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TITLE: Defining the role of the 5-HT4 receptor in the brain, behavior and gut abnormalities resulting from in utero SSRI exposure

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14. ABSTRACT: Depression during pregnancy is common. Because untreated maternal depression during pregnancy is associated with negative psychiatric and gastrointestinal (GI) developmental outcomes in children, treatment is paramount. The safest and first-line therapy, selective serotonin reuptake inhibitors (SSRIs), however, also cause GI and psychiatric issues. Our laboratories have validated the first mouse model of perinatal SSRI exposure that demonstrates an impact of perinatal SSRI exposure on all four parts of the brain-gut-behavior-microbiome (BGBM) axis. Utilizing this model, we generated key evidence confirming that developmental SSRI (fluoxetine) exposure leads to long-lasting alterations in brain wiring, behavior (depression, anxiety), enteric nervous system (ENS) development, GI functions (constipation, altered intestinal epithelial growth) and the intestinal microbiome. Importantly, the BGBM abnormalities demonstrated in our model mimic those demonstrated in children exposed to SSRIs perinatally, making a translational in-depth analysis of the SSRI-BGBM axis interplay now feasible. Further, we have utilized this model to demonstrate that treatment with a serotonin 4 receptor (5-HT4R) antagonist, piboserod, during early development, rescues intestinal, behavioral and enteric microbiota phenotypes in mice exposed to SSRIs during the perinatal period. In this proposal we aim to: (1) elucidate brain, gut and microbiome-based mechanisms that underlie the abnormalities associated with perinatal SSRI exposure, (2) extend our preclinical studies of piboserod to define its developmental effects and therapeutic window and (3) utilize novel transgenic mouse models that selectively eliminate the serotonin reuptake transporter (SERT), the critical protein antagonized by SSRIs, in the brain and the intestine, to delineate the distinct roles of brain and gut serotonin SERT in the BGBM axis.					
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- 1. INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Depression during pregnancy is common. Because untreated maternal depression during pregnancy is associated with negative psychiatric and gastrointestinal (GI) developmental outcomes in children, treatment is paramount. The safest and first-line therapy, selective serotonin reuptake inhibitors (SSRIs), however, also cause GI and psychiatric issues. Our laboratories have validated the first mouse model of perinatal SSRI exposure that demonstrates an impact of perinatal SSRI exposure on all four parts of the brain-gut-behavior-microbiome (BGBM) axis. Utilizing this model, we generated key evidence confirming that developmental SSRI (fluoxetine) exposure leads to long-lasting alterations in brain wiring, behavior (depression, anxiety), enteric nervous system (ENS) development, GI functions (constipation, altered intestinal epithelial growth) and the intestinal microbiome. Importantly, the BGBM abnormalities demonstrated in our model mimic those demonstrated in children exposed to SSRIs perinatally, making a translational in-depth analysis of the SSRI-BGBM axis interplay now feasible. Further, we have utilized this model to demonstrate that treatment with a serotonin 4 receptor (5-HT₄R) antagonist, piboserod, during early development, rescues intestinal, behavioral and enteric microbiota phenotypes in mice exposed to SSRIs during the perinatal period. In this proposal we aim to: (1) elucidate brain, gut and microbiome-based mechanisms that underlie the abnormalities associated with perinatal SSRI exposure, (2) extend our preclinical studies of piboserod to define its developmental effects and therapeutic window and (3) utilize novel transgenic mouse models that selectively eliminate the serotonin reuptake transporter (SERT), the critical protein antagonized by SSRIs, in the brain and the intestine, to delineate the distinct roles of brain and gut serotonin SERT in the BGBM axis.

- 2. KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Brain-gut-behavior-microbiome axis, serotonin, selective serotonin reuptake inhibitors, maternal depression, mouse models

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Define the relationship between brain and gut serotonergic signaling and the role of the 5-HT4R

Major Task: Determine how and where developmental 5-HT4 antagonism affects SSRI-induced BGBM dysfunction Mice used: 4 treatment groups: vehicle, fluoxetine, piboserod, and fluoxetine + piboserod.

Major Task: Characterize brain and intestinal 5-HT4R expression that occurs with developmental fluoxetine exposure +/- piboserod Mice used: Five groups of mice from HTR4-GFP+/+ pregnant mothers exposed to one of the following: (1) Naive controls or gavaged throughout pregnancy and breastfeeding with: (2) saline, (3) fluoxetine, (4) piboserod, and (5) fluoxetine and piboserod.

