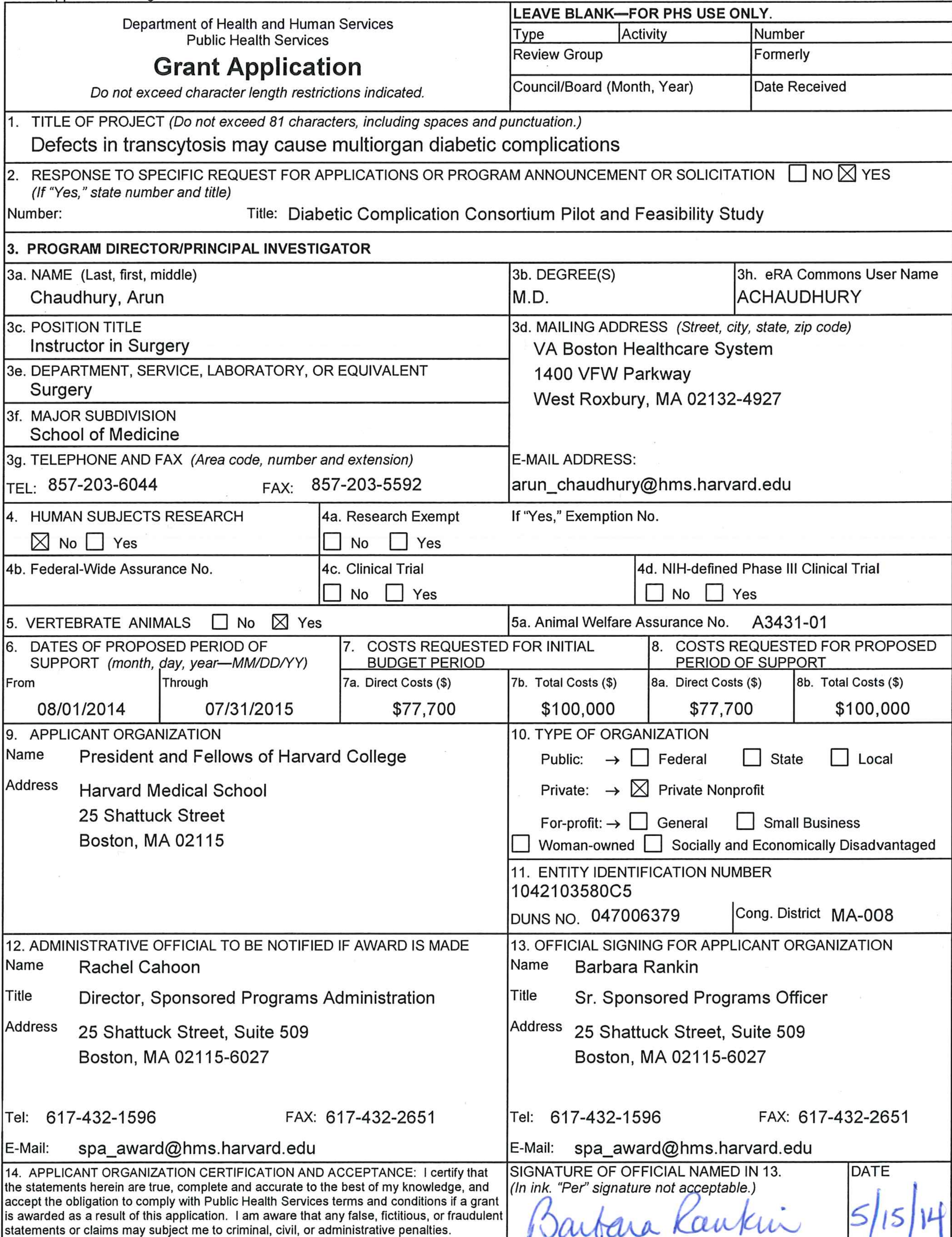

Form Approved Through 8/31/2015 — OMB No. 0925-0001

Department of Health and Human Services
Public Health Services

Grant Application

Do not exceed character length restrictions indicated.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Defects in transcytosis may cause multiorgan diabetic complications

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☐ NO ☒ YES
(If "Yes," state number and title)
Number:
Title: Diabetic Complication Consortium Pilot and Feasibility Study

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR

3a. NAME (Last, first, middle): Chaudhury, Arun
3b. DEGREE(S): M.D.
3h. eRA Commons User Name: ACHAUDHURY
3c. POSITION TITLE: Instructor in Surgery
3d. MAILING ADDRESS *(Street, city, state, zip code)*:
VA Boston Healthcare System
1400 VFW Parkway
West Roxbury, MA 02132-4927
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Surgery
3f. MAJOR SUBDIVISION: School of Medicine
3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: 857-203-6044 FAX: 857-203-5592
E-MAIL ADDRESS: arun_chaudhury@hms.harvard.edu

4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes
4a. Research Exempt ☐ No ☐ Yes
If "Yes," Exemption No.
4b. Federal-Wide Assurance No.
4c. Clinical Trial ☐ No ☐ Yes
4d. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes
5a. Animal Welfare Assurance No. A3431-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
08/01/2014	07/31/2015	$77,700	$100,000	$77,700	$100,000

9. APPLICANT ORGANIZATION
Name: President and Fellows of Harvard College
Address: Harvard Medical School
25 Shattuck Street
Boston, MA 02115

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
1042103580C5
DUNS NO. 047006379 Cong. District MA-008

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Rachel Cahoon
Title: Director, Sponsored Programs Administration
Address: 25 Shattuck Street, Suite 509
Boston, MA 02115-6027
Tel: 617-432-1596 FAX: 617-432-2651
E-Mail: spa_award@hms.harvard.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Barbara Rankin
Title: Sr. Sponsored Programs Officer
Address: 25 Shattuck Street, Suite 509
Boston, MA 02115-6027
Tel: 617-432-1596 FAX: 617-432-2651
E-Mail: spa_award@hms.harvard.edu

