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Evaluation of Mucosal 5-HT Signaling and Potential Sex Differences in Motor Activity in the Experimental Autoimmune Encephalomyelitis Mouse Model of Multiple Sclerosis

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Abstract

Multiple Sclerosis (MS) is an autoimmune disease that causes a myriad of symptoms, including cognitive, sensory, and motor deficits, as well as gastrointestinal (GI) dysmotility. We have previously demonstrated that male mice with experimental autoimmune encephalomyelitis (EAE), the predominant model for MS, have disrupted GI motility, but the mechanisms for this dysmotility are not understood, and potential sex differences have not been evaluated. Since 5-hydroxytryptamine (5-HT; serotonin) is a mediator of motor and secretory reflexes in the gut, we investigated key elements of mucosal 5-HT signaling in the EAE mouse model of MS. Enzyme linked immunosorbent assay (ELISA) was used to evaluate 5-HT tissue levels as normalized by a bicinchoninic acid assay and RT-qPCR was used to evaluate mRNA expression for the rate limiting molecule in 5-HT synthesis, tryptophan hydroxylase 1 (Tph1), and the serotonin reuptake transporter (SERT), both of which were normalized to the housekeeping gene beta 2 microglobulin (B2M). Motility and fecal composition were also compared in female versus male mice with EAE. The mRNA expression for Tph1 was decreased in EAE mice, but levels of 5-HT in the tissue and SERT mRNA expression were unchanged. Furthermore, no differences were detected in colonic motility, fecal water content, or whole GI transit in female versus male mice with EAE. However, it was noted that male mice with EAE displayed peak GI dysfunction at day 21, whereas female mice with EAE showed peak GI dysfunction at day 27. These findings indicate that female and male mice present with similar levels of GI dysfunction over time, representing comparable models for motility disruption in EAE, and suggest that mechanisms other than altered 5-HT signaling are responsible for altered motility in this model.

Introduction

Multiple Sclerosis and GI Dysfunction

Multiple Sclerosis (MS) is typically characterized as an inflammatory autoimmune disease of the central nervous system (CNS) in which T cells attack the myelin sheaths of axons resulting in scar tissue, sclerosis, and nerve fiber damage. This damage and loss of myelin creates a disruption of nerve signals resulting in a wide expanse of symptoms including movement disabilities (National Multiple Sclerosis Society, 2018). An overlooked component of these movement disabilities includes gastrointestinal (GI) motility, as up to two thirds of all MS patients report having bowel dysfunction, with constipation and fecal incontinence being the predominant symptoms (Future, Joy, & Richard B. Johnston, 2001; Levinthal et al., 2013; Preziosi et al., 2013). A recent report stated that approximately fifty percent of individuals with MS will experience constipation, with thirty percent of the MS population also experiencing fecal incontinence (Future et al., 2001). Despite the common prevalence of these symptoms and general GI dysfunction in the MS population, the exact etiology behind this dysfunction is still unclear.

A prominent experimental approach in attempting to understand these mechanisms is derived from the use of the experimental autoimmune encephalomyelitis (EAE) mouse model of MS. To this extent, a recent study by the Mawe laboratory (Spear et al., 2018) showed that male mice with EAE demonstrate altered GI function consistent with MS-related constipation, validating the model as a potential means of examining the molecular changes that might occur leading to the noted alterations in GI function. Furthermore, recent studies have indicated the involvement of the enteric nervous system (ENS) as a target of circulating auto-antibodies in MS and EAE (Spear et al., 2018; Wunsch et al., 2017). The ENS is the neuronal governing system of GI tract motor and secretory functions, and consists of cellular aspects and proteins that are similar

to those of the CNS. In this regard, the studies by the Mawe and Kürten labs (Spear et al., 2018; Wunsch et al., 2017) indicated that there is an involvement of autoantibodies in MS, which targets the neurons and glial cells of the ENS, similar to those that target the CNS, and might mediate components associated with GI dysfunction. However, despite these breakthrough findings, the exact neuronal mechanisms that lead to the alterations in motility are still unknown.

The Role of Serotonin in GI Motility

One possible mechanism leading to the alterations in MS-related GI motility involves the role of serotonin. Serotonin (5-hydroxytryptamine; 5-HT) is an intercellular signaling molecule that initiates, regulates, and modifies GI motility, secretion, and vasodilation (Spohn & Mawe, 2017). The majority of 5-HT in the body is synthesized in the GI tract, with a subtype of enteroendocrine cell, the enterochromaffin cell, being the primary source of 5-HT. Enterochromaffin cells function as sensory transducers and use the rate limiting molecule tryptophan hydroxylase 1 (Tph1) to initiate the synthesis of L-tryptophan to 5-HT. Unlike acetylcholine, 5-HT does not have a counterpart intercellular degrative enzyme to terminate signaling, and thus requires a specialized transport molecule, serotonin selective reuptake transporter (SERT), to remove 5-HT from the intercellular space and stop receptor activation. Once it is transported, 5-HT can be recycled into vesicles or degraded by monoamine oxidase (Mawe & Hoffman, 2013).

Serotonin signaling has been investigated in animal models of GI inflammatory diseases and in human samples from ulcerative colitis and diverticulitis patients. An alteration consistent in these conditions is decreased epithelial expression of SERT with the exact 5-HT signaling pathway alterations varying for each disease (Coates et al., 2017). As MS is associated with

inflammation, it stands to reason that a contributing factor of this inflammation and GI dysfunction could correspond to altered levels of 5-HT and SERT. This is supported by preliminary data from the Mawe lab examining 5-HT levels outside the epithelium in colon preparations of EAE versus control mice using amperometry. In these experiments, 5-HT was measured at the mucosal surface. These findings showed that there was a significantly higher concentration of 5-HT measured at the mucosal surface of colons from the EAE mice (figure 1 A), and that this difference was eliminated in the presence of fluoxetine, a SERT inhibitor (figure 1 B). These data suggest an alteration of 5-HT signaling in EAE in correlation to a decreased expression and/or function of SERT. Based on this preliminary data and previous literature it could be suggested that an alteration in 5-HT mechanisms might be associated with the GI dysfunction exhibited by the EAE model. ***This led to the hypothesis that there is an increase in 5-HT availability in the EAE GI tract driven by a decrease in the 5-HT transporter SERT.***

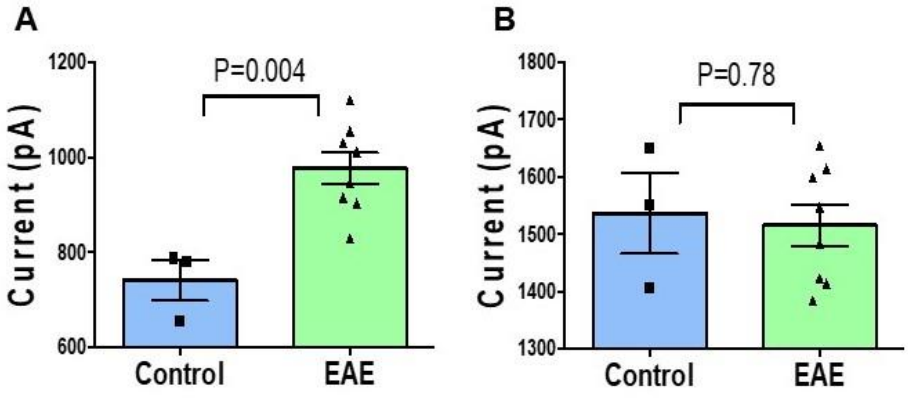

Figure 1 Preliminary data from the Mawe lab examining 5-HT levels outside the epithelium of EAE colon preparations using amperometry. There is a significantly higher concentration of 5-HT in EAE colon tissue as compared to controls (A, $^{**}P < 0.01$), which disappeared after the addition of fluoxetine, a SERT inhibitor (B, n.s.).

