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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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RECIPIENT: Columbia University Medical Center

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13. SUPPLEMENTARY NOTES

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 t he intestine, to delineate the distinct roles of brain and gut serotonin SERT in the BGBM axis.

15. SUBJECT TERMS
None Listed

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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 have generated key evidence confirming that developmental SSRI (fluoxetine) exposure leads to long-lasting alterations in brain wiring, behavior (depression, anxiety), cognition, enteric nervous system (ENS) development, GI functions (constipation, altered intestinal epithelial growth and permeability) and the intestinal microbiome. 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 have thus far found that (1) treatment with a 5-HT4 antagonist, piboserod throughoughout in utero development is critical to ameliorate the ENS abnormalities induced by developmental SSRI exposure; (2) Eliminating SERT selectively from the enteric epithelium or the ENS plays important roles in gastrointestinal motility and intestinal epithelial balance; (3) Pet1, previously thought to be exclusively in the brain, is located throughout the intestinal epithelium and also the ENS where it affects ENS-driven functions including ex vivo motility and enteric epithelial balance;(5) Constitutive Pet1 deletion causes mild anxiety- and depression-like phenotypes, (6) The microbiome does not appear to be significantly impacted by SERT deletion in the intestinal epithelium, ENS or Pet1+ cells. Our most recent studies have elucidated the more precise roles that epithelial and enteric neuronal SERT play in anxiety- and depression-like phenotypes. Studies are ongoing regarding (1) the evaluation of sympathetic and parasympathetic discharge in mouse models with selective SERT deletion; (2) 5-HT4 localization with fluoxetine +/- piboserod exposure in the brain and the intestine; (3) the role of piboserod administration during young adulthood in ameliorating the affects of developmental SSRI exposure; and (4) the change in the metabolome in mouse models in which SERT is conditionally knocked out.

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:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW.

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. ***In progress.***

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. ***Not initiated.***

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). ***In progress.***

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. ***In progress.***

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 ***In progress.***

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). ***In progress.***

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 had bred this mice up sufficiently to conduct the proposed experiments but were unable to complete them when our laboratories were shut down emergently once the COVID19 pandemic hit. What we were able to determine was that: (1) 5-HT4^{+} neurons are present in the myenteric plexus of the small and large intestine of the mice; (2) that there is some, but not 100% co-localization of 5-HT4 with serotonin in neurons of the myenteric plexus; (3) that 5-HT4 is located throughout >90% of the epithelial cells lining the colon and (4) Although 5-HT4 is on the epithelial cells lining the small intestine, it is present in much smaller numbers than that of the colon. These mice are now being bred up again with the intention of completing these experiments during the time provided for the no-cost extension.

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

We have thus far sought to determine whether prenatal or immediate postnatal exposure of the 5-HT4 antagonist, piboserod, prevents the abnormalities in enteric nervous system (ENS) development and function induced by perinatal SSRI (fluoxetine) exposure. There were four treatment groups: groups (1 and 2) saline +/- piboserod and (3 and 4) fluoxetine +/- no piboserod.

Our preliminary data showed that prenatal administration of the 5-HT4 antagonist, piboserod, could prevent the abnormalities in ENS development and long-term GI dysfunction caused by prenatal fluoxetine exposure. We extensively evaluated both the myenteric and submucosal plexuses of the enteric nervous system (ENS) and found an enteric neuronal hyperplasia induced in the myenteric and submucosal plexuses by fluoxetine exposure. In the mice exposed to fluoxetine there was a normalization of enteric neuronal number in the myenteric plexus to that of control mice. This was accompanied by a normalization in a critical ENS function (motility). Total gastrointestinal (GI) transit time and colonic motility were also normalized in these mice, to WT levels. This was accompanied by a decrease in anxiety- and depression-related phenotypes.

In contrast to our findings noted in mice that received piboserod prenatally, we found that postnatal administration of the 5-HT4 antagonist, piboserod, did not prevent the abnormalities in ENS development and long-term GI dysfunction caused by prenatal fluoxetine exposure. There was no difference noted in neuronal numbers in the myenteric or submucosal plexuses (figs 1a, 1c). There was also no difference in gastrointestinal motility parameters, including total GI transit time (fig 1b), colonic motility (fig 1d), small intestinal transit time or gastric emptying. Finally, there was no difference in enteric epithelial balance, measured as villus height and crypt depth and area, nor was there any difference in the outcomes of behavioral studies for depression or anxiety.

Since we did not see effects of Piboserod at the timepoints when the mouse pups were receiving it exclusively in the breastmilk (P1-P21), we set up an experiment where we sought to administer piboserod to 3 week old pups exposed to fluoxetine prenatally. We did this to determine whether the lack of piboserod effect was due to ineffective transfer through the breastmilk rather than a lack of effect of piboserod. The breedings for this set of experiments were not successful so the numbers achieved were smaller than we needed for our proposed experiments. We conducted pilot studies with the few mice available and, while these suggested there may be some effects, we need to evaluate larger numbers of mice, as laid out in our proposal, to truly determine if piboserod is effective.

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 thus far successfully crossed and begun to examine mice in whom SERT is knocked out specifically in the intestinal epithelium (*Villin-Cre::SERT*$^{fl/fl}$), the ENS (*Wnt1-Cre::SERT*$^{fl/fl}$) or the CNS (*Pet1::SERT*$^{fl/fl}$) at P42. To study the role of SERT in the ENS, we initially proposed and implemented studies in *PAF-Cre::SERT*$^{fl/fl}$ mice instead of *Wnt1-Cre::SERT*$^{fl/fl}$. Although the publications on the PAF-cre mice indicated that the PAF-cre driver was localized to the ENS (and not in the CNS), the mice we examined were functioning like SERT KO mice. Because of this we stained the CNS and found that PAF is actually located throughout the CNS in addition to the ENS. Since PAF is expressed throughout the CNS and thus lacks sufficient neural crest specificity as a cre driver, we substituted Wnt1-cre mice. Wnt1 has been the Cre driver most utilized to delete genes from neural crest derivates and the ENS. Although we were able to validate these mice and perform experiments on them, a newer version of Wnt1 was more recently created that has no effect on dopaminergic neurons in the brain (whereas the original Wnt1-cre was found to) so we again crossed SERT-floxed mice with mice harboring this newer, more ENS-focused version of Wnt1-cre. Utilizing both strains of mice, we have vastly expanded over the past year on the motility and behavioral studies in the *Villin-Cre::SERT*$^{fl/fl}$ and the *Wnt1-Cre::SERT*$^{fl/fl}$ mice. The results we have achieved in each line are noted below.

SERT-floxed/villin-cre mice:

GI Findings

Neurogenesis: When mucosal SERT is selectively deleted in *Villin-Cre::SERT*$^{fl/fl}$ mice, the 5-HT compartmentation of the gut wall is lost and the large amount of 5-HT that enterochromaffin (EC) cells secrete floods the developing ENS and alters neurogenesis and ENS function. The result is ENS hyperplasia that mimics that seen in SERT KO mice.

In vivo motility: Total GI transit time (TGIT) is not significantly faster in *Villin-Cre::SERT*$^{fl/fl}$ females or males than WT. There was also no difference in colonic motility in males or females. Small intestinal transit (SIT) trended towards being faster in females (p=0.1) but was not significant. SIT was not significant in males. For gastric emptying (GE,) there was not significance found in males nor females.

Ex vivo motility: Both male and female *Villin-Cre::SERT*$^{fl/fl}$ mice have faster and more frequent CMMCs than WT. CMMC length is not different.

Intestinal epithelial growth: Villus height and crypt depth were both significantly greater in males and trending towards significance in females but were not significant in either sex.

Microbiome: Alpha diversity and beta diversity were not significantly different when evaluating for sex or genotype. STAMP (Statistical analysis of taxonomic and functional profiles) was used for taxonomic abundance plots. For two group comparison at Phyla and Genera level , we used Welch's t-test with Benjamani Hochber correction. With multiple test correction, we did not see any significant differences at any taxonomic levels between the WT and *Villin-Cre::SERT*$^{fl/fl}$ mice.

Behavioral Outcomes: An extensive array of behavioral studies were completed evaluating for anxiety- and depressive-like phenotypes. To test for anxiety, the novelty-suppressed feeding test, morris water maze test, the light-dark box test, the elevated plus maze test, and the open-field test were completed. To test for depressive-like phenotypes, the forced swim test, the sucrose preference test and the tail suspension test were completed.
Anxiety-like phenotypes: To test for anxiety, the novelty-suppressed feeding test, morris water maze test, the light-dark box test, the elevated plus maze test, and the open-field test were completed by the Margolis and Ansorge laboratories. The light-dark box test and the open-field test were both significantly different in that the *Villin-Cre::SERT*$^{fl/fl}$ mice showed a less anxious phenotype than their WT counterparts. None of the other tests evaluating for anxiety were significantly different when comparing *Villin-Cre::SERT*$^{fl/fl}$ mice to their WT littermates.
Depression-like phenotypes: The forced swim test, the sucrose preference test and the tail suspension test were completed by both the Margolis and the Ansorge laboratories. The forced swim test was significantly different when comparing *Villin-Cre::SERT*$^{fl/fl}$ mice to their WT littermates in that the *Villin-Cre::SERT*$^{fl/fl}$ mice showed a less depressive-like phenotype than their WT counterparts. None of the other tests evaluating for depression were significantly different when comparing *Villin-Cre::SERT*$^{fl/fl}$ mice to their WT littermates.

These findings imply that the ENS hyperplasia seen in the *Villin-Cre::SERT*$^{fl/fl}$ mice results in faster ex vivo motility. These findings confirm prior studies of ours demonstrating the relationship between serotonin-driven ENS hyperplasia and ex vivo motility. There was no difference, however, in the in vivo motility studies. This could be the result of brain to gut communication and/or an increase in sympathetic and/or parasympathetic drive to the gut which would result in a slowing of motility. The behavior tests we have conducted show that the *Villin-Cre::SERT*$^{fl/fl}$ mice are less anxious and less depressed than WT mice. Because of these observed differences we are now revising our animal protocols and planning for experiments to look at the effect of this genetic abnormality and its associated changes in mood on sympathetic and parasympathetic drive to the gut (and gut-to-brain in the case of parasympathetic drive). These experiments will require chemical sympathectomy or vagotomy, respectively. It is not likely to be a difference based on microbial signaling because the intestinal microbial communities were not different between the WT and *Villin-Cre::SERT*$^{fl/fl}$ mice.