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
Barbara Rankin
DATE 5/15/14

Program Director/Principal Investigator (Last, First, Middle): Chaudhury, Arun

PROJECT SUMMARY (See instructions):

Directed transcellular movements of soluble proteins and vesicles are of fundamental relevance to cell physiology. This occurs during neurotransmission, insulin exocytosis and nNOS localization at the skeletal muscle membrane, which coordinates metabolic functions like glucose uptake. The presence of force generating proteins like myosin Va that facilitate transport of both vesicle and the soluble protein nNOS in the cell periphery is now being recognized. Recent preliminary data suggest that sixteen weeks after induction of diabetes by low-dose streptozotocin, myosin Va is nearly absent in the enteric nerve terminals and myenteric ganglia in jejunum of Wistar rat. On the contrary, nNOS expression was intact in the enteric nerve terminals and most myenteric ganglia showed cellular expression of nNOS. These data suggest that in the early weeks following induction of diabetes, genomic transcription of myosin Va is likely severely affected, resulting in inhibition of axonal transport of myosin Va. GLUT4 mediated glucose uptake in peripheral tissues relies on serine1650P-myosin Va and nitric oxide. Unlike all other cells in which nNOS appear diffusely across the cell and the sub-membranous zone, nNOS localizes discretely under the skeletal muscle membrane in a regular fashion. If myosin Va ubiquitously transports cellular nNOS, then skeletal muscle light microscopic imaging may be a simple but definitive approach to test mislocalization of membrane targeted nNOS in diabetes. Nitrergic neurotransmission is critical for oro-aboral progression of gut luminal contents. S-nitrosylation of glucokinase regulates insulin exocytosis in beta cells of pancreas. Nitric oxide synthesized in skeletal muscles facilitates GLUT4 mediated glucose uptake. Deregulation of these nitrergic functions are at the core of etiology of gastroparesis and pseudo-obstruction, progression of diabetes due to impairment of insulin release and peripheral insulin resistance that occurs in both NIDDM as well as a secondary complication of long standing type I diabetes. Specific aims are proposed to obtain evidence of alteration patterns of cellular myosin Va , its transcription factor snail and localization of nNOS at the cell membranes of enteric nerve terminals, beta cells and skeletal muscles in animal models of diabetes.

RELEVANCE (See instructions):

Cellular force generating proteins may be defective in diabetes due to early inhibition of its genomic synthesis. This may result in defective release of insulin from the pancreas. The same underlying defect may also contribute to diabetic complications like gastroparesis and constipation and peripheral insulin resistance, resulting in diabetic disease progression.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location

Organizational Name: President and Fellows of Harvard College

DUNS: 047006379

Street 1: VA Boston Healthcare System | Street 2: 1400 VFW Parkway

City: West Roxbury | County: | State: MA

Province: | Country: | Zip/Postal Code: 02132-4927

Project/Performance Site Congressional Districts: MA-008

Additional Project/Performance Site Location

Organizational Name:

DUNS:

Street 1: | Street 2:

City: | County: | State:

Province: | Country: | Zip/Postal Code:

Project/Performance Site Congressional Districts:

RESEARCH GRANT
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Appendix (*Five identical CDs.*) ☐ Check if Appendix is Included

* Follow the page limits for these sections indicated in the application instructions, unless the Funding Opportunity Announcement specifies otherwise.

Program Director/Principal Investigator (Last, First, Middle): Chaudhury, Arun

DETAILED BUDGET FOR INITIAL BUDGET PERIOD DIRECT COSTS ONLY	FROM	THROUGH
	08/01/2014	07/31/2015

List PERSONNEL *(Applicant organization only)*
Use Cal, Acad, or Summer to Enter Months Devoted to Project
Enter Dollar Amounts Requested *(omit cents)* for Salary Requested and Fringe Benefits

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Arun Chaudhury	PD/PI	8.04			75,000	50,475	13,225	63,700
SUBTOTALS →						50,475	13,225	63,700

Category	Amount
CONSULTANT COSTS	
EQUIPMENT *(Itemize)*	
SUPPLIES *(Itemize by category)* Antibodies and chemicals	4,000
TRAVEL	
INPATIENT CARE COSTS	
OUTPATIENT CARE COSTS	
ALTERATIONS AND RENOVATIONS *(Itemize by category)*	
OTHER EXPENSES *(Itemize by category)* Animal purchase and per diem: $8,000 Publications: $2,000	10,000
CONSORTIUM/CONTRACTUAL COSTS — DIRECT COSTS	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*	$ 77,700
CONSORTIUM/CONTRACTUAL COSTS — FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD	$ 77,700

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	2nd ADDITIONAL YEAR OF SUPPORT REQUESTED	3rd ADDITIONAL YEAR OF SUPPORT REQUESTED	4th ADDITIONAL YEAR OF SUPPORT REQUESTED	5th ADDITIONAL YEAR OF SUPPORT REQUESTED
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*	63,700				
CONSULTANT COSTS					
EQUIPMENT					
SUPPLIES	4,000				
TRAVEL					
INPATIENT CARE COSTS					
OUTPATIENT CARE COSTS					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	10,000				
DIRECT CONSORTIUM/ CONTRACTUAL COSTS					
SUBTOTAL DIRECT COSTS *(Sum = Item 8a, Face Page)*	77,700				
F&A CONSORTIUM/ CONTRACTUAL COSTS					
TOTAL DIRECT COSTS	77,700				
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD					$ 77,700

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel

Arun Chaudhury, M.D., Principal Investigator, (8.04 calendar months) will supervise the overall progress of the projects, conduct all experiments and endeavor to complete the proposed specific aims. Dr. Chaudhury is a physician-scientist with expertise in neuroanatomy and cell biology and has extensive training in animal microsurgery, nutritional neurosciences and has performed systematic investigations in nitrergic neurotransmission. Dr. Chaudhury has a Without Compensation appointment at VA Boston Healthcare System.