Sex Differences in Multiple Sclerosis/EAE

As previously mentioned, it has been validated that the EAE model demonstrates GI dysmotility consistent with that of MS (Spear et al., 2018; Wunsch et al., 2017). However, the strains of mice selected and sex of the animals used for studies have varied across experiments, yielding slightly different results in susceptibility to EAE depending on these variables (Constantinescu et al., 2011). This is somewhat consistent with the general heterogeneric nature of MS as a whole and its sexually dimorphic characteristics. In fact, the EAE model has been utilized to examine sex differences in disease presentation of MS (Spach et al., 2009; Teuscher et al., 2004, 2006). These studies have shown that there is both an involvement of the Y chromosome as well as an age dependent factor in disease susceptibility in the model (Spach et al., 2009; Teuscher et al., 2006). Similarly, investigations along these lines have indicated that the sex of the mouse is the strongest contributing factor in the development of the hallmark brain and spinal cord lesions associated with EAE when also taking into consideration the age of the mouse and season of induction of the disease (Teuscher et al., 2004). Despite this critical investigation into possible sex differences within the EAE model, most of the work to date has focused on differences within the CNS derived symptomology of EAE and does not indicate whether or not these differences in susceptibility and effectiveness as a model might exist in the ENS, specifically in terms of modeling GI dysmotility.

That said, previous data from the Mawe lab has predominately focused on the use of male mice. This focus was derived from the fact that male mice in the hands of the Mawe lab have demonstrated a high rate of consistent EAE disease presentation in terms of characteristic motor symptoms ranging from tail weakness to quadriplegic status and have therefore been most associated with GI dysfunction, whereas female mice tend to classify as asymptomatic. Despite

this, preliminary data from the Mawe lab has shown that female mice reported to be largely asymptomatic, or did not develop severe motor symptoms, still showed altered fecal water content (figure 2 A), which would be consistent with GI dysfunction. These data appear to indicate that GI symptoms can be associated with mice that present as asymptomatic, as do most female mice in the Mawe lab studies, supporting the idea that female and male mice could represent comparable MS GI dysmotility models despite the apparent difference in EAE susceptibility between the two sexes.

Further supporting this possibility, a population of asymptomatic male mice in a recent study by the Mawe lab showed that there was an increase in colonic transit time (slower motility) measured using the bead expulsion assay (figure 2 B) and therefore could be classified as having altered GI motility despite presenting without the classical EAE motor symptoms. Collectively, however, these data were not strong enough to conclude that asymptomatic mice will exhibit GI dysfunction, as there is still a strong correlation between an increase in motor symptom severity and GI dysfunction. This leaves much to speculation as to whether or not mice that are asymptomatic, such as the female population used in the Mawe lab, actually develop GI dysfunction comparable to the symptomatic male mice. Therefore, based on these preliminary data, ***we tested the hypothesis that female mice can in fact serve as comparable models to their male counterparts in terms of displaying GI dysfunction consistent to that of MS.***

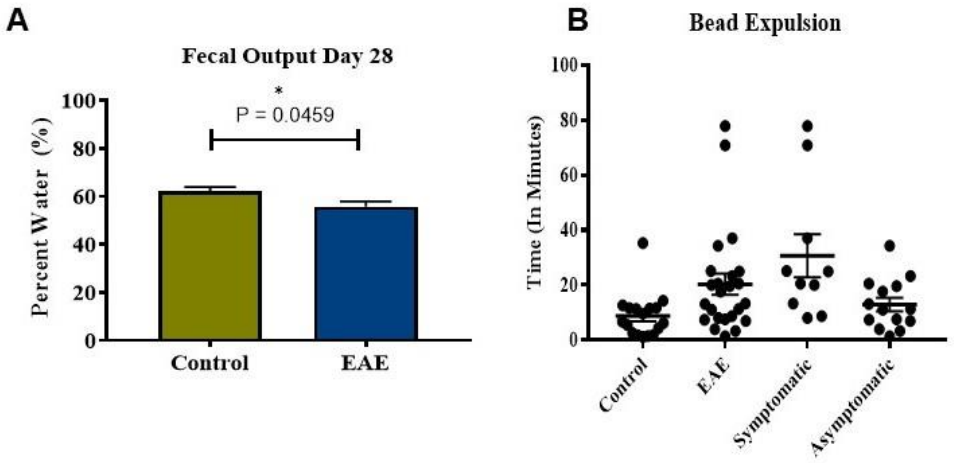

Figure 2 Preliminary data from the Mawe lab regarding potential sex derived differences in GI motility. C57BL/6J female mice that were largely asymptomatic for EAE or did not develop severe motor symptoms, presented with altered fecal water content (A). Male C57BL/6J mice reported to be asymptomatic for EAE presented with slowed colonic motility (**P* <0.05) as measured by bead expulsion (B). (A, control n = 6, EAE n = 8, **P* <0.05; B, control n = 17, EAE n = 24, symptomatic n = 10, asymptomatic n = 14). Data analyzed using an unpaired t test.

Study Aims

As can be seen from gaps in the literature, there is still much to be discovered in terms of the implications between the ENS and MS and the impact on GI dysfunction. In this regard, it is the goal of this study to contribute to the field by providing a novel assessment on the role of mucosal 5-HT signaling within the EAE GI tract and the potential sex differences in GI motility of EAE mice. This assessment was accomplished through both molecular and *in vivo* motility assays. The molecular assays, including RT-qPCR to examine Tph1 and SERT mRNA expression and an enzyme-linked immunosorbent assay (ELISA) to assess the tissue 5-HT content, were utilized to fulfil the goal of examining key elements of 5-HT signaling. In parallel, the *in vivo* GI motility assays, including an analysis of colonic motility as assessed by bead expulsion, constipation as measured by fecal water content, and an examination of whole GI transit, allowed for an assessment into the GI motor activity of both female and male mice.

Methods

Animals

For the *in vivo* GI motility assays used in this study, two cohorts of female and male C57BL/6J mice approximately 8 weeks of age were utilized. The first cohort of mice were obtained from within the Sox10/GCAMP transgenic C57BL/6J mouse line of the Mawe lab mouse colony and consisted of 26 mice (14 EAE and 12 controls, which comprised of 7 EAE female/males and 6 control female/males). Similarly, the second cohort of mice were C57BL/6J mice purchased from Jackson Labs, Bar Harbor, ME, and had a total of 38 mice (19 EAE and 19 controls, which comprised of 9 female controls, 10 male controls, 10 female EAE, and 9 male EAE). All experimental animal protocols used for this study were approved by University of Vermont Institutional Animal Care and Use Committee.

EAE Induction

Female and male C57BL/6J mice were randomly divided into control and experimental condition groups. The experimental conditioned mice were induced with EAE using the 2X MOG protocol, as follows. Mice were immunized with a sonicated emulsion of myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) and complete Freund's adjuvant (CFA) containing 200 μg *Mycobacterium tuberculosis* H37RA (Becton Dickinson & Co). The mice then underwent a 2X induction protocol, in which they received two posterior right and left flank subcutaneous injections of the emulsion each a week apart with the second injection being anterior to the initial injection site as previously described (Spear et al., 2018). The control mice followed the same protocol, however they only received CFA injections. Daily evaluations included recordings of weight and disease severity scores beginning 14 days postimmunization until the end of the trial

on day 28 as previously described (Spear et al., 2018). EAE disease scoring was based off of a 0 to 5 scale in which a 0 was asymptomatic, 1 was tail paralysis, 2 was tail paralysis and hind limb weakness, 3 was tail and hind limb paralysis, 4 was tail and hind limb paralysis and urinary incontinence, and 5 was moribund. Once a mouse reached a score of 3, food pellets and Napa Nectar were placed at the bottom of the cage to assure nutrient uptake. Fluid injections were also given to mice if they were noted to have lost 30 percent of their initial body weight, mice that consistently lost weight at this percentage and did not respond to fluid injections were euthanized. Cumulative disease scores were calculated by adding together the daily somatic disease scores across the span of the experiment to represent the total disease score accrued.

GI Motility Assays

Three assays were utilized to assess *in vivo* GI motility: bead expulsion, fecal output and water content, and an evaluation of whole GI transit. Bead expulsion and fecal water content were conducted both as a baseline measure on day 0 before induction of EAE as well as an experimental measure, whereas the whole GI transit was only conducted as an experimental measure. All motility assays were completed by day 28 of the experiment, with the fecal water and bead expulsion assays occurring on day 27 and the whole GI transit assay occurring on day 28. The second cohort of mice also underwent an additional GI motility time point assessment at day 21.

