Animal models of gastrointestinal and liver diseases. Animal models of visceral pain: pathophysiology, translational relevance, and challenges

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Submitted 23 December 2014; accepted in final form 11 March 2015

Greenwood-Van Meerveld B, Prusator DK, Johnson AC. Animal models of gastrointestinal and liver diseases. Animal models of visceral pain: pathophysiology, translational relevance, and challenges. Am J Physiol Gastrointest Liver Physiol 308: G885–G903, 2015. First published March 12, 2015; doi:10.1152/ajpgi.00463.2014.—Visceral pain describes pain emanating from the thoracic, pelvic, or abdominal organs. In contrast to somatic pain, visceral pain is generally vague, poorly localized, and characterized by hypersensitivity to a stimulus such as organ distension. Animal models have played a pivotal role in our understanding of the mechanisms underlying the pathophysiology of visceral pain. This review focuses on animal models of visceral pain and their translational relevance. In addition, the challenges of using animal models to develop novel therapeutic approaches to treat visceral pain will be discussed.

gastrointestinal; IBS; models; pain; rodent

CHRONIC ABDOMINAL PAIN IS the hallmark feature of multiple disorders, some with distinct organ pathology, including inflammatory bowel disease, pancreatitis, and interstitial cystitis/painful bladder syndrome, whereas in other disorders such as irritable bowel syndrome (IBS) and functional dyspepsia (FD) there is no evidence of any structural or histological abnormalities to explain the pain. However, abdominal pain represents one of the main reasons patients seek medical attention. Currently, despite large numbers of patients with chronic and often debilitating visceral pain, the clinical management of these patients is largely inadequate. There are few effective therapies to treat patients with chronic visceral pain and those that are available are limited by poor side-effect profiles such as addiction, fatigue, constipation, nausea, and liver damage. Notwithstanding the large number of patients complaining of chronic visceral pain, the mechanisms leading to this debilitating symptom require further investigation. Pain pathways innervating the gastrointestinal (GI) tract are shown in Fig. 1. Upon stimulation at the peripheral organ, extrinsic nociceptors (Aδ- and C-fibers) synapse in the dorsal horn of the spinal cord. Second order neurons then ascend to the brain through anterolateral pathways such as the spinothalamic and the spinoreticular tracts. Tertiary neurons in the thalamus distribute the pain signal to the primary somatosensory cortex to provide localization of the signal. Other regions, such as the insula and the anterior and midcingulate cortex, are activated to provide the “feel” of the stimulus (sharp, dull, aching, burning) and the perception of unpleasantness. Ascending brain stem connections also activate the amygdala to integrate the pain signal with outflow to the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. Descending projections from the anterior cingulate cortex modulate signaling within the periaqueductal gray to activate antinociceptive systems within the brain stem that lead to inhibition of the ascending pain signal within the dorsal horn of the spinal cord. Additionally, there is evidence from both the upper (266) and lower (256) GI tract for vagal modulation of nociception (13, 80). Sensitization of these central and/or peripheral pathways leads to visceral hypersensitivity, which can be affected by multiple mechanisms. For example, stressors can alter the physiology of nuclei that process pain signals, leading to central sensitization. Allelic variations can alter the function of neurotransmitter receptors or reuptake mechanisms, predisposing an individual to enhanced pain signaling in the central pain matrix. Inflammation can lead to long-term changes in the physiology of the affected organ due to immune mediators released at the site of injury that can sensitize peripheral afferents. Increased epithelial permeability permits luminal contents to directly activate afferent nerve endings, which can lead to sensitization. Shifts in commensal microbiota can lead to changes in fermentation products and/or low-grade immune activation, either of which produces mediators that can sensitize afferent nerves. Overlap between these mechanisms adds to the complex, multifactorial nature of visceral hypersensitivity, i.e., genetic variation may amplify stress responses, which increases permeability and changes immune responses to an acute infection, leading to a shift in microbial populations within the GI tract (Fig. 2).

This review summarizes the recent literature on the role that animal models have played in enhancing our understanding of the physiology and pathophysiology of visceral pain with a particular focus on abdominal pain of GI origin. We will discuss the translational relevance of the animal models and...
their importance in studying 1) the mechanisms of visceral pain and 2) the development of novel therapeutic approaches to treat visceral pain. As each animal model is described we have discussed how well the model mimics clinical characteristics of visceral pain (face validity), as well as the relevance to human situations (construct validity). We will also describe whether the drug studies performed in animal models of visceral pain can predict the efficacy of a new drug in patients with visceral pain (predictive validity). In addition, this review examines the challenges of using animal models with an emphasis on the specific strengths and weaknesses of each model. Together, this review provides an important summary of animal models to increase our understanding of the basic mechanisms involved in the pathophysiology of visceral pain, their translational relevance for the treatment of visceral pain disorders, and the important limitations that must be considered when interpreting the results from studies that have utilized animal models of visceral pain.

ANIMAL MODELS OF COLONIC HYPERSENSITIVITY RELEVANT TO IBS

Rats and mice are the most commonly used animal models used to assess colonic physiology, pathophysiology, and new treatment approaches for visceral pain. In general, there are multiple, well-established methodologies to universally quantify visceral nociception in rats and mice. The most commonly used technique involves recording devices such as electromyographic (EMG) electrodes or strain gauges implanted on the external oblique abdominal musculature, to quantify the number of reflex abdominal muscle contractions in response to graded colonic balloon distension. Measurement of isobaric distension pressures with a barostat offers numerous strengths, such as an objective assessment of pseudoaffective nociceptive reflexes, allowing for assessment of colonic compliance, as well as mimicking the approach used in clinical studies in which sensitivity is assessed in response to rectosigmoid distension. Measurement of isobaric distension pressures with a barostat offers numerous strengths, such as an objective assessment of pseudoaffective nociceptive reflexes, allowing for assessment of colonic compliance, as well as mimicking the approach used in clinical studies in which sensitivity is assessed in response to rectosigmoid distension. However, there are also weaknesses that must be considered in the data interpretation. For example, the animals may be exposed to stress during the procedure as a result of the novel laboratory environment or may be restrained to reduce movement artifact during EMG recording. An acclimatization period to the experimental environment has been shown to significantly reduce animal stress and allows for the assessment of visceral sensitivity in a freely moving animal. However, anesthesia will always be necessary to insert the colorectal balloon; it should be kept as brief as possible to avoid inter-