SERT-floxed/Wnt-cre mice:

GI Findings

Neurogenesis: Similar to what we saw in the Villin-*Cre::SERT*$^{fl/fl}$ mice The rate of enteric neurogenesis in *Wnt1-Cre::SERT*$^{fl/fl}$ mice is greater than that in WT controls and there is a specific increase in late-developing neuronal subsets (dopamine- and GABA-expressing), which are known to be serotonin-dependent.

In vivo motility: There was no significant difference in total GI transit time (TGIT) between the first or second generation *Wnt1-Cre::SERT*$^{fl/fl}$ and their WT littermates. Colonic motility is not significantly different in the first generation *Wnt1-Cre::SERT*$^{fl/fl}$ males or females but second generation *Wnt1-Cre::SERT*$^{fl/fl}$ males appear to have much slower colonic motility than WT littermates. Gastric emptying (GE) was also significantly slower in the second generation (but not the first generation) *Wnt1-Cre::SERT*$^{fl/fl}$ mice compared to WT. There was no difference in small intestinal transit (SIT) in males nor females.

Ex vivo motility: Colonic migrating motor complexes (CMMCs) are slower and less frequent in *Wnt1-Cre::SERT*$^{fl/fl}$ males which is consistent with slower in vivo colonic motility seen in these animals. *Wnt1-Cre::SERT*$^{fl/fl}$ females did not exhibit differences in CMMC frequency or velocity.

Intestinal epithelial growth: Villus height and crypt depth were not significantly greater in the first generation or the second generation *Wnt1-Cre::SERT*$^{fl/fl}$ mice than in WT mice.

Microbiome: Alpha diversity was not significantly different when evaluating for sex or genotype. Beta diversity trended towards significance for genotype and sex but was not significant. STAMP (Statistical analysis of taxonomic and functional profiles) was used for taxonomic abundance plots. For two group comparison at Phyla and Genera level , we used Welch's t-test with Benjamani Hochber correction. With multiple test correction, as with the *Villin-Cre::SERT*$^{fl/fl}$ mice we did not see any significant differences at any taxonomic levels between the WT and the *Wnt1-Cre::SERT*$^{fl/fl}$ mice.

Behavioral Outcomes: An extensive array of behavioral studies were completed evaluating for anxiety- and depressive-like phenotypes. To test for anxiety, the novelty-suppressed feeding test, morris water maze test, the light-dark box test, the elevated plus maze test, and the open-field test were completed. To test for depressive-like phenotypes, the forced swim test, the sucrose preference test and the tail suspension test were completed.
Anxiety-like phenotypes: To test for anxiety, the novelty-suppressed feeding test, morris water maze test, the light-dark box test, the elevated plus maze test, and the open-field test were completed by the Margolis and Ansorge laboratories. The elevated plus maze test and the novelty-suppressed feeding test were both significantly different in that the *Wnt1-Cre::SERT*$^{fl/fl}$ mice showed a more anxious phenotype than their WT counterparts. None of the other tests evaluating for anxiety were significantly different when comparing *Wnt1-Cre::SERT*$^{fl/fl}$ mice to their WT littermates.
Depression-like phenotypes: The forced swim test, the sucrose preference test and the tail suspension test were completed by both the Margolis and the Ansorge laboratories. None of these tests evaluating for depression were significantly different when comparing *Wnt1-Cre::SERT*$^{fl/fl}$ mice to their WT littermates.

These findings imply that , although the *Wnt1-Cre::SERT*$^{fl/fl}$ mice exhibit an ENS hyperplasia, they exhibit slower colonic motility both in vivo and ex vivo. These findings suggests that there is a defect in neuronal signaling in these mice which cause motility to slow down and that the defect is not dependent on enteric neuronal hyperplasia, as we have seen in other serotonin-driven models. This also suggests that the side effect of constipation, seen in SSRIs is the cause of neuronal serotonin, rather than epithelial serotonin. The microbiome does not appear to play a role in the differences noted. Because there is a significant difference in anxiety-like phenotypes between *Wnt1-Cre::SERT*$^{fl/fl}$ mice and WT littermates, studies are currently being designed to lok at the effects of gut to brain signaling, with vagotomy, on these mice.

SERT-floxed/Pet1-cre mice:

GI Findings: Although it had previously been reported that Pet1 is not located in the enteric neurons this report was over a decade ago in experiments utilizing antibodies instead of genetically tagged mice. We thus used the Pet1cre-EFP mouse to confirm these results. Unexpectedly, we identified Pet1 both in the murine enteric epithelium as well as in enteric neurons.

Neurogenesis: Enteric neurogenesis in *Pet1-Cre::SERT*$^{fl/fl}$ mice was greater than that in WT controls with a specific increase a specific increase in late-developing neuronal subsets (dopamine- and CGRP-expressing), which are known to be serotonin-dependent. These findings mimic what we found in the *Wnt1-Cre::SERT*$^{fl/fl}$ mice, implying that Pet1 may be an important transcription factor in the control of enteric neurogenesis.

In vivo motility: Despite the difference in enteric neuronal numbers and architecture, there was no significant difference in total GI transit (TGIT), colonic motility, gastric empty (GE) or small intestinal transit (SIT) between the *Pet1-Cre::SERT*$^{fl/fl}$ mice and WT controls of either sex.

Ex vivo motility: CMMCs are more frequent and faster in *Pet1-Cre::SERT*$^{fl/fl}$ females, and close to significance in males (also faster) which is consistent with the enteric neuronal hyperplasia seen in these animals but is not consistent with their in vivo colonic motility, which was not different.

Intestinal epithelial growth: Villus height and crypt depth were both significantly greater in male *Pet1-Cre::SERT*$^{fl/fl}$ mice than in WT mice. We have not yet measured villus height and crypt depth in females. These results are consistent with our prior findings, that enteric neuronal serotonin-driven hyperplasia affects epithelial growth.

Microbiome: Alpha and beta diversity were not significantly different when evaluating for sex or genotype although beta diversity was almost significant when considering sex, but not phenotype. In accordance with the *Wnt1-Cre::SERT*$^{fl/fl}$ and the *villin-Cre::SERT*$^{fl/fl}$ we did not see any significant differences at any taxonomic levels between the WT and the *Pet1-Cre::SERT*$^{fl/fl}$ mice.

Behavior: *Pet1-Cre::SERT*$^{fl/fl}$ mice have a mild behavioral phenotype. They exhibit slightly increased anxiety and depression-like behavior as assessed in the open field test, elevated plus maze, novelty suppressed feeding test, and sucrose preference test.

The findings show that the hyperplastic ENS these mice manifest is associated with, like the *Villin-Cre::SERT*$^{fl/fl}$ mice, the enhanced ex vivo motility and intestinal mucosal growth that these mice display. Because in vivo motility is unaltered, however, it is likely that external neuronal influence is playing an important role in affecting the in vivo motility in these animals. This could be attributable to excess sympathetic drive driven by the mild anxiety and or depressive phenotypes the *Pet1-Cre::SERT*$^{fl/fl}$ mice exhibit. The microbiome does not appear to play a prominent role in this differential communication.

SERT-floxed/Emx1-Cre mice:

GI Findings

To dissect the behavioral contribution of the transient and early postnatal SERT expression in a subpopulation of cortical glutamatergic neurons, we assessed their behavioral performance of Emx1-Cre::*SERT*$^{fl/fl}$ animals in adulthood. We found that *Emx1-Cre::SERT*$^{fl/fl}$ mice have a robust and specific behavioral phenotype, exhibiting decreased fear extinction learning. We are in the process of evaluating they performance in attention set shifting and find indicative evidence of impaired performance, but testing is ongoing.

While it is known that Emx1 is not expressed in raphe serotonergic neurons, the expression in the gut was not known. Hence, we used the Emx1-Cre::eYFP mouse to evaluate enteric expression. Unexpectedly, we identified Emx1in the myenteric plexus of wild-type mice. Future studies will be focused on understanding the role of Emx in behavior and GI function.

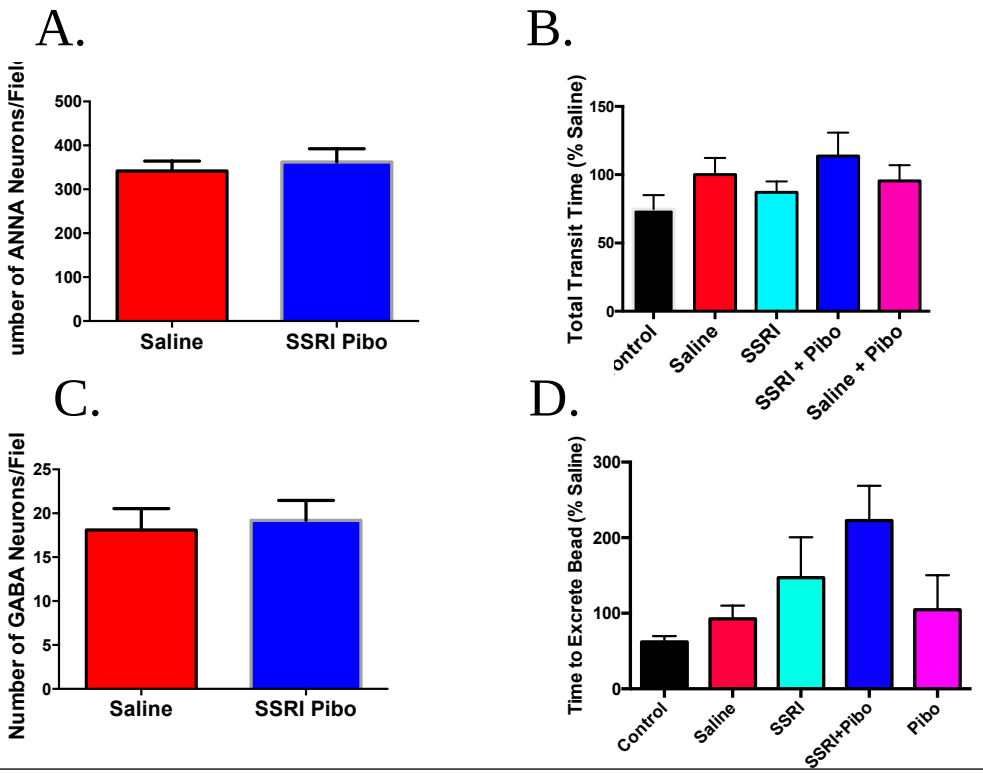


Figure 1: Postnatal administration of Piboserod does not normalize neuron counts (A,C), total GI transit (B) or colonic motility (D) in mouse pups who are exposed to fluoxetine during pregnancy and breastfeeding.