Bead Expulsion

The bead expulsion assay was utilized to assess colonic transit time. Mice were lightly anesthetized with isoflurane and a 2.3 mm bead was inserted approximately 20 mm into the distal colon using a blunt gavage needle. Time of bead insertion, time of recovery from anesthesia, and

time of bead expulsion were all recorded, allowing for an assessment of total colonic transit time as measured by the time elapsed between insertion and bead expulsion. This assay was conducted as a baseline measure before the induction of EAE and as an experimental measure at day 27 to allow for a comparison in terms of progression of symptoms. The second cohort of mice also underwent another time point assessment, in addition to the one at day 27, at day 21 to allow for a more in-depth analysis of colonic motility during the disease course.

Fecal Water Content

Each mouse was placed in an individual cage and monitored for 1 hour. During this time, deposited fecal pellets were collected and weighed. The pellets were then dried for a period of 24 hours at 50˚C and reweighed to allow for a calculation of fecal water content. The number of pellets was also recorded in order to better understand the changes in motility. This assay was conducted as a baseline measure before the induction of EAE and as an experimental measure at day 27. The second cohort of mice also underwent another time point assessment, in addition to the one at day 27, at day 21 to allow for a more in-depth analysis of potential GI dysmotility during the EAE disease course.

Whole GI Transit

Mice were placed in individual cages without bedding and administered an oral gavage of 300 µl 6% carmine red and 0.5% methylcellulose (Sigma Aldrich) in water. They were then given food and water *ad libitum* and monitored until carmine red was noted in the fecal pellets, indicating the total time elapsed for whole GI transit to occur.

Tissue Collection

After completion of the 28-day experimental period following previous Mawe lab procedures, the mice were euthanatized and their colons dissected and separated into six segments for potential use in molecular analysis.

Examination of 5-HT Signaling

For the purpose of this portion of the study previously obtained EAE and control colon segments from C57BL/6J were utilized. These colon segments were stored at -80˚C and originated from experiments related to the Spear et al. study, following the 2X MOG protocol. Key aspects of 5-HT signaling were assessed including mRNA expression of the synthetic enzyme, tryptophan hydroxylase 1 (Tph1), and serotonin selective reuptake transporter (SERT) using RT-qPCR, as well as tissue 5-HT content using an enzyme-linked immunosorbent assay (ELISA).

RT-qPCR

For the purpose of this study RT-qPCR was utilized in order to examine the mRNA expression of 5-HT, as measured by Tph1 and SERT (primers obtained from Thermofisher). RNA was extracted from colon preparations using a RNeasy Mini Kit (Qiagen) and analyzed using a NanoDrop Spectrophotometer in the COBRE laboratory. Samples then underwent RT-PCR to convert the isolated RNA to stable cDNA for use in qPCR experiments. All qPCR experiments were conducted using Applied Biosystems 7500 Fast Real-Time PCR System under Standard 7500 Mode in the COBRE laboratory, therefore yielding a quantification of mRNA expression of Tph1 and SERT in EAE samples relative to control samples. These data were then normalized to a selected housekeeper gene, beta 2 microglobulin (B2M), that demonstrated no significant

difference between the groups despite experimental condition, allowing for a more accurate analysis of mRNA expression between groups.

ELISA

A 5-HT ELISA kit (Beckman Coulter) was used to directly examine the tissue 5-HT content in the GI tract of EAE versus control mice as quantified by the microplate reader in the COBRE laboratory and standardized using a known concentration serotonin solution ranging from 0 nM – 200 nM. These data were then normalized to the total amount of protein present in the samples through use of bicinchoninic acid assay with the Pierce BCA kit (Thermofisher).

Statistical Analysis

All statistical analysis was completed using GraphPad Prism for Windows 7. Data were analyzed using an unpaired t test in order to determine statistical significance. Additionally, outliers were identified and excluded from the data sets using a Q test (2 standard deviations from the mean). Statistical significance is denoted as * P <0.05, ** P <0.01, *** P <0.001, and **** P <0.0001.

Results

Female and male mice have similar disease score characteristics

Cumulative disease score was obtained through an addition of the daily somatic disease scores across the total experimental period, allowing for an illustration of the total disease severity. There was no significant difference between the female EAE and male EAE cumulative disease scores in either the first or second cohort of mice (figure 3). These data suggests that both female and male EAE mice establish a similar degree of disease severity across the experimental period.

The day of disease onset was determined based on the first experimental day that a score other than 0, asymptomatic, was recorded. There was no significant difference between female and male EAE mice in the day of disease onset for either the first or second cohort (figure 4). These data suggest that female and male EAE mice have a similar systemic disease onset time course as measured by the EAE scoring protocol.

Average daily disease scores across the experimental period were examined in order to investigate the potential differences in disease activity for female and male mice. Visually the disease activity was closely mirrored between female and male mice in both cohorts (figure 5 A & B), with the caveat that female mice in the second cohort presented with disease activity that was generally slightly less severe than male mice (figure 5 B). Additionally, male mice were shown to have a slightly higher average daily disease score than female mice around experimental day 21 in both cohorts (figure 5 A & B). In contrast, female mice were shown to have a slightly higher average daily disease score than males on experimental day 17 and 18 for the first and second cohort respectively (figure 5 A & B). However, analysis of these individual time points shows no significant difference between female and male daily disease activity (figure 5 C - F). Taken together these data suggest that female and male mice have similar general daily disease activity.

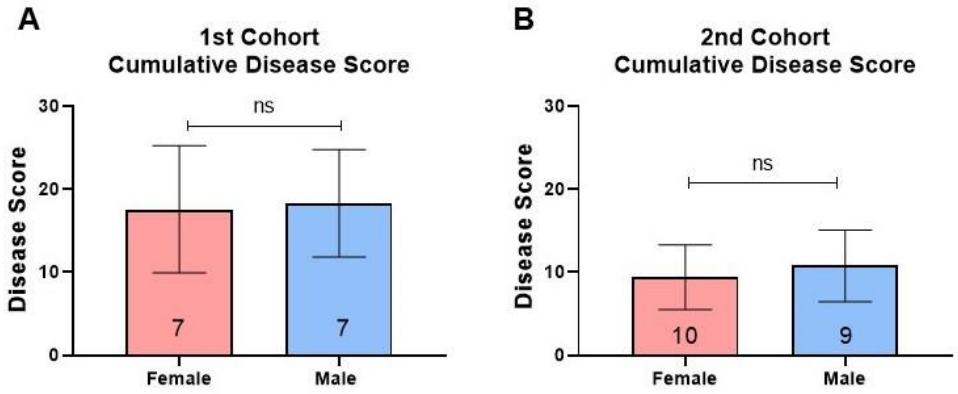

Figure 3 Representation of severity of EAE disease course through cumulative disease score. Female and male mice were shown to represent equal models in terms of EAE severity. (A, female n = 7, male n = 7 n.s.; B, female n = 10, male n = 9, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

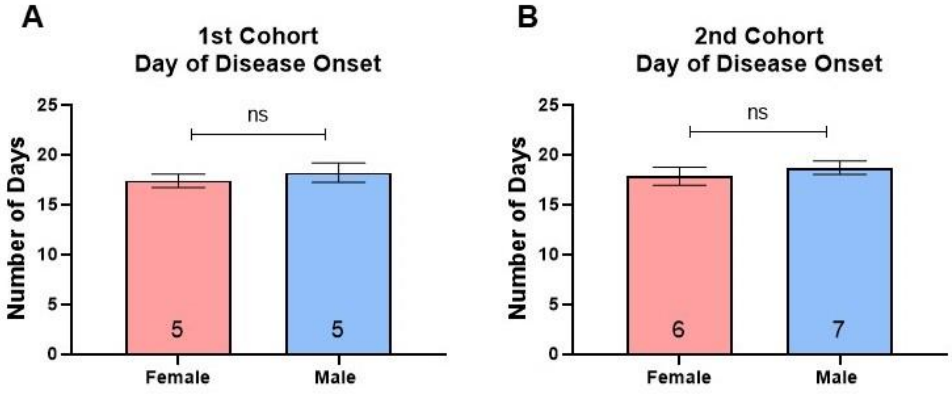

Figure 4 Day of disease onset was determined by the first day that a score other than 0 was recorded, mice that only received a score of 0 were excluded. Female and male mice were shown to represent equal models in terms of day of disease onset across the experimental period. (A, female n = 5, male n = 5, n.s.; B, female n = 6, male n = 7, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

