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TITLE: The Role of Lateral Hypothalamus Orexin Glucose-Inhibited Neurons in Binge-Eating Disorder

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14. ABSTRACT

Repeated patterns of intermittent calorie restriction followed by re-feeding of highly palatable foods can contribute to binge pathologies in some at risk populations. For this project we use a murine of model of dietary binge eating that investigates the interaction of intermittent calorie restriction (i.e., hypoglycemia bouts) and rapid consumption of highly palatable food (i.e., sweetened fat; SF) on lateral hypothalamus (LH) orexin neurons. Mice with repeated access to SF without the repeated calorie restriction do not demonstrate a binge phenotype. Behavioral data indicates that using chemogenetic activation of LH orexin neurons we determined that mice with SF access only developed a binge phenotype. In vitro electrophysiology experiments demonstrated intermittent calorie restriction increases glutamate transmission on LH orexin target dopamine-containing in the ventral tegmental area (VTA). This increased VTA glutamine current could be a mechanism to drive binge eating following repeated calorie restriction. Our research is designed to investigate how binge eating pathology is different from normal homeostatic eating, as it pertains to the addictive nature of highly palatable rewarding foods. Our goal is to assist in developing clinically effective treatment strategies for improving the quality of life for military service members and veterans afflicted with eating disorders.

15. SUBJECT TERMS

binge eating, bulimia nervosa, eating disorders, weight restriction, orexin, feeding

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1. INTRODUCTION: Military service members are at risk for developing eating pathologies. Disordered eating, in particular binge eating, can arise from establishing unhealthy dietary habits and maintaining lower than normal body weight. Intermittent feeding behaviors, such as caloric restriction and rapid consumption of highly palatable calorie dense foods dysregulate the neural controls of feeding. The purpose of this grant is to uncover the role of a distinct population of lateral hypothalamic (LH) orexin neurons, which are activated during calorie restriction or low blood glucose (i.e., glucose inhibited; GI). Engagement of LH orexin GI neurons are believed to be involved in dietary habits that establish binge-like eating behaviors. In particular, LH orexin-GI neurons send projections to ventral tegmental (VTA) dopamine neurons that are involved in reward and reinforcing behaviors. Our overall hypothesis is that intermittent caloric restriction alters the activity and glucose sensitivity of LH orexin-GI neurons to enhance the glutamatergic activity (i.e., AMPA/NMDA receptor ratio) of VTA dopamine neurons. These experiments will use a murine model of dietary-induced binge eating to further understand the neural circuitry involved in the maintenance of binge eating disorder and necessary for developing clinically effective treatment strategies for improving the quality of life for military service members and veterans afflicted with eating disorders.

2. KEYWORDS: binge eating, bulimia nervosa, eating disorders, weight restriction, orexin, feeding

3. ACCOMPLISHMENTS: What were the major goals of the project?

There are 3 major goals of this project

Goal #1. Determine whether the development of binge eating is associated with enhanced glutamatergic transmission onto VTA DA neurons. (Major Tasks 1 & 2)

Goal #2. Determine whether the development of binge eating alters the glucose sensitivity of LHA orexin neurons. (Major Task 3)

Goal #3. Determine whether inhibiting orexin neurons during caloric restriction suppresses binge-like eating behavior (Major Task 4)

What was accomplished under these goals?

Major Task 1, Subtask 1, month 1. We have obtained the new electrophysiology equipment and it is set up. This is subtask is completed. (Routh, NJMS, Rutgers, Newark,NJ)

