isoform (iii) confocal imaging (iv) proximity ligation assay (v) spectrophotometric and imaging methods to assay in vitro nitric oxide production (Chaudhury et al 2008, Chaudhury et al 2009, Chaudhury et al 2011, Chaudhury et al

2012). The investigator is conservative in approaches including utilization of resources and broad experience in timing of these experiments. These experiments may be well accomplished in a period of 6-9 months. Shank knockout mice are available from Jackson Labs. Antibodies against various proteins are available. Though there is no experience with shank3 antibodies, data from other laboratories will be utilized in making decision for the newer antibodies. Preliminary reports exist regarding the localization of shank in enteric nerve cell body (Raab et al 2010). Though unlikely, it is possible that shank may not be present in nitrergic nerve terminals, or even if present, may not play any role in nitrergic transmission. In diverse systems, myosin Va-LC8-nNOS has been shown to form complexes with Shank-GKAP-PSD95 (Yoshii et al 2013, Rodriguez-Crespo et al 2001, Navarro-Lerida et al 2004). This important issue merits examination in enteric motor terminals, and remains the mainstay of this proposal.

Shank has been reported to associate with cell adhesion molecules like neuroligins in the postsynaptic compartment. Changes through neuroligin-neurexin signaling have been proposed in the presynaptic compartment during neuronal activity (Aarons et al 2012, Sudhof 2008). Additionally, neuroligin defects have been suggested as a pathophysiological basis for defective gastrointestinal neurotransmission in autism (Gershon and Ratcliffe 2004). Though NO signaling may not be spatially localized because of the very high diffusion coefficient (Chaudhury et al 2013), specific role of cell adhesion molecules in nitrergic neurotransmission has only recently been suggested. Examining their role remains beyond the scope of the current proposal.

Shank2 may also play a role in nitrergic neurotransmission in enteric neuro-smooth muscle junctions. Shank2-guanine nucleotide exchange factor ArhGEF interactions has been reported (Park et al 2003). Deletion of ArhGEF has been shown to develop esophageal achalasia (Zizer et al 2010, Goyal and Chaudhury 2010). Both shank2 and shank1 mutations have also been reported recently to present with autism features (Guilmatre et al 2014, Sato et al 2012, Leblond et al 2012, Schmeisser et al 2012). The role of shank2/shank1 in enteric nitrergic neurotransmission may also be significant. As mentioned earlier, we remain aware of these potential issues.

**Specific Aim 2 To examine defects in nitrergic synthesis, including shank deficiency, in maternal immune activation mice, a model of autism spectrum disorder**

**Rationale for specific aim and background information** We propose to utilize an established animal model of autism spectrum disorder to examine defects in nitric oxide synthesis in enteric nerve terminals. This model of viral infection during pregnancy is called “maternal immune activation model.” (Hsiao et al 2013, Naviaux et al 2013) Briefly, to develop these pups, mice are injected with poly-inosinic-polycytidylic acid sodium salt (Poly-I:C) at one or two fixed time points during pregnancy. Pups born after 6 weeks consistently demonstrate autistic behavior including repetitive grooming, automatic behavior and lack of vocalization. These mice have recently been reported to show abnormalities in gastrointestinal physiology, including leaky epithelium and evidence of altered gut microbiota (Hsiao et al 2013). **We hypothesize that neurotransmitter release, including nitric oxide synthesis, may be disrupted in enteric nerve terminals in this MIA mice model.**

**Proposed experiments** Whole gastrointestinal tracts from sham (saline) injected C57BL/6J and MIA (maternal immune activation) postnatal mice between 6-12 weeks of age will be utilized to obtain enteric nerve terminals by a combination of ultracentrifugation and sucrose gradient purification. To examine nitrergic neurotransmission mechanisms, the following will be examined and compared between MIA and wild type: (i) nNOS total protein in nerve terminals by comparative western blots. Cold SDS PAGE may reveal any alteration in dimer/monomer ratios. (ii) Shank, PSD95, LC8 and myosin Va contents in nerve terminals (iii) nNOS-Shank protein binding (iv) membrane bound nNOSα (iv) NO production assays of DAF loaded terminals.

