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Review

Animal models of visceral pain and the role of the microbiome

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ABSTRACT

Visceral pain refers to pain arising from the internal organs and is distinctly different from the expression and mechanisms of somatic pain. Diseases and disorders with increased visceral pain are associated with significantly reduced quality of life and incur large financial costs due to medical visits and lost work productivity. In spite of the notable burden of illness associated with those disorders involving increased visceral pain, and some knowledge regarding etiology, few successful therapeutics have emerged, and thus increased attention to animal models of visceral hypersensitivity is warranted in order to elucidate new treatment opportunities.

Altered microbiota-gut-brain (MGB) axis communication is central to the comorbid gastrointestinal/psychiatric diseases of which increased visceral (intestinal) sensitivity is a hallmark. This has led to a particular focus on intestinal microbiome disruption and its potential role in the etiology of heightened visceral pain. Here we provide a review of studies examining models of heightened visceral pain due to altered bidirectional communication of the MGB axis, many of which are conducted on a background of stress exposure. We discuss work in which the intestinal microbiota has either been directly manipulated (as with germ-free, antibiotic, and fecal microbial transplantation studies) or indirectly affected through early life or adult stress, inflammation, and infection. Animal models of visceral pain alterations with accompanying changes to the intestinal microbiome have the highest face and construct validity to the human condition and are the focus of the current review.

1. Introduction

Visceral pain refers to pain arising from the internal organs or 'viscera' and is distinct from somatic pain in terms of the neurological mechanisms and perception of pain processing (Cervero and Laird, 1999; Cervero, 2000). While increased pain perception can occur at many regions of the viscera, for the purposes of this review we are concerned with that affecting the gastrointestinal (GI) tract specifically. GI pain shares main features with all other types of visceral pain. Namely, five characteristics distinguish visceral pain from other types of pain: not all internal organs evoke pain, pain is not always linked to injury of the viscera, the pain is poorly localized and diffuse, pain is referred or perceived in a location away from its origin, and the pain is accompanied by motor and autonomic reflexes like vomiting and nausea that may serve as a warning (Cervero and Laird, 1999).

Functional gastrointestinal disorders (FGID) are the most prevalent disorders underlying the experience of visceral pain (Sikander and Dickenson, 2012; Drewes et al., 2020). FGID is diagnosed by characteristic GI symptoms occurring for a period of at least 3 months in the absence of any obvious organic cause (Thompson et al., 1998). Included in this is irritable bowel syndrome (IBS) which affects 1.1–29.2% of the general population and underlies a significant proportion of abdominal symptoms and pain in patients seeking medical care (Oshima and Miwa, 2015; Enck et al., 2016). Visceral pain is also a hallmark of inflammatory bowel disorders (IBD), with this symptom affecting up to 70% of young adult IBD patients (Wagtmans et al., 1998; Zeitz et al., 2016) during active inflammation at the onset of disease and relapse, and 30–50% of patients with no discernible inflammation during remission (Bielefeldt et al., 2009). While pain can initially be a consequence of inflammation due to the induction of pro-inflammatory cytokines that sensitize primary afferent sensory neurons, it is more challenging to explain visceral hypersensitivity that can remain post-inflammation and during remission (Bielefeldt et al., 2009). In these cases visceral hypersensitivity is characterized as persisting even after 4–6 weeks of recovery and in the absence of obvious inflammation (Esquerre et al., 2020; Johnson et al., 2020). Given then the prevalence

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of gut-related pain disorders, and that much is still unknown regarding etiology and pathophysiology particularly when pain occurs in the absence of overt organic indicators (Farrell et al., 2014), the need for appropriate animal models is clearly indicated. Traditional animal models of visceral pain can fall into two broad categories, as summarized by Mayer and Collins (2002): visceral pain triggered by central targeted stimuli (neonatal stress and post-traumatic stress disorder) or peripherally targeted stimuli (infection and inflammation). Given our more recent understanding of the microbiota-gut-brain axis (Foster and McVey Neufeld, 2013; Margolis et al., 2021), current animal models may involve direct manipulation of intestinal bacteria.