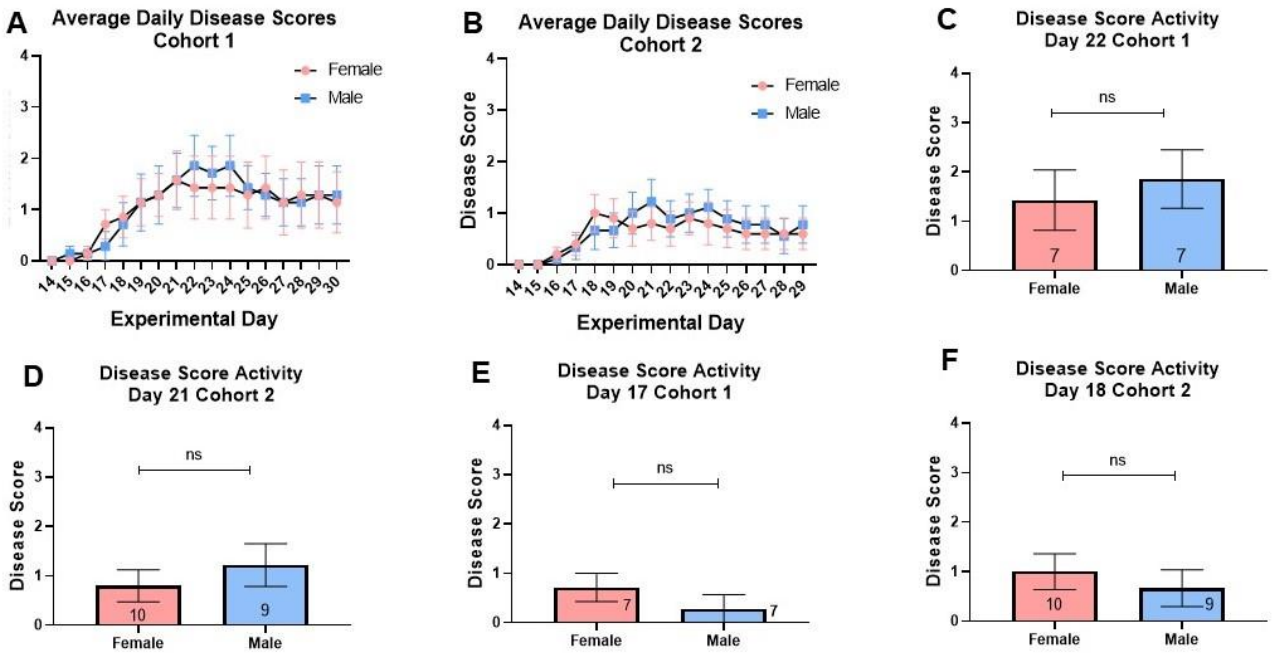

Figure 5 Average daily disease scores were reported over the course of the experiment. Daily disease activity was closely mirrored between both sexes (A & B). However, male mice were shown to have a slightly higher but not significantly different disease activity around experimental day 21 for both cohorts (C & D) and female mice were shown to have slightly higher but not significantly different disease activity on experimental day 17 and 18 for the first and second cohort respectively (E & F). (A, female n = 7, male n = 7; B, female n = 10, male n = 9; C, female n = 7, male n = 7, n.s.; D female n = 10, male n = 9, n.s.; E female n = 7, male n = 7, n.s.; F female n = 10, male n = 9, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

Female and male mice represent comparable GI motility models

Bead expulsion, fecal water content, and whole GI transit were all utilized to assess the possibility of sex derived motility differences in the EAE mouse model. Bead expulsion allowed for an assessment of colonic motility, measured through the time elapsed between bead insertion and expulsion. As previously demonstrated by experiments in the Mawe lab, bead expulsion on experimental day 27 for the combined EAE female and male mice was shown to be significantly slower than control mice for both cohort one, $P = 0.0268$, and cohort two, $P = 0.0037$ (figure 6), signifying delayed colonic motility in EAE mice. However, no significant difference was noted amongst both studied cohorts of mice when comparing female EAE to male EAE mice (figure 7).

These data suggest that both female and male mice exhibit comparable levels of GI dysfunction in terms of decreased colonic transit time.

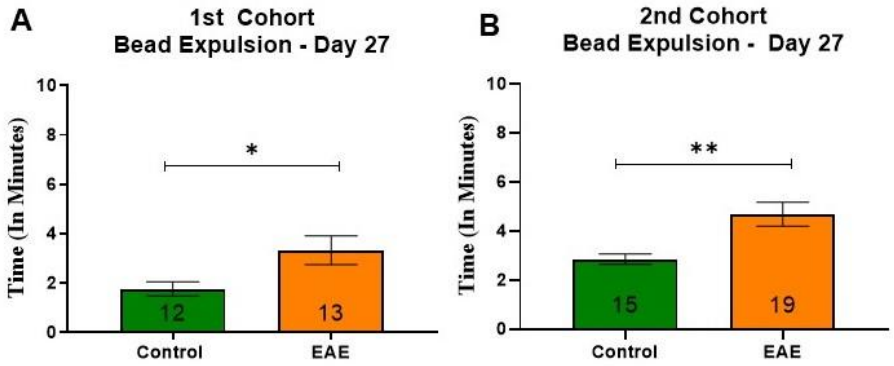

Figure 6 Colonic motility was measured by the time elapsed between bead insertion and expulsion. EAE mice showed significantly slower colonic motility in both cohorts as compared to controls. (A, control n = 12, EAE n = 13, *P <.05; B, control n = 15, EAE n = 19, ** P <0.01). Data represented as mean ± SEM and was analyzed using an unpaired t test.

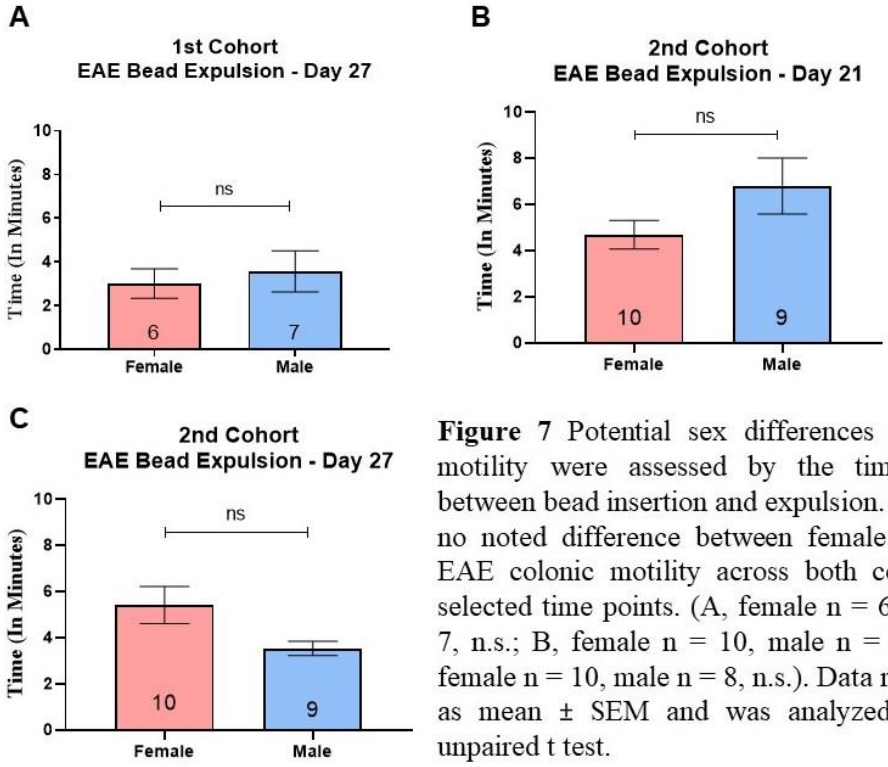

Figure 7 Potential sex differences in colonic motility were assessed by the time elapsed between bead insertion and expulsion. There was no noted difference between female and male EAE colonic motility across both cohorts and selected time points. (A, female n = 6, male n = 7, n.s.; B, female n = 10, male n = 9, n.s.; C, female n = 10, male n = 8, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