**Anticipated results** Though exploratory in approach, the proposed experiments may systematically dissect defects in nitric oxide synthesis in a mouse model of autism, the major neurotransmitter for inhibitory neuromuscular communication. We aim to examine nitrergic defects in systematic way. First, we will examine whether there are changes in total nNOS expression, which will indicate transcriptional failure. Staining for whole mounts can reveal whether there are defects in transport of nNOS from the cell body to the nerve terminals. Western blots shall reveal whether there are alterations in dimeric (active) (Chaudhury et al 2009, Rao et al 2008, Welsh et al 2013) and monomeric (inactive) nNOS (Rao et al 2008, Chaudhury et al 2009, Gangula et al 2007), or whether there is a reduction of membrane-bound nNOSα (active zone of nitric oxide synthesis) (Chaudhury et al 2009, Chaudhury et al 2011) in MIA mice enteric nerve terminals. Thereafter, we

shall test whether there have been any alterations in concentrations in shank proteins (shank3) in MIA mice, which may explain deficiency of nitrergic neurotransmission. It is possible that maternal immune activation by dsRNA leads to enhanced immune-mediated degeneration of myenteric neurons in the pups. Inflammation is a well-known mechanism of damage to enteric neurons and impact on nitrergic neurotransmission (Goyal and Chaudhury 2010).

**Feasibility, anticipated problems and troubleshooting**

Generation of this mouse model of maternal immune activation is relatively easy. We will perform these experiments in specific-pathogen free environment in our dedicated animal house facility. The pups will be tested by routine behavioral assays for development of repetitive behavior (Ey et al 2011). This will serve as a marker for the efficiency of induction of autistic behavior by poly-I:C injections. Poly-I:C is available from Sigma-Aldrich and standard concentrations for inducing maternal immune activation has been described (Naviaux et al 2013). Maternal or pup mortality has been reported in this model with the double injections. We will increase number of animals injected if we encounter significant loss of pups. MIA pups examined between 6-12 weeks may not have any molecular defects at all, but may develop at a later time. If such a condition arises, we shall extend the time of examination. Incipient data show gut inflammation in this model resulting from defective intercellular junctions, which per se may cause motility disturbances (Hsiao et al 2013). This kind of intercellular adhesion defect has also been described in gastrointestinal samples obtained from irritable bowel syndrome (Martinez et al 2013; Camilleri et al 2012). We will focus mainly in relation to the role of shank proteins in enteric nitrergic neurotransmission.

As well known, enteric inhibitory neuromuscular transmission, the major neurophysiological mechanism for accommodating luminal contents, involves release of a vesicular neurotransmitter ATP, apart from the non-vesicular neurotransmitter NO (Chaudhury 2013, Gallego et al 2013, Gallego et al 2008, Pluja et al 1999, Burnstock 2013). In this model of maternal immune activation, it has been reported that the CNS manifestations result from excessive release of purines, creating a state of hyperpurinergia (Naviaux et al 2013, Page and Coleman 2000). It is possible that the actin cortical cytoskeleton may be affected, thus resulting in aberrant vesicular ATP release. We hypothesize that excess purinergic release may occur, in coherence with the observation of enhanced release of ATP in the peripheral blood. This may be a compensatory mechanism to a possibly reduced nitrergic neurotransmission. Excess ATP release may desensitize purinergic P2Y1 receptors, which mediate inhibitory effects of ATP on neuromuscular transmission (Gallego et al 2012, Hwang et al 2012). Thus, we plan to assay P2Y1 receptors in smooth muscle extracts by quantitative western as a surrogate measure for enhanced vesicular ATP release. Experiments related to specific Aim 2 are planned in the later half of the first year. The earlier part of the second year will be focused on all the trouble shooting issues, as well as examining alternate avenues if the first level observations are not coherent with the proposed hypotheses.

**High risk high reward opportunity if specific aims are achieved:** Shank protein may not be involved in nitrergic neurotransmission at all. However, it is likely that myosinVa-LC8-nNOS interacts with shank proteins prior to attaching with PSD95 at the membrane. Pepscan assays have shown that LC8 potentially interacts with actin (Rodriguez 2004). LC8 may play a fundamental role in shuttling nNOS in the peripheral cortical zone for membrane binding with palmitoyl-PSD95. There is a possibility of existence of an actin diffusion barrier to nNOS from the cytosolic compartment to its localization in active zone of the membrane. If defects in shank proteins are detected as a cause for impairment of nitrergic neurotransmission, then methods for pharmacological management for **shankopathies** (Wang et al 2014, Carbonetto 2014, Canitano 2013), such as replacement of shank proteins that are being reported for management of autism, may provide benefits for gastrointestinal symptoms as well. Insulin like growth factor (IGF1) have been shown to enhance shank protein expression (Bozdagi et al 2013, Shcheglovitov et al 2013). IGF1 may have a potential therapeutic role in treatment of functional bowel disorders in patients with autism spectrum disorders. Shank proteins are also known to respond to enteric-specific neurotrophic factor GDNF via Ret tyrosine kinase signaling (Schuetz et al 2004). These neurotrophic factors may also have impact on management of gastrointestinal motility problems that may result from defective shank signaling. These are some of the most early thought approaches to development of rational pharmacotherapy for functional bowel disorders. This proposal may also be viewed as a developmental project that supports examining these shank proteins in impairment of nitrergic neurotransmission in other functional bowel disorders like irritable bowel syndrome and idiopathic gastroparesis. These concepts may be tested in separate proposals.