Animal Costs

Funds in the amount of $8000 are requested for animal purchase and per diem related costs over a one year period. We anticipate purchasing 90 mice, with price ranging at 20$/C5JBI, $36/DBA, $98/db and $195/Akita mice. Mice will be euthanized and tissue harvested in the same week of their arrival to the animal facility.

Budget Justification continued:

Antibodies and Chemicals
$4,000 is requested for antibodies and chemicals for laboratory assays. University of California Davis (Antibodies Inc; neuromab.ucdavis.edu) and Developmental Studies Hybridoma Bank at the University of Iowa antibody sources (dshb.biology.uiowa.edu) will be explored for reduced pricing. Details of the antibodies and chemicals and their possible vendor source are described in facilities and resources. The PI is well versed in optimal utilization of resources and detailed planning for balanced budget for investigations. The core facilities (confocal microscopy, vibratome, cryosection, dark room facility) operate at no additional charge at the VA Boston HealthCare System (West Roxbury).

Publication Costs
$2,000 over the one year period is requested for open access costs related to two anticipated publications from these investigations.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Arun Chaudhury	POSITION TITLE 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	9/2000	Medicine
All India Institute of Medical Sciences, New Delhi	MD (Residency)	6/2004	Anatomy
Monell Chemical Senses Center, Philadelphia, PA	Postdoctoral	2/2007	Nutritional Neurosciences
Harvard Medical School, Boston, MA	Research Fellow	6/2013	Neurogastroenterology
ECFMG Certification		3/2013	

A. Personal Statement

My investigative interests are focused on physiology and pathology of cellular secretions, time scales of secretion and signal sensing. In this proposal, I propose to investigate disease progression of diabetes and multiorgan complications, based on the hypothesis that defective cellular cytosolic movements of proteins may be a fundamental pathophysiological aspect of diabetes mellitus.

B. Positions and Honors

Positions and Employment

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

Honors

1994 Ranked 5th 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. 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
12. 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
13. 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
14. Chaudhury A, Cristofaro V, Carew JA, Goyal RK, Sullivan MP. Myosin Va plays a role in nitrergic smooth muscle relaxation in gastric fundus and corpora cavernosa of penis. PLoS One. 2014 Feb 6;9(2):e86778. doi: 10.1371/journal.pone.0086778. eCollection 2014.
15. Chaudhury A. Molecular handoffs in nitrergic neurotransmission. Front. Med., 1:8, 10 April 2014 | doi: 10.3389/fmed.2014.00008

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

RESOURCES

Follow the 398 application instructions in Part I, 4.7 Resources.

1. Laboratory and office space including computers are available 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.

5. Spectrophotometers and western blotting apparatus are available.

6. All diabetic mice and controls will be obtained from Jackson Labs.

7. Source of chemicals, antibodies and other useful reagents

- Western Blotting supplies: BioRad
- Snail antibody: Cell Signaling
- Insulin antibody
- nNOSk-20, LC8, actin, myosin Va antibodies: Santa Cruz Biotech & Sigma (extensively characterized in the lab)
- Buffers & chemicals: Sigma
- Glass slides and coverslips
- DAF, FM1-43: Invitrogen

Program Director/Principal Investigator (Last, First, Middle): Chaudhury, Arun

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☒ NEW application. *(This application is being submitted to the PHS for the first time.)*

☐ RESUBMISSION of application number: ____
(This application replaces a prior unfunded version of a new, renewal, or revision application.)

☐ RENEWAL of grant number: ____
(This application is to extend a funded grant beyond its current project period.)

☐ REVISION to grant number: ____
(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of program director/principal investigator.

Name of former program director/principal investigator: ____

☐ CHANGE of Grantee Institution. Name of former institution: ____

☐ FOREIGN application ☐ Domestic Grant with foreign involvement List Country(ies) Involved:

INVENTIONS AND PATENTS *(Renewal appl. only)* ☐ No ☐ Yes

If "Yes," ☐ Previously reported ☐ Not previously reported

1. PROGRAM INCOME ***(See instructions.)***
All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***
In signing the application Face Page, the authorized organizational representative agrees to comply with the policies, assurances and/or certifications listed in the application instructions when applicable. Descriptions of individual assurances/certifications are provided in Part III and listed in Part I, 4.1 under Item 14. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☒ DHHS Agreement dated: 05/31/2013 ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with ____ Regional Office.

☐ No DHHS Agreement, but rate established with ____ Date ____

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

	Amount of base $	x Rate applied	% = F&A costs $
a. Initial budget period:	77,700	28.70	22,300
b. 02 year			
c. 03 year			
d. 04 year			
e. 05 year			
		TOTAL F&A Costs	22,300

*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct cost base ☐ Other base *(Explain)*

☒ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*:

