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Title of Project: Serotonin and GI motility: The effect of 5-HTP Slow Release (5-HTP SR) therapy on Treatment resistant depression (TRD) associated chronic constipation

Study Purpose and Rationale :

There is a well-established connection between functional gastrointestinal disorders and depression, anxiety and somatoform disorders. Functional gastrointestinal disorders (FGIDs) are disorders of gut–brain interaction, classified by symptoms related to any combination of the following throughout the GI tract: motility disturbance, visceral hypersensitivity, altered mucosal and immune function, altered gut microbiota, and altered central nervous system processing [1]. Irritable bowel syndrome (IBS) is a well-studied FGID and many patients with irritable bowel syndrome (IBS) experience comorbid psychiatric disorders [2]. While there is no single causal factor in functional gastrointestinal disorders such as irritable bowel syndrome, many exacerbating factors have been identified. Both stress and depression have been shown to affect the gastrointestinal manifestations of IBS [3].

Several animal models have demonstrated the link between behavioral disorders and gastrointestinal motility. For instance in one study, rats with a heightened response to stress had altered visceral pain sensitivity, gastrointestinal motility and fecal transit [4]. In another study, mice with chronic depression were observed to have changes in colonic transit and a shift in the profile of resident gut microbiota [5].

One likely explanation for the altered GI function and morphology seen in some patients with psychiatric disorders is found in the neurotransmitter serotonin. Serotonin, or 5-Hydroxytryptamine (5-HT) is an important neurotransmitter in both the central and enteric nervous systems with many diverse functions. Serotonin has been shown to modulate gut motility and secretion as well as play a role in the development of the enteric nervous system (ENS), and thereby the abnormal ENS observed in patients with intestinal inflammatory diseases. Enteric 5-HT has been shown to promote neurogenesis, with the largest influence on late-developing neuronal subsets that express  $\gamma$ -Aminobutyric acid (GABA), calcitonin gene-related peptide (CGRP) and tyrosine hydroxylase (dopaminergic) and nitric oxide synthase [6]. Serotonin also acts as a local paracrine messenger in the gut, used by enterochromaffin cells, which function as sensory transducers. Different 5-HT receptors are present throughout the GI tract, each with specific downstream effects. These receptors are both pre and post synaptic. They are present in both inhibitory and excitatory intrinsic motor pathways as well as in extrinsic afferent pathways to the central nervous system. They are also present on smooth muscle cells. Research on medications (antagonists and agonists) that affect the 5-HT receptor has shown us the different effects that serotonin has on gastrointestinal tone, motility and visceral hypersensitivity [7]. Serotonin is also strongly suspected to play a role in the pathogenesis of emotional disorders such as depression and anxiety [8].

Selective serotonin reuptake inhibitors (SSRIs) are widely used for the treatment of depression today and these work by inhibiting the serotonin transporter's (SERT) reuptake of 5-HT into the raphe nuclei neurons of the brain thereby increasing 5-HT levels throughout the central nervous system [9]. Due to the fact that SSRIs globally increase serotonin availability (in the ENS and CNS), it has been difficult to elicit the precise role of SSRIs in the control of GI function. No consistent effects of SSRIs on sensation throughout the gastrointestinal tract have been found to date. However the effects of SSRIs on GI motility are more consistently observed [7]. Three out of four small placebo controlled studies on patients with irritable bowel syndrome

have shown symptomatic benefit of SSRI over the placebo [10-13]. A placebo controlled study on healthy male volunteers showed the SSRI Citalopram to have a significant effect on motility with greater preprandial gastric relaxation, lower postprandial volume increase, and lower satiation scores affecting liquid meal intake [14].

Over the last decade, mouse models with targeted deletion of genes encoding mediators of serotonergic transmission have contributed greatly towards understanding the role of 5HT in the regulation of emotion [8]. More recently, such mouse models have been used to show the effects of serotonin on intestinal development and function [15, 16] & Gershon Lab, unpublished). Disturbances in serotonin signaling have been shown to affect the development of the enteric nervous system leading to abnormalities in GI transit, peristaltic reflex activity and the proliferation and integrity of the intestinal mucosal epithelium [6, 15-17]. These abnormalities in critical gasterointestinal structure and function may predispose individuals to intestinal inflammatory disease [16].