FGID are often comorbid with psychological disorders (Wu, 2012). Many of these patients experience a reduced health-related quality of life associated with both FGID-like symptoms (including those with inactive IBD) and mood disorders (Farrokhyar et al., 2006; Piche et al., 2010). The interplay between the intestinal microbiome and the brain is a factor in both the development and the management of these disorders and their comorbidities (Defaye et al., 2020). It is now recognized that the microbiota-gut-brain axis is central to the comorbid gastrointestinal/psychiatric diseases of which increased visceral sensitivity is a hallmark (Foster and McVey Neufeld, 2013; Pusceddu and Gareau, 2018). Looking with hindsight and new knowledge of the many factors that impact intestinal microbial status, we can now appreciate that the majority of animal models of altered visceral pain also demonstrate changes to the intestinal microbiome. Even more persuasively we now know that feeding both pre- and pro-biotics can attenuate visceral pain in rodents (Liebregts et al., 2005; Johnson et al., 2011; Kannampalli et al., 2014). Moving forward, the most comprehensive models of visceral pain alterations will actively demonstrate changes to the intestinal microbiome, and these are the focus of the current review. Here we will detail animal models of visceral pain that also demonstrate alterations to the intestinal microbiome, alterations resulting from both direct and indirect manipulation. We begin with a brief discussion of the methods for measuring visceral pain in rodent models, then go on to discuss studies that directly manipulate the intestinal microbiota, using germ-free mice, antibiotic treated mice, and fecal microbial transplantation studies. Animal models that indirectly result in altered intestinal bacteria will also be discussed such as early life and adult stress studies, as well as both inflammation and infection studies.

2. Measuring visceral pain in animal models

Typically, visceral pain is assessed both clinically (Bouin et al., 2002; Drewes et al., 2020) and in animal models (Moloney et al., 2015) using colorectal distention (CRD), which involves the phasic distention of a balloon in the colorectal cavity, while monitoring pressure with a barostat, to determine pain sensation, tolerance, and threshold. In the laboratory, physical distension of the colon in rodents is a mechanical stimulus thought to closely replicate the human experience of visceral pain and its pattern of referral (Ness and Gebhart, 1988; Christianson and Gebhart, 2007) and has largely replaced other methods of inducing pain such as injection of irritant chemicals. (For a summary of the advantages and disadvantages of different methods of assessing rodent visceral pain, please see Table 1.) Measurable responses to noxious intensities of CRD can be assessed such as increases in heart rate, respiration and blood pressure (Christianson and Gebhart, 2007). Further, CRD is thought to be a preferable method of assessing GI pain as it mimics a natural stimulus and is restricted to the viscera itself (Christianson and Gebhart, 2007). This technique has thus been extremely useful in providing a quantifiable and reproducible benchmark when modelling disorders involving visceral hypersensitivity. Humans exposed to CRD in clinical trials report the sensation of pain once certain threshold distensions are exceeded, while rodents exposed to CRD display cardiovascular responses such as pressor response and tachycardia that are graded with increasing intensities of distension. Both of these responses are thought to be due to increased sympathetic and decreased parasympathetic outflow. In addition, electophysiologic studies have demonstrated graded responses in the spinal neurons after balloon distension in rats (Ness and Gebhart, 1987). In more recent years, CRD has been successfully paired with brain imaging in order to

Table 1
Advantages and Disadvantages of Techniques for Measuring Visceral Pain in Animal Models.

Method to Induce Visceral Pain for Measurement	Description	Advantages	Disadvantages
Colorectal Distension (CRD)	A balloon is inserted via the anus to the distal colon of the rodent under sedation and then pressure is applied in an ascending stepwise fashion via a customized barostat. Repeatable air inflation and pressures can be applied to the distal colon region.	• Closely replicates human experience of visceral pain (Christianson and Gebhart 2007) • Used for high throughput studies • Relatively simple • Widely accepted	
Response to Colonic Instillation of Algesic Substances	Algesic compounds such as acetic acid, capsaicin, mustard oil, or zymosan are applied intracolonically	• Simple to administer • Used for high throughput studies	• Poor reproducibility • Questionable relationship to human pathology • Long-lasting and inescapable pain
Technique for Measuring Visceral Pain			
Electromyography	Quantifies magnitude of abdominal contractions in response to CRD or colonic instillation of algesic substances	• Quantifies the visceromotor response • Non-invasive	
Manometry	Measures pressure and pattern of muscle contractions in visceral organs in response to CRD or to evaluate analgesic substances. In CRD, monitors pressure changes within the descending balloon (Arvidsson et al., 2006).	• Reliable noninvasive, non-surgical method in mice (Arvidsson et al., 2006).	
Brain Imaging in Response to CRD (microPET, fMRI	Examination of pain processing in the brain in response to CRD	• Non-invasive (Lazovic et al., 2005; Johnson et al., 2010; Wouters et al., 2012) • Allows examination of the brain in live animals	• Expensive • Requires specialized equipment/expertise
Abdominal Withdrawal Reflex and other visually assessed rodent pain behaviours	AWR is an involuntary motor reflex in response to CRD. The animal is graded on a scale ranging from immobility, to mild contraction of the abdomen to severe contraction including body arching and lifting of the pelvis (Al-Chaer et al., 2000)	• Does not require surgery like some measures of the visceromotor reflex (O'Mahony et al., 2012)	• Labour intensive • Time consuming • Lacks objectivity and reproducibility (Regmi and Shah 2020)