Offsite rate: Harvard Medical School at VA Boston Healthcare System

4. DISCLOSURE 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

Background Numerous lines of evidence indicate directed transcellular movements of soluble proteins as well as vesicles of fundamental relevance to cell physiology.[1-4] For example, enteric nerve terminal-smooth muscle neurotransmission involves the tandem release of neurotransmitters, soluble adenosine triphosphate (ATP) and gaseous nitric oxide (NO) after electrical field stimulation (EFS).[5] This necessitates positioning of ATP containing SLC17A9-positive vesicles and the enzyme neuronal nitric oxide synthase (nNOS) at the membrane of nerve terminals.[6-7] In the beta cells of pancreatic islets, glucose responsive release of insulin occurs at the membrane from large dense core vesicles (LDCVs).[8-11] Glucose sensing in the beta cells occurs with the help of enzyme glucokinase. The activity of glucokinase is closely regulated by nNOS.[12] nNOS has been demonstrated to be present on the membranes of insulin secretory granules.[13-18] Recent evidence hint towards the presence of force generating unconventional myosins that can facilitate transport of both vesicle and the soluble protein nNOS in the cell periphery due to their biophysical ability of undertaking long step sizes on actin with the cargo attached to these myosins.[19-22] For example, it has been demonstrated that myosin Va coordinates tandem release of the inhibitory neurotransmitters ATP and NO in the enteric nerve terminals.[6-7] Myosin Va is also present in beta cells and facilitate insulin exocytosis.[10-11] Myosin Va has been speculated to function as nNOS transporter in beta cells, though direct evidence for this is lacking.[23] Myosin Va protein has the ability to carry both vesicular and non-vesicular cargo.[6-7] It has been shown that the tail region of myosin Va potentially binds with vesicular cargo. A small portion in the N terminal region of the tail spanning amino acids 1282-1284 binds to dynein light chain of molecular weight 8 kDa (LC8).[24] Numerous lines of evidence suggest the ability of LC8 to binds to nNOS.[7,25] LC8 is present in both enteric nerve terminals and pancreatic islet beta cells.[7,13,26]

Recent preliminary evidence has been obtained that sixteen weeks after induction of diabetes by streptozotocin, myosin Va is nearly absent in the enteric nerve terminals in jejunum of Wistar rat.[27] This study showed that myosin Va expression was significantly reduced or absent in most cell bodies of myenteric ganglia. On the contrary, nNOS expression was intact in the enteric nerve terminals and most myenteric ganglia showed cellular expression of nNOS, though there were some variations in nNOS expression patterns. These data suggest that in the early weeks following induction of diabetes, genomic transcription of myosin Va is likely severely affected, resulting in inhibition of axonal transport of myosin Va. The current proposal aims to examine whether reduction of myosin Va is a global early phenomena involving multiple organs in diabetes mellitus, affecting myosin Va dependent functions critical for multicellular physiology. These investigations are based on the hypothesis that in either pharmacologic or genetic model of diabetes, early reduction of myosin Va will contribute towards impairment of insulin release and contribution towards development of peripheral insulin resistance. Reduction of myosin Va may also result in impairment of inhibitory neuro-smooth muscle neurotransmission, resulting in gastrointestinal complications arising from stasis of luminal contents. We propose to examine myosin Va expression in two different time points in animal models of both insulin-dependent and non-insulin dependent diabetes mellitus (NIDDM).

Cytosolic streaming of proteins are critical in peripheral glucose uptake.[28-30] For example, the glucose transporter GLUT4 mediated glucose uptake relies on serine1650^{P}-myosin Va.[31] Myosin Va facilitates glucose-transported mediated glucose uptake in adipocytes.[32] Thus, if reduction in myosin Va is an early molecular pathology in diabetes, it is likely that alteration of transcription of myosin Va and consequent reduced protein expression may also occur in skeletal muscles. In fact, myosin Va expression has been shown to be diminished in a rat model of diabesity (obese Zucker fa/fa rat model).[33] Interestingly, nitric oxide synthesized by nNOS plays a major physiological role in glucose metabolism and uptake by the skeletal muscle fibers.[34-37] There is a unique expression pattern of nNOS in the skeletal muscles. Unlike all other cells in which nNOS appears diffusely across the cell and the sub-membranous zone, nNOS localizes discretely under the skeletal muscle membrane in a regular fashion.[38] The entire cytosol is nearly free of any nNOS signal, though some nNOS may be stained in the nuclei. While this peripheral concentration of nNOS occurs in skeletal muscle by macromolecular complex of syntrophins and dystrophins,[39-47] almost nothing is known about nNOS transport in skeletal muscles. It may likely result from a discrete transcellular transport system and efficient binding of nNOS in the periphery by dystrophin. Note that syntrophin associated nNOS interaction may be seen in diverse tissues.[48-50] This unique cellular biology of nNOS in the skeletal muscle makes it a unique model organ to test membrane distribution of nNOS. Membrane bound nNOS in different organs has been examined by

western blotting of membrane lysates, electron microscopic approach and super-resolution microscopy.[7,51-53] If myosin Va ubiquitously transports cellular nNOS, and if myosin Va expression is affected in early diabetes, then skeletal muscle light microscopic imaging may be a simple but definitive approach to test alteration of myosin Va expression and membrane mislocalization of nNOS in diabetes. Myosin Va and LC8 expression has been examined in skeletal muscles, and reduction in membrane localization of nNOS has been shown in Zucker rats.[33] Though the isoforms of nNOS are different in nerve terminals (nNOSα) and skeletal muscles (nNOSμ), it seems likely that the molecular transport mechanisms are similar and dependent on LC8-myosin Va interactions. LC8 associates with nNOS in its N-terminal region within the first 300 amino acids, which are similar in nNOS α and μ.[26,54] We have recently demonstrated that nitrergic relaxations are impaired in cavernosal tissues of dilute DBA/2J mice,[55] inbred mice that are deficient in myosin Va. In cavernosal nerve fibers, nNOSμ and myosin Va localize.[55] Thus, it is likely that nNOSμ may be transported by myosin Va, similar to nNOSα. **Significance** The significance of appropriate cellular distribution of nNOS in signaling domains at the membrane by myosin Va is paramount. In the gastrointestinal tract, nitrergic neurotransmission is critical for oro-aboral progression of luminal contents.[56] S-nitrosylation of glucokinase regulates insulin exocytosis in beta cells of pancreas.[12] Nitric oxide synthesized in skeletal muscles facilitates GLUT4 mediated glucose uptake.[36,37] Deregulation of these nitrergic functions are at the core of etiology of gastroparesis and pseudo-obstruction, progression of diabetes due to impairment of insulin release, peripheral insulin resistance that occurs in both NIDDM as well as a secondary complication of long standing type I diabetes. As a pilot project, specific aims are proposed here to obtain evidence of alteration patterns of cellular myosin Va and localization of nNOS at the cell membranes of different organs in animal models of diabetes mellitus.