The rate limiting enzymes Tryptophan hydroxylase-1 (TPH1) and Tryptophan hydroxylase-2 (TPH2) are required for serotonin synthesis, both of which have profound effects on intestinal development and function [6, 17-19]. TPH2 is found in the neurons of the enteric nervous system and the central nervous system; it is the rate-limiting enzyme of neuronal serotonin [6, 18]. There is evidence that, by influencing serotonergic function, genetic variations in TPH2 directly impact the response to antidepressant treatment [20-22]. Nearly one-third of patients with clinical depression cannot achieve remission even after trying available therapies [23]. These individuals have treatment resistant depression (TRD). TRD remains a clinically unmet need that affects a significant portion of the population [24]. It is possible that TPH2 polymorphisms are present in individuals with TRD, thereby making these patients resistant to SSRI and other therapies. Due to the fact that both the ENS and CNS both share a functional dependence on 5-HT, these same mutations may explain the comorbid GI symptoms that occur in patients with depression. One such mutation is the severe loss-of-function mutation in human tryptophan hydroxylase-2 (hTPH2). In one study, this mutation was found to be 10-fold more prevalent in patients with severe, SSRI resistant unipolar depression than in healthy individuals without depression. This rare mutation in TPH2 (G1463A) decreased 5-HT synthesis by about eighty percent [22]. One genome analysis study of human post mortem amygdala samples showed the TPH2 (G1463A) mutation in the edited form of RNA samples from individuals with various psychiatric disorders (drug abuse and suicide victims, schizophrenic patients) [25]. When the equivalent loss of function mutation is knocked into mice (TPH2KI mice), these mice exhibit SSRI-resistant anxiety and depression-like behaviors similar to humans with TRD [21], [26]. These mice demonstrate markedly reduced (approximately 80% in one study) brain 5-HT production [27]. The Gershon lab has recently demonstrated that these TPH2KI mice also experience reduced gut motility and enteric neurogenesis, resulting in a constipation-like phenotype characteristic of the chronic constipation seen in some patients with SSRI-resistant depression (Gershon lab, unpublished).

5-hydroxytryptophan (5-HTP) is a therapeutically relevant precursor of 5-HT. Acute adjunct 5-HTP has been reported to enhance the 5-HT-elevating effects of SSRI treatment in human and animal models [23], [28]. Moreover, small, double-blind trials have demonstrated that adjunct 5-HTP therapy improves the efficacy of SSRI treatment in previously resistant forms of TRD [28], [29],[30]. In addition, 5-HTP supplementation has been shown to prevent the exacerbation of 5-HT deficiency that occurs during chronic SSRI treatment in patients with this TPH2 mutation [31]. Acute adjunct 5-HTP, however, has poor pharmacokinetics, which reduce the efficacy of acute therapy in maintaining 5-HT levels long-term. Recent advances in pharmacotherapeutic design have enabled the production of 5-HTP slow-release (SR). 5-HTP

SR has been shown to successfully maintain therapeutically relevant levels of CNS 5-HT in animal models of TRD [23], [28].

Due to the fact that TPH2KI mice suffer from low 5-HT levels that lead to enteric hypoplasia and reduced gut motility, and 5-HTP has been shown to rescue CNS 5-HT levels in SSRI-resistant TPH2KI mice, we hypothesize that treatment with 5-HTP SR will rescue ENS dysfunction in TPH2KI mice as well [31], [28]. If our hypothesis is correct, this data would suggest that 5-HTP could serve as the first single drug therapy for both SSRI-resistant TRD and associated chronic constipation.

#### Aims:

Aim 1: Investigate whether the slowed intestinal motility in TPH2KI mice is corrected in those that receive 5-HTP SR therapy.

Aim 2: Determine whether TPH2KI mice that receive 5-HTP SR therapy will demonstrate a normalization of ENS neuroanatomy.

#### 2) Study Design:

All animal studies are approved by the Columbia University Institute of Comparative Medicine and methods are based on our labs prior study [16].