*This is also well reviewed in Regmi and Shah 2020 (doi: https://doi.org//10.1002/ame2.12130).

assess central responses to painful stimuli (Lazovic et al., 2005; Wang et al., 2008; Johnson et al., 2010; Wouters et al., 2012). CRD has been used extensively to evaluate drug efficacy, and to investigate differences in animal strain and sex following various manipulations designed to increase distal colon sensitivity including the effects of stressors and intestinal microbial manipulation (O'Mahony et al., 2012). In brief, under sedation a balloon is inserted via the anus to the distal colon of the rodent and then pressure is applied in an ascending stepwise fashion via a customized barostat. In this manner consistent, repeatable air inflation and pressures can be applied to the distal colon region, and animal responses can be assessed (see Table 1). This can be done quantitatively via heart rate or visceromotor (electromyographic recordings of the abdominal muscles) responses, or alternately in a more qualitative manner by visually assessing rodent pain behaviours by a trained, blinded scorer. Visual assessment involves observation of abdominal withdrawal reflexes which include total abdominal withdrawal and stretching behaviours (O'Mahony et al., 2012). Much of the data collected on visceral sensitivity in the animal models discussed below has been collected through the use of CRD and is summarized in Table 2.

3. Intestinal microbial disruption - Germ-free and antibiotic treated mice.

Disrupting the intestinal microbiota through the use of germ-free (GF) (Luczynski, 2017) and antibiotic (AB) treated rodents (Verdu et al., 2006; Hoban et al., 2016) has provided useful animal models of altered pain perception. Microbial disruption either from the beginning of life (as in the case of GF animals) or at various timepoints throughout development and into adulthood (as with AB treatment regimes) results in changes to visceral pain sensitivity. GF rodents are those bred and maintained in the complete absence of microbial exposure and input. As a result these animals have demonstrated changes to immune, brain, gut and metabolic systems (Sudo et al., 2004; Backhed et al., 2005; Hooper and Macpherson, 2010; Tremaroli and Backhed, 2012; Heijtz et al., 2011; Neufeld et al., 2011; Luczynski et al., 2016). GF mice demonstrate profound disturbances in immune function, with absent lymphoid architecture, significantly decreased IgA antibodies, and T cell deficiencies as examples. For an excellent review on the interplay between the microbiota and immune system please see Zheng et al., 2020 and Round and Mazmanian, 2009. Interestingly, these mice also exhibit heightened visceral pain sensitivity (Luczynski, 2017). In a recent study, pain responses of GF mice were compared to conventionally colonized control mice following CRD, with GF animals showing significantly increased visceromotor responses to the painful stimuli, as well as significantly lower pain thresholds. In addition, GF mice showed significantly increased mRNA gene expression in spinal cord tissue of several Toll-like receptors (TLR) and cytokines related to pain response (Luczynski, 2017). When brain tissue was examined, the GF mice exhibited volume reductions in the anterior cingulate cortex and increases in the periaqueductal grey, brain regions implicated in the emotional component of pain and inhibition of pain respectively. Importantly, when adult GF mice were colonized with bacteria from control animals, visceral pain hypersensitivity to CRD was normalized with a concomitant normalization of some TLR and cytokine gene expression in the spinal cord thus demonstrating the importance of the intestinal microbiota in the normal perception of pain.

It is important to note that aside from the obvious advantages of GF mouse models in allowing us to ascertain a role for the microbiota in visceral pain, there are certain disadvantages to this model as well. GF models have been criticized for their evident lack of clinical validity and for their distinct physiological deficits including but not limited to immune and metabolic functioning. For a more in-depth discussion of the strengths and weaknesses of the GF model, we direct the reader to a comprehensive review by Luczynski et al., 2016. As a result of the limitations of GF models in teasing apart mechanisms of action of the microbiota-gut-brain axis in etiology and pathophysiology of disorders, there has been a move to more clinically relevant experimental manipulations of gut bacteria disruption, with many focusing instead on AB treated animals.

