SEROTONERGIC SIGNALING AT 5-HT 3 RECEPTORS IN SERTOTONIN TRANSPORTER (SERT) KNOCKOUT (KO) RAT, A SEX SPECIFIC ANIMAL MODEL OF VISCERAL HYPERSENSITIVTY

By

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ABSTRACT

SEROTONERGIC SIGNALING AT 5-HT 3 RECEPTORS IN SERTOTONIN TRANSPORTER (SERT) KNOCKOUT (KO) RAT, A SEX SPECIFIC ANIMAL MODEL OF VISCERAL HYPERSENSITIVTY

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The irritable bowel syndrome (IBS) is a functional gastrointestinal motor and visceral sensation disorder that is more common in women compared to men. 5-Hydroxytryptamine (5-HT, serotonin) signaling is disrupted in some IBS patients possibly due to polymorphic variations in the gene encoding the serotonin transporter (SERT) which result in increased extracellular 5-HT availability. Female SERT knockout (KO) rats exhibit visceral hypersensitivity to colonic distention that mimics colonic hypersensitivity known to occur in female IBS patients. Studies performed herein focused on understanding the role of 5-HT3 receptor signaling in SERT KO rats. The visceromotor response (VMR) to colorectal distension (CRD) was determined following inhibition of peripheral and central 5-HT3 receptors in SERT KO and WT rats. In female SERT KO rats spinal 5-HT3 receptor inhibition with alosetron caused an increase in VMR to CRD. In Male SERT KO rats activation of spinal 5-HT3 receptors increased VMR to CRD. Depletion of descending serotonergic input from the brainstem reversed the effects of 5-HT 3 receptor inhibition in SERT KO female rats and activation in SERT KO male rats. Based on these studies, I concluded that this sex specific response observed in SERT KO rats is due to differential pattern of 5-HT3 receptor expression in male and female SERT KO rats.

The effects of ovarian hormone on VMR to CRD were also investigated in SERT KO and WT female rats. Ovariectomy and estrogen receptor (ER) antagonist studies were performed in SERT KO and WT female rats. VMR to CRD was enhanced in the proestrus phase of the estrous

cycle in SERT KO but not WT female rats. Ovariectomy increased discomfort to CRD in SERT KO female rats in a time dependent manner. VMR to CRD increased on day 7 post-ovariectomy and lasted for up to 3 weeks. Intrathecal administration of ICI 182 780 (ER-α and ER-β) antagonist increased VMR to CRD in SERT KO female rats. The G-protein coupled ER (GPR30) antagonist, G15, did not affect VMR to CRD in SERT KO and WT female rats. These studies suggest that in SERT KO female rats classical ERs (ERα and ERβ) play an antinociceptive role in the presence of serotonergic dysfunction.

This dissertation is dedicated to my dear uncle Eddie Chaaban.
Thank you for being my role model.

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Chapter 1

General Introduction

1.1 Pain transmission

Sensory information is transmitted from visceral organs to the brain along a three neuronal network as shown in Figure 1.1. Once a stimulus is applied to the peripheral terminals of primary afferent neurons, cell depolarization occurs. This leads to the release of excitatory neurotransmitters at central terminals of the primary afferent neurons located in the dorsal horn of the spinal cord. In turn, the excitatory neurotransmitters will activate second order neurons which relay the signal to the thalamus along the spinothalamic tract (STT) and dorsal column (DC) (Palecek, Paleckova et al. 2002) and excitatory interneurons. STT and spinoreticulothalamic tract collaterals relay sensory information to subcortical structures that are critical in pain modulation (Chen, Li et al. 2001). From the thalamus, third order neurons project to different areas of the brain for pain perception. This ascending pain signal is subject to extensive modulation in the dorsal horn of the spinal cord where the primary and secondary order neurons synapse (Millan 2002; Blackshaw, Brookes et al. 2007). Descending modulation can either enhance or dampen pain transmission. A detailed explanation of anatomical organization and neurochemical signaling in visceral pain transmission is discussed in this chapter.

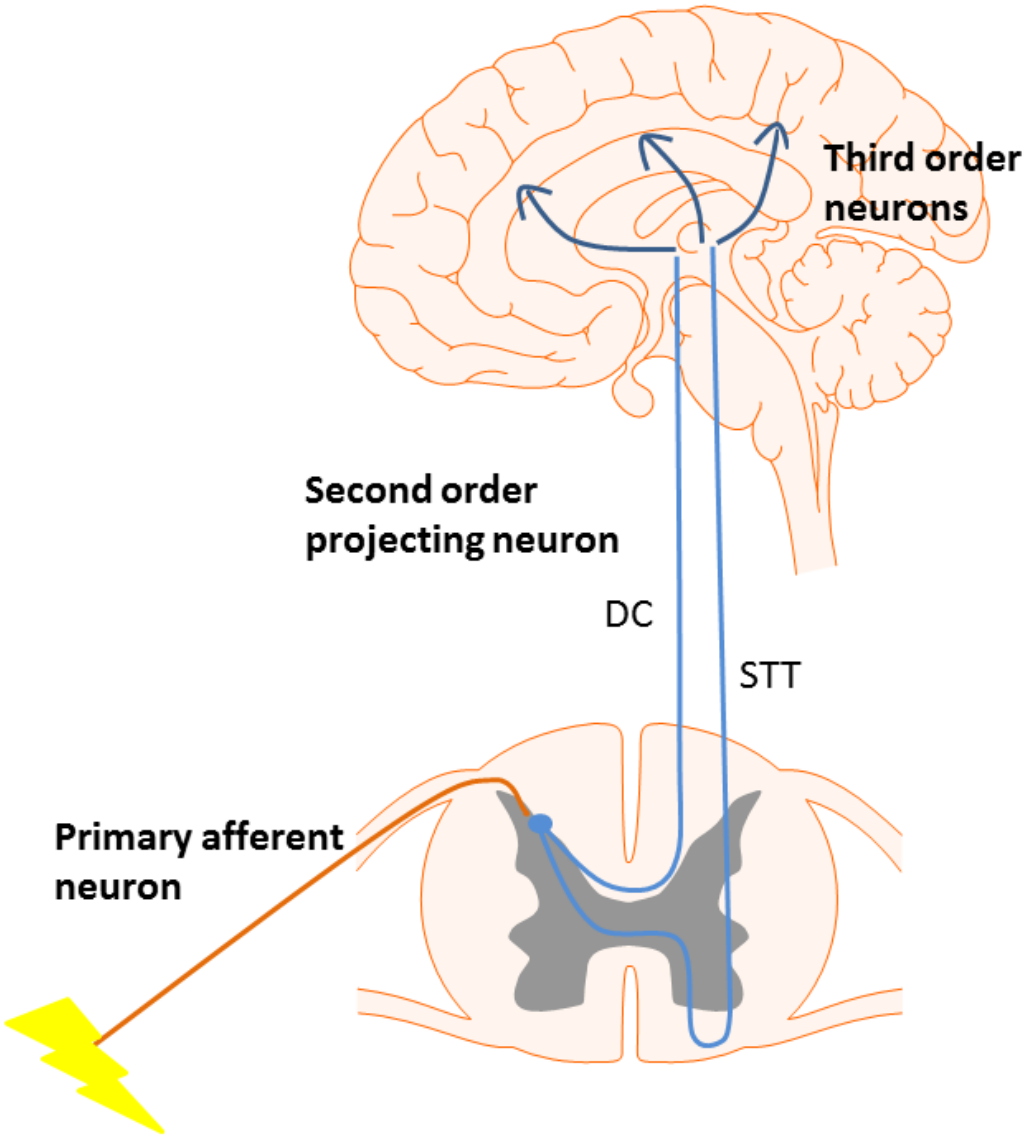

Figure 1.1 Ascending pain pathway. Upon activation by a stimulus, primary afferent neurons transmit sensory information to the dorsal horn where they synapse on second order projecting neurons. Second order neurons carry the information to the thalamus via the dorsal column (DC) and spinothalamic tract (STT). From the thalamus, third order neurons carry the sensory information to various brain regions for pain perception.

1.1.1 Anatomical organization of pain pathways originating from the colon

The gastrointestinal (GI) tract is innervated by an intrinsic network of neurons housed within the GI tract known as the enteric nervous system (ENS). The ENS controls motor and intrinsic sensory function of the GI tract to regulate nutrient absorption, secretion, and motility. Cells bodies of the ENS neurons are located in the myenteric and submucosal plexuses. The GI tract is also innervated by extrinsic neurons that modulate gut function and transmit sensory information from the gut to the central nervous system (CNS). (Blackshaw, Brookes et al. 2007).

Two types of extrinsic primary afferent neurons innervate the GI tract including vagal afferents and spinal afferents. The peripheral terminals of vagal afferents innervate the proximal GI tract (esophagus, stomach, small intestine and proximal colon). Cell bodies of the vagal afferents are located in the nodose ganglia and their central terminals are found in the nucleus tractus solitarious (NTS) in the brainstem. Spinal afferents innervate the entire length of the GI tract and are divided into three groups. The thoracolumbar, lumbosacral and sacral spinal afferents innervate the proximal, middle and distal GI tract, respectively (Figure 1.2). The cell bodies of spinal afferents are found in the dorsal root ganglia and their central terminals are located in the superficial lamina of the dorsal horn in the spinal cord (Furness, Rivera et al.).

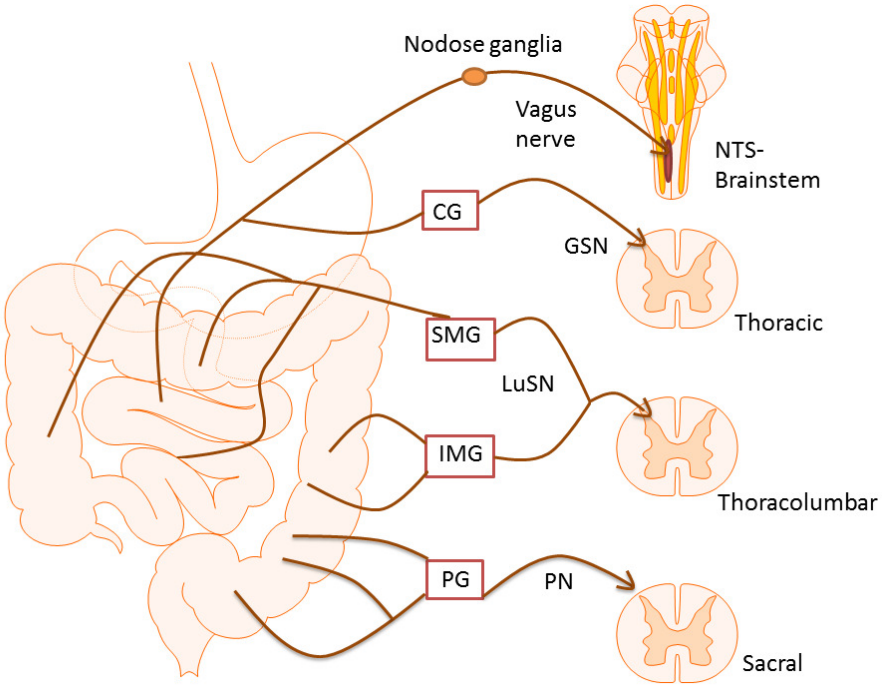

Figure 1.2 Extrinsic sensory innervation of the gastrointestinal tract. Sensory information from the gastrointestinal tract is carried to the central nervous system via the vagus and spinal nerves. The vagus nerve innervates the proximal gastrointestinal tract and transmits non-noxious stimuli. On the other hand, spinal nerves innervate the entire gastrointestinal tract and travel alongside sympathetic neurons. CG: celiac ganglion, GSN: greater splanchnic nerve, IMG: inferior mesenteric ganglion, LuSN: lumbosacral nerve, PG: pelvic ganglion, PN: pelvic nerve, SMG: superior mesenteric ganglia.

The receptive (peripheral) terminals of extrinsic afferent neurons are classified according to their response to different types of stimuli and intensities (Brookes, Spencer et al. 2013; Gebhart and Bielefeldt 2016) . The peripheral terminals include: enteric intraganglionic laminar afferents ending (IGLEs), vascular afferents, mucosal, muscular-mucosal and intramuscular afferents (Figure 1.3). All types of afferent endings respond to probing and thus must express mechanoreceptors. Only mucosal and muscular-mucosal afferents respond to stroking of the mucosa. IGLEs, muscular-mucosal and intramuscular afferents respond to stretch. Vascular nerve ending respond to high threshold but not low threshold probing

suggesting that they convey noxious stimuli. A subset of vascular afferents is called silent or mechanically insensitive afferents (MIA) because they lack mechanosensitivity unless sensitized by capsaicin or inflammatory mediators in different experimental settings (Furness, Rivera et al. ; Blackshaw, Brookes et al. 2007; Brookes, Spencer et al. 2013).

Vascular afferents innervate blood vessels located in the mesentery and submucosa of the GI tract and send collaterals to myenteric and submucosal ganglia, mucosa and muscle layers. Vascular afferents are exclusively spinal and are believed to be the predominant type of neurons capable of transmitting noxious stimuli to the spinal cord. They exhibit varicose branching axons (VBAs) and can be activated both mechanically and chemically (Furness, Rivera et al. ; Su and Gebhart 1998).

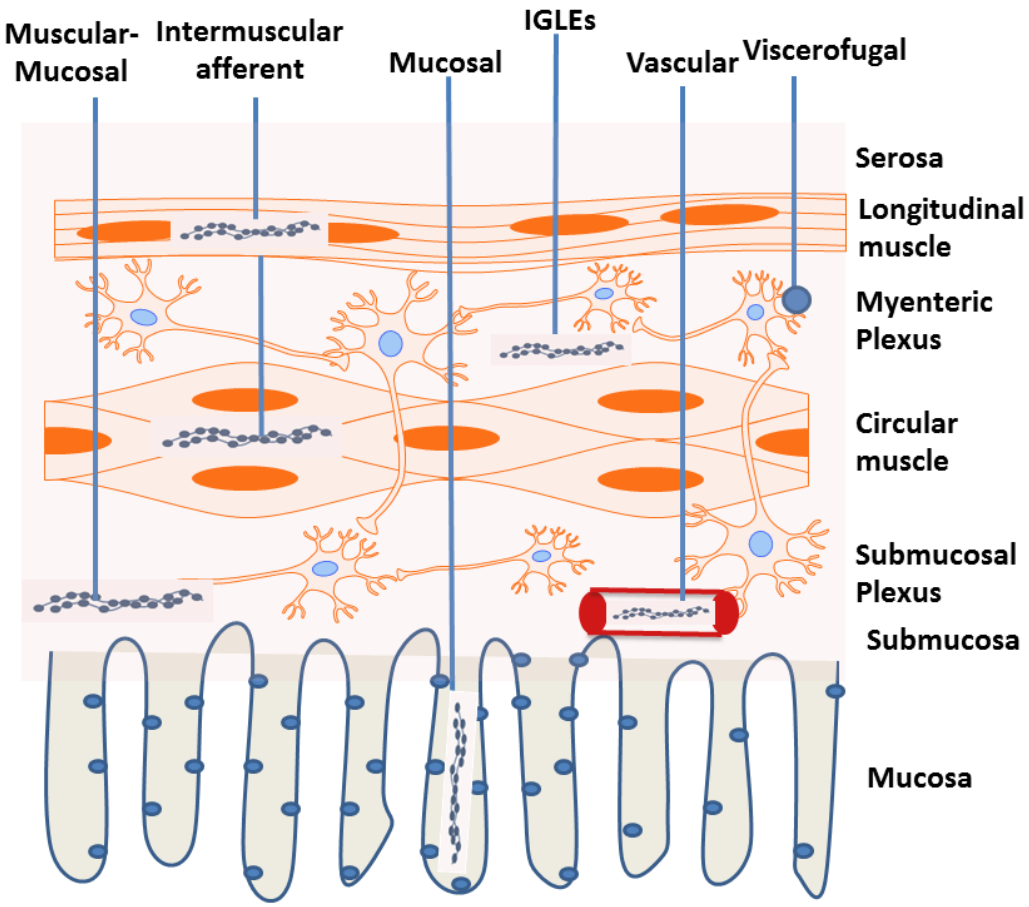

Figure 1.3 Extrinsic afferent ending types innervating the colon wall. The colon wall is innervated by extrinsic primary afferent neurons at various locations. The muscularis mucosa is innervated by muscular-mucosal nerve endings. Intermuscular afferent ending innervate the longitudinal and circular muscles in the colon. Mucosal afferent endings extend to the villi to innervate the mucosa. Intraganglionic laminar ending (IGLEs) are found in the myenteric plexus in close proximity to enteric neurons. Vascular endings terminate on blood vessels in the mucosa as well as blood vessels in the mesentery and serosa (not depicted). Viscerofugal afferents are not technically extrinsic since their cell bodies are found in the myenteric plexus, however they project to the spinal cord.

Studies conducted in endothelin-3 knockout mice which lack enteric nerves in the distal colon suggest that high threshold vascular nerve endings are not the only type of nerve ending capable of transmitting noxious stimuli. Rather, low threshold, wide dynamic range muscular and muscular-mucosal nerve endings play an important role in pain transmission (Zagorodnyuk, Kyloh et al. 2011). This theory is in line with the fact that low threshold stimulation can cause

abdominal pain in patients with visceral hypersensitivity due to inflammation or other pathological states (Gebhart 1999).

Regardless of the type of afferent nerve being stimulated, when sympathetic axons (where thoracolumbar and lumbosacral spinal afferent are located) supplying the GI tract have been severed, pain transmission is prevented indicating that spinal afferents covey noxious signals to the CNS (Furness, Rivera et al.) .

Noxious sensation is carried to the spinal cord via c-fibers and Aδ fibers. Both have small diameter cell bodies and are unmyelinated (c-fibers) or lightly myelinated (Aδ) fibers. The central terminals of these afferents terminate on ascending projecting neurons or excitatory interneurons in the layers of the dorsal horn. Descending cortical and subcortical structures send direct and indirect projections to the dorsal horn of the spinal cord to modulate pain transmission as shown in Figure 1.4. Descending pain modulation can either decrease (descending inhibition) or increase (descending facilitation) pain transmission depending on the neurotransmitter released and type/location of the receptors modulated (Millan 2002).

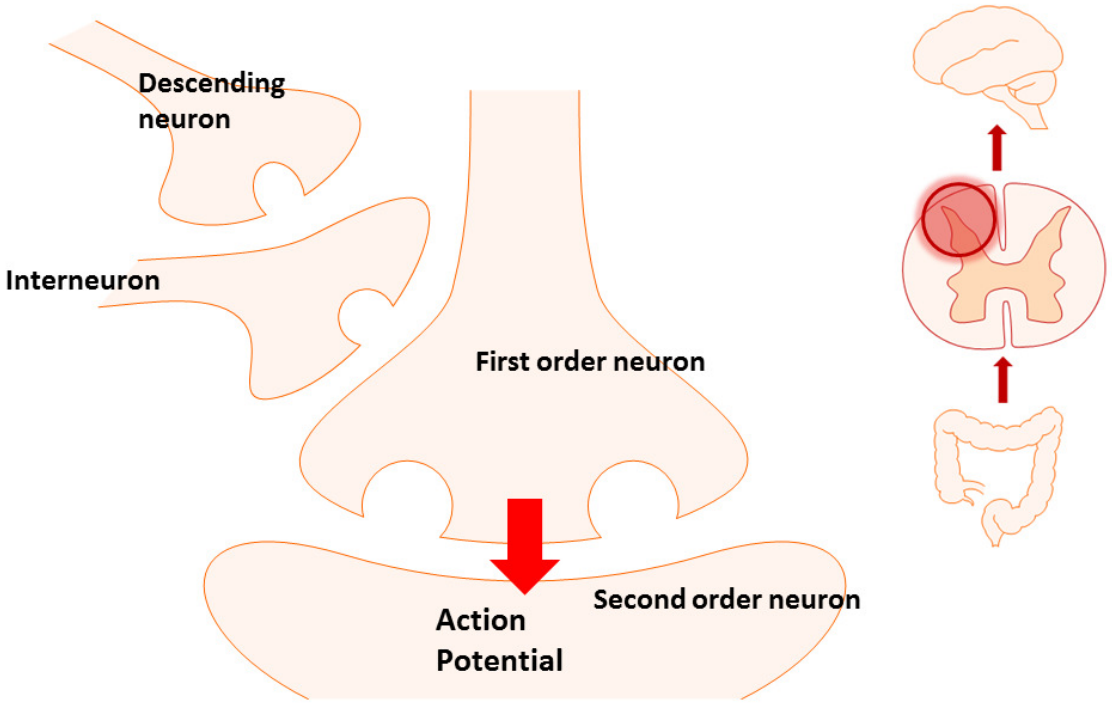

Figure 1.4 Descending innervation of the dorsal horn of the spinal cord. Descending innervation from cortex and brainstem terminate at various regions in the dorsal horn. For instance, interneuron activity can either be enhanced or decreased depending on the neurotransmitter released and the receptor modulated.

Cortical structures such as the anterior cingulate cortex (ACC) increase pain perception by enhancing descending facilitation. Frontal cortex nuclei also send projections to the subcortical regions such as nucleus raphe magnus (NRM) which supplies serotonergic input to the dorsal horn. Serotonergic neurons in the NRM receive input from multiple regions including direct innervation from deep layers of the dorsal horn (Layer, Keller et al. 2007). NRM neurons are found in the rostroventral medial medulla (RVM) which is an important region of the brain stem where ON and OFF cells are located. ON cells enhance pain perception and are inhibited by opioids, while OFF cells are part of the descending inhibition and are activated by opioids but

inhibited by pain. The hypothalamus and parabrachial nucleus (PBN) integrate autonomic and sensory information to modulate the autonomic response to pain. Nucleus tractus solitarious (NTS) receives major input from the vagus nerve and spinal afferents of the dorsal horn and extensively communicates with other subcortical structures such as the periaqueductal grey (PAG) to influence pain perception. The PAG plays the most pivotal role in modulating pain processing at the level of the brain (Millan 2002). The PAG modulates the emotional response to pain due to its extensive input into the amygdala. It also sends projections to other cortical structures, hypothalamus, RVM, NRM, PBN, NTS and pontine noradrenergic cell groups such as A5, A6 (locus coeruleus) and A7 (subcoeruleus) (Millan 2002). Noradrenergic nuclei send projections to the spinal cord and also play an important role in pain modulation at the spinal level (Llorca-Torralba, Borges et al. 2016).

1.1.2 Neurochemical signaling along the pain pathway

Mechanical distension of the gut wall can activate neurons directly via action of mechanoreceptors and indirectly by causing release of signaling molecules from specialized enteroendocrine cells (EEC) such as enterochromaffin cells (EC) (Kirkup, Brunsden et al. 2001; Blackshaw, Brookes et al. 2007; Gunawardene, Corfe et al. 2011). Serotonin, ATP, and capsaicin activate cation channels to cause neuronal depolarizations. While inflammatory signaling molecules such adenosine, thrombin, histamine, bradykinin, prostaglandins, and mast cell tryptase activate various G-coupled receptors to increase intracellular cAMP to sensitizes primary afferents (Kirkup, Brunsden et al. 2001). These mediators can be released from various inflammatory cells including mast cells. Mast cell activation via IgE, IgG, Toll Like Receptors (TLRs) and CD 161 can lead to the release of inflammatory mediators which will in turn activate

receptors such as 5-HT3, PPARs, and TRPV channels on primary afferents (Lee and Lee 2016). Gut microbiota can affect activity of neurons, EC cells, mast cells and macrophages which influence gut barrier function and neuronal activation (Bhattarai, Muniz Pedrogo et al. 2017). Short chain fatty acids, such as butyrate are produced by the gut microbiome secondary to fiber fermentation, can enhance primary afferent neuron activation via upregulation of ERK1/2 pathways (Xu, Wu et al. 2013).

Once activated, the central terminals of primary afferent neurons will release excitatory neurotransmitters to activate centrally projecting neurons that are located in Lamina I-III and X or excitatory interneurons in lamina II in the dorsal horn of the spinal cord as depicted in Figure 1.5. The main excitatory neurotransmitter released by all primary afferent neurons is glutamate which acts on ionotropic glutamate receptors for fast transmission. A subset of primary afferents release peptides, such as, substance P and calcitonin-gene related peptide (CGRP) in addition to glutamate. The expression of different excitatory neurotransmitters indicates functional differences among nociceptive neurons however the significance of such differences remains unclear (Iyengar, Ossipov et al. 2017). Eighty percent of centrally projecting neurons express neurokinin 1 (NK1) receptors suggesting that noxious transmission is mediated predominantly by substance P expressing primary afferent neurons (Todd 2010).

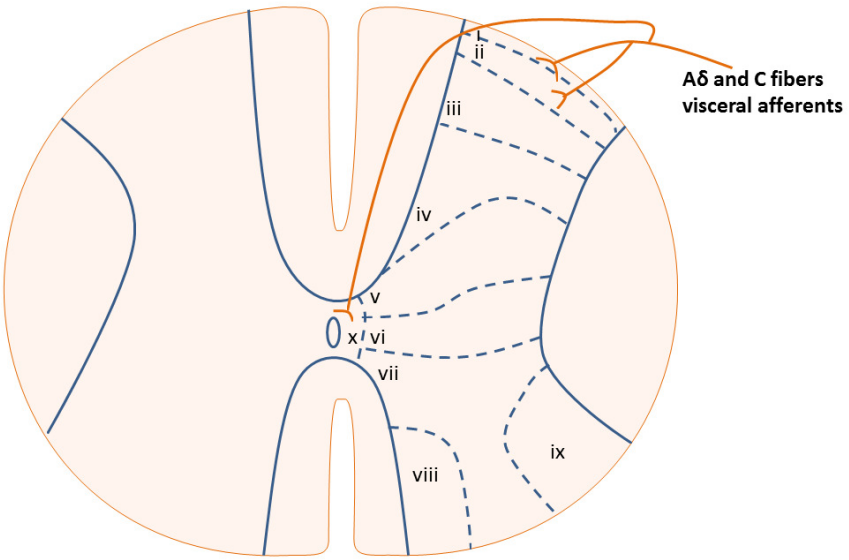

Figure 1.5 Organization of the grey matter of the spinal cord. Lamina i, ii, and x of the spinal cord receive noxious sensory input from somatic and visceral organs. The grey matter of the spinal cord is divided according to the cellular structure of the neurons present in each lamina. Aδ and C- fibers terminate in lamina i, ii, and x as depicted.

Central terminals of primary afferent neurons, interneurons and dendritic processes of projection neurons are all subject to descending modulation from cortical and subcortical structures. In addition to the direct synaptic interactions between neurons in the dorsal horn, volume transmission also occurs. In volume transmission, neurotransmitters diffuse from the site of release to act on distant receptors resulting in a widespread and persistent modulation of neuronal activity. This mechanism of neurotransmitter release is similar to that of intrathecal drug application which allows activation of receptors and neurons that are not part of a synapse (Millan 2002; Todd 2010) .

While multiple brain structures have been implicated in descending modulation, the specific neurotransmitters and mechanisms of pain modulation are unknown. The most extensively studied neurotransmitters involved in descending modulation are serotonin,

norepinephrine (NE) and gamma amino butyric acid (GABA). The net effect of each neurotransmitter on pain signaling depends on the type and location of receptor being activated or inhibited. For instance, NE is predominantly involved in descending inhibition via activation of alpha-2 adrenergic receptors (2ARs) on central terminals of primary afferent neurons. At the same time, it could also enhance facilitation by inhibiting GABA release from interneurons (2ARs -mediated) or by increasing glutamate release from excitatory interneurons (α1AR- mediated) (Millan 2002; Llorca-Torralba, Borges et al. 2016).

1.1.3 Serotonergic signaling in pain transmission

Serotonin activates intrinsic and extrinsic primary afferent neurons in the GI tract to regulate gut absorption, secretion, motility and sensory functions. The gut is the most abundant source of 5-HT in humans and other animal species including rodents. 5-HT is synthesized by specialized neuroendocrine cells called enterochromaffin cells (ECs). Serotonin is synthesized from the amino acid tryptophan in EC cells and serotonergic neurons. Tryptophan hydroxylase (TPH) is the rate limiting enzyme in 5-HT synthesis. TPH isoform 1 is expressed in EC cells while isoform 2 is expressed in 5-HT neurons (Gershon 2004; Spohn and Mawe 2017) . The release of 5-HT from ECs can be accomplished via mechanical or chemical stimuli of ECs as shown in Figure 1.7. Serotonin released from EC cells activates different types of 5-HT receptors to modulate its actions (Gershon 2004). To date seven classes of 5-HT receptor types have been identified some of which have subtypes as shown in Figure 1.6.

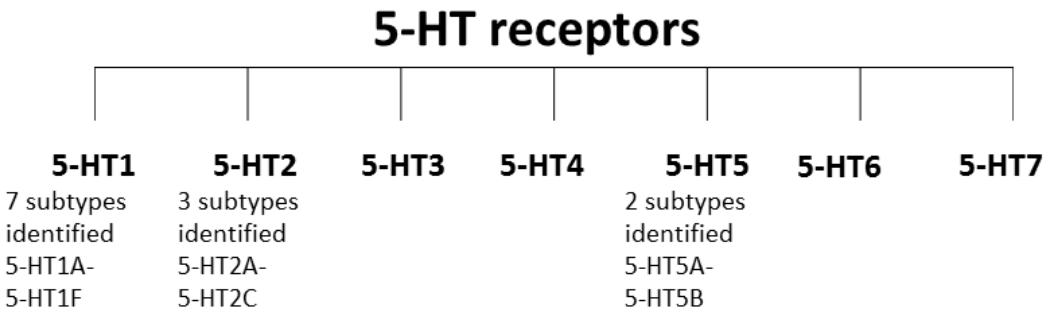

Figure 1.6 5-HT receptors types. Seven different 5-HT receptors have been identified. 5-HT1, 5-HT2 and 5-HT5 receptors have different subtypes. 5-HT1, 5-HT2, 5-HT3, 5-HT4 and 5-HT7 have been identified in the GI tract.

5-HT1 and 5-HT5 receptors are Gi- coupled receptors, 5-HT2 receptors are Gq-coupled, 5-HT4, 5-HT6, and 5-HT7 receptors are Gs-coupled, and the 5-HT3 receptor is a pentameric cation channel (Jorgensen 2007). The activity of 5-HT is terminated via reuptake by the serotonin transporter (SERT) which is expressed on enterocytes and serotonergic neurons in the ENS (figure 1.7). Serotonin can also be cleared by high capacity organic cation transporter (OCT) and dopamine transporters in the gut. Once inside the cell, 5-HT is metabolized into 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase (MOA) in enterocytes and serotonergic neurons. (Gershon 2004; Gershon and Tack 2007).

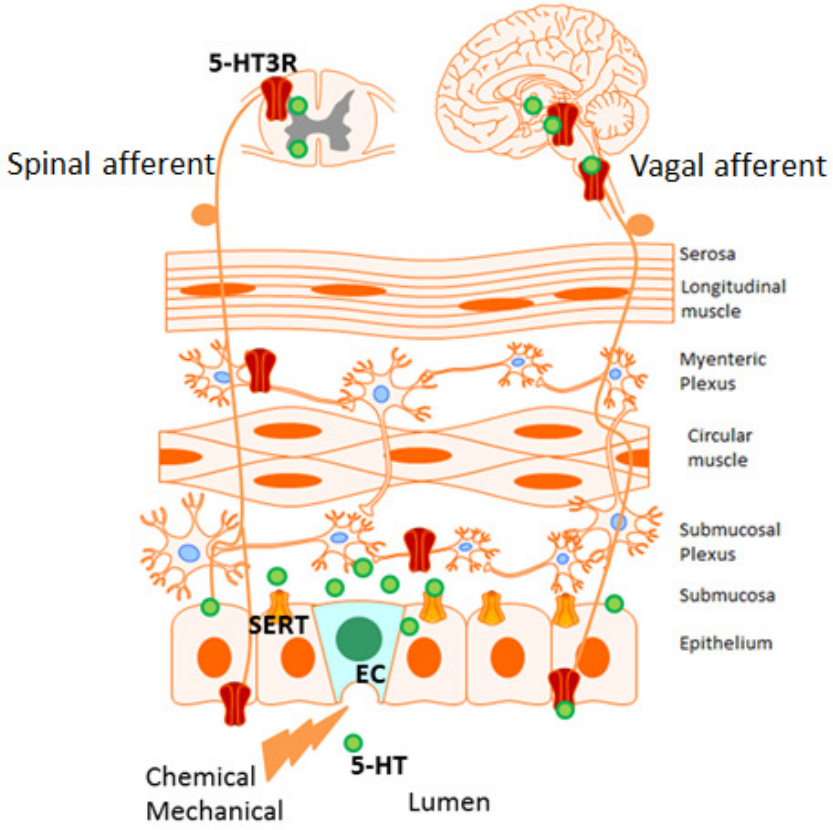

Figure 1.7 5-HT homeostasis in the gut. 5-HT is released from enterochromaffin (EC-cells) upon chemical or mechanical stimulation. The activity of 5-HT is terminated via reuptake by the serotonin transporter (SERT) expressed on epithelial cell and serotonergic neurons in the gastrointestinal tract. 5-HT3R indicates 5-HT3 receptor.

The net effects of 5-HT signaling in the GI tract depend on the type and location of the receptors being activated. Intrinsic primary afferent neurons express 5-HT1p, 5-HT3 and 5-HT4 receptors. Since the publication of Gershon, 2004, 5-HT1p receptors have been identified as 5-HT7 receptors (Kim and Khan 2014). 5-HT7 receptors are expressed on submucosal intrinsic primary afferent neurons (IPANs) and mediate peristaltic stimulation and secretory reflexes. Similarly, 5-HT4 receptors are also expressed in IPANs and once activated enhance the release of acetylcholine and CGRP from excitatory interneurons in the myenteric plexus to cause smooth muscle contraction and increased gut motility (Gershon 2004). 5-HT3 receptors are

expressed on IPAN and myenteric motor neurons. Their activation leads to initiation of fast excitatory neurotransmission leading to increased gut motility. In addition, 5-HT3 receptors are also expressed on extrinsic primary afferents neurons and once activated lead to neuronal depolarization and signal transmission to the brainstem(via the vagus nerve) and spinal cord (Gershon 2004; Sengupta 2009).

Centrally, 5-HT is synthesized by neurons localized to the raphe nuclei (RN). Rostral RN nuclei send serotonergic output to various structures in the brain, while the caudal nuclei send serotonergic projections to the spinal cord (Hornung 2003) . Many 5-HT receptors have been identified in the brain and spinal cord and can either enhance or dampen pain perception. Experiments done in rodents suggest that 5-HT1A receptors are predominantly antinociceptive since their activation leads to reduction in glutamatergic signaling and reduction in pain behaviors (Jeong, Mitchell et al. 2012; Choi, Cho et al. 2013). Recent work done using GAD 65/GAD 67 knock-in mice show expression of 5-HT1A receptors on GABAergic neurons as well. 5-HT2 receptors have also been implicated in central pain modulation. Pharmacological studies suggest that 5-HT2A receptors are pronociceptive, while 5-HT2C receptors are anti-nociceptive (Rahman, Bannister et al. 2011). Similarly, 5-HT3 receptors appear to have a dual role in pain modulation and will be discussed in great detail in chapter 2.

1.2 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal disorder (FGID) characterized by sensory and motility dysfunction. The sensory dysfunction results in abdominal pain while the motility disorder manifests as diarrhea and/or constipation. The diagnosis of IBS

is based on the Rome criteria which were updated in 2016. The most updated Rome IV criteria placed a greater emphasis on abdominal pain as a main diagnostic symptom that must occur at least once a week versus 3 times per month per Rome III criteria (Simren, Palsson et al. 2017). IBS is not a life threating disorder but it negatively impacts the quality of life of affected individuals. In fact, IBS patients have a quality of life scores worse than patients suffering from depression, diabetes mellitus and end stage renal disease (Gralnek, Hays et al. 2000; Buono, Carson et al. 2017) . In addition, IBS imposes a large economic burden resulting from doctors' visits and work absenteeism (Hoekman, Rutten et al. 2015; Buono, Carson et al. 2017; Buono, Mathur et al. 2017).