Specific Aims Achieving the goals of the proposed aims will help establish a common pathophysiologic basis for multiorgan dysfunction arising out of untreated progressive diabetes.

Specific Aim 1 To examine myosin Va protein and myosin Va-specific transcription factor "Snail" expression in myenteric motor neurons, pancreas and skeletal muscles in diabetic mice

Specific Aim 2 To examine membrane localized neuronal nitric oxide synthase (nNOS) in myenteric motor neurons, pancreas and skeletal muscles in diabetic mice

Preliminary Data

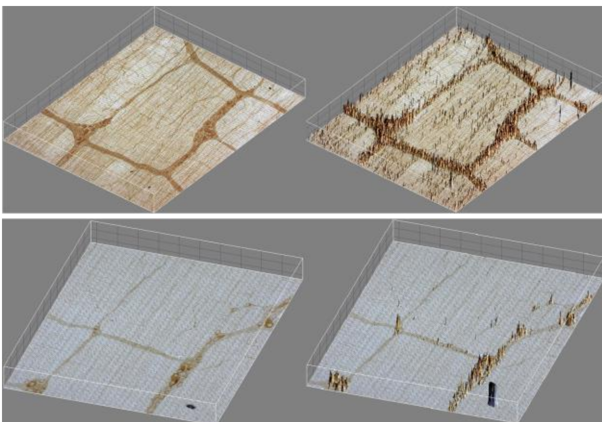


Fig 1. 3D surface plot of DAB staining intensity of myosin Va in WT (upper) and diabetic jejunum **(lower panel)** Note that in vehicle treated rats, staining spikes present in the muscle segments interposed between the ganglia and plexuses have dense distribution of myosin Va spikes in the nerve terminals, while these spikes within the muscularis is almost absent in diabetic tissues (16 weeks after STZ treatment). These nerve terminals are the sites of neuro-smooth muscle neurotransmission.

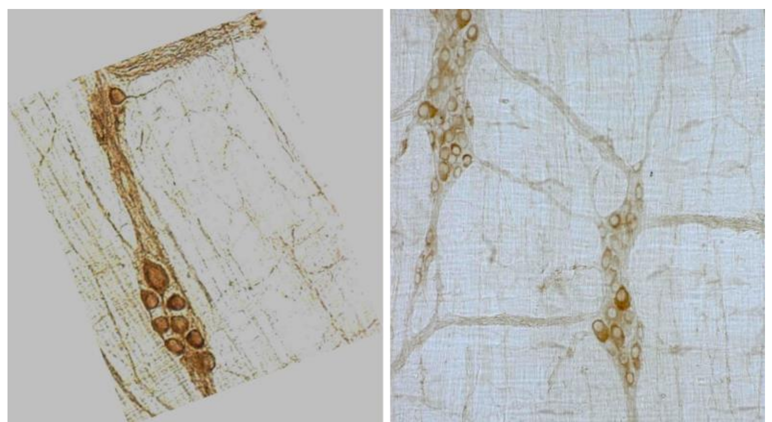
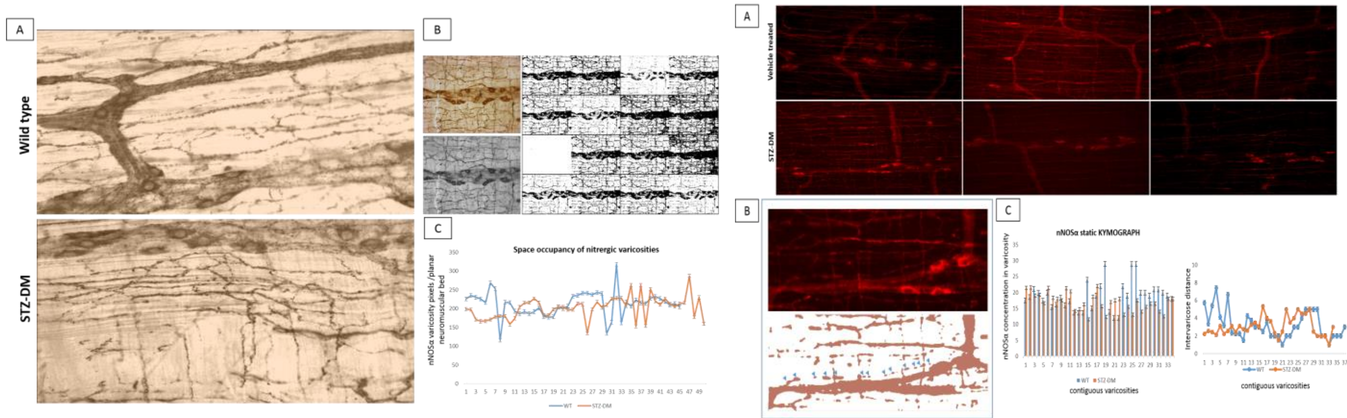


Fig 2. Note myosin Va immunoreactivity extending from the cell body to the processes of Dogiel type 2 neurons emanating from myenteric plexus In vehicle treated group (left panel), myosin Va is present in the initial segment, as well as axonal processes extending to the muscle layers. Myosin Va is also present in the interneuronal processes within the ganglion. In contrast, note that in the diabetic rats, despite the presence of secondary plexus linking the ganglia and tertiary extensions of nerve processes into the muscle layers, the brown DAB reaction product representative of myosin Va presences is scant or nearly absent in these processes.