In early AB work, adult mice gavaged with a combination of non-absorbable antibiotics developed visceral hypersensitivity to CRD, increased myeloperoxidase activity and substance P immunoreactivity in the colon, with a concomitant disruption of the intestinal microbiota (Verdu et al., 2006). Feeding probiotic *Lactobacillus paracasei* in combination with the antibiotic cocktail normalized both visceral hypersensitivity and substance P reactivity but did not restore the intestinal microbiota (Verdu et al., 2006). In support of these findings, a separate study in infant rats has also demonstrated that feeding either the single antibiotic vancomycin or an antibiotic cocktail from postnatal days 4–13 resulted in long-term heightened visceral pain sensitivity as measured by CRD in adult male rats (O'Mahony et al., 2014), which was accompanied by changes to mRNA expression of pain-related receptors in the lumbo-sacral region of the spinal cord. This study is of note as while the antibiotics were delivered for a finite period of time during early life, the measures of visceral hypersensitivity were observed later in adulthood following a long period of no antibiotic exposure.

In contrast, some antibiotic studies have shown decreased visceral sensitivity following treatment. One such study by Hoban *et al.*, showed that feeding adult male rats a cocktail of antibiotics via drinking water resulted in a number of cognitive deficits as well as decreased visceral sensitivity, with higher balloon pressures required in order for pain behaviours to be displayed (Hoban et al., 2016). Similarly, another study has shown that treatment of adult mice with broad-spectrum antibiotics for two weeks resulted in bacterial disruption characterized by increased *Bacteroides* spp, *Clostridium coccoides*, and *Lactobacillus* spp and reduced *Bifidobacterium* spp (Aguilera et al., 2015). Visceral pain-related responses to intraperitoneal acetic acid and intracolonic capsaicin were attenuated in antibiotic-treated mice, as measured by visceral pain related behaviours such as licking and stretching of the abdomen (Aguilera et al., 2015). While this work contrasts with reports that antibiotic treatment may increase visceral hypersensitivity, it should be noted that visceral pain was induced in this particular study via application of a chemical stimulus vs mechanical colorectal distension.

It is important to note that many studies have used pre- and probiotics feeding in order to demonstrate the potential involvement of intestinal bacterial change in altered visceral pain sensitivity (Larauche et al., 2012; Kannampalli et al., 2014; Wang et al., 2017; O'Mahony et al., 2020). While these types of feeding studies are not animal models of visceral pain per se, they are often used on a background of various animal models of increased visceral pain such as the AB studies discussed above, or the stress and inflammation studies which will be discussed below. For an excellent summary of probiotic feeding and its impact of visceral pain perception we direct the reader to a recent review by Lomax et al., 2019.

4. Fecal microbial transfer and visceral pain

An intriguing study involving fecal microbial transfer (FMT) from human to rat has furthered the evidence that visceral sensitivity could be in part programmed by the resident intestinal microbiota. This hallmark study found that inoculating GF rats with fecal microbiota from IBS patients, either with or without hypersensitivity to CRD, resulted in a transfer of increased sensitivity to distension in those animals colonized with microbiota from the hypersensitive group (Crouzet et al., 2013). Further, the researchers found that the ultimate microbial composition established in the recipient rats closely resembled the donor human fecal microbiota, and remained so for the 7-week study duration. There were no specific alterations to gastrointestinal mucosa observed in recipient rats and so the authors speculated that hypersensitivity was conferred via bacterial metabolites.

Table 2
Review of Animal Models of Visceral Hypersensitivity and Effects on the Microbiota.