Treatment of IBS depends on the associated bowel dysfunction and is often inadequate due to its' multifactorial pathogenesis. Patients with IBS-diarrhea (IBS-D) are treated with drugs that slow gut transit and motility to decrease diarrhea. IBC-constipation (IBS-C) patients are treated with pro-kinetic drugs and/or stool bulking agents to alleviate the constipation. IBS-alternating or mixed are treated with a combination of drugs to address the symptoms as they present. Of the available IBS-C treatments only linaclotide, which is a CG-receptor agonist, relieves the abdominal pain. The exact mechanism by which linaclotide relives pain is unknown but it may simple be due to treating the constipation. The major side effect of linaclotide is diarrhea resulting in lower patient compliance. Eluxadoline is a peripherally restricted δ-opioid receptor antagonist and μ-opioid receptor agonist that has been shown to treat diarrhea and reduce visceral sensitivity in IBS-D patients. While eluxadoline is effective in treating IBS-D symptoms it has a black box waring due to increased risk pancreatitis in patients with gall bladder diseases. Other conventional pain medications such as NSAIDS, acetaminophen, aspirin

and narcotics do not adequately treat the pain associated with IBS due to complication that result from long term use. Many clinical studies have been conducted to assess the efficacy of benzodiazepine, tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRI) and serotonin norepinephrine reuptake inhibitors (SNRI)for treating abdominal pain in IBS patients. It appears that only TCAs are efficacious at reducing pain when used in IBS patients with comorbid psychological disorders. Other pain medications such as clonidine, gabapentin, and pregabalin have been shown to reduce overall pain in IBS. Unfortunately, all three lead to drowsiness and sedation making them unsuitable for long term pain management in IBS. Drugs that target the serotonergic system have also been used to treat IBS. Alosetron, a 5-HT3 receptor antagonist is FDA approved for the treatment of severe IBS-D in women. Alosetron can only be prescribed under risk management protocol due to serious side effect of severe constipation and ischemic colitis. Teagserod, a 5-HT4 receptor agonist, was also used to treat IBS-C. However, it was pulled of the market due to increased risk of ischemic heart disease. (Chen, Ilham et al. 2017) The adverse events of the effective drugs and lack of efficacy of the other treatments discussed underscores the importance of developing medications to effectively treat the visceral pain associated with IBS.

1.3 Visceral Hypersensitivity

The cause of pain in some IBS patients is thought to be due to neuronal dysfunction which leads to visceral hypersensitivity. Visceral hypersensitivity is defined as enhanced perception of pain to noxious stimuli or pain perception to non-noxious stimuli. IN 1973, the first account of increased pain perception to rectal distension was made. Since then multiple clinical and basic science studies have shown that IBS patients and IBS animal models have lower pain threshold

than healthy controls. It has been reported that 50-80% of IBS patients exhibit VHS which does not correlate with IBS symptom severity (Drossman, Camilleri et al. 2002). A more recent study done in Japan confirmed that visceral hypersensitivity is observed in IBS patients and significantly correlated with IBS symptom severity (Kanazawa, Hongo et al. 2011). This group also found that rectal distention in IBS patients caused increased blood flow in specific brain structures implicated in pain processing such as ACC, prefrontal cortex, insula and thalamus. In pediatric patients pain threshold to rectal distension was also lower in IBS patients compared to healthy controls (CAMILLERI, COULIE et al. 2001; Camilleri, Coulie et al. 2001; Halac, Noble et al. 2010). A study performed by Boubin et al. showed that VHS should be used as a diagnostic marker to distinguish between different causes of abdominal pain since patients with IBS, unlike patients suffering from other GI disorders exhibited lower pain threshold to rectal distension (Bouin, Plourde et al. 2002).

1.4 Serotonin signaling dysfunction in IBS

In the early 2000s multiple case-control studies showed that certain polymorphisms in the SLC6A4 gene which codes for serotonin transporter (SERT) could be associated with IBS (Camilleri 2004; Jin, Cao et al. 2016). Of the identified polymorphisms the 5-HTT gene linked polymorphic region (5-HTTLRP) upstream of the transcription start site is the most extensively studied. This polymorphism is 41 base-pair deletion or insertion in the promotor region of the SERT gene (park, choi et al. 2006; Jin, Cao et al. 2016). The short (s) allele leads to a reduction in SERT expression compared to the long (l) allele (park, choi et al. 2006). In 2004, Patel et al. showed that the s-allele is associated with Turkish IBS patients with a strong association of l/s genotype in IBS-D patients. Other studies performed in Korea and India also showed a strong

association between s-allele and IBS-D patients (Yeo 2004; park, choi et al. 2006). The largest study was performed in the United States and showed no association between SERT polymorphism and IBS. Experts in the field speculated that this might be due to ethnic heterogeneity of the subjects(Camilleri 2004). In addition, other SERT gene polymorphisms have been identified and could also alter SERT expression regardless of 5-HTTLRP polymorphism(Jin, Cao et al. 2016). Of specific importance is the rs25531 polymorphism which is a functional single nucleotide polymorphism found in intron 2 of the SERT gene. The G allele increases the odds of developing IBS by 3-fold in carriers and is capable of reducing SERT expression even in patients with l/l allele (Kohen, Jarrett et al. 2009). Therefore the SERT gene deserves further investigation to understand its role in IBS pathogenesis.

In addition to the polymorphisms in the SERT gene, other alterations in serotonergic signaling in IBS patients have been identified. For instance, IBS patients exhibit higher mucosal and plasma 5-HT levels, decreased SERT expression, and increased TPH1 expression (Foley, Garsed et al. 2011). Additional studies showed a reduction in 5-HT uptake by platelets from IBS patients compared to healthy controls suggesting aberrant SERT function (Cremon, Carini et al. 2011).

Another clinical observation that corroborates serotonergic dysfunction in IBS is the effectiveness of the 5-HT3 receptor antagonist, alosetron, in relieving global IBS symptoms (Mangel and Northcutt 1999; Houghton, Foster et al. 2000; Viramontes, Camilleri et al. 2001; Zheng, Yu et al. 2017). Alosetron can cause severe constipation and ischemic colitis in IBS-C patients and as a result was withdrawn from the market in 2000. However, the FDA

reintroduced it back to the marker under restricted prescription following patients' request to treat severe IBS-D in women (Zheng, Yu et al. 2017). On the other hand, the 5-HT3 receptor antagonists approved in Europe are used to treat nausea but not IBS symptoms. In the far east, ramosetron and azasetron are approved for the treatment of IBS-diarrhea (Thompson and Lummis 2007). Tegaserod, a 5-HT_4 receptor agonist, is efficacious in treating IBS-C but was pulled of the market due to cardiovascular risks in 2007 (Layer, Keller et al. 2007).

1.5 Sex differences in IBS

IBS is considered a women's health issue because it is twice as prevalent in females as in males. Until recently this female predominance was thought to occur in western countries only (Meleine and Matricon 2014; Mulak, Tache et al. 2014). However, a study done in China showed that IBS is indeed more prevalent in female patients, especially those in more affluent regions (Chang, Lu et al. 2010; Bhattarai, Muniz Pedrogo et al. 2017). The female predominance in IBS is associated with the severity of constipation relative to that of diarrhea (Herman, Pokkunuri et al. 2010). In addition to the female predominance in IBS, women IBS patients are more likely to have IBS-C, comorbid anxiety, depression, and somatic pain. Furthermore, alosetron has been shown to be more efficacious in female patients in comparison to male IBS-D patients (Mulak, Tache et al. 2014).

The incidence of IBS in women decreases with age to equal that of males after the 6th decade of life. Studies comparing IBS symptom severity across the menstrual cycle, pregnancy, and menopause are equivocal. For instance, while the incidence of IBS decreases with age, symptoms severity increases in menopause. Another study showed that post-menopausal

women experience IBS symptoms more frequently compared to men. However, the difference was lost when the data was corrected for age (Cain, Jarrett et al. 2009). It has been established that the high levels of ovarian hormones during pregnancy result in an analgesic state. It has also been shown that during the third trimester of pregnancy gastrointestinal transit is slowed (Chiloiro, Darconza et al. 2001). However, no studies were performed to specifically determine the effect of pregnancy on IBS. In female IBS patients, abdominal is exacerbated during menses when ovarian hormone levels reach their nadir (Houghton, Lea et al. 2002). Despite these observations, the exact role of ovarian hormones in modulating abdominal pain remains unclear. The role of estrogen in mediating visceral hypersensitivity is discussed in chapter 3.

1.6 Serotonin transporter knockout (SERT KO) rat, sex specific animal model of VHS

Clinical studies emphasized the importance of serotonergic signaling in the pathogenesis of IBS inspiring the investigation of serotonergic dysfunction in animal models. For instance, infusion of 5-HTP caused an increase in pain response to colorectal distension (CRD) in rats following an inflammatory insult (Choi, Sung et al. 2008). Similarly, SERT KO mice have been used to study gastrointestinal dysfunction. SERT KO mice exhibit GI motility dysfunction which manifests as alternating periods of diarrhea and constipation (Chen, Li et al. 2001). However, assessment of VHS in mice is difficult due to small size. Therefore, studies assessing visceral sensation in these animals were not made.

Homberg et al developed a global SERT KO rat via N-ethyl-N-nitrourea (ENU) mutagenesis which resulted in a single point mutation leading to premature stop codon in the part of the gene that codes for the second extracellular loop of SERT (Figure 1.8) (Homberg, Olivier et al.

2007). While SERT KO rats exhibits decreased whole tissue levels of 5-HT in the brain, they have a 9-fold increase in extracellular 5-HT concentrations in the hippocampus which was the only brain region investigated. This is thought to occur due to the reduction in reuptake of 5-HT in the absence of increased expression or activity of TPH. These rats also exhibit anxiety and depression-like behaviors in a sex-independent manner which recapitulates the comorbidities observed in some IBS patients (Olivier, Van Der Hart et al. 2008). Galligan et al. showed that SERT KO rats is as an animal model to study sex-specific visceral hypersensitivity since the female but not male rats exhibit increased visceromotor response (VMR) to colorectal distension (CRD) (Galligan, Patel et al. 2013). A recent article written by Meleine and Matricon highlighted the need for an integrated animal model that can recapitulate the multifactorial and complex nature of the pathogenesis of IBS (Meleine and Matricon 2014). SERT KO rat serves as such a model since it will allow us to understand how interactions between ovarian hormones in presence of a dysfunction in serotonergic signaling contribute to the development of visceral hypersensitivity.

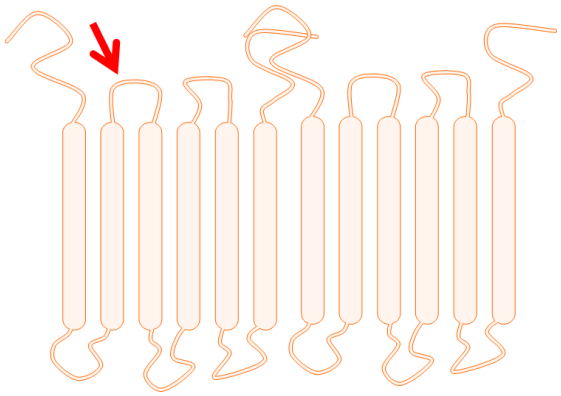

Figure 1.8 Location of stop codon in SERT protein. In SERT KO rat, a stop codon in the part of the gene that codes for the second extracellular loop (red arrow) results in a nonfunctional SERT protein.

1.7 Hypothesis and specific aims

The overall aim of this study was to understand the paradoxical increase in visceral sensitivity observed in SERT KO female rats following the administration of some 5-HT3 receptor antagonists and how estrogen could be modulating these effects. We hypothesized that the location of 5-HT3 receptors and their interaction with estrogen receptors mediated this response. We tested this hypothesis in 2 specific aims.

Specific Aim 1: To determine the location of 5-HT3 receptors mediating the enhanced pain perception by testing VMR to CRD following subcutaneous, intrathecal and intracerebroventricular administration of 5-HT3 receptor antagonists and to determine the mechanism by which inhibition of 5-HT3 receptors increased VMR to CRD.

Specific Aim 2: To determine the mechanism by which estrogen could modulate the increase in VMR to CRD in SERT KO rats by measuring VMR following 1) ovariectomy and 2) inhibition of genomic and non-genomic estrogen receptors in intact SERT KO and WT female rats.

BIBLIOGRAPHY

BIBLIOGRAPHY

Bhattarai Y, Muniz Pedrogo DA, Kashyap PC (2017) Irritable bowel syndrome: a gut microbiota-related disorder? American Journal of Physiology-Gastrointestinal and Liver Physiology 312:G52-G62.

Blackshaw LA, Brookes SJ, Grundy D, Schemann M (2007) Sensory transmission in the gastrointestinal tract. Neurogastroenterology Motility 19:1-19.

Bouin M, Plourde V, Boivin M, Riberdy M, Lupien F, Laganière M, Verrier P, Poitras P (2002) Rectal distention testing in patients with irritable bowel syndrome: Sensitivity, specificity, and predictive values of pain sensory thresholds. Gastroenterology 122:1771-1777.

Brookes SJ, Spencer NJ, Costa M, Zagorodnyuk VP (2013) Extrinsic primary afferent signalling in the gut. Nature Reviews of Gastroenterology and Hepatolology 10:286-296.

Buono JL, Carson RT, Flores NM (2017a) Health-related quality of life, work productivity, and indirect costs among patients with irritable bowel syndrome with diarrhea. Health Quality Life Outcomes 15:017-0611.

Buono JL, Mathur K, Averitt AJ, Andrae DA (2017b) Economic Burden of Irritable Bowel Syndrome with Diarrhea: Retrospective Analysis of a U.S. Commercially Insured Population. Journal of Managed Care and Specialty Pharmcy 23:453-460.

Camilleri M (2004) Is there a SERT-ain association with IBS? Gut 53:1396-1399.

Camilleri M, Coulie B, Tack JF (2001a) Visceral hypersensitivity facts sepculations and challenges camelliri. Gut 48:7.

Camilleri M, Coulie B, Tack JF (2001b) Visceral hypersensitivity: facts, speculations, and challenges. Gut 48:125-131.

Chang FY, Lu CL, Chen TS (2010) The current prevalence of irritable bowel syndrome in Asia. J Neurogastroenterology and Motility 16:389-400.

Chen JJ, Li Z, Pan H, Murphy DL, Tamir H, Koepsell H, Gershon MD (2001) Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. Journal of Neuroscience 21:6348-6361.

Chen L, Ilham SJ, Feng B (2017) Pharmacological Approach for Managing Pain in Irritable Bowel Syndrome: A Review Article. Anesthesiology and Pain Medicine 7.

Choi IS, Cho JH, Jang IS (2013) 5-Hydroxytryptamine 1A receptors inhibit glutamate release in rat medullary dorsal horn neurons. Neuroreport 24:399-403.

Choi YD, Sung TS, Kim HJ, La JH, Kim TW, Yang IS (2008) Increased 5-hydroxytryptamine mediates post-inflammatory visceral hypersensitivity via the 5-hydroxytryptamine 3 receptor in rats. Digestive Disease and Sciences 53:2909-2916.

Cremon C, Carini G, Wang B, Vasina V, Cogliandro RF, De Giorgio R, Stanghellini V, Grundy D, Tonini M, De Ponti F, Corinaldesi R, Barbara G (2011) Intestinal Serotonin Release, Sensory Neuron Activation, and Abdominal Pain in Irritable Bowel Syndrome. The American Journal of Gastroenterology 106:1290-1298.

Drossman DA, Camilleri M, Mayer EA, Whitehead WE (2002) AGA technical review on irritable bowel syndrome. Gastroenterology 123:2108-2131.

Foley S, Garsed K, Singh G, Duroudier NP, Swan C, Hall IP, Zaitoun A, Bennett A, Marsden C, Holmes G, Walls A, Spiller RC (2011) Impaired uptake of serotonin by platelets from patients with irritable bowel syndrome correlates with duodenal immune activation. Gastroenterology 140:1434-1443 e1431.

Furness JB, Rivera LR, Cho HJ, Bravo DM, Callaghan B The gut as a sensory organ: Nature Reviews Gastroenterology and Hepatolology 2013 Dec;10(12):729-40. doi: 10.1038/nrgastro.2013.180. Epub 2013 Sep 24.

Galligan JJ, Patel BA, Schneider SP, Wang H, Zhao H, Novotny M, Bian X, Kabeer R, Fried D, Swain GM (2013) Visceral hypersensitivity in female but not in male serotonin transporter knockout rats. Neurogastroenterololgy and Motililty 25:e373-381.

Gebhart GF (1999) Peripheral contributions to visceral hyperalgesia. Canadian Journal of Gastroenterology 13:37A-41A.

Gebhart GF, Bielefeldt K (2016) Physiology of Visceral Pain. Comprehensive Physiology 6:1609-1633.

Gershon MD (2004) Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. Alimentary Pharmacology and Therapeutics 7:3-14.

Gershon MD, Tack J (2007) The serotonin signaling system: from basic understanding to drug development for functional GI disorders. Gastroenterology 132:397-414.

Gralnek IM, Hays RD, Kilbourne A, Naliboff B, Mayer EA (2000) The impact of irritable bowel syndrome on health-related quality of life. Gastroenterology 119:654-660.

Gunawardene AR, Corfe BM, Staton CA (2011) Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. International Journal of Experimental Pathology 92:219-231.

Halac U, Noble A, Faure C (2010) Rectal sensory threshold for pain is a diagnostic marker of irritable bowel syndrome and functional abdominal pain in children. Journal of Pediatrics 156:60-65 e61.

Herman J, Pokkunuri V, Braham L, Pimentel M (2010) Gender distribution in irritable bowel syndrome is proportional to the severity of constipation relative to diarrhea. Gender Medicine 7:240-246.

Hoekman DR, Rutten JM, Vlieger AM, Benninga MA, Dijkgraaf MG (2015) Annual Costs of Care for Pediatric Irritable Bowel Syndrome, Functional Abdominal Pain, and Functional Abdominal Pain Syndrome. Journal of Pediatrics 167:1103-1108.

Homberg JR, Olivier JD, Smits BM, Mul JD, Mudde J, Verheul M, Nieuwenhuizen OF, Cools AR, Ronken E, Cremers T, Schoffelmeer AN, Ellenbroek BA, Cuppen E (2007) Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system. Neuroscience 146:1662-1676.

Hornung JP (2003) The human raphe nuclei and the serotonergic system. Journal of Chemical Neuroanatomy 26:331-343.

Houghton LA, Foster JM, Whorwell PJ (2000) Alosetron, a 5-HT3 receptor antagonist, delays colonic transit in patients with irritable bowel syndrome and healthy volunteers. Alimentary Pharmacology and Therapeutics 14:775-782.

Iyengar S, Ossipov MH, Johnson KW (2017) The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine. Pain 158:543-559.

Jeong HJ, Mitchell VA, Vaughan CW (2012) Role of 5-HT(1) receptor subtypes in the modulation of pain and synaptic transmission in rat spinal superficial dorsal horn. British Journal of Pharmacology 165:1956-1965.

Jin D-C, Cao H-L, Xu M-Q, Wang S-N, Wang Y-M, Yan F, Wang B-M (2016) Regulation of the serotonin transporter in the pathogenesis of irritable bowel syndrome. World Journal of Gastroenterology 22:8137.

Jorgensen HS (2007) Studies on the neuroendocrine role of serotonin. Danish Medical Bulletin 54:266-288.

Kanazawa M, Hongo M, Fukudo S (2011) Visceral hypersensitivity in irritable bowel syndrome. Journal of Gastroenterology and Hepatology 26:119-121.

Kirkup AJ, Brunsden AM, Grundy D (2001) Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. American Journal of Physiology-Gastrointestinal and Liver Physiology 280:G787-794.

Kohen R, Jarrett ME, Cain KC, Jun S-E, Navaja GP, Symonds S, Heitkemper MM (2009) The Serotonin Transporter Polymorphism rs25531 Is Associated with Irritable Bowel Syndrome. Digestive Diseases and Sciences 54:2663-2670.

Layer P, Keller J, Loeffler H, Kreiss A (2007) Tegaserod in the treatment of irritable bowel syndrome (IBS) with constipation as the prime symptom. Therapeutic Clinical Risk Management 3:107-118.

Lee KN, Lee OY (2016) The Role of Mast Cells in Irritable Bowel Syndrome. Gastroenterology Research and Practice 2031480:28.

Llorca-Torralba M, Borges G, Neto F, Mico JA, Berrocoso E (2016) Noradrenergic Locus Coeruleus pathways in pain modulation. Neuroscience 338:93-113.

Mangel AW, Northcutt AR (1999) Review article: the safety and efficacy of alosetron, a 5-HT3 receptor antagonist, in female irritable bowel syndrome patients. Alimentary Pharmacology and Therapeutics 2:77-82.

Meleine M, Matricon J (2014) Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones. World Journal of Gastroenterology 20:6725-6743.

Millan MJ (2002) Descending control of pain. Progress Neurobiology 66:355-474.

Mulak A, Tache Y, Larauche M (2014) Sex hormones in the modulation of irritable bowel syndrome. World Journal of Gastroenterology 20:2433-2448.

Olivier JD, Van Der Hart MG, Van Swelm RP, Dederen PJ, Homberg JR, Cremers T, Deen PM, Cuppen E, Cools AR, Ellenbroek BA (2008) A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. Neuroscience 152:573-584.

Palecek J, Paleckova V, Willis WD (2002) The roles of pathways in the spinal cord lateral and dorsal funiculi in signaling nociceptive somatic and visceral stimuli in rats. Pain 96:297-307.

Park J. M, Choi M-G, Park J-A, Oh JH, Cho YK, Lee IS, Kim SW, Choi KY, Chung I-S (2006) Serotonin transporter gene polymorphism and irritable bowel syndrome. Neurogastroenterology and Motility 18:995-1000.

Rahman W, Bannister K, Bee LA, Dickenson AH (2011) A pronociceptive role for the 5-HT2 receptor on spinal nociceptive transmission: an in vivo electrophysiological study in the rat. Brain Research 25:29-36.

Sengupta JN (2009) Visceral Pain: The Neurophysiological Mechanism. 194:31-74.

Simren M, Palsson OS, Whitehead WE (2017) Update on Rome IV Criteria for Colorectal Disorders: Implications for Clinical Practice. Current Gastroenterology Reports 19:017-0554.

Spohn SN, Mawe GM (2017) Non-conventional features of peripheral serotonin signalling - the gut and beyond. Nature Reviews of Gastroenterology and Hepatology 14:412-420.

Su X, Gebhart GF (1998) Mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat are polymodal in character. Journal of Neurophysiology 80:2632-2644.

Todd AJ (2010) Neuronal circuitry for pain processing in the dorsal horn. Nature Reviews of Neuroscience 11:823-836.

Viramontes BE, Camilleri M, McKinzie S, Pardi DS, Burton D, Thomforde GM (2001) Gender-related differences in slowing colonic transit by a 5-HT3 antagonist in subjects with diarrhea-predominant irritable bowel syndrome. American Journal of Gastroenterology 96:2671-2676.

Xu D, Wu X, Grabauskas G, Owyang C (2013) Butyrate-induced colonic hypersensitivity is mediated by mitogen-activated protein kinase activation in rat dorsal root ganglia. Gut 62:1466-1474.

Yeo A (2004) Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women. Gut 53:1452-1458.

Zagorodnyuk VP, Kyloh M, Nicholas S, Peiris H, Brookes SJ, Chen BN, Spencer NJ (2011) Loss of visceral pain following colorectal distension in an endothelin-3 deficient mouse model of Hirschsprung's disease. Journal of Physiology 589:1691-1706.

Zheng Y, Yu T, Tang Y, Xiong W, Shen X, Jiang L, Lin L (2017) Efficacy and safety of 5-hydroxytryptamine 3 receptor antagonists in irritable bowel syndrome: A systematic review and meta-analysis of randomized controlled trials. PLoS One 12.

Chapter 2

Spinal 5-HT3 receptor inhibition increases visceral hypersensitivity in SERT KO female rats.

2.1 Abstract

The irritable bowel syndrome (IBS) is a functional gastrointestinal motor and visceral sensation disorder that is more common in women compared to men. 5-Hydroxytryptamine (5-HT, serotonin) signaling is disrupted in some IBS patients possibly due to polymorphic variations in the gene encoding the serotonin transporter (SERT) which result in increased extracellular 5-HT availability. Female SERT knockout (KO) rats exhibit visceral hypersensitivity to colonic distention that mimics colonic hypersensitivity known to occur in female IBS patients. Alosetron, a 5-HT_3 receptor antagonist, is used to treat severe IBS-D in female patients. The exact mechanism by which alosetron reduced abdominal pain is not well understood since it is capable of crossing the blood brain barrier (BBB). Thus, it can act on peripheral and central 5-HT3 receptors to reduce pain. We measured the visceromotor response (VMR) to colorectal distension (CRD) in SERT KO and wild type (WT) rats following treatment with several 5-HT3 receptor antagonists and agonist. Specifically, we tested subcutaneous (s.c.), intrathecal (i.t.), and intracerebroventricular (icv) administration of various BBB-permeable 5-HT_3 receptor antagonists and the partially BBB-permeable 5-HT3 receptor antagonist ramosetron (s.c.). We also tested the effects of intrathecal administration of the 5-HT3 receptor agonist SR 57227 in SERT KO and WT rats. Depletion of spinal serotonin via intrathecal administration of the neurotoxin 5, 7-dihydroxytryptamine (5,7-DHT) was performed to determine the role of descending serotonergic input in modulating pain transmission in the dorsal horn of the spinal cord. Immunohistochemistry (IHC) studies were performed to determine the pattern of 5-HT_3 A

receptor subunit and □-aminobutyric acid (GABA) expression in the spinal cord of SERT KO and WT rats. The 5-HT3 receptor antagonists alosetron and granisetron caused an increase in VMR to CRD when given peripherally in SERT KO female rats. Alosetron also caused an increase in VMR to CRD in WT male rats. Intrathecal alosetron increased VMR to CRD in SERT KO female rats only, while alosetron (25 nmol, i.c.v) had no effect on VMR to CRD in all rats. In SERT KO male rats intrathecal administration of SR 52772 increased visceral sensitivity. Depletion of spinal 5-HT by 5, 7-DHT prevented the increase in VMR to CRD in SERT KO female and male rats treated with intrathecal alosetron and SR 52772, respectively. IHC studies showed increased 5-HT A subunit and GABA colocalization in the dorsal horn of the spinal cord in SERT KO female rats compared to SERT KO males rats and WT rats. In conclusion, the increase in visceral hypersensitivity in SERT KO female rats treated with 5-HT_3 receptor antagonists is mediated by blockade of spinal 5-HT_3 receptors that are likely expressed on inhibitory GABAergic interneurons. While in male SERT KO rats activation of 5-HT3 receptors caused an increase in visceral sensitivity since 5-HT3 receptors are likely predominantly expressed on central terminals of primary afferent neurons.

2.2 Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by altered bowel habits and abdominal pain (Chey, Kurlander et al. 2015). The abdominal pain is believed to occur due to visceral hypersensitivity secondary to neuronal dysfunction. 5-HT signaling at 5-HT3 receptors may contribute to visceral pain observed in IBS patients. 5-HT3 receptors are expressed in the central and peripheral nervous system. (Jensen, Davies et al. 2008; Lummis 2012). They are found on ENS neurons and the peripheral and central terminals

of spinal and vagal extrinsic primary afferent neurons that innervate the gut (Figure 2.1)(Glatzle, Sternini et al. 2002). 5-HT3 receptors may also be expressed by colonic enterocytes in humans as suggested by one study. (Kapeller, Houghton et al. 2008). Thus, the 5-HT3 receptors can contribute to gut motility and pain transmission from the gut (Morales, Battenberg et al. 1998; Browning 2015).

In the CNS 5-HT3 receptors are expressed with the highest density in the nucleus tractus solitarious and area postrema of the brainstem where the vomit reflex is mediated. In fact, antagonists targeting 5-HT3 receptors are a mainstay for the treatment of post-surgical, chemotherapy and radiation induced nausea (Barnes, Hales et al. 2009; Kovac 2016; Simino, Marra et al. 2016).

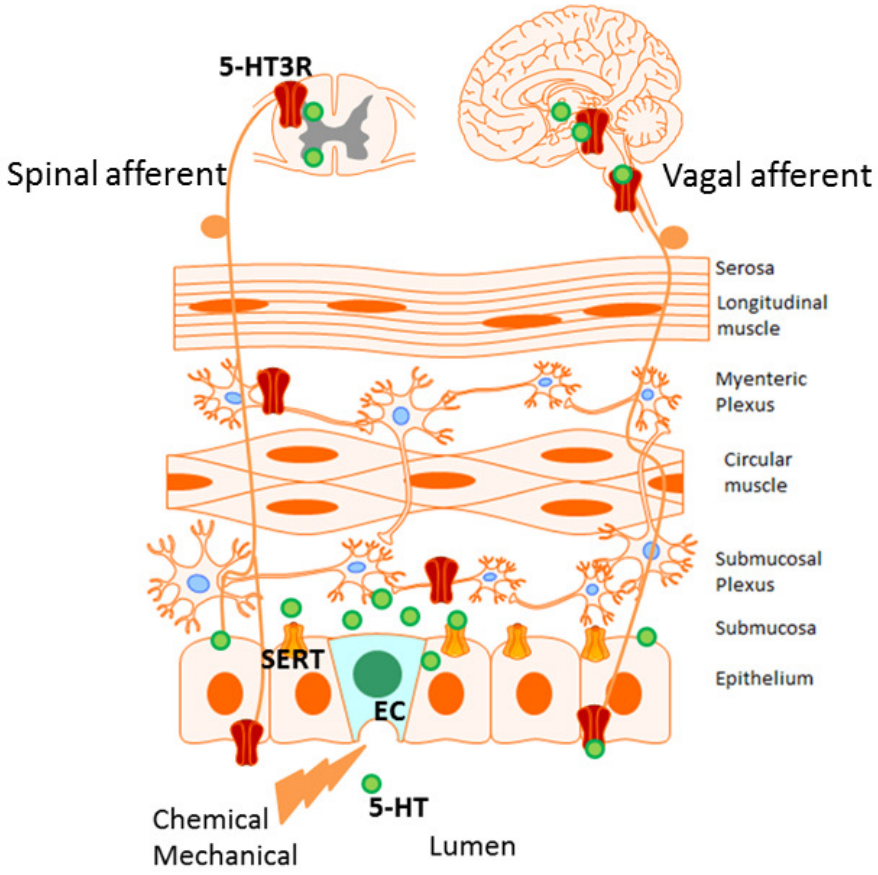

Figure 2.1 5-HT3 receptor expression along the pain pathway. 5-HT3 receptors are expressed in the peripheral and central nervous system. Extrinsic spinal and vagal primary afferents express 5-HT3 receptors on their peripheral and central terminals.

5-HT3 receptors are ligand-gated cation channels composed of 5 subunits. They belong to the Cys-loop receptor family which includes ionotropic GABAergic receptor and nicotinic acetylcholine receptors (Jensen, Davies et al. 2008). At the time of their discovery it was believed that pentameric 5-HT3 receptors where comprised of one type of subunit. However, the electrophysiological properties of 5-HT3 receptors composed of only □-subunits expressed in cell lines differ from the properties of 5-HT3 receptors expressed in the nervous system of laboratory animals suggesting the existence of other subunits. Butler et al. noticed differences

in 5-HT ED50 values in electrophysiological studies performed across species and different tissues within a species(Butler, Hill et al. 1988; Wolf 2000). Since the 5-HT3A receptor subunit was cloned, several other subunits and splice variants have been discovered confirming the existence of multiple types of 5-HT3 receptors. In humans, 5 different subunits (A-E) have been identified some of which have splice variants. The A-C subunits are expressed centrally and peripherally, while the D and E subunits have been only identified in the gastrointestinal tract (Barnes, Hales et al. 2009). It is important to note that for a 5-HT3 receptor to be functional it must express at least one A-subunit. Polymorphisms in the A and E subunits have been associated with increased IBS-diarrhea risk (Cheung and Wu 2014; Gu, Zhang et al. 2015). The functional polymorphisms in the 5-HT3A and 5-HT3E gene result in increased 5-HT3 receptor density in the cell membrane of transfected HEK293 cells suggesting that receptor upregulation might occur in patients with these polymorphisms (Kapeller, Houghton et al. 2008).

In rodents only A and B subunits have been identified. While it is agreed that the 5-HT3A receptor subunit is expressed both centrally and peripherally, the expression of 5-HTB subunit in the central nervous system remains debatable due to discrepancies between electrophysiological and immunohistochemistry studies (Morales and Wang 2002; Jensen, Davies et al. 2008). Furthermore, in rodents the 5-HT3A subunit has two splice variants, a short allele (5-HT3b or 5-HT3s) and long allele (5-HT3a or 5-HT3l) (Miquel, Emerit et al. 1995). The short allele is more ubiquitous with 90% expression versus the long allele and is similar to the 5-HT3A subunit in humans. For a 5-HT3 receptor to be functional, it must have an at least one A-subunit both in humans and other animals. Furthermore, the single channel conductance of 5-HT3 receptors composed of 5 A-subunits is less than 1 picosecond. 5-HT3 receptors composed

of A and B-subunits exhibit a slower single channel conductance (Lummis 2012). . The permeability of 5-HT3 receptors to cations is also dependent on their subunit composition. 5-HT3A receptors are permeable to divalent and monovalent cations, while 5-HT3AB receptor are selective for monovalent cations (Barnes, Hales et al. 2009; Thompson, Verheij et al. 2013). These properties support the importance of distinguishing the different types of 5-HT3 receptors.

Alosetron is FDA approved for the treatment of severe IBS-diarrhea in female patients only. Alosetron is not approved for the treatment of IBS-diarrhea in males because it failed to improve symptoms. The reasons behind this observation remains unclear (Rahimi, Nikfar et al. 2008). Furthermore, the mechanism by which alosetron exerts its antinociceptive effect in females is unknown. Positron emission topography (PET) scans showed that alosetron treatment reduced cerebral blood flow in the amygdala and other limbic structures involved in pain processing during rectal distension. These data suggest that central 5-HT3 receptor antagonism caused pain relief in IBS patients. Furthermore, the authors also deduced from their data that peripheral inhibition of 5-HT3 receptors by alosetron is unlikely to produce analgesia since the regions of the brain receiving input from visceral afferent such as the thalamus had either unchanged or reduced blood flow during distension (Mayer, Berman et al. 2002).

