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THE EVALUATION OF THE PROKINETIC ACTION OF A LUMINALLY
RESTRICTED 5-HT₄ RECEPTOR AGONIST IN MICE

A Thesis Presented

By

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Abstract

Serotonin (5-HT) is a neurotransmitter mainly produced in the enteric nervous system (ENS) of the gastrointestinal (GI) system. Serotonin has widespread function throughout the body including regulating mood, cognition, sleep, sex, and appetite. In the context of the gut, the monoamine modulates vasodilation, mucus secretion, pain, and peristaltic reflexes for motility. The 5-HT₄ receptor is expressed on enteric epithelial cells and nerve terminals in the intestinal mucosa. The agonists available to act on these receptors are a favorable target to promote epithelial healing, recovery from colitis or constipation, and epithelial cell proliferation.

The non-absorbable, luminally restricted, 5-HT₄R agonist (5HT4-LA1; Takeda Pharmaceuticals, 10mg/kg) was found to aid mice in recovery from constipation through the ability to assist in the excretion of materials (motility). However, these studies do not directly test if the agonist is acting on epithelial or neuronal receptors. In this experimentation to further investigate the necessary and/or sufficient site of action to elicit prokinetic action, mice are bred to conditionally knock out the epithelial 5-HT₄R. This was done by generating epithelial 5-HT₄R CKO mice by the crossing of a cre recombinase mouse (Villin-Cre) with a *loxP* mouse. The cre recombinase will excise the 5-HT₄R gene, rendering it non-functional with all other sites intact, creating the CKO and wildtype littermates. The mice underwent three motility assays at baseline and after agonist treatment. The first was whole gut transit involving the oral gavage of agonist solution and unabsorbable red dye, the mice were monitored until a red fecal pellet was found. The next two assays were run in conjunction, the fecal water content assay involved the collection of pellets for one hour after gavage for wet and dry measurements. The colonic motility assay involved the time to expel a bead inserted into the distal colon to determine the speed of propulsion. The wildtype littermates responded to the agonist in comparison to the baseline with an accelerated time to excrete the red pellet and time to expel the bead as well as an increase in the amount of water present in the pellets – indicating faster transit. The CKO mice did not experience a change in all three assays, demonstrating that the epithelial 5-HT₄R is necessary for the agonist to mediate prokinetic action and that the neuronal 5-HT₄R does not mediate motility. The second investigation involves the testing of the 5-HT₄ receptor agonist (5HT4-LA1) and antagonist (GR113808) on constipation in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis (MS) patients. MS is a neurodegenerative disease of the CNS manifesting as an autoimmune disorder resulting in axonal demyelination with constipation as a co-morbidity element. It was hypothesized that the agonist upon EAE induction will rescue symptoms of constipation by stimulation of 5-HT₄ receptors and this effect will be blocked by the 5-HT₄R antagonist. The whole gut transit and colonic motility assays were able to confirm constipation symptoms at the height of disease. We observed no significant changes for the same three motility assays in response to the agonist or agonist + antagonist which is contrasting to the literature available. As demonstrated, the 5HT4-LA1 agonist is found to act primarily on the epithelial 5-HT₄ receptors using the VilCre CKO model and does not act on neuronal receptors. Future studies conducted may conditionally knock out the neuronal receptor to determine any potential differences or effects. The results of the agonist and antagonist administration in EAE mouse model was overall nonconclusive due to the small final cohort and large groups will be needed to determine if 5-HT₄R agonists may be an effective treatment for constipation.

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CHAPTER I: Literature Review

1.1 The Gastrointestinal System

The gastrointestinal (GI) system is crucial to the ability to process dietary nutrients to provide energy for the organism. The GI tract is highly conserved and one of the first systems to develop in the embryonic stage. During gastrulation, invagination occurs forming the blastopore to become one end of the digestive system. The formation of the invagination causes the differentiation of cells into the three germ layers the endoderm, mesoderm, and ectoderm forming a primitive gut tube. This primitive gut tube undergoes morphological changes forming the major regions: the foregut, midgut, and hind gut. In the orientation of the gut, the endoderm gives rise to the epithelial lining of the GI tract and luminal gastrointestinal organs from the pharynx to the anterior duodenum (Willet & Mills, 2016). The mesoderm gives rise to smooth muscle and the connective tissue in the wall of the GI tract. The ectoderm differentiates into the peripheral nervous system (PNS), namely the enteric nervous system, which populates the neurons in the GI tract (Lake & Heuckeroth, 2013).

The GI tract is a complex system regulating digestion, absorption, and excretion. This organ system from the oral to anal end is comprised of specialized regions that differentiate into the esophagus, stomach, small intestine, and large intestine. The layers of the intestines include, from the lumen laterally: the mucosa, submucosa, muscularis externa, and serosa with a slight differentiation in the cells that are present (Figure 1.1). The small intestine – the longest portion of the tract – is divided into three segments: the

duodenum, the jejunum, and the ileum. The duodenum is rich in enteroendocrine cells and is the segment into which bile and pancreatic secretions enter the gut tube. The jejunum continues from the duodenum and functions to carry out most of the digestion and absorption of nutrients into the vascular and lymphatic systems. The ileum is the last and longest portion of the small intestine and functions to absorb any remaining nutrients and stomach bile acids. The small intestine is characterized by transverse circular folds of the mucosa and submucosa also known as the plicae circulares. These folds are lined with intestinal villi and microvilli that project into the lumen and increase surface area to promote more efficient digestion and absorption (Hornbuckle, et al., 2008).

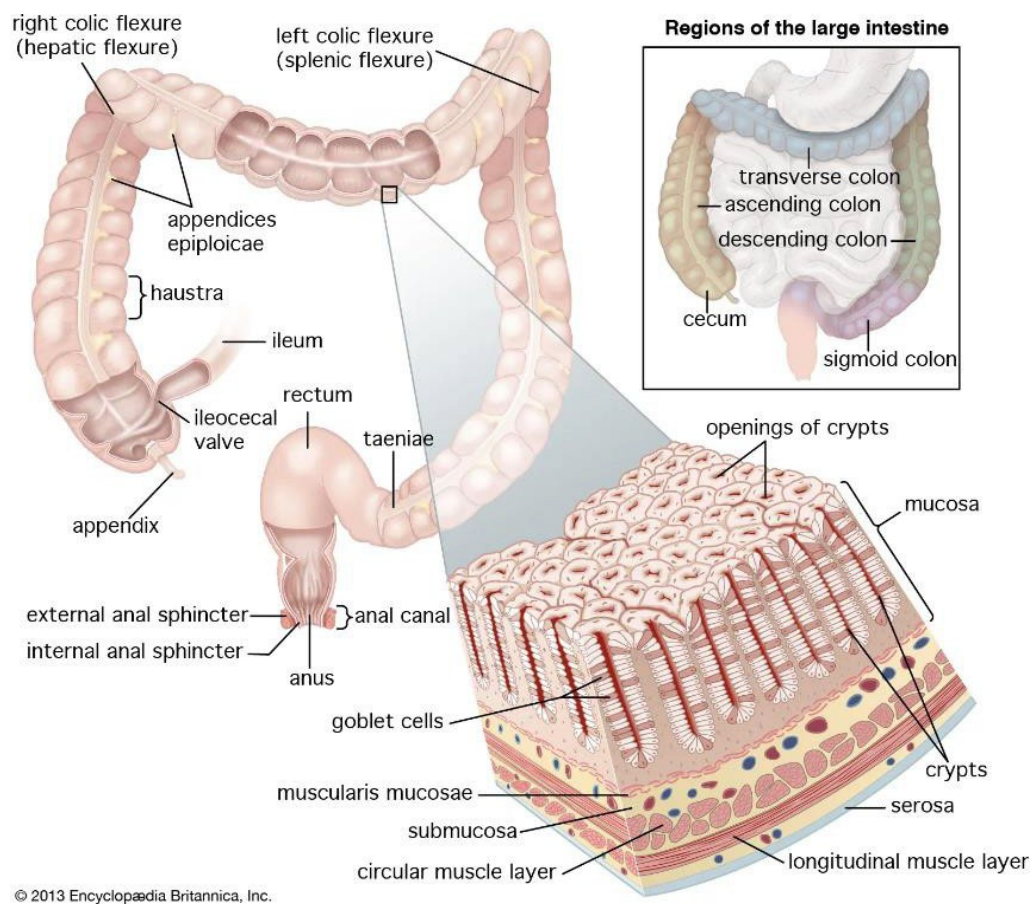


Figure 1.1: Gastrointestinal Structure: Overview of the segmentation sections of the large intestine with a cross section of the circular morphology of the gut tube including the mucosa, goblet cells, and muscularis layer.

The large intestine, also known as the colon, is divided into sections (in humans) starting with the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum, and anus (Figure 1.1). In the colon, the epithelial layer and a layer of mucous secreted from goblet cells forms a physical barrier between the non-sterile environment found in the lumen and tissues of the body (Figure 1.2). The mucosal layer of the colon does not have villi, instead it has invaginations referred to as crypts or colonic glands that contain enteroendocrine cells, goblet cells, and enterocytes (Chandan, et al.,

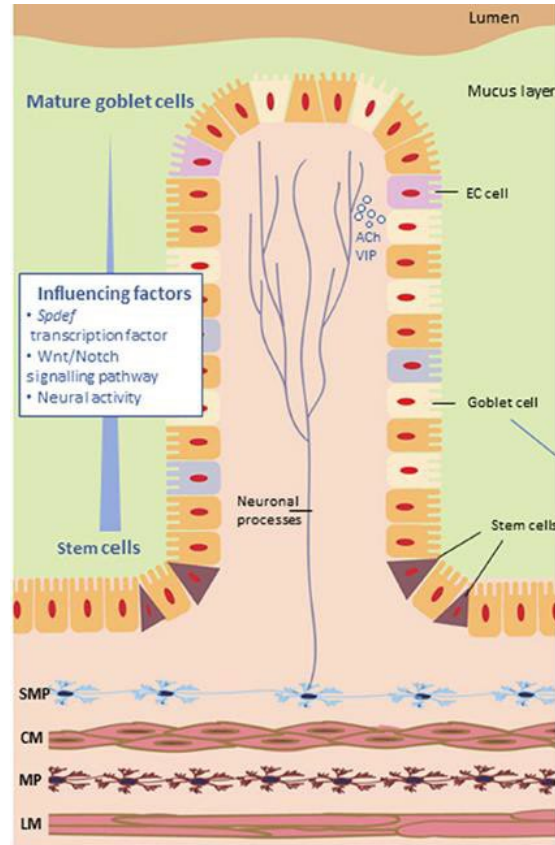


Figure 1.2: Schematic of the Cell Layers of a Singular Intestinal Crypt: The EC and goblet cells in the mucosal layer contain epithelial 5-HT₄R and the neuronal 5-HT₄R are located on the muscularis. The neuronal dendrites receive activation stimulus and synapse to communicate with the CNS.

2019) (Figure 1.2). The overall function of the colon is to absorb any remaining water and electrolytes, then condense and concentrate indigestible material to move into the rectum for the elimination of waste. The colon is one of the most common sites for dysfunction and disease to occur, primarily irritable bowel syndrome, ulcerative colitis, Crohn's disease, and colorectal cancer (Saffrey, 2014).