Animal Models of Visceral Hypersensitivity	Visceral Pain Assessed by:	Antibiotics	Probiotics	Alterations to Microbiome	Effect on Visceral Pain
Post-Inflammatory Models					
Dextran sodium sulfate (DSS) to induce colitis (Esquerre et al., 2020)	CRD	Mice treated with 2.5% DSS in drinking water for 5 days or controls recovered for 5 weeks. Both control and DSS mice were treated with antibiotics (ampicillin 1 g/L, Neomycin 1 g/L, Vancomycin 0.5 g/L, and Metronidazole 1 g/L) for the last 2 weeks of recovery	N/A	FMT of DSS-recovered stool into antibiotic treated mice induced VH. FMT of control stool reversed antibiotic induced VH. Post-DSS mice had increased SCFA-producing bacteria.	DSS mice developed VH. Antibiotics during DSS recovery induced VH independent of inflammation.
5% v/v colorectal TNBS vs. maternal stress (Zhou, 2016)	Abdominal withdrawal reflex and electromyography in response to CRD	N/A	N/A	Fecal microbiota dysbiosis occurred in both models. Fusobacterium increased in the MS group and Clostridium XI increased in the TNBS rats	Both models developed VH
TNBS (Song et al., 2020)	Abdominal withdrawal reflex	N/A	N/A	Greater abundances of *Empedobacter, Psychrobacter, Enterococcus, Butyricimonas, Vampirovibrio, Kurthia, Intestinimonas, Neisseria, Falsiporphyromonas, Bilophila, Fusobacterium, Alistipes, Veillonella, Flavonifractor,* and *Clostridium XlVa* compared to controls	TNBS rats had greater VH. VH to CRD was decreased after electroacupuncture.
Mustard oil (Wang et al., 2018)	AWR and CRD	N/A	N/A	Higher relative abundance of *Bacteroidetes* and lower relative abundance of *Firmicutes* at phylum level and elevated relative abundance of *Prevotella, Bacteroides, Barnesiella, Paraprevotella, Clostridium XI* and *Sphingomonas* at genus level	Model rats had increased AWR scores. Moxibustion treatment reduced AWR scores and reversed changes in microbiota profiles.
Intracolonic TNBS (Johnson et al., 2011)	CRD	N/A	Oral *Bifidobacterium infantis* 35,624	N/A	Probiotic administration normalized VH produced by TNBS
Intracolonic zymosan for 3 days in neonatal rats (Kannampalli et al., 2014)	VMR to CRD	N/A	*Lactobacillus rhamnosus* GG	N/A	Probiotic administrastion attenuated chronic visceral pain
TNBS (Liebregts et al., 2005)	Visceromotor reflex to CRD	N/A	*Escherichia coli Nissle* 1917 in drinking water	N/A	Developed hyperalgesia that was attenuated by probiotic treatment
DSS (Wang et al., 2020)	Colitis assessed by weight loss, colon shortening, and histopathological damage	N/A	*Lactobacillus reuteri* I5007 given before and during DSS treatment	Altered colonic microbiota composition	Probiotic improved DSS-induced colitis
Post-Infectious Models					
*Trichenella spiralis (*Bai et al., 2018)	Abdominal withdrawal reflex	N/A	*Bifidobacterium longum* HB55020 for 7 days	Gavage with fecal microbiota for 7 days	B. longum mice had a higher pain threshold as did fecal microbiota-treated mice
Stress-Induced Models					
2-hr partial restraint stress (Ait-Belgnaoui et al., 2006)	CRD	N/A	10^11 CFU/day *L. farciminis* orally over 15 days	N/A	Prevented stress induced hypersensitivity and prevented colonic paracellular permeability
Repeated water avoidance (WA) stress for 10 consecutive days (Fourie et al., 2017)	Not tested	N/A	N/A	WAS treated rats exhibit higher alpha-diversity and increased beta-diversity than unstressed controls, including specific increases in *Proteobacteria*	WA stressor previously shown to increase VH
Chronic unpredictable mild stress (CUMS) paired with TNBS (Ma et al., 2019)	CRD	N/A	N/A	Significant disturbance of gut microbiota	Heightened VH
Early Life Stress-Induced Models					
Neonatal maternal separation for 3 h/d on postnatal days	Abdominal muscle electromyography to CRD	N/A	Bifidobacterium lactis NCC362, Lactobacillus johnsonii NCC533, or	MS enhanced gut paracellular permeability	MS significantly increased colonic VH to CRD. Only Lactobacillus paracasei

(*continued on next page*)

Table 2 (*continued*)