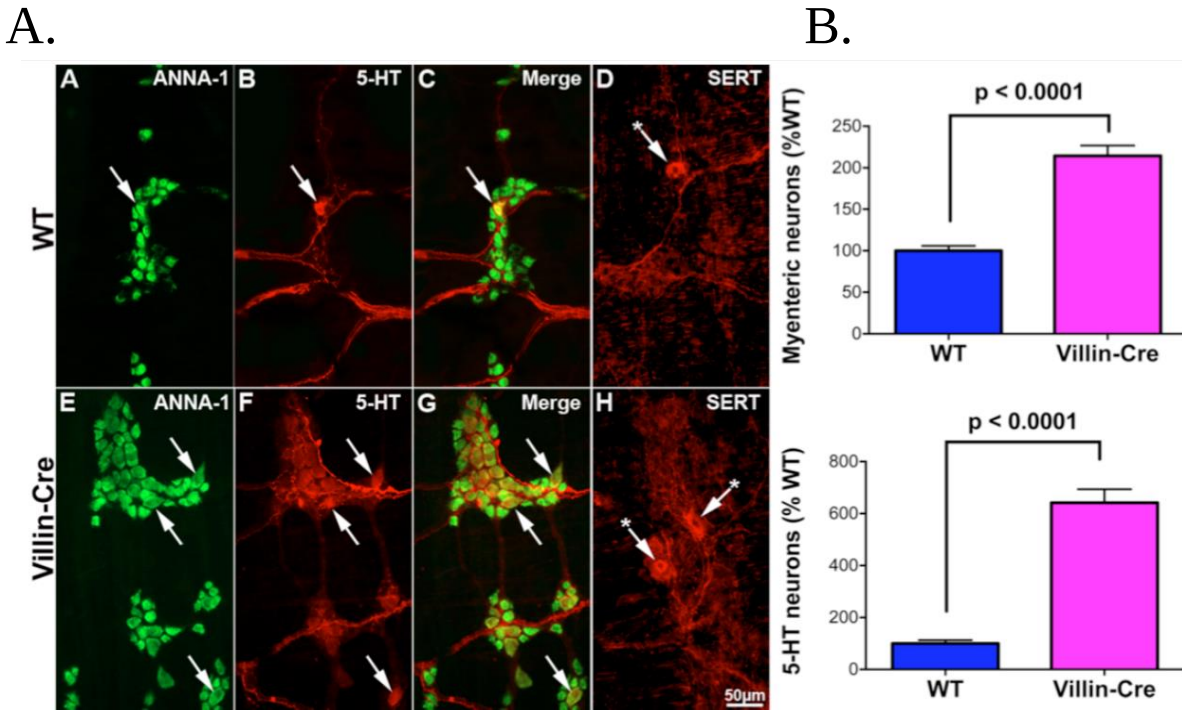


Figure 2: (A) *Villin-Cre::SERT*$^{fl/fl}$ mice possess SERT in the ENS but not in the intestinal mucosa (B) Villin-Cre/SERT flx/flx^{-} mice have more enteric neurons and serotonergic neurons in the myenteric plexus

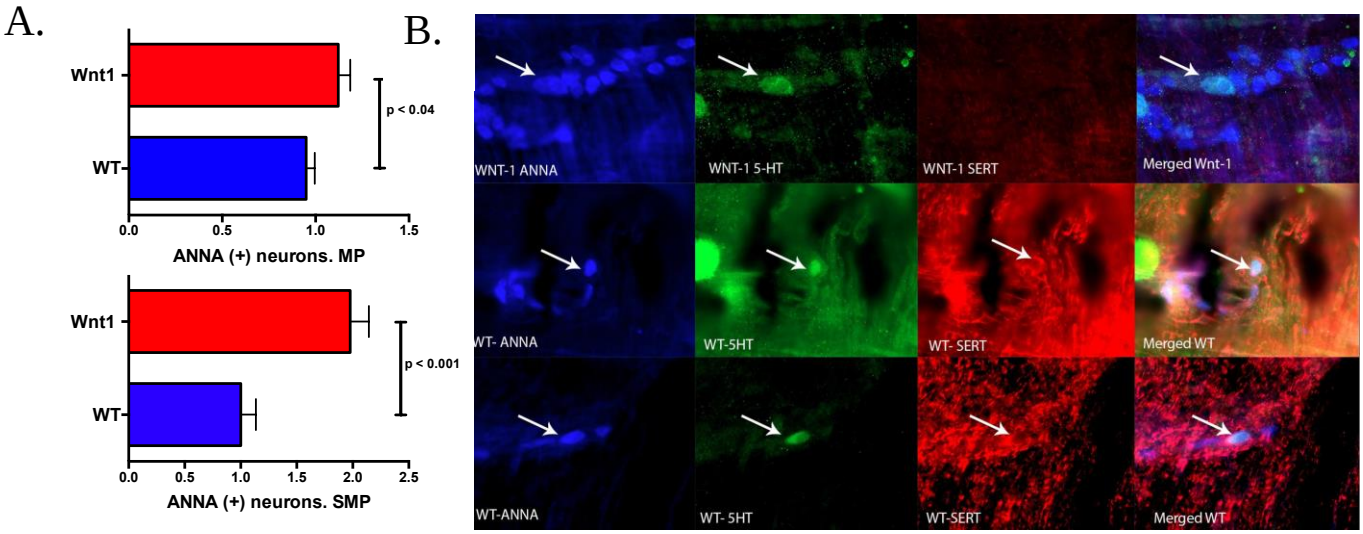


Figure 3: (A) *Wnt1-Cre::SERT*$^{fl/fl}$ mice have more enteric neurons in the myenteric and submucosal plexuses (B) *Wnt1-Cre::SERT*$^{fl/fl}$ mice possess SERT in the intestinal mucosa but not in the ENS

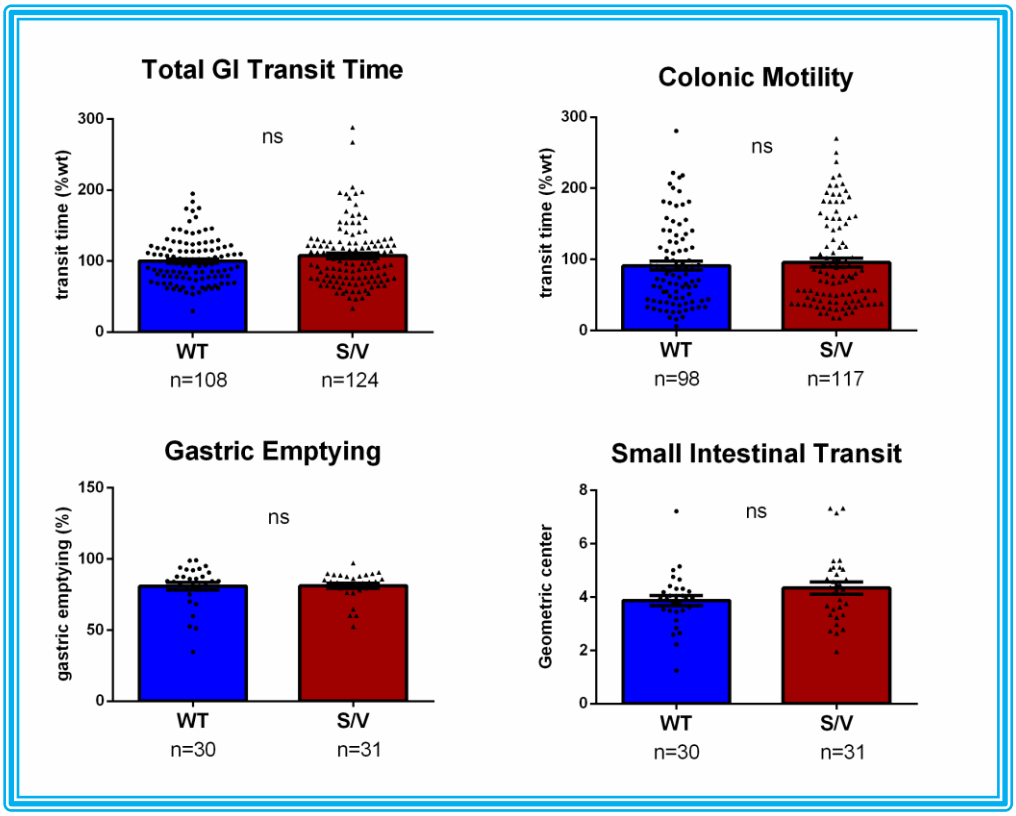


Figure 4: Total GI transit time, colonic motility, gastric emptying and small intestinal transit are not significantly different between *Villin-Cre::SERT*$^{fl/fl}$ mice and their WT counterparts

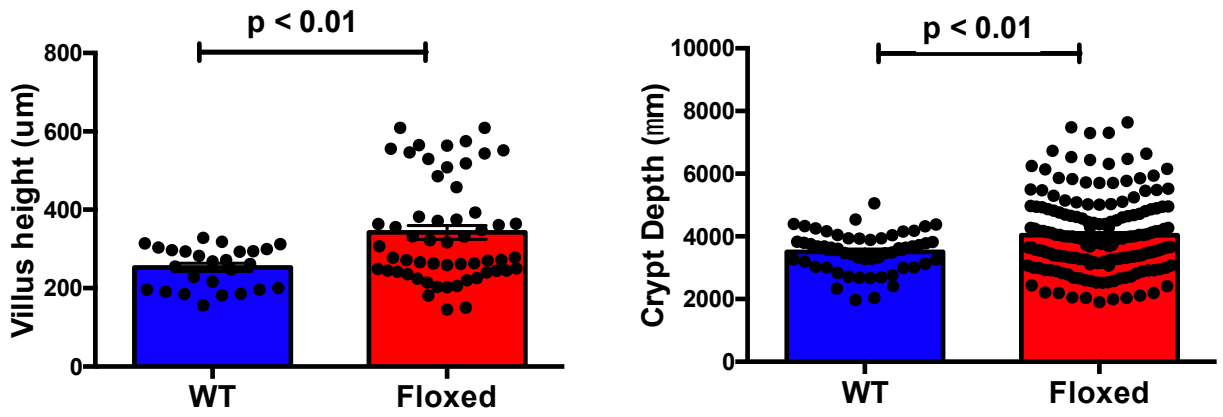


Figure 5: Villus height and crypt depth are significantly greater in male *Villin-Cre::SERT*$^{fl/fl}$ mice than male WT mice