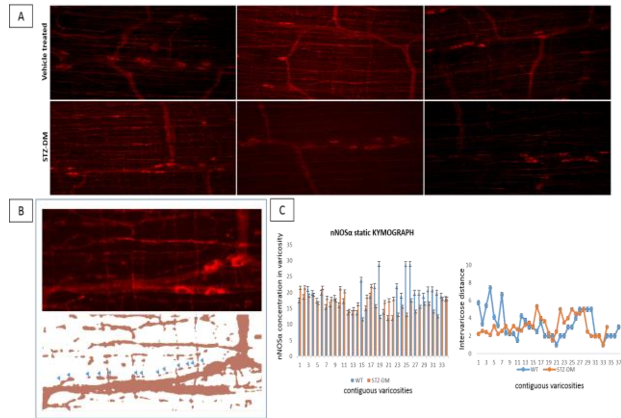


Fig 3. Comparable levels of ramifications of nitrergic axonal processes and varicosities on the muscularis of vehicle treated and diabetic jejunum In panel A, the 16 bit images were pseudocolored sepia to focus on expression of the nitrergic varicosities and ignoring appearance of background during visualization. Panel B shows repetitive iterations of thresholding performed with NIH ImageJ. Panel C shows comparative levels of space occupancy of nitrergic varicosities in wild type and diabetes, suggesting that no axonal retraction has taken place in diabetic tissues.

Fig 4. Comparative levels of expression of nNOSα in nerve terminals of both wild type and diabetic tissues A. Upper panel shows variance in appearance of nNOSα in wild type and diabetes. B. Fluorescent images were thresholded to digitally isolate individual nitrergic varicosities. Varicosity areas were estimated with ImageJ. C. nNOSα static kymograph shows normal axonal transport of nNOSα in diabetic jejunum. Comparative quantitation demonstrated similar nNOSα levels within varicosities.

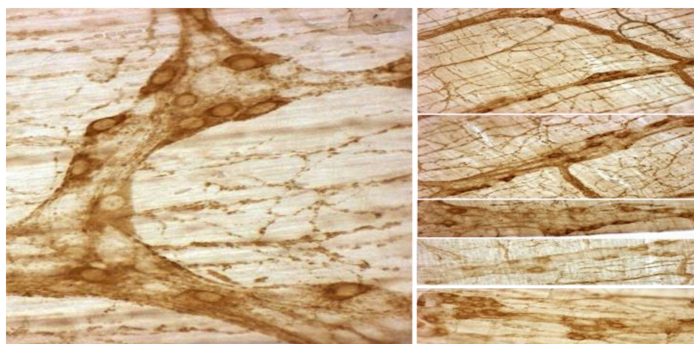


Fig 5. Variance of nNOSα expression in vehicle treated jejunum of Wistar rats

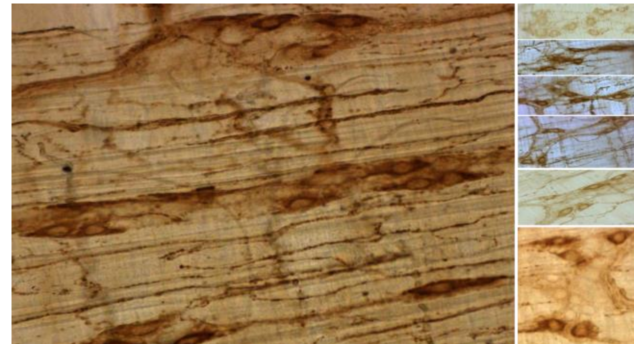


Fig 6. nNOSα expression in STZ treated diabetic jejunum Note reduction of nNOS expression in some cell bodies. Note that the lowest panel show some degenerating neuronal cell bodies.

Research Design and Methods

Specific Aim 1 To examine myosin Va protein and myosin Va-specific transcription factor "Snail" expression in myenteric motor neurons, pancreas and skeletal muscles in diabetic mice

Rationale Myosin Va is ubiquitously distributed in cell bodies of myenteric ganglia,[57] nerve terminals of nitrergic axons emanating from myenteric ganglia,[7] pancreatic beta cells[10,13] and skeletal muscles.[33] Preliminary data suggest significant reduction of myosin Va expression in myenteric neuronal soma in a rat model of streptozotocin-induced diabetes (figs 1-2). We propose to examine whether this reduction of myosin Va is a general phenomenon occurring in multiple tissues. Specifically, we will use Ins2Akita, db/db and BKS (db/db) mice as models of insulin-dependent and non-insulin dependent diabetes. BKS (db/db) is a model for lean diabetes, a clinical condition that is being recognized in increasing epidemiologic proportions. Young mice at 12 weeks of age and retired breeder mice will be used for the experiments. Retired breeders of Ins2Akita will serve as a model for peripheral insulin resistance occurring as a result of long-standing diabetes. Age matched C57BL/6J and DBA/2J, the background on which the diabetic mice were generated, will serve as controls. These studies will enable monitoring of progressive decrease (if any) of myosin Va. Myosin Va expression will be examined in myenteric ganglia, enteric nitrergic nerve terminals, pancreatic beta cells and skeletal muscles.