Animal Models of Visceral Hypersensitivity	Visceral Pain Assessed by:	Antibiotics	Probiotics	Alterations to Microbiome	Effect on Visceral Pain
2–14 (Eutamene et al., 2007)			Lactobacillus paracasei NCC2461 for 2 weeks		NCC2461 improved MS-induced VH
Neonatal maternal separation for 3 h/day on postnatal days 2-12_(O'Mahony et al., 2009)	Behavioural response to CRD	N/A	N/A	MS induced changes to the microbiome as compared to unstressed controls	Maternal separation resulted in colonic VH to CRD
Neonatal maternal separation for 3/day on postnatal days 2–14 (Distrutti et al., 2013)	Behavioural response to CRD	N/A	VSL#3 for 12–57 days	N/A	VSL#3 reversed the hypersensitivity and allodynia induced by MS
Neonatal maternal separation for 3 h/day on postnatal days 2–12 (O'Mahony et al., 2020)	Behavioural response to CRD	N/A	Milk fat globule membrane (MFGM) and polydextrose/galacto-oligosaccharide prebiotic blend provided from postnatal day 21	MS induced microbial dysbiosis at a family level. MFGM, prebiotic blend or combo influenced abundance at family and genus levels and beta-diversity levels	MS-induced VH was ameliorated by MFGM and the combination
Antibiotic-Treated Models					
Non-absorbable antibiotics for 10 days (Verdu et al., 2006)	CRD	Mice treated with a mixture of bacitracin (4 mg/ml), neomycin (4 mg/ml), and primaricin (0.2 g/ml) by gavage for 5 days, then reduced to 2 mg/ml for bacitracin and neomycin and 0.1 g/ml for primaricin for an additional 5 days	100 μL of 10E10 L paracasei NCC2461/ml for 10 days by oral gavage	Antibiotic combination disrupted the microbiome	Mice gavaged with antibiotic combination developed VH to CRD. Feeding of probiotic with antibiotic cocktail normalized VH but did not restore intestinal microbiota.
Single antibiotic vancomycin or antibiotic cocktail postnatal (O'Mahony et al., 2014)	CRD	Infant rats treated with a single antibiotic vancomycin or a cocktail of bacitracin, neomycin, and primaricin from post-natal days 4–13	N/A	Microbiota was significantly altered by Vancomycin but was restored by 8 weeks of age	Long-term heightened visceral pain sensitivity in adulthood, following a long period of no antibiotic exposure
Antibiotic cocktail via drinking water (Hoban et al., 2016)	CRD	Rats treated by antibiotic cocktail in drinking water	N/A	Antibiotic-induced gut dysbiosis results in deficits in spatial memory and increased depressive-like behaviour	Reduced visceral sensitivity after antibiotic treatment requiring higher balloon pressures to produce pain behaviours
Broad-spectrum antibiotics for two weeks (Aguilera et al., 2015)	Response to algesic substance	Mice treated with mixture of Bacitracin A and Neomycin (0.4 mg/mouse/day) (Amphotericin B included to prevent yeast overgrowth; 0.1 mg/mouse/day) for 14 days by oral gavage	N/A	Colonic dysbiosis-increase Bacteroides spp, Clostridium coccoides, and Lactobacillus spp and reduction in Bifidobacterium spp	Visceral pain related responses attenuated in antibiotic treated mice

See Anthony et al. 2020 for an excellent review of the advantages and disadvantages of these models.
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5. Early life stress and lifelong changes to visceral pain perception

While previously discussed work involved direct manipulation of the intestinal microbiome to provide models of visceral pain sensitivity, some experimental conditions result in indirect changes to the intestinal bacteria with corresponding changes to visceral pain responses. One such example is early life stress in rodents, such as that induced by maternal separation, which is known to result in long-term behavioural and physiological changes to offspring (O'Mahony et al., 2011). Maternal separation is a well-established model of early life stress and involves removing the rodent dam from offspring for a series of consecutive days for a constant period of time. Typically, the dam is removed from the home cage, and pups are then also removed and put into clean, new housing containers over heated blankets in an experimental room. The dam is returned alone to the housing room, with separation usually occuring for a few hours. This is repeated typically for 10 days, however timing for separations can vary between experiments.

A number of studies have now demonstrated that maternal separation in rodents results in adult offspring demonstrating long-term visceral hypersensitivity (Coutinho et al., 2002; Schwetz et al., 2005; Ren et al., 2006 with accompanying intestinal microbial alterations (Eutamene et al., 2007; O'Mahony et al., 2009, 2020; Distrutti et al., 2013; De Palma et al., 2015). Previous work has shown that animals exposed to neonatal stress through maternal separation demonstrate altered gene expression in both the colon and spinal cord that could be linked to pain (Distrutti et al., 2013; McVey Neufeld et al., 2020). Microarray examination paired with functional analysis revealed that less than 3% of colonic genes showed up- or down-regulated mRNA gene expression in pups exposed to maternal separation, with many of these genes falling into the category of metabolism or pain/inflammation categories. Furthermore, feeding probiotic VSL#3 was able to counter-regulate expression of many of these genes in the pain/inflammation category (Distrutti et al., 2013). In addition to microbial change and visceral hypersensitivity, rats that have been subjected to neonatal maternal separation show a variety of long-term cognitive and behavioural

deficits, including increased anxiety behaviour, stress hyper-responsivity, altered brain neurochemistry and gastrointestinal dysfunction (O'Mahony et al., 2011). Combined, these deficits make them an ideal model for many of the comorbid gastrointestinal and psychiatric disorders involving a disrupted microbiota-gut-brain axis.