Subtask : Localization of intestinal 5-HT4R

Specific Aim 2: Refine the time window for rescue of fluoxetine-exposed mice with piboserod

Major Task: Determine whether postnatal administration of piboserod can reverse BGBM axis phenotypes once they occur Mouse line used: Mice exposed to fluoxetine throughout the perinatal period will be administered piboserod, for four weeks beginning at ages equivalent in humans to (1) the immediate postnatal period (P1), (2) children (3 weeks), and (3) adolescents/young adults (6-8 weeks). There will be four treatment groups at each timepoint: saline/no piboserod, saline/piboserod, fluoxetine/no piboserod and fluoxetine/piboserod.

Subtask: Behavioral studies for depression (sucrose preference, forced swim test), anxiety (open field and elevated plus maze, novelty-suppressed feeding test)

Subtask: ENS neuroanatomy will be determined by immunocytochemistry and ENS-mediated GI functions (enteric motility, epithelial permeability) will be examined to determine whether 5-HT4R antagonism changes ENS development and functions influenced by developmental SSRI exposure.

Specific Aim 3: Examine which components of the BGBM axis are mediated by brain SERT, intestinal SERT and/or differences in the microbiome and metabolome

Major Task 1: Determine whether the phenotypic consequences of developmental SSRI exposure are caused by intestinal versus central SERT blockade Mouse lines used: Mice where SERT is knocked out specifically in brain 5-HT neurons (SERT-floxed/Pet1-Cre), intestinal epithelium (SERT-floxed/villin-cre) or the ENS (SERT-floxed/ PAF-cre) will be examined at P21 and P42

Subtask: Similar behavioral studies as those described in aim 2 will be performed to determine if CNS- or GI- derived SERT influences behaviors

Subtask: ENS neuroanatomy and ENS-mediated GI functions, as described in aim 2, will be examined to determine whether CNS- or GI-derived SERT influences ENS development or ENS-derived GI functions. Milestone(s) achieved: Results will provide critical insight into which aspects of the BGBM axis are modulated by intestinal versus central SERT. Publish 1-2 manuscripts that elucidate the relationship between brain and intestinal SERT to BGBM function (and dysfunction).

What was accomplished under these goals?

Specific Aim 1: Define the relationship between brain and gut serotonergic signaling and the role of the 5-HT4R

As noted in 5 (below), because of the delay in ACURO approval and the initial difficulty in breeding the 5HT4-GFP mice, we proceeded with Aims 2 and 3 in order to continue to progress on the grant aims. We are currently working on Aim 1 and anticipate results within the next several months.

Specific Aim 2: Refine the time window for rescue of fluoxetine-exposed mice with piboserod

To determine whether postnatal administration of piboserod can reverse BGBM axis phenotypes once they occur, we exposed mice to fluoxetine throughout the perinatal period who were then administered piboserod, for four weeks beginning at the immediate postnatal period (P1). We chose this timepoint because it is still a period of the most active ENS development. There were four treatment groups: saline +/- piboserod and fluoxetine +/-no piboserod. We extensively evaluated: (1) ENS neuroanatomy, (2) enteric motility (total gastrointestinal [GI] transit, colonic motility, small intestinal transit or gastric emptying) and (3) enteric epithelial balance (villus height and crypt depth). We did not find preliminary behavioral studies, for depression and anxiety, also determined no significant differences. This could be attributed to (1) the failure of piboserod to pass successfully through breastmilk or (2) the possibility that piboserod only affects ENS development during the fetal stages. These possibilities will be examined (see next year goals).

Specific Aim 3: Examine which components of the BGBM axis are mediated by brain SERT, intestinal SERT and/or differences in the microbiome and metabolome

To determine whether the phenotypic consequences of developmental SSRI exposure are caused by intestinal versus central SERT blockade we have developed and begun to examine the mice in whom SERT is knocked out specifically in: (1) the intestinal epithelium (SERT-floxed/villin-cre) or (2) the ENS (SERT-floxed/ Wnt1-cre) at P42.

We have determined that: (1) in both SERT-floxed/villin-cre and SERT-floxed/ Wnt1-cre mice, the myenteric and submucosal plexuses of the ENS are hyperplastic compared to WT, (2) The ENS hyperplasia in the SERT-floxed/ Wnt1-cre manifests as faster total GI transit time (TGIT) in female mice specifically. There were no differences seen thus far in either gender in colonic motility, gastric emptying or small intestinal transit. There were consistent trends in all motility studies, however, which indicate that in vivo and in vitro may actually be faster in the SERT-floxed/ Wnt1-cre mice. These mice tended, however, to have smaller litters (approximately 50% of what we were expecting) so the variability was high and we will need to repeat these studies on an additional cohort of animals to fully determine whether these results are true. Measures of intestinal epithelial balance (villus height, crypt depth, measures of enteroendocrine and enterochromaffin cells, enteric permeability) were also not significantly different but also presented with smaller numbers and higher variability. We bred another cohort which are currently being tested for anxiety but these results are not yet available. Further, stool has been collected from these animals for microbiome and metabolome analysis.