The brain and the gut engage in bidirectional communication to regulate neurotransmitters, hormones, entero-endocrine signaling, and immune activation to optimize homeostasis. The enteric nervous system (ENS) is the third major division of the

autonomic nervous system (ANS), acting autonomously from the central nervous system (CNS) giving it the nickname “the second brain”. The ENS innervates the GI tract and digestive organs to intrinsically control muscular and mucosal activity. Interneurons, sensory, and motor neurons process gut sensations such as swelling, distension, volume of fecal content, and pain through visceral afferents to the CNS. The efferent signaling to the GI tract is a result of stress and emotional responses from the CNS to indirectly influence hormone production through neurotransmitter release. Interestingly, the gut has the ability to independently (devoid of extrinsic afferents) remain active through secretory and peristaltic reflexes; which are responsible for the movement of luminal contents (Lake & Heuckeroth, 2013). A major trigger of these reflexes is serotonin, which is released from a specialized group of enteroendocrine cells and activates the intrinsic primary afferent neurons of the intrinsic reflex circuitry (Mawe & Hoffman, 2013) (Figure 1.2). The coordinated contraction and relaxation of the gut is responsible for the removal of waste when appropriate pressure in the gut is detected (Hao, et al., 2016).

1.2 Role of Serotonin and Disease

1.2.1 Overview

Serotonin (5-Hydroxytryptamine; 5-HT) is commonly thought of as the happiness neurotransmitter due to its role in regulating and stabilizing mood. Many commonly used antidepressants inhibit the reuptake of serotonin to increase the concentration of serotonin in the synaptic cleft to improve mood and decrease symptoms of depression and anxiety. However, this is not the only function of serotonin – as the monoamine is known to

influence many functions in the body such as cognition, sleep, sex, and appetite (Mawe & Hoffman, 2013). Serotonin is also the primary paracrine signaling molecule of the GI tract modulating vasodilation, perception of pain and nausea, mucus secretion, and motility (Konen, et al., 2020). Current research estimates serotonin in the brain only accounts for a maximum of 5% of the body's serotonin, whereas 95% of serotonin is synthesized and stored in the gut, without the ability to cross the blood brain barrier (Kanova & Kohout, 2021). Serotonin is derived from tryptophan, an essential amino acid obtained from food consumption. In the ENS, neuronal serotonin is synthesized by the enzyme tryptophan hydroxylase 2 (TPH2) whereas in epithelial cells, tryptophan hydroxylase 1 (TPH1) is the rate limiting enzyme for serotonin synthesis by enterochromaffin cells (EC) (Spohn & Mawe, 2017). Enterochromaffin cells are specialized enteroendocrine cells located in the intestinal mucosa and function to synthesize, store, and release serotonin (Kendig & Grider, 2015). EC cells are sensory transducers that are triggered by a chemical or mechanical stimulus of the lamina propria to interact with serotonin receptors on afferent nerve fibers (Spohn & Mawe, 2017). Epithelial 5-HT4Rs located in the luminal mucosal layer can interact with neuronal 5-HT4Rs to transduce signals to modify motility. Consequently, very low levels of serotonin due to the lack of tryptophan, serotonin receptors, or EC cells for proper signaling leads to gastrointestinal distress or dysregulation.

1.2.2 5-HT4 receptor and available 5-HT4 agonists influencing GI function

There are seven subtypes of serotonin receptors and many variants within each subtype. Two of the subtypes important in GI function are, the 5-HT3 receptor, a ligand gated ion channel, and 5-HT4 receptor, a G-protein coupled receptor (Kanova & Kohout,

2021). The 5-HT₃ receptor mediates nausea, bloating and vomiting by relaying visceral hypersensitivity from the gut to the CNS. 5-HT₄ receptors, the most widely studied, are expressed in a variety of tissues and cell types including GI neurons, epithelial cells, urinary tract, and cardiac muscle (Spohn

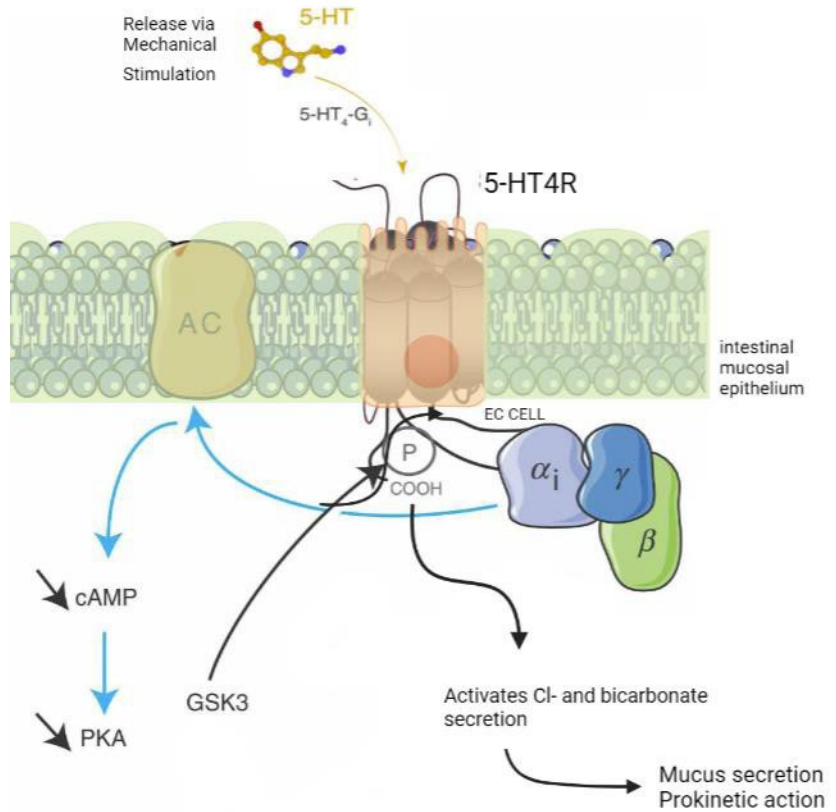


Figure 1.3: Schematic of Epithelial 5-HT₄R Activation and Signaling. 5-HT₄ release from mechanical or chemical stimulation binds to the epithelial 5-HT₄ G-protein coupled receptor located in an EC cell of the mucosal epithelial layer. Adenylate cyclase (AC) for cAMP production and PKA is activated to cause the secretion of Cl⁻ and elicit mucus secretion and prokinetic action.

& Mawe, 2017), as well as on goblet cells in the gut which act to stimulate mucus secretion near the epithelium of the lumen (Kendig & Grider, 2015). Importantly, in the orientation of GI function, the 5-HT₄ receptor is located on the nerve terminals of enteric intrinsic primary afferent neurons (IPANs) which transduce physiological stimuli (villi movement or muscle contraction). The 5-HT₄R is a G-protein coupled receptor, so upon activation from the binding of serotonin, the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) activates the stimulatory G protein (Gs) (Mawe & Hoffman, 2013) (Figure 1.3). In turn, this activates the adenylate cyclase (cAMP production) pathway and

protein kinase A (PKA) pathway to cause a conformational change and the activation of downstream signaling such as epithelial cell (goblet cell) mucus secretion, enterocyte chloride (Cl⁻) secretion, bicarbonate secretion, and enhanced acetylcholine release from myenteric neurons for intestinal contractions (peristaltic reflex) (Mawe & Hoffman, 2013) (Figure 1.3). Overall, the activation of the secretory and peristaltic reflexes leads to expelling waste – this process is responsible for the prokinetic action of 5-HT₄ receptor agonists (Kendig & Grider, 2015). Termination of serotonin signaling occurs through reuptake mediated by the serotonin transporter (SERT) which is expressed in the epithelial cells of the intestinal mucosa (Spohn & Mawe, 2017). SERT functions by internalizing serotonin through a sodium and chloride- dependent mechanism to regulate the amount of available serotonin.

A study conducted by Hoffman and colleagues (2012), indicate that 5-HT₄Rs are located in the epithelial layer of the colon in mice, rats, guinea pigs, and humans and their targeted activation elicits serotonin release and prokinetic and nociceptive effects. Human mucosal biopsy specimens from the gastric corpus, duodenum, ileum, proximal and distal colon underwent reverse transcription-polymerase chain reaction (RT-PCR). 5-HT₄ mRNA transcripts were present in all five regions, confirming the density of receptor expression, with the highest expression in the human terminal ileum. In the mouse model, immunostaining data from 5-HT₄(BAC)-eGFP suggest that goblet cells (one of the epithelial cell types) express 5-HT₄Rs visualized as cavitations in the epithelial layer in response to 5HT₄-LA1 activation leading to mucous secretion.

Current 5-HT₄ receptor agonists such as cisapride and tegaserod were developed for targeting the intrinsic reflex circuitry to treat constipation, namely constipation

predominant irritable bowel syndrome (IBS-C). However, both cisapride and tegaserod were pulled from the market due to concern for cardiovascular side effects (Spohn et al., 2016). Tegaserod, a non-selective 5-HT ligand, was pulled for concerns of ischemic cardiovascular problems due to off-target stimulation of 5-HT1D and 5-HT1B receptors and inhibition to the 5-HT2B receptor with antagonist properties (Madia, et al., 2020). Cisapride was pulled because of patient fatalities due to ventricular fibrillation or cardiac arrhythmias, specifically involving cardiac inhibition of the human ether-ago-go related gene (hERG) leading to a prolonged QT phase or ventricular tachycardia, also known as Torsades-de-pointes (Spohn & Mawe, 2017). The new generation of 5-HT4 receptor agonists, such as prucalopride, have greater selectivity and affinity to the 5-HT4 receptor, minimizing but not eliminating absorption to other areas of the body (Madia, et al., 2020). Prucalopride binds with a high affinity to the 5-HT4 receptor isoforms A and B, activating adenylate cyclase increasing intracellular cAMP levels (Wong, et al., 2010). Takeda pharmaceuticals developed an agonist 5HT4-LA1 based on prucalopride but is lumenally restricted and non-absorbable in an effort to minimize the side effects experienced by other 5-HT4 agonists. Studies conducted in the Mawe Laboratory by Konen and colleagues (2020), demonstrated the prokinetic actions of 5HT4-LA1 in healthy mice, and showed evidence of its ability to alleviate constipation two mouse models of constipation.

1.2.3 Constipation

Constipation is a dysfunction of the GI tract is characterized by the inability to eliminate waste due to structural and/or functional abnormalities. The pathophysiology of chronic constipation is unknown, but associated with various risk factors such as age, diet,

drug use, organic diseases, physical activity, and some genetic factors. Stress is well recognized as a condition that leads to altered GI function, but rather than causing constipation, it is typically associated with enhanced motility and secretion leading to diarrhea (Camilleri, et al, 2017).

The activity of the GI tract is dependent on the ENS for regulation through peristalsis which is activated by mechanical or chemical stimuli. Enteroendocrine cells synthesize and release serotonin in response to stimuli such as bile salts, nutrient content, fatty acids, and mechanical stimulation (Hao, et al., 2016). In turn, these cues activate interneurons send signals along the myenteric plexus to activate excitatory motor neurons and downstream inhibitory motor neurons. This interaction triggers the contraction of upstream smooth muscle cells and the relaxation of downstream smooth muscles to mediate propulsion (Mawe & Hoffman, 2013). Colonic propulsion primarily depends on mass contractions of the smooth muscle and the inhibition of small pouches (haustra or segmentation) of the bowel wall. The excretion of waste is dependent on the excitation and inhibition of the pelvic floor muscles and sphincters. The inability to coordinate abdominal, pelvic, rectal, and anal muscles results in dysfunction in the necessary force to propel waste from the body (Camilleri, et al., 2017).

Primary chronic constipation is the most common diagnosis of constipation which involves a disturbance in bowel dysfunction due to intrinsic problems such as diet, lifestyle, or irregular colonic propulsion and emptying. Secondary chronic constipation arises from drug use, colon cancer, and neurological disorders such as Parkinson's disease and multiple sclerosis (Andrews, & Storr, 2011). Constipation is characterized by symptoms such as infrequent bowel movements, abdominal pain, bloating, hard stool and excessive straining,

and is frequently associated with other disorders such as irritable bowel syndrome (IBS), dyspepsia, and mood disorders. Research indicates that patients with primary chronic constipation do not have evidence of slow colonic transit or dyssynergic muscle propulsion for excretion (Camilleri, et al., 2017).