6. Adult stress and changes to visceral pain

Stress occurring later in life has also been shown to affect visceral pain perception, and when used in conjunction with certain genetic rodent models bred for high anxiety-like behaviour, has been useful for modelling pain disorders with associated changes to microbiome. Studies make use of physical stress, such as acute or chronic restraint stress or psychological water avoidance stress (WAS) as well as chronic unpredictable mild stress (CUMS), and have all been shown to result in increased pain sensitivity in rodents as well as alterations to the intestinal microbiota. WAS is perhaps the most widely used adult rodent stressor in models of visceral sensitivity (Bradesi et al., 2005; Hong et al., 2009; Larauche et al., 2008) and exposure of male Wistar rats to one hour of WAS has been shown to result in increased visceral sensitivity 24 h but not immediately following stress exposure with accompanying increased activation of corticotropin-releasing factor type 1 receptor signaling (Schwetz et al., 2004). WAS has also been employed on a more chronic scale, with animals subjected to the stressor for 10 consecutive days, and again findings show heightened visceral pain responses follow stress exposures (Larauche et al., 2008). A more recent study examining intestinal microbial alterations in rats exposed to WAS has shown that stressed rats exhibit higher alpha-diversity and increased beta-diversity as compared to unstressed control rats. In addition, WAS rats showed specific increases in *Proteobacteria* and decreases in bacteria responsible for energy- and lipid-metabolism (Fourie et al., 2017). In a study combining acute restraint stress, colorectal distension and probiotic feeding, researchers fed female Wistar rats the beneficial bacteria *Lactobacillus farciminis* for 15 days before subjecting them to a 2 hr partial restraint stress, following by CRD. They found that while restraint stress significantly increased VMR responses to CRD, feeding probiotic for 15d prior to stress resulted in an attenuation of stress-induced hyperresponsivity (Ait-Belgnaoui et al., 2006). CUMS has similarly been used to successfully induce visceral hypersensitivity in susceptible genetic strains of rat, most recently coupled with a gut inflammatory stimulus. Treated rats demonstrated increased depressive-like behaviour, heightened visceral sensitivity, increased corticotropin hormone receptor expression in the brain, significantly increased plasma stress hormones and altered intestinal microbiota (Ma et al., 2019).

7. Animal models of post-inflammatory visceral pain

The microbiome is a critical component in the development of post-inflammatory visceral hypersensitivity. Several animal models have been useful in demonstrating the relationship between previous inflammation, perturbations to the microbiome, and alterations in visceral pain response. The most widely used chemically induced post-inflammatory animal models in the literature are established models for IBD and post-infectious IBS. These models employ the use of trinitrobenzene sulfonic acid (TNBS), dextran sulphate sodium (DSS), oxazolone, or other irritants to induce colitis that mimics the pathognomonic morphology, histopathology, and visceral pain observed in IBD (Randhawa et al., 2014). Colonic inflammation lasts for several weeks after intracolonic TNBS or DSS causing immunological dysfunction and chronic microbial perturbations that contribute to visceral hypersensitivity.

The role of the microbiome in post-inflammatory animal models of visceral pain has been investigated by both the improvement or exacerbation of visceral hypersensitivity by FMT and through microbiome sequencing. FMT from DSS-treated mice increased visceral hypersensitivity in already sensitized antibiotic-treated mice, while control stool reversed this, indicating that the microbiome plays a substantial role in post-inflammatory visceral pain (Esquerre et al., 2020). This was attributed to an increase in fecal acetate/butyrate from short-chain fatty acid (SCFA)-producing bacteria in the DSS model and which increased sensitivity of nociceptive neurons (Esquerre et al., 2020). It should be noted that the data on the role of SCFAs on visceral pain is conflicting, with some studies arguing for an exacerbation of pain in presence of SCFAs (Xu et al., 2013) and others a reduction (Russo et al., 2016).

Several studies have characterized perturbations in microbial diversity in post-inflammatory models of visceral pain sensitivity. Zhou (2016) compared maternal stress and post-TNBS (5% v/v colorectal) inflammatory rat models and showed that while both models developed comparable levels of visceral hypersensitivity, fecal microbial diversity (as measured by the Shannon index and the inverse Simpson index) was reduced and time-variable in only the maternal stress model. However, compared to human IBS patient sequencing data, *Clostridium XI* colonized at higher levels in both the human IBS group and the TNBS rats (Zhou, 2016). In another model of TNBS-induced visceral hypersensitivity, model rats exhibited increased levels of IL-18 and altered microbiome diversity, with greater abundances of *Empedobacter, Psychrobacter, Enterococcus, Butyricimonas, Vampirovibrio, Kurthia, Intestinimonas, Neisseria, Falsiporphyromonas, Bilophila, Fusobacterium, Alistipes, Veillonella, Flavonifractor,* and *Clostridium XlVa* compared to controls (Song et al., 2020). Another post-inflammatory model of visceral hypersensitivity induced by mustard oil resulted in a higher relative abundance of *Bacteroidetes* and lower relative abundance of *Firmicutes* at phylum level and elevated relative abundance of *Prevotella, Bacteroides, Barnesiella, Paraprevotella, Clostridium XI* and *Sphingomonas* at genus level (Wang et al., 2018).