Proposed experiments Stomach, jejunum and colon whole mounts will be stained for myosin Va expression and imaged by confocal microscope. Pancreatic sections will be co-labeled for insulin and myosin Va for

examining alterations of myosin Va expression in Ins2 Akita mice. Skeletal muscle cross-sections will be labeled for myosin Va expression. All sections will also be co-labeled for LC8, the protein that mediates interaction of myosin Va with nNOS.[24] Additionally, all these tissues will be examined for expression of "Snail", a transcription factor that binds to myosin Va promoter E-box.[58] Snail is a zinc finger protein belonging to basic helix-loop-helix (bHLH) family of transcription factors.[59-67] It was originally demonstrated to repress genomic expression of E-cadherin during gastrulation.[66-67] However, it was also demonstrated that snail could activate genes that transcribe proteins for cell movements during epithelial-mesenchymal transition (EMT).[59,68] In fact, it was demonstrated that snail binds upstream of the myosin Va promoter and directly activates myosin Va gene expression.[58] Interestingly, the transcription factor snail can undergo O-glycNation.[69] Snail undergoes complex set of regulation, with glycosylation stabilizing the protein and retention within the nucleus, and phosphorylation at several sites that cause nuclear export of snail to the cytoplasm for ubiquitinylation.[70-71] At a first level, we hypothesize that early phases of diabetes and hyperglycemia may cause decrease in expression of the transcription factor snail in multiple organs, which in turn causes repression of myosin Va transcription and reduction in myosin Va protein expression. We propose to examine this by labeling myenteric ganglia, pancreatic islets and skeletal muscle with snail-specific antibody and examining its location within the nuclei. Blood glucose estimates will be done in all mice prior to harvesting tissues.***Anticipated outcomes*** The anticipated results are that in early phase as well as late stage diabetes, myosin Va expression may be suppressed in myenteric ganglia, enteric neuromuscular nerve terminals, beta cells of pancreas and skeletal muscles. We will have standardized and similar protocols for staining and use NIH ImageJ for analyzing quantitative differences in protein expression. If needed, we shall perform quantitative western blots for comparisons. Snail normally appears as speckled appearance within the nuclei, probably as a result of its association with splice sites. We will compare snail expression in the nuclei and cytoplasm of myenteric ganglia, pancreatic beta cells and skeletal muscles between wild type and diabetic mice. Because of limited time scope, we shall not examine glycosylation or phosphorylation of snail in the current proposal. The current proposal shall focus on generating preliminary evidence for future examination of snail regulation by reciprocal glycosylation and phosphorylation. ***Limitations*** It is possible that myosin Va may not be decreased in all the organs examined. The reduction in myosin Va observed in the neuronal soma of myenteric ganglia may be only restricted to the enteric nervous system. There may be compensatory changes. In fact, in the central nervous system, STZ-induced diabetes caused reduction of myosin Va,[72] but compensated with an increase in expression of the non-muscle myosin II (NMMII).[73] In DBA/2J mice, myosin Va is deficient in enteric nerve terminals.[7] Because of general deficiency of myosin Va, these mice would be expected to have frank diabetes. However, in the initial stages, the DBA/2J mice actually represent a hypersecretor phenotype, with increased release of insulin.[74-75] This has been reported to result from plastic changes within the beta cells. However, a quarter of DBA/2J mice develop frank diabetes in the long duration.[76] The nature of the compensatory changes that may occur in beta cells in a NIDDM mouse model is almost unknown.[77] Similarly, expression of the transcription factor snail may not be affected in all organs. There could be deregulation of a more upstream located transcriptional network. The limited proposal may not be able to address all these issues. However, the proposed specific aim is based on preceding evidence. Expertise in pathophysiology and technical aspects of confocal imaging, image analyses, protein blotting and animal handling will ensure success of the proposed aim.

Specific Aim 2 To examine membrane localized neuronal nitric oxide synthase (nNOS) in myenteric motor neurons, pancreas and skeletal muscles in diabetic mice

Rationale nNOS located within nerve terminals of nitrergic axons arising from myenteric ganglia, pancreatic beta cells and skeletal muscles perform critical function of gastrointestinal smooth muscle relaxation,[7,54] feedback inhibitory loop of glucose sensing by beta cells[12] and GLUT4 mediated glucose uptake by skeletal muscles[36,37] respectively, thus contributing to reduction of glucose in peripheral circulating blood. Preliminary data suggest significant reduction of myosin Va expression but not nNOS in enteric nerve terminals in a rat model of streptozotocin-induced diabetes (figs 1-6). Slow inhibitory junction potential (sIJP) is recorded using impaled electrodes within gastrointestinal smooth muscles in response to nitric oxide released from prejunctional nerve terminals after electrical field stimulation (EFS). Independent studies have confirmed impairment of sIJP in muscle samples obtained from all portions of the gastrointestinal tract of animals with pharmacologically induced or genetically acquired diabetes mellitus.[78-80] Mechanical studies have confirmed