1.2.4 Multiple Sclerosis

Multiple sclerosis (MS) is a neurodegenerative autoimmune disease of the central nervous system (CNS) with a prevalence in the United States of nearly 1 million people in 2020 which doubled from 2019 (MSAA, 2020). It is an autoimmune disorder in which recurrent inflammatory lesions occur through autoreactive lymphocytes that are able to cross the blood-brain-barrier (BBB). These lesions perpetuate demyelination, axonal damage, and neuronal damage, which in turn, causes the formation of plaques, resulting in disability. The onset of symptoms is very rapid, distinctively presented as sensory disturbances, motor deficits, constipation, fatigue, and visual disturbances. Additionally, the autonomic system is also affecting bladder, cardiac, sexual, and gastrointestinal dysfunction, which have been minimally studied. A very debilitating autonomic symptom reported is constipation, affecting up to 73% of MS patients, and it may predate the motor complications (Khanna, et al., 2022; Spear, et al., 2018). MS typically develops and becomes symptomatic in individuals between 20-40 years old. MS is the most common in those of European descent and women are 3 times more likely than men to be affected. The etiology of MS is unknown and hypothesized to be a combination of genetic susceptibility and environmental factors such as infection, lifestyle, metabolism, or vitamin D exposure (amount of sunlight) (MSAA, 2020).

Diagnosis is determined using MRI at the first presentation of a clinical attack, characterized by evidence of one or two lesions on MRI scans and cerebral spinal fluid analysis to distinguish from other disorders. There are four recognized categories of MS that are dependent on the course of the disease. The most common, occurring in 85% of patients, is relapsing-remitting MS (RRMS), characterized by the relapse of symptoms followed by periods of remission in which symptoms improve or disappear (Goldenberg, 2012). These patients may further regress and develop into the second category, secondary progressive MS (SPMS) in which there are no longer periods of remission, only a continuous worsening of symptoms (Huang, et al., 2017). Primary progressive MS (PPMS) affects 10-15% of patients where symptoms begin abruptly and gradually worsen (Goldenberg, 2012). The rarest form, affecting <5% of patients, is progressive-relapsing MS (PRMS) in which there is a progressive worsening of symptoms with periodic exacerbations and no periods of remission (Goldenberg, 2012).

In early stages of the disease, the most commonly reported debilitating symptom is fatigue and motor muscle dysfunction, secondarily reported is GI disturbances – including the lack of coordination of intestinal muscles for proper propulsion of materials. Despite the increased prevalence of constipation in MS patients compared to the general population; inflammatory dysfunction of the GI tract in these patients is poorly understood and lacking available effective treatments (Preziosi, et al., 2018). Typically, treatment for chronic constipation is centered around lifestyle management such as diet and increasing physical activity, with the introduction of manual clearance and laxatives in cases where lifestyle management is ineffective. This treatment regimen is relatively ineffective for patients with MS due to the motor disturbances impacting lifestyle and secondary

complications such as: sepsis, uremia, and respiratory failure due to the bedridden state (Scalfari, 2013).

1.3 Experimental Autoimmune Encephalomyelitis

Constipation is an established complication of multiple sclerosis, and studies conducted in the Mawe Laboratory have demonstrated that this condition is present in the experimental autoimmune encephalomyelitis (EAE) model of clinical MS in mice. In this model, immunization is achieved with dosing of mouse spinal cord homogenate (harvested in house from retired breeders), complete Freund's adjuvant (CFA) and pertussis toxin (PTX). Successful induction will produce clinical symptoms of paralysis between days 10 and 13 and the height of disease (including constipation symptoms) is around day 21. The clinical scoring and assessment of symptoms is synonymous to the neurological presentation marked by motor deficits such as a lack of coordination and spasticity as seen in MS patients during an acute attack (Spear et al., 2018).

Studies conducted by Spear and colleagues (2018), demonstrated that EAE mice demonstrated characteristics of GI dysfunction such as slowed gastric emptying, slowed GI transit of a full bowl movement, slowed colonic motility, and exceptionally dry fecal pellets. The *ex vivo* spatiotemporal analysis of the isolated colon in segments from EAE mice indicated there was a shift in the contractility intervals in which there were longer periods of inactivity in comparison to the healthy control mice. Additionally, EAE mice demonstrated decreased glial fibrillary acidic protein (GFAP) distribution within the myenteric ganglia which is known to result in altered GI motility. The EAE mice did not

demonstrate neuronal loss by having no change in neuron number, density, and ganglion size in comparison to the control mice, determined with immunoreactivity (pan-neuronal marker HuD). A later study by Konen and colleagues were able to replicate similar findings of slowed motility for a full bowel movement, dysfunctional propulsive ability of the distal colon, and low water content of fecal pellets in the EAE model indicating constipation. The EAE mice were administered 5-HT4-LA1 and the symptoms of constipation were reversed, demonstrating the mice were able to recover prokinetic action on all three of the assays. In addition, in healthy mice, the antagonist was administered 10 minutes prior to the agonist during the colonic motility assays and 30 minutes prior to the agonist for the whole gut transit assay. The antagonist was found to inhibit prokinetic action and the agonist did not alter the rate of motility. To further this investigation during the current study conducted, the EAE condition was induced, and the antagonist was administered 30 min before the agonist.

The experimentation conducted by Spear and colleagues (2018) also demonstrated that B cell-deficient EAE mice did not present symptoms of constipation, supporting the hypothesis that B cell-generated autoantibodies contribute to slowed motility. Autoantibodies and several of their antigens are expressed in the ENS including over 20 CNS autoantibodies identified in MS patients. In one study of GI antibodies, 28% of patients were found to have antibodies that target ENS structures such as mucosal antigens (Spear, et al., 2018). Therefore, it is suggested autoantibodies have a distinct role in contributing to constipation as seen in MS patients that is B-cell mediated. This study claims that EAE is associated with molecular and physiological alterations specifically in the ENS itself causing gastrointestinal distress. Therefore, the goal of the EAE motility

study is to demonstrate if constipation is a measurable feature of the EAE model of MS and determine the association between changes in the ENS and that the pathology is accurately represented clinically.

1.4 Specific Aims

Serotonin is a very viable target for the treatment of constipation as an important signaling molecule in the enteric nervous system. The present study focuses on the evaluation of the effects of the non-absorbable, luminally restricted, 5-HT4 receptor agonist, 5-HT4-LA1 on patterns of motility in the conditional 5-HT4R CKO mice and restoration of intestinal motility from constipation and the EAE model. 5-HT4-LA1, the drug developed by Takeda pharmaceuticals in collaboration with the Mawe Laboratory is restricted to the lumen of the GI tract and expected to be acting solely on epithelial 5-HT4 receptors. This specific binding is expected to increase effectiveness and minimize the off-target effects or toxicities exhibited by previously approved 5-HT4 receptor agonists, cisapride and tegaserod.

Aim I: Effect of luminally restricted 5-HT4R agonist on intestinal motility in conditional epithelial 5-HT4 KO mice

We investigated that the site of action of the 5-HT4R agonist 5-HT4-LA1 involves the activation of epithelial 5-HT4 receptors versus neuronal receptors in the modulation of prokinetic reaction. Mice were bred with the epithelial 5-HT4 receptor conditionally knocked out using the *Cre-loxP* system, while the expression of the neuronal receptor is

left intact with the goal of identifying if 5-HT4 receptors expressed by epithelial cells are necessary and/or sufficient to elicit prokinetic action. 5-HT4-LA1 will be administered to observe potential differences in motility in the conditional knock out (CKO) mice in comparison to their wildtype (WT) littermates, with the expectation that the agonist will have little or no effect on motility in the CKO mice.

Aim II: Effect of luminally restricted 5-HT4 receptor agonist and antagonist on intestinal motility in EAE mice

5-HT4-related compounds are used to determine whether symptoms of constipation can be rescued by the stimulation of 5-HT4 receptors, and the degree to which this effect is blocked by a 5-HT4 receptor antagonist. The EAE mouse model is induced, and animals are evaluated for symptoms of constipation by comparing whole gut transit time, fecal water content, and colonic motility time under variable conditions. Animals presenting symptoms of constipation are treated with 5-HT4-LA1 and 5HT4-LA1 + GR113808 and evaluated for any differences in response from baseline. We hypothesize that 5H4-LA1 will improve motility by recovery from constipation and the antagonist GR113808 is binding in the same site as the agonist and would inhibit the prokinetic action of 5HT4-LA1.

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CHAPTER II: Effect of 5-HT4 Agonist and Antagonist on Intestinal Motility in the Treatment of Constipation in EAE Mice and Conditional 5-HT4 Receptor Knock-out Mice.

2.1 Introduction

Serotonin (5-Hydroxytryptamine; 5-HT) is a major signaling molecule that is important for the regulation of motility in the gastrointestinal (GI) tract. Serotonin, released from enterochromaffin (EC) cells, stimulates intrinsic reflexes such as vasodilation, propulsive motility, and mucus secretion. Additionally, the stimulation of serotonin receptors on vagal and spinal afferent nerve fibers promotes extrinsic reflexes modulating gastric emptying, pain, and nausea (Mawe & Hoffman, 2013).

Due to the importance of serotonin in gut function and sensation, compounds that interact with 5-HT receptors have been developed to treat a multitude of GI disorders such as irritable bowel syndrome (IBS) and constipation. One of these targets is the 5-HT4 receptor, which when stimulated promotes secretion and propulsive motility. 5-HT4 receptor agonists have been approved for the treatment of constipation but some of these initially developed compounds have been associated with adverse cardiovascular side effects due to the low selectivity for intestinal 5-HT4 receptor (Spohn et al., 2016). The Mawe laboratory has demonstrated that epithelial cells in the colon express 5-HT4 receptors and when these receptors are stimulated, motility and secretion are enhanced (Hoffman et al., 2012). To improve the safety profile of these agonists, the Mawe Laboratory, in collaboration with Takeda Pharmaceuticals have developed a non-

absorbable, luminally acting 5-HT₄ receptor agonist (5HT₄-LA1) to treat symptoms of constipation. In vivo, serum distribution after agonist administration was assessed to determine bioavailability and there was found to be a negligible detection of serum in the plasma and no detection in the plasma after 2 hours, indicating minimal systemic absorption. Konen and colleagues (2020) demonstrated that 5HT₄-LA1 is a potent and selective agonist in vitro, in which applying 5HT₄-LA1 to isolated colons are found to have increased contractility in comparison to the vehicle. The contractile activity was monitored through imaging, determining the average diameter along the length of the colon. The distal colon was particularly found to have the greatest degree of difference between the agonist and control. The primary outcome measures of motility were: whole gut transit, fecal water content, and colonic motility, and they were assessed for changes in response to administration of the agonist and antagonist in healthy and diseased animals. The results indicate the agonist accelerated motility in healthy mice and in EAE mice improve symptoms of constipation by the accelerating whole gut transit, fecal output, and fecal water content. The administration of the antagonist assessed on the same outcome measures were found to successfully inhibit prokinetic action.

The 5HT₄R-CKO study reported here furthers this investigation by confirming the location/site of action of the agonist by conditionally knocking out the epithelial 5-HTR and evaluating any changes in motility; it is expected to have no change if primarily acting upon the epithelial receptor instead of the neuronal 5-HT₄ receptor. The EAE study endeavors to confirm the prokinetic action of the agonist by testing the ability of the drug to recover symptoms of constipation. We utilized the antagonist administration to confirm the specificity of the 5-HT₄R agonist and any blocking ability or prokinetic action

mediated by the 5-HT4R due to response in motility and not as an effect of the mice recovering over time.