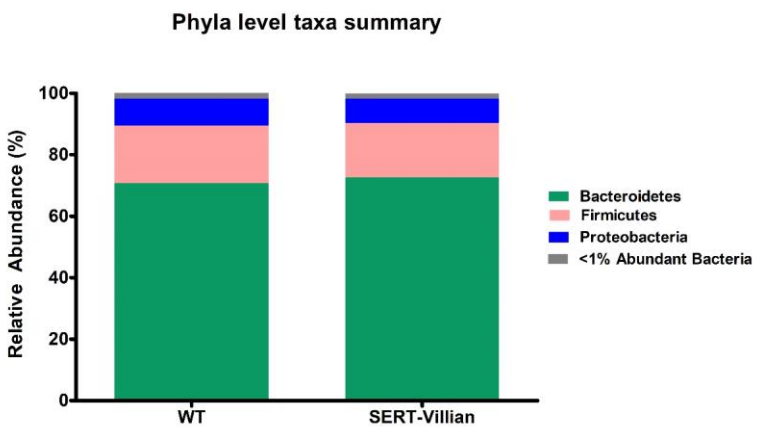
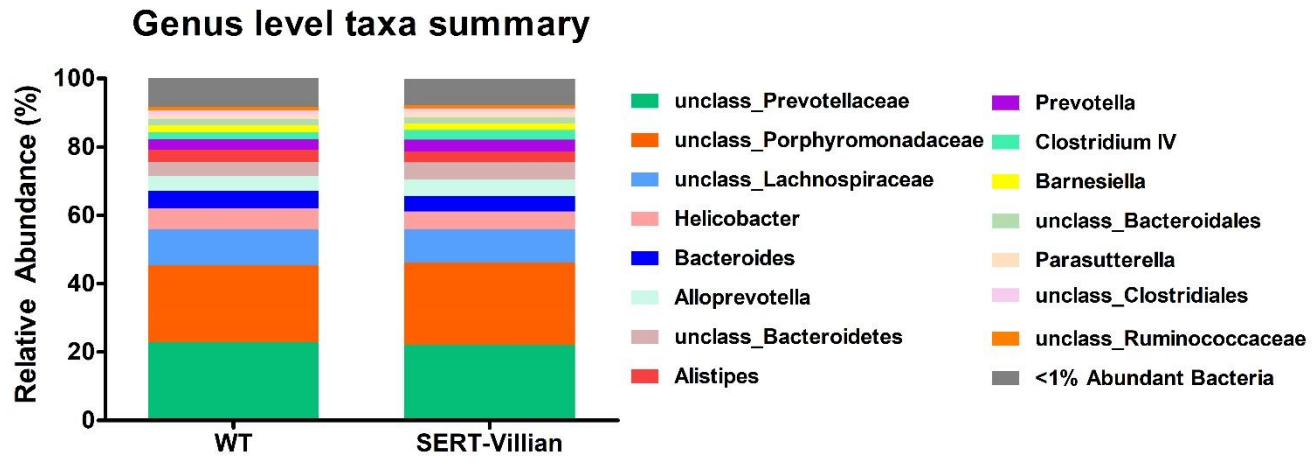


Figure 6: Two group comparison at the phyla and genera level did not reveal any significant differences at any taxonomic levels between *Villin-Cre::SERT*$^{fl/fl}$ *and WT mice*



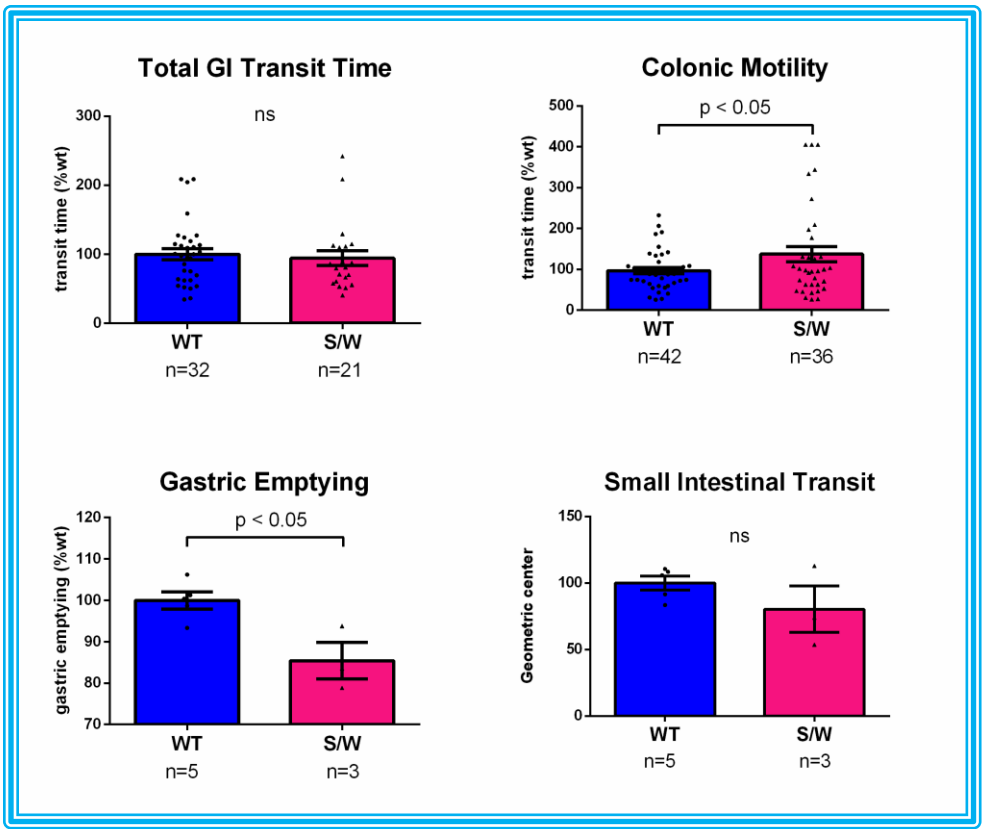


Figure 7: Colonic motility and gastric emptying are significantly slower in *Wnt1-Cre::SERT*$^{fl/fl}$ mice than in their WT counterparts. In contrast, total GI transit time, and small intestinal transit are not significantly different between *Wnt1-Cre::SERT*$^{fl/fl}$ mice and their WT counterparts

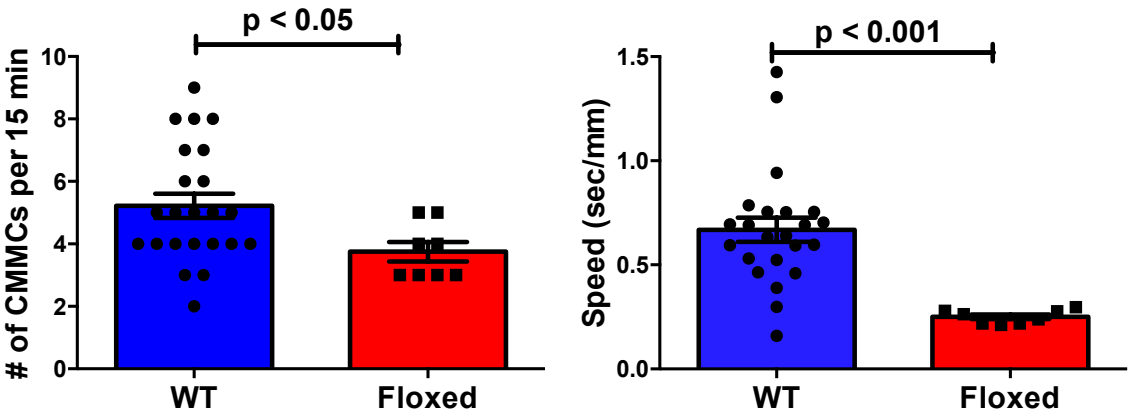


Figure 8: CMMC frequency and velocity are significantly slower in *Wnt1-Cre::SERT*$^{fl/fl}$ mice of both sexes relative to their WT counterparts

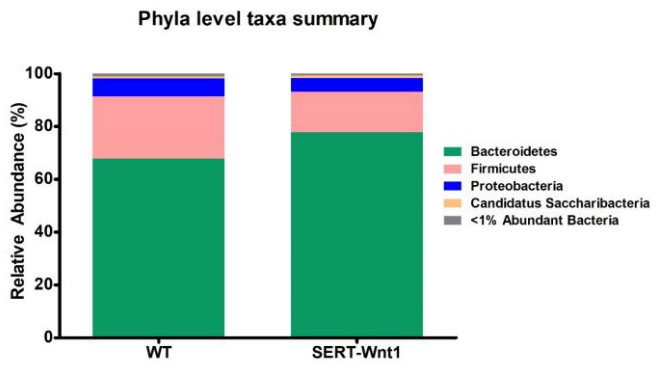
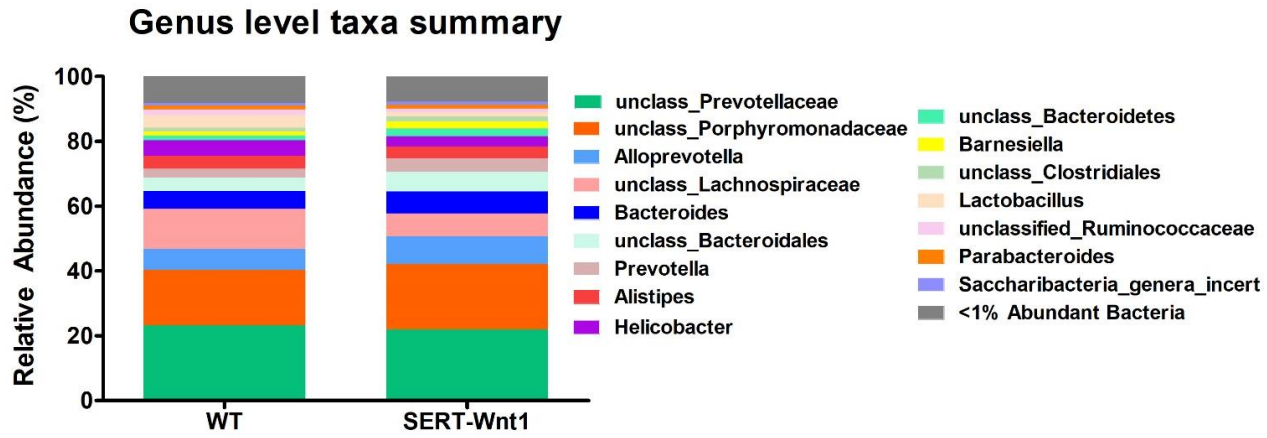


Figure 9: Two group comparison at the phyla and genera level did not reveal any significant differences at any taxonomic levels between *Wnt1-Cre::SERT*$^{fl/fl}$ and WT mice.