Fecal water content was also used as a measure of GI dysfunction through an assessment of constipation, in that a smaller percentage of water indicates drier fecal pellets consistent with constipation. For this particular assay, the first and second cohort results for the combined female and male EAE mice as opposed to the controls presented differently. Consistent with the previous experiments from the Mawe lab, the EAE mice in the first cohort demonstrated a significant decrease in water content as compared to controls, $P = 0.0444$ (figure 8 A), whereas, the second cohort showed no significant difference between the control and EAE mice (figure 8 B). These data suggest that the first cohort of EAE mice showed GI dysfunction consistent with constipation while the second cohort of EAE mice did not present with decreased fecal water content. When examined by sex, it was found that there was no significant difference between the female EAE and male EAE mice across both cohorts (figure 9). These data then suggest that both female and male EAE mice present with the comparable levels of GI dysfunction to one another.

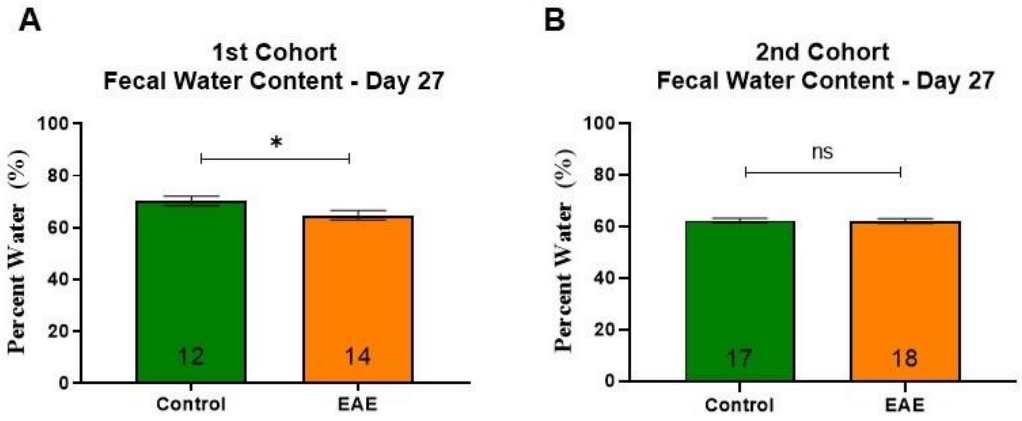

Figure 8 Constipation was assessed through an examination of the percentage of water content in fecal pellets collected over the span of an hour. EAE mice in the first cohort were shown to have a smaller percentage of water content than controls (A), however there was no noted difference in water percentage for the second cohort (B). (A, control n = 12, EAE n = 14, $*P < 0.05$; B, control n = 17, EAE n = 18, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

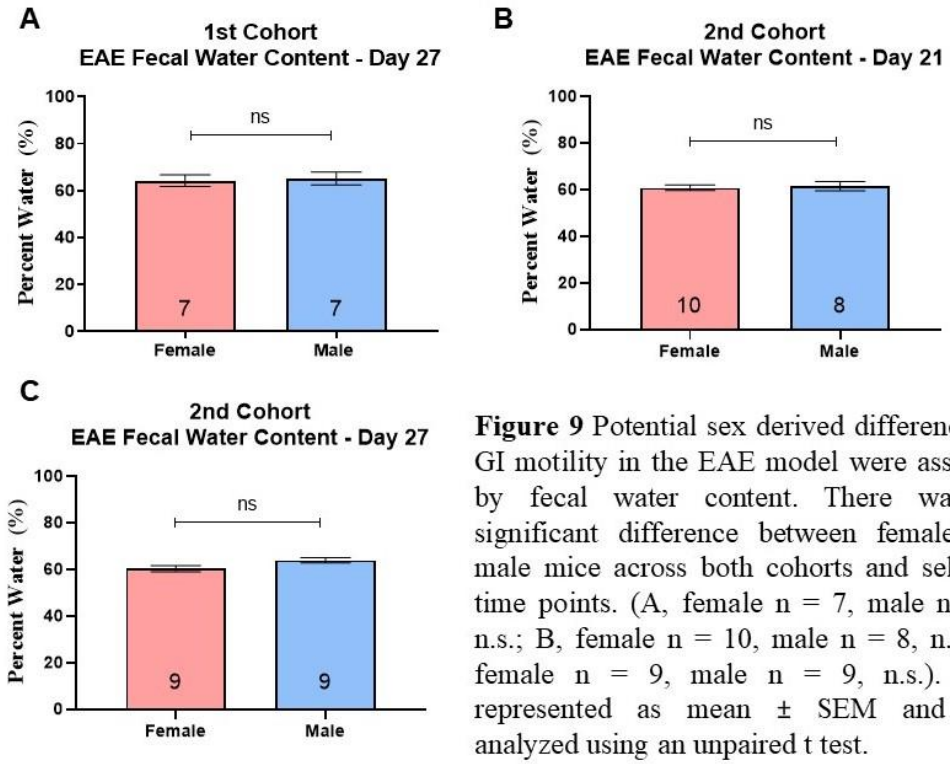

Figure 9 Potential sex derived differences in GI motility in the EAE model were assessed by fecal water content. There was no significant difference between female and male mice across both cohorts and selected time points. (A, female n = 7, male n = 7, n.s.; B, female n = 10, male n = 8, n.s.; C, female n = 9, male n = 9, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

Finally, whole GI transit, as measured by the time after oral gavage of carmine red dye to the presence of the dye within the fecal pellets, was noted to not be significantly different in either cohort when examining the total female and male EAE mice as compared to control mice (figure 10 A & C). These data would suggest that for these two cohorts of mice, at the time point that was studied, GI dysfunction did not reach an extent of decreased whole GI transit as has been previously seen in experiments by the Mawe lab. Additionally, there was no significant difference noted between the first cohort of female EAE versus male EAE mice in terms of whole GI transit time (figure 10 B). In contrast, the second cohort of female EAE versus male EAE mice that showed a slower whole GI transit in female EAE mice as opposed to males (figure 10 D). In this regard, the data from the first cohort of mice suggests that both female and male EAE mice present with a comparable degree of GI dysfunction. However, the data from the second cohort of mice suggests that female EAE mice have a slower GI transit time than males, presenting a possible sex

derived difference in the model, the implications of which will be discussed in greater detail bellow.