Fig. 1. Visceral pain signaling pathways. A: noxious visceral stimulation activates nociceptors that synapse in the superficial lamina of the dorsal horn of the spinal cord. Ascending pain signals are transmitted through the anterolateral pathways in the dorsal column. Tertiary neurons in the thalamus distribute the pain signal to cortical areas for localization while other regions provide intensity and emotional components of the pain stimulus. The amygdala is also activated to integrate the responses of the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. B: pain inhibition is modulated by descending projections from the anterior cingulate cortex that activate the periaqueductal gray (PAG) to further activate serotonergic (caudal raphe) and opioidergic (rostral ventral medulla) antinociceptive systems. Pain facilitatory systems can be engaged by amygdala modulation of the paraventricular nucleus of the hypothalamus for HPA axis activity and of the locus coeruleus for the sympathomedullary axis. The amygdala can also modulate descending antinociception through connections to the PAG. ACC, anterior cingulate cortex; pACC, perigenual ACC; MCC, midcingulate cortex. The figure is adapted with permission from Grover and Drossman (79).
ference with the nociceptive reflex. Additionally, the balloon distension paradigm should include randomization of the pressures (0–60 mmHg). An additional approach to assess colonic sensitivity is by the visual assessment of the abdominal withdrawal reflex (AWR) in response to colorectal distension (CRD). A benefit of this technique is that the stereotyped nociceptive behavior is induced by a brief distension; however, the AWR is a subjective behavioral measure, requiring larger samples sizes to demonstrate significant differences between treatment groups. An alternative approach to the use of a colonic balloon distension paradigm is to directly stimulate visceral nociceptors through colonic infusion of algesic chemicals, such as capsaicin or mustard oil. Such substances produce spontaneous nocifensive behaviors (perianal licking, abdominal retraction or compression, and hindlimb stretching) and take advantage of spontaneous evoked nocifensive behaviors. A note of caution in the data interpretation is that the inflammatory component of the stimulus suggests that the model may more relevant to inflammatory pain rather than functional visceral pain. In summary, reflex abdominal contractions in response to colonic distension and quantification of nocifensive behaviors in response to colonic infusion of algesic chemicals represent the most widely used approaches for quantifying visceral pain in the specific models that will be described in this section of the review and summarized in Fig. 3 or Table 1.

**Genetic/Spontaneous Models of Colonic Hypersensitivity**

**Knockout animals.** Knockout models provide the opportunity to investigate the role of a specific gene in the regulation of colonic sensitivity. We have previously shown that there is a significant decrease in colonic sensitivity to CRD in corticotropin-releasing factor (CRF)-1 receptor knockout mice, suggesting the importance of CRF in colonic sensitivity (226). Further studies in knockout mice have demonstrated the importance of other signaling molecules and transporters in the regulation of colonic sensitivity, including brain-derived neurotrophic factor (BDNF), guanylate cyclase C (GC-C), serotonin (5HT), and interleukin 10 (IL-10), human excitatory amino acid transporter 2 (EAAT2), and the serotonin reuptake transporter (SERT). These models have also been used to identify key receptors and ion channels involved in visceral sensitivity such as the glial cell line-derived neurotrophic factor family receptor α-3 (GFRα3), G protein-coupled receptor kinase 6 (GRK6), the protease-activated receptor-2 (PAR2), PAR4, purinergic receptor-3 (P2X3), sigma-1 (σ1) receptor, Toll-like receptor 4 (TLR4), the short transient receptor potential channel 4 (TRPC4), the transient receptor potential cation channel, subfamily A, member 1 (TRPA1), and the transient receptor potential cation channel, subfamily V, member 1 (TRPV1).
WKY rat. Multiple studies have confirmed the original observation that the Wistar-Kyoto (WKY) rat displays a hypersensitive response to colonic distension (81). No single mechanism has been demonstrated to be responsible for the hypersensitive response in WKY rats. Recent studies have shown that both central (21) and peripheral (165) CRF receptors are differentially expressed and that selective CRF antagonists can inhibit colonic hypersensitivity (23, 101). Additionally, treatment with immune modulators (26, 243), probiotics (141), calcium channel inhibition (162), and 5HT receptor inhibition (160) reduced colonic hypersensitivity in WKY rats.

Challenges. Interpretation of data in genetic models is complicated and represents a significant challenge. For example, gene deletion may affect the overall health of the animal along with endogenous compensatory and/or redundant mechanisms that mask the true effect of the loss of the gene. An issue with the WKY rats is that, to our knowledge, they are the only rat strain exhibiting spontaneous colonic hypersensitivity. However, the inbred nature of this strain may limit the translational relevance because they may only model the pain experienced by a subgroup of patients with functional visceral pain.

Early Life Stress Models of Colonic Hypersensitivity

Early life stress (ELS), which includes childhood neglect, physical abuse, and sexual abuse, is extremely common in the
United States, affecting ~40% of the population before adolescence (48, 60, 68). Increasing evidence from clinical studies suggests that a history of ELS serves as a risk factor for the development of adult pathologies including but not limited to GI disorders such as IBS, with affected patients being two to four times more likely to report an adverse experience during childhood (20). In conjunction with these reports, a history of childhood abuse has been correlated with abnormal bidirectional communication between the brain and the gut, providing a potential explanation for the linkage between ELS and the symptoms of adult GI disorders (25, 184). Despite the strong correlation between ELS and decreased health-related quality of life in adults due to GI-related abnormalities, the mechanism by which ELS underlies these changes is still unknown. Although the complex nature of the human ELS experience cannot be completely simulated in animal models, we consider that animal models of ELS are important tools to develop our understanding of how adverse neonatal experiences alter brain-gut communication that may lead to the development of abnormal visceral perception.