2.2 Methods

2.2.1 Conditional Epithelial 5-HT4R Knock-Out Mice

Generation of Transgenic Mice

Male and female C57BL/6J mice and their littermates on a SV129 background are bred to obtain conditional knockout mice using the Cre-*loxP* system (Figure 2.1). Cre recombinase is tyrosine site-specific, which allows for the control of gene expression by

the recognition of specific DNA fragment sequences (*loxP*) resulting in the manipulation or deletion of genes. *LoxP* sites flank the target gene and Cre recombinase causes the excision of the genetic sequence in

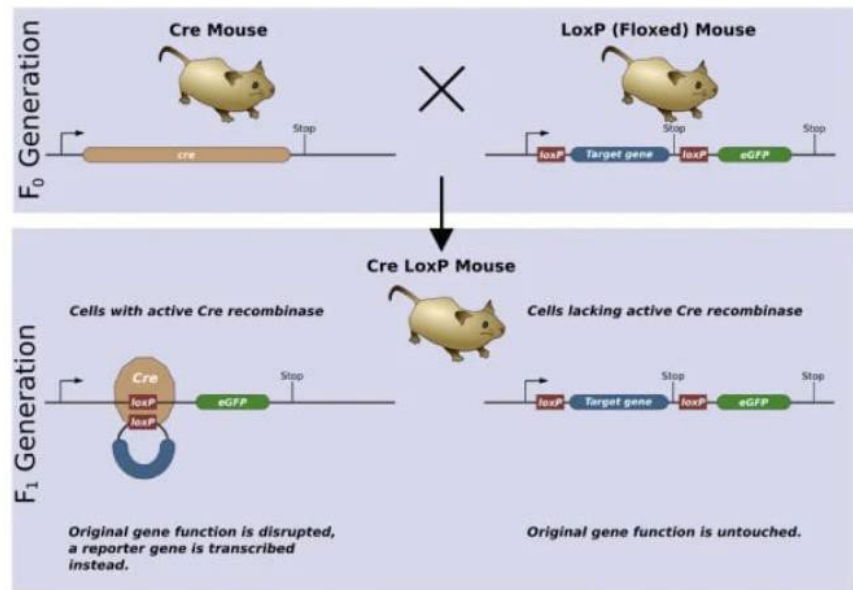


Figure 2.1: Conditional Epithelial 5-HT4R Knock-Out Mice Model: The development of the CKO model through the crossing of a cre mouse and LoxP mouse. Cre recombinase recognizes the *loxP* sites flanking the epithelial 5-HT4R and causes gene excision (exon 5), rendering the gene/receptor non-functional.

between the two sites. Cre recombinase is only present in cells that express villin, which is found in intestinal epithelial cells located in the mucosal layer. These mice are generated

by breeding (in-house) one mouse that expresses active villin-cre recombinase with a mouse where the target gene for the epithelial 5-HT₄ receptor is flanked with two *loxP* sites, resulting in the excision of exon 5, rendering a mutation via the transcription of a non-functional gene. In the conditional knock-out mice in which this mutant gene is expressed, the neuronal 5-HT₄R and other systemic 5-HT₄R's are left intact while the epithelial 5-HT₄R is downregulated in villin-CRE-positive cells (VilCre). Of the litter, 7 wildtype and 6 conditional knock-out mice were obtained. The mutant gene is characterized during polymerase chain reaction (PCR) by two bands (612bp and 1214bp) whereas the wildtype has one band (1214bp) to verify a successful knock-out during gel electrophoresis (Supplemental Figure 2.12). The degree of mutation or non-functionality of the gene is not known due to the lack of an antibody available for immunostaining to conduct immunohistochemistry or western blot to confirm the degree of downregulation.

2.2.2 Experimental Autoimmune Encephalomyelitis Mice

The investigation utilized a total of 15 male 7-8-week-old C57BL/6J mice (Jackson Labs) in the EAE study conducted. The EAE condition is variable in presentation visually by clinical signs and gastrointestinal distress. The constipation symptom requirements are defined by the colonic motility/bead expulsion assay in which the mice have at least a 20% increase in expulsion time at the height of disease in comparison to the baseline scoring, indicating gastrointestinal distress. The target region of the colonic motility assay is the distal colon, which has the highest density of the 5-HT₄R of the mouse intestines, thus the primary area of drug interaction and most accurate to establish the determination of constipation. In the final cohort of 15 mice, 6 mice were excluded due to lack of meeting

the constipation symptom requirements. All experimental protocols follow proper guidelines and were approved by the Institutional Animal Care and Use Committees of the University of Vermont. Animals were euthanized by isoflurane overdose followed by cervical dislocation as the secondary method of euthanasia.

EAE Induction

The control mice received three subcutaneous injections (50ul) containing (100uL of 4X complete Freund’s adjuvant (CFA) and 100ul 1X phosphate buffered saline (PBS)). The experimental mice were immunized and received three subcutaneous injections (50ul) containing an emulsion of 100uL of 4X CFA, 100ul 1X PBS, 4mg/ml mycobacterium tuberculosis, and 50mg/ml of mouse spinal cord homogenate (MSCH) distributed equally in the scruff between the shoulders and in the posterior right and left flank. Thereafter, the mice received a 100ul

dose of solution of 200ng pertussis toxin (PTX) diluted in 100ul 1X PBS by intraperitoneal injection and an additional dose two days later (Figure 2.2).

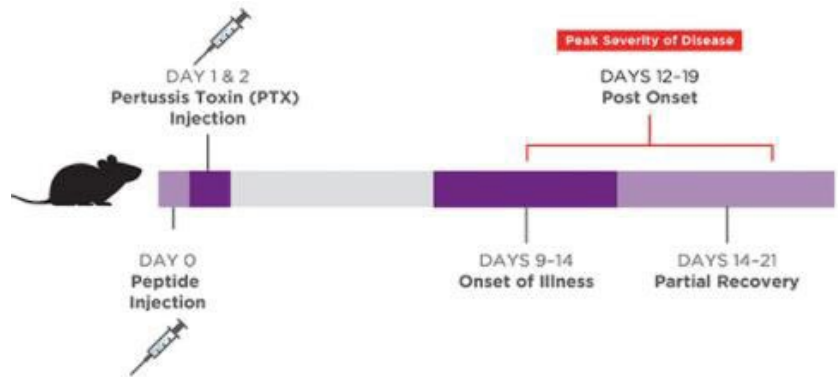


Figure 2.2: Timeline of EAE condition: During EAE induction the peptide injection used is an emulsion of CFA, PBS, mycobacterium tuberculosis, and MSCH. PTX was dosed twice.

Clinical Scoring

Clinical EAE monitoring began 10 days after induction in which the mice were weighed and scored daily until euthanized. EAE scoring is monitored by a rating of 0 – no symptoms, 1 – tail paralysis, 2 – tail paralysis and hind limb weakness, 3 – tail and hind limb paralysis, 4 – paralysis and urinary incontinence, and 5 – moribund or death. If a score of 3 is reached, food pellets and Napa Nectar is placed at the bottom of the cage for access to nutrients due to limb paralysis. Mice with a score of 3 that rapidly lost more than 2 grams of weight, 1mL of saline was administered to restore lost fluid and slow disease progression. Mice that lost greater than 30% of their initial weight or reached a score of 5 were euthanized. Any mice that did not take to the treatment indicating a clinical score of 0 and a lack of evidence of constipation ($\geq 20\%$ increase in time to expel the bead during the colonic motility assay) were excluded from the study.

2.2.3 Timelines and Motility Assays

Conditional Knock-Out Mice

The motility assay data collection timeline for the conditional 5-HT4 knock-out mice is a direct comparison with two different points a week apart per respective assay for the baseline and agonist treatment (administration of 5HT4-LA1 via oral gavage). Whole Gut Transit (WGT) was conducted on a single day and after a week of rest, the fecal water content and bead expulsion were conducted on the same day with an hour of pellet collection/acclimation before the bead expulsion assay. The mice are placed in separate

cages without bedding containing only a food pellet and napa nectar during experimentation.

EAE Mice

The three motility assays testing efficacy of 5-HT4-LA1 were performed at four different points, the baseline (prior to induction), at the height of disease (21 days post-induction), and two treatments: 1. administration of agonist or 2. administration of antagonist, then agonist 30 minutes later via oral gavage. The treatments were administered on day 25 (whole gut transit) and day 27 (colonic motility) with half of the mice receiving agonist and the other half receiving the antagonist treatment. On day 34 (whole gut transit) and day 36 (colonic motility) the mice received the opposite treatment than previously received – for comparison controlling for any differences in individual mice. WGT was conducted on a single day and after a day of rest, the fecal water content and bead expulsion were conducted together. One hour was allotted for fecal water content pellet collection followed by the bead expulsion assay. The mice are placed in individual cages without bedding during testing with a food pellet and napa nectar.

2.2.3.1 Whole Gut Transit (WGT)

WGT is to determine the amount of time it takes for luminal contents to travel the entire length of the GI tract (full bowel movement). The mice received 300ul of solution via oral gavage. The vehicle solution is prepared as 6% carmine red, 0.5% methyl cellulose, dissolved in warm tap water and vortexed. The agonist solution includes 10mg/kg of 5HT4-LA dissolved in the vehicle solution. The antagonist (1 mg/kg GR113808) is

administered 30 minutes before the agonist solution. The difference in the time elapsed between the gavage time and the expulsion of the first carmine red pellet determines transit speed and subsequent dysfunction (Figure 2.3).

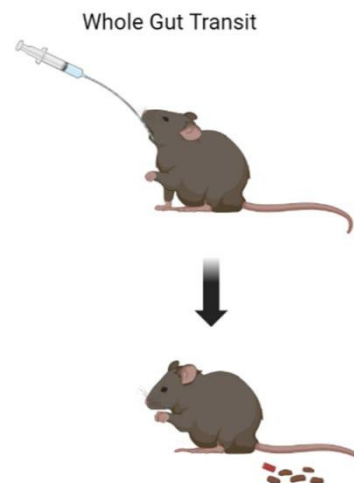


Figure 2.3 Schematic of Whole Gut Transit. The time elapsed from gavage of solution to excretion of the red pellet.

2.2.3.2 Fecal Water Content

Fecal water content is to determine the amount of water in the feces with the idea that agonist treatment would increase transit through the colon allowing less time for water absorption leading to higher water content. The mice received through oral gavage 100ul of vehicle solution (5mM citrate, 1% Tween, 0.5% methylcellulose). The agonist solution consists of 10mg/kg of 5HT4-LA dissolved in vehicle. The antagonist (1 mg/kg GR113808) is administered 30 minutes before the agonist solution. After gavage, the mice are placed in separate and all expelled feces are collected for

cages for one hour for the water content analysis. The expelled pellets are collected in pre-weighed airtight Eppendorf tubes, counted, and weighed at the end of one hour as wet specimens. Pellets are then dried for 24 hours in a chamber at 50°C and weighed again as dry pellets to determine the amount of water that was present (Figure 2.4).

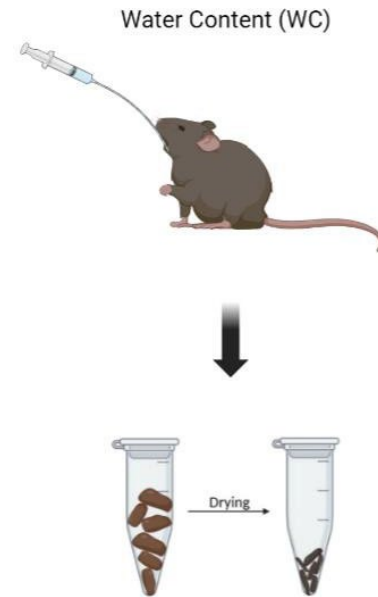


Figure 2.4 Schematic of Water Content Assay: The oral gavage of solution and collection of fecal pellets for one hour. They are weighed as wet specimens and after 24hrs of drying, weighed again to determine water content by water loss.