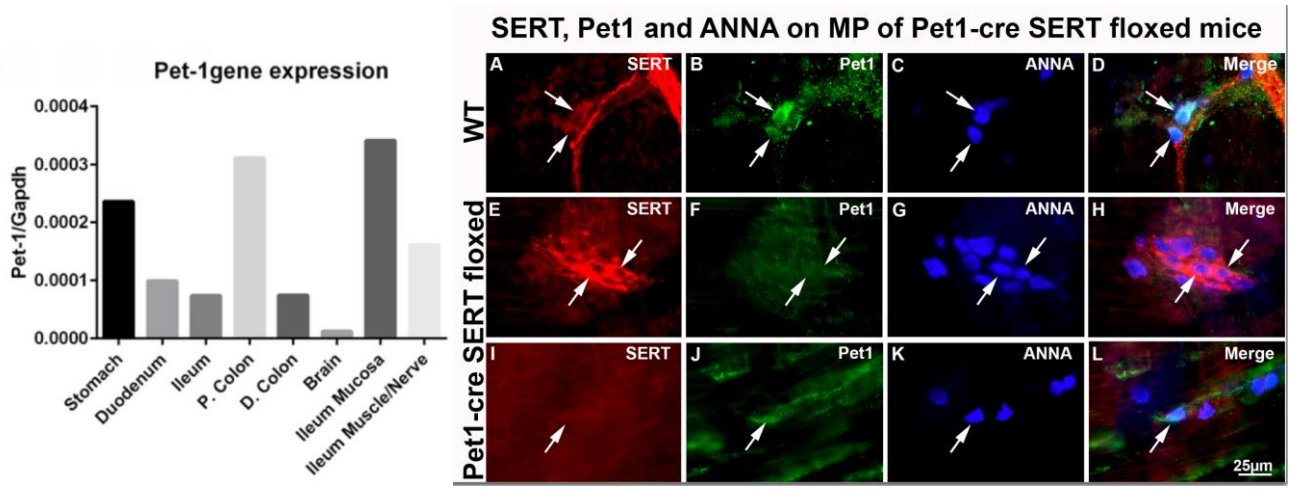


Figure 10: (A) Pet1 transcripts are located throughout the intestine , in both the epithelium and the ENS; (B) Pet1 protein is located in the myenteric neurons of WT male mice and *Pet1-Cre::SERT*$^{fl/fl}$ mice lack SERT is Pet1+ neurons.

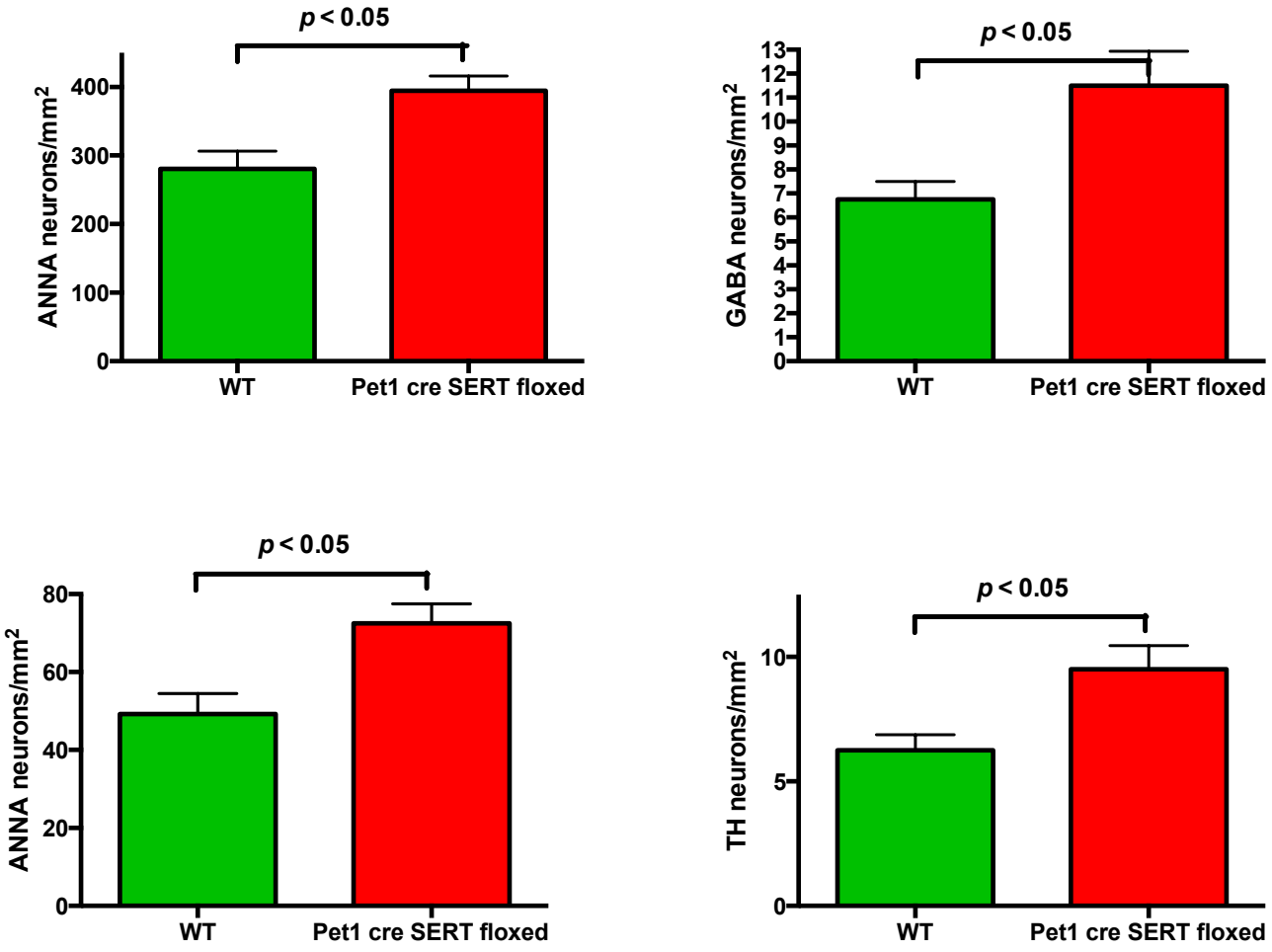


Figure 11: *Pet1-Cre::SERT*$^{fl/fl}$ mice have more total neurons in the myenteric (A) and submucosal (C) plexuses than WT mice. *Pet1-Cre::SERT*$^{fl/fl}$ mice have more GABAergic (B) neurons in the myenteric plexus and more dopaminergic (D) neurons in the submucosal plexus than WT mice.

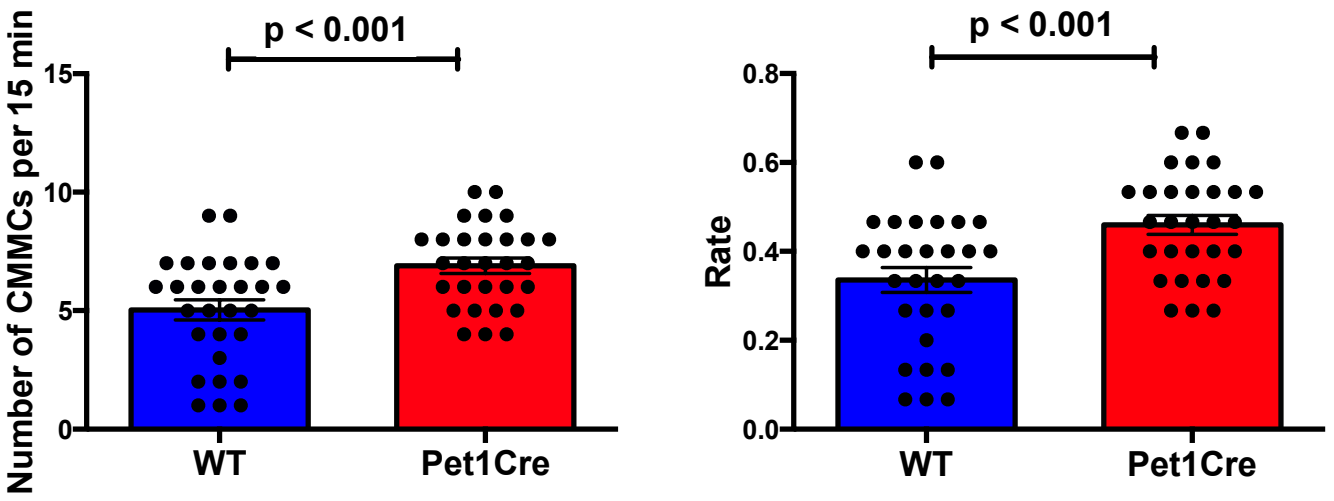


Figure 12: CMMC number and rate are significantly greater in *Pet1-Cre::SERT*$^{fl/fl}$ mice relative to WT mice.