The role of 5-HT3 receptors in mediating pain transmission is even more controversial in animal studies. Over the past 20 years many studies have been performed, while most support a pronociceptive role of 5-HT3 receptors, some indicate that 5-HT signaling at 5-HT3 receptors

is antinociceptive. What is agreed upon is the importance of 5-HT3 receptors in mediating pain transmission. 5-HT3A subunit knockout mice exhibit reduced pain response following intraperitoneal 5-HT injection indicating that 5-HT3 receptors on primary afferent neurons are required to mediate pain transmission. Furthermore, treatment of the 5-HT3A KO mice with intrathecal 5-HT resulted in a damped scratching behavior compared to WT mice. This further supports a pronociceptive role of spinal 5-HT3 receptors (Zeitz, Guy et al. 2002). Mice treated with dextran sulfate sodium solution to induce colitis, exhibit increased pain behavior and increased 5-HT3 receptor expression in dorsal root ganglia. (Matsumoto, Lo et al. 2012). Similarly, rats treated with intracolonic acetic acid also develop colitis and exhibit 5-HT3 mediated increases in visceral sensitivity (Choi, Sung et al. 2008; Greenwood-Van Meerveld, Mohammadi et al. 2014). Sengupta et al, 2009 showed that rats treated with an acidic saline (pH4) intramuscular injection into gastrocnemius muscle develop somatic and visceral hypersensitivity. Treatment with chronic intravenous or intrathecal alosetron reduces the somatic pain but didn't affect the visceral pain experienced in response to colorectal distension. The authors concluded that while the inhibition of 5-HT3 receptors didn't reduce the pain response compared to controls it prevented the sensitization observed in acid treated rats (Miranda, Peles et al. 2006). In a study where mast cell degranulation was induced by BrX-537A to cause visceral hypersensitivity, 5-HT3 receptor inhibition didn't affect the pain response. However, 5-HT1A receptor inhibition reduced the pain response associated with mast cell degranulation. (Coelho, Fioramonti et al. 1998). Infusion of 5-HT precursor 5-hydroxytryptophan (5-HTP) caused bladder hyperalgesia in female rats and this response was attenuated by intrathecal but not subcutaneous ondansetron suggesting a pronociceptive role

of 5-HT (Hall, DeWitte et al. 2015). While, 5-HT3 receptors on the vagus nerve play an antinociceptive role in intact rats, spinal 5-HT3 receptors play a nociceptive role in rats subjected to water avoidance stress (Bradesi, Lao et al. 2007). Coelho et al and Bradesi et al concluded that upregulation of a spino-bulbo-spinal loop leads to increased activity of primary afferent neurons to enhance pain perception via signaling at 5-HT3 receptors as shown in Figure 2.2.

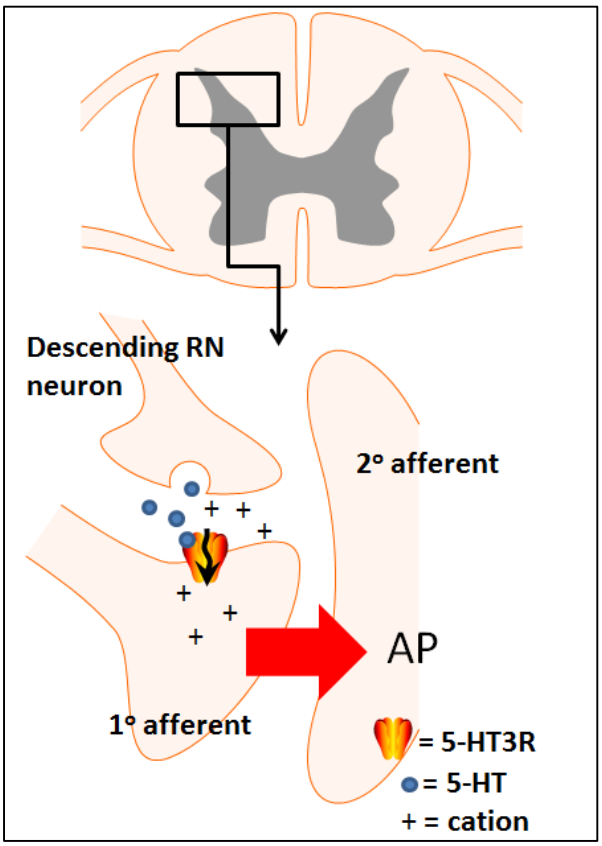

Figure 2.2 Spino-bulbo-spinal loop in the dorsal horn of the spinal cord. Descending serotonergic input can cause pain facilitation via 5-HT3 receptors activation on central terminal of primary afferent neurons. This will lead to increased excitatory neurotransmitter release and enhanced nociceptive signaling via projecting neurons.

Saria et al showed that activation of spinal 5-HT3 receptors caused the release of substance P, neurokinin A and calcitonin-gene related peptide, all of which are excitatory

neurotransmitters that cause increased pain transmission to corroborate the spino-bulbo-spinal loop theory (Saria, Javorsky et al. 1990). Other 5-HT receptors are expressed in the spinal cord and have been shown to modulate pain. For instance, 5-HT1A receptor agonists cause a reduction in pain in response to hind paws formalin injection, while 5-HT3 receptors mediate nociception (Oyama, Ueda et al. 1996). Depletion of 5-HT in the spinal cord by the neurotoxin 5, 7-dihydroxytryptamine reversed the antinociceptive effects of 5-HT3 receptor antagonists suggesting that endogenous 5-HT activates 5-HT3 receptors to enhance pain transmission. Replacing 5-HT via intrathecal injection in the 5, 7-DHT treated rats at a high and low dose resulted in a variable response. Rats treated with a high dose of 5-HT intrathecally developed hyperalgesia via 5-HT3 receptor activation. On the other hand, low dose 5-HT resulted in antinociception by acting of 5-HT1A receptors (Oyama, Ueda et al. 1996; Sawynok and Reid 1996).

Other studies suggested that 5-HT signaling at spinal 5-HT3 receptors is antinociceptive. Mice treated with intrathecal 5-HT or 5-HT3 receptor agonist (2-methy-5-HT) exhibit increased latency of tail flick and decreased pain behaviors in response to intrathecal administration of N-methyl-D-aspartate (NMDA) or substance P. This increase in latency and pain behaviors were reversed following treatment with 5-HT3 receptor antagonists (Alhaider, Lei et al. 1991). In rats, hot plate latency was increased following treatment with intrathecal 5-HT or 2-methy-5-HT and was reversed with 5-HT3 receptor inhibition (Glaum, Proudfit et al. 1990). In both studies it was concluded that 5-HT3 receptor mediated analgesia was produced by release of GABA from inhibitory interneurons since the $GABA_A$ receptor antagonist, ICS-205-930 blocked the analgesic effects of 5-HT3 receptor agonist.

It is clear from clinical and basic research discussed that the role of 5-HT signaling at 5-HT3 receptors in pain modulation is not well understood. The discrepancies arise from animal model, pain assay and route of administration of various 5-HT3 receptor antagonists and agonist. For instance, animal models used to study IBS rely on inflammatory insults such as intracolonic instillation of acetic acid or dextran sulfate to induce visceral pain. This is a good animal model to study post-infectious IBS. However, post-infectious IBS is seen in only 10% of IBS patients (Beatty, Bhargava et al. 2014). Therefore, relying on inflammation in animal models to study IBS pathophysiology is not optimal. Stress-induced visceral hypersensitivity animal models are also used extensively as 50 % of IBS patients have a comorbid anxiety disorder (Grzesiak, Beszlej et al. 2014). Despite the increased prevalence of IBS in female patients most basic research conducted relies on male animals. SERT KO rats circumvent these issues since inflammatory insult, treatment with 5-HTP or stress stimuli are not required to induce visceral hypersensitivity. Galligan et al. 2013 showed that SERT KO rat serves as a sex-specific animal model of visceral hypersensitivity. Therefore the SERT KO rat serves as a great resource to help us understand the mechanism by which a dysfunction in serotonergic system, which is observed in some IBS patients, can lead to sex-specific visceral hypersensitivity. The studies performed herein will test the hypothesis that that inhibition of 5-HT3 receptor will decrease VMR to CRD in SERT KO female rats. This will allow us to gain insights on the specific role of 5-HT3 receptors in mediating visceral hypersensitivity with underlying disruption in serotonergic signaling.

2.3 Methods

2.3.1 Animals

All animal use protocols were approved by the Institution Animal Use and Care Committee at Michigan State University. The serotonin transporter (SERT) knockout (KO) and wild type (WT) control rats were purchased under license from Genoway, Inc (http://genoway.com/). The WT rats used in this study and the background of SERT KO rats are the Wistar strain. Following surgery all animals were singly housed to prevent post-surgical complications. Age matched 3-4 months old rats were used for all experiments described in this chapter.

2.3.2 Surgery

Electromyographic (EMG) electrode implantation. Rats were deeply anaesthetized with 4% isoflurane gas and placed on a heating pad (~30^{o}C). A midscapular incision was made followed by a ventral midline incision. A hemostat was used to gently separate the skin from the muscles. Teflon coated silver wires (A-M systems, Sequim, WA) were stitched into the external oblique muscles above the inguinal ligaments and the incision was closed with wound clips. The electrodes were tunneled subcutaneously, secured to the back musculature and eternalized in the midscapular region for future access. Sutures were used to close the midscapular incision. Rats were allowed to recover for 3 days before assessing visceromotor response to colorectal distension. During recovery the rats were acclimatized to a plexiglass rat restrainer for an hour a day over two consecutive days. Carprofen (5mg/kg, s.c.) and piperacillin/tazobactam (120mg/kg, i.m.) were administered at the time of surgery to control pain and reduce risk of infection respectively.

ICV cannula implantation. Rats were deeply anaesthetized with 4% isoflurane gas and positioned in a stereotactic apparatus on a heating pad (~30^{o}C). An incision was made to expose the skull and a hole was drilled to place the cannula in the lateral ventricle. Jeweler's screws and dental cement were used to anchor the cannula in place. Carprofen (5mg/kg, subcutaneous) and piperacillin/tazobactam (120mg/kg, intramuscular) were administered at the time of surgery to control pain and reduce risk of infection respectively.

2.3.3 Visceromotor response (VMR) to colorectal distension (CRD) and volume pressure loop data acquisition.

Under light Isoflurane anesthesia, a flexible latex balloon (4.5 cm, females; 7 cm, males) was inserted intra-anally into the colon. The distal end of the balloon was located in the rectum 1 cm away from the anal sphincter and secured to the base of the tail using surgical tape to prevent excretion of the balloon during experiments. After recovery from anesthesia, the animals were placed in the plexiglass restrainer and allowed to acclimate for 30 minutes before the initiation of the colorectal distension procedure. The balloon was connected to an animal barostat (G&J electronics, Toronto, Ontario, Canada) and the electrode leads were connected to amplifier (7P1, Grass-Instruments-Astro-Med Inc., West Warwick, RI, USA). The barostat and amplifier were connected to an A/D converter amplifier (Digidata 1223A, Axon Instruments-Molecular Devices, Sunnyvale, CA, USA) and signals digitized at 1KHz and recorded using Axoscope 10 software (Axon-instruments-Molecular Devices).

The VMR to CRD was assessed before and after treatment with the drug being studied. Each CRD procedure consisted of a series of 5 phasic distensions at pressures of 10, 20, 40, 60,

80 mmHg (10-s duration; 5-min interval between distension). The VMR to CRD was measured by recording electromyographic (EMG) activity of the external oblique muscles 10 s before, 10 s during, and 10 s after each distension episode. The EMG activity was analyzed using Clampfit 10 (Axon-instruments-Molecular Devices). The signal was rectified and the area under the curve calculated and normalized to baseline which is measured 10-s before each distension episode. The data is reported as normalized visceromotor response (VMR).

Volume pressure loop data were collected by measuring the pressure of the colon and rectum in response to increased volume. The volume inside of the balloon was increased by 0.1 mL increments to reach a maximum of 1 mL. The pressure inside the colon recorded throughout the balloon inflation period. This protocol was performed before and after drug treatments before the start of VMR recoding. Using the barostat, the volume pressure loop data was collected by recording the volume needed to increase the intracolonic pressure by 1.0 mmHg up to 10 mmHg.

2.3.4 Intrathecal injection

Rats were lightly anesthetized with 4% isoflurane, the hair along the dorsal aspect of the lower spine was clipped. The L5-L6 intervertebral space was exaggerated by placing a 50 mL centrifuge tube under the lower limbs. A 27 G ½ inch needle connected to a 50 µL glass syringe was gently inserted into the vertebral canal. Tail flick confirmed entry of the needle into the vertebral canal. Drugs were dissolved in 5-10 µL solvent and administered using a 50 µL glass syringe to minimize back flow of CSF upon removal of the needle following injection.

2.3.5 Cerebrospinal fluid (CSF) fluid collection

Rats were deeply anaesthetized with 4% isoflurane gas and position in a stereotactic apparatus to exaggerate the atlanto-occiptal (AO) joint. A midline incision was made at the base of the skull and retractor was used to expose the AO joint. A pulled capillary was used to puncture the ligaments and access the CSF in the cisterna magna. CSF samples were collected in Eppendorf tubes and stored at – 80 degrees Celsius until time of analysis.

2.3.6 High-performance liquid chromatography (HPLC)

For ramosetron detection a Phenomenex Luna 5u C-18 250x4.6mm column was used. A Photo Diode Array detector was employed and ramosetron was read at 213 nm. The mobile phase was 70% 0.05M sodium phosphate (pH 5.2) and 30% acetonitrile with flow rate at 1.0 ml/min. 5-HT detection was performed using a Thermo Scientific ODS Hypersil 3 u 150 x 3mm column with detection at 200 mV. The mobile phase used was 90mM Dihydrogen Sodium Phosphate, 50 mM Citrate, 50 uM EDTA, and 1.7mM sodium octyl sulfate with 10% acetonitrile with flow rate at 0.6 ml/min. Norepinephrine was detected using a Thermo Scientific HR-80 3u 80X4.6mm column with Cat-A-Phase II mobile phase with flow rate at 1.1 ml/min at detection at -300mV.

2.3.7 Immunohistochemistry

The lumbosacral spinal cord and DRG were collected and fixed in 4 % paraformaldehyde (PFA) overnight. Following fixation the tissue was cryopreserved in 30% (w/vol) sucrose solution. Optimal cutting temperature (OCT) matrix compound was used to embed the tissue for sectioning using a cryostat. Ten-20 µm sections were collected on glass slides and stored at -

80 ^{o}C. Thawed sections were washed with 0.01 M PB-T (PB with 0.4 % Triton X-100) 3 times for a total of 30 minutes. To prevent non-specific binding, sections were incubated in 5% animal serum (GS) for 1 hr at room temperature. Primary antibody incubations were performed overnight at 4 ^{o}C. Following incubation with primary antibody, the sections were washed 3 times with PB for 10 minutes each and incubated with secondary antibody at room temperature for 1 hr. Secondary antibody was washed off and sections were rinsed with PB 3 times for 10 minutes each. Prolong gold anti-fade reagent was applied to sections and cover slips were placed to preserve slides for future imaging.

All primary antibodies were diluted in 1% animal serum and all secondary antibodies were diluted in 0.01 M PB.

Table 2.1 Primary and secondary antibodies used for IHC

Antibody type	Vendor	Catalogue #	Lot #	Dilution
Primary: 5-HT3 receptor antibody	BIOSS	Bs-12051R	AE090712	1:10
Primary: GABA antibody	Abcam	Ab17413	GR3175268-1	1:300
Secondary: Alexa 488	Life technologies	A11073	1841755	1:500
Secondary: Alexa 594	Life technologies	A21207	1668652	1:500

2.3.8 Statistics

Electromyographic recordings from visceromotor response to colorectal distension were analyzed using two way-repeated measures ANOVA and Bonferonni's post hoc test to determine the effects of drugs on VMR at each distending pressure. We used 6-8 animals per group to achieve a power of 0.8 unless specified otherwise for a given experiment. Data will be

presented as mean ± SEM. P-values less than 0.05 were considered statistically significant. HPLC data were analyzed using Student's-t test. $P < 0.05$ was considered statistically significant.

2.3.9 Time-line of studies performed

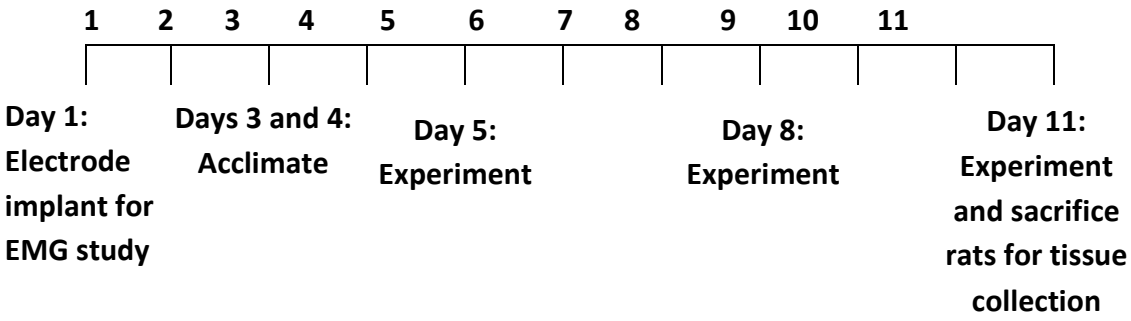

Figure 2.3 Time-line for studies of subcutaneous drug administration. Alosetron (0.1 mg/kg), granisetron (0.1 mg/kg), and sterile saline vehicle were administered in random order. Rats were equipped with electrodes on day one and allowed to recover on day 2. On day 3 and 4 the rats were acclimated to the restrainer for one hour each day. On day 5, 8 and 11 VMR to CRD was assessed before and after treatment with the drug or vehicle. Rats were euthanized at day 11 following the final experiment.

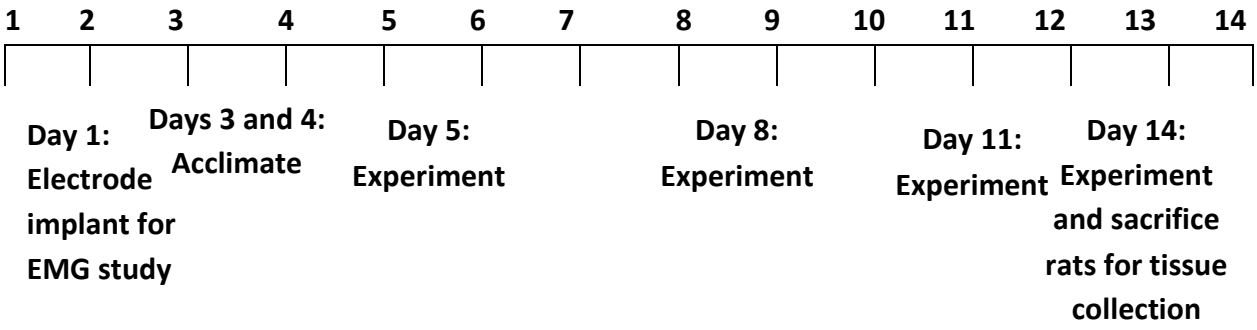

Figure 2.4 Ramosetron and alosetron dose response and intrathecal study time-lines. Three different doses of ramosetron (0.01, 0.1 and 1 mg/kg) and sterile saline vehicle were administered to each rat in random order. Rats were equipped with electrodes on day one and allowed to recover on day 2. On day 3 and 4 the rats were acclimated to the restrainer for one hour each day. On day 5, 8, 11 and 14 VMR to CRD was assessed before and after treatment with the drug or vehicle. Rats were euthanized at day 14 following the final experiment. A similar procedure was performed for another group of rats to determine the alosetron dose response curve. Alosetron (0.01, 0.1 and 1 mg/kg) or sterile saline vehicle were given to each rat. For intrathecal studies alosetron, granisetron and ondansetron were administered in random order at a dose of 25 nmol in 10 µl of sterile saline.

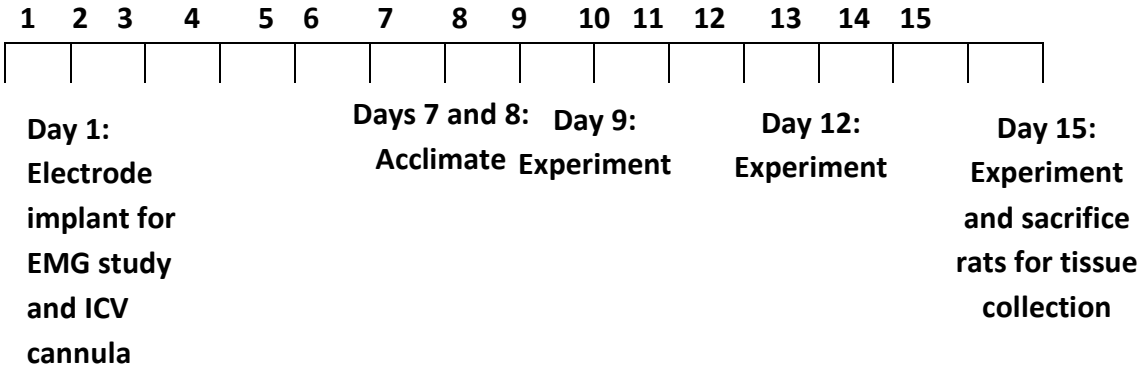

Figure 2.5 Intracerebroventricular study timeline. On day 1, rats were equipped with electrodes for electromyographic recording and cannula in the right lateral cerebral ventricle. Rats were allowed to recover for 6 days post-surgery. On days 7 and 8 rats were acclimated to the restrainer for an hour each day. On days 9, 12 and 15 rats were treated with alosetron 25 nmol/10µl), alosetron (2.5 nmol/ 10 µl) or 10 µL of sterile saline vehicle in random order.

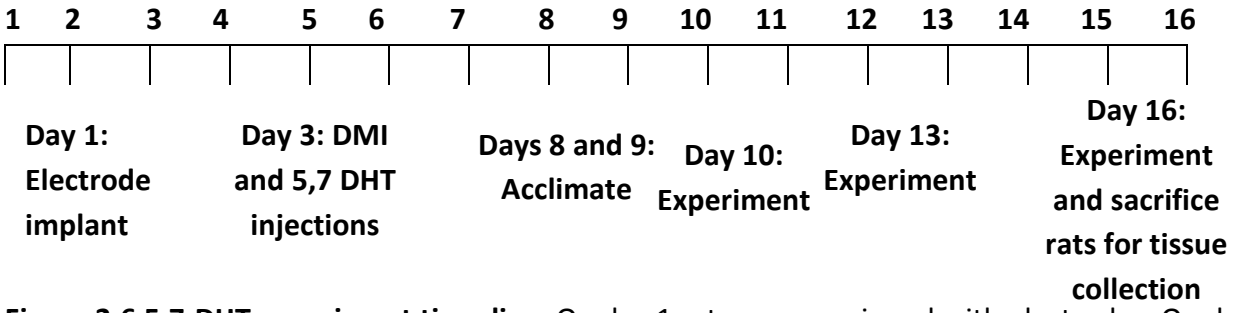

Figure 2.6 5,7-DHT experiment time-line. On day 1 rats were equipped with electrodes. On day 3 rats were treated with desipramine (25 mg/kg) via an intraperitoneal injection (i.p). Forty five minutes after desipramine treatment rats were treated with 100 µg of 5, 7-DHT dissolved in 10 µL of sterile saline or sterile saline vehicle (10 µL). Rats were allowed to recover for 7 days following 5, 7-DHT treatment. On days 8 and 9 rats were acclimated to the restrainer and on days 10, 13 and 16 rats were treated with alosetron (25 nmol, i.t.), SR57227 (100 pmol, i.t.) or sterile saline vehicle in random order. Visceromotor response to colorectal distension was recorded before and after treatment.

2.4 Results

To test the hypothesis that inhibition of 5-HT3 receptors decreases VMR to CRD we administered various 5-HT3 receptor antagonists via subcutaneous injection. Alosetron and granisetron increased VMR to CRD in SERT KO but not WT female rats as shown in Figure 2.7. Alosetron increased VMR to CRD in WT male rats only and granisetron did not enhance pain perception in WT and SERT KO male rats (Figure 2.8). Volume-pressure loop analysis which measures the compliance of the GI tract indicates that inhibition of 5-HT3 receptors doesn't increase the VMR by reducing the relaxation of the gut walls in response to increased volume as shown in Figure 2. 9.

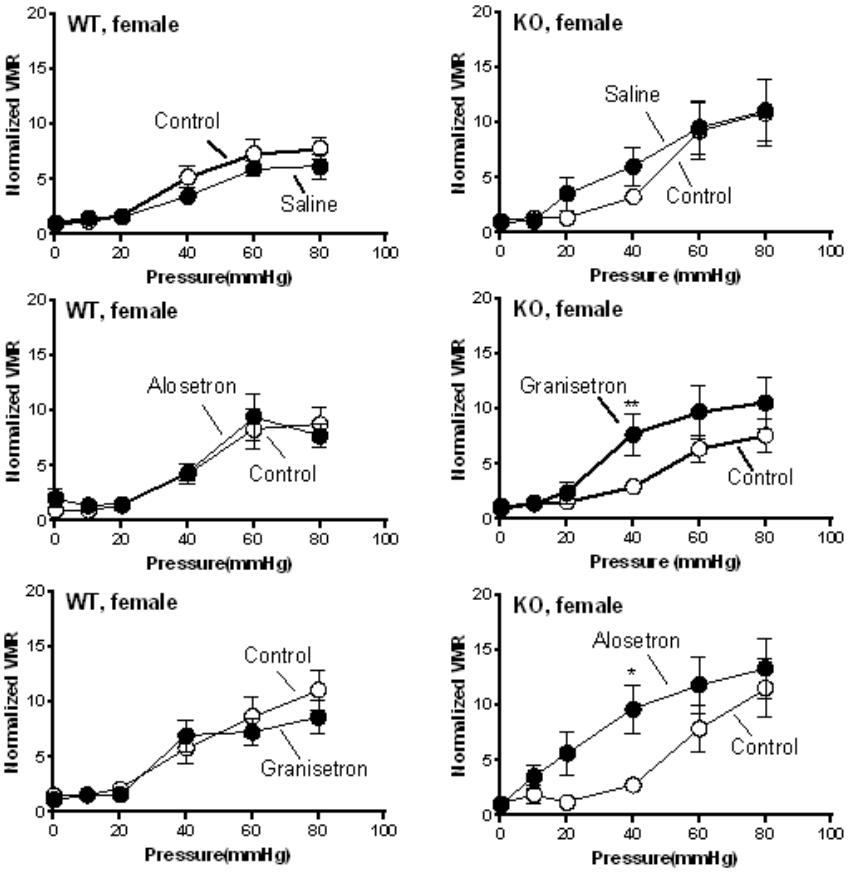

Figure 2.7 Subcutaneous administration of 5-HT3 receptor inhibitor increased VMR to CRD in SERT KO but not WT female rats. VMR recorded at the indicated pressure before (control) and after treatment with 5-HT3R antagonists granisetron (0.1 mg/kg, s.c.), alosetron (0.1 mg/kg, s.c.) or saline. Saline didn't affect VMR to CRD in WT (top left) and SERT KO female rats (top right). Granisetron didn't affect VMR to CRD in WT female rats (bottom left) but increased VMR to CRD in SERT KO female rats (middle right). Alosetron didn't affect VMR to CRD in WT female rats (middle left) but increased VMR to CRD in SERT KO female rats (bottom right). *, ** Indicate significantly different from control (* $P<0.05$,** $P<0.01$). Data are mean ± SEM.

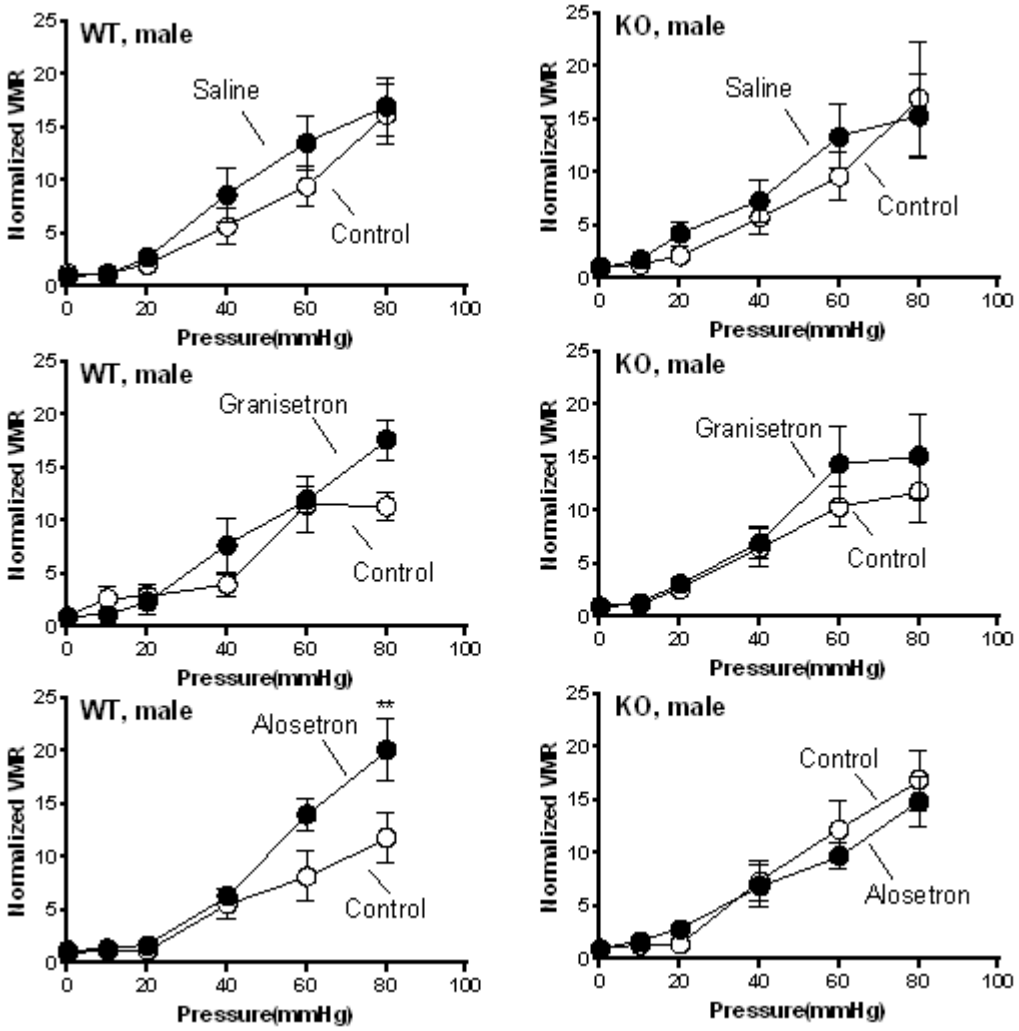

Figure 2.8 Subcutaneous administration of the 5-HT3 receptor inhibitor, alosetron, increased VMR to CRD in WT but not SERT knockout (KO) male rats. VMR recorded at the indicated pressure before (control) and after treatment with 5-HT3R antagonists granisetron (0.1 mg/kg, s.c.), alosetron (0.1 mg/kg, s.c.) or saline. Saline didn't affect VMR to CRD in WT (top left) and SERT KO male rats (top right). Granisetron didn't affect VMR to CRD in WT (middle left) and SERT KO male rats (middle right). Alosetron increased VMR to CRD in WT (bottom left) but not SERT KO male rats (bottom right). **Indicates statistical significance from control ($P<0.01$). Data are mean ± SEM.

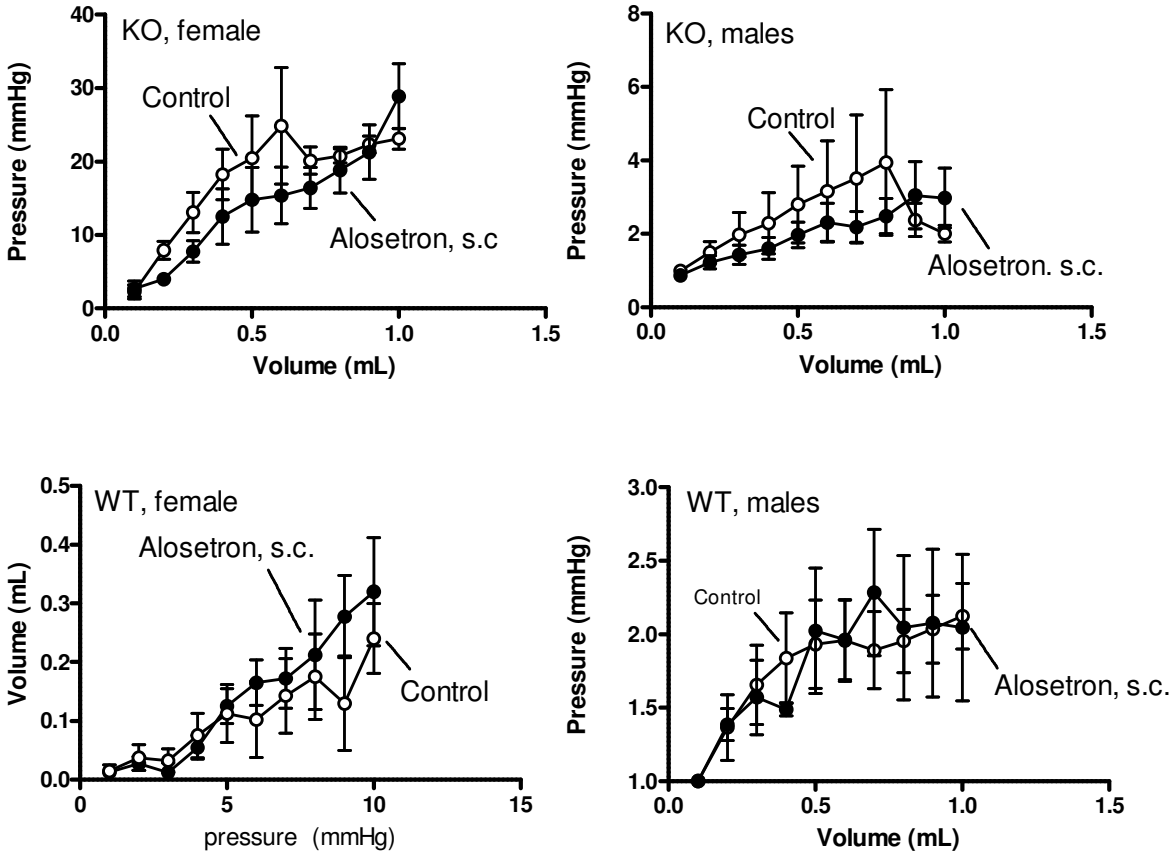

Figure 2.9 Volume-Pressure loop analysis. Inhibition of 5-HT3R does not affect the compliance of the colon and distal rectum in WT and SERT KO female and male rats. Data are mean ± SEM.

Alosetron (0.01 mg/kg, s.c) and (1mg/kg, s.c.) didn't not affect VMR to CRD.

However, an intermediate dose of 0.1 mg/kg alsoetron caused an increase in VMR to CRD as shown in Figure 2.10.

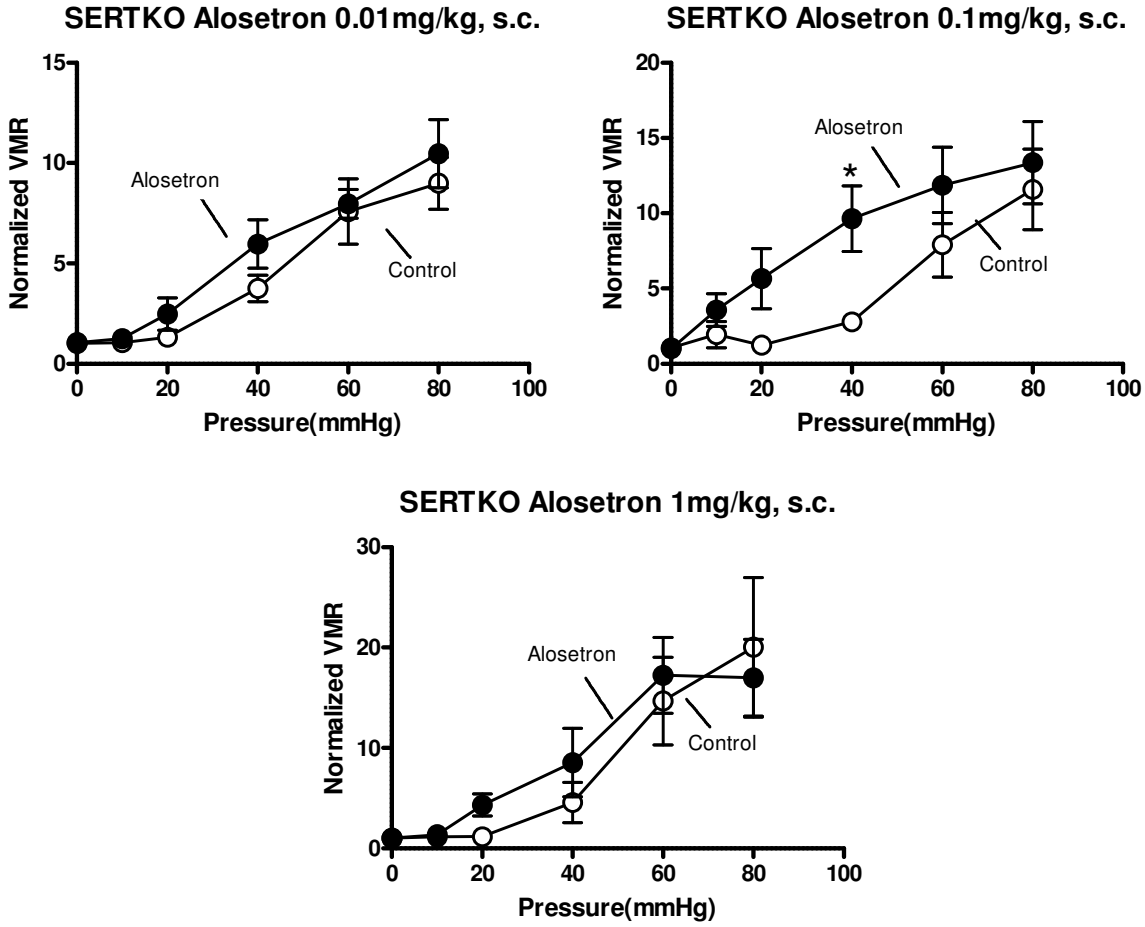

Figure 2.10 Alosetron dose-response in SERT KO female rats. VMR recorded at the indicated pressure before (control) and after treatment with alosetron at 0.01 mg/kg, 0.1 mg/kg, and 1.0 mg/kg, s.c. *Indicate significantly different from control (* $P<0.05$), Data are mean ± SEM.