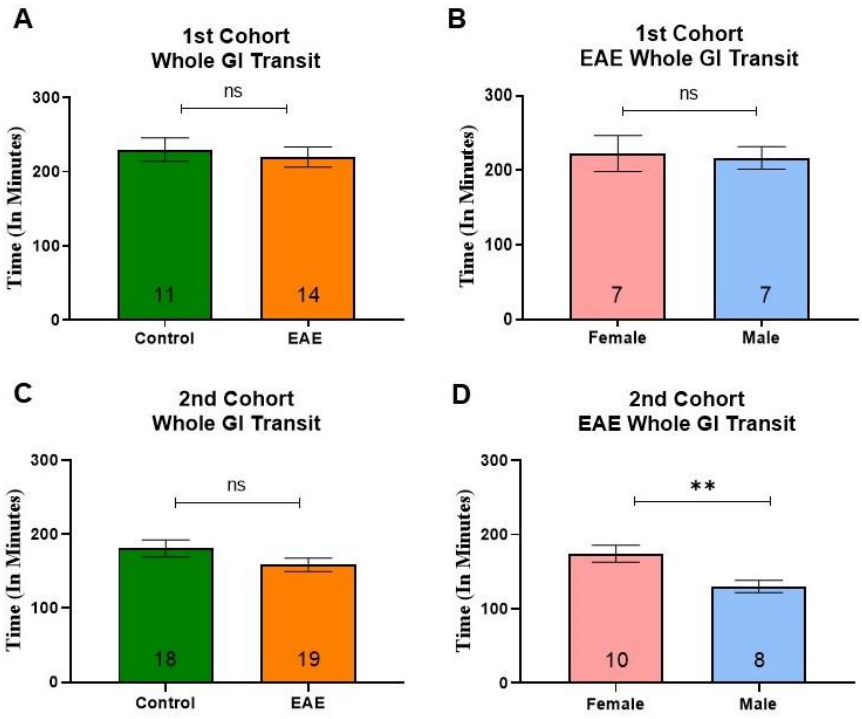

Figure 10 Whole GI transit time as measured by the time elapsed between oral gavage of carmine red dye to presence of the dye in fecal pellets. EAE mice were not shown to have altered whole GI transit in either cohort as compared to controls (A & C). There was no difference in female or male EAE whole GI transit time in the first cohort (B), however in the second cohort female mice were shown to have slower whole GI transit (D). (A, control n = 11, EAE n = 14, n.s.; B, female n = 7, male n = 7, n.s.; C, control n = 18, EAE n = 19, n.s.; D, female n = 10, male n = 8, $**P < 0.01$). Data represented as mean ± SEM and was analyzed using an unpaired t test.

Female and male mice have different peak colonic dysmotility time points

As mentioned previously, colonic motility was assessed using bead expulsion. For the second cohort of mice two separate time points, 21 and 27 days into the experimental protocol, were utilized in order to assess the level of colonic dysmotility, spurred by separate concurrent experimentation in the Mawe lab which will be discussed in greater detail bellow. Bead expulsion at day 21 showed a significant increase, $P = 0.0411$, in expulsion time for male EAE mice as opposed to male control mice (figure 11 B), indicating slowed colonic motility and GI dysfunction. This is in contrast to the female EAE and control mice which did not significantly differ from each other on day 21 (figure 11 A). Bead expulsion at day 27 showed a significant increase, $P = 0.006$, in expulsion time for female EAE mice as opposed to female control mice (figure 11 C), indicating

slowed colonic motility and GI dysfunction. These data at day 27 are in contrast with the male EAE and control mice which did not significantly differ from each other at the selected time point (figure 11 D). Taken together the bead expulsion data from day 21 and day 27 suggest that female and male EAE mice differ in the peak onset of GI dysfunction, with male EAE mice most prominently presenting with slowed colonic motility at day 21 and female EAE mice predominantly presenting with slowed colonic motility at day 27.

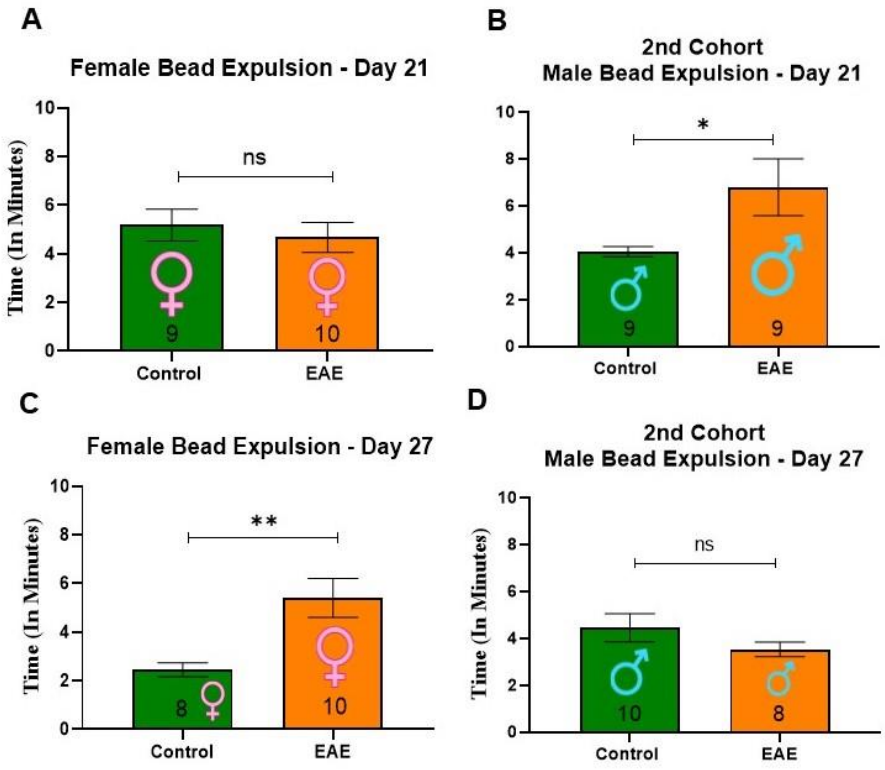

Figure 11 Female and male EAE mice have different peak colonic dysmotility time points as measured by bead expulsion. Male mice were shown to have significantly slower colonic motility at day 21 (B) but not day 27 (D). Female mice were shown to have significantly slower colonic motility at day 27 (C) but not day 21 (A). (A, control n = 9, EAE n = 10, n.s.; B, control n = 9, EAE n = 9, $*P < 0.05$; C, control n = 8, EAE n = 10, $**P < 0.01$; D, control n = 10, EAE n = 8, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

Altered GI motility is not directly tied to changes in 5-HT signaling

To assess the role of 5-HT in GI dysfunction of EAE mice, molecular assays were utilized to examine mucosal 5-HT signaling in various stages including mRNA expression for critical 5-HT signaling molecules and for and tissue 5-HT content. RT-qPCR was utilized to assess the mRNA expression of serotonin selective reuptake transporter (SERT) and the synthetic enzyme, tryptophan hydroxylase 1 (Tph1) to elucidate an understanding of 5-HT mRNA expression. mRNA expression was normalized to the housekeeper gene beta 2 microglobulin (B2M), as this gene showed no significant difference between both the experimental and control conditions. Final analysis showed that there was a significant, albeit modest, decrease in Tph1 expression between EAE and control mice, $P = 0.046$ (figure 12 A); however, there was no significant difference in SERT expression between EAE and control mice (figure 12 B). These data indicate that there is a potential alteration in 5-HT synthesis, due to the decrease in Tph1 expression, however this is not mirrored in the expression of SERT, suggesting that any possible changes to the 5-HT pathway would not be mediated by SERT.

Tissue 5-HT content was assessed using an enzyme-linked immunosorbent assay (ELISA) and the obtained data was normalized against the total quantity of proteins present in the tissue, as determined by a bicinchoninic acid assay. These data showed that there was no significant difference in tissue 5-HT content in EAE mice as compared to controls (figure 13). Taken together with the data from the RT-qPCR assay, these data suggest that the dysmotility noted in the EAE model is not driven through alterations in 5-HT signaling.

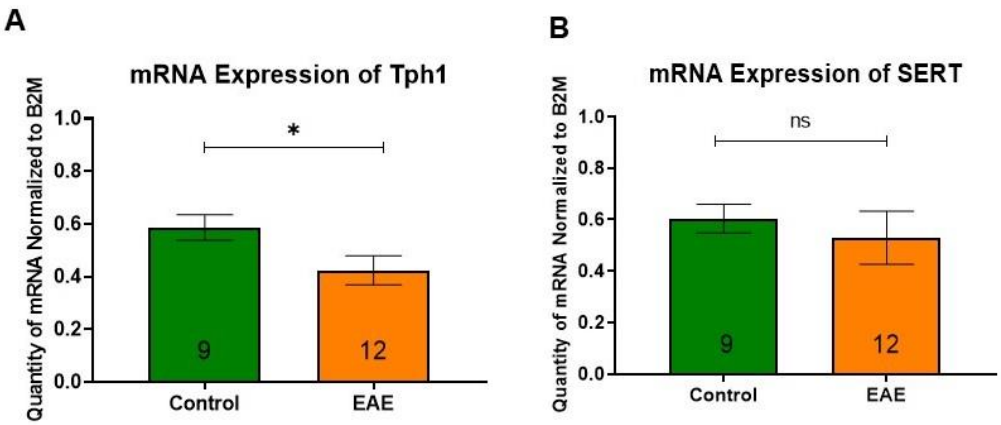

Figure 12 RT-qPCR analysis of Tph1 and SERT mRNA expression normalized to B2M. Tph1 expression was significantly lower in EAE than control mice (A), however SERT expression did not significantly differ from controls (B). (A, control n = 9, EAE n = 12, **P* <0.05; B, control n = 9, EAE n = 12, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