Maternal separation. The most well studied model of ELS is maternal separation (MS), which involves removal of pups from mother and nest, most commonly for 3 h/day on postnatal (PN) day 2–14 (PN2-14) (175). The purpose of this paradigm is to mimic childhood neglect and abuse through separation and subsequent alterations in maternal care, including altered licking and grooming behaviors and arched-back nursing (173). MS pups exhibit decreased weaning body weight at PN22 compared with controls that are left undisturbed in their home cage on PN2, potentially introducing the effects of malnutrition as a side effect of neglect in this ELS model. In adulthood, there are contradictory results on visceral sensitivity depending on the duration of separation. However, a 3 h/day separation has been shown to result in visceral hypersensitivity, as evidenced by an increased visceromotor response (VMR) to CRD in male Long-Evans rats (49). This model presents an opportunity to investigate the relationship between ELS and subsequent development of visceral hypersensitivity, in conjunction with hyper-reactivity of the HPA axis, two commonly comorbid symptoms in disorders such as IBS (57, 240).

Odor-attachment learning. The odor-attachment learning (OAL) model of ELS is a classical conditioning model that utilizes predictable or unpredictable odor-shock pairings to mirror an attachment to an abusive caregiver (207). Conditioning occurs from PN8-12 wherein rat pups are experiencing both a sensitive and a hypersensitive period. These are evolutionary advantages that allow the pups to be more sensitive to maternal odors to find the dam in the cage for care and nursing. This behavior coincides with an inability to initiate a stress response, ensuring that pups do not learn an aversion to the dam when she is stepping on or moving them around the cage by their scruff (208). Therefore, by utilizing an odor and modest shock, this model is able to mimic patterned interactions between pup and dam, thus creating an odor attachment to the conditioned odor in response to predictable or paired odor/shock presentations. This paradigm also utilizes an unpaired or unpredictable odor/shock presentation, and an odor-only presentation as a control, neither of which presentation results in pups learning an attachment or aversion to the conditioned odor. In adulthood, this is the first model to our knowledge that induces female-specific visceral hypersensitivity in adult Long-Evans rats. Following OAL, female rats with a history of unpredictable ELS exhibit an increased VMR to CRD, an effect that has been shown to be estrogen dependent (31). This model of ELS has far reaching relevance to translational research as it parallels the female predominance found in patients who experience visceral pain, and thus far it is the only rat model capable of linking ELS to adult visceral hypersensitivity in women (20).
From PN2 to PN9, all bedding material is removed and the dam and pups are placed on a wire cage bottom with only a single paper towel for nesting material. This limitation of bedding material causes disruptions in normal maternal care similar to those exhibited by dams during the MS protocol; however, the LN model does not require removal of pups from the dam at any time during the experimental paradigm. This advantage also eliminates differences in weaning weights seen in the MS model, since male and female animals who experience neonatal LN weigh the same at weaning as their control counterparts who are left undisturbed until PN22 (175). Furthermore, this particular paradigm produces increased visceral sensitivity in adult male rats, as quantified by increased VMR to CRD (175). The LN model is relevant to investigation of the human situation, because this mirrors neglect and abuse that occurs in the presence and not as a result of absence of the mother (97).

Neonatal colonic irritation. Another model of ELS utilizes neonatal colonic irritation (nCI) using colonic infusion of mustard oil or repeated CRD in neonates (4). This model relies strictly on manipulation of the colon; however, there are no

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| Table 1. Rodent models of colonic hypersensitivity (2009–2014) |
|------------------|------------------|------------------|
| Model | Mice | Rats |
| Genetic/spontaneous models | | |
| BDNF HET | 260, 264 | |
| GC-C KO | 62 | |
| GFRα3 KO | 213 | |
| GRK6 KO | 61 | |
| IL-10 KO | 276 | |
| Mast Cell (Ws/Ws) KO | | 258 |
| PAR2 KO | 6 | |
| PAR4 KO | 9 | |
| P2X2/P2X3 KO | 185 | |
| P2X3 KO | 197 | |
| P2X3/TRPV1 KO | 107 | |
| P2X7 KO | 106 | |
| SERT KO | | 69 |
| α1 Receptor KO | 74 | |
| TLR4 KO | 220 | |
| TRPA1 KO | 22, 30 | |
| TRPC4 KO | | 239 |
| TRPV1 KO | 155 | |
| EAAT2 OE | 125 | |
| Wistar-Kyoto | 21, 23, 26, 70, 101, 141, 160–162, 164, 165, 243 | |
| Early life stress models | | |
| Limited nesting | | 175 |
| Maternal separation | 12, 15, 40, 44, 58, 63, 75, 90, 91, 93, 119, 134, 162, 164, 202, 216–218, 227–231, 248, 269 | |
| Odor attachment learning | | 31 |
| Stress-induced models | | |
| CeA implants | 82, 149–152, 222, 223, 225 | |
| Restraint stress | 1, 2, 24, 62, 167, 196, 200, 209, 243, 250, 270 | |
| Variable stress | 219, 220 | |
| Colonic irritation models | | |
| Acetic acid | 77, 85, 115, 247 | 8, 54, 138, 139, 251 |
| Butyrate | 3, 14, 35, 61, 66, 74, 83, 116, 140, 259 | 181, 188, 231 |
| Capsaicin | 16, 89, 94, 124 | |
| DSS | 9, 30, 34, 35, 66, 116, 117, 155, 172, 237 | 98, 170, 171, 198, 199, 239 |
| Mustard oil | 5, 22, 29, 30, 62, 124, 237, 260 | 1, 62, 103, 146, 179, 191, 200, 203 |
| TNBS | 64, 107, 197 | 115 |
| Zymosan | | |
| Postinflammatory models | | |
| Acetic acid | 51, 169, 194, 209, 214 | |
| C. rodentium | 95, 96 | |
| C. jejuni | 92 | |
| DSS | 61, 113 | 36 |
| TNBS | 27, 59, 65, 100, 142, 155, 166, 167, 206, 213, 275 | 59, 100, 142, 155, 166, 167, 206, 275 |
| T. spiralis | 46, 106, 130, 131, 185, 235 | 257, 258, 261 |