It is evident from clinical and basic research that despite their similar affinities to 5-HT3 receptors, 5-HT3 receptor antagonists have variable effectiveness in treating pain and nausea. For instance, ondansetron and granisetron reduce chemotherapy induced nausea, but alosetron has no effect on nausea. Instead nausea is a side effect of alosetron (Cremonini, Nicandro et al. 2012). Therefore, we decided to treat SERT KO females with ondansetron to determine if we will observe a similar response to granisetron and alosetron. Ondansetron did not increase VMR to CRD in SERT KO female rats (data not shown).

Alosetron and granisetron cross the BBB. Therefore, the increase in pain might be mediated by central inhibition of 5-HT3 receptors. To test this hypothesis we administered ramosetron, a partially BBB permeable 5-HT3 receptor antagonist at 3 doses. We hypothesized that at the lower dose of 0.1 mg/kg, the drug will not have sufficient central nervous system penetration while the higher dose will be able to accumulate in the CNS. To confirm our hypothesis we measured ramosetron concentration in cerebrospinal fluid (CSF) using HPLC. Ramosetron (1 mg/kg, s.c) was detected in CSF and caused an increase in VMR to CRD. The lower dose of ramosetron (0.1 mg/kg, s.c.) was not detected in CSF and didn't affect the pain response in SERT KO rats as shown in Figure 2.11.

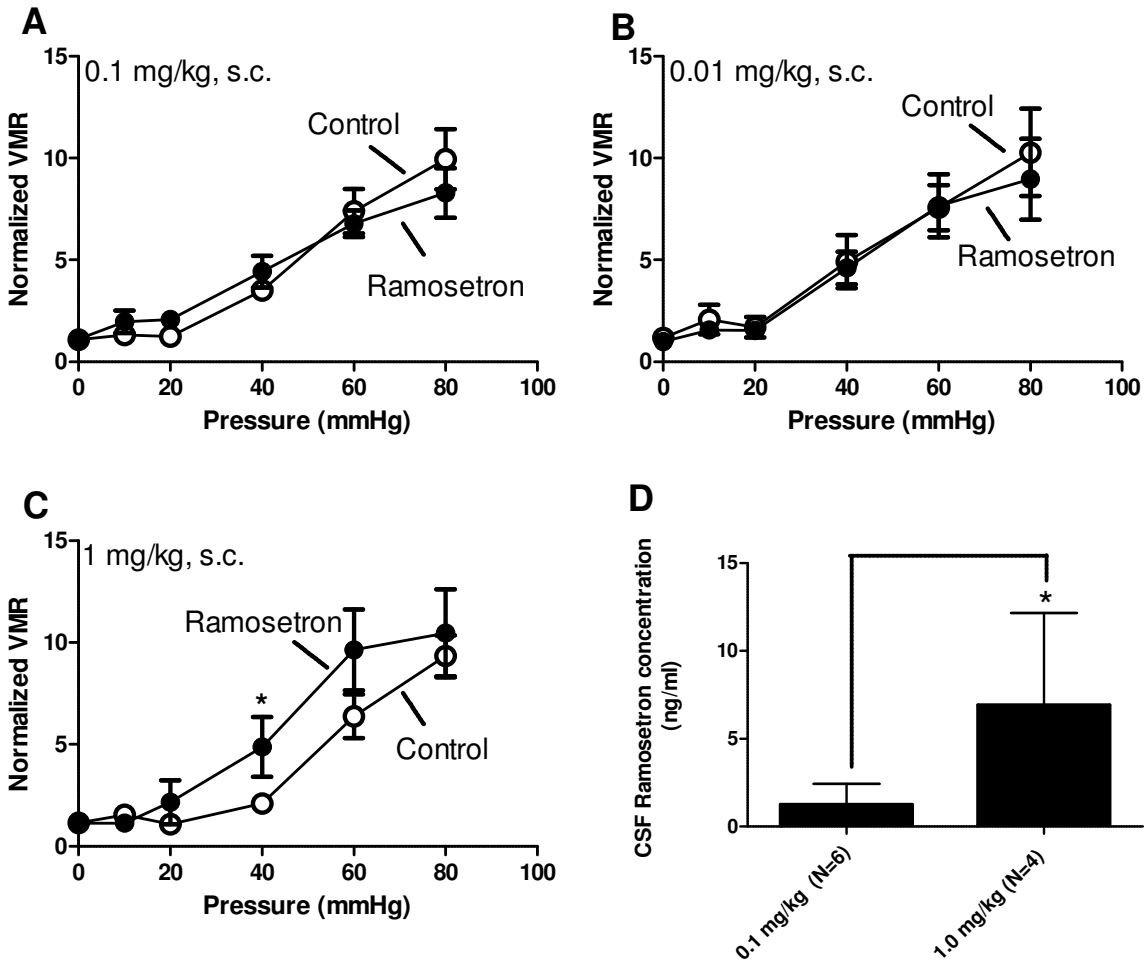

Figure 2.11 Dose-response curve of s.c. ramosetron in SERT KO female rats. VMR recorded at the indicated pressure before (control) and after treatment with ramosetron at 0.01 mg/kg (A), 0.1 mg/kg (B), and 1.0 mg/kg (C), s.c. HPLC analysis of CSF indicates that at 1.0 mg/kg, s.c. dose, Ramosetron was detected (D). *Indicate significantly different from control (* $P<0.05$), Data are mean ± SEM.

A more direct way of testing the effects of central inhibition of 5-HT3 receptors is to directly administer the drugs into the CNS. This was accomplished by i.t. and i.c.v. injections. Alosetron (25 nmol), granisetron (25 nmol), ondansetron (25 nmol) and saline vehicle were administered i.t. to SERT KO and WT rats. Alosetron increased VMR to CRD in SERT KO female rats only (Figure 2.12). Intrathecal administration of alosetron, granisetron and ondansetron did not affect VMR to CRD in WT female rats, and male rats regardless of genotype (Figure 2.13).

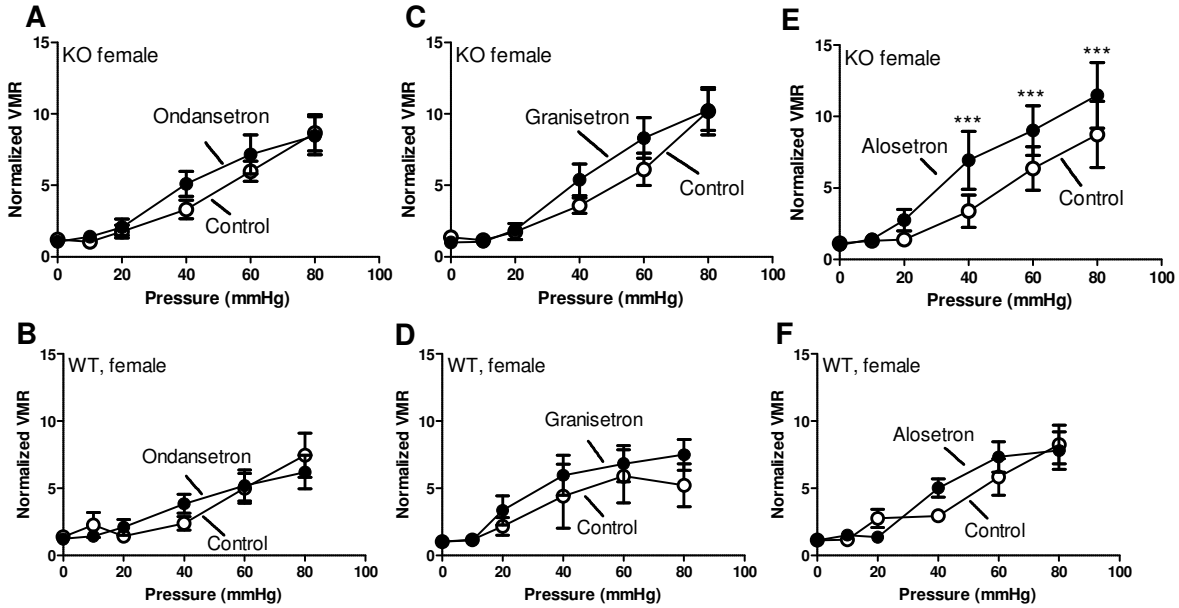

Figure 2.12 Intrathecal administration of alosetron increased VMR to CRD in serotonin transporter (SERT) knockout (KO) but not WT female rats. VMR recorded at the indicated pressure before (control) and after treatment with 5-HT3R antagonists granisetron (25 nmol, i.t.), alosetron (25 nmol, i.t.) and ondansetron (25 nmol, i.t.). Ondansetron didn't affect VMR to CRD in WT (N=6) (A) and SERT KO female rats (B). Granisetron didn't affect VMR to CRD in WT (N=5) (C) and SERT KO female rats (D). Alosetron didn't affect VMR to CRD in WT female rats (N=5) (E) but increased VMR to CRD in SERT KO female rats. N=8 for SERT KO rats. *** indicates significantly different than control ($P<0.001$) (F). Data are mean ± SEM.

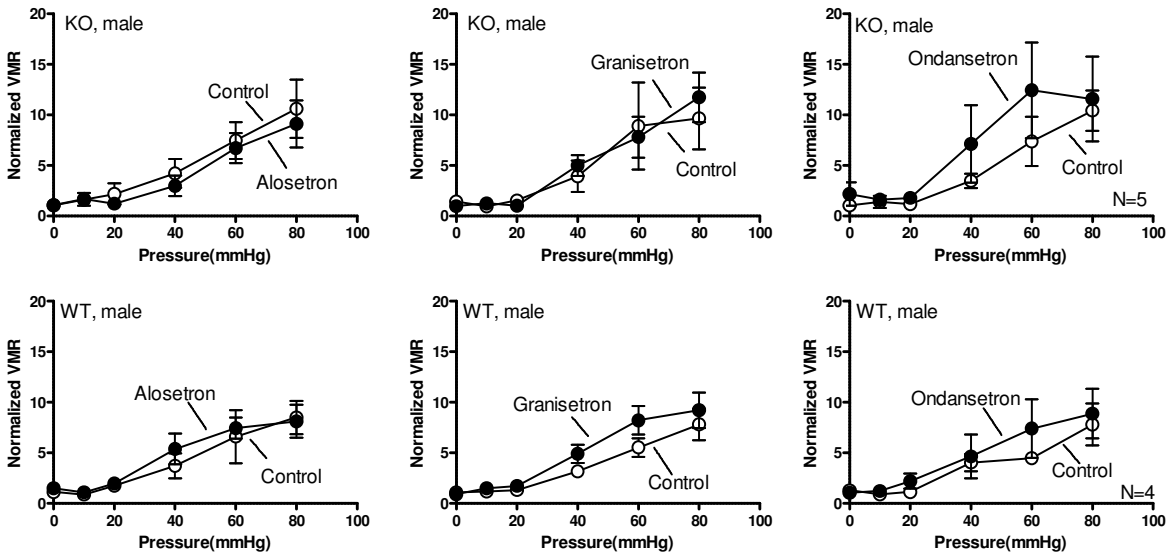

Figure 2.13 Inhibition of spinal 5-HT3 receptors did not affect VMR to CRD in SERT KO or WT male rats. VMR recorded at the indicated pressure before (control) and after treatment with 5-HT3R antagonists granisetron (25 nmol, i.t.), alosetron (25 nmol, i.t..), .) or saline. Data are mean ± SEM.

Drugs administered i.t. circulate throughout the central nervous system via CSF. Therefore, the increase in VMR to CRD observed with i.t. alosetron could be mediated in the brain or brainstem. To test this possibility we administered alosetron via i.c.v. cannula implanted into the right lateral ventricle of the brain in SERT KO and WT rats. Confirmation of proper placement of the ICV cannula was determined at the end of the last experiments by injecting methylene blue and grossly inspecting the ventricles. ICV administration of alosetron did not increase in VMR to CRD in all rats (Figure 2.14).

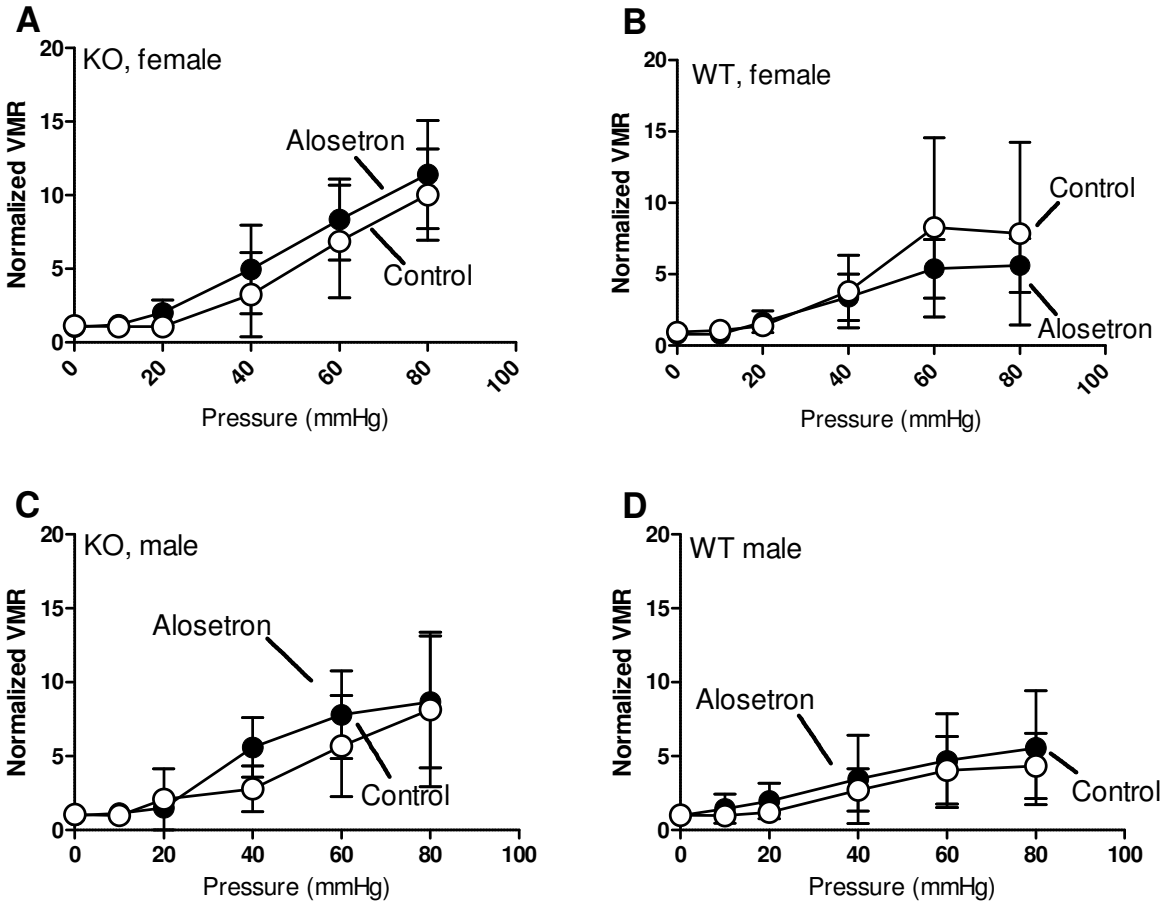

Figure 2.14 Intracerebroventricular administration of alosetron didn't affect VMR to CRD in SERT KO and WT rats. VMR recorded at the indicated pressure before (control) and after treatment with 5-HT3R antagonists alosetron (25nmol, i.t.) or saline. Data are mean ± SEM. KO female N=7, WT female N=5, KO male N=6, WT male N=5.

Since the inhibition of 5-HT3 receptors caused an increase in VMR to CRD in SERT KO female rats we wanted to test if activation of 5-HT3 receptors using an agonist could decrease the pain response. The 5-HT3 receptor agonist SR 57227 caused an increase in VMR to CRD in SERT KO males but not SERT KO female rats and WT rats (Figure 2.15).

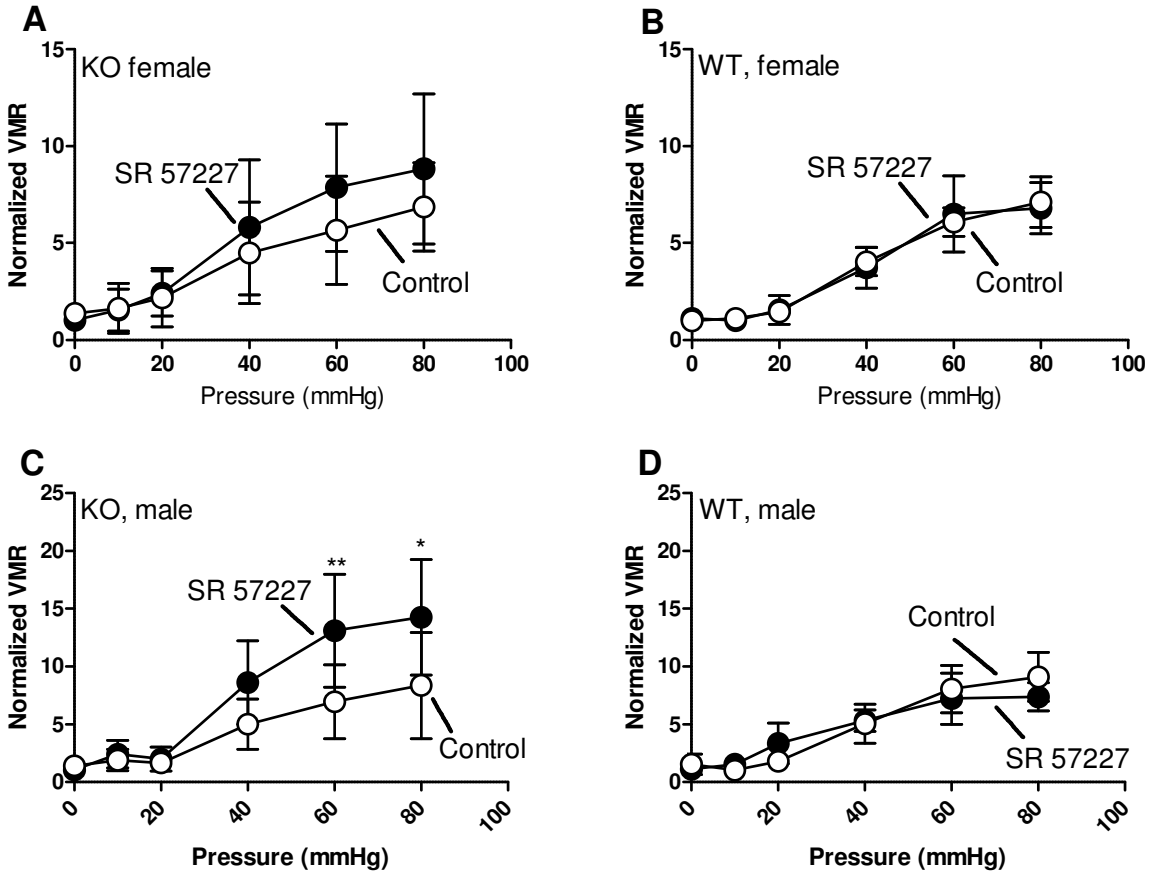

Figure 2.15 Activation of 5-HT3 receptor increased VMR to CRD in male SERT KO rat. The 5-HT3 receptor agonist SR 57227 (100 pm/10µl) increased visceral sensitivity in SERT KO male rats. VMR recorded at the indicated pressure before (control) and after treatment with 5-HT3R agonist SR 57227 (100 pmol, i.t.).). *, ** Indicate significantly different from control (* P<0.05), (** P<0.01). Data are mean ± SEM.

Blocking the action of 5-HT from descending serotonergic neurons at 5-HT3 receptors on inhibitory interneurons might be the mechanism by which alosetron increases VMR to CRD in SERT KO female rats. To test this hypothesis the effects of intrathecal alosetron on VMR to CRD were assessed after reduction of descending 5-HT neurons using the neurotoxin 5,7-dihydroxytrptamine (5,7-DHT). Intrathecal treatment with 5,7 DHT reduced serotonin levels in the lumbosacral spinal cord (figure 2.16). The reduction of 5-HT was more prominent in the WT animals. HPLC analysis of lumbosacral spinal cord homogenate showed that 5-HT was reduced

by 60 % in WT female rats, 40% in WT male rats, 32 % in SERT KO male rats and 25% in SERT KO female rats. Norepinephrine levels were not affected by 5,7 DHT treatment as shown in figure 2.17. IHC analysis of spinal cord samples from 5,7-DHT treated and untreated rats exhibited a reduction in 5-HT staining in the treated rats versus untreated rats regardless of genotype. Functional studies showed that the 25% reduction in 5-HT was sufficient to prevent the increase in VMR to CRD in SERT KO female rats (figure 2.18). 5,7-DHT did not affect the response to 5-HT3 receptor inhibition in WT female rats and male rats as shown in Figure 2.19.

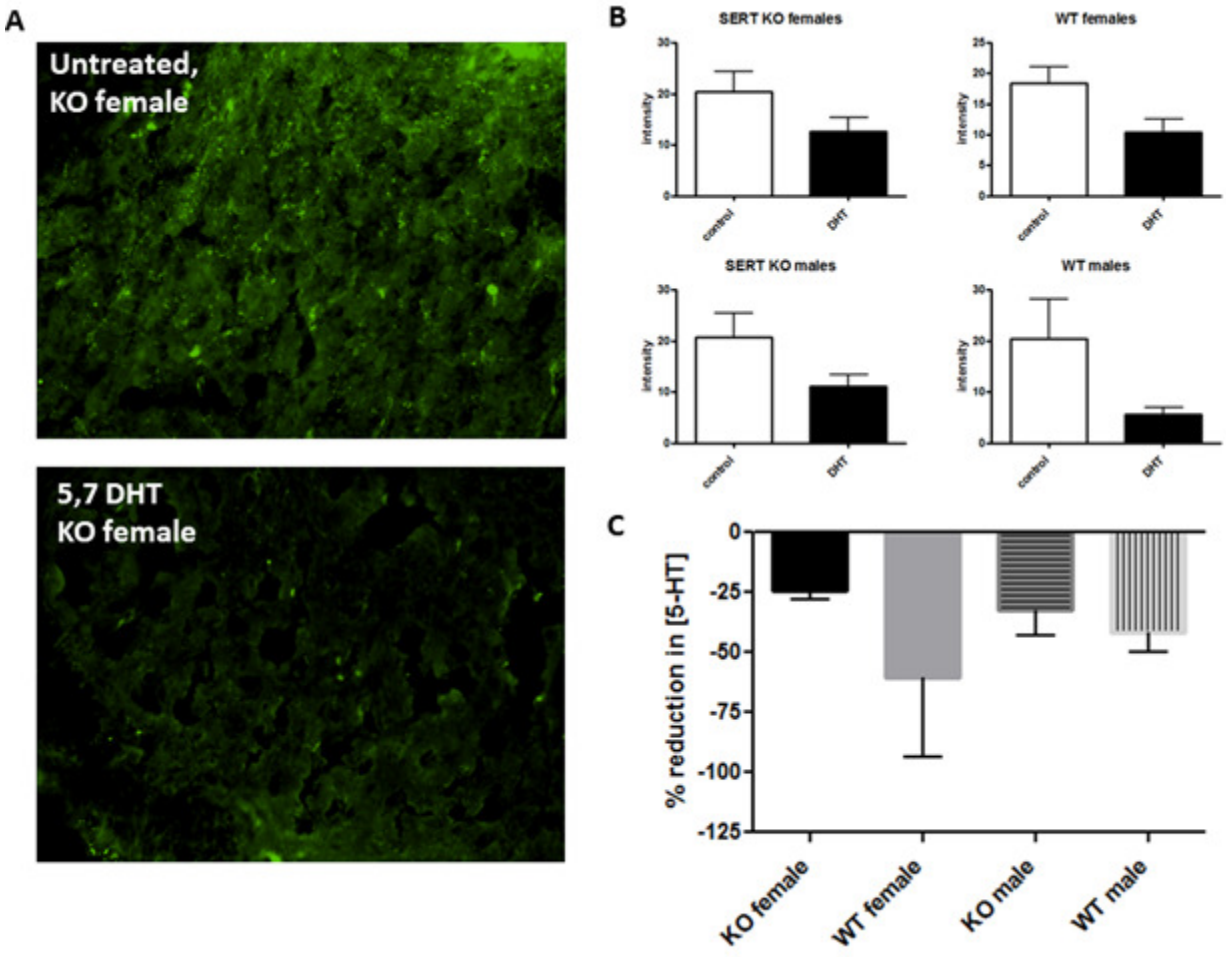

Figure 2.16 5,7 DHT (100 µg, i.t.) treatment decreased 5-HT levels in the lumbar and sacral spinal cord of KO and WT rats. IHC studies showed a reduction in 5-HT intensity in SERT KO female rat treated with 5,7 DHT (A). Quantification of intensity of 5-HT signal from IHC experiments shows a reduction in intensity in 5,7 DHT treated rats (B). HPLC analysis of lumbar and sacral spinal cord indicates a reduction in 5-HT levels following 5,7 DHT treatment (C).

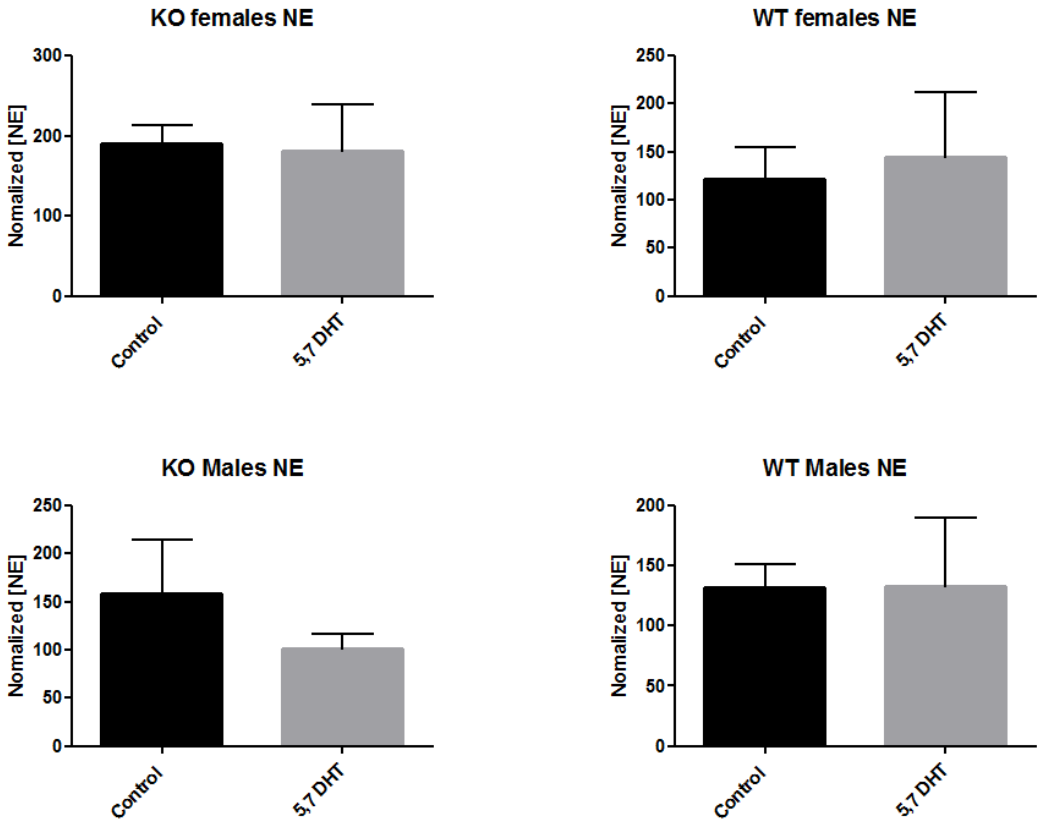

Figure 2.17 Norepinephrine levels are not affected by 5, 7-DHT. HPLC analysis was used to determine the amount of norepinephrine in lumbosacral spinal cord. Desipramine treatment prevented the uptake of 5, 7-DHT by noradrenergic neurons in WT and SERT KO rats. Data are mean ± SEM

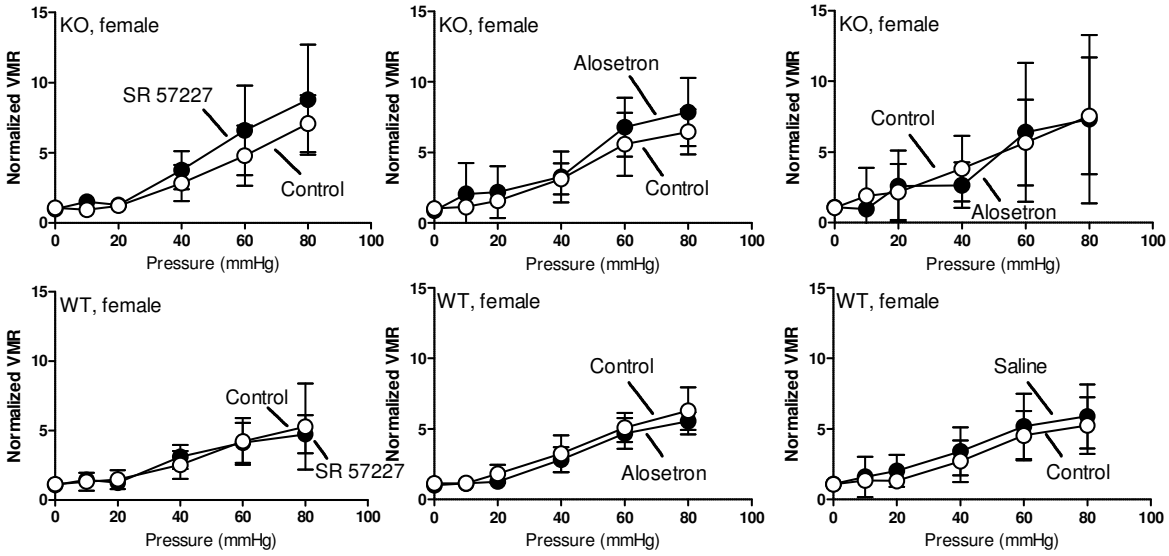

Figure 2.18 Treatment with 5,7-DHT prevented the increased VMR to CRD in SERT KO female rats following alosetron treatment. SERT KO and WT female rats showed no change in VMR to CRD following treatment with alosetron (25 nmol. i.t.), SR 57227 (100 pmol, i.t.) and sterile saline vehicle (10 µL). Data are mean ± SEM

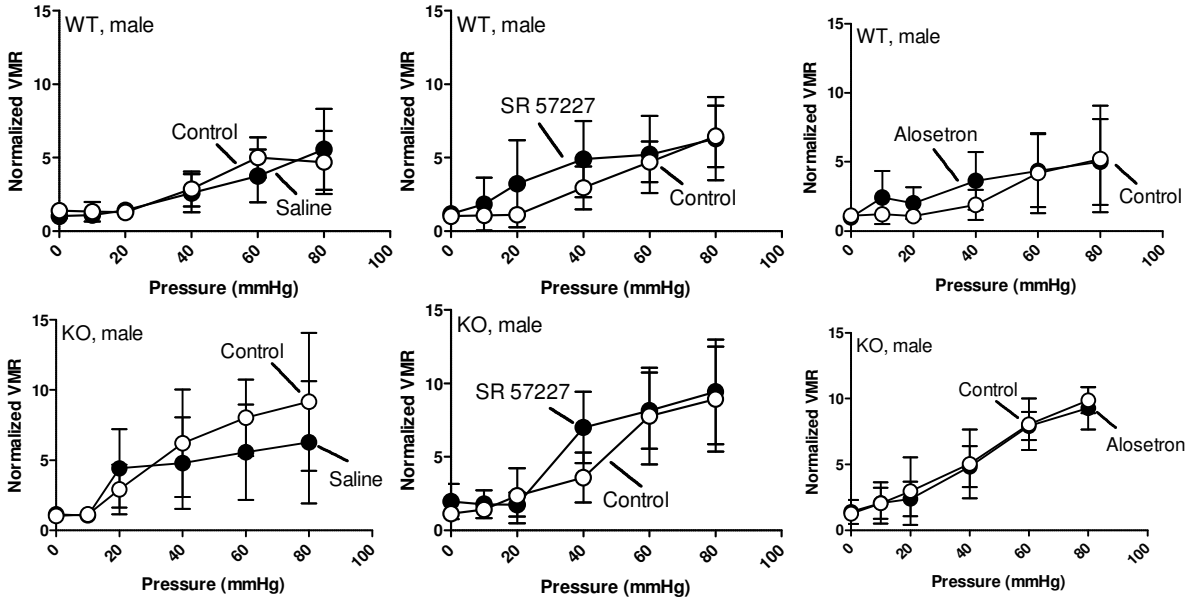

Figure 2.19 Treatment with 5,7-DHT prevented the increase in the VMR to CRD in SERT KO male rats following SR 57227 treatment. SERT KO and WT female rats showed no change in VMR to CRD following treatment with alosetron (25 nmol. i.t.), SR 57227 (100 pmol, i.t.) and sterile saline vehicle (10 µL). Data are mean ± SEM

To determine the pattern of 5-HT3 receptor expression we used IHC. We also stained spinal cord sections with GABA antibody to determine if 5-HT3 receptors are expressed on GABAergic inhibitory interneurons (Figure 2.20). 5-HT3A receptor subunit and GABA are colocalized to in all rats. A higher degree of colocalization is observed in SERT KO female rats as shown in Figure 2.20 and Figure 2.21.