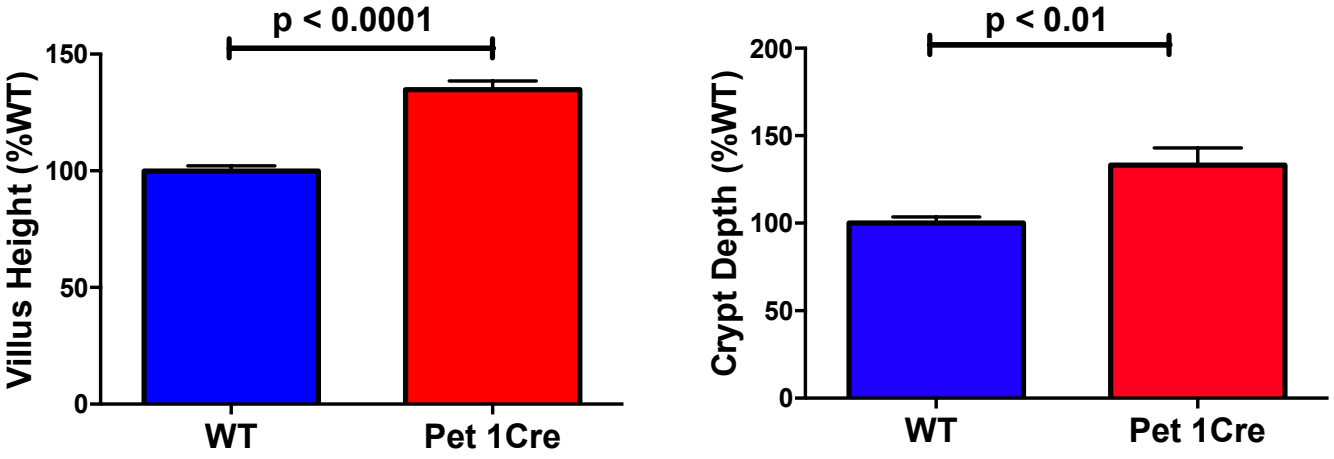


Figure 13: Villus height and crypt depth are significantly greater in male *Pet1-Cre::SERT*$^{fl/fl}$ mice than male WT mice

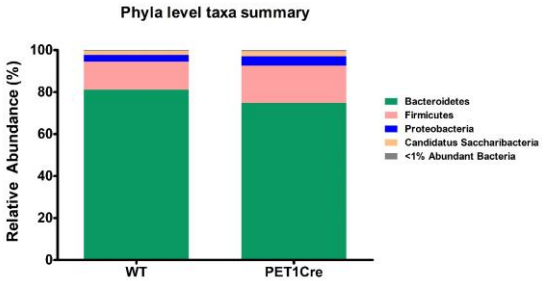
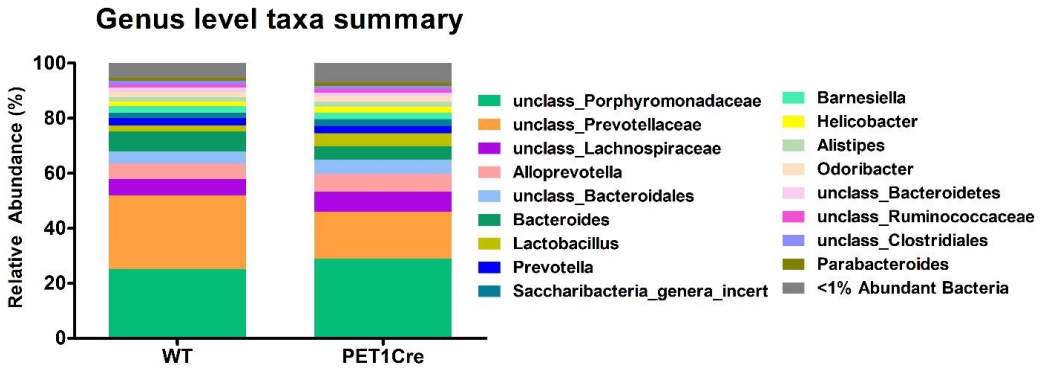


Figure 14: Two group comparison at the phyla and genera level did not reveal any significant differences at any taxonomic levels between *Pet1-Cre::SERT*$^{fl/fl}$ and WT mice.



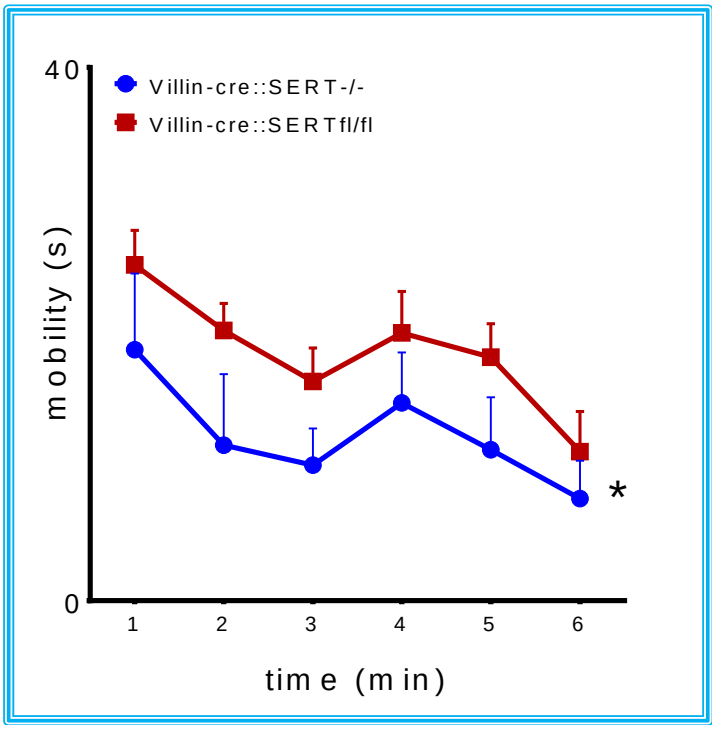


Figure 15: *Villin-Cre::SERT*$^{fl/fl}$ mice express an anti-depressive phenotype in the tail suspension test

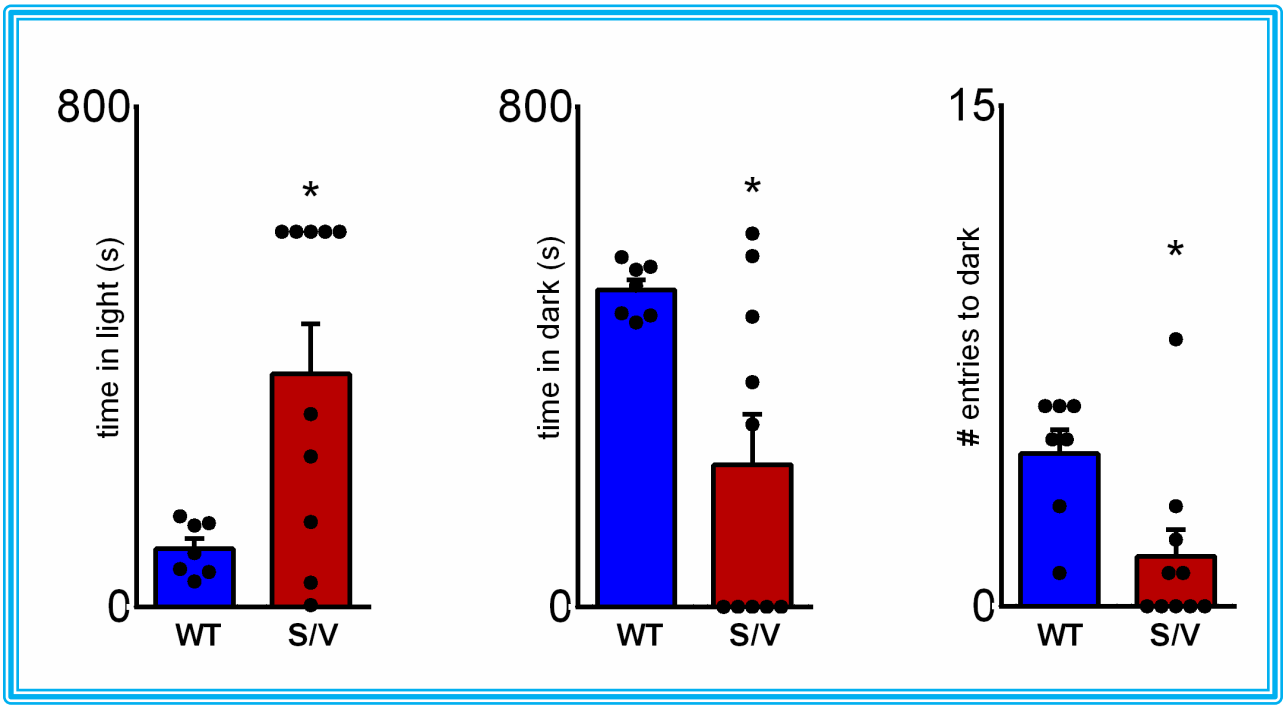


Figure 16: *Villin-Cre::SERT*$^{fl/fl}$ mice express an anxiolytic phenotype in the dark-light box test.