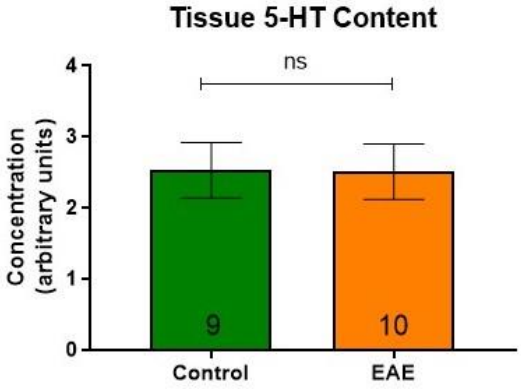

Figure 13 Tissue 5-HT content was quantified using an ELISA and normalized to the total quantity of proteins in the sample using a BCA assay. There was no significant difference in tissue expression of 5-HT between EAE and control mice (control n = 9, EAE = 10, n.s.). Data represented as mean ± SEM and was analyzed using an unpaired t test.

Discussion

This study aimed to gain a deeper understanding of the involvement of the ENS in GI dysfunction in the EAE mouse model of MS, specifically examining the role of mucosal 5-HT signaling and the potential sex derived motor activity differences within the model. The findings presented suggest that the alterations in GI motility seen in the EAE model are not driven by changes to mucosal 5-HT signaling. Additionally, the results demonstrated that both female and male EAE mice induced under the 2X MOG protocol present with similar extents of GI dysfunction though the time course of said dysfunction differs by 6 days between the sexes.

Female and male mice can both serve as models of MS-related GI dysfunction

As mentioned previously, many studies utilizing the EAE mouse model of MS have differed in the use of the selected strain, sex, and method of EAE induction. The Mawe lab is no exception to this claim, as most of the EAE studies completed have predominately utilized male mice, as they were previously shown to be most consistent in demonstrating EAE symptomology and are therefore thought to be the ideal model for assessments of GI dysfunction. However, this study suggests that female and male EAE C57BL/6J mice induced utilizing the 2X MOG protocol are comparable models in terms of the extent of presented GI dysfunction based upon the *in* vivo assays as well as the disease activity characteristics and can therefore be used in conjunction with one another. These findings then alter the paradigm for the selected use of one sex over the other to model GI dysfunction in MS.

Multiple time point analysis

While the results of this study suggest that female and male mice represent comparable models, these findings also suggest that there are transient differences between the two sexes. Most notably the discovery that female and male mice have differing peak time points for colonic dysmotility as analyzed by bead expulsion. As previously mentioned, these findings were spurred by separate concurrent research in the Mawe lab. This separate experimentation was designed with the purpose of discovering the optimal experimental time course to examine consistent GI dysfunction under the 2X MOG induction method of EAE in male mice. Data from that experimentation revealed that colonic dysmotility could be noted at day 21, which was earlier than the previously utilized time point in the Mawe lab (data not shown). Following this trend of data, the new checkpoint was added to the second cohort of mice with the expectation that there would be notable colonic dysmotility in males on day 21. As this concurrent investigation utilized only male mice however, the motility outcome for female mice was unknown. The finding that female and male mice differ in peak colonic dysmotility added a new aspect to the analysis of potential sex derived differences in the motor activity of EAE mice. This once again shifts the paradigm and understanding of the utilization of the EAE mouse model for examination of GI dysmotility. While it can be said that these models are comparable to one another based on the fact that no significant difference was noted between the selected *in vivo* assays and can therefore be used in conjunction with one another, it cannot be discounted that there are still possible mechanistic differences between the two sexes as evidenced by this difference in peak colonic dysmotility time points.

Differences in in vivo motility assays

Whole GI transit

Previous studies by the Mawe lab, specifically reported in Spear et al., demonstrated that EAE mice present with delayed whole GI transit, however these findings were not mirrored in this study at the selected time point analysis. This difference between these two studies is most likely derived from mechanisms concerning gastric emptying. As shown in Spear et al. it has been suggested that EAE mice present with accelerated gastric emptying and no changes in small intestinal motility in relation to control mice (Spear et al., 2018). In turn, based on these findings it is possible that an increase in gastric emptying without a change in small intestinal motility could compensate for a reduction in colonic motility, resulting in a whole GI transit that is not significantly different from the controls, as was seen in this study.

Similarly, the fact that female EAE mice had a significantly slower whole GI transit than male EAE mice in the second cohort again implicates the suggested time component difference of GI motor activity between female and male EAE mice. As was suggested by the findings of the bead expulsion assay, female mice experience peak colonic dysmotility at day 27 as opposed to males which experience peak colonic dysmotility at day 21. Since the whole GI transit assay is conducted at day 28, only one day after the stated peak colonic dysmotility time point for females, it stands to reason that female mice would have a slower GI transit time compared to males. This could be explained through the mechanism that the noted increased gastric emptying in EAE mice might not be able to compensate as strongly for the greater extent of delayed colonic motility seen in females as compared to males. While this difference does exist between female and male mice in the second cohort for whole GI transit, the general results of the assay are unchanged. Female mice when compared to their respective controls show no significant difference in whole GI transit,

nor do males against their respective controls, leading to the general reported nonsignificant results of the assay. Additionally, these findings in conjunction with the difference in peak colonic motility time points suggest that there might be alterations in the molecular underpinnings of the 2X MOG EAE model between female and male mice, however these differences do not impact the general results of the *in vivo* motility assays allowing for both sexes to serve as comparable models.

Fecal water content

Previous results concerning fecal water content of EAE mice have generally shown a smaller percentage of water content in EAE mice than controls. These findings suggest that EAE mice tend to have alterations in secretion and absorption in conjunction to the noted dysmotility features (Spear et al., 2018). However, the results from the current study in terms of fecal water content are much more dichotomized between the two cohorts for the total EAE and control groups of mice. In this sense, the first cohort of mice is consistent with the previously conducted studies by the Mawe lab, however the second cohort presents a different story. The results from the second cohort suggest that there is not a difference in the levels of absorption or secretion within the GI tract between EAE and control mice. However, these findings also need to be examined in conjunction to the assessment of colonic motility through bead expulsion. These data show that for the selected time point, day 27, there is a significant difference between the total EAE and control groups, indicating that there is colonic dysmotility present within the EAE group. Therefore, the findings of the fecal water content assay indicate that for that the second cohort of mice, the mice presented with colonic dysmotility without alterations to secretion and absorption.

Variability of data from EAE models

While the variability of EAE presentation between the sexes and strains of mice has been commonly recognized in the literature, there has also been noted to be a level of variability in EAE presentation and GI dysfunction from experiment to experiment as well as between studies utilizing the same induction method. This particular phenomenon was noted in this current study, as was just mentioned in the fecal water content assays, as well as when comparing this study to previous investigations in the Mawe lab utilizing the 2X MOG induction protocol. These variability differences are most likely derived from the complexity of EAE pathogenesis. It is currently thought that the EAE mechanism for the MOG protocol involves the activation of the immune system, through the foreign presentation of MOG. This immune response, specifically the activated T cells primed against MOG, then enter the CNS and cause the destruction of myelin, due to the presence of the MOG peptide. In turn, the effects of the immune response cause the production of antibodies by B cells which can interact with enteric neurons and glial mediating the GI dysfunction observed in the model, as was supported by Spear et al., that showed that B cell deficient EAE mice did not demonstrate altered GI motility (Spear et al., 2018). It is from this multitude of processes involved in EAE pathogenesis that many levels of variability can exist, purely based on immune system signaling, regulation, and response to the MOG induction, creating the possibility of differing extents of disease severity and GI dysfunction while utilizing the same experimental protocol.