**Proposed use of animals**

**Species, strain, age, sex, numbers:** The study will be conducted in **thirty shank3 knockout mice**, in accordance with a protocol pending approval from Animal Studies Subcommittee (IACUC), VA Boston Healthcare System. Wild type C57BL/6J mice will be used to obtain enteric varicosities for comparative studies (n=30) by high speed ultracentrifugation. Another **10 wild-type female mice** will be used to generate pups (**n=20, 10 males and 10 females**). Assuming a litter size of 8 per pregnancy and 30% loss of pups due to maternal immune activation, we anticipate development of 50 pups for experiments proposed in specific aim 2. 30 mice will be used for initial experiments proposed in specific aim 2. 20 mice will be aged if deficiencies of nitric oxide production are not initially detected in pups aged 6 weeks. Thus, **a total of 50 wild type mice are anticipated to be ordered from Jackson labs**.

**Justification of use of animals, choice of species and numbers:** Based on previous protocol standardization (Chaudhury et al 2009, Chaudhury et al 2011, Chaudhury et al 2012), all experiments assessing the role of shank in nerve terminals will be performed in 3 independent groups. Nerve terminals from 10 animals in each group will be pooled to reuse biological variance. **This justifies the use of 30 animals in each group (wild type, shank knockout or MIA mice).** The study proposes to examine the role of shank in nitrergic neurotransmission in the intestines and its deficit in autism, so shank knockout mice and MIA mice, a model of autism, are proposed to be studied.

Statistically significant differences among groups will be determined by t test. A $p$ value$<0.05$ will be considered statistically significant. Per diem veterinary care of mice is only moderate. Mice will be used as soon as they arrive, except for aging MIA mice proposed in specific aim 2.

**Euthanasia protocol: Mice will be euthanized by CO2 over inhalation according to AVMA guidelines**. This is the standard protocol used at the VA for rapid euthanasia without causing distress, discomfort or anxiety to the animal. **After confirmation of death, median laparotomy will be performed to dissect and obtain whole gastrointestinal tract from subdiaphragmatic esophagus to anus. Lumen will be opened up along antimesenteric border, cleaned of luminal contents and spun at low speeds. Supernatants and muscular debris will be cold stored for future experiments. Pieces of intestinal tissues will also be used to create whole mounts for staining experiments.**

Animal facilities are specific pathogen free and no problems are anticipated in developing MIA (maternal immune activation) mice. If there is significant fetal demise due to poly-I:C injections, only one dose will be administered instead of the proposed two injections.

**Veterinary care:** Animals in this study will be under general care and supervision of VA veterinarian on staff.

**Pain Assessment, pain minimization and Control:**
Assessment of pain or distress will be based on many different criteria. We will look for the following signs and symptoms when assessing the pain in the pregnant mice:

- Decreased activity
- Abnormal postures, muscle flaccidity or rigidity
- Poor grooming
- Decreased food or water consumption
- Decreased fecal or urine output

- Weight loss (generally 20-25% of baseline), failure to grow, or loss of body condition (cachexia)
- Dehydration
- Physical response to touch (withdrawal, lameness, abnormal aggression, vocalizing, abdominal splinting, increase in pulse or respiration)

**If sick animals are detected, they will be immediately euthanized per standard protocols**. The specific endpoint criteria to be used for identifying sick animals will include weight loss (20% of initial weight), inactivity, and inability to ambulate. Decision to euthanize will be based on condition of the animal, and the team taking care of the animals, in consultation with the staff veterinarian. Pups of MIA model will be separated after birth and housed independently and closely observed for any abnormality. If animals are sick for greater than 2-3 continuous days, they will be euthanized per AVMA protocol.

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