BDNF, brain-derived neurotrophic factor; CeA, central nucleus of the amygdala; DSS, dextran sodium sulfate; EAAT2, excitatory amino-acid transporter type 2; GC-C, guanylate cyclase C; GFRα3, GDNF family receptor alpha-3; GRK6, G protein-coupled receptor kinase 6; HET, heterozygous; IL-10, interleukin 10; KO, knockout; OE, overexpression; PAR2, protease activated receptor 2; PAR4, protease activated receptor 4; P2X2, P2X purinoceptor 2; P2X3, P2X purinoceptor 3; P2X7, P2X purinoceptor 7; SERT, serotonin reuptake transporter; TLR4, Toll-like receptor 4; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TRPA1, transient receptor potential cation channel, subfamily A, member 1; TRPC4, short transient receptor potential channel; TRPV1, transient receptor potential cation channel, subfamily V, member 1.

or lower socioeconomic standing (72). From PN2 to PN9, all bedding material is removed and the dam and pups are placed on a wire cage bottom with only a single paper towel for nesting material. This limitation of bedding material causes disruptions in normal maternal care similar to those exhibited by dams during the MS protocol; however, the LN model does not require removal of pups from the dam at any time during the experimental paradigm. This advantage also eliminates differences in weaning weights seen in the MS model, since male and female animals who experience neonatal LN weigh the same at weaning as their control counterparts who are left undisturbed until PN22 (175). Furthermore, this particular paradigm produces increased visceral sensitivity in adult male rats, as quantified by increased VMR to CRD (175). The LN model is relevant to investigation of the human situation, because this mirrors neglect and abuse that occurs in the presence and not as a result of absence of the mother (97).
long-lasting observed changes in the colonic mucosa of adult animals. nCI results in altered neuronal excitability and permeability within the colon, as well as visceral hypersensitivity of adult animals (4, 32, 122, 123). This model of ELS may be relevant in instances of repeated physical or sexual abuse, and potentially in patients who experience some type of colonic inflammation during childhood.

Challenges. Rodent models of ELS aim to mimic specific facets of childhood adverse experiences reported by patients and are pivotal tools in the investigation of the mechanisms that underlie visceral pain following early life trauma. However, ELS studies as a whole, as well as each paradigm, are not without challenges. Overall, the initial consideration of protocol, strain, and sex differences present major considerations. Within the MS model alone there are many variations of separation duration in terms of hours per day and length of protocol across the neonatal period (163), and this is further complicated by the use of varying strains of rats including Long-Evans, Sprague-Dawley (SD), and Wistar (114, 173, 186). The other models, LN, OAL, and nCI, have not yet been so widely studied; however, these same modifications to the original model developed may become an important factor. A second overarching challenge is the decision to study males, females, or both. Because some models have been shown to affect strictly males or strictly females, it is important to decide the merits of studying only an affected group vs. investigating potential protective mechanisms within the seemingly unaffected portion of a study. Also, it is important to recognize how each model may be different and include that in the interpretation of results. Although MS and LN produce abnormalities in maternal care, the MS model potentially includes the effects of malnutrition not observed in LN or OAL models. Challenges of a more concrete nature in regards to animal models of ELS include time and resources. Whereas the LN protocol requires little interaction with the observer, the MS, nCI, and OAL models require significant time spent by personnel for separation of pups, manipulation, and conditioning, respectively. This does not include further time to allow pups to mature into adult animals, which ranges from PN60 to PN90 depending on the study. All in all, from arrival to first experiment this can be 4 mo before data can be obtained. In conjunction with time, development of ELS models can be costly in terms of animal housing for the duration of the study, which is a major consideration for budgetary resources. Despite these challenges, the importance and relevance of ELS models to investigate the relationship between childhood adverse experiences and adult visceral pain remain.

Stress-Induced Models of Colonic Hypersensitivity in Adulthood

CeA implants. As a model of stress targeting only the amygdala, implantation of corticosterone (CORT) micropellets on the dorsal surface of the central nucleus of the amygdala (CeA) were initially shown to increase colonic sensitivity to innocuous distension (76). An interesting feature of this model is its relevance to patients with IBS based on imaging studies showing heightened activation of the amygdala in IBS patients in response to colonic distension (154, 242). Careful stereotoxic targeting of the CeA is necessary, since placement of the CORT micropellet in adjacent nuclei does not reproduce the colonic hypersensitivity (148). Follow-up studies demonstrated that the CORT-induced colonic sensitivity was mediated by nonredundant mechanisms involving both glucocorticoid (GR) and mineralocorticoid receptors (MR) (148, 149), and the effect on colonic sensitivity persisted in the absence of the CORT stimulus (150) and was concurrent with increased CRF expression (222). Further investigation of the persistent effects of the CORT implant has recently demonstrated that an epigenetic mechanism involving deacetylation of the GR promoter induces the increase in CRF expression, leading to the colonic hypersensitivity (225). Thus central dysfunction of stress-integrative neurocircuity is sufficient to induce colonic hypersensitivity, in the absence of peripheral manipulation of the colon.

Restrain stress-induced colonic hypersensitivity. The most typical protocol is a 1- or 2-h acute stress protocol in which the animal either has its forelimbs wrapped with tape to restrict movement and grooming behavior or is placed in a restraint apparatus (cage or tube) that prevents turning and grooming. A strength of the model is the robust and reproducible nature of the colonic hypersensitivity induced by restraint stress. However, a notable weakness of all stress models is the translational relevance to clinical stressors. This acute restraint stress induces an increase in colonic sensitivity to distension as measured by EMG or AWR quantification. Restraint stress-induced hypersensitivity to colonic distension can be modulated by serotonergic receptors (167), GC-C receptors (62, 200), PARs (270), or peripheral nociception/orphanin FQ receptors (1). Additionally, although an endothelial cell adhesion molecule inhibitor failed to inhibit the stress-induced hypersensitivity (243), a combination treatment of simethicone and alverine citrate (24) or a probiotic product (2) significantly inhibited poststress hypersensitivity. Using a repeated restraint stress protocol, 4 or 7 days of daily 2-h restraint, increased the EMG response to colonic distension, and the hypersensitivity was inhibited by either a cannabinoid receptor 1 (CB1) agonist (196) or by cannabinoid administration (250).