2.2.3.3 Colonic Motility

Colonic motility is to measure the propulsive ability of the distal colon to effectively excrete pellets. This assay is conducted after the fecal water content measurement and the mice have acclimated to the environment for one hour (duration of

pellet collection for water content) which assists in reducing stress on the mice. An isoflurane machine is used to lightly anesthetize the mice for 50 seconds, then a small bead is inserted 20 mm into the distal colon using a blunt gavage needle. The time between bead insertion and bead expulsion is recorded to determine speed of propulsion and distal colonic transit time (Figure 2.5).

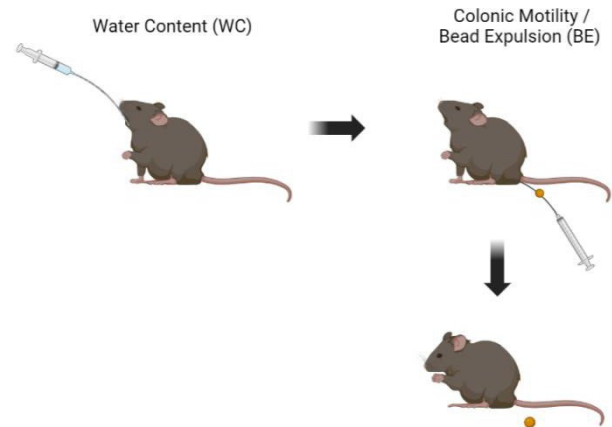


Figure 2.5: Schematic of Colonic Motility: After the WC assay is complete, the mice are anesthetized, and a bead is inserted 20mm into the distal colon to determine speed of propulsion by excretion of the bead.

Data Analysis

Data analysis for the CKO mice was run using GraphPad Prism Software. The data is presented as a mean \pm SEM for n number of animals, there are 7 wildtype (WT) and 6 knock-out (KO) mice, no mice were excluded. Statistical significance was assessed for each group with a two-tailed paired-t test of baseline vs agonist. A p value of less than 0.05 was defined as statistically significant.

Data analysis for the EAE mice was run using GraphPad Prism Software and the data is presented as mean \pm SEM for n number of animals. 6 mice were excluded for not meeting constipation criteria – leaving 9 mice in the cohort. EAE symptoms and constipation were analyzed with a two-tailed paired t-test in order to stringently determine

the height of constipation if present for assay testing. The statistical significance between the vehicle and treatment conditions were assessed with a repeated measures one-way ANOVA with Tukey's multiple comparisons test. A p value of less than 0.05 was defined as statistically significant.

2.3 Results

2.3.1 5-HT4-LA1 Agonist Effect on Intestinal Motility in Conditional 5-HT4R Knock-out Mice

The successful conditional knock-out of the epithelial 5-HT4R in a litter of CKO and wildtype littermates serving as a control was confirmed with PCR. Upon this confirmation, we are able to evaluate if the 5HT4-LA1 agonist is non-absorbable and lumenally restricted by primarily acting upon the epithelial receptors. Through the conditional epithelial knock-out, all other systemic 5-HT4R are intact as well as the neuronal 5-HT4 receptors located in the muscularis that are believed to assist in prokinetic action. We are able to evaluate if the epithelial 5-HT4R activation alone is necessary and sufficient to elicit prokinetic action and accelerate motility.

5-HT4-LA1 agonist had no effect on WGT in 5HT4R-CKO mice

The 5HT4-LA1 agonist had an accelerating effect on wildtype mice but no effect on 5HT4R-CKO mice during whole gut transit excretion. The wildtype mice experienced accelerated motility in the presence of the agonist in comparison to the baseline at $p < 0.05$ (Figure 2.6A) indicating drug interaction with the receptor. The knock-out mice exhibited

no change in motility upon 5HT4-LA1 treatment administration (Figure 2.6B). Some knock-out mice experienced improved motility but not to a degree significantly different from the baseline, this is expected since the receptor is no longer fully functional to interact with the drug. The baseline scoring for the wildtype mice was not significantly different from the baseline scoring CKO mice.

5-HT4 did not alter water content in 5HT4R-CKO mice

The wildtype mice experienced an increase in the water content of fecal pellets from baseline to agonist treatment at $p < 0.01$ (Figure 2.7A). Conversely, the 5-HT4 agonist had no effect on percent fecal water retention in knock-out mice (Figure 2.7B). The knock-out mice did not exhibit a significant change in the percentage of water in fecal pellets between the baseline and treatment. The baseline scoring for the wildtype mice was significantly different from the baseline CKO mice at $p < 0.0001$.

5-HT4 agonist had no effect on colonic motility in 5HT4R-CKO mice

As expected, the administration of the 5HT4-LA1 had no effect in the 5HT4-CKO mice whereas in the wildtype mice, the agonist had a significant effect on distal colonic motility. The wildtype mice upon treatment administration exhibited a faster bead expulsion (Figure 2.8A) indicating agonist interaction with the functional target 5-HT4 receptor. The knock-out mice on average did not experience an accelerated time of bead expulsion indicating no change in propulsive motility from baseline to agonist treatment

(Figure 2.8B). The baseline scoring for the wildtype mice was significantly different from the baseline CKO mice at $p < 0.05$.

2.3.2 Efficacy of agonist 5HT4-LA1 and antagonist GR113808 on motility in EAE model

The successful induction of the EAE condition to model constipation was able to be achieved in the whole gut transit and colonic motility assays. However, the fecal water content assay was not successful in showing symptoms of constipation. We are evaluating the effectiveness of agonist 5HT4-LA1 in mediating prokinetic action (shown in the wildtype mice during the CKO study) in a disease model for constipation. The antagonist serves as a way to determine the site of action and binding ability of the agonist in the presence of the antagonist GR113808. We hypothesize that the agonist will recover symptoms of constipation and will be inhibited by the antagonist.

5HT4-LA1 agonist and whole gut transit

Mice exhibited a slower whole gut transit time at the height of disease (21 days after EAE induction) in comparison to the baseline, confirming difficulty in motility. A paired t-test performed to compare the two time points, indicated a significant difference in response to the baseline and height of disease $p < 0.01$ (Figure 2.9A). An increased amount of time to expel the red pellet during the height of disease condition indicates a state of constipation and intestinal dysfunction due to a slowed bowel movement. The difference in the time taken to expel the red pellet between the vehicle and 5HT4-LA1

treatment or 5HT4-LA1 + GR113808 treatment was not significantly different when assessed by a one-way ANOVA (Figure 2.9B). The 5HT4-LA1 + GR113808 treatment was not significantly different from the vehicle in which the treatment did not increase the time to expel the red dye further than the vehicle.

5-HT4 agonist and fecal water content

In this assay, there is not significant evidence to determine constipation since the mice did not exhibit a difference in water content between the baseline and height of disease during a two-tailed paired t-test (Figure 2.10A). There was no significant difference between the vehicle and the treatment conditions when assessed with a one-way ANOVA (Figure 2.10B).

5-HT4 agonist and colonic propulsive motility

There is a significant difference in motility between baseline and height of disease during a paired t-test, indicating that the time taken to expel the bead increased $p < 0.01$, indicating constipation and subsequent dysfunction (Figure 2.11A). The treatment conditions did not exhibit a significant effect in comparison to the vehicle as assessed with a one-way ANOVA (Figure 2.11B). There was not a significant difference between the 5HT4-LA1 condition and the 5HT4-LA1 + GR113808 condition.

2.3.3 Figures

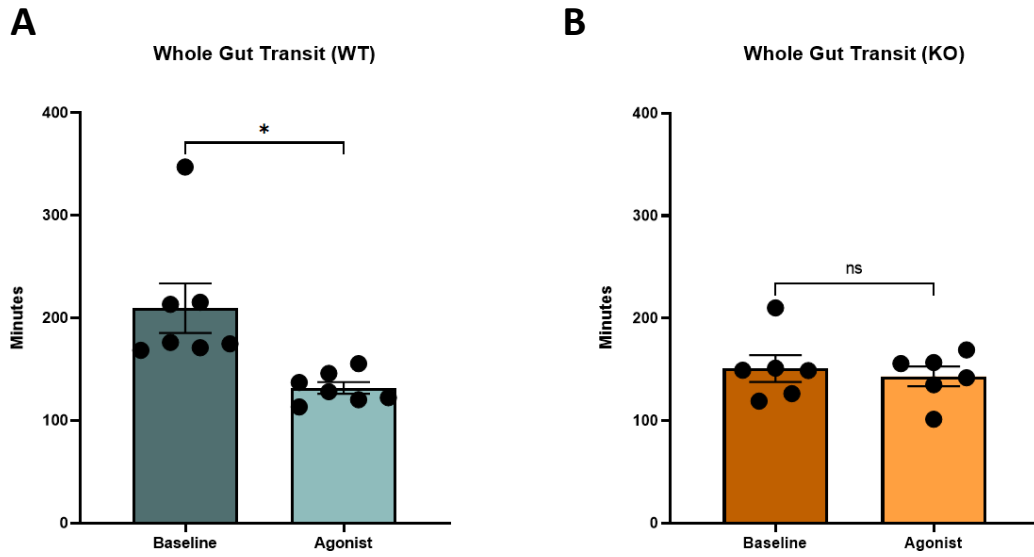


Figure 2.6: WT vs CKO Whole gut Transit. Whole gut transit was assessed via oral gavage of treatment and the time elapsed to expel the red dye. In the wildtype mice (A), the agonist accelerated transit time indicating interaction with the functional 5-HTR $t(6) = 2.784$, $p^* = 0.0318$. The knock-out mice (B) are $p < ns$, indicating no change in the knock-out mice with the mutant receptor $t(5) = 0.5261$, $p < 0.6213$. Data represented as mean \pm SEM and significance assessed for each group between the baseline and agonist a with two-tailed paired t-test.

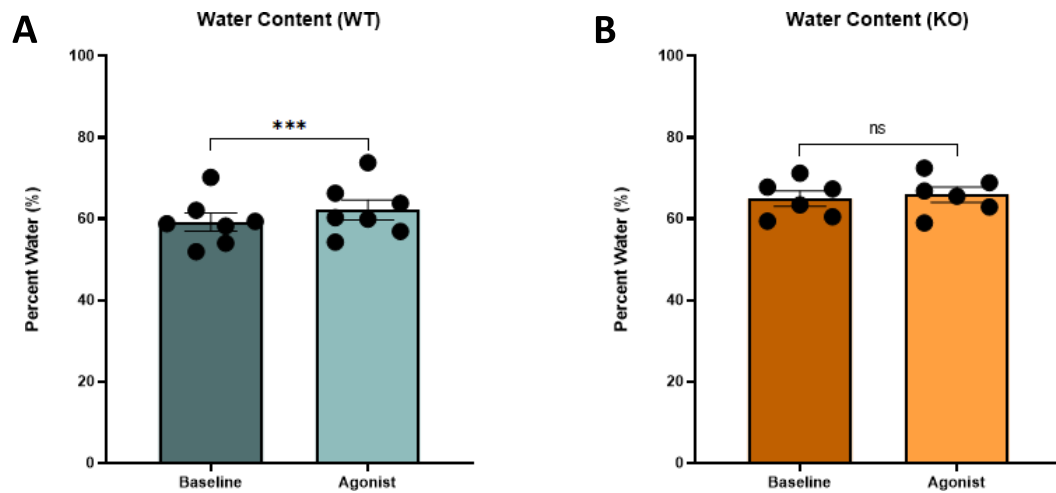


Figure 2.7: WT vs CKO water content. Fecal water content was assessed by capturing fecal pellets and weighing them as wet specimens and dry specimens to determine the percentage of water in the pellets. (A) The agonist effected the wildtype mice in which the amount of water present in the pellets increased $p^{**} < 0.001$ in comparison to the baseline $t(6) = 6.617$, $p = 0.0006$. (B) The knock-out mice did not have a change in the percent of water from the baseline to agonist treatment at $p < ns$, $t(5) = 1.310$, $p = 0.2470$. Data represented as mean \pm SEM and significance assessed between the baseline and agonist for each group with a two-tailed paired t-test.