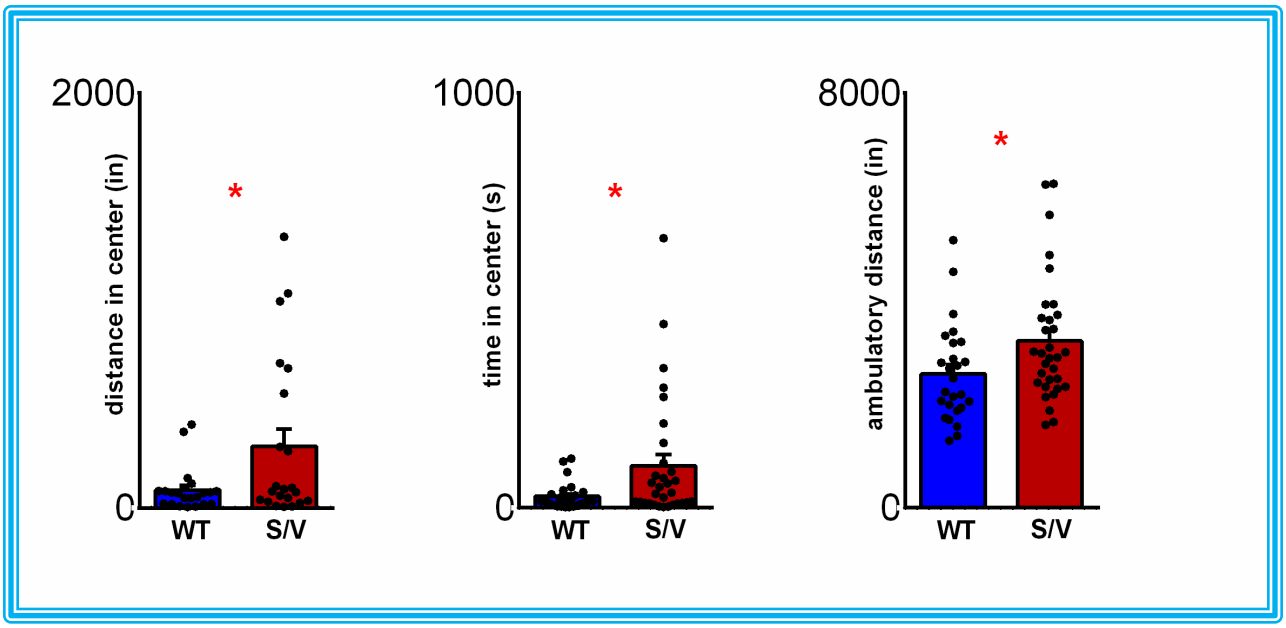


Figure 17: *Villin-Cre::SERT*$^{fl/}$mice express an anxiolytic phenotype in the open field test.

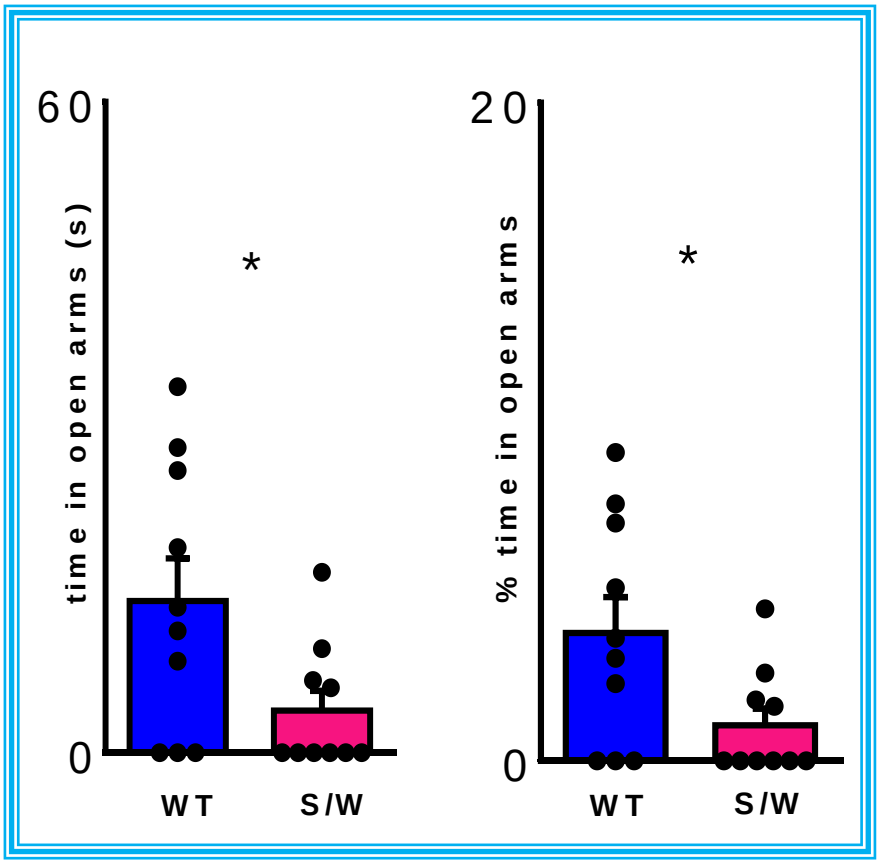


Figure 18: *Wnt1-Cre::SERT*$^{fl/}$mice express an anxiogenic phenotype in the elevated plus maze test

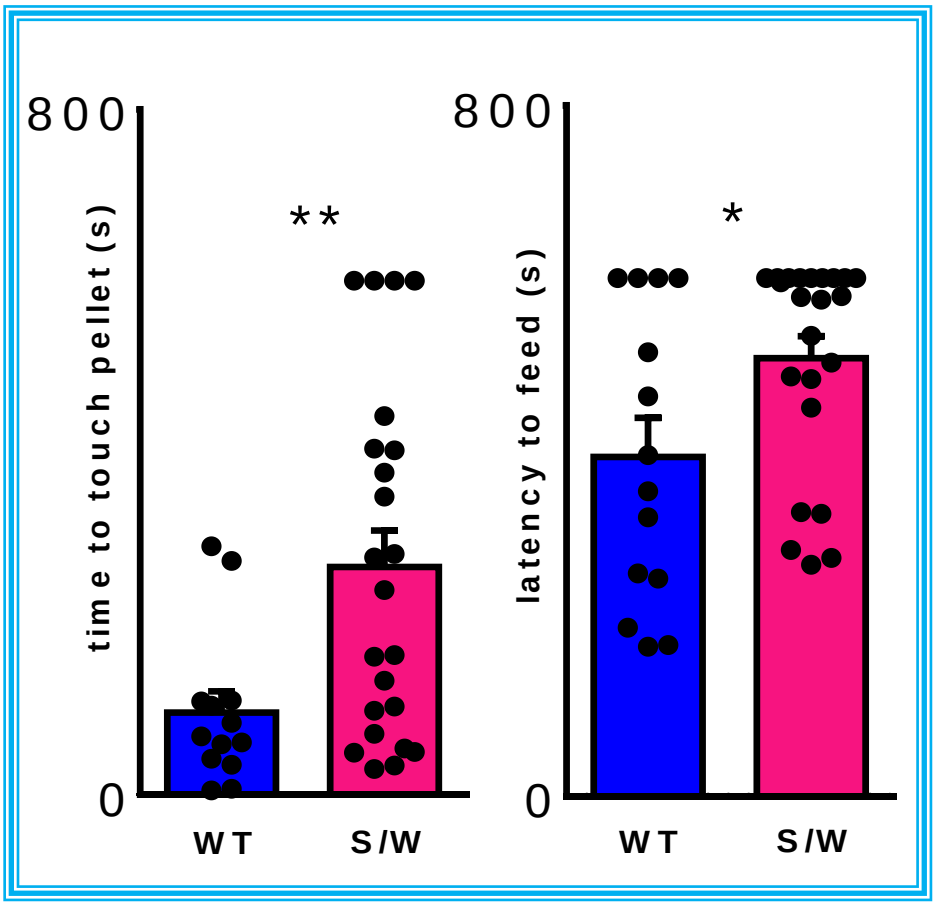


Figure 19: *Wnt1-Cre::SERT*$^{fl/}$ mice express an anxiogenic phenotype in the novelty suppressed feeding test.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report. This project was not intended to provide training and support.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report yet. We plan to disseminate information once the data is complete.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

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

These mice took a great deal of time to start breeding successfully and we were again stalled during the COVID19 pandemic. We made progress in the examination of 5-HT4 distribution in the gut and need to focus more on the brain. Now that we are again increasing our numbers of 5-HT4-GFP positive mice, we will be able to again implement the stated objectives shortly.

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

Although we were disappointed to not be 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 breastmilk 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 in utero. The experiment we set up to determine whether the lack of piboserod effect was due to ineffective transfer through the breastmilk rather than a lack of effect of piboserod, was to administer piboserod to 3 week old pups exposed to fluoxetine prenatally. Since the number of pups required for these experiments was not adequately achieved through our breedings for this set of experiments, we need to evaluate larger numbers of mice, as laid out in our proposal, to truly determine if piboserod is effective. We will start breeding these mice as soon as we are allowed to re-expand this colony (limited because of the COVID19 pandemic)

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

Our most intriguing findings over the past 1-2 years have been identified in the *Villin-Cre::SERT*$^{fl/fl}$ mice and the *Wnt1-Cre::SERT*$^{fl/fl}$ mice. We will thus focus on these mice in the current next phase of our studies.

For *Villin-Cre::SERT*$^{fl/fl}$ mice, the most interesting finding is the difference in in vivo versus ex vivo motility which, as stated above, could be the result of the microbiota, brain to gut communication and/or an increase in sympathetic drive to the gut which would result in a slowing of motility. The microbiome did not show major differences and evaluation of the metabolome should take place over the following year and we are also preparing to conduct chemical sympathectomy or vagotomy on these mice to evaluatethe role of sympathetic and parasymp[athetic control over motility as well as our newly discovered differences in mood.

For *Wnt1-Cre::SERT*$^{fl/fl}$ mice, we have now confirmed in the second generation of mice that colonic motility and gastric emptying are slower relative to WT despite the ENS hyperplasia. In order to look at neuronal signaling, we are currently identifying subsets of excitatory and inhibitory neurons and will ultimately evaluate neuronal transmission through calcium channel signaling. We will also prepare to evaluate sympathetic and parasympathetic signaling, as noted above for the *Villin-Cre::SERT*$^{fl/fl}$ mice, to determine if the motility abnormalities and mood dysfunction are connected through brain-gut axis signaling.

Given that *Pet1-Cre::SERT*$^{fl/fl}$ mice do not demonstrate abnormalities in in vivo motility it appears as though it is primarily an in vivo brain-modulatory model, at least in adulthood so can still be evaluated as a CNS-centric model. It's role in development, and how it affects brain-gut communication development will also be important for future studies. We will also need to examine in greater depth the role that Pet1 plays in ENS function. We will initially need to identify the percentage of neurons that are Pet1^{+}, which neurotransmitters Pet1 co-localizes with and if, as in the brain, all Pet1^{+} neurons are also 5-HT^{+}. In a future grant we will focus on the developmental trajectory of enteric Pet1 as well as the differentiation of its brain and intestinal effects in mice that lack Pet1 in the brain versus the gut.