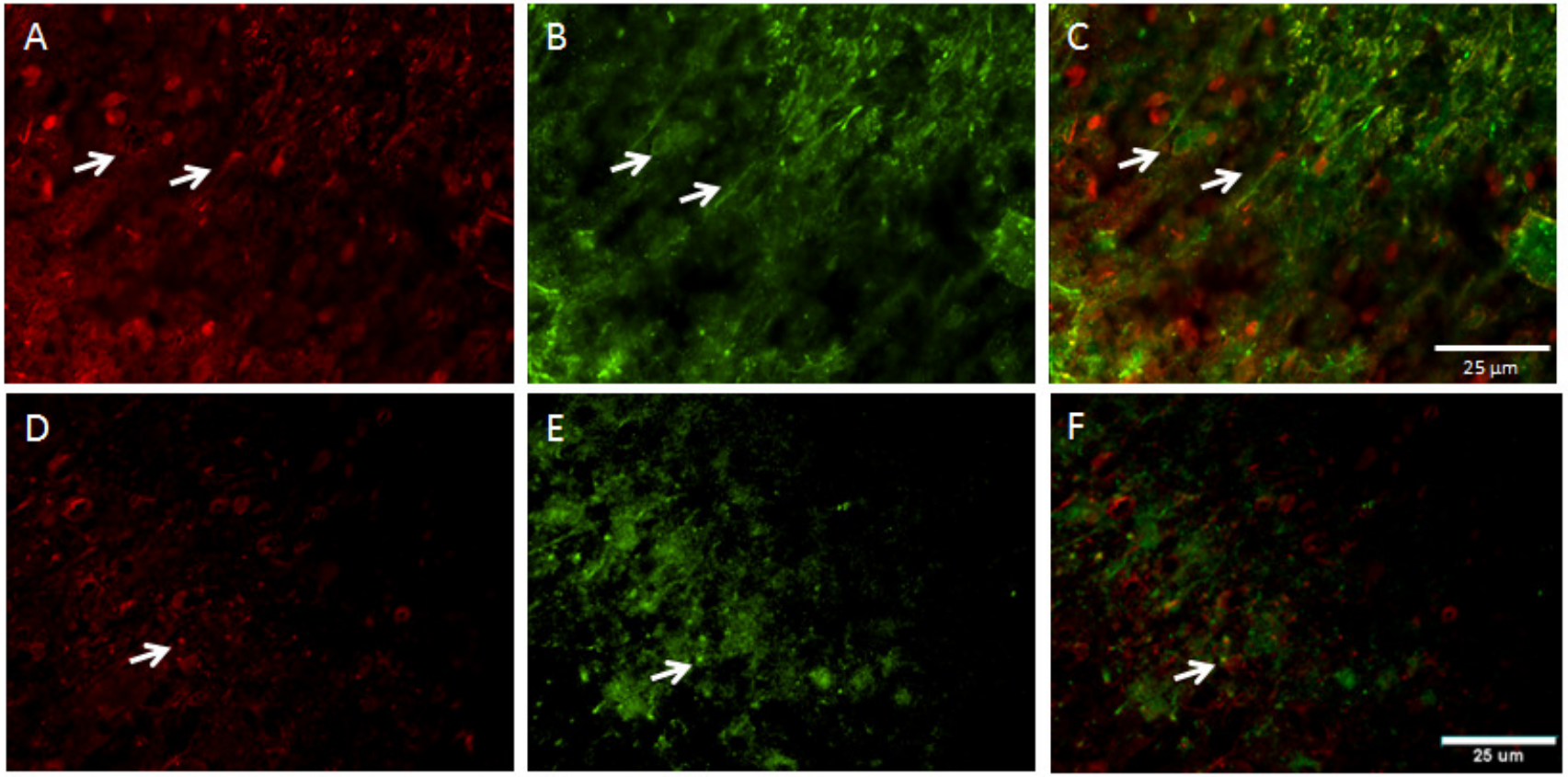

Figure 2.20 5-HT3A subunit and GABA expression in the superficial dorsal horn of the spinal cord in SERT KO rats. Alexa 594 (red) was used to visualize 5-HT3A subunits expression. Alexa 488 (green) was used to visualize GABA expression. The top panel shows 5-HT3A (A), GABA (B) and superimposed 5-HT3A and GABA images (C) in SERT KO female rats. The bottom panel shows 5-HT3A (D), GABA (E) and superimposed 5-HT3A and GABA images (F) in SERT KO male rats. White arrows indicate regions of colocalization.

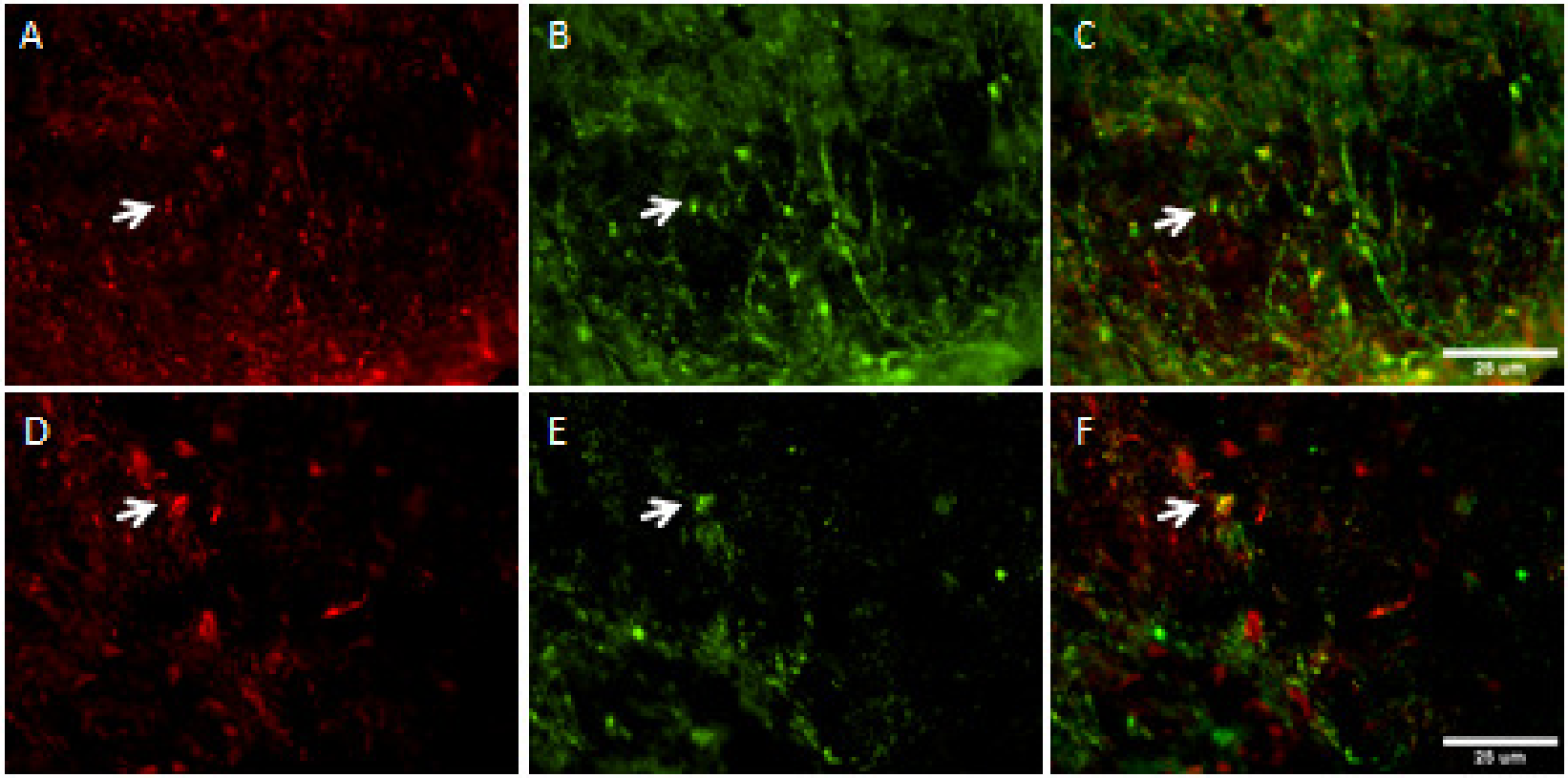

Figure 2.21 5-HT3A subunit and GABA expression in the superficial dorsal horn of the spinal cord in WT rats. Alexa 594 (red) was used to visualize 5-HT3A subunits expression. Alexa 488 (green) was used to visualize GABA expression. The top panel shows 5-HT3A (A), GABA (B) and superimposed 5-HT3A and GABA images (C) in WT female rats. The bottom panel shows 5-HT3A (D), GABA (E) and superimposed 5-HT3A and GABA images (F) in WT male rats. White arrows indicate regions of colocalization.

2.5 Discussion

Peripheral administration of the 5-HT3 receptor antagonists alosetron and granisetron at 0.1 mg/kg dose via subcutaneous injection resulted in an increase in VMR to CRD in SERT KO female rats only (table 2.2). Alosetron but not granisetron caused an increase in VMR to CRD in WT male rats. Volume-pressure loop analysis shown in figure 2.9 indicates that the increase in VMR to CRD in response to alosetron in SERT KO female rats and WT male rats is not due to reduced compliance of the colon and rectum. Compliance is a measure of wall tension in response to change in pressure. (Gregersen and Kassab 1996). A reduction in compliance would suggest that inhibition of 5-HT3 receptors within the gastrointestinal tract prevents the normal relaxation reflex of the colon walls in response to increased pressure leading to increased pain. A similar experiment to ours was performed by Bradesi et al, 2007 where they determined the effects of alosetron (0.3 mg/kg, s.c.) on VMR to CRD. Their data was identical to our data which showed an increase in VMR to CRD following subcutaneous administration of alosetron. They showed that capsaicin induced vagal ablation prevented the increase in VMR to CRD observed following treatment with alosetron. They concluded that inhibition of 5-HT3 receptors on peripheral terminals of the vagus nerve decreased descending inhibition at the level of the spinal cord which increased the VMR to CRD. They ruled out the possibility that alosetron is acting centrally by showing that intrathecal administration of alosetron didn't affect VMR to CRD. This is in line with our intrathecal studies. In WT male rats, inhibition of spinal 5-HT3 receptors with alosetron, granisetron and ondansetron didn't affect the VMR to CRD ruling out direct central effects. Similarly, intrathecal 5-HT3 receptor inhibition did not affect the pain response in WT female rats and SERT KO male rats. In SERT KO female rats, intrathecal

alosetron caused an increase in VMR to CRD. However, granisetron and ondansetron didn't affect the pain response.

5-HT3 receptor antagonists administered intrathecally can circulate to supraspinal structures via CSF to exert their effects at the level of the brain. To determine if the effects of alosetron are mediated in the brain we administered alosetron directly into the lateral ventricle of the brain via an icv cannula. Alosetron (2.5 nmol and 25 nmol) did not affect VMR to CRD in all rats when administered directly into the brain.

In SERT KO female rats we also tested the effects of the 5-HT3 receptor inhibitor, ramosetron. Unlike the other 5-HT3 receptor antagonist tested, ramosetron is a partially BBB permeable. While it does cross the BBB it is transported out of CNS via P-glycoprotein efflux pumps (Yamamoto, Murakami et al. 2002). We tested the VMR to CRD in SERT KO female rats after administration of 3 doses of ramosetron (0.01, 0.1 and 1 mg/kg, s.c.). The low dose of 0.1 mg/kg was not detected in CSF and did not affect VMR to CRD. The 1.0 mg/kg dose was detected in CSF samples and increased visceral sensitivity confirming that central but not peripheral inhibition of 5-HT3 receptors in SERT KO female rats leads to the increase in discomfort.

Table 2.2 Summary of results from 5-HT3 receptor antagonist studies

	SERT KO females	SERT KO males	WT females	WT males
Alosetron, s.c.	Increased VMR			Increased VMR
Granisetron, s.c	Increased VMR			
Ondansetron, s.c		np	np	np
Alosetron, it	Increased VMR			
Granisetron, it				
Ondansetron, it				
Alosetron, ICV				
Ramosetron, s.c. Low dose		np	np	np
Ramosetron, s.c High dose	Increased VMR	np	np	np

Blank cells indicate no change; np indicates not performed

Our results indicate that alosetron causes an increase in visceral sensitivity in SERT KO female rats by inhibiting lumbosacral spinal 5-HT3 receptors. However, while granisetron caused an increase in visceral sensitivity with peripheral administration, it failed to increase VMR following intrathecal and ICV application. Furthermore, alosetron but not granisetron caused an increase in VMR to CRD in male rats. These observations are in line with multiple animal studies which showed different 5-HT3 receptor antagonists exhibit variable responses. Rats treated with intracolonic acetic acid show reduction in visceral hypersensitivity with s.c granisetron but not ondansetron even when administered at a dose 10-fold higher than granisetron (Langlois, Pascaud et al. 1996). In another study where visceral sensitivity was induced via systemic injection of 5-hydroxytrptophan, granisetron but not ondansetron was able to relief visceral sensitivity (Banner and Sanger 1995). In both studies it was speculated that the variable response to 5-HT3 receptor antagonists is most likely due to the different potencies for different 5-HT3 receptors. Perhaps granisetron has higher affinity for 5-HT3 receptors composed of 2-A and 3-B subunits, while ondansetron has higher potency for 5-HT3

receptor composed of A subunits only. This hypothesis has yet to be tested. It is worth mentioning that studies characterizing 5-HT3 receptor antagonists are performed in 5-HT3 receptors composed of the A-subunit only (Banner and Sanger 1995). Clinically, variable responses to 5-HT3 receptor antagonists have also been observed as summarized below.

In a study with 5 participants ondansetron increased pain threshold to rectal electric stimulation but decreased maximum tolerated volume in rectal distension studies (Goldberg, Kamm et al. 1996). A recent study assessing clinical endpoints in 120 IBS patients showed that while ondansetron decreased stool frequency, urgency, and bloating it failed to improve pain scores (Garsed, Chernova et al. 2014). However, granisetron was able to reduce pain response in 12 IBS patients by assessing responses to rectal distension. Aside from the fact that this study had only 12 participants, pain as a clinical endpoints was not measured in this study which weakens the claim of the authors that granisetron reduces pain in IBS patients (Chen, Ilham et al. 2017). Alosetron has been extensively studied in IBS patients with sufficient number of participants and is the only FDA-approved 5-HT3 receptor antagonist shown to reduce abdominal pain in female IBS-diarrhea patients (Rahimi, Nikfar et al. 2008; Cremonini, Nicandro et al. 2012). The efficacy of alosetron in treating IBS in male patients has been questioned. A recent study with over 600 IBS-D male patients showed that while alosetron improved stool consistency, it failed to reduce the number of days with increased stool frequency, bloating and pain (Grover and Camilleri ; Chang, Ameen et al. 2005). The difference in efficacy of alosetron between males and females could be due to sex differences in drug metabolism (CYP2C19), SERT gene polymorphism, and limbic system activation (women exhibit higher activity of limbic system during rectal distension) (Berman, Munakata et al. 2000; Naliboff, Berman et al. 2003;

Koch, Corrigan et al. 2004). Furthermore, alosetron is more potent in female IBS-D patients expressing the l/l allele of the gene encoding SERT (Acosta and Camilleri 2015). It has been postulated that this due to increased 5-HT clearance in l/l carriers. Our studies support the observation that the effect of alosetron depends on polymorphisms in the SERT gene since in SERT KO rats, alosetron caused an increase in visceral sensitivity in female rats.

In SERT KO female rats alosetron exhibited a bell-shaped dose response curve as shown in Figure 2.10. This was observed in more than one study where the potency of alosetron was studied (Langlois, Pascaud et al. 1996; Clayton, Sargent et al. 1999). We can explain this antagonist response by assuming that at high dose steric hindrance at the binding site of the drug or action at other types of receptors could result in reduced potency (Wolf 2000). Indeed, alosetron has been shown to bind albeit with low affinity to H3 histamine receptors and 5-HT1C receptors (Clayton, Sargent et al. 1999).

Data from my i.t. studies indicate that the mechanism by which alosetron causes an increase in visceral sensitivity in SERT KO female rats is different than that in WT male rats. Descending serotonergic input to the dorsal horn of the spinal cord has been extensively studied and plays a pivotal role in pain modulation in the presence of inflammation, nerve injury or serotonergic dysfunction (Yoshimura 2006). SERT KO mice exhibit mechanical allodynia but not thermal hyperalgesia following sciatic nerve injury. This is thought to be due to specific changes in Aδ fibers (Vogel, Mossner et al. 2003). When assessing visceral pain produced by intraperitoneal injection of acetic acid, SERT KO mice showed a similar response to WT mice. (Hall, Schwarzbaum et al. 2011). Since sex-specific response to visceral pain is observed in SERT

KO rats, it is worth noting that the sex of the mice used in this study was not reported. A potential mechanism by which 5-HT3 receptor inhibition could mediate increased pain perception could be due to blocking the effects of 5-HT released from raphe neurons onto 5-HT3 receptors expressed on spinal dorsal horn inhibitory interneurons as shown in Figure 2.22. To test this hypothesis, we measured the effect of alosetron on visceral sensitivity in rats treated with the neurotoxin 5,7-DHT. Treatment with 5,7-DH reduced spinal 5-HT levels suggesting decreased descending 5-HT innervation to the lumbosacral spinal cord. Forty five minutes before administration of 5,7 DHT, rats were treated with norepinephrine transporter blocker, desipramine to prevent damage to nerve terminals of descending noradrenergic neurons. Reduction in spinal 5-HT prevented the increase in visceral sensitivity observed with intrathecal alosetron treatment suggesting that endogenous 5-HT signaling at 5-HT3 receptors in SERT KO female rats is antinociceptive. A similar observation was reported in somatic pain animal models where formalin was injected into the hind paw to induce somatic pain. Oyama, Ueda et al. 1996 showed that reduction of spinal 5-HT reduced 5-HT3 receptor mediated analgesia. In their study 5,7-DHT caused a 15% reduction in spinal 5-HT which is comparable to the 5-HT reduction we saw in SERT KO female rats. In another study, HPLC analysis of microdialysis samples from rat spinal cord treated with intrathecal 5-HT3 receptor agonist showed an increased in GABA release compared to control rats (Kawamata, Omote et al. 2003). Therefore, a potential mechanism by which 5-HT3 receptors mediate nociception is via activating inhibitory GABAergic interneurons (Alhaider, Lei et al. 1991; Song, Meyerson et al. 2011). To test this possibility we performed immunohistochemistry analysis to determine co-expression of 5-HT3A subunits and GABA in dorsal horn of lumbar spinal cord. IHC experiments

showed that SERT KO female rats exhibit increased colocalization of 5-HT3A and GABA in the superficial dorsal horn of the spinal cord compared to SERT male rats and WT rats.

Since inhibition of 5-HT3 receptors led to an increase in the VMR in SERT KO female rats, we wanted to test if activation of 5-HT3 receptors will produce analgesia. We treated rats with the selective 5-HT3 receptor agonist, SR 57227, via intrathecal injection. 5-HT3 receptor activation didn't affect the VMR in SERT KO female rats or WT rats. However, SR 57227 caused an increase in VMR to CRD in SERT KO male rats. These data are summarized in table 2.3. This may be observed due to expression of 5-HT3 receptors on central terminals of primary afferent neurons in SERT KO male rats as shown in Figure 2.2. Depletion of 5-HT from descending serotonergic neurons prevented the increase in visceral sensitivity in SERT KO male rats.

Table 2.3 Summary of 5-HT3 receptor antagonist and agonist studies

	SERT KO females	SERT KO males	WT females	WT males
Alosetron, s.c.	Increased sensitivity			Increased sensitivity
Granisetron, s.c	Increased sensitivity			
Ondansetron, s.c		np	np	np
Alosetron, it	Increased sensitivity			
Granisetron, it				
Ondansetron, it				
Alosetron, ICV				
Ramosetron, s.c. Low dose		np	np	np
Ramosetron, s.c High dose	Increased sensitivity	np	np	np
SR 57277, it		Increased sensitivity		
Alosetron, it In 5,7-DHT treated rats				

Blank cells indicate no change; np indicates not performed

In conclusion, our data suggest that sex-difference in 5-HT3 receptor mediated pain transmission is observed in SERT KO rats. In SERT KO female rats inhibition of spinal 5-HT3 receptors led to an increase in visceral sensitivity. On the other hand, spinal 5-HT3 receptor activation caused an increase in pain transmission in SERT KO males rats. This may be due to differential expression of 5-HT3 receptors in a sex-dependent manner. Functional and IHC data suggest that 5-HT3 receptors are predominantly expressed on inhibitory GABAergic neurons in SERT KO female rats and on central terminals of primary afferent neurons in SERT KO male rats.

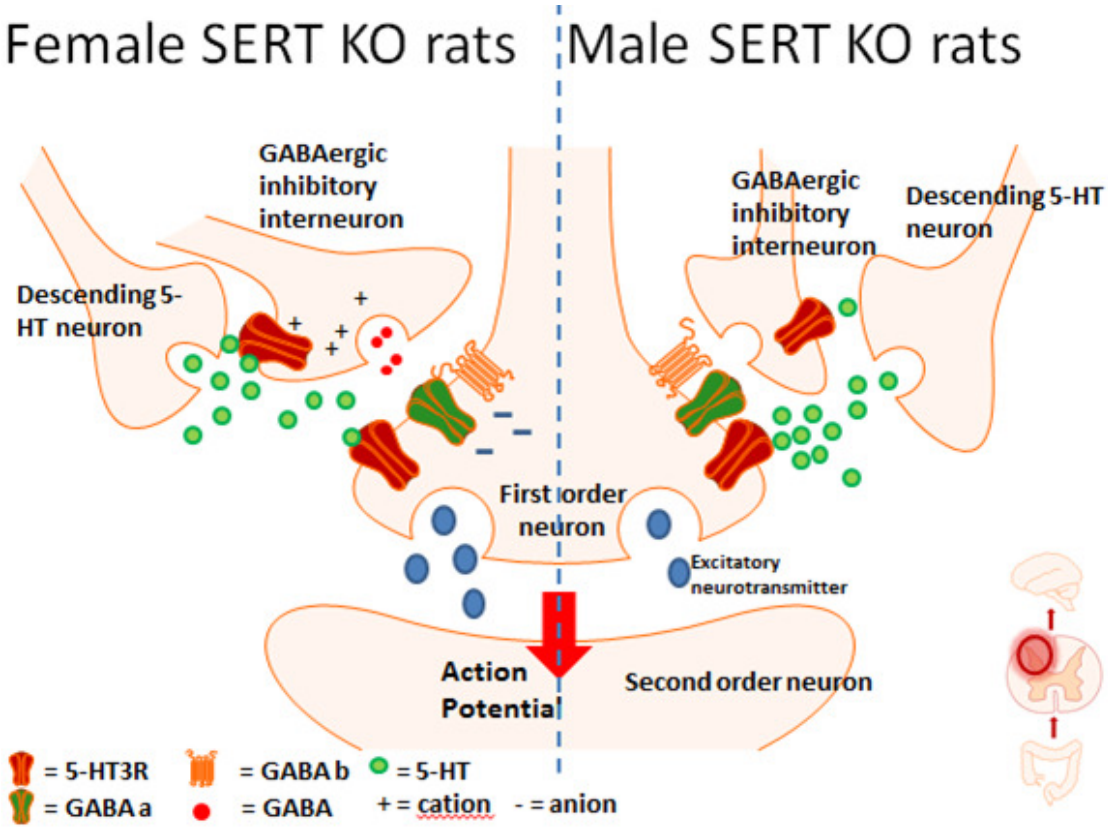

Figure 2.22 Differential 5-HT3 receptor expression in female and male SERT KO rats. 5-HT from descending serotonergic raphe nuclei acting on 5-HT3 receptors expressed on GABAergic interneurons causes the release of GABA to reduce pain transmission.

BIBLIOGRAPHY

BIBLIOGRAPHY

Acosta, A. and M. Camilleri (2015). "Pharmacogenetics in irritable bowel syndrome." Expert Opinion on Drug Metabolisim and Toxicology 11(8): 1187-1191.

Alhaider, A. A., S. Z. Lei, et al. (1991). "Spinal 5-HT3 receptor-mediated antinociception: possible release of GABA." Journal of Neuroscience 11(7): 1881-1888.

Banner, S. E. and G. J. Sanger (1995). "Differences between 5-HT3 receptor antagonists in modulation of visceral hypersensitivity." British Journal of Pharmacology 114(2): 558-562.

Barnes, N. M., T. G. Hales, et al. (2009). "The 5-HT3 receptor--the relationship between structure and function." Neuropharmacology 56(1): 273-284.

Beatty, J. K., A. Bhargava, et al. (2014). "Post-infectious irritable bowel syndrome: mechanistic insights into chronic disturbances following enteric infection." World Journal of Gastroenterology 20(14): 3976-3985.

Berman, S., J. Munakata, et al. (2000). "Gender differences in regional brain response to visceral pressure in IBS patients." European Journal of Pain 4(2): 157-172.

Bradesi, S., L. Lao, et al. (2007). "Dual role of 5-HT3 receptors in a rat model of delayed stress-induced visceral hyperalgesia." Pain 130(1-2): 56-65.

Browning, K. N. (2015). "Role of central vagal 5-HT3 receptors in gastrointestinal physiology and pathophysiology." Frontiers of Neuroscience 9(413).

Butler, A., J. M. Hill, et al. (1988). "Pharmacological properties of GR38032F, a novel antagonist at 5-HT3 receptors." British Journal of Pharmacology 94(2): 397-412.

Chang, L., V. Z. Ameen, et al. (2005). "A dose-ranging, phase II study of the efficacy and safety of alosetron in men with diarrhea-predominant IBS." American Journal of Gastroenterology 100(1): 115-123.

Chen, L., S. J. Ilham, et al. (2017). "Pharmacological Approach for Managing Pain in Irritable Bowel Syndrome: A Review Article." Anesthesiology and Pain Medicine 7(2).

Cheung, C. K. and J. C. Wu (2014). "Genetic polymorphism in pathogenesis of irritable bowel syndrome." World J Gastroenterology 20(47): 17693-17698.

Chey, W. D., J. Kurlander, et al. (2015). "Irritable bowel syndrome: a clinical review." Journal of Ameican Medical Association 313(9): 949-958.

Choi, Y. D., T. S. Sung, et al. (2008). "Increased 5-hydroxytryptamine mediates post-inflammatory visceral hypersensitivity via the 5-hydroxytryptamine 3 receptor in rats." Digestive Disease and Sciences 53(11): 2909-2916.

Clayton, N. M., R. Sargent, et al. (1999). "The pharmacological properties of the novel selective 5-HT3 receptor antagonist, alosetron, and its effects on normal and perturbed small intestinal transit in the fasted rat." Neurogastroenterology and Motility 11(3): 207-217.

Coelho, A. M., J. Fioramonti, et al. (1998). "Mast cell degranulation induces delayed rectal allodynia in rats: role of histamine and 5-HT." Digestive Disease and Sciences 43(4): 727-737.

Cremonini, F., J. P. Nicandro, et al. (2012). "Randomised clinical trial: alosetron improves quality of life and reduces restriction of daily activities in women with severe diarrhoea-predominant IBS." Alimentary Pharmacology Ther 36(5): 437-448.

Garsed, K., J. Chernova, et al. (2014). "A randomised trial of ondansetron for the treatment of irritable bowel syndrome with diarrhoea." Gut 63(10): 1617-1625.

Glatzle, J., C. Sternini, et al. (2002). "Expression of 5-HT3 receptors in the rat gastrointestinal tract." Gastroenterology 123(1): 217-226.

Glaum, S. R., H. K. Proudfit, et al. (1990). "5-HT3 receptors modulate spinal nociceptive reflexes." Brain Research 510(1): 12-16.

Goldberg, P. A., M. A. Kamm, et al. (1996). "Modification of visceral sensitivity and pain in irritable bowel syndrome by 5-HT3 antagonism (ondansetron)." Digestion 57(6): 478-483.

Greenwood-Van Meerveld, B., E. Mohammadi, et al. (2014). "Synergistic effect of 5-hydroxytryptamine 3 and neurokinin 1 receptor antagonism in rodent models of somatic and visceral pain." Journal of Pharmacology and Experimental Therapeutics 351(1): 146-152.

Gregersen, H. and G. Kassab (1996). "Biomechanics of the gastrointestinal tract." Neurogastroenterology and Motility 8(4): 277-297.

Grover, M. and M. Camilleri Ramosetron in irritable bowel syndrome with diarrhea: new hope or the same old story?, Clinical Gastroenterology and Hepatology. 2014 Jun;12(6):960-2. doi: 10.1016/j.cgh.2013.12.025. Epub 2014 Jan 3.

Grzesiak, M., J. A. Beszlej, et al. (2014). "The lifetime prevalence of anxiety disorders among patients with irritable bowel syndrome." Advanced Clinical Experimental Medicine 23(6): 987-992.

Gu, Q. Y., J. Zhang, et al. (2015). "Association of genetic polymorphisms in HTR3A and HTR3E with diarrhea predominant irritable bowel syndrome." International Journal of Clinical and Experimental Medicine 8(3): 4581-4585.

Hall, F. S., J. M. Schwarzbaum, et al. (2011). "A greater role for the norepinephrine transporter than the serotonin transporter in murine nociception." Neuroscience 175: 315-327.

Hall, J. D., C. DeWitte, et al. (2015). "Serotonin enhances urinary bladder nociceptive processing via a 5-HT3 receptor mechanism." Neuroscience Letters 604: 97-102.

Jensen, A. A., P. A. Davies, et al. (2008). "3B but which 3B and that's just one of the questions: the heterogeneity of human 5-HT3 receptors." Trends in Pharmacological Sciences 29(9): 437-444.

Kapeller, J., L. A. Houghton, et al. (2008). "First evidence for an association of a functional variant in the microRNA-510 target site of the serotonin receptor-type 3E gene with diarrhea predominant irritable bowel syndrome." Human Molecular Genetics 17(19): 2967-2977.

Kawamata, T., K. Omote, et al. (2003). "The activation of 5-HT(3) receptors evokes GABA release in the spinal cord." Brain Research 978(1-2): 250-255.

Koch, K. M., B. W. Corrigan, et al. (2004). "Alosetron repeat dose pharmacokinetics, effects on enzyme activities, and influence of demographic factors." Alimentary Pharmacology and Therapeutics 20(2): 223-230.

Kovac, A. L. (2016). "Comparative Pharmacology and Guide to the Use of the Serotonin 5-HT3 Receptor Antagonists for Postoperative Nausea and Vomiting." Drugs 76(18): 1719-1735.

Langlois, A., X. Pascaud, et al. (1996). "Response heterogeneity of 5-HT3 receptor antagonists in a rat visceral hypersensitivity model." European Journal of Pharmacology 318(1): 141-144.

Lummis, S. C. (2012). "5-HT(3) receptors." Journal of Biological Chemistry 287(48): 40239-40245.

Matsumoto, K., M. W. Lo, et al. (2012). "Experimental colitis alters expression of 5-HT receptors and transient receptor potential vanilloid 1 leading to visceral hypersensitivity in mice."

Laboratory Investigation 92(5): 769-782.

Mayer, E. A., S. Berman, et al. (2002). "The effect of the 5-HT3 receptor antagonist, alosetron, on brain responses to visceral stimulation in irritable bowel syndrome patients." Alimentary Pharmacology and Therapeutics 16(7): 1357-1366.

Miquel, M. C., M. B. Emerit, et al. (1995). "Developmental changes in the differential expression of two serotonin 5-HT3 receptor splice variants in the rat." Journal of Neurochemistry 65(2): 475-483.

Miranda, A., S. Peles, et al. (2006). "Effects of the 5-HT3 receptor antagonist, alosetron, in a rat model of somatic and visceral hyperalgesia." Pain 126(1-3): 54-63.

Morales, M., E. Battenberg, et al. (1998). "Distribution of neurons expressing immunoreactivity for the 5HT3 receptor subtype in the rat brain and spinal cord." Journal of Comparative Neurology 402(3): 385-401.

Morales, M. and S. D. Wang (2002). "Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system." Journal of Neuroscience 22(15): 6732-6741.

Naliboff, B. D., S. Berman, et al. (2003). "Sex-related differences in IBS patients: central processing of visceral stimuli." Gastroenterology 124(7): 1738-1747.

Oyama, T., M. Ueda, et al. (1996). "Dual effect of serotonin on formalin-induced nociception in the rat spinal cord." Neuroscience Research 25(2): 129-135.

Rahimi, R., S. Nikfar, et al. (2008). "Efficacy and tolerability of alosetron for the treatment of irritable bowel syndrome in women and men: a meta-analysis of eight randomized, placebo-controlled, 12-week trials." Clinical Therapeutics 30(5): 884-901.

Saria, A., F. Javorsky, et al. (1990). "5-HT3 receptor antagonists inhibit sensory neuropeptide release from the rat spinal cord." Neuroreport 1(2): 104-106.

Sawynok, J. and A. Reid (1996). "Interactions of descending serotonergic systems with other neurotransmitters in the modulation of nociception." Behavioral Brain Research 73(1-2): 63-68.

Simino, G. P., L. P. Marra, et al. (2016). "Efficacy, safety and effectiveness of ondansetron compared to other serotonin-3 receptor antagonists (5-HT3RAs) used to control chemotherapy-induced nausea and vomiting: systematic review and meta-analysis." Expert Review of Clinical Pharmacology 9(9): 1183-1194.

Song, Z., B. A. Meyerson, et al. (2011). "Spinal 5-HT receptors that contribute to the pain-relieving effects of spinal cord stimulation in a rat model of neuropathy." Pain 152(7): 1666-1673.

Thompson, A. J., M. H. Verheij, et al. (2013). "Structure-activity relationships of quinoxaline-based 5-HT3A and 5-HT3AB receptor-selective ligands." ChemMedChem 8(6): 946-955.

Vogel, C., R. Mossner, et al. (2003). "Absence of thermal hyperalgesia in serotonin transporter-deficient mice." Journal of Neuroscience 23(2): 708-715.

Wolf, H. (2000). "Preclinical and clinical pharmacology of the 5-HT3 receptor antagonists." Scandinavian Journal Rheumatology Suppl 113: 37-45.

Yamamoto, C., H. Murakami, et al. (2002). "Contribution of P-glycoprotein to efflux of ramosetron, a 5-HT3 receptor antagonist, across the blood-brain barrier." Journal of Pharmacy and Pharmacology 54(8): 1055-1063.

Yoshimura, M. (2006). "Plastic changes of the descending serotonergic system in the spinal dorsal horn." Current Anaesthesia & Critical Care 17(1): 3-8.

Zeitz, K. P., N. Guy, et al. (2002). "The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors." Journal of Neuroscience 22(3): 1010-1019.

Chapter 3

Classical estrogen receptors play an antinociceptive role in SERT KO female rats

3.1 Abstract

Serotonin transporter knockout (SERT KO) female rats exhibit an increase in visceromotor response (VMR) to colorectal distension (CRD) compared to wild type (WT) female rats. This recapitulates clinical studies that show female IBS-D patients carrying the s/s polymorphism in the SERT gene experience more severe abdominal pain. Studies performed in this chapter investigated the effects of fluctuating ovarian hormone levels during the estrous cycle on VMR to CRD. Ovariectomy and estrogen receptor (ER) antagonist studies were also performed in SERT KO and WT female rats. VMR to CRD was enhanced in the proestrus phase of the estrous cycle in SERT KO but not WT female rats. Ovariectomy increased discomfort to CRD in SERT KO female rats in a time dependent manner. VMR to CRD increased on day 7 post-ovariectomy and lasted for up to 3 weeks. Intrathecal administration of ICI 182 780 (ER-α and ER-β) antagonist increased VMR to CRD in SERT KO female rats. The G-protein coupled ER (GPR30) antagonist, G15, did not affect VMR to CRD in SERT KO and WT female rats. These studies suggest that in SERT KO female rats classical ERs (ERα and ERβ) play an antinociceptive role in the presence of serotonergic dysfunction.

3.2 Introduction

Irritable bowel syndrome (IBS) is considered a women's health issue since it is more prevalent in females compares to males. Questionnaire based and epidemiological studies show a 2:1 and 4:1 female IBS predominance, respectively (Meleine and Matricon 2014; Mulak, Tache et al. 2014; Harris, Umar et al. 2016). The motility disorder in IBS also exhibits a sex difference. Female IBS patients are more likely to suffer from constipation while males have more diarrhea (Adeyemo, Spiegel et al. 2010). Furthermore, women experience more abdominal pain and as a result have lower IBS quality of life scores compared to males. (Cain, Headstrom et al. 2006; Choghakhori, Abbasnezhad et al. 2017). In addition to the abdominal pain, female IBS patients often present with comorbid chronic pain disorders such as fibromyalgia and chronic pelvic pain (Meleine and Matricon 2014).