Animals: TPH2KI heterozygote males and females are currently being bred at CUMC (mice acquired from laboratory of Marc Caron, Duke University). The WT and homozygote TPH2KI mice that result from these breedings will be used for our studies. The four experimental groups evaluated in all proposed studies will be: (1) WT mice fed normal chow for four weeks (WT control), (2) TPH2KI mice fed normal chow for four weeks (TPH2KI control), (3) WT mice fed specially prepared chow with SR 5-HTP (same as used in TRD studies) for four weeks (4) TPH2KI mice fed specially prepared chow with SR 5-HTP (same as used in TRD studies) for four weeks. Male mice will be given control or 5-HTP chow for four weeks prior to the onset of studies, and this duration of time was selected because research has demonstrated that ENS regeneration can be observed in adult mice after 2-3 weeks [32]. Therefore mice will be 10-11 weeks old at the time of proposed study. Euthanasia for all experiments will be done via CO<sub>2</sub> asphyxiation. The study population size was determined based on the required sample size for significance in our prior experiments using similar methods. In this study, we will focus on male mice as they are the gender used in prior TRD studies. Female mice will be studied in a separate set of experiments.

Aim 1: Investigate whether the slowed intestinal motility in TPH2KI mice is corrected in those that receive 5-HTP SR therapy (n=10/group). All GI functions that are ENS-mediated will be examined: in vivo total intestinal transit, small intestinal motility, colonic motility, gastric emptying and in vitro intestinal peristaltic activity, by measurement of colonic migrating motor complexes (CMMCs) from constructed spatiotemporal maps.

In vivo total intestinal transit time (TGIT): Carmine red (300 ul; 6%; Sigma-Aldrich), a nonabsorbable dye, is gavaged at T<sub>0</sub>. TGIT is the interval between T<sub>0</sub> and time carmine red appears in stool [16, 33].

In vivo colonic motility: Mice anesthetized with 1-5% isoflurane via nasal cone (Baxter) have a 3mm glass bead placed 2 cm into the colon. Time required for glass bead expulsion estimates colonic motility.

In vivo gastric emptying and small intestinal (SI) motility: Mice will be euthanized, by CO<sub>2</sub> inhalation and cervical dislocation, 15 minutes after oral gavage of rhodamine B dextran (100 ul;10 mg/ml; Invitrogen). Fluorescence is measured in supernatant (VersaFluor Fluorometer; Bio-Rad) from stomach and segments of small intestine. Gastric emptying and SI transit is calculated using an established formula.

In vitro colonic motility by measurement of CMMC: Colons mounted in an organ bath and maintained in physiologic conditions will allow motor patterns to be video-imaged that will then be used to calculate frequency, velocity and length of CMMCs.

Aim 2: Determine whether TPH2KI mice that receive 5-HTP SR therapy will demonstrate a normalization of ENS neuroanatomy (n=6/group). Whole mount immunocytochemistry of the myenteric plexus (MP) and submucosal plexus (SMP) of all four groups of mice will be used to identify and quantify total neurons [ANNA-1; gift] and late-developing neuronal subsets (dopamine-, CGRP-, and GABA-expressing neurons; all antibodies are from Cell Signaling Technology). These phenotypes were chosen for this study because serotonergic neurons are born early and 5-HT enhances development of late-born neurons expressing tyrosine hydroxylase (dopaminergic neurons),  $\gamma$ -aminobutyric acid (GABA), and calcitonin gene-related peptide (CGRP) [18, 34, 35]. We have successfully used all of these antibodies in prior studies [16]. Primary antibodies will be visualized with species-specific secondary antibodies labeled with contrasting fluorophores (Alexa Fluor<sup>TM</sup> 350, 488, or 594; diluted 1:200). Mounted preparations will be imaged with a Leica CTR 6000 or Leica SP8 multiphoton microscope and analyzed with computer assistance (Volocity 6.0 software, Improvion/Perkin Elmer Life or Analytical Sciences or MobileMatriX software, respectively). Results will be confirmed by RT-PCR with primers previously used by our group (Applied Biosystems, NY).

3) Data analysis: Assuming a normal distribution of data, pairwise t tests will be used to compare means between pairs of groups, using a standard deviation that is pooled from all of the groups. The pairwise.t.test function in the R program will conduct a multiplicity of t-tests, on more than two samples, and will adjust the p-value to compensate for this multiplicity by various methods.

Note: Pairwise t tests were done to rather than t-test on each set of groups as the latter can lead to compound uncertainty.

*4 Experimental groups: n=10 mice per group for Aim1, and n=6 mice per group for Aim2.*

The study population size was determined based on the required sample size for significance in our prior experiments using similar methods [16].