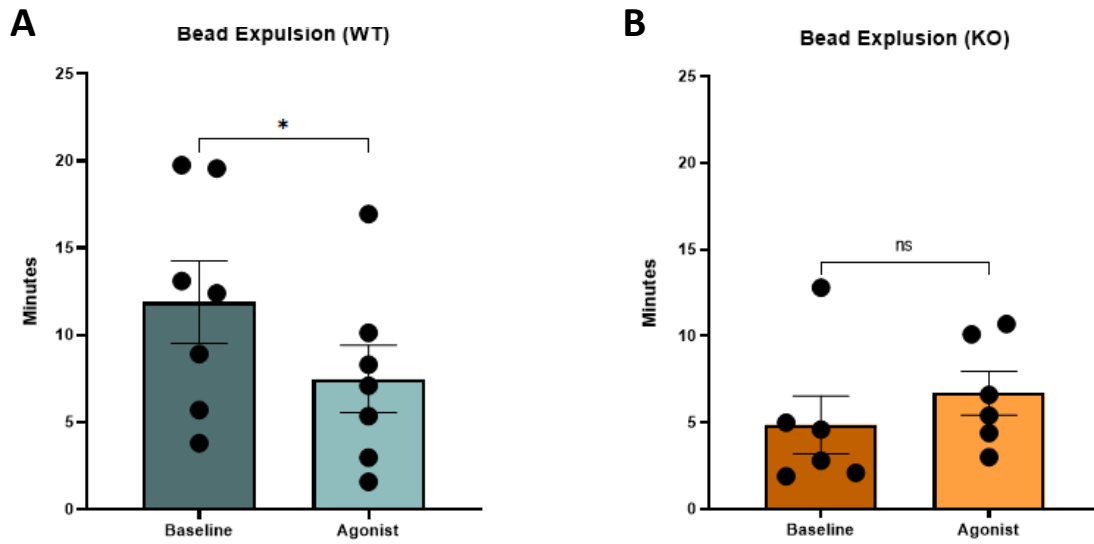


Figure 2.8: WT vs CKO Bead Expulsion. Colonic motility was assessed via oral gavage of treatment or vehicle and the time elapsed from insertion of the bead and time to expel the bead. The agonist in the wildtype mice (A) with the functional 5-HT₄R accelerated the time needed to expel the bead at $p < 0.05$, $t(6) = 2.623$, $p = 0.03$. The mice expressing the non-functional gene, do not have a significant change in the time elapsed to expel the bead $p < ns$, $t(5) = 0.7940$, $p = 0.4632$. Data represented as mean \pm SEM and significance assessed for each group between the baseline and agonist with a two-tailed paired t-test.

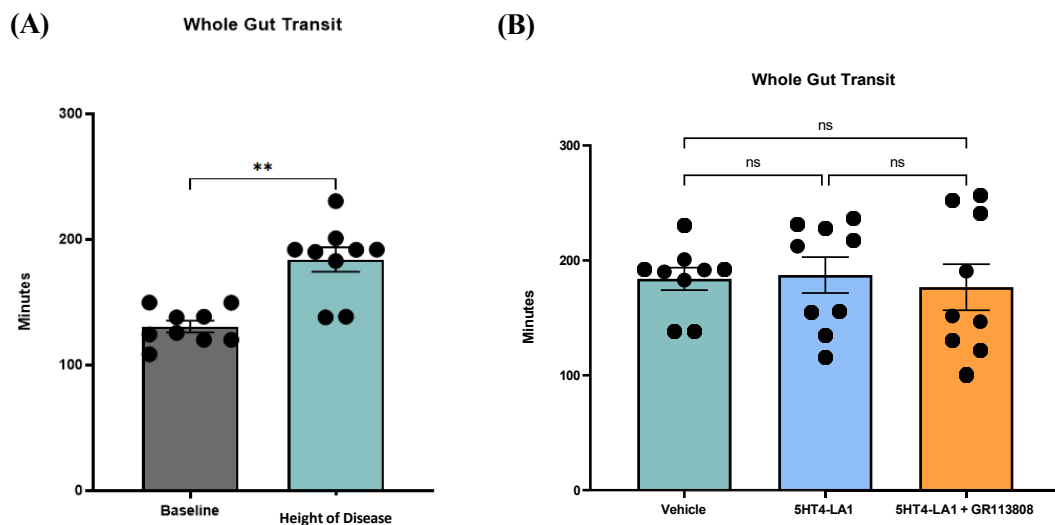


Figure 2.9: EAE mouse and Agonist Efficacy During Whole Gut Transit. Whole gut transit was assessed via oral gavage of treatment or vehicle and the time elapsed to expel the red dye. (A) Constipation was confirmed by a slower transit time to the baseline. A paired t-test was performed to assess for constipation between the baseline and the vehicle conditions $p < 0.01$. There was a significant difference in response to the baseline ($M = 130.5$, $SD = 14.18$) and vehicle ($M = 184.0$, $SD = 29.27$); $t(8) = 4.780$, $p < 0.0014$ indicating that the time taken to expel the dye increased, establishing

symptoms of constipation. Data represented as mean \pm SEM and analyzed by a two-tailed paired t-test. (B) The treatment conditions did not have an effect in comparison to the constipated state (vehicle) ($p < ns$). Data represented as mean \pm SEM and significance assessed with a one-way ANOVA with Tukey's multiple comparison tests to compare the treatment conditions to the vehicle $p < ns$, $n = 9$.

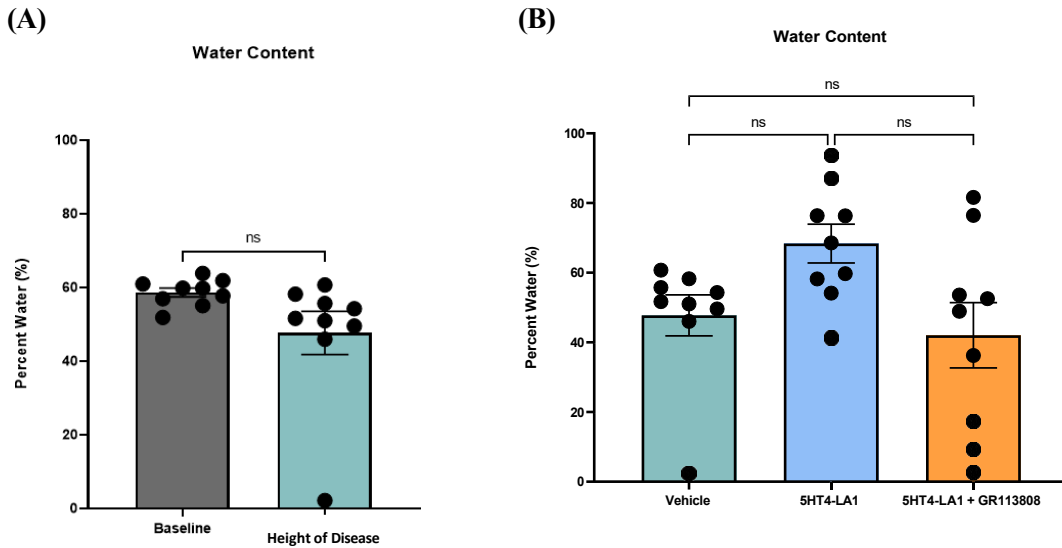


Figure 2.10: Fecal water content was assessed by capturing fecal pellets and weighing them as wet specimens and dry specimens to determine the percentage of water in the pellets. (A) Water content was assessed for constipation between the baseline and the vehicle conditions with a paired t-test $p < ns$. There was not a significant difference in response to the baseline ($M = 58.73$, $SD = 3.663$) and vehicle ($M = 47.74$, $SD = 17.65$); $t(8) = 1.864$, $p = 0.0993$ indicating no change in the time taken to expel the dye and no symptoms of constipation. Data represented as mean \pm SEM and analyzed by a two-tailed paired t-test. (B) There is no significant difference between the vehicle and treatment conditions $p < ns$. Data represented as mean \pm SEM and significance assessed with a one-way ANOVA with Tukey's multiple comparison tests to compare the treatment conditions to the vehicle $p < ns$, $n = 9$.

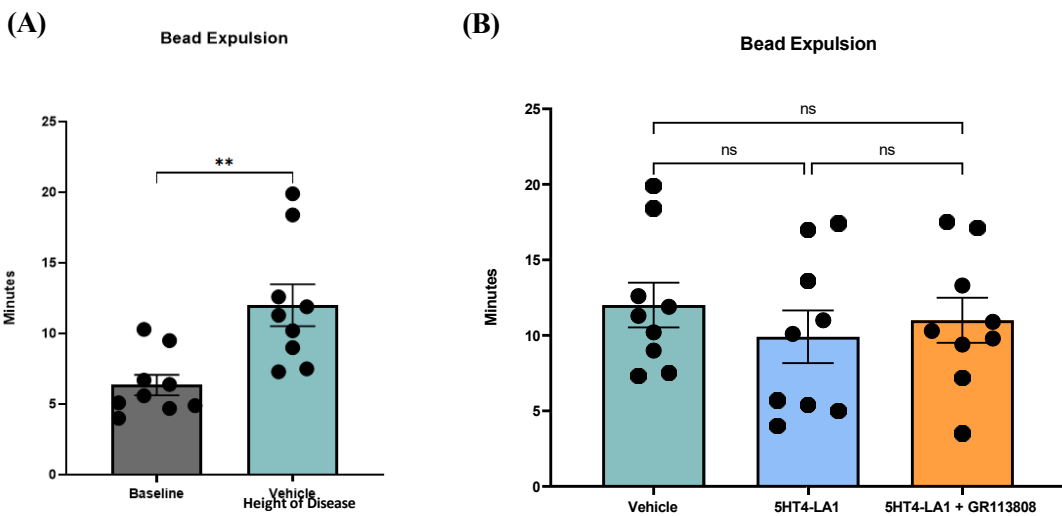
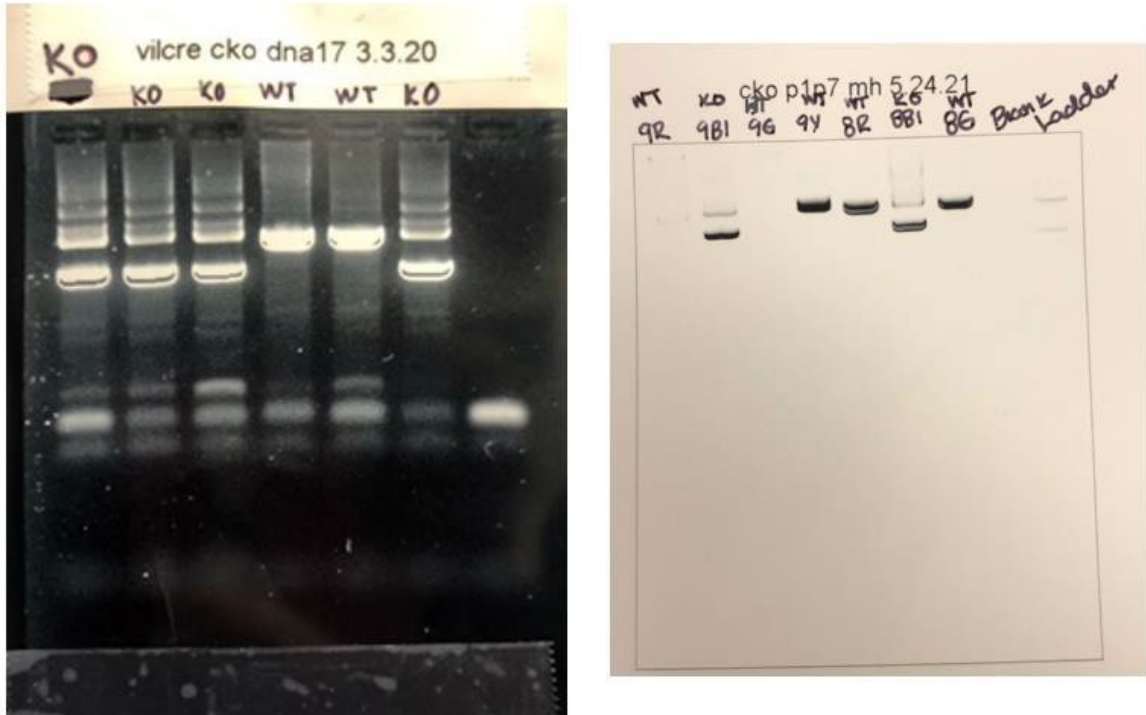