Major Task 1, Subtask 2, months 2-7. Measure excitatory the AMPA/NMDA receptor ratio after 1 and 5 episodes of restriction. We were significantly delayed in this subtask as noted in the last progress report. The delay was primarily due to COVID-19 personnel restrictions in conjunction with having to cease animal breeding and cut our colonies to 50%. This issue was worsened by the loss of an experienced electrophysiologist and the challenge of training a new individual when we were not able to allow staff to be in the lab full time until late 2020 (even now there are some restrictions to maintain social distance). However, despite these issues in the past year we have made excellent progress and have nearly completed this subtask. Moreover, our preliminary findings are not only supportive of our hypothesis that the restrictive episodes which trigger binge eating enhance VTA glutamate signaling but provide compelling insight into mechanism. We found that after 5 intermittent episodes of 24 h dietary restriction the NMDA receptor current amplitude was increased on VTA DA neurons compared to control (non-restricted, naïve) mice (Fig 1). Interestingly, the total current (Fig 2) and AMPA receptor current (Fig 3) were not increased, nor was there a change in the AMPA/NMDA receptor current ratio (Fig 4). These data contrast with our original expectations based on the data from weight loss studies described below in "related work from other projects". In the other project investigating chronic weight loss we found the exact opposite of intermittent restriction. That is, total and AMPA receptor currents as well as the AMPA/NMDA receptor current ratio were increased, while NMDA current amplitude remained unchanged. These latter data are consistent with the standard view of positive glutamate plasticity (long term potentiation) being mediated by AMPA receptors. On the

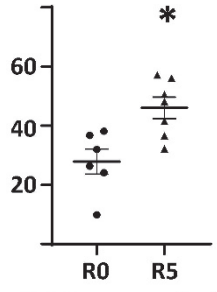

Figure 1. NMDA receptor currents on VTA DA neurons in control (R0) mice and after 5 intermittent episodes of 24h food restriction (R5). * P = 0.007; R0 n = 6, R5 n = 6)

other hand, increased NMDA currents would increase neuronal excitability to incoming stimuli such as would occur with increased orexin release during the low glucose associated with an overnight fast. This is consistent with our published data (Teegala et al., 2018) showing that an overnight fast increased total glutamate current onto VTA DA neurons only in the presence of low glucose. It would be interesting to determine whether we see something similar after intermittent restriction. Another intriguing consequence of increased NMDA vs AMPA receptor currents is that the former is associated with the change from burst to tonic firing in VTA DA neurons. Burst firing may play a role in the value judgement regarding salience of environmental stimuli according to Zaharov et al., 2016. Thus, our findings suggest not only that intermittent restriction increases glutamate transmission onto VTA DA neurons, but also that the increased NMDA current could potentially affect decision making about palatable food and lead to bingeing. This may contrast with a simple drive to increase intake of palatable food in response to weight loss. To complete this subtask, we will directly compare individual glutamate receptor mediated currents following 1 and 5 intermittent restrictive episodes. (Routh, NJMS, Rutgers, Newark,NJ)

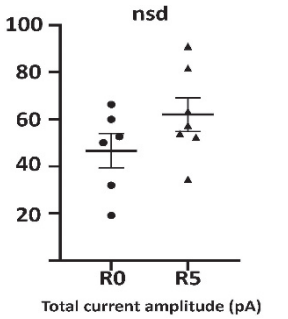

Figure 2. Total NMDA + AMPA receptor currents on VTA DA neurons in control (R0) mice and after 5 intermittent episodes of 24h food restriction (R5). * P = 0.16; R0 n = 6, R5 n = 6)

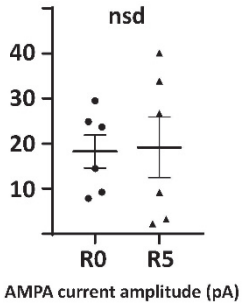

Figure 3. Total AMPA receptor currents on VTA DA neurons in control (R0) mice and after 5 intermittent episodes of 24h food restriction (R5). * P = 0.91; R0 n = 6, R5 n = 6)

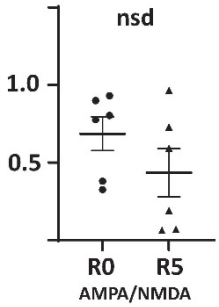

Figure 4. AMPA/NMDA receptor currents on VTA DA neurons in control (R0) mice and after 5 intermittent episodes of 24h food restriction (R5). * P = 0.21; R0 n = 6, R5 n = 6)