- (1) WT mice fed normal chow for four weeks (WT control)
- (2) TPH2KI mice fed normal chow for four weeks (TPH2KI control)
- (3) WT mice fed specially prepared chow with SR 5-HTP for four weeks
- (4) TPH2KI mice fed specially prepared chow with SR 5-HTP for four weeks

Aim 1 Data analysis: Investigate whether the slowed intestinal motility in TPH2KI mice is corrected in those that receive 5-HTP SR therapy (n=10/group).

Pairwise t-tests comparing the mean values of quantitative data (see directly below) between all 4 groups, and particular interest will be paid to the following sets of groups:

- 1) WT mice fed normal chow **and** TPH2KI mice fed normal chow
- 2) TPH2KI mice fed normal chow **and** TPH2KI mice fed chow with SR 5-HTP

- 3) WT mice fed normal chow **and** TPH2KI mice fed chow with SR 5-HTP
- Null Hypothesis:
    - H<sub>1</sub>: The wild type and TPH2KI mice both fed normal show will have equal mean motility measures.
    - H<sub>2</sub>: The TPH2KI mice fed normal chow and the TPH2KI fed chow with 5-HTP SR have equal mean motility measures.
  - H<sub>3</sub>: The WT mice fed normal chow and the TPH2KI fed chow with 5-HTP SR will have equal mean motility measures.
  - Quantitative data (5 types of motility measurements):
    - **in vivo total intestinal transit time:** time required for a nonabsorbable dye (carmine red) to appear in the stool after its gavage into the stomach
    - **gastric emptying & small intestinal motility transit time:** Mice will be gavage fed rhodamine B dextran in methylcellulose. Mice will be sacrificed and their gut removed 15 minutes after gavage. The percentage of dye remaining in the stomach as well as the geometric center of the rhodamine B dextran in the intestine is determined and a formula is used to calculate the time.
    - **colonic motility:** Time required for glass bead expulsion from the colon
    - **in vitro intestinal peristaltic activity:** Evaluated by measurement of colonic migrating motor complexes (CMMCs) from constructed spatiotemporal maps created via video imaging analyzing CMMCs in isolated colon.

Alternative for Aim 1 Data analysis: Two way ANOVA would also be appropriate as this experiment has a quantitative outcome (motility measurements) and we have two categorical explanatory variables (the variables are not independent for certain).

- explanatory/independent variables, with their respective levels:
  - 1) Genetic makeup:
    - Mutation (TPH2KI mutation with a severe loss-of-function R441H mutation)
    - Wild Type
  - 2) Medication:
    - No medication in chow
    - 5-HTP SR: 5-hydroxytryptophan slow-release in chow
- Treatment groups are all possible combinations of the factors:  $2 \times 2 = 4$  treatment groups.
- Null Hypothesis:
  - H<sub>01</sub>: The wild type and TPH2KI mice have equal mean motility measures.
  - H<sub>02</sub>: The mice fed normal chow and those fed chow with 5-HTP SR have equal mean motility measures.

Aim 2 Data Analysis: Determine whether TPH2KI mice that receive 5-HTP SR therapy will demonstrate a normalization of ENS neuroanatomy *n=6 mice per group*

Pairwise t-tests comparing the mean values of quantitative data (see directly below) between all 4 groups, and particular interest will be paid to the following sets of groups:

- 1) WT mice fed normal chow **and** TPH2KI mice fed normal chow
  - 2) TPH2KI mice fed normal chow **and** TPH2KI mice fed chow with SR 5-HTP
  - 3) WT mice fed normal chow **and** TPH2KI mice fed chow with SR 5-HTP
- Quantitative data: Collected images will be computer-processed in order to estimate numbers of immunoreactive cells and measured as cells per square millimeter of ganglionic area.

- Number of total neurons in small intestine submucosal plexus and small intestine and large intestine myenteric plexus [ neuronal marker ANNA-1; gift] [36]
- Number of late-developing neuronal subsets (using antibody staining)\*:
  - Dopaminergic neurons (tyrosine hydroxylase - immunoreactive)
  - CGRP-immunoreactive neurons
  - GABA-expressing neurons

\*The above subtypes were chosen for this study because 5-HT enhances the development of late-born neurons that express tyrosine hydroxylase (dopaminergic neurons),  $\gamma$ -aminobutyric acid (GABA), and calcitonin gene-related peptide (CGRP) [18, 34, 35].

4) Study Drugs: 5-hydroxytryptophan Slow Release (5-HTP SR), currently an experimental drug.

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