We have conducted a similar set of studies in the SERT-floxed/villin-cre mice and found that: (1) there was a significant increase in both males and female SERT-floxed/villin-cre over WT mice, for in vitro colonic peristaltic contractions. TGIT, in vivo colonic motility, gastric emptying and small intestinal transit were not different between groups. One of the more interesting aspects of these results is that in vivo colonic motility showed no difference while in vitro colonic motility (peristaltic contractions) were significantly faster in SERT-floxed/villin-cre mice. Because peristaltic contractions are a direct measure of ENS function, this suggests that the CNS may be modulating motility in these mice. Dr. Ansorge will be conducting the CNS physiology tests in

these mice to determine whether signaling is altered.

Measures of intestinal epithelial balance (villus height, crypt depth, measures of enteroendocrine and enterochromaffin cells, enteric permeability) demonstrated consistent trends in that crypt depth was smaller and permeability was less in SERT-floxed/villin-cre of both sexes, relative to WT. Like the SERT-floxed/Wnt1-cre mice, however, these results were not significantly different because of high variability. We recently bred another cohort which are being tested for anxiety and also for enteric epithelial measures but these results are not yet available. Stool has been collected from these animals for microbiome and metabolome analysis.

We currently breeding, the mice in whom SERT is knocked out specifically in brain 5-HT neurons (SERT-floxed/Pet1-Cre). Our data thus far indicate that neurogenesis is increased in the ENS of these mice.

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

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

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

Specific Aim 1: Define the relationship between brain and gut serotonergic signaling and the role of the 5-HT4R

Now that our breeding has been successful with these mice, we have started to isolate and stain the intestines from the postnatal mice. We anticipate being able to complete a large portion of this aim over the next year.

Specific Aim 2: Refine the time window for rescue of fluoxetine-exposed mice with piboserod

Although we were disappointed that we were not able to achieve significant results in the first set of experiments for this aim we are still optimistic. Given that we did not see effects of piboserod specifically at the timepoints when the mouse pups were receiving it exclusively in the breast milk (P1-P21) this could mean that the drug is not effectively transmitted to the pups through the breastmilk. The effects we saw in our initial studies may thus be the result of maternal transfer. Our next set of studies will thus include exposing dams (and thus progeny) to fluoxetine throughout the perinatal period and then administering piboserod directly to the progeny at 3-7 weeks of age. Because the drug will be administered directly to the progeny, rather than indirectly through the breastmilk, this will elucidate two things: (1) whether direct exposure to piboserod has an effect on the brain-gut axis, and (2) whether piboserod may be an effective drug for the reversal of brain-gut symptoms elicited by perinatal SSRI exposure. If we do see differences in piboserod administration during this time period we will then proceed to test the drug on 6-8 week old mice to determine if reversal of brain +/- gut manifestations are remain reversible in older animals. Studies of behavior, ENS neuroanatomy and ENS-mediated GI functions will be evaluated, as have been done in our prior studies.

Specific Aim 3: Examine which components of the BGBM axis are mediated by brain SERT, intestinal SERT and/or differences in the microbiome and metabolome

Because we were delayed in being able to conduct experiments in the 5-HT₄-GFP mice, we progressed further in completing our experiments for Aim 3.

Over the next year, we are planning to repeat the motility and enteric epithelial studies that we conducted in the SERT-floxed/villin-cre and SERT-floxed/ Wnt1-cre mice to increase our numbers and decrease our variability. We will also implement these experiments in the SERT-floxed/ Pet1-cre mice. Further, we will have all stool analyzed at Baylor, by Dr. Luna, for microbiome and metabolome analyses.

We should have our first set of behavioral test results, for depression and anxiety, available for the SERT-floxed/villin-cre mice in the next 1-2 months. We are currently breeding a larger cohort of the SERT-floxed/ Wnt1-cre mice in order to conduct these experiments which we also plan to implement this year.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

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:**

We have not had any experimental problems and do not anticipate changes to the protocol. We had to start the proposed experiments several months later than anticipated, however, because of the delay in ACURO approval. This delay resulted in a postponement of required breeding and thus proposed experiments, and particularly the experiments proposed in aim 1. Now that we have approval, we have proceeded with all planned experiments. Because of the initial difficulty in breeding the 5HT₄-GFP mice, we proceeded with Aims 2 and 3 in order to continue to progress the grant aims. Results are noted above.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

It took several months before our ACURO protocols were approved by the DoD. This delayed our work and we therefore did not spend all of the animal and supply expenditures that we anticipated. Our anticipated budget remains the same.