diminution of EFS-mediated nitrergic relaxations in diabetic enteric tissues like ileum and mid-colon.[80-84] Some of these studies have also demonstrated restoration of sIJP with externally added nitric oxide donor to the organ bath in diabetic tissues,[81-83] indicating that the diminution of sIJP in diabetic gut tissues resulted primarily from reduction or inhibition of nitric oxide synthesis within the prejunctional nerve terminals. Controversy exists in the reports regarding the content of nNOS within the enteric nerve terminals in diabetic gut tissues. Limited data showing only a single low power microscopic field have been used to report reduced number of nitrergic axons traversing the neuromuscular wall in streptozotocin induced diabetes.[85] Other studies have reported reduced dimer/monomer ratio of nNOS[86] or increase in total nNOS levels in diabetes,[86,87] but the nNOS blots were run with extracts obtained from whole gut tissues, thus precluding specific information about nNOS contents within the nerve terminals per se. Studies have also reported normal nNOS enzymatic activity of diabetic whole gut extracts during in vitro assays.[88] However, none of these studies provide unequivocal information about nNOS contents within the nerve terminals, the site of inhibitory enteric neuromuscular nitrergic neurotransmission. Recently, evidence has been provided that mere presence of nNOS within nerve terminals is not adequate for prejunctional nitric oxide synthesis.[7,19,55] The regulation of nNOS within the nerve varicosities require multiple allosteric interactions, most notably, its positioning at PDZ-rich active zones that allow interfacing of water soluble nNOS with membrane-bound palmitoyl-PSD95.[19,51] Intriguingly, these binding of nNOS is not stochastic and dependent on a Brownian kind of diffusion but rather relies on specific molecular interactions involving motor proteins like myosin Va that have the ability to deliver nNOS to membrane-binding sites.[7] Using a mouse model of myosin Va mutation, the dilute DBA/2J mice, it was shown that in vitro nitric oxide synthesis, NO-mediated sIJP and L-NAME sensitive mechanical relaxations were impaired in gastric tissues of dilute mice.[7,55] Our preliminary data[27] (figs 3-6) show that nNOSα staining in diabetic jejunum neuromuscular strips obtained 16 weeks after injection with streptozotocin (35 mg/kgx5 days), in comparison to vehicle treated Wistar rats, showed (a) near intact expression of nNOSα in neuronal cell bodies, with reduction of expression in few cell bodies (b) intact presence of nitrergic nerve fibers, with normal ramification and arborization patterns of nitrergic nerve fibers, normal density of nitrergic nerve fibers in comparison with untreated animals and normal concentration of nNOSα within a majority of nerve terminals and intact axonal transport of nNOSα to distant nerve terminals. We now propose to examine membrane-bound nNOS in diabetes. ***Proposed experiments*** Cold SDS PAGE helps visualize membrane bound nNOS dimers.[7,23,33,51] If myosin Va is deficient, then it may result in inadequate transport of nNOS to the membrane, thus causing deficiency of membrane-bound nNOS in diabetic Ins2 Akita, db/db and BKS(db/db) mice. Membrane lysates of enteric varicosities, pancreatic beta cells and skeletal muscle extracts will be examined for nNOS and expression levels compared between control C57BL/6J and DBA/2J mice and diabetic mice. Furthermore, direct visualization of submembranous nNOS will be performed by imaging skeletal muscle sections stained for nNOSμ. nNOSα specific antibody also identifies nNOSμ isoform, as the N terminus antibody sequences are same. ***Anticipated outcomes*** Because of earlier studies that show a role for myosin Va in transposition of nNOS to the periphery of the cell membrane,[7] we hypothesize that myosin Va will cause mislocalization of nNOS in different tissues in diabetes. Namely, we anticipate decrease in membrane associated nNOS in enteric nerve terminals, beta cells and skeletal muscles. In the skeletal muscles, confocal imaging should demonstrate decreased nNOS fluorescence signals at the membrane in diabetic mice, in comparison to wild type. These experiments aim to logically identify a common mechanism of directed movements of cellular proteins like nNOS by force generating motor proteins like myosin Va and their functional disruption as an early molecular pathophysiology, leading to multiorgan diabetic complications. ***Limitations*** Our preliminary evidence in the diabetic enteric nerve terminals show that nNOS expression is mostly intact, though some myenteric ganglia showed evidence of decreased nNOS expression (figs 5&6). Other studies have reported nitrergic neuronal loss and activation of transcription factors like FoxO that cause cell death.[89] Importantly, there are a number of transcription factors that regulate and cause alternate splicing for nNOS.[90-92] It may be possible that nNOS transcription may also be affected by hyperglycemia, though our preliminary data suggests that even if it is the case, nNOS transcription is reduced at much slower rate in comparison to myosin Va expression in the early stages of diabetes. It is possible that in the retired breeders, we may find significant reduction or absence of nNOS in various tissues. The retired breeder Ins2Akita show severe gait difficulties and while arthritic changes or sensory nerve conduction may contribute to this, it may also result from skeletal muscle fatigue due to loss of nitric oxide production. Overall, the investigator possesses expertise in nNOS blotting, ultracentrifugation and confocal imaging and experiments are proposed based on feasibility of accomplishment within the timeframe of the proposal. The lab possesses expertise for nitric oxide imaging by DAF (diaminofluorescein) and exocytosis assay by FM1-43. Functional experiments in the tissues being examined will be planned depending upon outcomes of the initial myosin Va, snail and nNOS expression assays.

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Proposed use of animals

The study will be conducted using a total of 90 male mice (9 mice in each group, experiments performed in triplicates; two different age groups, 12 weeks and retired breeders, respectively), in accordance with a protocol pending approval from Animal Studies Subcommittee (IACUC), VA Boston Healthcare System. C57BL/6J and DBA/2J mice will be used as controls. db/db, BKS(db/db) and Ins2Akita mice will be used as models of diabetes. Gastrointestinal tissues will be used to obtain enteric varicosities for comparative studies. Small pieces of tissues will be used for preparing gut whole mounts. Pancreas and gastrocnemius sections will be used for imaging studies. Skeletal muscles and pancreas will also be pooled for preparing whole tissue lysates. 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 to the animal facility.

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 will be cold stored for future experiments. Pancreas and skeletal muscles will be stored for proposed experiments.

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

Pain Assessment 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 diabetic mice (for example, retired breeders of Ins2Akita):

- 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)
- 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. Importantly, mice will be used within a week of arrival to the animal facility. 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.

Resource sharing plans

All images and data arising out of the completion of the projects will be submitted for public archival at the Diabetic Complications Consortium Site.