Most female IBS patients are of childbearing age. In fact the prevalence of IBS begins to decrease by the 4^{th} decade of life and equals that of males by the 7^{th} decade (Heitkemper and Chang 2009). Studies were performed to determine the influence of menstrual cycle, oral contraceptive use, pregnancy and menopause on IBS symptoms. Unfortunately, no conclusions were reached from these studies due to variation in study design. In pre-menses and menses when ovarian hormone levels reach their nadir, IBS symptoms worsen (Heitkemper and Chang 2009; Mulak, Tache et al. 2014; Harris, Umar et al. 2016). Balloon rectal distension studies showed that in IBS patients but not healthy controls rectal sensitivity is enhanced during menses. In addition to the enhanced rectal sensitivity these patients also reported increased abdominal pain and bloating during menses (Houghton, Lea et al. 2002).

Ovarian hormones can influence pain by affecting gut motility, permeability and the stress response. It has been shown that patients with IBS have increased gut permeability which correlates with symptom severity and that this is mediated via an estrogen dependent mechanism (Harris, Umar et al. 2016). Unlike male IBS patients, female IBS patients exhibit decreased activity of brain regions that mediate the hypothalamic-pituitary-axis response and increased activation of the limbic system in response to colorectal distension (Naliboff, Berman et al. 2003). These data suggest that there are sex differences in central processing of abdominal pain. Ovarian hormone effects on motility are more complex. Studies suggest that estrogen slows gut motility by upregulating nitric oxide signaling in the GI tract of pregnant women and progesterone increases SERT expression to decrease extracellular 5-HT to slow gut motility (Meleine and Matricon 2014).

While experiments done in rodents corroborate the clinical observation that females exhibit higher pain response compared to males, the mechanisms by which this occurs remain unclear. Like humans, rodents also exhibit fluctuations in their response to CRD across different phases of the estrous cycle. An artificial stone implanted in the ureter of rats resulted in increased pain behaviors in the metestrus and diesterus stages of the estrous cycle. Similarly, noxious stimulation of the uterus increased escape behaviors during the metestrus and diestrus stages of the estrous cycle (Giamberardino, Affaitati et al. 1997; Bradshaw, Temple et al. 1999). On the other hand, visceral hypersensitivity to colorectal distension in rats is the highest during proestrus phase (Ji, Tang et al. 2008) or proestrus and diestrus (Gustafsson and Greenwood-Van Meerveld 2011; Galligan, Patel et al. 2013; Moloney, Sajjad et al. 2016).

There is strong evidence from the studies discussed herein that estrogen plays an important role in pain modulation. However, the mechanisms by which estrogen modulates nociceptive signal transmission are poorly understood. Estrogen signaling is mediated via steroidal estrogen receptors (ER) and membrane-bound G-protein coupled estrogen receptors (mER). The steroidal estrogen receptors ERα and ERβ mediate classical estrogen signaling which affects protein expression. On the other hand, mERs mediate rapid estrogen receptor signaling and include GPR30, ERα, ERβ, ER-αΔ4, ER-X and mER-Gαq (Wierman 2007; Sinchak and Wagner 2012). In addition to rapid signaling via activation of various protein kinases, m-ERs also affect gene expression via downstream cyclic adenosine monophosphate (cAMP) and response element protein (pCREP)(Wierman 2007).

Similar to the strong evidence linking estrogen to pain modulation, it is clear that an interaction between estrogen and 5-HT also plays a role in mediating pain. Most of the clinical studies investigating the link between the two signaling systems aim at understanding their role in headaches. Data from a study investigating the pathogenesis of migraines suggests that estrogen directly affects serotonergic signaling in the brain by upregulating tryptophan hydroxylase 1 (TPH1) expression and down regulating SERT expression (Gupta, McCarson et al. 2011). Other studies suggest a direct effect of estrogen on 5-HT1 and 5-HT2 receptors in mediating depression, osteoporosis and blood clotting disorders (Rybaczyk, Bashaw et al. 2005). Few experiments focus on signaling mechanisms that link 5-HT and ovarian hormones in the pathogenesis of abdominal pain. Male and female IBS-diarrhea patients have higher fasting and postprandial platelet-depleted plasma 5-HT levels compared to healthy controls. Furthermore female IBS-diarrhea patients have higher 5-HT plasma levels during the luteal

phase when progesterone and estrogen levels are high compared to female IBS-diarrhea patients during menses where ovarian hormone levels are low (Houghton, Brown et al. 2009). This study suggests that 5-HT metabolism differs across phases of the menstrual cycle. Together the data from this study and the clinical observation that 5-HT3 receptor inhibition reduces abdominal pain in females strongly suggests that an interaction between ovarian hormones and 5-HT signaling contribute to the pathogenesis of IBS.

Galligan et al, 2013 showed that the SERT KO female rat exhibits increased visceral hypersensitivity compared to WT female rats. SERT KO and WT male rats have similar VMR to CRD. Furthermore, the increase is visceral sensitivity in SERT KO female rats is estrous cycle depend since VMR to CRD is enhances in the proestrus and estrus phase. In this chapter I will test the hypothesis that ovarian hormones cause the increase in visceral hypersensitivity in SERT KO rat by exerting their effects on spinal estrogen receptors specifically.

3.3 Methods

3.3.1 Animals

All animal use protocols were approved by the Institution Animal Use and Care Committee at Michigan State University. The serotonin transporter (SERT) knockout (KO) and wild type (WT) control rats were purchased under license from Genoway, Inc (http://genoway.com/). The WT rats used in this study and the background of SERT KO rats are the Wistar strain. Following surgery all animals were singly housed to prevent post-surgical complications. Age matched 3-4 months old rats were used for all experiments described in this chapter.

3.3.2 Surgery

Electromyographic (EMG) electrode implantation. Rats were deeply anaesthetized with 4% isoflurane gas and placed on a heating pad (~30^{o}C). A midscapular incision was made followed by a ventral midline incision. A hemostat was used to gently separate the skin from the muscles. Teflon coated silver wires (A-M systems, Sequim, WA) were stitched into the external oblique muscles above the inguinal ligaments and the incision was closed with wound clips. The electrodes were tunneled subcutaneously, secured to the back musculature and eternalized in the midscapular region for future access. Sutures were used to close the midscapular incision. Rats were allowed to recover for 3 days before assessing visceromotor response to colorectal distension. During recovery the rats were acclimatized to a plexiglass rat restrainer for an hour a day over two consecutive days. Carprofen (5mg/kg, s.c.) and piperacillin/tazobactam (120mg/kg, i.m.) were administered at the time of surgery to control pain and reduce risk of infection respectively.

Ovariectomy. Ovariectomies or sham operation of rats (n=48, 24 WT and 24 SERT KO rats) were performed under isoflurane anesthesia (4%) and aseptic conditions. Surgical sites were scrubbed with chlorhexidine and alcohol wipes. Bilateral flank incisions 5 mm in length were made through the skin and then muscle wall. The ovaries were pulled through the incisions, and the ovarian artery and vein were tied off tightly with suture silk before cutting off the ovaries. The muscle wall incision was closed with suture silk and the skin incision was closed with wound clips. Uterine weight was recorded at the time of euthanasia to confirm ovariectomy.

3.3.3 Visceromotor response (VMR) to colorectal distension (CRD)

Under light Isoflurane anesthesia, a flexible latex balloon (4.5 cm, females; 7 cm, males) was inserted intra-anally into the colon and secured to the base of the tail using surgical tape. After recovery from anesthesia, the animals were placed in the plexiglass restrainer and allowed to acclimate for 30 minutes before the initiation of the colorectal distension procedure. The balloon was connected to an animal barostat (G&J electronics, Toronto, Ontario, Canada) and the electrode leads were connected to amplifier (7P1, Grass-Instruments-Astro-Med Inc., West Warwick, RI, USA). The barostat and amplifier were connected to an A/D converter amplifier (Digidata 1223A, Axon Instruments-Molecular Devices, Sunnyvale, CA, USA) and signals digitized at 1KHz and recorded using Axoscope 10 software (Axon-instruments-Molecular Devices).

The VMR to CRD was assessed before and after treatment with the drug being studied. Each CRD procedure consisted of a series of 5 phasic distensions at pressures of 10, 20, 40, 60, 80 mmHg (10-s duration; 5-min interval between distension). The VMR to CRD was measured by recording electromyographic (EMG) activity of the external oblique muscles 10 s before, 10 s during, and 10 s after each distension episode. The EMG activity was analyzed using Clampfit 10 (Axon-instruments-Molecular Devices). The signal was rectified and the area under the curve calculated and normalized to baseline which is measured 10-s before each distension episode. The data is reported as normalized VMR.

3.3.4 Intrathecal injection

Rats were lightly anesthetized with 4% isoflurane, the hair along the dorsal aspect of the lower spine was clipped. The L5-L6 intervertebral space was exaggerated by placing a 50 mL centrifuge tube under the lower limbs. A 27 G ½ inch needle connected to a 50 µL glass syringe was gently inserted into the vertebral canal. Tail flick confirmed entry of the needle into the vertebral canal. Drugs were dissolved in 5-10 µL solvent and administered using a 50 µL glass syringe to minimize back flow of CSF upon removal of the needle following injection.

3.3.5 Vaginal lavage and cytology

The vaginal canal was flushed with a few drops of sterile saline 2-3 times. The resulting solution was placed on a glass slide and visualized at 20X magnification to determine the phase of the estrous cycle.

3.3.6 Statistics

Electromyographic recordings of the visceromotor response to colorectal distension were analyzed using two way-repeated measures ANOVA and Bonferonni's post hoc test to determine the effects of drugs on VMR at each distending pressure. We used 6-8 animals per group to achieve a power of 0.8 unless specified otherwise for a given experiment. Data will be presented as mean ± SEM. P-values less than 0.05 were considered statistically significant.

3.3.7 Time-line of studies performed

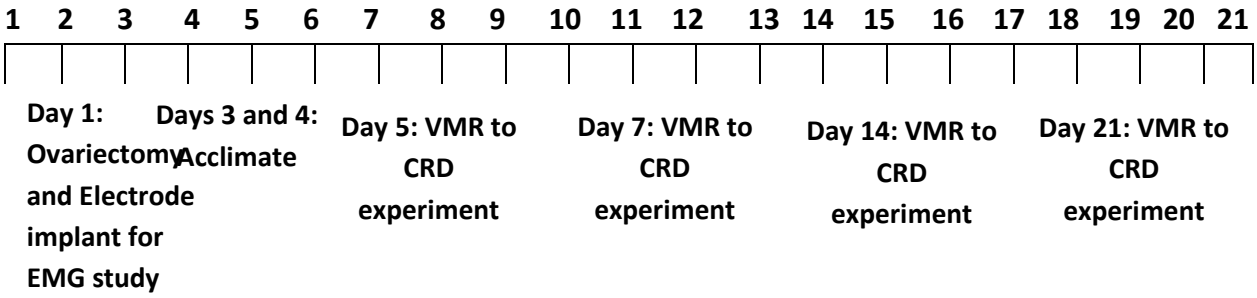

Figure 3.1 Timeline of ovariectomy studies. On day 1 rats underwent ovariectomy or sham ovariectomy and electrode implant. Following a 2 day recovery, rats were acclimated to the restrainer on days 3 and 4 for one hour each day. On days 5, 7, 14 and 21 VMR to CRD experiments were performed and the rats were euthanized at the end of the last experiment.

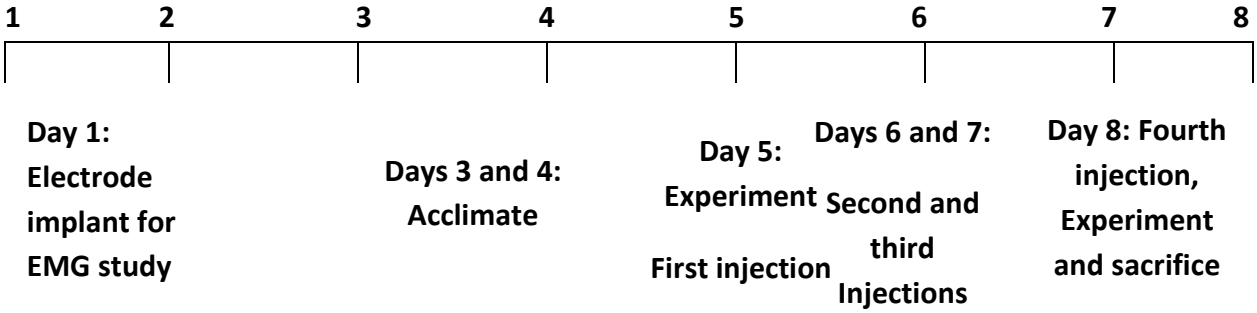

Figure 3.2 Estrogen receptor antagonist experiment timeline. Electrode implants were performed on day 1. On days 3 and 4 rats were acclimated to the restrainer. On day 5 VMR to CRD was performed before and 30 minutes after the first injection of ER antagonist (ICI 182 780, 100 nM/10 µL, i.t) or G15 10ng/10 µL, i.t.) or vehicle (20% (2-Hydroxypropyl)-β-cyclodextrin). Injections were repeated on days 6 and 7. On day 8 animals received final injection and VMR to CRD experiments were performed. At the end of the experiment the animals were euthanized for tissue collection.

3.4 Results

Female SERT KO rats exhibit an enhanced VMR to CRD compared to WT female rats. On the other hand, SERT KO and WT male rats do not demonstrate a difference in their response to CRD (Figure 3.3). The discomfort in response to CRD is dependent on the estrus cycle as shown in Figure 3.4. SERT KO female rats experience increased VMR to CRD compared to WT female rats in the proestrus phase of estrous cycle.

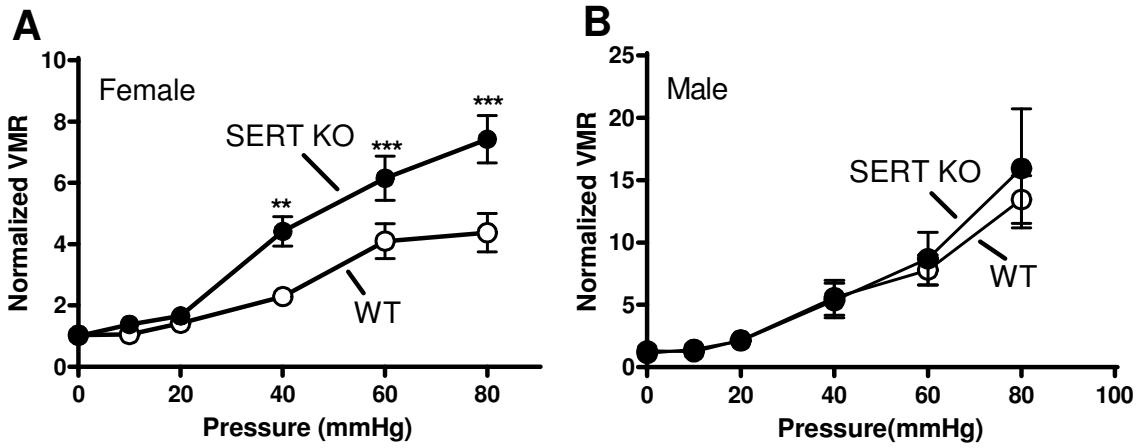

Figure 3.3 SERT KO rats exhibit a sex dependent increase in VMR to CRD. Female SERT KO rats exhibit an increase in VMR to CRD compared to WT female rats (n=7) (A). There is no difference in visceral sensitivity to CRD between SERT KO and WT male rats (n=7) (B). **, *** indicates significantly different than control (** P<0.01), (***P<0.001) (F). Data are mean ± SEM.

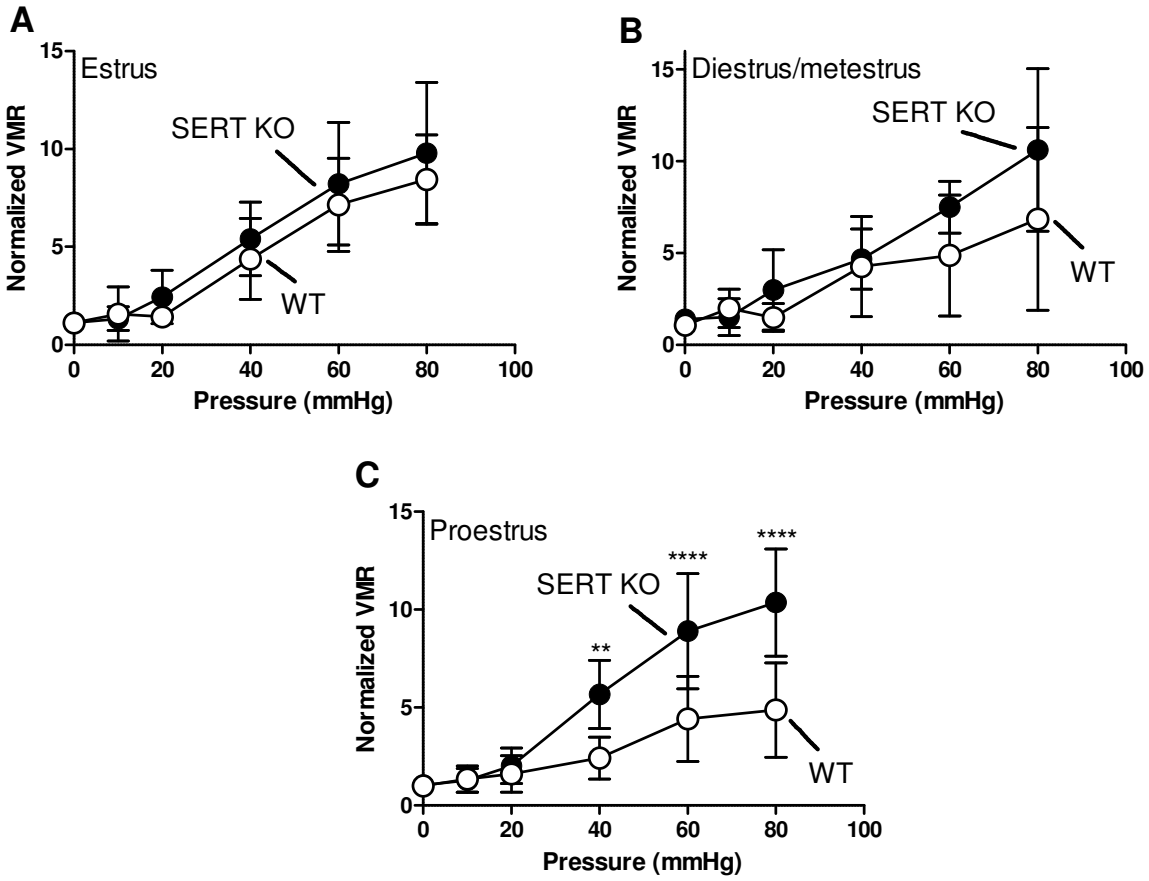

Figure 3.4 The increase VMR to CRD is estrous cycle dependent. In the estrus and diestrus/metestrus phase of the estrous cycle VMR to CRD did not differ between SERT KO and WT female rats(n=9) (A,B). In the proestrus phase SERT KO female rats exhibit increased VMR to CRS compared to WT female rats (n-=8) (C). **, *** indicates significantly different than control (** P<0.01), (***P<0.001) (F). Data are mean ± SEM.

Ovariectomy further enhanced VMR to CRD in SERT KO female rats but not WT female rats. The increase in VMR to CRD occurred 7 days post-ovariectomy and persisted for up to 3 weeks as shown in Figure 3.5. On the other hand, ovariectomy did not affect VMR to CRD in WT female rats (Figure 3.6). Sham WT female rats were unable to be maintained for 3 weeks since animals damaged their electrodes or aggravated the back incision where the electrodes were exteriorized.

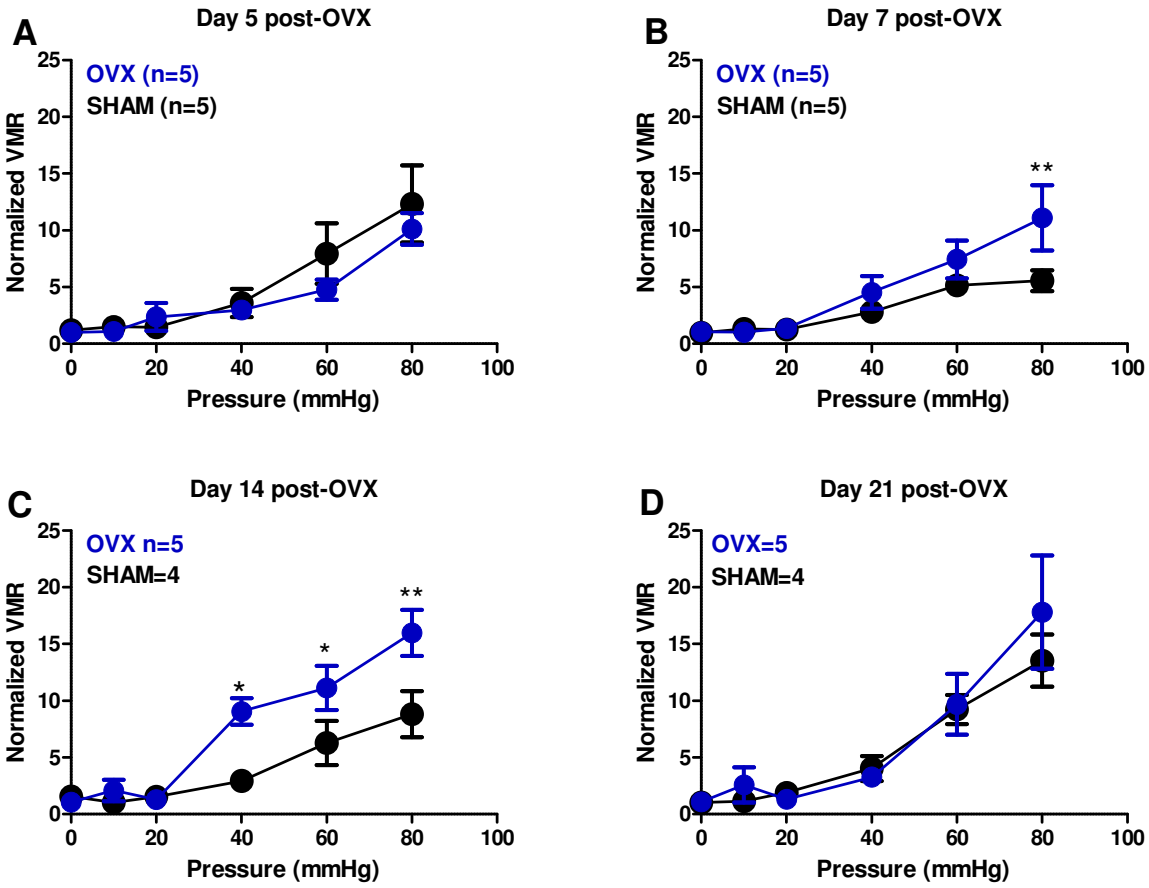

Figure 3.5 Ovariectomy increased VMR to CRD in SERT KO female rats in a time dependent manner. Ovariectomy caused an increase in VMR to CRD in SERT KO female rats compared to sham SERT KO female rats on days 7 and 14 post-ovariectomy (B –C). VMR to CRD was not different between ovariectomized and sham rats on days 5 and 21 (A, D). *, ** indicates significantly different than control (* P<0.05), (**P<0.01) (F). Data are mean ± SEM. OVX: ovariectomy.

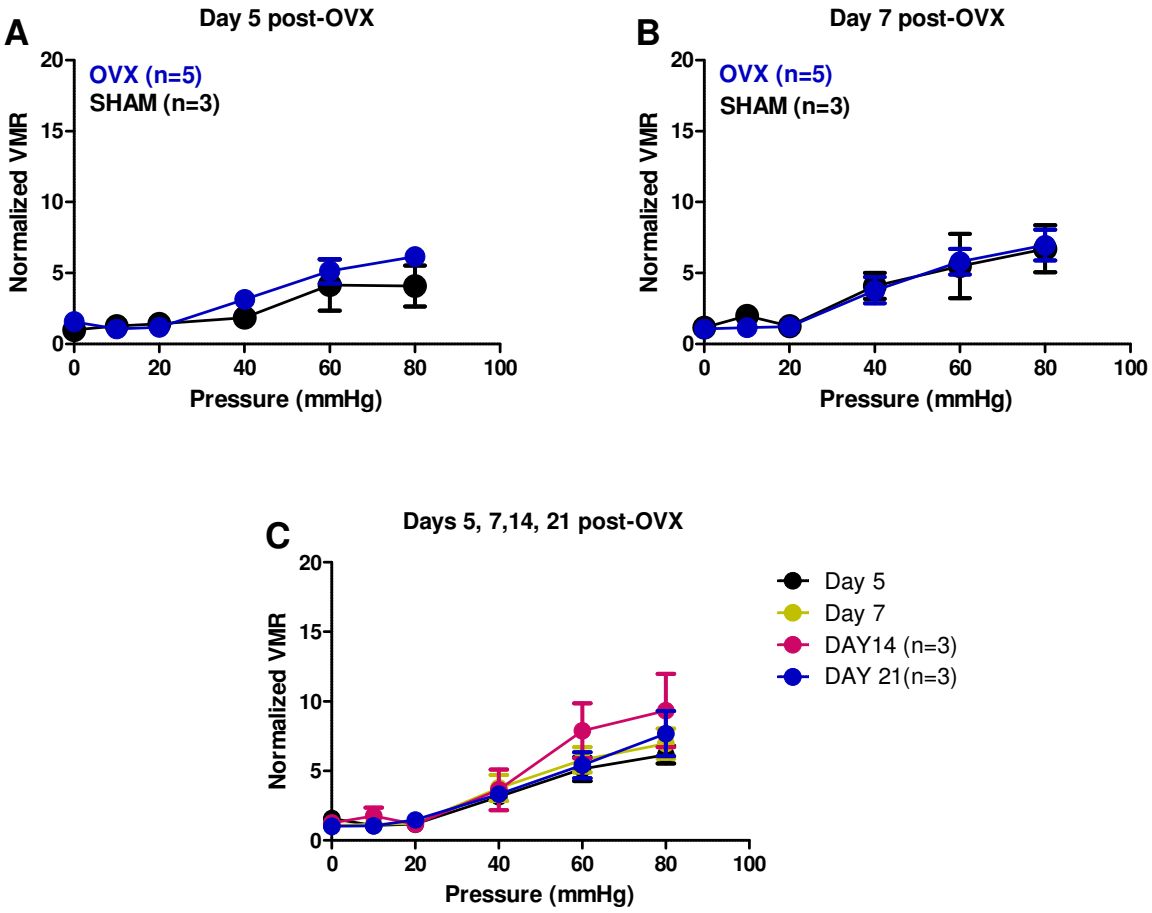

Figure 3.6 Ovariectomy did not affect VMR to CRD in WT female rats. VMR to CRD did not differ between ovariectomized and sham operated WT female rats on days 5 and 7 pots-ovariectomy (A,B). VMR to CRD did not differ among ovariectomized rats on day 5, 7, 14 and 21. Data are mean ± SEM. OVX: ovariectomy.

Intrathecal administration of the steroidal estrogen receptor antagonist ICI 182 780 (100 nM/10µL) caused an increase in VMR to CRD in SERT KO female rats one hour after the first injection. The increase in VMR to CRD was maintained following the fourth injection as shown in Figure 3.7. On the other hand, the mER receptor antagonist G15 did not affect VMR to CRD in SERT KO and WT female rats.

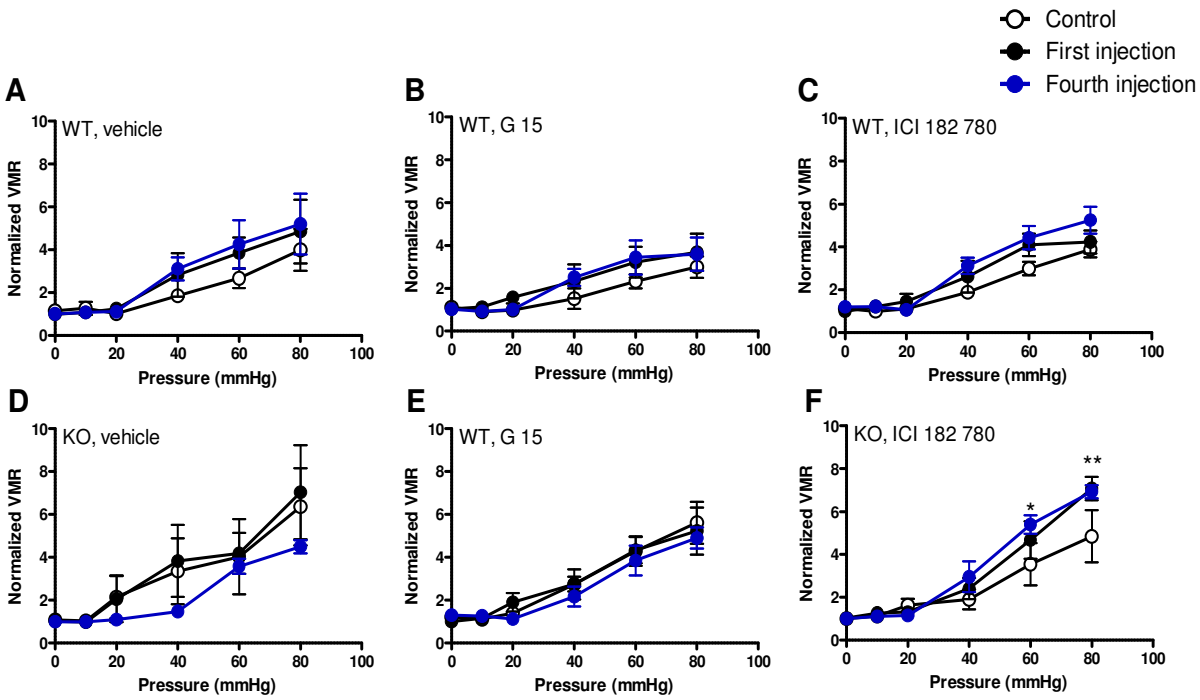

Figure 3.7 The effect of intrathecal administration of ER receptor antagonists. The vehicle and GPR30 antagonist G15 (10 ng/ 10µL, i.t.) did not affect VMR to CRD in SERT KO and WT female rats (A, B, E, D). The steroidal estrogen receptor antagonist ICI 182780 (100 nM/ 10 µL, i.t.) did not affect VMR to CRD in WT female rats (E) but increased VMR to CRD in SERT KO female rats following the first injection and fourth injection (F). *, ** indicates significantly different than control (* P<0.05), (**P<0.01) (F). Data are mean ± SEM.

3.5 Discussion

The SERT KO female rat exhibits increased visceral sensitivity to CRD compared to WT female rats. This recapitulates the clinical observation that female IBS patients with s/s allele of the SERT gene experience severe abdominal pain compared to IBS patients expressing s/l and l/l allele (park, choi et al. 2006). Fluctuations in ovarian hormone levels occurring during the estrous cycle affect the level of discomfort experienced during CRD. In intact Sprague-Dawley rats VMR to CRD was highest during the proestrus phase of the estrous cycle (Ji, Tang et al. 2008). Similarly, Moloney et al, 2016 showed that the Sprague-Dawley female rat exhibited increased pain behaviors during CRD in the proestrus and estrus phase of estrus cycle.

However, this response is lost in rats subjected to maternal separation stress as they exhibit increased pain behaviors in all phases of the estrus cycle (Moloney, Sajjad et al. 2016). Galligan et al, 2013 showed that the VMR to CRD is enhanced in the proestrus and estrus phase of the estrous cycle in SERT KO female rats. However, in my hands I was able to show an increased in pain response only in the proestrus phase of the estrous cycle as shown in Figure 3.4. Most studies equate the proestrus cycle with high estrogen levels and the rapid change in estrogen levels is often overlooked (Goldman, Murr et al. 2007). Estrogen levels begin to decline at in the early afternoon in the proestrus stage. Therefore, the fluctuation in estrogen levels might contribute to the increase in VMR and pain behaviors in response to CRD (Figure 3.8). Studies discussed herein, similar to my studies, were performed between 9am-2pm. Therefore the declining levels of estrogen in early afternoon might contribute to the increase in discomfort. Clinically, abdominal pain in IBS patients is exaggerated in pre-menses and menses when estrogen levels drop to reach their nadir (Houghton, Lea et al. 2002; Heitkemper and Chang 2009). Experts in the field suggest that the fluctuations in ovarian hormones levels leads to the increase in pain observed in peri-menses and menses.

The ovaries produce both estrogen and progesterone. While many studies investigated the role of estrogen which will be discussed later, the role of progesterone in mediating visceral pain has not been adequately investigated. Thus, its role in mediating pain is equivocal. In one study progesterone administration into the amygdala of ovariectomized rats caused an increases in abdominal contraction in response to VMR (Myers, Schulkin et al. 2011). On the other hand, progesterone reduced estrogen induced visceral hypersensitivity in ovariectomized rats treated with intracolonic mustard oil to induce inflammation (Ji, Tang et al. 2005). In

another study progesterone administration to ovariectomized rats did not affect VMR to CRD indicating that progesterone signaling in not important in pain modulation (Lu, Hsieh et al. 2009).

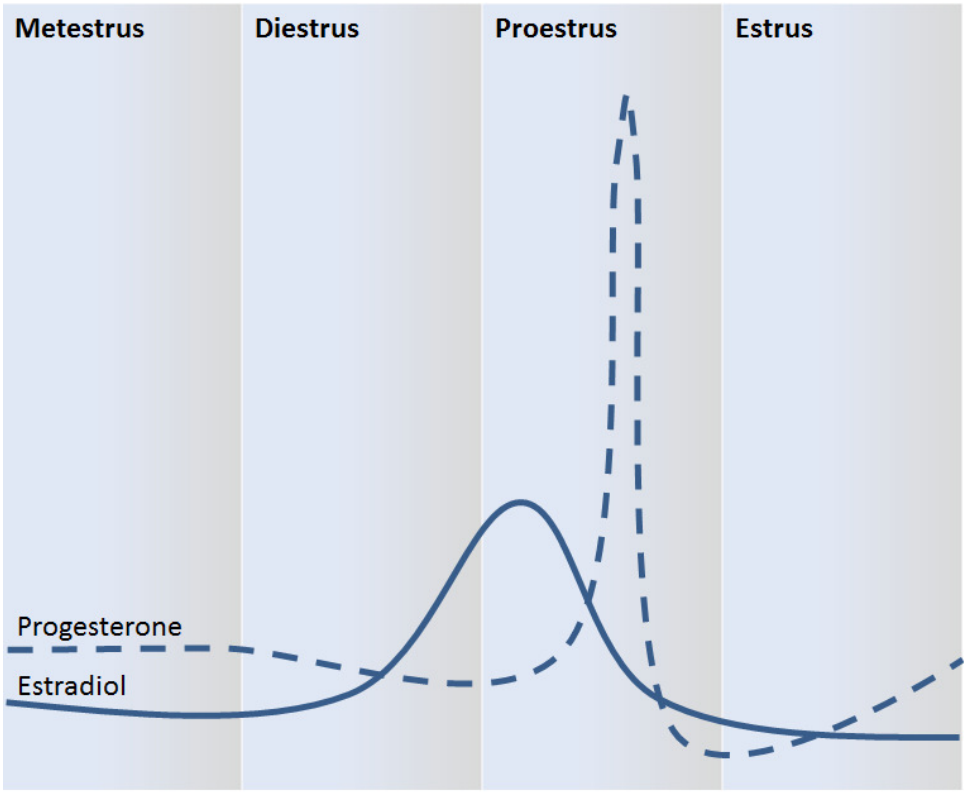

Figure 3.8 Fluctuation of ovarian hormones during the estrus cycle. Estrogen levels peak during the morning of the estrus cycle and begin to drop during the afternoon. While progesterone peaks in the early afternoon and rapidly drops before the evening hours. Modified from (Goldman, Murr et al. 2007).