Water avoidance stress-induced colonic hypersensitivity. In this experimental model of stress, the rat is placed on a dry platform surrounded by water in attempt to mimic a psychological stressor. However, this model may also engage the fear neurocircuity, which could evoke freezing behaviors in the colonic distension paradigm and affect interpretation of data using this model. With the exception of two studies (111, 112), water avoidance stress (WAS) protocols in rats induce colonic hypersensitivity to distension. A single exposure to WAS has been demonstrated to induce colonic hypersensitivity either immediately, within 60-min of the acute stressor (151, 155), or after recovery from the stressor, 24-h poststress (62, 237), which could be modulated by GC-C (62), TRPV1, and 5HT3 receptors (155). A single WAS exposure can also induce a prolonged colonic hypersensitivity in rats previously exposed to maternal separation (204, 229–231, 248), mediated in part by mast cells (229, 230), peripheral histamine receptors (204), and TRPV1 (231). An extension of this model has been to perform daily 1-h exposure to the WAS for multiple days in an attempt to mirror a chronic stressor. A note of caution when using this model is to measure fecal pellet output or systemic CORT daily to ensure that the rats do not habituate to the
WAS. We have shown in this model that stress-responsive limbic areas of the brain regulate colonic hypersensitivity induced by 7 days of WAS through an interaction of GR/MR (151) and CRF receptors (224) and an epigenetic modulation their expression (221). Epigenetic mechanisms have also been implicated in regulating reciprocal changes in CB1 and TRPV1 in the dorsal root ganglion (DRG), further modulated by GR, that induce colonic hypersensitivity following 10 days of WAS (86–88, 271). Ten-day WAS-induced hypersensitivity can also be inhibited by treatment with CRF-1 antagonists (19, 71) or antibiotics (250). In recent literature, the effect of WAS on colonic sensitivity in mice has been variable. Two reports have demonstrated that a 4-day exposure to WAS induced colonic hypersensitivity to distension, which could be inhibited by pretreatment with probiotics (156) or with a PAR4 agonist (5). A 7-day WAS protocol also increased total pain behaviors induced by intracolonic capsaicin, which were reduced by predosing of broad-spectrum antibiotics (3). In contrast, measuring colonic sensitivity noninvasively through colonic manometry, 10 days of WAS produced either hyper- or hyposensitivity, depending on the housing and the surgical status of the mouse (110). With use of a similar noninvasive measurement technique, 10 days of WAS only produced a transient increase in colonic hypersensitivity in mice also exposed to an acute dextran sodium sulfate (DSS)-induced colitis, with no apparent direct effect of WAS on colonic sensitivity (113).

Variable-stress-induced colonic hypersensitivity. To eliminate the possibility of rats habituating to a single repetitive stressor, several variable stress protocols have been developed that expose animals to a set of randomly presented stressors. The unpredictable nature of the stressors prevents acclimatization to the procedures. One protocol used in rats, termed heterotypic intermittent stress or heterotypic chronic stress (HeCS), randomizes presentation of three stressors: 45 min of cold (4°C) restraint, 60 min of WAS, or 20 min of forced swim. Each stressor is presented once or twice daily for 9 days. The duration of colonic hypersensitivity to distension, measured through EMG or AWR, was strain dependent with a duration of action of up to 48 h poststress. Stress-induced colonic hypersensitivity was inhibited by administration of a cystathionine β-synthetase antagonist (an enzyme responsible for H2S production) (236), a selective β3-adrenergic antagonist (265), antibodies targeting nerve growth factor (NGF) (246), anti-sense oligodeoxynucleotides targeting NGF receptor (246), or through electroacupuncture activation of endorphin-mediated antinociception (277). Colonic hypersensitivity was further increased when HeCS was combined with DSS colitis, which was also inhibited with anti-NGF antibodies (36). Colonic hypersensitivity was also demonstrated in adult male and female rats whose dams were exposed to HeCS during the last 9–11 days of gestation (244). A further exposure of the adult rats to the HeCS protocol produced a colonic hypersensitivity that persisted for 1 wk in the male rats, and for 2 wk in the female rats, and could be inhibited by administration of a histone acetyltransferase antagonist, or through inhibition of BDNF or BDNF receptor (244). An alternative protocol of chronic unpredictable stress applied over 21 days induced colonic hypersensitivity to distension that was prevented by neonatal denervation with capsaicin and inhibited by administration of a mast cell stabilizer (37). A third protocol has been developed in mice that randomly exposes the animals to either social defeat or overcrowding stress for 19 days to induce colonic hyperalgesia (219). The colonic hyperalgesia in the mice was decreased in mice deficient for TLR4 or following peripheral or central administration of a TLR4 antagonist (220).

Challenges. Rodent models of acute stress produce increases in plasma CORT that are similar in magnitude to the CORT response in stressed humans. However, an issue with the stress models is their construct validity, i.e., the experimental stressors do not mirror the types of stress that humans experience. In rat models of stress-related visceral pain the nature of the stressors, including whether they are physical vs. psychological, acute vs. chronic, or predictable vs. unpredictable, can have a profound influence not only on the outcome of the investigation but also on the biological processes involved. Many rat strains, particularly the commonly used SD, will habituate to the repeated presentation of a homotypic stressor (56, 73), which may affect the development of visceral pain responses; therefore, it is recommended that strains be employed that do not readily habituate (56, 232) or the models used are unpredictable to better mimic the human pathophysiological condition.

Colonic Irritation Models of Colonic Hypersensitivity

Noninflammatory irritation: acetic acid and butyrate. Acetic acid has been used to induce colonic hypersensitivity to distension through two different mechanisms. Low-concentration (<1.0%) acetic acid produces a transient sensitization of colonic afferents (109, 174). Higher concentrations of acetic acid produce a mild damage to the colon, producing inflammation-associated hypersensitivity. Although there are multiple receptors and transmitters that can participate in acute afferent sensitization, compounds targeting TRPV1 (247), neurokinin-1 receptor (77), 5HT3 (77), and 5HT4 (85, 115) have demonstrated efficacy in inhibiting acetic acid induced colonic hypersensitivity.