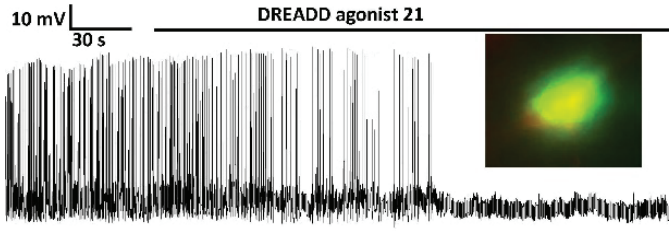

Figure 5. Brain slice current clamp recording of an orexin neuron transfected with hM4D(Gi)-mCherry. The DREADD agonist 21 (1 uM) inhibited this neuron. Membrane potential at start was -50 mV, hyperpolarizing to -54 mV with the agonist. The recorded cell was labelled with lucifer yellow (green) in the pipette and expressed mCherry (red) (insert).

Major Task 2, Subtask 1, months 8- 29. Perform the experiments with DREADDs, which require stereotaxic surgery. For this subtask we were to hire a technician in the Routh laboratory to learn and perform surgeries; however, we could not hire during the COVID-19 restrictions. We were able to recruit an individual while under restrictions who was willing to wait and this individual is now in place. Due to COVID-19 restrictions the training veterinarians were not allowed to conduct in person training and/or validation of skills in aseptic technique and suturing (required by the IACUC for surgeries at Rutgers). However, they have recently begun validating surgeons and we have conducted some pilot studies with the DREADDs. Figure 5 indicates successful transfection of an orexin neuron with an inhibitory DREADD. When the brain slice was exposed to the DREADD agonist, C21, action potential frequency was reduced and the neuron was inhibited. This pilot experiment provides confidence that we will be able to modulate neuronal activity in these neurons in vivo. In addition, it has taken a significant amount of time to restore our colony of mice expressing cre-recombinase in their orexin neurons but these animals are now breeding well. Thus, we will proceed with these experiments as we are wrapping up Major Task 1 Subtask 2. (Routh, NJMS, Rutgers, Newark, NJ).

Major Task 3, Subtask 1, months 30-35, This was not scheduled to begin during this funding period. (Routh, NJMS, Rutgers, Newark, NJ). However, we have finally been able to increase our colony of mice that express green fluorescent protein in their orexin neurons (orexin-GFP mice). These mice are critical for this task.

Major Task 4, Subtask 1, months 1-34. Major Task 4, Subtask 1, months 1-34. For this task there are two sets of chemogenetic experiments reliant on adeno-associated viral vectors containing Gi inhibitory construct (pAAV- hSyn-DIO-hM4D-mCherry) or Gq excitatory construct (pAAV- hSyn-DIO-hM3D-mCherry) from Addgene (Watertown, MA). After completing some initial experiments (reported in our last progress report), we noticed reduced or no expression of the Gi inhibitory vector in the OREXIN: CRE mice. There was some back and forth discussion with Addgene and confirmation of the

vector expression in another mouse line (TH:CRE), it was determined that package of inhibitory AAV vectors must have been exposed to room temperatures for an extended period of time. This was likely a caused by the re-routing of the package in compliance with the University COVID-19 shipment receipt protocols. This issue has been resolved and validation of a new shipment of inhibitory Gi vector has been confirmed to have adequate expression in the OREXIN:CRE mice. While we were troubling shooting the Gi inhibitory vector expression, we focused on completing the proposed experiments using Gq excitatory vector. For the Gq excitatory experiments, all male and female OREXIN:CRE mice (n = 7-8 group/sex; N = 62) underwent Gq excitatory AAV injections aimed at the LH. One week later all mice were pre-exposed to binge food (sweetened fat, SF; vegetable shortening blended with 10% sucrose), the BINGE or NAIVE feeding protocol. Mice exposed to BINGE feeding protocol, are exposed to 30 min access to SF twice a week, whereas the NAIVE group does not receive repeated access to the SF. All mice received ad libitum standard chow. Notably, mice with repeated SF, but without the repeated restriction do not demonstrate a binge phenotype (i.e., escalation in 30 min intake over 5 episodes; Sachdeo et al, Frontiers in Psychology, 2019). For this experiments, excitation of LH orexin neurons would mimic the repeated calorie restriction. Our results indicated for the BINGE mice there was an effect for binge episodes [$F(4, 112) = 7.81$, $p < 0.0001$] and dose X binge episodes [$F(4, 112) = 2.6$, $p < 0.05$] on 30 min Kcal intake. Post-hoc Tukey HSD testing revealed increase total Kcal intake Binge 5 compared with Binge 1 in C21 treated mice ($p < 0.005$), there were no escalation in intake in saline treated mice. There were no significant effects in 30 min calorie intake in NAIVE mice receiving C21 compared with saline. All mice used in the analysis demonstrated mCherry expression in the LH region.