As stated earlier the role of estrogen in mediating abdominal pain has been extensively studied. Ovariectomy is often used to study the effects of ovarian hormones in modulating pathogenesis of various diseases. Most ovariectomy experiments done in rats to study visceral pain mechanisms suggest that ovarian hormones are pronociceptive. For instance, ovariectomy decreased VMR to CRD in unsensitized and sensitized rats treated with either intracolonic

mustard-oil , maternal separation or subcutaneous 5-HTP injections (Ji, Murphy et al. 2003; Ji, Tang et al. 2005; Lu, Hsieh et al. 2009; Gustafsson and Greenwood-Van Meerveld 2011) . However, few studies suggest that estrogen signaling can be antinociceptive. A study performed in mice showed that ovariectomy increased pain behavior induced by intracolonic mustard-oil administration and was reversed by estrogen but not progesterone administration (Sanoja and Cervero 2010). Similarly, a study done in rats showed that ovariectomy decreased pain response to repeated 20 mmHg colorectal balloon distension and that this response was reversed with estrogen replacement. Similarly, electrophysiological recording from dorsal horn neurons showed that ovariectomy reduced firing of neurons in the dorsal horn that was reversed with estrogen replacement suggesting that estrogen modulates pain transmission at the level of the spinal cord (Ji, Murphy et al. 2003). In SERT KO rats ovariectomy enhanced VMR to CRD in a time dependent manner as shown in figure 3.5. Visceral hypersensitivity was increased between days 7-21 post-ovariectomy. This suggests that in presence of serotonergic dysfunction, ovarian hormones play an antinociceptive role. However the mechanism by which this occurs is unclear.

My 5-HT3 receptor antagonist studies discussed in Chapter 2 suggest that inhibition of spinal 5-HT3 receptors contributes to the enhanced VMR to CRD observed in SERT KO female rats. Similarly, studies focused on understanding the role of estrogen in mediating visceral pain also suggest that spinal estrogen receptors play an important role in pain transmission. In the rat lumbosacral dorsal root ganglia (DRG) neurons ER-α receptors are found in 17% of L6-S1 DRG neurons, ER-β receptors are found in 23% of L6-S1 DRG neurons and 5% of these neuron express both receptors (Papka and Storey-Workley 2002). Furthermore, it has been shown that

ER-α receptors are predominantly expressed in the superficial layers (lamina i and ii) of dorsal horn of the spinal cord. ER-β receptors are expressed in lamina iii and iv but not lamina i and ii were most primary afferent terminate (Papka, Storey-Workley et al. 2001; Papka and Storey-Workley 2002) (Vanderhorst, Gustafsson et al. 2005). Ji et al 2011 showed that treatment of ovariectomized rats with ER-α receptor agonist, propyl pyrazole triol (PPT), caused an increased in VMR to CRD. While treatment with steroidal ERs antagonist ICI 182 780 decreased VMR to CRD observed in ovariectomized rats treated with estrogen. Furthermore, they performed in-vivo electrophysiological recordings from dorsal root and dorsal horn neurons responsive to CRD and showed that activation of ER-α receptors increased neuronal firing of Abrupt neurons whose response to CRD is terminated after 4 seconds (Ji, Tang et al. 2011). Estrogen signaling at ER-β receptors is believed to be antinociceptive in animal models of visceral hypersensitivity. The ER-β receptor agonists diarylpropionitrile (DPN) and WAY-200070 reduced VMR to CRD in intact and ovariectomized rats when administered subcutaneously. DPN was also able to reduce VMR to CRD when administered intrathecally suggesting that spinal ER-β receptors are antinociceptive (Cao, Ji et al. 2012). The antinociceptive effects of ER-β agonist took 4 hours to occur, therefore the authors concluded that in their animal model genomic ER-β signaling is responsible for the reduction in VMR. Lu et al , 2009 showed that estrogen signaling at spinal GPR30 membrane bound estrogen receptor is pronociceptive in 5-HTP-induced visceral hypersensitivity animal model (Lu, Hsieh et al. 2009).

Downstream mechanisms that mediates estrogen signaling in visceral hypersensitivity include activation of neurokinin 1 (NK1) receptor and upregulation of cAMP signaling in the dorsal horn of the spinal cord (Bradesi, Eutamene et al. 2003; Lu, Hsieh et al. 2007). (Bradesi,

Eutamene et al. 2003). It has been shown that ERα receptor activation increased the activity of NMDA receptors via phosphorylation on NR1 subunit and up regulation of NMDA receptor expression in the lumbosacral dorsal horn to enhance VMR to CRD (Tang, Ji et al. 2008). Furthermore, ER-α and ER-β KO mice exhibit an increase in P2X3 and TRPV1 receptors in lumbosacral DRG neurons indicating that estrogen might play an antinociceptive role since both receptors are found on primary afferent neurons and mediate enhanced pain transmission (Cho and Chaban 2012).

Intrathecal administration of the steroidal estrogen receptor antagonist ICI 182780 caused an increase in VMR to CRD following the first and fourth injection. Inhibition of GPR30 via intrathecal administration of the GPR30 antagonist G15 did not affect VMR to CRD as shown in figure 3.7. These data suggest that inhibition of either ER-α or ER-β increases discomfort in SERT KO female rats similar to the increase in discomfort observed in ovariectomy studies. Since VMR to CRD is measured one hour following the first intrathecal injection non-genomic estrogen signaling at ER receptors is likely to be responsible for the increase in VMR to CRD. This response was maintained on the fourth day following treatment with the antagonist for 4 days.

Estrogen signaling can be mediated via a genomic response where cytoplasmic estrogen receptor dimerization followed by translocation to the nucleus is required to affect gene expression. This response required multiple hours to occur (McEwen and Alves 1999; Marino, Galluzzo et al. 2006). Estrogen signaling at ERα and ERβ receptors can also occur via rapid non-genomic signaling. In fact, the ER-α agonist PPT exerts it effects 15 minutes following drug

administration. The exact mechanism by which activation leads to increase in pain appears to be mediated via MAPK activation in this case. (Amandusson, Hallbeck et al. 1999) (Ji, Tang et al. 2011).

In conclusion, SERT KO female rats exhibit an increase in VMR to CRD compared to WT female rats. This increase in discomfort occurs in the proestrus phase of the estrus cycle. Ovariectomy increased VMR to CRD in SERT KO female rats which developed 7 days post-ovariectomy and lasted for up to 3 weeks. Similarly, inhibition of spinal ER-α and ER-β estrogen receptors with ICI 1822 780 enhanced discomfort in SERT KO female rats. However, the inhibition of GPR30 did not affect VMR to CRD. This suggests that the spinal ER-α and/or ER-β signaling in the spinal cord is antinociceptive. ER-α is expressed on inhibitory enkephalin interneurons in the spinal cord (Amandusson, Hermanson et al. 1996; Amandusson, Hallbeck et al. 1999). Therefore, might mediate antinociception by activating inhibitory opioid neurons located in the dorsal horn of the spinal cord. Another study showed that activation of ER-β receptors increased the expression of TPH2 in dorsal raphe nuclei suggesting that estrogen signaling at ER-β receptors can increase inhibitory descending serotonergic input to the spinal cord. Furthermore, ER-δ and ER-β receptors have been colocalized to inhibitory GABAergic interneurons (Blurton-Jones and Tuszynski 2002). Thus, estrogen can exert its antinociceptive effect via GABAergic interneurons located in the spinal cord.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adeyemo, M. A., B. M. Spiegel, et al. (2010). "Meta-analysis: do irritable bowel syndrome symptoms vary between men and women?" Alimentary Pharmacology and Therapeutics 32(6): 738-755.

Amandusson, A., M. Hallbeck, et al. (1999). "Estrogen-induced alterations of spinal cord enkephalin gene expression." Pain 83(2): 243-248.

Amandusson, A., O. Hermanson, et al. (1996). "Colocalization of oestrogen receptor immunoreactivity and preproenkephalin mRNA expression to neurons in the superficial laminae of the spinal and medullary dorsal horn of rats." European Journal of Neuroscience 8(11): 2440-2445.

Blurton-Jones, M. and M. H. Tuszynski (2002). "Estrogen receptor-beta colocalizes extensively with parvalbumin-labeled inhibitory neurons in the cortex, amygdala, basal forebrain, and hippocampal formation of intact and ovariectomized adult rats." Journal of Comparative Neurology 452(3): 276-287.

Bradesi, S., H. Eutamene, et al. (2003). "Stress-induced visceral hypersensitivity in female rats is estrogen-dependent and involves tachykinin NK1 receptors." Pain 102(3): 227-234.

Bradshaw, H. B., J. L. Temple, et al. (1999). "Estrous variations in behavioral responses to vaginal and uterine distention in the rat." Pain 82(2): 187-197.

Cain, K. C., P. Headstrom, et al. (2006). "Abdominal pain impacts quality of life in women with irritable bowel syndrome." American Journal of Gastroenterology 101(1): 124-132.

Cao, D. Y., Y. Ji, et al. (2012). "Estrogen receptor beta activation is antinociceptive in a model of visceral pain in the rat." J Pain 13(7): 685-694.

Cho, T. and V. V. Chaban (2012). "Expression of P2X3 and TRPV1 receptors in primary sensory neurons from estrogen receptors-alpha and estrogen receptor-beta knockout mice." Neuroreport 23(9): 530-534.

Choghakhori, R., A. Abbasnezhad, et al. (2017). "Sex-Related Differences in Clinical Symptoms, Quality of Life, and Biochemical Factors in Irritable Bowel Syndrome." Digestive Disease and Science 62(6): 1550-1560.

Galligan, J. J., B. A. Patel, et al. (2013). "Visceral hypersensitivity in female but not in male serotonin transporter knockout rats." Neurogastroenterology and Motility 25(6): e373-381.

Giamberardino, M. A., G. Affaitati, et al. (1997). "Changes in visceral pain reactivity as a function of estrous cycle in female rats with artificial ureteral calculosis." Brain Research 774(1-2): 234-238.

Goldman, J. M., A. S. Murr, et al. (2007). "The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies." Birth Defects Research B Developmental Reproduction and Toxicology 80(2): 84-97.

Grzesiak, M., J. A. Beszlej, et al. (2014). "The lifetime prevalence of anxiety disorders among patients with irritable bowel syndrome." Advances in Clinical and Experimental Medicine 23(6): 987-992.

Gupta, S., K. E. McCarson, et al. (2011). "Mechanisms of pain modulation by sex hormones in migraine." Headache 51(6): 905-922.

Gustafsson, J. K. and B. Greenwood-Van Meerveld (2011). "Amygdala activation by corticosterone alters visceral and somatic pain in cycling female rats." American Journal of Physiology-Gastrointestinal and Liver Physiology 300(6): 31.

Harris, L. A., S. B. Umar, et al. (2016). "Irritable Bowel Syndrome and Female Patients." Gastroenterology Clinics of North America 45(2): 179-204.

Heitkemper, M. M. and L. Chang (2009). "Do fluctuations in ovarian hormones affect gastrointestinal symptoms in women with irritable bowel syndrome?" Gender Medicine 2: 152-167.

Heitkemper, M. M. and L. Chang (2009). "Do fluctuations in ovarian hormones affect gastrointestinal symptoms in women with irritable bowel syndrome?" Gender Medicine 6 Suppl 2: 152-167.

Houghton, L. A., H. Brown, et al. (2009). "5-hydroxytryptamine signalling in irritable bowel syndrome with diarrhoea: effects of gender and menstrual status." Alimentary Pharmacology and Therapeutics 30(9): 919-929.

Houghton, L. A., R. Lea, et al. (2002). "The menstrual cycle affects rectal sensitivity in patients with irritable bowel syndrome but not healthy volunteers." Gut 50(4): 471-474.

Ji, Y., A. Z. Murphy, et al. (2003). "Estrogen modulates the visceromotor reflex and responses of spinal dorsal horn neurons to colorectal stimulation in the rat." Jornal of Neuroscience 23(9): 3908-3915.

Ji, Y., B. Tang, et al. (2005). "Modulatory effects of estrogen and progesterone on colorectal hyperalgesia in the rat." Pain 117(3): 433-442.

Ji, Y., B. Tang, et al. (2008). "The visceromotor response to colorectal distention fluctuates with the estrous cycle in rats." Neuroscience 154(4): 1562-1567.

Ji, Y., B. Tang, et al. (2011). "Spinal estrogen receptor alpha mediates estradiol-induced pronociception in a visceral pain model in the rat." Pain 152(5): 1182-1191.

Lu, C. L., J. C. Hsieh, et al. (2009). "Estrogen rapidly modulates 5-hydroxytrytophan-induced visceral hypersensitivity via GPR30 in rats." Gastroenterology 137(3): 1040-1050.

Lu, C. L., J. C. Hsieh, et al. (2007). "Estrogen rapidly modulates mustard oil-induced visceral hypersensitivity in conscious female rats: A role of CREB phosphorylation in spinal dorsal horn neurons." American Journal of Physiology Gastrointestinal Liver Physiology 292(1): 14.

Marino, M., P. Galluzzo, et al. (2006). "Estrogen signaling multiple pathways to impact gene transcription." Current Genomics 7(8): 497-508.

McEwen, B. S. and S. E. Alves (1999). "Estrogen actions in the central nervous system." Endocrine Reviews 20(3): 279-307.

Meleine, M. and J. Matricon (2014). "Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones." World Journal of Gastroenterology 20(22): 6725-6743.

Moloney, R. D., J. Sajjad, et al. (2016). "Estrous cycle influences excitatory amino acid transport and visceral pain sensitivity in the rat: effects of early-life stress." Biology of Sex Differences 7(33): 016-0086.

Mulak, A., Y. Tache, et al. (2014). "Sex hormones in the modulation of irritable bowel syndrome." World Journal of Gastroenterology 20(10): 2433-2448.

Myers, B., J. Schulkin, et al. (2011). "Sex steroids localized to the amygdala increase pain responses to visceral stimulation in rats." Journal of Pain 12(4): 486-494.

Naliboff, B. D., S. Berman, et al. (2003). "Sex-related differences in IBS patients: central processing of visceral stimuli." Gastroenterology 124(7): 1738-1747.

Papka, R. E. and M. Storey-Workley (2002). "Estrogen receptor-alpha and -beta coexist in a subpopulation of sensory neurons of female rat dorsal root ganglia." Neuroscience Letters 319(2): 71-74.

Park, J. M., Choi, et al. (2006). "Serotonin transporter gene polymorphism and irritable bowel syndrome." Neurogastroenterology and Motility 18(11): 995-1000.

Rybaczyk, L. A., M. J. Bashaw, et al. (2005). "An overlooked connection: serotonergic mediation of estrogen-related physiology and pathology." BMC Womens Health 5: 12.

Sanoja, R. and F. Cervero (2010). "Estrogen-dependent changes in visceral afferent sensitivity." Autonomic Neuroscience 153(1-2): 84-89.

Sinchak, K. and E. J. Wagner (2012). "Estradiol signaling in the regulation of reproduction and energy balance." Frontiers in Neuroendocrinology 33(4): 342-363.

Tang, B., Y. Ji, et al. (2008). "Estrogen alters spinal NMDA receptor activity via a PKA signaling pathway in a visceral pain model in the rat." Pain 137(3): 540-549.

Vanderhorst, V. G., J. A. Gustafsson, et al. (2005). "Estrogen receptor-alpha and -beta immunoreactive neurons in the brainstem and spinal cord of male and female mice: relationships to monoaminergic, cholinergic, and spinal projection systems." Journal of Comparative Neurology 488(2): 152-179.

Wierman, M. E. (2007). "Sex steroid effects at target tissues: mechanisms of action." Advances in Physiology Educaction 31(1): 26-33.

Chapter 4

General discussion

4.1 Ovarian hormones, serotonin and visceral hypersensitivity

Clinical studies suggest that the abdominal pain observed in some IBS patients may be due to serotonergic dysfunction. However, the mechanism by which this occurs is not understood. SERT gene polymorphisms have been identified in a subset of IBS patients (Camilleri 2004; Acosta and Camilleri 2015). Female IBS-patients carrying the s/s allele exhibit decreased SERT function and increased abdominal pain severity (Kumar, Ranjan et al. 2012). Furthermore, variations in plasma 5-HT levels across the menstrual cycle have been identified (Houghton, Atkinson et al. 2003). In the luteal phase of the menstrual cycle when estrogens and progesterone levels are high, post-prandial plasma 5-HT levels were higher in female IBS-diarrhea patients compared to healthy controls (Houghton, Atkinson et al. 2003). A follow up study showed that this increase in post-prandial 5-HT does not occur during menses when abdominal pain is reported to be the worst (Heitkemper and Chang 2009). Furthermore, the 5-HT3 receptor antagonist alosetron is effective in reducing IBS-D symptoms in female but not male patients. While some speculate that this could be due to reduced alosetron clearance in women resulting in 30-50 percent increased concertation of alosetron following a given dose (Koch, Palmer et al. 2002). This could also be due to differences in serotonergic signaling between males and females since alosetron results in increased 5-HT synthesis in specific brain regions in males only (Nakai, Diksic et al. 2005).

In rodents it has been shown that estrogen upregulates TPH2, SERT and 5-HT2A receptor expression in the brain (Blurton-Jones and Tuszynski 2002; Sumner, Grant et al. 2007).

In a partial restraint-stress induced rat animal model of visceral hypersensitivity, 17β-estradiol and progesterone replacement in ovariectomized rats caused down regulation in 5-HT3 receptor expression in the colon. This suggests that estrogen plays an antinociceptive role since 5-HT3 receptor are expressed on primary afferent neuron terminals and lead to neuronal activation (Li, Yu et al. 2004).

These clinical and basic research observations demonstrate the importance of studying the interaction between ovarian hormones and serotonergic signaling in mediating nociceptive signaling. The SERT KO rat serve as an excellent animal model to investigate the interaction between ovarian hormones and 5-HT signaling. Galligan et al, 2013 showed that the SERT KO female rat exhibits increased discomfort in response to CRD. Furthermore, the increase in discomfort is estrous cycle dependent. SERT KO rats exhibit an increased visceral sensitivity to colorectal distension (CRD) in the proestrus (high estrogen) and estrus (low estrus) stage of the estrus cycle. While colonic mucosal levels of 5-HT are the same in SERT KO and WT rats, amperometric studies showed that extracellular 5-HT levels are higher in SERT KO female and male rats compared WT rats. Whole-cell patch-clamp analysis of colon projecting DRG neurons showed that SERT KO female DRG neurons exhibit increased rate of action potential firing compared to WT female and male rats (Galligan, Patel et al. 2013). These data suggest that in presence of serotonergic dysfunction, ovarian hormones might modulate increased visceral sensitivity by affecting neuronal signaling at the peripheral or central terminals of primary afferent neurons.

As discussed in chapter 2, 5-HT3 receptors play an important role in mediating sensory information from the gastrointestinal tract to the central nervous system. We showed that in the SERT KO female rat spinal 5-HT3 receptors play an antinociceptive role. On the other hand, in the SERT KO male rat spinal 5-HT3 receptor activation led to enhanced VMR to CRD. These functional differences may be due to differential 5-HT3 receptor expression in the dorsal horn of the spinal cord. In female SERT KO female rats 5-HT3 receptors appear to be predominantly expressed on inhibitory interneurons in the dorsal horn of the spinal cord. While in SERT KO male rats 5-HT3 receptors are likely to be expressed predominantly on the central terminals of primary afferent neurons where their activation results in an increase in excitatory neurotransmitters release (Figure 4.1).

It has been shown that the competitive GABAa receptor antagonist SR95531 was able to reverse receptor desensitization in cell culture (Xu, Roberts et al. 2016). Therefore we could also hypothesize that in female SERT KO rats, alosetron which is a competitive receptor antagonist could reverse 5-HT3 receptor desensitization leading to increased discharge of excitatory neurotransmitters from central terminals of primary afferent neurons (Machu 2011).

Furthermore, the SERT KO female rat exhibits increased VMR to CRD in the proestrus phase of the estrous cycle where estrogen levels reach their peak in the late morning and drop to their minimum by the end of the day (Goldman, Murr et al. 2007). This indicates that perhaps the fluctuations in ovarian hormones rather than the absolute concentration at a given time contributes to the development of visceral hypersensitivity. This is also observed clinically where female IBS patients report increased abdominal pain during around menses where

ovarian hormone levels begin to drop. Ovariectomy increased VMR to CRD between days 7 and 21 post ovariectomy. Inhibition of the steroidal estrogen receptors ERα and ERβ in the spinal cord caused an increase in VMR to CRD one hour following intrathecal administration of the ICI 182 780. This increase in discomfort was also observed after intrathecal administration of the drugs for 4 days. This data suggest that the increase in VMR to CRD could be mediated by non-genomic and genomic signaling of the ERα and ERβ. In fact, ER α and ER β have been reported to mediate non-genomic signaling. Specifically, it has been shown that membrane ER-α receptors forms a complex with metabotropic glutamate receptors to mediate its non-genomic signaling (Sinchak and Wagner 2012). Estrogen mediated analgesia could also occur via GABAergic neurotransmission. For instance, ER-α receptors have been colocalized with GABAergic inhibitory interneurons (Lu, Willcockson et al. 2005). It has also been shown that in proestrus phase membrane estrogen receptors (ER-α and ER-β) cause heterodimerization of μ-opioid and κ-opioid receptors to mediate the antinociceptive effects of spinal dynorphins. In the diestrus/estrus phase spinal dynorphins mediate nociception via κ-opioid receptor monomer signaling (Gintzler and Liu 2012). Therefore, we can hypothesize that blockade of membrane ER-α and/or ER-β can result in hyperalgesia due to a reduction in opioid receptor heterodimerization.

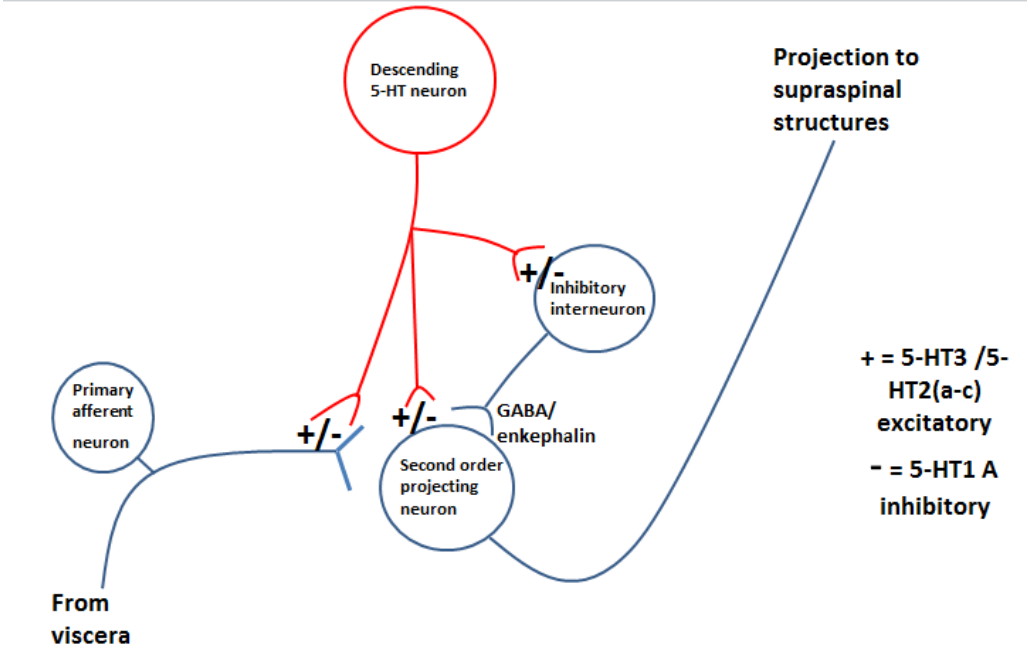

Figure 4.1 Inhibitory and excitatory 5-HT signaling in the dorsal horn of the spinal cord. Various 5-HT receptors are expressed on inhibitory interneuron, primary afferent and second order neurons. For instance decreased pain transmission occurs when descending 5-HT activates 5-HT3 receptors on inhibitory interneurons and 5-HT1A receptors on primary and secondary afferents. On the other hand increased nociceptive signaling occurs when 5-HT1A receptors are activated on inhibitory interneurons and 5-HT3 receptors are activated on primary and secondary afferent neurons.

4.2 Technical considerations

In this section I will discuss some of the techniques used to perform my experiments and discuss the strength and limitation of some of these techniques.

4.2.1 Visceromotor response (VMR) to colorectal distension (CRD)

CRD is a reliable method to study acute visceral pain since it is minimally invasive and unlike electrical or chemical stimulation is reproducible. Noxious response to CRD can be measured by monitoring changes in blood pressure, heart rate and aversive behavior. VMR is also used to measure noxious effects of CRD. VMR is an abrupt contraction of the abdominal

and hind-limb muscles in response to CRD. It is equivalent to the abdominal muscle rigidity that occurs in humans experiencing abdominal pain. The VMR to CRD is diminished following injury to the spinal cord at either the thoracic or cervical level but is not affected by midcollicular lesion which interrupts the communication between the brainstem and the brain. This suggests that the VMR is mediated at the level of the brainstem. CRD results in both a non-noxious response caused by low pressure distensions of 20 mmHg or lower and noxious response occurring above 30 mmHg (Ness and Gebhart 1988). Morphine and clonidine both of which are known for their analgesic effects diminished the VMR to CRD as shown by Ness, et al 1988. We were also able to show that morphine when given subcutaneously and intrathecally diminished the VMR to CRD (Figure 4.2). Similarly, clonidine was also able to completely inhibit VMR to CRD as shown in Figure 4.3. This suggests that increased intraabdominal pressure at or above 40 mmHg results in noxious stimulus that can be employed to study visceral hypersensitivity.

It is worth noting that while the VMR to CRD is reproducible and reliable, differences in noxious response have been observed in the rat depending on the strain of the rat. For instance, Sprague-Dawley rats do not exhibit noxious response to CRD at intracolonic pressure lower than 60 mmHg and show minimal number of pain behaviors at higher pressure. On the other hand Wistar Kyoto rats exhibit pain response at 40 mmHg and increased number of pain behaviors (Hyland, O'Mahony et al. 2015). The control rats used in our studies were Wistar strain which is also the background of the SERT KO rats.

4.2.2 5,7-DHT mechanism of action.

The neurotoxin 5 7 DHT results in destruction of 5-HT nerve terminals and axons at the site of injection. At 200 µg dose, 5,7 DHT reduced 5-HT levels by up to 50% in the rat brain when administered via an intracerebroventricular cannula (Choi, Jonak et al. 2004). Pretreatment with the norepinephrine transport (NET) blocker desipramine prevented the destruction of norepinephrine neurons. We were also able to reproduce this observation as shown in chapter 2 confirming that uptake of 5,7-DHT via NET is required to cause toxicity to noradrenergic neurons. However, the use of selective 5-HT transporter inhibitors (SSRI) such as fluoxetine and other 5-HT blocker (chlorimipramine, alaproclate)) were unable to prevent 5-HT neuronal toxicity by 5,7-DHT suggesting that uptake via SERT is not required for toxicity. Furthermore, the blockade of 5-HT auto-receptors using metergoline was also unable to block 5,7 DHT toxicity (Choi, Jonak et al. 2004). Pretreatment with reserpine failed to protect 5-HT neurons from 5,7 DHT induced neurotoxicity suggesting that synaptic vesicles are not the site of neurotoxicity. In fact, studies performed with labeled 5,7-DHT indicate that the toxin accumulates in the mitochondria of 5-HT neurons (Baumgarten 1994).

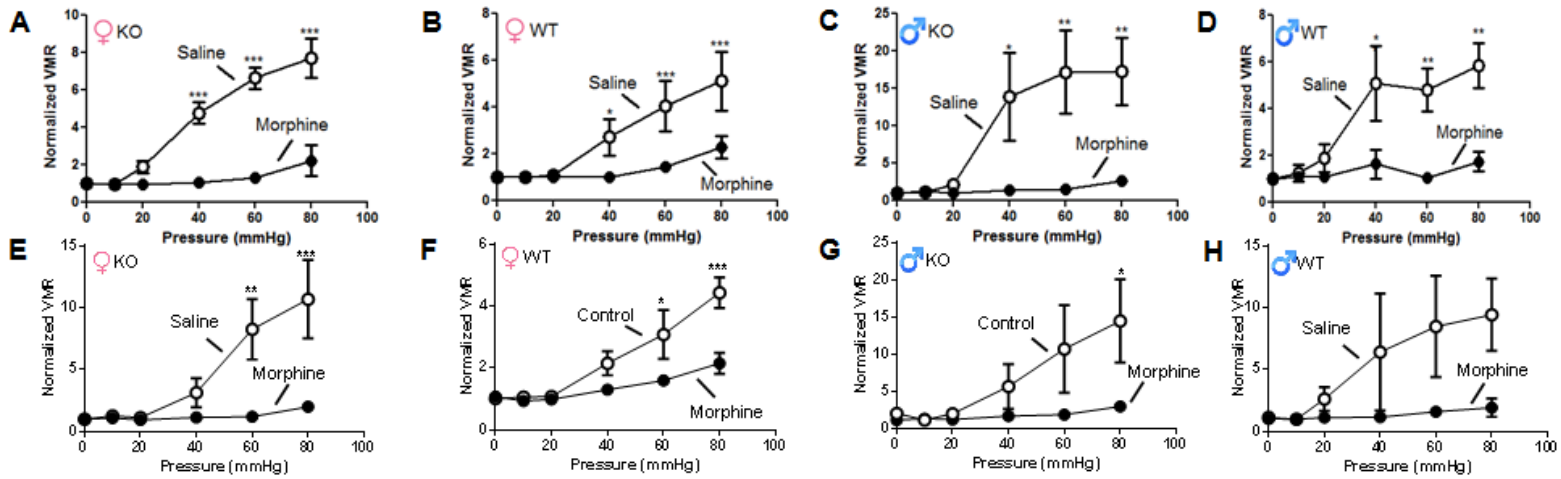

Figure 4.2 Effects of morphine on VMR to CRD. Morphine (3mg/kg, s.c.) suppresses VMR to CRD in SERT KO female rats (n=3) (A) WT female rats (saline, n=4; morphine, n=7) (B) SERT KO male rats (n =4) (C) and WT male rats (saline, n=4; morphine, n=7) (D). . Morphine (10µg, i.t.) suppresses VMR to CRD in SERT KO female rats (=3) (E) WT female rats (n=4) (F) SERT KO male rats (n =4) (G) and WT male rats (n=2) (H). Two-Way ANOVA was used to detect statistical significance. *,**,*** Indicate significantly different from control (*P< 0.05),(** P<0.01) and (***P<0.001). Data are mean ± SEM.

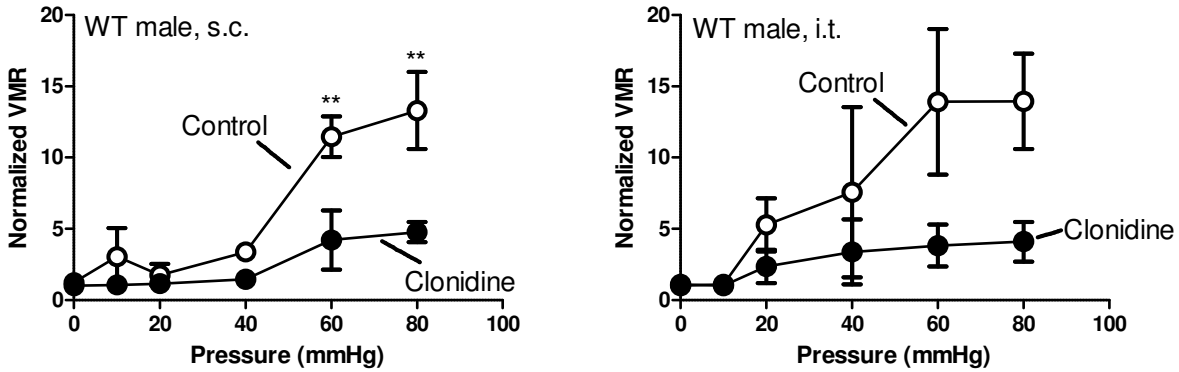

Figure 4.3 Effect of clonidine on VMR to CRD in WT male rats. Clonidine (α2 adrenergic agonist) reduced VMR to CRD in WT male rats when administered subcutaneously at done of 100µl/kg (A) and intrathecally at dose of 8 µg/10 µL (B). Two-Way ANOVA was used to detect statistical significance (n=2). ** Indicate significantly different from control (* P<0.05), (** P<0.01). Data are mean ± SEM.

5,7-DHT produced a robust decrease in spinal 5-HT levels in both SERT KO and WT rats. Our studies, like the studies described above suggest the SERT is not required for 5,7 DHT neurotoxicity One mechanism which has not been investigated and could account for uptake of 5,7-DHT by serotonergic neurons when SERT is inhibited or absent is uptake via organic cation transporters (OCTs) . OCTs are capable of transporting a wide variety of cations including 5-HT. In SERT KO mice, OCT3 expression is upregulated in the hippocampus but not other brain regions. However, OCT1 which is predominantly expressed in the liver and kidney was not upregulated in SERT KO mice (Schmitt, Mossner et al. 2003). Unpublished work performed in our lab showed that OCT3 expression in the colon is not upregulated in SERT KO rats compared to WT rats. However, this may not be reflective of OCT3 expression in the central nervous

system. Therefore, we cannot rule out the possibility of 5,7-DHT uptake via OCTs expressed in the spinal cord.

4.2.3 Ovariectomy limitations

Ovariectomy increased VMR to CRD between 7 and 21 post-ovariectomy. The reversal of the increase in VMR to CRD 3 weeks after ovariectomy suggests that the increase in pain was due to physiological adaptations in response to the rapid reduction in ovarian hormones. Indeed, it has been shown that estrogen levels reach their nadir 1-2 weeks post-ovariectomy in rodents. Therefore, the endpoints measured during the first month post-ovariectomy may be an reflection of physiological adaptations and should be considered carefully (Diaz Brinton 2012).

Furthermore, the assumption that the levels of steroids measured in the plasma are reflective of steroid concentration in organs after ovariectomy has also been challenged. In a study where neuroactive steroid levels were measured in plasma, peripheral nervous system and central nervous system after gonadectomy in rats showed that the levels of neuroactive hormones were significantly different in plasma compared to levels in sciatic nerve, cerebral cortex, brainstem and spinal cord (Diaz Brinton 2012). In fact, aromatase is expressed in lamina I and V in the spinal cord in neurons that stain positive for inhibitory interneuron markers. This suggests that estrogen can be synthesized locally in the spinal cord to modulate pain transmission (Tran, Kuhn et al. 2017). These studies suggest that neuroactive steroidal levels are controlled locally and therefore, interpretation of ovariectomy studies should be performed with these caveats in mind.

4.3 Clinical significance and future directions

Our studies showed that inhibition of spinal 5-HT3 receptors caused an increase in VMR to CRD in SERT KO female rats which is in line with the clinical observation that patients with reduced SERT activity (s/s allele carriers) respond poorly to alosetron (Acosta and Camilleri 2015). On the other hand, activation of spinal 5-HT3 receptors in male SERT KO rats caused an increase in discomfort to CRD. However, inhibition of 5-HT3 receptors didn't affect VMR to CRD in SERT KO male rats. This recapitulates the clinical observation that alosetron does not alleviate abdominal pain in male IBS-D patients (Koch, Palmer et al. 2002).