Similarly, for female mice in particular, the stage of the estrous cycle can impact the presentation of EAE severity and symptomology. A recent study suggested that female mice in proestrus, the stage of the estrus cycle characterized by an elevated expression of estrogen and progesterone, demonstrated attenuated EAE symptomology as induced using MOG, both in terms

of disease score and neurological motor impairment. Likewise, the same study reported that the administration of CFA or an emulsion of CFA and MOG had a tendency to prolong the amount of time spent in diestrus, therefore altering the timing of the normal estrous cycle (Rahn, Iannitti, Donahue, & Taylor, 2014). Viewed together, these findings implicate the role of the estrous cycle on EAE variability, as desynchrony in the estrous cycle between females could alter individual EAE disease presentation and impact the obtained experimental data.

On a more environmental level however, the involvement of the gut microbiome has also been implicated in EAE susceptibility and severity (Chu et al., 2018). That said, it is well known that the gut microbiome also has a predominant influence on GI activity. Taken together, these factors suggest that separate cohorts of mice could respond differently to an induction of EAE depending on the expression of bacteria within their GI tract, even if they are from the same strain of mice, demonstrating another potential avenue for variability of data from EAE models.

5-HT signaling and the search for a stable housekeeper gene

While alterations in 5-HT signaling have been implicated in various GI inflammatory diseases, including ulcerative colitis and diverticulitis (Coates et al., 2017), the current study suggests that changes in mucosal 5-HT signaling are not a causal agent behind the reported GI dysfunction in the EAE mouse model of MS. This assessment was primarily obtained through the use of RT-qPCR as this allowed for an mRNA derived analysis of SERT, which was the hypothesized driving force behind the possible alterations in 5-HT signaling and has been confirmed to be altered in the previously mentioned GI inflammatory diseases.

The caveat to the use of RT-qPCR in this study however, was the ability to find a stable housekeeper gene which could be used to normalize the results between both the EAE and control

conditions. Previous experimentation in the Mawe lab has commonly relied on the use of hypoxanthine phosphoribosyltransferase (HPRT) and ubiquitin (UBC) as the housekeeper genes, since both of these genes have been shown to be stable between the different conditions. However, when these genes were tested for this particular study there was a significant decrease in the mRNA expression for both HPRT and UBC in EAE mice, negating their use for normalizing RT-qPCR data in the current study. An in-depth literature search was conducted in order to determine other possible suitable housekeeper genes. As very little work has been completed utilizing RT-qPCR to examine mRNA expression in EAE colon tissue, the chosen reviews by Eissa et al. focused on RT-qPCR in inflamed colonic tissue from the mouse model of colitis, as this model would hypothetically be similar to the GI inflammation and dysmotility in EAE mice (Eissa et al., 2016; Eissa, Kermarrec, Hussein, Bernstein, & Ghia, 2017). From these reviews, as well as previous Mawe lab experimentation, six other possible housekeeper genes were selected as potential candidates. These genes included 18s ribosomal RNA, eukaryotic translation elongation factor 2 (Eef2), phosphomannomutase 1 (PMM1), TATA-box binding protein (Tbp), glucuronidase beta (Gusb), and beta 2 microglobulin (B2M). Of these tested genes, only B2M was shown to have no significant difference between the EAE and control group. All other tested genes demonstrated a significant decrease in mRNA expression of the selected gene in EAE samples as compared to controls (data not shown). This appears to indicate that there might be a mechanism of molecular degradation that is higher or altered in EAE mice as compared to controls, leading to this trend. Therefore, a more thorough analysis of RT-qPCR techniques utilizing EAE colon tissue is needed in order to better understand future applications of RT-qPCR in EAE derived samples and to determine the ideal housekeeper gene for EAE colonic tissue.

Choice of statistical analysis

The statistical analysis and conclusions for this study were possible due to the ability to examine the data in a sub-grouped manner, examining both the total EAE and control populations as well as the specific subsets of female and male EAE and control mice. It can be recognized that at a glance there could be issues with the selected sub-grouped methodology instead of looking at all four data sets (female EAE, female control, male EAE and male control) as a whole entity using a two-way ANOVA following statistical conventions. However, this study was designed with purpose of determining the possible differences between two specific groups, female and male EAE mice, therefore justifying the separation of data analysis.

Future directions

Deeper examination of 5-HT signaling

As mentioned previously, this study's design relied on an examination of mRNA expression to ascertain possible alterations in SERT. However, this analysis method only allowed for an indirect evaluation of the SERT mechanism and its involvement in 5-HT signaling. In this sense, it is possible that there are alterations in the protein level, post translational modifications, or protein function of SERT which could corroborate with the amperometry preliminary data seen in the Mawe lab, in which addition of a SERT inhibitor normalized 5-HT release between EAE and control tissue. Therefore, a functional study of SERT changes is necessary in order to better understand the true implications behind the involvement of SERT in mucosal 5-HT signaling in the EAE model.

Likewise, the results from this study concerning the decreased mRNA expression of Tph1 also provides an area of future study. 5-HT is synthesized in enterochromaffin cells which use the

rate limiting enzyme Tph1 to convert L-tryptophan to 5-HT (Mawe & Hoffman, 2013). In this sense, an analysis of enterochromaffin cells would provide insight into a possible explanation as to why this study found that there was a decrease in Tph1 mRNA expression without any noted alterations to tissue 5-HT content. One possible avenue for exploration would include the use of immunohistochemistry to stain enterochromaffin cells utilizing a goat anti-5-HT polyclonal antibody labeled with donkey anti-goat Cy3 as previously described (Hoffman et al., 2012). These stained cells could then be manually counted based on numbers of cells per colonic gland as previously described (Linden et al., 2005), allowing for an assessment in the prevalence of enterochromaffin cells for 5-HT synthesis. The potential of including this work as a future aim in studies has already been discussed in the Mawe lab.

Sex derived difference experimentation

The difference in findings between cohorts for *in* vivo GI motility assays demonstrated in this study alludes to the concept that there are transient effects in examining GI dysfunction within the EAE model using the 2X MOG protocol. Both of these two cohorts were induced under the same protocol and followed the same time course assessment, with the exception of an additional time point for bead expulsion and fecal water content at day 21 utilized for the second cohort. However, despite this similarity in protocol there are differences between the two cohorts in terms of reported significance of the results, the possible reasoning behind this being previously addressed. Similarly, the fact that this current study failed to replicate the results for whole GI transit as was seen in Spear et al. utilizing the same strain of mice, induction method, and time course again illustrates the transient nature of GI symptoms in the 2X MOG EAE model. Taken together, it is therefore suggested that future studies explore the possible sex differences in motor

activity in a more consistently robust induction method of the EAE model in terms of GI dysfunction in order to truly ascertain the impact of sex on GI motor activity across multiple variations of the EAE model.

In addition to the use of different EAE models for investigation into the greater translatable potential effects of sex on motor activity, this study also spurred many questions about the possible effects of estrogen on EAE in the GI tract. Previous studies have suggested that elevated levels of estrogen can reduce EAE symptomology (Haghmorad et al., 2016; Polanczyk et al., 2003) and that the position within the estrous cycle can have an impact on EAE presentation and neurological motor impairment (Rahn et al., 2014). However, very little research has specifically examined the implications of estrogen on GI motility and symptomology in the GI tract of EAE mice. Therefore, future aims of this research might attempt to quantify estrogen levels during the EAE experimental period to potentially correlate estrogen expression with the GI symptomology time line. Similarly, it might be beneficial to study mice with synchronized estrous cycles in order to potentially negate estrous cycle derived variability in the EAE model. This line of inquiry would therefore allow for a better understanding of the possible differences in peak GI dysmotility time points noted between female and male mice.

Along these lines it is also suggested that a more thorough molecular examination into the potential sex derived GI dysmotility peak onset times be investigated. Previous work in the Mawe lab has indicated that the GI dysfunction seen in EAE mice is B cell mediated and involves the targeting of autoantibodies (Spear et al., 2018). In this sense, future research might aim to examine if there are any differences in the circulation of cytokine factors, which are involved in inflammation, or B cell maturation between female and male mice that might account for the difference in onset of peak GI dysfunction between sexes.

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