Major Task 4, Subtask 2, month 1-34. We gave begun these experiments, we have initial data examining the influence of activation of the LH orexin and the effectiveness of sibutramine feeding suppression. Previously, we demonstrated that sibutramine feeding suppression in our dietary-induced binge eating paradigm (Sachdeo et al, Frontiers in Psychology, 2019). We observed that mice exposed to the repeated episodes of calorie restriction and SF access were resistant to sibutramine feeding suppression. The purpose of these experiments was to determine whether activation or inhibition of the LH orexin system influence the differential feeding suppression of sibutramine. For this, male and female BINGE and NAIVE mice (n = 3-4; N= 22) underwent a within study design and received and each animal received vehicle, 1 mg/kg sibutramine , and 3 mg/kg sibutramine 1 week apart. Sibutramine was oral gavaged 1 hour prior to the start of the SF access (i.e., 30 minutes prior to C21 or saline injection). In the BINGE mice, there was an effect for sibutramine [$F(3, 21) = 4.5$, $p < 0.05$], sibutramine X sex [$F(3, 21) = 5.0$, $p < 0.05$], sibutramine X dose [$F(3, 21) = 9.8$, $p < 0.005$], and sex, sibutramine x dose [$F(3, 21) = 11.2$, $p < 0.005$]. There was a trend for male C21 treated BINGE mice to have higher intake with sibutramine treatment than saline treated male BINGE mice, but post-hoc tests were not significant. We are in the process of adding more animals to this c a few more animals to this cohort. There were no significant effects in 30 min calorie intake in NAVE mice receiving C21 compared with saline. We recently (August 10, 2021) upgraded our BioDAQ system to 24 cages with the ability to accommodate 32 cages. This will allow us to more effectiveness collect the data meal pattern and thermoregulation. In the Bello lab, a laboratory technician, Lori Scarpa, and a graduate student, Gregory Berger are fully committed to this project. Assistance in the Bello lab has also been provided by Dr. Lihong Hao, Research Associate. Dr. Hao received her Ph.D. under research direction of Dr. Routh and bring a complementary skill set to strengthen the collaborative partnership between the Bello and Routh lab and provide assistance for Major Task 3.

Related work from other projects: Because the orexin inhibitory experiments would require a continuous inhibition of the orexin system during the restriction period, we have proposed as an alternative to provide the water-soluble DREADD agonist, C21 in drinking water. In the Bello laboratory, we conducted a pilot experiment to determine the concentration of C21 that was detectable by taste in drinking water compared of mice. For this, male and female C57Bl/6J mice (n = 6) in automated gustometer (i.e., Davis Rig; Med Associates, Fairfax, VT), which is used to measure taste response (number of licks) to a brief access (5s) of a solution for C21 (0.32-32mg/kg; ½ log increments) in drinking water. There was no overall effect of concentration [$F(6, 30) = 1.8$, $p < 0.13$]. However, planned comparison revealed that 32 mg/kg concentration was significant different from water ($p < 0.05$). It should be noted that mice were tested in calorie deprived condition to mimic experimental conditions in the restriction protocol. Therefore, moving forward we will use 10 mg/kg for experiments using oral C21 for Gi inhibitory experiments. In the Routh laboratory, a graduate student working on a related project which evaluates chronic food restriction and weight loss on glutamatergic signaling in the VTA has made observations relevant to this project. In 2018 (Teegala et al), he found that after an overnight 24 hour fast, the amplitude of excitatory currents