Furthermore, we showed that ovariectomy and inhibition of classocal ERs also increased discomfort. While clinical studs assessing oral contraceptives for treatment of IBS are sparse. One study showed that progestin only birth control is associated with increased IBS diagnosis. This suggests that disruption of ovarian hormone balance may contribute to the development of IBS (Bird, Liu et al. 2012). We observed that SERT KO female rats exhibit normal estrous cycle and are capable of reproducing without any complications or reduced capacity. This suggests that the dysfunction in serotonergic signaling does not alter reproductive function in SERT KO rats. Yet, ovarian hormones appear to influence response to noxious stimulation of the colon.

The sex difference in VMR to CRD response in SERT KO rats and the response to 5-HT3 receptor inhibition suggest that SERT KO rats serves as a valuable resource to understand the connection between ovarian hormones and 5-HT in mediating visceral hypersensitivity. Future work should focus on determining how inhibition of ERs leads to increase visceral sensitivity.

Employing various estrogen receptor antagonist and agonist to intact rats is a better approach than ovariectomy given the ovariectomy limitations discussed above.

Furthermore, Investigation the role of other 5-HT receptors in mediating visceral hypersensitivity in SERT KO rats will further enhance our understanding of the role of 5-HT signaling in mediating pain. Specifically, 5-HT1A, 5-HT2 and 5-HT7 receptors have been shown to mediate pain transmission at the level of the spinal cord (figure 4.1) (Millan 2002). Such work will allow us to combine different 5-HT receptor mediators to effectively treat pain instead of focusing on once receptor in a clinical setting.

As discussed in chapter 2, reducing 5-HT levels in the spinal cord from descending 5-HT neurons prevented the increase in VMR to CRD observed following alosetron treatment in SERT KO female rats and SR 57227 in SERT KO male rats. This could be observed due to unmasking the antinociceptive effects of descending noradrenergic signaling at α2-adregenrgic receptors. In fact, co-administration of clonidine (α2 agonist) and alosetron prevented the increase in VMR to CRD in SERT KO female rats (data not shown). Therefore, we can employ SERT KO rats to further investigate the interactions between descending noradrenergic and serotonergic pain modulation in mediating visceral hypersensitivity. Most studies performed to determine descending pain modulation employ somatic pain assays such as hot plate, tail flick and sciatic nerve ligation. Very few experiments focus on understanding descending modulation of visceral pain. Therefore, performing such experiments will fill a large gap in the field.

BIBLIOGRAPHY

BIBLIOGRAPHY

Acosta, A. and M. Camilleri (2015). "Pharmacogenetics in irritable bowel syndrome." Expert Opinion on Drug Metabolism & Toxicology 11(8): 1187-1191.

Adeyemo, M. A., B. M. Spiegel, et al. (2010). "Meta-analysis: do irritable bowel syndrome symptoms vary between men and women?" Alimentary Pharmacology & Therapeutics 32(6): 738-755.

Alhaider, A. A., S. Z. Lei, et al. (1991). "Spinal 5-HT3 receptor-mediated antinociception: possible release of GABA." Journal of Neuroscience 11(7): 1881-1888.

Amandusson, A., M. Hallbeck, et al. (1999). "Estrogen-induced alterations of spinal cord enkephalin gene expression." Pain 83(2): 243-248.

Amandusson, A., O. Hermanson, et al. (1996). "Colocalization of oestrogen receptor immunoreactivity and preproenkephalin mRNA expression to neurons in the superficial laminae of the spinal and medullary dorsal horn of rats." European Journal of Neuroscience 8(11): 2440-2445.

Banner, S. E. and G. J. Sanger (1995). "Differences between 5-HT3 receptor antagonists in modulation of visceral hypersensitivity." British Journal of Pharmacology 114(2): 558-562.

Barnes, N. M., T. G. Hales, et al. (2009). "The 5-HT3 receptor--the relationship between structure and function." Neuropharmacology 56(1): 273-284.

Baumgarten, H. G., Zimmerman B (1994). Neurotoxic phenylalkylamines and indolealkylamines. Selective Neurotoxicity, Springer-Verlag: 225-229.

Beatty, J. K., A. Bhargava, et al. (2014). "Post-infectious irritable bowel syndrome: mechanistic insights into chronic disturbances following enteric infection." World Journal of Gastroenterology 20(14): 3976-3985.

Berman, S., J. Munakata, et al. (2000). "Gender differences in regional brain response to visceral pressure in IBS patients." European Journal of Pain 4(2): 157-172.

Bhattarai, Y., D. A. Muniz Pedrogo, et al. (2017). "Irritable bowel syndrome: a gut microbiota-related disorder?" American Journal of Physiology -Gastrointestinal and Liver Physiology 312(1): G52-G62.

Bird, S. T., W. Liu, et al. (2012). "Irritable bowel syndrome and drospirenone-containing oral contraceptives; a comparative-safety study." Current Drug Safety 7(1): 8-15.

Blackshaw, L. A., S. J. Brookes, et al. (2007). "Sensory transmission in the gastrointestinal tract." Neurogastroenterololgy and Motility 19(1 Suppl): 1-19.

Blurton-Jones, M. and M. H. Tuszynski (2002). "Estrogen receptor-beta colocalizes extensively with parvalbumin-labeled inhibitory neurons in the cortex, amygdala, basal forebrain, and hippocampal formation of intact and ovariectomized adult rats." Journal of Comparative Neurology 452(3): 276-287.

Bouin, M., V. Plourde, et al. (2002). "Rectal distention testing in patients with irritable bowel syndrome: Sensitivity, specificity, and predictive values of pain sensory thresholds." Gastroenterology 122(7): 1771-1777.

Bradesi, S., H. Eutamene, et al. (2003). "Stress-induced visceral hypersensitivity in female rats is estrogen-dependent and involves tachykinin NK1 receptors." Pain 102(3): 227-234.

Bradesi, S., L. Lao, et al. (2007). "Dual role of 5-HT3 receptors in a rat model of delayed stress-induced visceral hyperalgesia." Pain 130(1-2): 56-65.

Bradshaw, H. B., J. L. Temple, et al. (1999). "Estrous variations in behavioral responses to vaginal and uterine distention in the rat." Pain 82(2): 187-197.

Brookes, S. J., N. J. Spencer, et al. (2013). "Extrinsic primary afferent signalling in the gut." Nature Reviews Gastroenterology & Hepatology 10(5): 286-296.

Browning, K. N. (2015). "Role of central vagal 5-HT3 receptors in gastrointestinal physiology and pathophysiology." Frontiers in Neuroscience 9(413).

Buono, J. L., R. T. Carson, et al. (2017). "Health-related quality of life, work productivity, and indirect costs among patients with irritable bowel syndrome with diarrhea." Health and Quality of Life Outcomes 15(1): 017-0611.

Buono, J. L., K. Mathur, et al. (2017). "Economic Burden of Irritable Bowel Syndrome with Diarrhea: Retrospective Analysis of a U.S. Commercially Insured Population." Journal of Managed Care and Specialty Pharmcy 23(4): 453-460.

Butler, A., J. M. Hill, et al. (1988). "Pharmacological properties of GR38032F, a novel antagonist at 5-HT3 receptors." British Journal of Pharmacology 94(2): 397-412.

Cain, K. C., P. Headstrom, et al. (2006). "Abdominal pain impacts quality of life in women with irritable bowel syndrome." The American Journal of Gastroenterology 101(1): 124-132.

Cain, K. C., M. E. Jarrett, et al. (2009). "Gender Differences in Gastrointestinal, Psychological, and Somatic Symptoms in Irritable Bowel Syndrome." Digestive Diseases and Sciences 54(7): 1542-1549.

Camilleri, M. (2004). "Is there a SERT-ain association with IBS?" Gut 53(10): 1396-1399.

Camilleri, M., B. Coulie, et al. (2001). "Visceral hypersensitivity facts sepculations and challenges camelliri." Gut 48: 7.

Cao, D. Y., Y. Ji, et al. (2012). "Estrogen receptor beta activation is antinociceptive in a model of visceral pain in the rat." Journal of Pain 13(7): 685-694.

Chang, F. Y., C. L. Lu, et al. (2010). "The current prevalence of irritable bowel syndrome in Asia." J Neurogastroenterology and Motility 16(4): 389-400.

Chang, L., V. Z. Ameen, et al. (2005). "A dose-ranging, phase II study of the efficacy and safety of alosetron in men with diarrhea-predominant IBS." The American Journal of Gastroenterology 100(1): 115-123.

Chen, J. J., Z. Li, et al. (2001). "Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters." Journal of Neuroscience 21(16): 6348-6361.

Chen, L., S. J. Ilham, et al. (2017). "Pharmacological Approach for Managing Pain in Irritable Bowel Syndrome: A Review Article." Anesthesiology and Pain Medicine 7(2).

Cheung, C. K. and J. C. Wu (2014). "Genetic polymorphism in pathogenesis of irritable bowel syndrome." World Journal of Gastroenterology 20(47): 17693-17698.

Chey, W. D., J. Kurlander, et al. (2015). "Irritable bowel syndrome: a clinical review." Journal of American Medical Association 313(9): 949-958.

Chiloiro, M., G. Darconza, et al. (2001). "Gastric emptying and orocecal transit time in pregnancy." Journal of Gastroenterology 36(8): 538-543.

Cho, T. and V. V. Chaban (2012). "Expression of P2X3 and TRPV1 receptors in primary sensory neurons from estrogen receptors-alpha and estrogen receptor-beta knockout mice." Neuroreport 23(9): 530-534.

Choghakhori, R., A. Abbasnezhad, et al. (2017). "Sex-Related Differences in Clinical Symptoms, Quality of Life, and Biochemical Factors in Irritable Bowel Syndrome." Digestive Diseases and Sciences 62(6): 1550-1560.

Choi, I. S., J. H. Cho, et al. (2013). "5-Hydroxytryptamine 1A receptors inhibit glutamate release in rat medullary dorsal horn neurons." Neuroreport 24(8): 399-403.

Choi, S., E. Jonak, et al. (2004). "Serotonin reuptake inhibitors do not prevent 5,7-dihydroxytryptamine-induced depletion of serotonin in rat brain." Brain Research 8: 1-2.

Choi, Y. D., T. S. Sung, et al. (2008). "Increased 5-hydroxytryptamine mediates post-inflammatory visceral hypersensitivity via the 5-hydroxytryptamine 3 receptor in rats." Digestive Diseases and Sciences 53(11): 2909-2916.

Clayton, N. M., R. Sargent, et al. (1999). "The pharmacological properties of the novel selective 5-HT3 receptor antagonist, alosetron, and its effects on normal and perturbed small intestinal transit in the fasted rat." Neurogastroenterology and Motility 11(3): 207-217.

Coelho, A. M., J. Fioramonti, et al. (1998). "Mast cell degranulation induces delayed rectal allodynia in rats: role of histamine and 5-HT." Digestive Diseases and Sciences 43(4): 727-737.

Cremon, C., G. Carini, et al. (2011). "Intestinal Serotonin Release, Sensory Neuron Activation, and Abdominal Pain in Irritable Bowel Syndrome." The American Journal of Gastroenterology 106(7): 1290-1298.

Cremonini, F., J. P. Nicandro, et al. (2012). "Randomised clinical trial: alosetron improves quality of life and reduces restriction of daily activities in women with severe diarrhoea-predominant IBS." Alimentary Pharmacology & Therapeutics 36(5): 437-448.

Diaz Brinton, R. (2012). "Minireview: translational animal models of human menopause: challenges and emerging opportunities." Endocrinology 153(8): 3571-3578.

Drossman, D. A., M. Camilleri, et al. (2002). "AGA technical review on irritable bowel syndrome." Gastroenterology 123(6): 2108-2131.

Foley, S., K. Garsed, et al. (2011). "Impaired uptake of serotonin by platelets from patients with irritable bowel syndrome correlates with duodenal immune activation." Gastroenterology 140(5): 1434-1443 e1431.

Furness, J. B., L. R. Rivera, et al. The gut as a sensory organ, Nature Reviews of Gastroenterology and Hepatology. 2013 Dec;10(12):729-40. doi: 10.1038/nrgastro.2013.180. Epub 2013 Sep 24.

Galligan, J. J., B. A. Patel, et al. (2013). "Visceral hypersensitivity in female but not in male serotonin transporter knockout rats." Neurogastroenterology and Motility 25(6): e373-381.

Garsed, K., J. Chernova, et al. (2014). "A randomised trial of ondansetron for the treatment of irritable bowel syndrome with diarrhoea." Gut 63(10): 1617-1625.

Gebhart, G. F. (1999). "Peripheral contributions to visceral hyperalgesia." Canadian Journal of Gastroenterology & Hepatology 13: 37A-41A.

Gebhart, G. F. and K. Bielefeldt (2016). "Physiology of Visceral Pain." Comprehensive Physiology 6(4): 1609-1633.

Gershon, M. D. (2004). "Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility." Alimentary Pharmacology & Therapeutics 7: 3-14.

Gershon, M. D. and J. Tack (2007). "The serotonin signaling system: from basic understanding to drug development for functional GI disorders." Gastroenterology 132(1): 397-414.

Giamberardino, M. A., G. Affaitati, et al. (1997). "Changes in visceral pain reactivity as a function of estrous cycle in female rats with artificial ureteral calculosis." Brain Research 774(1-2): 234-238.

Gintzler, A. R. and N. J. Liu (2012). "Importance of sex to pain and its amelioration; relevance of spinal estrogens and its membrane receptors." Frontiers in Neuroendocrinology 33(4): 412-424.

Glatzle, J., C. Sternini, et al. (2002). "Expression of 5-HT3 receptors in the rat gastrointestinal tract." Gastroenterology 123(1): 217-226.

Glaum, S. R., H. K. Proudfit, et al. (1990). "5-HT3 receptors modulate spinal nociceptive reflexes." Brain Research 510(1): 12-16.

Goldberg, P. A., M. A. Kamm, et al. (1996). "Modification of visceral sensitivity and pain in irritable bowel syndrome by 5-HT3 antagonism (ondansetron)." Digestion 57(6): 478-483.

Goldman, J. M., A. S. Murr, et al. (2007). "The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies." Birth Defects Research Part B: Developmental and Reproductive Toxicology 80(2): 84-97.

Gralnek, I. M., R. D. Hays, et al. (2000). "The impact of irritable bowel syndrome on health-related quality of life." Gastroenterology 119(3): 654-660.

Greenwood-Van Meerveld, B., E. Mohammadi, et al. (2014). "Synergistic effect of 5-hydroxytryptamine 3 and neurokinin 1 receptor antagonism in rodent models of somatic and visceral pain." Journal of Pharmacology and Experimental Therapeutics 351(1): 146-152.

Gregersen, H. and G. Kassab (1996). "Biomechanics of the gastrointestinal tract." Neurogastroenterology and Motility 8(4): 277-297.

Grover, M. and M. Camilleri Ramosetron in irritable bowel syndrome with diarrhea: new hope or the same old story?, Clinical Gastroenterology and Hepatology. 2014 Jun;12(6):960-2. doi: 10.1016/j.cgh.2013.12.025. Epub 2014 Jan 3.

Grzesiak, M., J. A. Beszlej, et al. (2014). "The lifetime prevalence of anxiety disorders among patients with irritable bowel syndrome." Advances in Clinical and Experimental Medicine 23(6): 987-992.

Gu, Q. Y., J. Zhang, et al. (2015). "Association of genetic polymorphisms in HTR3A and HTR3E with diarrhea predominant irritable bowel syndrome." International Journal of Clinical and Experimental Medicine 8(3): 4581-4585.

Gunawardene, A. R., B. M. Corfe, et al. (2011). "Classification and functions of enteroendocrine cells of the lower gastrointestinal tract." International Journal of Experimental Pathology 92(4): 219-231.

Gupta, S., K. E. McCarson, et al. (2011). "Mechanisms of pain modulation by sex hormones in migraine." Headache 51(6): 905-922.

Gustafsson, J. K. and B. Greenwood-Van Meerveld (2011). "Amygdala activation by corticosterone alters visceral and somatic pain in cycling female rats." American Journal of Physiology-Gastrointestinal and Liver Physiology 300(6): 31.

Halac, U., A. Noble, et al. (2010). "Rectal sensory threshold for pain is a diagnostic marker of irritable bowel syndrome and functional abdominal pain in children." Journal of Pediatrics 156(1): 60-65 e61.

Hall, F. S., J. M. Schwarzbaum, et al. (2011). "A greater role for the norepinephrine transporter than the serotonin transporter in murine nociception." Neuroscience 175: 315-327.

Hall, J. D., C. DeWitte, et al. (2015). "Serotonin enhances urinary bladder nociceptive processing via a 5-HT3 receptor mechanism." Neuroscience Letters 604: 97-102.

Harris, L. A., S. B. Umar, et al. (2016). "Irritable Bowel Syndrome and Female Patients." Gastroenterol Clin North Am 45(2): 179-204.

Heitkemper, M. M. and L. Chang (2009). "Do fluctuations in ovarian hormones affect gastrointestinal symptoms in women with irritable bowel syndrome?" Gender Medicine 6 Suppl 2: 152-167.

Heitkemper, M. M. and L. Chang (2009). "Do fluctuations in ovarian hormones affect gastrointestinal symptoms in women with irritable bowel syndrome?" Gender Medicine 2: 152-167.

Herman, J., V. Pokkunuri, et al. (2010). "Gender distribution in irritable bowel syndrome is proportional to the severity of constipation relative to diarrhea." Gender Medicine 7(3): 240-246.

Hoekman, D. R., J. M. Rutten, et al. (2015). "Annual Costs of Care for Pediatric Irritable Bowel Syndrome, Functional Abdominal Pain, and Functional Abdominal Pain Syndrome." Journal of Pediatrics 167(5): 1103-1108.

Homberg, J. R., J. D. Olivier, et al. (2007). "Characterization of the serotonin transporter knockout rat: a selective change in the functioning of the serotonergic system." Neuroscience 146(4): 1662-1676.

Hornung, J. P. (2003). "The human raphe nuclei and the serotonergic system." Journal of Chemical Neuroanatomy 26(4): 331-343.

Houghton, L. A., W. Atkinson, et al. (2003). "Increased platelet depleted plasma 5-hydroxytryptamine concentration following meal ingestion in symptomatic female subjects with diarrhoea predominant irritable bowel syndrome." Gut 52(5): 663-670.

Houghton, L. A., H. Brown, et al. (2009). "5-hydroxytryptamine signalling in irritable bowel syndrome with diarrhoea: effects of gender and menstrual status." Alimentary Pharmacology & Therapeutics 30(9): 919-929.

Houghton, L. A., J. M. Foster, et al. (2000). "Alosetron, a 5-HT3 receptor antagonist, delays colonic transit in patients with irritable bowel syndrome and healthy volunteers." Alimentary Pharmacology & Therapeutics 14(6): 775-782.

Houghton, L. A., R. Lea, et al. (2002). "The menstrual cycle affects rectal sensitivity in patients with irritable bowel syndrome but not healthy volunteers." Gut 50(4): 471-474.

Hyland, N. P., S. M. O'Mahony, et al. (2015). "Early-life stress selectively affects gastrointestinal but not behavioral responses in a genetic model of brain-gut axis dysfunction." Neurogastroenterol Motil 27(1): 105-113.

Iyengar, S., M. H. Ossipov, et al. (2017). "The role of calcitonin gene-related peptide in peripheral and central pain mechanisms including migraine." Pain 158(4): 543-559.

Jensen, A. A., P. A. Davies, et al. (2008). "3B but which 3B and that's just one of the questions: the heterogeneity of human 5-HT3 receptors." Trends in Pharmacological Sciences 29(9): 437-444.

Jeong, H. J., V. A. Mitchell, et al. (2012). "Role of 5-HT(1) receptor subtypes in the modulation of pain and synaptic transmission in rat spinal superficial dorsal horn." British Journal of Pharmacology 165(6): 1956-1965.

Ji, Y., A. Z. Murphy, et al. (2003). "Estrogen modulates the visceromotor reflex and responses of spinal dorsal horn neurons to colorectal stimulation in the rat." Journal of Neuroscience 23(9): 3908-3915.

Ji, Y., B. Tang, et al. (2005). "Modulatory effects of estrogen and progesterone on colorectal hyperalgesia in the rat." Pain 117(3): 433-442.

Ji, Y., B. Tang, et al. (2008). "The visceromotor response to colorectal distention fluctuates with the estrous cycle in rats." Neuroscience 154(4): 1562-1567.

Ji, Y., B. Tang, et al. (2011). "Spinal estrogen receptor alpha mediates estradiol-induced pronociception in a visceral pain model in the rat." Pain 152(5): 1182-1191.

Jin, D.-C., H.-L. Cao, et al. (2016). "Regulation of the serotonin transporter in the pathogenesis of irritable bowel syndrome." World Journal of Gastroenterology 22(36): 8137.

Jorgensen, H. S. (2007). "Studies on the neuroendocrine role of serotonin." Danish Medical Bulletin 54(4): 266-288.

Kanazawa, M., M. Hongo, et al. (2011). "Visceral hypersensitivity in irritable bowel syndrome." Journal of Gastroenterology and Hepatology 26: 119-121.

Kapeller, J., L. A. Houghton, et al. (2008). "First evidence for an association of a functional variant in the microRNA-510 target site of the serotonin receptor-type 3E gene with diarrhea predominant irritable bowel syndrome." Human Molecular Genetics 17(19): 2967-2977.

Kawamata, T., K. Omote, et al. (2003). "The activation of 5-HT(3) receptors evokes GABA release in the spinal cord." Brain Research 978(1-2): 250-255.

Kim, J. J. and W. I. Khan (2014). "5-HT7 receptor signaling: improved therapeutic strategy in gut disorders." Frontiers in Behavioral Neuroscience 8(396).

Kirkup, A. J., A. M. Brunsden, et al. (2001). "Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents." American Journal of Physiology-Gastrointestinal and Liver Physiology 280(5): G787-794.

Koch, K. M., B. W. Corrigan, et al. (2004). "Alosetron repeat dose pharmacokinetics, effects on enzyme activities, and influence of demographic factors." Alimentary Pharmacology & Therapeutics 20(2): 223-230.

Koch, K. M., J. L. Palmer, et al. (2002). "Sex and age differences in the pharmacokinetics of alosetron." British Journal of Clinical Pharmacology 53(3): 238-242.

Kohen, R., M. E. Jarrett, et al. (2009). "The Serotonin Transporter Polymorphism rs25531 Is Associated with Irritable Bowel Syndrome." Digestive Diseases and Sciences 54(12): 2663-2670.

Kovac, A. L. (2016). "Comparative Pharmacology and Guide to the Use of the Serotonin 5-HT3 Receptor Antagonists for Postoperative Nausea and Vomiting." Drugs 76(18): 1719-1735.

Kumar, S., P. Ranjan, et al. (2012). "Serotonin transporter gene (SLC6A4) polymorphism in patients with irritable bowel syndrome and healthy controls." Journal of Gastrointestinal Liver Disease 21(1): 31-38.

Langlois, A., X. Pascaud, et al. (1996). "Response heterogeneity of 5-HT3 receptor antagonists in a rat visceral hypersensitivity model." European Journal of Pharmacology 318(1): 141-144.

Layer, P., J. Keller, et al. (2007). "Tegaserod in the treatment of irritable bowel syndrome (IBS) with constipation as the prime symptom." Therapeutics and Clinical Risk Management 3(1): 107-118.

Lee, K. N. and O. Y. Lee (2016). "The Role of Mast Cells in Irritable Bowel Syndrome." Gastroenterology Research and Practice 2031480(10): 28.

Li, T. J., B. P. Yu, et al. (2004). "Ovarian hormone modulates 5-hydroxytryptamine 3 receptors mRNA expression in rat colon with restraint stress-induced bowel dysfunction." World Journal of Gastroenterology 10(18): 2723-2726.

Llorca-Torralba, M., G. Borges, et al. (2016). "Noradrenergic Locus Coeruleus pathways in pain modulation." Neuroscience 338: 93-113.

Lu, C. L., J. C. Hsieh, et al. (2009). "Estrogen rapidly modulates 5-hydroxytrytophan-induced visceral hypersensitivity via GPR30 in rats." Gastroenterology 137(3): 1040-1050.

Lu, C. L., J. C. Hsieh, et al. (2007). "Estrogen rapidly modulates mustard oil-induced visceral hypersensitivity in conscious female rats: A role of CREB phosphorylation in spinal dorsal horn neurons." American Journal of Physiology-Gastrointestinal and Liver Physiology 292(1): 14.

Lu, C. R., H. H. Willcockson, et al. (2005). "Ionotropic glutamate receptors are expressed in GABAergic terminals in the rat superficial dorsal horn." Journal of Comparative Neurology 486(2): 169-178.

Lummis, S. C. (2012). "5-HT(3) receptors." J Biol Chem 287(48): 40239-40245.

Machu, T. K. (2011). "Therapeutics of 5-HT3 receptor antagonists: current uses and future directions." Pharmacology and Therapeutics 130(3): 338-347.

Mangel, A. W. and A. R. Northcutt (1999). "Review article: the safety and efficacy of alosetron, a 5-HT3 receptor antagonist, in female irritable bowel syndrome patients." Alimentary Pharmacology & Therapeutics 2: 77-82.

Marino, M., P. Galluzzo, et al. (2006). "Estrogen signaling multiple pathways to impact gene transcription." Current Genomics 7(8): 497-508.

Matsumoto, K., M. W. Lo, et al. (2012). "Experimental colitis alters expression of 5-HT receptors and transient receptor potential vanilloid 1 leading to visceral hypersensitivity in mice." Laboratory Investigation 92(5): 769-782.

Mayer, E. A., S. Berman, et al. (2002). "The effect of the 5-HT3 receptor antagonist, alosetron, on brain responses to visceral stimulation in irritable bowel syndrome patients." Alimentary Pharmacology & Therapeutics 16(7): 1357-1366.

McEwen, B. S. and S. E. Alves (1999). "Estrogen actions in the central nervous system." Endocrine Reviews 20(3): 279-307.

Meleine, M. and J. Matricon (2014). "Gender-related differences in irritable bowel syndrome: potential mechanisms of sex hormones." World Journal of Gastroenterology 20(22): 6725-6743.

Millan, M. J. (2002). "Descending control of pain." Progress Neurobiology 66(6): 355-474.

Miquel, M. C., M. B. Emerit, et al. (1995). "Developmental changes in the differential expression of two serotonin 5-HT3 receptor splice variants in the rat." Journal of Neurochemistry 65(2): 475-483.

Miranda, A., S. Peles, et al. (2006). "Effects of the 5-HT3 receptor antagonist, alosetron, in a rat model of somatic and visceral hyperalgesia." Pain 126(1-3): 54-63.

Moloney, R. D., J. Sajjad, et al. (2016). "Estrous cycle influences excitatory amino acid transport and visceral pain sensitivity in the rat: effects of early-life stress." Biology of Sex Differences 7(33): 016-0086.

Morales, M., E. Battenberg, et al. (1998). "Distribution of neurons expressing immunoreactivity for the 5HT3 receptor subtype in the rat brain and spinal cord." Journal of Comparative Neurology 402(3): 385-401.

Morales, M. and S. D. Wang (2002). "Differential composition of 5-hydroxytryptamine3 receptors synthesized in the rat CNS and peripheral nervous system." Journal of

Neuroscience 22(15): 6732-6741.

Mulak, A., Y. Tache, et al. (2014). "Sex hormones in the modulation of irritable bowel syndrome." World Journal of Gastroenterology 20(10): 2433-2448.

Myers, B., J. Schulkin, et al. (2011). "Sex steroids localized to the amygdala increase pain responses to visceral stimulation in rats." Journal of Pain 12(4): 486-494.

Nakai, A., M. Diksic, et al. (2005). "The effects of the 5-HT3 antagonist, alosetron, on brain serotonin synthesis in patients with irritable bowel syndrome." Neurogastroenterology and Motility 17(2): 212-221.

Naliboff, B. D., S. Berman, et al. (2003). "Sex-related differences in IBS patients: central processing of visceral stimuli." Gastroenterology 124(7): 1738-1747.

Ness, T. J. and G. F. Gebhart (1988). "Colorectal distension as a noxious visceral stimulus: physiologic and pharmacologic characterization of pseudaffective reflexes in the rat." Brain Research 450(1-2): 153-169.

Olivier, J. D., M. G. Van Der Hart, et al. (2008). "A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders." Neuroscience 152(3): 573-584.

Oyama, T., M. Ueda, et al. (1996). "Dual effect of serotonin on formalin-induced nociception in the rat spinal cord." Neuroscience Research 25(2): 129-135.

Palecek, J., V. Paleckova, et al. (2002). "The roles of pathways in the spinal cord lateral and dorsal funiculi in signaling nociceptive somatic and visceral stimuli in rats." Pain 96(3): 297-307.

Papka, R. E. and M. Storey-Workley (2002). "Estrogen receptor-alpha and -beta coexist in a subpopulation of sensory neurons of female rat dorsal root ganglia." Neuroscience Letters 319(2): 71-74.

Papka, R. E., M. Storey-Workley, et al. (2001). "Estrogen receptor-alpha and beta-immunoreactivity and mRNA in neurons of sensory and autonomic ganglia and spinal cord." Cell and Tissue Research 304(2): 193-214.

Park, J. M., Choi, et al. (2006). "Serotonin transporter gene polymorphism and irritable bowel syndrome." Neurogastroenterology and Motility 18(11): 995-1000.

Rahimi, R., S. Nikfar, et al. (2008). "Efficacy and tolerability of alosetron for the treatment of irritable bowel syndrome in women and men: a meta-analysis of eight randomized,

placebo-controlled, 12-week trials." Clinical Therapeutics 30(5): 884-901.

Rahman, W., K. Bannister, et al. (2011). "A pronociceptive role for the 5-HT2 receptor on spinal nociceptive transmission: an in vivo electrophysiological study in the rat." Brain Research 25: 29-36.

Rybaczyk, L. A., M. J. Bashaw, et al. (2005). "An overlooked connection: serotonergic mediation of estrogen-related physiology and pathology." BMC Women's Health 5: 12.

Sanoja, R. and F. Cervero (2010). "Estrogen-dependent changes in visceral afferent sensitivity." Autonomic Neuroscience: Basic and Clinical 153(1-2): 84-89.

Saria, A., F. Javorsky, et al. (1990). "5-HT3 receptor antagonists inhibit sensory neuropeptide release from the rat spinal cord." Neuroreport 1(2): 104-106.

Sawynok, J. and A. Reid (1996). "Interactions of descending serotonergic systems with other neurotransmitters in the modulation of nociception." Behavioural Brain Research 73(1-2): 63-68.

Schmitt, A., R. Mossner, et al. (2003). "Organic cation transporter capable of transporting serotonin is up-regulated in serotonin transporter-deficient mice." Journal of Neuroscience Research 71(5): 701-709.

Sengupta, J. N. (2009). "Visceral Pain: The Neurophysiological Mechanism." 194: 31-74.

Simino, G. P., L. P. Marra, et al. (2016). "Efficacy, safety and effectiveness of ondansetron compared to other serotonin-3 receptor antagonists (5-HT3RAs) used to control chemotherapy-induced nausea and vomiting: systematic review and meta-analysis." Expert Review of Clinical Pharmacology 9(9): 1183-1194.

Simren, M., O. S. Palsson, et al. (2017). "Update on Rome IV Criteria for Colorectal Disorders: Implications for Clinical Practice." Current Gastroenterology Reports 19(4): 017-0554.

Sinchak, K. and E. J. Wagner (2012). "Estradiol signaling in the regulation of reproduction and energy balance." Frontiers in Neuroendocrinology 33(4): 342-363.

Song, Z., B. A. Meyerson, et al. (2011). "Spinal 5-HT receptors that contribute to the pain-relieving effects of spinal cord stimulation in a rat model of neuropathy." Pain 152(7): 1666-1673.

Spohn, S. N. and G. M. Mawe (2017). "Non-conventional features of peripheral serotonin signalling - the gut and beyond." Nature Reviews Gastroenterology & Hepatology 14(7): 412-420.

Su, X. and G. F. Gebhart (1998). "Mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat are polymodal in character." Journal of Neurophysiology 80(5): 2632-2644.

Sumner, B. E., K. E. Grant, et al. (2007). "Raloxifene blocks estradiol induction of the serotonin transporter and 5-hydroxytryptamine2A receptor in female rat brain." Neuroscience Letters 417(1): 95-99.

Tang, B., Y. Ji, et al. (2008). "Estrogen alters spinal NMDA receptor activity via a PKA signaling pathway in a visceral pain model in the rat." Pain 137(3): 540-549.

Thompson, A. J. and S. C. Lummis (2007). "The 5-HT3 receptor as a therapeutic target." Expert Opinion in Therapeutic Targets 11(4): 527-540.

Thompson, A. J., M. H. Verheij, et al. (2013). "Structure-activity relationships of quinoxaline-based 5-HT3A and 5-HT3AB receptor-selective ligands." ChemMedChem 8(6): 946-955.

Todd, A. J. (2010). "Neuronal circuitry for pain processing in the dorsal horn." Nature Reviews of Neuroscience 11(12): 823-836.

Tran, M., J. A. Kuhn, et al. (2017). "Neuronal aromatase expression in pain processing regions of the medullary and spinal cord dorsal horn." Journal of Comparative Neurology 525(16): 3414-3428.

Vanderhorst, V. G., J. A. Gustafsson, et al. (2005). "Estrogen receptor-alpha and -beta immunoreactive neurons in the brainstem and spinal cord of male and female mice: relationships to monoaminergic, cholinergic, and spinal projection systems." Journal of Comparative Neurology 488(2): 152-179.

Viramontes, B. E., M. Camilleri, et al. (2001). "Gender-related differences in slowing colonic transit by a 5-HT3 antagonist in subjects with diarrhea-predominant irritable bowel syndrome." The American Journal of Gastroenterology 96(9): 2671-2676.

Vogel, C., R. Mossner, et al. (2003). "Absence of thermal hyperalgesia in serotonin transporter-deficient mice." Journal of Neuroscience 23(2): 708-715.

Wierman, M. E. (2007). "Sex steroid effects at target tissues: mechanisms of action." Advances in Clinical and Experimental Medicine 31(1): 26-33.

Wolf, H. (2000). "Preclinical and clinical pharmacology of the 5-HT3 receptor antagonists." Scandinavian Journal of Rheumatology Suppl 113: 37-45.

Xu, D., X. Wu, et al. (2013). "Butyrate-induced colonic hypersensitivity is mediated by mitogen-activated protein kinase activation in rat dorsal root ganglia." Gut 62(10): 1466-1474.

Xu, X. J., D. Roberts, et al. (2016). "Competitive antagonists facilitate the recovery from desensitization of alpha1beta2gamma2 GABAA receptors expressed in Xenopus oocytes." Acta Pharmacologica Sinca 37(8): 1020-1030.

Yamamoto, C., H. Murakami, et al. (2002). "Contribution of P-glycoprotein to efflux of ramosetron, a 5-HT3 receptor antagonist, across the blood-brain barrier." Journal of Pharmacy and Pharmacology 54(8): 1055-1063.

Yeo, A. (2004). "Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women." Gut 53(10): 1452-1458.

Yoshimura, M. (2006). "Plastic changes of the descending serotonergic system in the spinal dorsal horn." Current Anaesthesia & Critical Care 17(1): 3-8.

Zagorodnyuk, V. P., M. Kyloh, et al. (2011). "Loss of visceral pain following colorectal distension in an endothelin-3 deficient mouse model of Hirschsprung's disease." Journal of Physiology 589(Pt 7): 1691-1706.

Zeitz, K. P., N. Guy, et al. (2002). "The 5-HT3 subtype of serotonin receptor contributes to nociceptive processing via a novel subset of myelinated and unmyelinated nociceptors." Journal of Neuroscience 22(3): 1010-1019.

Zheng, Y., T. Yu, et al. (2017). "Efficacy and safety of 5-hydroxytryptamine 3 receptor antagonists in irritable bowel syndrome: A systematic review and meta-analysis of randomized controlled trials." PLoS One 12(3).