Figure 2.11: Colonic motility was assessed via oral gavage of treatment or vehicle and the time elapsed from insertion of the bead and time to expel the bead. (A) Constipation was confirmed by a slower transit time than the baseline. A

paired t-test was performed to assess for constipation between the baseline and the vehicle conditions $p < 0.01$. There was a significant difference in response to the baseline ($M = 6.356$, $SD = 2.183$) and vehicle ($M = 12.01$, $SD = 4.455$); $t(8) = 4.797$, $p = 0.0014$. indicating that the time taken to expel the bead decreased, indicating constipation. Data represented as mean \pm SEM and analyzed by a two-tailed paired t-test. (B) The treatment conditions did not have an effect compared to the vehicle $p < ns$. Data represented as mean \pm SEM and significance assessed with one-way ANOVA with Tukey's multiple comparison tests, $p < ns$, $n = 9$.



Supplemental Figure 2.12: CKO and WT PCR confirmation of epithelial 5-HT4Rs. Polymerase Chain Reaction (PCR) verification of the successful knock out (mutation) of the 5-HT4 receptor is indicated by two bands expressing the non-functional gene at 612bp and the wildtype by one band at 1214bp. The wildtype mice have a single band at 1214bp.

2.4 Discussion

These studies were conducted in order to understand the role underlying the site of binding of the agonist drug to the 5-HT4 receptor to modulate motility. The receptor that is activated can have an effect via neuronal or epithelial 5-HT4R but by knocking out the epithelial receptor in mice and observing the effects upon drug administration; the CKO findings conclude that the agonist binds to the epithelial receptor, deeming it necessary to elicit prokinetic action. The 5HT4-LA1 agonist is confirmed to be lumenally restricted and unable to significantly interact with the neuronal 5-HT4 receptors. The EAE studies were conducted to investigate the effectiveness of the lumenally restricted 5-HT4 receptor agonist and antagonist on constipation, a feature exhibited in EAE mice as a model of multiple sclerosis. Patients with multiple sclerosis often experience difficulty with intestinal function involving excretion consistent with constipation. The enteric nervous system controls the coordinated peristalsis of the intestines which can be activated by chemical or mechanical stimulus – in these patients the autonomic dysregulation prevents proper function leading to an inability to move materials and cause pain (Camilleri, et al., 2017). Symptoms of constipation or lack of motility are thought to be rescued by the agonist stimulation of 5-HT4R and an action that can be inhibited by an antagonist in wildtype and EAE mice although not demonstrated in our study, but in the literature.

2.4.1 5-HT4R Agonist Effect on Intestinal Motility in Conditional 5-HT4R Knock-out Mice

Serotonin is known to modulate aspects of GI motility and peristalsis – such as exciting enteric inhibitory neurons which slow gastric emptying. Additional studies with 5-HT4 agonists indicate that stimulation of 5-HT4 receptors enhances the generation and survival of new enteric neurons. 5-HT4 stimulation enhances synaptic transmission through a presynaptic mechanism and also promotes neurite outgrowth and the formation of neural networks such as the transdifferentiation of glial cells and enteric neurons (Belkind-Gerson, et al., 2015). These extensive projections could influence recovery from colitis or inflammation by releasing increased serotonin. Spohn and colleagues (2018), found that agonist treatment increased epithelial proliferation, recovery from colitis, wound healing, and resistance to oxidative stress. 5-HT4 receptors could be influenced by the neurogenesis involving the creation of new extensive glial projections and the repair of neural circuits which has shown to cause improvement in the regeneration and recovery of enteric and excretion reflexes (Terry & Margolis, 2016). Furthermore, studies in mice where the 5-HT4 receptor was knocked out to simulate a deficiency during embryonic development, did not alter the density of enteric neurons but did lead to a postnatal deficiency in enteric neurons (Mawe & Hoffman, 2013). 5-HT4 receptor stimulation is demonstrated to recover these neural networks and excretion reflexes, in practice, an isolated glia in an avian hindgut gave rise to a generic neural network (Belkind-Gerson, et al., 2015).

Overall, in the present conditional knock-out study, upon drug administration, the wildtype mice responded to the 5HT4-LA1 agonist treatment and the epithelial 5HT4R-CKO mice exhibited no change in motility in comparison to the baseline scoring. The wildtype mice had increased and faster whole gut and distal colon transits as well as increased water content indicating that the agonist is acting on 5-HT4 receptors. The

knock-out mice express mutant, non-functional 5-HT₄ epithelial receptors which prevent the binding of the agonist to this receptor in epithelial cells. The presence of the mutant gene was confirmed using PCR indicating two bands for the mutant gene at 612bp and the wildtype receptor at 1214bp (Supplemental Figure 2.12). The presence of the band at 1214bp for the KO mice indicates that the 5-HT₄ neuronal receptors and 5-HT₄ receptors in alternate locations are intact and functional. Due to the lack of an antibody for immunostaining or western blot, it is not possible to confirm the degree of mutation or downregulation of the epithelial 5-HT₄R. Therefore, it is possible that some receptors are left intact for interaction with the drug but there was no evidence of a significant interaction with the data collected from all three motility assays. The lack of change between the baseline and agonist scoring indicates that the prokinetic effects seen in the wildtype mice are working predominately on epithelial 5-HT₄ receptors since the neuronal receptors are functional for potential interaction with 5-HT₄R agonists. 5HT₄-LA1 is lumenally restricted so the neuronal population of receptors is expected to not be directly activated as these receptors lie in the muscularis. Epithelial 5-HT₄R action is demonstrated as the primary receptor modulating prokinetic action.

The GI tract is innervated extrinsically by the CNS and intrinsically by the ENS. Serotonin can activate signaling to the CNS to stimulate digestive and homeostatic reflexes that can cause abdominal pain and satiety. Mechanical and chemical stimulation causes the release of 5-HT from enterochromaffin cells (EC) which activates the vago-vagal reflex (the vagal afferent fibers arising in the upper GI tract and nerve endings in the gut mucosal layer) to regulate fluid secretion and inhibit gastric emptying (Mawe & Hoffman, 2013). The knockout of the epithelial serotonin receptors is to emphasize that the signaling is not

being processed or sent down via the CNS and it is directly acting on epithelial receptors. This drug is demonstrated to be unaided by the CNS or acting on the intact neuronal 5-HT₄ receptors in the muscularis because there was no change in motility without the functional epithelial receptors, deeming the main target. Cisapride – another 5-HT₄R agonist – was found to elicit mucosal serotonin release by directly activating the target serotonin receptors on EC cells and persisted in the presence of tetrodotoxin (TTX), which is blocking the use of a neural mechanism by blocking the sodium channels for action potentials to fire (Hoffman, et al., 2012). Additionally, a separate recent study by Kashyap and colleagues (2018), identified a ligand for the epithelial 5-HT₄R, which induces increased colonic mucus secretion and accelerated whole gut transit, supporting that prokinetic action lies in the epithelium, as hypothesized in this study.

2.4.2 Efficacy of agonist 5HT₄-LA1 and antagonist GR113808 on motility in EAE model

In preceding studies of GI function in EAE mice (Spear et al., 2018; Konen et al., 2020) the EAE mice exhibited symptoms of constipation at the height of disease with a slower whole gut transit time, expulsion of the bead, and lower water percentage of fecal contents. The literature successfully confirms the EAE condition as a model of MS and gastrointestinal distress. In the study conducted by Spear and colleagues (2018), it was determined that the EAE mice exhibit structural and functional changes at the level of the ENS. The GI motility of the EAE mice is demonstrated to be digressive as shown by the motility assays indicating the inability of the GI to efficiently excrete material. In an assay for glial fibrillary acidic protein (GFAP), there was a decreased distribution within the

colonic myenteric ganglia which is associated with gastrointestinal disease and phenotypic changes in neurons and altering motility.

5-HT₄ analogues such as Cisapride, Renzapride, Tegaserod, and Prucalopride have been found to possess prokinetic action in accelerating gastric emptying and alleviating constipation. Some 5-HT₄ agonists that are no longer available, were initially created to be systemically absorbed via the blood stream to access the enteric neural plexuses of the intestines and stomach. This lack of selectivity and systemic distribution resulted in the binding to other 5-HT₄ receptors located in the adrenal cortex, urinary bladder, and to ERG K⁺ channels in cardiac muscle leading to fatal arrhythmias. Luminally restricted 5-HT₄ agonists have shown minimal or negligent absorption in other peripheral tissues or circulation. Konen and colleagues (2020) confirmed that two agonist compounds (including 5HT₄-LA1) were luminally restricted by bioavailability studies in which negligible concentrations (10 ng/ml) of the drug were found in the plasma after agonist administration but after 2 hours, the agonist was no longer detected at all. The concentration in the colon content itself was around 100,000 ng/ml at two hours. Increased 5-HT release from the agonist binding during luminal administration can activate the peristaltic reflex, mucus release to decrease intestinal friction, and fluid secretion can facilitate softer stool and effective propulsion (Hoffman, et al., 2012).

However, the results obtained in the current EAE study did not exhibit an effect on the mice upon agonist or antagonist administration in comparison to the vehicle (height of disease). The mice were shown to be induced with EAE with signs of constipation in only two of the assays – the whole gut transit and bead expulsion. On trend, it was demonstrated that there was some change in the average speed of excretion for the bead expulsion assay

that is consistent with the literature. The agonist minimally accelerated the amount of time needed to expel fecal pellets, indicating an ability to rescue the symptoms of constipation. The antagonist effectively inhibited the binding of the agonist to the 5-HT₄ receptor to a similar degree of the constipated state. This can indicate that the duration of binding the antagonist to the receptor is exceeding 30 minutes and a potent antagonist even in the presence of the agonist. The agonist had the most extensive response in the bead expulsion assay since the targeted region is where the primary serotonin receptors are located. The receptors are densely located in the mouse distal colon, given that fluorescent labeling in BAC transgenic mice and 5-HT₄ mRNA transcription levels are significantly greater in this region than the stomach, duodenum, ileum, and proximal colon in the mouse mucosa (Hoffman et al., 2012). The other two assays focus on the whole GI transit which allows for other factors and distribution resulting in potentially not as strong of an effect from the agonist. A caveat is that the drug may yield different results from the mouse mucosa model to the human mucosa since the human ileum has the highest mRNA transcript levels in contrast to the distal mouse colon. The target effectivity of the drug may vary from assay to assay and the human model, but the agonist and antagonist bind to and have an effect on the 5-HT₄ receptor and motility in the literature and marketed agonist compounds such as prucalopride (Konen et al., 2020).

The lack of a statistically significant response to the agonist could be due to the small number of mice in each cohort, inaccurate preparation of the agonist emulsion, and the EAE recovery of the mice over time and lessening symptoms of constipation naturally. The small cohort of mice included in the final evaluation were much less than the entire group due to excluding animals that did not meet the criteria for symptoms of constipation for

testing. Therefore, with a larger cohort to eliminate outlier variation there may be a significant effect from the agonist and agonist + antagonist treatment conditions. The EAE condition of the mice gradually improves over time, and in the experiment, the mice are not all receiving the same drug treatment on the same day, this was to attempt to control for the factor of time and recovery but was not effective.