In an attempt to produce a noninflammatory model of colonic hypersensitivity with potential translational relevance to IBS, Bourdu et al. (18) characterized the effect of repeated butyrate enemas on both colonic hypersensitivity to distension and colonic histology. Six enemas of butyrate induced colonic hypersensitivity, reversible with morphine and requiring C-fibers, without evidence of inflammation or histological damage (18). Recent studies have also demonstrated that antagonists of acid-sensing ion channels (138), anti-NGF antibody (138), serotonin-norepinephrine reuptake inhibitors (54), or mitogen-activated protein kinase (MAPK) kinase inhibitors (251) can also prevent butyrate-induced colonic hypersensitivity.

Acute inflammatory irritation: capsaicin, mustard oil. Colonic installation of a low volume of capsaicin or mustard oil, which induce pain through direct activation of receptors on afferents as well as establishing an acute inflammation through tissue damage, is typically used to evaluate the analgesic properties of novel therapeutics. Stereotypic pain behaviors, such as abdominal licking, stretching, retraction, or compressing on the cage floor, are counted for 20–30 min after irritant administration. Studies of potential mechanisms for capsaicin-induced visceral pain found that pain behaviors were enhanced in a mouse with a transgenic mutation of a potassium channel in the forebrain (14) and decreased in σ1 receptor knockout.
mice (74) or in mice with altered sensory nerve development (259). Mustard oil-induced spontaneous nociceptive behaviors can be inhibited by morphine (66), melatonin receptor antagonists (35), α-bisabolol (116, 117), and an extract from red algae (34). Behaviors were also decreased in TRPC4 knockout rats (239) and increased in PAR4 knockout mice (9).

**DSS, TNBS, and zymosan-induced colitis.** DSS produces a mucosal colitis throughout the colon when administered as a 1–5% solution in the drinking water of rats or mice for 5–7 days. Daily monitoring of a disease activity index (DAI), composed of changes in body weight, stool consistency, and blood in the stool, is used to determine the progression of the colitis. If animals are then returned to normal tap water, recovery from the colitis (measured as decreases in the DAI) will begin within 1–2 days. Colonic sensitivity can be assessed in response to either distension or acute infusion of an algogenic compound in the inflamed colon. Mechanistic studies have demonstrated a role for transient receptor potential cation channel, subfamily M, receptor 8 (TRPM8) (89), glutamate transporter (GLT-1) (124), and endorphins produced by CD4+ T cells (16) in the regulation of the colitis-induced hypersensitivity.

In another model, 2,4,6-trinitrobenzenesulfonic acid (TNBS) is a hapten that produces an acute colonic inflammation when administered as an enema in combination with 25–50% ethanol (EtOH) to disrupt the mucosal barrier, in rats and mice. Although inflammatory changes in the colonic tissue can be measured within hours of the enema, most protocols investigate colonic sensitivity to distension, with EMG or AWR quantification, 3–7 days postenema, which will represent different amounts of inflammatory damage depending on the concentration of TNBS and EtOH in the enema. In mice, PAR4 (5), TRPA1 (30), and GC-C receptors (62), cathepsin S (29) and BDNF (260), and GLT-1 (124) have been found to modulate TNBS-induced colonic hypersensitivity. In a similar manner, GC-C (62, 200), estrogen (146), nociception/orphanin FQ (1), prokineticin (237), or 5HT4 receptors (191), as well as spinal L- and R-type calcium channels (179) or microglia (103), and TNF-α (203) all modify TNBS-induced colonic hypersensitivity in rats.

Colonic sensitivity in response to intracolonic zymosan-induced colitis is measured in response to distension either acutely (2–3 h postinfusion) or following once-daily infusion for 3 days. Recent studies have demonstrated roles for GC-C (64), 5HT4 (115), P2X3 (197), and TRPV1 (107) in the development of zymosan-induced colonic hypersensitivity.

**Challenges.** Although noninflammatory afferent sensitization models pain experienced in functional bowel disorders, the mechanisms responsible for the acute sensitization may not be directly translatable to the chronic pain experienced by the patient. However, tegaserod, which had efficacy in patients, was able to inhibit acetic acid-induced hypersensitivity (78), providing evidence that noninflammatory afferent sensitization is a model of colonic hypersensitivity with good face validity. Although DSS typically affects the whole of the colon, TNBS can produce focal damage depending on the volume of the administered enema. Assessing colonic sensitivity in rats and mice with active colitis likely increases the risk for perforation by the balloon catheter due to the potential presence of ulcerated and/or necrotic tissue. Additionally, selection of mouse strain is an importation factor with the TNBS model of colitis, since the C57BL/6 mouse strain, used as the genetic background for many knockout models, is not as susceptible to TNBS-induced colitis (215).

**Postinflammatory Models of Colonic Hypersensitivity**

**Irritant-induced inflammation: acetic acid, DSS, and TNBS.** Following recovery from an acute colitis, determined by gross morphology, histology, and/or tissue immune activation markers (cytokines), some animals might develop a hypersensitive response to colonic distension. These models attempt to mirror the postinflammatory/infecive hypersensitivity that develops in some IBS patients. The standard protocol for acetic acid-induced postinflammatory colonic hypersensitivity is to administer and enema of 4% acetic acid, followed by a buffered saline enema, with colonic sensitivity testing 7 days postenema. Interestingly, although multiple therapeutic targets have been shown to inhibit acetic acid-induced postinflammatory colonic hypersensitivity (51, 169, 194, 214), common to each study was the ability of nitric oxide synthase inhibitors to reverse the effect of the investigational therapy.

Strong evidence for postinflammatory colonic hypersensitivity following DSS colitis is lacking in recent literature. Although hypersensitivity to distension was demonstrated at 10 days post-DSS administration (36), colonic sensitivity was similar to control animals at 32 (113) or 49 days (61) postcolitis. In rats, postinflammatory colonic hypersensitivity has been investigated 14–112 days post-TNBS enema. Post-TNBS colonic hypersensitivity is associated with changes in glutamate receptor expression (206, 275) and can be inhibited by treatment with probiotics (100) or with a compound that activates both opioid and nitric oxide signaling (59). In mice, the response to TNBS-colitis had been studied at 14–28 days postenema, where activation of GC-C (27) or GFRα3 (213) was able to reduce postinflammatory colonic hypersensitivity.