Greater abundance of *Clostridium XI* appears to be a shared biomarker among post-inflammatory models of visceral hypersensitivity. Interestingly, in an analysis of gut microbial composition from IBS patients, there was an increased relative abundance of *Firmicutes* taxa, including *Bacilli* and *Clostridia* at the class level (Labus et al., 2017). *Clostridia Cluster IV* and *XIVa* and other specific spore-forming bacteria have been found to induce synthesis of serotonin by enterochromaffin cells in the colon (Yano et al., 2015). Indirect associations involving connectivity of subcortical brain regions using tripartite network analysis were found between *Clostridium XIVa* from healthy controls and several visceral sensitivity measures, such as rectal discomfort threshold and intensity, rectal pain threshold and pain during the nutrient and lactulose challenge test, which were not found in IBS groups (Labus et al., 2019). This finding suggested that *Clostridium XIVa* may influence the respective subcortical regions which contributes to visceral sensitivity and pain in IBS (Labus et al., 2019). However, it should be noted that *Clostridium* are a prominent species of commensal bacteria with probiotic potential, producing large amounts of SCFAs and influencing intestinal homeostasis (Guo et al., 2020).

As previously touched upon, targeting of the microbiota by specific probiotic bacteria administration, or pharmabiotics, can improve post-inflammatory visceral pain responses. For example, oral *Bifidobacterium infantis* 35624 normalized the sensitivity to colorectal distention produced by intracolonic TNBS (Johnson et al., 2011). Furthermore, treatment with *Lactobacillus rhamnosus* GG attenuated chronic visceral pain in neonatal rats that received intracolonic zymosan for three days (Kannampalli et al., 2014). In another study, rats previously instilled with TNBS developed hyperalgesia that was reduced by *Escherichia coli* Nissle 1917 in drinking water (Liebregts et al., 2005). Beneficial resident bacteria can also alter the microbiome to improve inflammatory outcomes. *Lactobacillus reuteri* I5007 given before and during DSS treatment altered colonic microbiota composition and improved colitis (Wang et al., 2020). Overall it is important to recognize that the microbiome is an ever-changing ecosystem with potential to modulate intestinal function and sensation for better or worse.

8. Animal models of post-infectious visceral pain

Visceral hypersensitivity can also be induced following acute gastrointestinal infection, leading to post-infectious IBS. Post-infectious animal models are produced by infection with pathogenic bacteria like *Campylobacter* or *Salmonella* strains, or parasites such as *Trichinella spiralis* (systematically reviewed in (Qin et al., 2011)). These models also develop transient inflammation and visceral hypersensitivity that persists after inflammation has resolved (Bercik et al., 2004; Long et al., 2010) and thus can be referred to as post-inflammatory models in the literature (Keating et al., 2011). One study found that the $P2X_7$-receptor, an important inflammatory mediator that regulates IL-1ß release, increased afferent mechanosensitivity in *T. spiralis* infected wild-type mice, but not in $P2X_7R$ mice and thus may trigger visceral hypersensitivity (Keating et al., 2011). Gastrointestinal infection with pathogenic bacteria, such as *Salmonella enterica* serovar *Typhimurium*, has been documented to disrupt the microbiome (Barman et al., 2008). Administration of *Bifidobacterium longum* HB55020 or fecal microbiota from healthy controls increased pain threshold in mice that developed visceral hypersensitivity after infection with intragastric *T. spiralis* larvae (Bai et al., 2018).

9. Conclusion

Clinical disorders with symptoms of increased visceral pain have a significant impact on quality of life, and many of these are considered functional disorders, in that there is no obvious organic cause for their etiology. This has made the creation of reliable and reproducible animal models of visceral pain both pressing and challenging. However, there is great promise in models that represent visceral hypersensitivity accompanied by an altered intestinal microbiota. Such animal models of increased visceral pain can involve direct disruption of intestinal microbes such as with GF or AB treated mice, or those subjected to FMT. Alternately models may involve indirect disruption of resident intestinal bacteria, such as that observed when rodents are subjected to acute or chronic stress, or intestinal inflammation or infection. While the intestinal microbiome has become a major focus of research in many aspects of gut-brain physiology in the last couple of decades, including the examination heightened visceral pain, many questions still remain unanswered. We now know that alterations to visceral pain perception is accompanied by disruption of the intestinal microbiome, however future work needs to focus on mechanisms by which the intestinal microbiome communicate with the host in order to affect changes to pain processing. In spite of still unanswered questions, it is abundantly clear that animal models of heightened visceral pain should show strong validity to the human condition, and a critical role for the intestinal microbiota in the etiology and pathophysiology of those disorders involving altered visceral pain perception is now fittingly reflected in our most promising animal models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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