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CHAPTER III: Concluding Remarks

3.1 Overview and Conclusion

Multiple Sclerosis (MS) is a neurodegenerative disease of the CNS manifesting as an autoimmune disorder in which inflammatory lesions are caused by autoreactive lymphocytes (t-cells). These lesions result in the demyelination of axons causing neuronal damage and formations of plaques leading to autonomic dysregulation (Goldenberg, 2012). The axonal damage leads to a rapid onset of symptoms presenting as motor weakness and gastrointestinal dysfunction such as constipation. The intestines are unable to effectively excrete materials by the coordination of muscle contractions and proper mucus secretion. The exact etiology is unknown, but lack of vitamin D levels and metabolism are known factors for immune system regulation and inadequate presentation of self-antigens (Goldenberg, 2012). The treatment options for patients usually involve the slowing of disease progression and preventing further disability. Recommendations include lifestyle changes such as outdoor activity and diet. However, given the debilitating nature of the disease this can be very difficult for late-stage patients. The best treatment options currently available act on improving symptoms but not the disease itself such as the 5-HT₄ receptor agonist 5HT₄-LA1 for constipation and regaining involuntary muscle control of the gut to excrete materials properly. 5-HT₄R agonistic compounds may also assist accelerated wound healing, resistance to oxidative stress, neuroprotective function, and epithelial cell proliferation as known functions of the 5-HT₄ receptor in literature (Konen, et al., 2020).

Serotonin is known to have major influence in various regions of the body from CNS to the PNS. A majority of the serotonin in the body is synthesized, stored, and released in the enteric nervous system with the activation of serotonin release stemming

both intrinsically and extrinsically to maintain homeostasis through bidirectional communication. There are seven types of 5-HT receptors with the main two receptors 5-HT₃ (ligand gated ion channel) and 5-HT₄ (G-protein coupled receptor) that greatly affect the GI system in relaying sensations of pain, bloating, and rate of excretion (peristaltic and secretory reflexes) (Kanova & Kohout, 2021). In the intestinal mucosa a subset of enteroendocrine cells – enterochromaffin cells (EC) – that are specialized to release stores of serotonin upon chemical or mechanical stimulation to interact with 5-HT₄ receptors (Mawe & Hoffman, 2013). The 5-HT₄ receptor agonist is known to mimic serotonin release and increase receptor activation to subsequently improve recovery from inflammation, epithelial healing, constipation, and propulsive motility (Konen, et al., 2020). The agonist 5HT4-LA1 upon binding could mediate these effects but is studied specifically for recovery from constipation through the modulation of prokinetic action.

It was hypothesized in the current study conducted that agonist 5-HT₄LA1 is acting upon epithelial 5-HT₄Rs to improve the speed of motility. Utilizing the VilCre epithelial CKO mouse model in comparison to wildtype mice of the same litter; we were able to confirm the epithelial receptor is necessary to elicit prokinetic action. After conducting the assays for whole gut transit, fecal water content, and colonic motility, all three yielded the same results in which the wildtype mice responded to 5HT4-LA1 agonist treatment with improved and faster motility. Whereas the CKO mice did not respond to the agonist treatment. The CKO mice express non-functional epithelial 5-HT₄Rs, unable to interact with the epithelial receptors in the intestinal mucosal layer or the neuronal 5-HT₄Rs located in the muscularis. The agonist 5HT4-LA1 is confirmed to be lumenally restricted due to a lack of interaction with neuronal receptors or other receptor types to induce prokinetic

action. The following arm of the study is evaluating the efficacy of 5HT4-LA1 in the recovery of symptoms of constipation utilizing the EAE model. Secondly, the evaluation of the site of action of the agonist 5HT4-LA1 by use of the antagonist GR113808 ability to inhibit the prokinetic action of the agonist. Symptoms of constipation were present in 2 of 3 assays, the whole gut transit and colonic motility assays without a response indicating constipation from the fecal water content assay. However, in all three of the assays the agonist and agonist + antagonist treatment conditions did not have a significant effect in comparison to the height of disease. We are not able to conclude from this data that the agonist is able to recover symptoms of constipation, contrasting the available literature.

3.2 Limitations

The 5-HT4R agonist 5HT4-LA1 stimulates epithelial 5-HT4Rs in order to mediate prokinetic action. The 5-HT4R is a G-protein coupled receptor, so upon activation from the binding of serotonin by the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) activates the stimulatory G protein (Gs) (Mawe & Hoffman, 2013). In turn, this activates the adenylate cyclase and protein kinase A (PKA) pathway to cause a conformational change and the activation of downstream signaling such as epithelial cell (goblet cell) mucus secretion, enterocyte chloride (Cl⁻) secretion, bicarbonate secretion, and enhanced acetylcholine release from myenteric neurons for intestinal contractions (peristaltic reflex) (Mawe & Hoffman, 2013). Additionally, the ligand-gated ion channel 5-HT3R can be stimulated due to the location on mucosal projections from myenteric primary afferent neurons. Interestingly, the administration of agonist in a bath solution with direct access to the myenteric plexus did not improve

motility (Hoffman, et al., 2012). However, the drug 5HT4-LA1 is lumenally restricted and should not be able to directly access or act presynaptically on the nerve terminals to enhance the release of acetylcholine as a naturally occurring reflex instead of through neurotransmission without the activation of epithelial 5-HT4Rs (Mawe & Hoffman, 2013). The epithelial 5HT4R-CKO data supports this conclusion, however, it is noted that the wildtype mice and conditional knock-out mice did not have similar baseline scoring for the fecal water content and colonic motility assay. There was no significant difference between the baseline scoring for the whole gut transit assay. The litter of mice obtained for experimentation was very small and the groups are uneven by one mouse (7 wildtype, 6 CKO), with a larger litter the data may shift to be significantly different across all three assays. It is hypothesized that the CKO mice are deficient in the epithelial 5-HT4Rs at birth and compensated with another method of excretion since the GI system is highly conserved and necessary for survival. The compensation could involve the reliance and genesis of excess 5-HT3Rs and an emphasis or ease of activation involving neuronal 5-HT4Rs. Additionally, the body may choose to produce more prostaglandins or hormones such as motilin or gastrin to stimulate intestinal muscle contractions.

The EAE efficacy experimentation was not able to establish symptoms of constipation for all of the assays at the height of disease. The EAE condition is variable in symptom presentation and essential that the mice survive through the length of the experimentation as in the previous pilot experimentation, most mice were too sick and required saline injections. This cohort during preparation of the emulsion there is room for experimental error and some mice received less of a dose than expected due to loss of solution during injection. The final cohort was much smaller than the initial litter which

places greater significance on outliers due to the inability to distinguish true outliers that are skewing the data. A larger cohort could shift the results of the agonist or agonist + antagonist treatments towards significance as shown in the literature. Additionally, the timeline for the experimentation during the second treatment administration is near day 35 and the mice are naturally recovering from EAE symptoms at this time. Natural disease recovery affects the degree of constipation the animals are experiencing at each assay administration, which creates a confounding factor for comparing the effect of the drug against symptoms of constipation.

Despite the findings of the EAE study, 5-HT4-LA1 is a promising therapeutic method for the treatment of constipation since similar compounds are approved medications and promising data collected from other studies. The differences between the human GI system and the mouse GI system can raise concerns about efficacy and toxicology experimentation. The intestinal 5-HT4Rs in the human gut has the highest density in the terminal ileum contrasting to the highest density in the mouse is the distal colon (Hoffman et al., 2012). Additionally, the human GI system is more complex and lined by glandular units, whereas the mouse stomach is lined with squamous epithelium (Willet, 2016). The structural and functional differences between the human small intestine vs the mouse large intestine may be even less comparable when testing the drug. The drug could be absorbed and metabolized differently, which would pose the greatest concern due to other 5-HT4Rs such as located on the heart. The EAE model cannot replicate certain aspects of human disease, mouse models rarely fully replicate the complexity and heterogeneity of the human condition. The results provide an estimation of the effects a drug will have on the body but can potentially lead to misleading conclusions in practice.

3.3 Future Direction

The EAE condition can be induced in multiple ways to yield constipation symptoms such as changing the type of peptides used and the age of the mice. The symptom presentation is variable and the factor of natural recovery over time limits the timeline of experimentation effectivity. In a study conducted by Liang and colleagues (2016), a model for constipation could be utilized in which irritation with ice-cold saline via intragastric administration. Ice-cold saline is administered for 14 days with stable signs of constipation and return to normalcy 6 days after termination of irritation. Immunohistochemistry results demonstrated that ice-cold saline causes morphological changes of the enteric nervous system in the jejunum, ileum, and colonic myenteric plexus, thus affecting epithelial and neuronal serotonin receptors. The results of GI irritation resulted in a significantly lower percent change in intestinal propulsion rate in comparison to control scoring. Additionally, rate of defecation was significantly prolonged in the amount of time and amount of fecal pellets produced. The changes in stool presentation are significant in which the weight, size, and water content of fecal pellets is significantly less than the control (Liang, et al., 2016). However, with the saline injections the water content assay may not be a reliable assay due to the nature of experimentation adding water to the GI system. This method of experimentation could be viably tested for the whole gut transit and colonic motility assay. This method of experimentation is promising due to low variability in symptom presentation such as with the EAE induction and model.

The epithelial conditional knock-out model could be further evaluated with a larger cohort to determine if the differences in the baseline scoring between the wildtype

and CKO mice is due to chance or morphological or hormone differences. This could be tested by immunostaining to determine the density of 5-HT₃ receptors or neuronal 5-HT₄ receptors if possible. The evaluation of any hormonal changes or amount of prostaglandins through blood or urine testing could be conducted to assess for the changes in baseline motility. Furthermore, the binding action of the agonist 5HT4-LA1 and level of assistance by potential interaction of neuronal 5-HT₄R_s could be evaluated by conditionally knocking out the neuronal 5-HT₄R, leaving the epithelial 5HT4R_s intact. Any changes from the wildtype baseline scoring could indicate that the neuronal receptors are being activated by some degree, which is not desired by a non-absorbable, lumenally restricted serotonin agonist.

3.4 Application

The agonist 5HT4-LA1 is expected to be nonabsorbable and lumenally restricted acting primarily upon epithelial receptors to mediate prokinetic action and recovery from symptoms of constipation. Similar drugs such as prucalopride act as a highly selective 5-HT₄ agonist promoting cholinergic neurotransmission by enteric neurons, similarly to 5HT4-LA1 (Konen, et al., 2020). In a study conducted by Wong and colleagues (2010), the selectivity for the 5-HT₄R was confirmed for prucalopride by an antagonist inhibitor GR113808 (the same antagonist used for the current experimentation). In patients with multiple sclerosis and constipation, prucalopride loosens stool consistency, increased the frequency of bowel movements, and decreased severity of constipation or need for laxatives. In the clinical trial cohort of ~4,000 patients there were no adverse cardiac events except for in two healthy patients who received 10x the therapeutic dosage. This finding

could indicate that the drug is able to bind to the 5-HT₄R isoforms in the heart at very high concentrations. Overall, prucalopride is well tolerated and patients reported an improved quality of life with medium- and long-term treatment. It would be expected that 5HT₄-LA1 would yield similar clinical results for patients with multiple sclerosis and chronic constipation. Prucalopride binds with high affinity to 5-HT₄ receptor isoforms but is not lumenally restricted for access and affinity to receptors in the cardiac tissue and other receptors such as the human dopamine receptor (D₄R), deeming the 5HT₄-LA1 agonist a potentially better candidate for treatment (Wong, et al., 2016).

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