(AMPA and NMDA combined) increased compared to control only when exposed to glucose levels seen in the brain during fasting (0.7 mM) but not when exposed to glucose levels seen in the brain during the fed state (2.5 mM). This was associated with a slight (~30%) but significant increase in the AMPA/NMDA receptor current ratio (an index of in vivo glutamate plasticity). He has now found that diet restriction to 85% body weight and maintenance of this weight for 1 week significantly increased the amplitude of total glutamate currents in 2.5 mM (fed) glucose (control: 46.1 +/- 7 pA vs weight loss: 70.8 +/- 5 pA; P=0.014). Moreover, the AMPA/NMDA receptor current ratio was increased 100% (control: 0.68+/-0.11 vs weight loss: 1.5 +/- 0.2 pA; P=0.017). Interestingly, this was entirely due to changes in the AMPA receptor mediated currents (control: 18.28 +/- 3.7pA vs weight loss 41.5 +/-4.2 pA; P = 0.002) with no change in the NMDA receptor mediated current (control 28 +/-4.2 pA vs weight loss 29 +/- 2.3 pA; P = 0.37). These data indicate first that changes in glutamate signaling are maintained over an extended time course. More importantly, the increase that we saw after one overnight fast were not representative of the maximum output of the system. Weight loss substantially enhanced glutamate signaling in the VTA. This suggests that similar changes will not only be maintained but enhanced after multiple episodes of food restriction. Interestingly, weight loss was associated with increased AMPA signaling whereas intermittent restriction increased NMDA but not AMPA receptor mediated circuits. Further comparison of these differences should provide interesting information regarding the triggers for binge eating behavior specifically as opposed to a simple drive to increase caloric intake following weight loss.

What opportunities for training and professional development has the project provided?

Lab personnel involved with this project have the opportunity to learn and expand their technical skills. These are related to *in vivo* survival surgeries in mice, in vitro electrophysiology, and chemogenetic platforms. As we generate more data for this project, personnel will have professional opportunities to present findings at national and international scientific conferences, invited talks, and publication in peer-reviewed journals. Graduate students from both labs

How were the results disseminated to communities of interest?

At this stage, preliminary results of the project have been disseminated as departmental seminars open to the public. These seminars were virtual and well attended (~50 participants). Gregory Berger, a graduate student in the Bello lab, presented a seminar entitled “Chemogenetic Manipulation of Orexin Neuronal Activity in Binge-like Eating” on December 4, 2020, which updated the progress on the Major Task 4, subtask 1 experiments. Suraj Teegala, a graduate student in the Routh lab presented a seminar entitled “The role of lateral hypothalamic area orexin –glucose inhibited neurons in reward-based feeding” on June 17, 2021, which updated the progress on the Major Task 1, subtask 2 experiments. As noted in our previous progress report, as results are generated the findings will be presented at national and international scientific conferences (i.e., Society for Neuroscience, Society for the Study of Ingestive Behavior, etc., Endocrine workshop at Rutgers), invited talks, and publication in peer-reviewed journals. Scientific conference over last year has been virtual, which has made attendance and dissemination of research findings challenging. As scientific conference move to “in person” or hybrid forums, we look forward to presenting and disseminating our findings to a wider audience.

What do you plan to do during the next reporting period to accomplish the goals?

We are currently working on systematic review article of the current literature of rodent model of binge eating, which is due 30 Sept 2021. The title is “Rodent models for investigating the attentional and arousal impairments in bulimia nervosa and binge eating disorder” contribution to a Special Issue of Obesities (MDPI journal) edited by Nicholas Bello (PI) entitled “Recent Advances in Eating Pathologies in At-Risk Populations” with relevance to at-risk military personnel. We received a waiver for the open access charge and this article will be freely accessible to the general public. For the Bello laboratory, for Major task 4, Subtask 1 we will finish the Gi inhibitory male and female mice in the next 6 weeks and continue to make progress on Major Task 4, subtask 2. To date, the Routh laboratory has trained an electrophysiologist and has successfully recruited an animal behaviorist to ensure completion of Major Task 2 & 3. The mouse colonies have been incrementally increasing since the Rutgers return to research, according to breeding restrictions the University imposed based on COVID-19. Based on our current progress, we have established a strong foundation to continue to make progress on the subtasks on these projects as described in the Statement of Work.