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:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

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 proposed experiments and particularly the experiments proposed in aim 1. Some of these experiments (noted above) were further delayed because the lab was shut down for 4 months due to the COVID19 pandemic. We are now proceeding with all experiemnts, as described 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.

We have had significant delays because the lab had to shut down due to safety concerns during the COVID19 pandemic. In order to complete our work, we have received a no cost extension and will use this time to fulfill the stated objectives.

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. These delays were further exacerbated because of COVID19. In order to resolve our delay in work, we are having an additional post-doctoral student experienced in these techniques work on this grant over the course of the no-cost extension so that we can fulfill the objectives in an efficient manner.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not applicable.

There have been no significant changes in use or care of vertebrate animals.

Significant changes in use of biohazards and/or select agents

There have been no significant changes in use of biohazards and/or select agents.

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Madra M, Ringel R and **Margolis KG**. Gastrointestinal Issues and Autism Spectrum Disorder. Child and Adolescent Psychiatry Clinics of North America 2020 April; 29(3):501-513.

2. Saurman V, **Margolis KG*** and Luna RA*. Autism Spectrum Disorder as a Brain-Gut-Microbiome Axis Disorder. Digestive Disease Sciences 2020 March; 65(3): 818-828. *Special microbiome edition dedicated to Eamonn Quigley*. ***co-senior authors**

3. Hoffman JM and **Margolis KG**. Building community in the gut: serotonin as a mediator of bacterial colonization and host-microbe interactions. Nature Reviews Gastroenterology and Hepatology; *Online October 17, 2019;* doi:https://doi.org/10.1038/s41564-019-0540-4.

4. Del Colle A, Israelyan N and **Margolis KG**. Novel aspects of Serotonin Signaling in Health and Brain-Gut Disease. American Journal of Physiology 2019; 318(1):G130-G143.

5. Ferguson BJ, Dovgan K, Severns D, Famuliner S, Marler S, **Margolis KG**, Bauman ML, Veenstra-Vanderweele J, Sohl K, Beversdorf DQ. Lack of associations between dietary intake and gastrointestinal symptoms in autism spectrum disorders. Frontiers in Psychiatry 2019; Online July 25, 2019: https://doi.org/10.3389/fpsyt.2019.00528.

6. Israelyan N, Del Colle EA, Li ZS, Park YJ, Xing A , Jacobsen JPR , Luna RA, Jensen D, Madra M, Law K, Rahim R, Saurman V, Latorre R , Bunnett N, Caron MG, and **Margolis KG**. A Novel Role for Neuronal Serotonin in the Brain-Gut Axis. Gastroenterology 2019; doi: https://doi.org/10.1053/j.gastro.2019.04.022.

7. **Margolis KG****, Buie T, Turner, JB, Silberman AE, Feldman JF, Murray KF, Maureen McSwiggan M, Levy J, Bauman ML, Veenstra-VanderWeele J, Whitaker AH, and Winter HS. Development of a Brief Parent-Report Screen for Common Gastrointestinal Disorders in Autism Spectrum Disorder". Journal of Autism and Developmental Disorders 2018. J Autism Dev Disord. 2019 Jan;49(1):349-362. doi: 10.1007/s10803-018-3767-7. ****corresponding author. Paper highlighted on "Medscape".**

8. Robson MJ, Quinlan MA, **Margolis K**, Gajewski-Kurdziel PA, Veenstra-Vanderweele J, Gershon MD and Blakely RD. P38alpha MAPK Signaling Drives Pharmacologically Reversible Brain and Gastrointestinal Phenotypes in the SERT Ala56 Mouse. PNAS 2018 Oct 23;115(43): E10245-E10254.

9. Lavoie B, Roberts JA, Haag MM, Spohn SN, **Margolis KG**, Sharkey KA, Lian JB and Mawe GM. Gut-derived serotonin contributes to bone deficits in colitis. Pharmacol Res. 2018. Pii S1043-6618(18)30234-2.

10. Demireva EY, Suri D, Morelli E, Mahadevia D, Chuhma N, Teixeira CM, Ziolkowski A, Hersh M, Fifer J, Bagchi S, Chemiakine A, Moore H, Gingrich JA, Balsam P, Rayport S, **Ansorge MS**. 5-HT2C receptor blockade reverses SSRI-associated basal ganglia dysfunction and potentiates therapeutic efficacy. Mol Psychiatry. 2018 Aug 17.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Beversdorf DQ, Stevens HE, Jones KL, **Margolis KG**, Van de Water J. Prenatal stress, maternal immune dysregulation, the microbiome and autism spectrum disorders. In: Gregory Barnes & Ayman El Baz, Eds. The Clinical and Basic Neuroscience of Autism Spectrum Disorders. CRC Press/Taylor & Francis Group, Oxfordshire, *in press*.

2. Law KM, Ferguson BJ, **Margolis KG*,** Beversdorf DQ* (2020). Gastrointestinal symptoms, anxiety, and autism spectrum disorder. S.M. Edelson & J.B. Johnson (Eds.), *Understanding and treating anxiety in autism*. London: Jessica Kingsley Publishers, *in press*.***co-senior authors**

Other publications, conference papers and presentations.

Oral Abstract Presentations

1. Hung LY, Del Colle EA, Law K, Burly C, Ringel R and **Margolis KG.** Maternal Separation Results in Long-Term Deficits in ENS Development and Function. International Neurogastroenterology Conference (Federation of Neurogastroenterology and Motility) 2020; Adelaide, Australia (oral presentation-conference cancelled due to Covid pandemic). Acknowledgement of federal support: yes.

2. Law K, Del Colle EA, Burly C, Ringel R and **Margolis KG**. Maternal Separation Results in Long-Term Deficits in ENS Development and Function. NASPGHAN 2019 (oral presentation). Acknowledgement of federal support: yes.

3. Evan Del Colle EA, Israelyan N, Park Y, Xing A, Li ZS, Caron M, Jacobson J, Gershon MD and **Margolis KG**. Genetic and Functional Analysis of Neuronal Serotonin in a Murine Model of Depression. Digestive Disease Week 2019 (oral presentation). Acknowledgement of federal support: yes.

4. Evan Del Colle EA, Israelyan N, Park Y, Xing A, Li ZS, Caron M, Jacobson J, Gershon MD and **Margolis KG**. Genetic and Functional Analysis of Neuronal Serotonin in a Murine Model of Depression. Digestive Disease Week 2019 (oral presentation). Acknowledgement of federal support: yes.

5. Israelyan N, Park Y, Del Colle EA, Xing A, Li ZS, Caron M, Jacobson J, Gershon MD and **Margolis KG**. A Role for Sustained-Release 5-HTP in Constipation. North American Society for Pediatric Gastroenterology, Hepatology and Nutrition 2018. Acknowledgement of federal support: yes.

State of the Art/Symposium Presentations

"Gastrointestinal Problems in Autism Spectrum Disorders"; Pediatric Grand Rounds, Children's Mercy Hospital, Kansas City; Kansas City, Missouri (presented by webinar due to Covid pandemic). Acknowledgement of federal support: yes.

"The Enteric Nervous System in the Context of Behavioral Disorders", State of the Art Talk for the International Gastronauts Symposium: the goal of the symposium is to bring experts from multiple disciplines to set the vision for future directions and collaborations in the field of gut-brain biology; Nantes, France (postponed due to Covid pandemic). Acknowledgement of federal support: yes.

"Gastrointestinal Dysfunction in autism spectrum Disorders", State of the Art Talk for the International Neurogastroenterology and Motility Meeting; Adelaide, Australia (postponed due to Covid pandemic). Acknowledgement of federal support: yes.

Serotonin as Brain-Gut Connection in Autism"; Pediatric Research Grand Rounds for the New York University School of Medicine; New York, NY. Acknowledgement of federal support: yes.

"A Genetic and Functional Analysis of Neuronal Serotonin in Constipation and Depression"; Baylor School of Medicine, Research Grand Rounds; Houston, Texas. Acknowledgement of federal support: yes.

"The Role of Peripheral Serotonin Modulation in Depression"; Neuroscience and Neurology Research Seminar Series, University of Vermont; Burlington, VT. Acknowledgement of federal support: yes.

"The Role of the Serotonin in the Brain, the Gut and the Microbiome in Brain-Gut Axis Disease"; Neuroscience Grand Rounds, Florida Atlantic University; Jupiter, Florida. Acknowledgement of federal support: yes.

"The Role of Serotonin in Brain-Gut Axis Disease"; Autism research Institute Autism Think Tank; Dallas, TX. Acknowledgement of federal support: yes.

"Autism as a Brain-Gut Axis Disorder: The Role of Serotonin", State of the Art Talk for the

International Neurogastroenterology and Motility Meeting; Amsterdam, Netherlands. Acknowledgement of federal support: yes.

"Serotonin as a Brain-Gut Link in Depression"; Symposium Speaker at the International Serotonin Conference; Cork Ireland. Acknowledgement of federal support: yes.

Ansorge MS. Speaker: "Monoamines and brain development - sensitive developmental periods impacting adult emotional behaviors and cognitive function". From Sensory Maps to Serotonin and Developmental Disorders. In Honor of Patricia Gaspar. Paris, France. October 2018. Acknowledgement of federal support: yes.

Ansorge MS. Speaker: "Serotonin modulates maturation of afferent projections to the amygdala". International Society for Serotonin Research. Cork, Ireland. July 2018. Acknowledgement of federal support: yes.

Ansorge MS. Speaker: "Monoaminergic circuit mechanisms mediating developmental malleability of emotional and cognitive function". New York, CUNY, Advanced Science Research Center, May 2019. Acknowledgement of federal support: yes.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  None to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  None to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  None to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

  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".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

There are minor changes in the personnel that has worked on this project

Name:	Ruth Anne Luna, PhD
Name:	Mark Ansorge, PhD
Name:	Zi Shan Li, PhD
Name:	Yeji Park, MS left so Evan Del Colle, MS took over with her responsibilities

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Kara Margolis currently receiving 10% salary support from a current RO1:

4RO1NS015547-34 (PI: Margolis and Gershon) 1/1/2018 – 12/31/2023 NIH/NIN
Microenvironment in Enteric Neuron Development

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

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: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A

duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. **APPENDICES:** Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.