**Pathogen-induced inflammation: C. rodentium, C. jejuni, and T. spiralis.** In an attempt to model postinfective IBS, colonic sensitivity has been assessed in rat and mouse models following an acute bacterial gastroenteritis. In rats, *Citrobacter rodentium* induced a significant increase in AWR score, which was inhibited by treatment with a traditional herbal medicine or allosetenin (92). In mice, *Campylobacter jejuni* infection caused long-term hyperexcitability of colonic DRG neurons (95); however, a clear increase in the EMG response to balloon distension was found only if the mouse was exposed to an additional stressor (96).

**Trichinella spiralis infection** has also been used to produce a long-term colonic hypersensitivity to distension in rats and mice. At 8 wk postinfection, the EMG response to distension was significantly increased along with increases in glutamatergic receptor expression (261) in rats, and the AWR score was increased in mice (130, 131), which could be inhibited by treatment with multiple probiotic strains (235). Similarly, a decrease in withdrawal threshold and an increase in total glutamatergic positive cells in colonic DRGs (257) were found at 100 days postinfection.

**Challenges.** In each of the irritant-induced inflammations, recovery from the colitis does not guarantee the existence for colonic hypersensitivity to balloon distension (275). Thus the acute effects of the initial inflammatory insult (loose stool/diarrhea, weight loss, occult or explicit bleeding) needs to be
monitored during the recovery period to aid in predicting which animals may develop postinflammatory colonic hyper-sensitivity. Optimally, colonic sensitivity to distension should be assessed before testing a therapeutic intervention with only those animals with verified colonic hypersensitivity being used for subsequent testing. Furthermore, additional precautions should be exercised when using the postinfictive models to protect the experimenters from the pathogens. Both tegaserod (78) and linaclotide (27) have been shown to inhibit TNBS-induced postinflammatory colonic hypersensitivity, providing evidence for the translational relevance of the model.

ANIMAL MODELS OF VISCERAL HYPERSENSITIVITY RELEVANT TO FUNCTIONAL DYSPEPSIA

There are at least three rat models of FD that have been investigated in the literature (Table 2). The first uses oral gavages of 0.1% iodoacetamide in 2% sucrose administered to male neonatal SD pups on PN10-16 to induce in adulthood FD-like responses to gastric balloon distension (128). In the second model, an enema of TNBS (130 mg/kg in 10% EtOH) is administered to PN10 male SD pups, resulting in a hypersensitive response to stomach distension in adulthood (245). The third model manipulates the stomach of adult male SD rats with 15–20 injections of 20% acetic acid (10 µl/site) into the submucosal layer of the glandular portion of the stomach (52). For all three models, the change in sensitivity in the stomach is measured as skeletal muscle contractions in response to isobaric balloon distension. In the adult rat, a balloon catheter is surgically implanted through the fundus and exteriorized through the base of the neck. Additionally, EMG electrodes are implanted in the acromiotrapezius and exteriorized through the base of the neck. The distension paradigm for each study is 20-s per distension in conscious rats, with pressures ranging from 0–120 mmHg. In addition to the EMG quantification, AWR score can also be determined (128). Studies investigating possible mechanisms and therapeutic interventions in the FD models have shown that acute predosing of baclofen (γ-aminobutyric acid receptor B agonist sc) or desvenlafaxine succinate (serotonin-norepinephrine reuptake inhibitor po) in adulthood dose dependently inhibited the EMG responses (52, 128). In the neonatal TNBS-induced model of FD, neonatal treatment (PN9-17) with mifepristone (GR antagonist sc) prevented the development of adult gastric hypersensitivity (245). Increased BDNF and decreased voltage-gated potassium channel 1.1 (Kv1.1) within the DRG as well as increased NGF in the fundus in adulthood were also demonstrated to be part of the potential mechanism in the TNBS-induced model of FD (245).

Challenges. The first challenge in these models is the manipulation of the neonatal pups with either po dosing of iodoacetamide or the TNBS enema. None of the papers reported any measure of maternal care or whether the manipulations changed weaning weight of the pups, which can be factors on overall adult health. Additionally, the studies did not report whether female pups developed gastric hypersensitivity in adulthood. Another significant challenge is the surgeries needed to implant a chronic balloon catheter within the fundus of the stomach and the EMG electrodes necessary for the assessment of gastric sensitivity. Although the chronic nature of the balloon catheter and the EMG electrodes allows for repeated studies within the same animal, care has to be taken to ensure the rat does not damage the instrumentation and that the balloon does not interfere with eating and gastric emptying. Additionally, although the iodoacetamide and acetic acid models reported complete penetrance, the TNBS-enema model only produced a chronic gastric hypersensitivity in 50% of the treated animals. Finally, although the use of mice would allow access to the multiple knockout models that exist, adapting the FD models to the mouse scale would be technically difficult.

TABLE 2. Other rodent models of visceral hypersensitivity

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CYP, cyclophosphamide; DBTC, dibutyltin dichloride; PPQ, para-phenylquinone; PS, protamine sulfate.
hypersensitivity is assessed as mechanical or thermal somatic thresholds with either von Frey filaments or hot plate withdrawal, respectively, or as the number of nocifensive behavioral responses to electrical stimulation of the inflamed pancreas. Studies investigating potential mechanisms for DBTC-induced pancreatitis have demonstrated the decrease in withdrawal response was associated with increased spinal dynorphin content, which could be blocked with anti-dynorphin antisera or lidocaine in the nodose ganglion (234). Sumatriptan, a mixed 5HT1B/D agonist, dose dependently inhibited the decrease in withdrawal threshold, and the effect was reversed by selective 5HT1B or 5HT1D antagonists (233). Another study demonstrated an increase in calcium channel expression within the spinal cord following induction of pancreatitis along an inhibition of the somatic withdrawal response following administration of gabapentin (calcium channel antagonist ip) (121). Selective endothelin receptor A or B antagonists (ip) have also been shown to increase mechanical and thermal withdrawal thresholds in the same model (168). In the TNBS-induced pancreatitis model, decreased abdominal withdrawal thresholds for both mechanical and thermal stimuli were demonstrated to be modulated by TRPV1 (253), PAR2 (267), and activated microglia.