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

There have been no significant changes in use or care of vertebrate animals, biohazards and/or select agents. Human subjects are not being evaluated in this study. The applicable IACUC expiration date is 7/2019.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Israelyan N and Margolis KG. Serotonin as a Link Between the Gut-Brain-Microbiome Axis in Autism spectrum Disorders. *Pharmacological Research*. June 2018, Vol 132; pp. 1-6. Acknowledgement of federal support: yes.

Margolis KG. A role for the serotonin reuptake transporter in the brain and intestinal features of autism spectrum disorders and developmental antidepressant exposure. *J Chem Neuroanat*. 2017 Oct; 83-84:36-40. doi: 10.1016/j.chemneu.2017.02.001.. pii: S0891-0618(16)30203-4. Acknowledgement of federal support: yes.

Books or other non-periodical, one-time publications.

Margolis KG. When Adult Gastrointestinal Disease is Really Pediatric. Published in *AGA Perspectives* on March 20, 2017. *AGA Perspectives* is a bimonthly magazine published by the AGA that “provides an opinion forum for noted gastroenterologists to debate today’s most controversial topics and provide brief updates on other topics relevant to academic and practicing physicians and scientists”. Acknowledgement of federal support: no.

Other publications, conference papers and presentations.

Publications

Margolis KG**, Vittori J, Talaver M, Gluck K, Iuga A, Stevanovic K, Saurman V, Israelyan N, Welch MG and Gershon MD. Enteric serotonin and oxytocin: endogenous regulation of severity in a murine model of necrotizing enterocolitis. *Am J Physiol Gastrointest Liver*. 2017 Nov 1;313(5):G386-398. doi:10.1152/ajpgi.00215.2017. **corresponding author

Israelyan N and Margolis KG. Kruppel-like Factor 5 extends its fingers to desmosomes: a new frontier in research? *Am J Physiol*. 2017 November 1;313(5):G476-G477. doi: 10.1155/ajpgi.00172.2017. Acknowledgement of federal support: yes.

Oral Abstract Presentations

Israelyan N, Lorimer T, Park Y, Xing A, Li ZS, Caron M, Jacobson J, Gershon MD and Margolis KG. Serotonin as a Modulator of the Gut-Brain-Microbiome Axis. International Symposium for Enteric Nervous System Development 2018. Acknowledgement of federal support: yes.

Israelyan N, Lorimer T, Park Y, Li ZS, Caron M, Jacobson J, Gershon MD and Margolis KG. The Role of Serotonin in the Gut-Brain-Microbiome Axis in a Mouse Model of constipation-Predominant IBS. NASPGHAN 2017. Acknowledgement of federal support: yes.

State of the Art/Symposium Presentations

“Serotonin as a Peripheral Modulator in Autism Spectrum Disorders”; Keystone Conference series; Park City, Utah. Acknowledgement of federal support: yes.

“Translating Mouse Models of Autism to Human Disease”; Symposium Speaker at American Gastroenterological Association Annual Meeting; Washington, DC. Acknowledgement of federal support: yes.

“Implications of Serotonin in Brain-Gut Axis Disease”; Speaker for the Texas Digestive Disease Centers National Conference on Brain-Gut Axis Disorders; Houston, TX. Acknowledgement of federal support: yes.

“Gastrointestinal Problems in Children with Autism: Serotonin as a Link in the Brain-Gut Axis”, speaker for the annual “National Conference on Autism”, University of Washington; Saint Louis, MO. Acknowledgement of federal support: yes.

- **Website(s) or other Internet site(s)**

None to report.

- **Technologies or techniques**

None to report.

- **Inventions, patent applications, and/or licenses**

None to report.

- **Other Products**

We have created mice in which the serotonin reuptake transporter is selectively eliminated from the intestinal epithelium, enteric neurons or serotonergic neurons from the brain.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

There are no changes in any of the personnel that has worked on this project	
<u>Name:</u>	Ruth Anne Luna, PhD
<u>Name:</u>	Mark Ansorge, PhD
<u>Name:</u>	Zi Shan Li, PhD
<u>Name:</u>	Yeji Park, MS

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

I am currently receiving 10% salary support from a previously pending grant:

4RO1NS015547-34 (PI: Gershon) 1/1/2018 – 12/31/2023 NIH/NINDS
Microenvironment in Enteric Neuron Development
(Role: co-I)

My NIH KO8 award has been completed. I have, however, received a no cost extension:

K08 DK093786-01A1 (PI: Margolis) 7/1/2013 – 6/30/2018 NIH NIDDK
The Role of ENS Development in the Immunology of Intestinal Inflammation

What other organizations were involved as partners?

No organizations were involved as partners. There is a co-Investigator from the Baylor School of Medicine (Dr. Luna; listed above).

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Mark Ansorge, the co-PI on this award, will submit an independent report.

9. APPENDICES: N/A