4. Impact

As data are generated, we will report distinctive contributions, major accomplishments, innovations, successes, and/or any change in practice as a result of the project. .

What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. Changes/Problems

Changes in approach and reasons for change.

Despite the COVID-19 restrictions and associated setbacks, there are no changes in our approach. Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them.

COVID-19 restrictions have significantly delayed progress due to closures and restrictions on breeding our mouse colonies. Other problems were noted elsewhere and have been resolved.

Changes that had a significant impact on expenditures.

There are some lingering issues regarding not being able to hire or work during the COVID-19 restrictions, we expect to have some funds remaining for a no-cost extension to pay salaries to complete the final studies and publish the results. As previously stated, we do not anticipate this delay in these expenditures will significantly impact complete the goals of the project, especially in light of our exciting new data which strongly support our hypothesis.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

Significant changes in use or care of human subjects

Not Applicable

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report. One minor change we have amended our animal protocol to include the use of DREADD agonist compound 21 (C21), since Clozapine-N-oxide has been demonstrated to be bioconverted to clozapine (Gomez et al., Science 2017). Clozapine is atypical antipsychotic has biological actions on dopaminergic activity and feeding behaviors, since C21

remains biological inert this minimizes the off-target effects. C21 is also water-soluble. As shown in Figure 2, we have been successful in modulating neuronal activity using C21. As reported in Major Task 4, we have been successful in modulating behavior with C21.

6. Products

Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

What individuals have worked on the project?

Name:	Nicholas T. Bello, Ph.D.
Project Role:	Investigator (Collaborating)
Researcher Identifier (e.g. ORCID ID):	0000-001-5300-5604
Nearest person months worked	6 months
Contribution to Project:	Oversees research on Major Tasks 4 (and assistance on Major Tasks 3)
Funding Support:	In addition to the current project, NIH R01 AT0008933

Name:	Lori Scarpa, M.S.
Project Role:	Laboratory Technician
Researcher Identifier (e.g. ORCID ID):	0000-0002-4421-1948
Nearest person months worked	12 months
Contribution to Project:	Small animal surgeries, viral injections, binge-eating protocols
Funding Support:	Current project only

Name:	Gregory Berger, B.S.
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0003-0537-4493
Nearest person months worked	12 months
Contribution to Project:	Small animal surgeries, binge-eating protocols
Funding Support:	Current project, Rutgers Department of Animal Sciences Teaching Assistantship

Name:	Lihong Hao, Ph.D.
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	0000-0001-7795-6981
Nearest person months worked	2 months
Contribution to Project:	Small animal surgeries, immunohistochemistry, behavioral tests.
Funding Support:	Current project and NIH/NCCIH R01 AT0008933

Name:	Vanessa H. Routh, Ph.D.
Project Role:	Investigator (Collaborating)
Researcher Identifier (e.g. ORCID ID):	0000-0003-3644-970X
Nearest person months worked	6 months
Contribution to Project:	Oversees research on Major Tasks 1-3

Funding Support:	In addition to the current project, NIH R01 DK103676, 2 R01 GM097000 and JDRF 3 SRA 2017 488SB

Name:	Pallabi Sarkar, Ph.D.
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	0000-0003-4837-0791
Nearest person months worked	6 months (mid-March to Aug 15 allowed to work 50%, currently allowed at 75%)
Contribution to Project:	Electrophysiologist on Major Tasks 1-3
Funding Support:	In addition to the current project, JDRF 3 SRA 2017 488SB

Name:	Dashiel Siegel
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person months worked	2 months (hired in early June but not allowed to work until late June due to COVID-19 restrictions, late June to Aug 15 allowed to work 50%, currently allowed at 75%)
Contribution to Project:	Technical support on Major Tasks 1-3
Funding Support:	In addition to the current project, NIH R01 DK103676

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

Nothing to Report

8. Special Reporting Requirements

COLLABORATIVE AWARDS: This is a duplicate report for Initiating PI (Routh) and Partnering PI (Bello).

9. Appendices

Nothing Included