One study adapted the TNBS-induced pancreatitis model to mice and tested the nociceptive behavioral responses in wild-type and TRPA1 knockout mice (28). Aside from increased mortality following the induction of the pancreatitis, TRPA1 knockout mice demonstrated increased withdrawal thresholds, increased voluntary wheel running, and more activity in the open field compared with the wild-type controls (28). The other model of pancreatitis developed in mice is induced by 6–12 injections (ip) of caerulein. The caerulein-induced pancreatitis model has been used to explore the role of TRPA1 and TRPV1 through the use of selective antagonists (190) and cyclooxygenase receptor 2 with selective knockout animals (211).

Challenges. In each of the models, histology of the pancreas verifies that the induced inflammation appears clinically relevant on the basis of the extent of damage and types of inflammatory infiltrate present in the tissue. However, each model has particular challenges associated with the development of the inflammation. For DBTC, pancreatitis develops following an iv injection, typically tail vein, and the inflammation can be enhanced with ethanol in the drinking water. For the TNBS-induced pancreatitis models in the rat and mouse, the TNBS is administered slowly through the pancreatic ducts, via the duodenum, which necessarily requires a surgery. When performed by experienced researcher, rats tolerate the TNBS instillation, whereas mortality in the mouse is ~20% with TNBS and 10% in the sham surgery group (28). The caerulein model of pancreatitis in the mouse requires multiple intraperitoneal injections that can be stressful for the mouse if not properly acclimated. All of the pancreatitis models also rely on measuring referred somatic pain responses as an indirect indication of the visceral pain, although stimulation electrodes can be attached to the pancreas after TNBS infusion to directly induce visceral nociceptive responses.

ANIMAL MODELS OF VISCERAL PAIN RELEVANT TO CYSTITIS/PAINFUL BLADDER SYNDROME

In rats and mice, intraperitoneal cyclophosphamide (CYP) had been used to induce an acute or chronic model of cystitis (Table 2). The acute model uses a single bolus injection of 150 mg/kg CYP to produce bladder inflammation and edema, with sensitivity measured within 48 h of the injection. The chronic model uses repeated injections of 75 mg/kg CYP, typically three to four total injections over 8–12 days, with testing 24–48 h following the final injection. Commonly, referred visceral hyperalgesia is tested in conscious animals as withdrawal to von Frey filament probing on the lower abdomen, although some studies have also measured EMG responses to bladder distension in anesthetized animals. In mice, the cystitis-induced increased number of withdrawals to von Frey filaments or increased the EMG response to bladder distension can be inhibited by TRPA1 antagonists (53), a MAPK kinase inhibitor (108), or thrombomodulin (212). An elegant study in rats demonstrated that nociceptive behaviors induced by acute CYP cystitis could be inhibited by aspirin, ibuprofen, or morphine without affecting the CYP-induced histological damage to the bladder (10). Intrathecal botulinum toxin A also had an analgesic effect on CYP-induced decreases in withdrawal threshold (45). Similar to the effect in mice, CYP cystitis induces increases in the MAPK signaling in DRG (47) and changes in glutamatergic signaling within the spinal cord of rats (33, 105).

In adult rats a 30-min intrabladder infusion of a 1% zymosan solution is used to produce an acute cystitis. A similar concentration is used in neonatal rats; however, three total infusions are performed, once each on PN14-16. In the adult rat, zymosan sensitizes lumbosacral spinal neurons to distension (158) and produces an estrous cycle-dependent increase in the EMG response to bladder distension (11). In the absence of persistent histological damage, adult hypersensitivity to bladder distension was demonstrated in the animals exposed to neonatal zymosan, with differences in sensitivity demonstrated following adult challenge with zymosan (183, 193). The neonatal model has also been used to investigate visceral-organ cross talk by measuring the EMG response to colonic distension in adult rats (143, 192).

Challenges. Histological damage to the bladder in these models of cystitis suggests that the inflammation is relevant to human pathology, with the caveat that these induced models do not model clinical etiologies. Although intraperitoneal injections are easily performed in rodents, CYP has not been studied outside of the effect on the bladder, and thus its effect on other organs is unknown. Intravesical administration of irritants is technically more difficult than intraperitoneal injections and generally requires study of only female animals to permit easier access to the bladder.

ANIMAL MODELS OF NONSPECIFIC ABDOMINAL PAIN

Although still highly reported in recent literature, abdominal writhing can be induced with multiple intraperitoneal irritants, usually acetic acid (Table 2). However, the excessive intensity, the inescapable nature, and the inability to target a specific visceral organ reduce the translational relevance of this approach.
SUMMARY

Animal models have provided multiple lines of pivotal evidence to enhance our understanding of the physiology and pathophysiology of visceral pain. However, visceral pain is a complex, multifactorial disorder involving higher cognitive and cortical function as well as complicated interactions between biological, psychological, and sociological variables that no animal model is able to mimic completely. Together there are multiple challenges and limitations that must be considered when interpreting the results of studies using animal models to address questions of disease pathology and translational relevance. For example, many of the models used to study visceral pain tend to induce exaggerated pain responses or hyperalgesia; however, many patients also exhibit allodynia to innocuous stimuli. This matter has relevance for any translational work focused on the development of novel therapeutics to treat visceral pain as different neurobiological mechanisms are involved in hyperalgesia and allodynia. Additionally, mechanisms responsible for acute sensitization in the various animal models may only have low-moderate predictive validity for chronic pain conditions; however, the acute models are excellent screening tools, since efficacy against acute nociceptive behaviors will provide an indication of potential benefit in chronic pain models. To address the somewhat poor face validity of individual models, and because of the multifactorial nature of the clinical condition, efficacy of novel therapeutics should be assessed in multiple models with different etiologies (e.g., stress-induced, acute sensitization, and postinflammation) whenever possible and scientifically justified.

ACKNOWLEDGMENTS

B. Greenwood-Van Meerveld acknowledges the generous funding support for her Research Career Scientist and Merit Review Awards from the Department of Veterans Affairs.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

B.G.-V.M., D.K.P., and A.C.J. edited and revised manuscript; B.G.-V.M., D.K.P., and A.C.J. approved version of manuscript; A.C.J. prepared figures.

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