Stuart M. Brierley ·
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Visceral Pain

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Preface

In 2014, a number of scientists working on the Enteric Nervous System (ENS) and Visceral Pain gathered in Adelaide to discuss the advances and future of their research. That meeting was very successful and involved all the leading researchers in the field of enteric neuroscience from across the world. The proceedings of the meeting were published by Springer in a very successful book *The Enteric Nervous System 30 Years Later*, edited by Drs Marcello Costa and Stuart Brierley.

Six years later, as part of the International Federation of Neurogastroenterology and Motility (FNM) meeting to be held in Adelaide on March 25–29th 2020, a special satellite on "Visceral Pain and the Gut-Brain axis" was planned. This satellite was timely as these topics represented one of the most rapidly advancing fields of science, including the gut-brain axis and mechanisms involving how the microbiome communicates with the brain. However, both FNM2020 and the satellite meeting were postponed by one year because of the COVID-19 pandemic. Due to the extraordinary conditions, these international conferences were conducted one year later (April 2021) when international borders to and from Australia remained shut. Determined to go ahead with these meetings, all overseas speakers pre-recorded talks, and participants could access the talks and ask questions via an online portal.

Researchers from around the globe presented their latest findings as a review of the current state of the art in the field from both the clinical and scientific points of view. This included long-established authorities who significantly contributed to the advances in visceral pain research over the past two decades and the new generation that will continue to contribute to advancing our understanding of visceral pain. These proceedings cover a very broad spectrum of research on visceral afferent systems that are now appreciated as being critical for shaping our well-being, and their disorders underlie chronic clinical conditions of significant morbidity and mortality. When disordered, these mechanisms contribute to clinical diseases such as irritable bowel syndrome, inflammatory bowel disease, bladder pain syndrome, endometriosis, and persistent cough.

We very much thank the financial support of Ironwood Pharmaceuticals, and the Australasian Neurogastroenterology and Motility Association (ANGMA). Without

their support, this satellite meeting may not have gone ahead. We are indebted to the professional help of Danny Brookes for the pre-recordings of presentations, and the postproduction that allowed participants to access all presentations.

The organizing committee members of the meeting were Drs Stuart Brierley and Nick Spencer.

Adelaide, SA, Australia

Stuart M. Brierley
Nick J. Spencer

Contents

In Vivo Non-linear Optical Microscopy as a Multidimensional Approach to Decipher Sensory Coding

Feng Wang and Yves De Koninck

Abstract Every type of sensation is mediated by diversified groups of sensory neurons, organized in multifaceted ensembles and complex temporal dynamics. It follows that understanding how sensory input is encoded requires monitoring neuronal activity through all aspects of this multidimensionality in relevant context: i.e., simultaneous recording of multiple cells within spatially and genetically identified populations and following the temporal profile of their responses to various natural stimuli applied to their receptive field. With the development of cutting-edge microscopy and optimization of genetically-encoded sensors, fast-scanning non-linear optical approaches have become a technique of choice to study the physiology of sensory neurons and how they encode different stimuli at both the single cell and population level. Here we illustrate this through applications of in vivo calcium imaging from thermoreceptive primary sensory neurons, which revealed a rich population-level coding strategies, opening novel avenues to understand somatosensation.

Keywords In vivo Ca^{2+} imaging · Non-linear optical imaging · Pain · Thermosensation · Polymodality · Combinatorial sensory coding · Dorsal root ganglion

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1 Introduction

Primary sensory neurons in the somatosensory system, including dorsal root ganglion (DRG) and trigeminal ganglion (TG) neurons, transduce environmental stimuli into action potential and encode the characteristics of the stimuli (Woolf and Ma 2007; Basbaum et al. 2009). Innocuous and noxious stimuli are encoded, represented, and decoded to induce innocuous and pain sensation, respectively. Normal sensation is highly dependent on the faithful coding of the stimuli by primary sensory neurons. After a long debate, there are still competing theories about how sensory neurons represent and encode different stimuli, with the existence and prevalence of polymodality being a pivot issue (Wang et al. 2018; Craig 2003; Ma 2010; Perl 2007; Prescott et al. 2014). In this chapter, we first discuss the main sensory coding theories. Then we will review the recent development of both molecular sensors and fluorescent microscopy, which made in vivo non-linear optical microscopy the very tool to crack the neural code and perhaps even the pain code (Wang et al. 2016; Chen et al. 2019; Ran et al. 2016; Sekiguchi et al. 2016; Steffens et al. 2012; Stosiek et al. 2003). As an example, we will summarize recent studies applying in vivo functional Ca^{2+} imaging to investigate the physiology and coding from a large population of DRG neurons, which revealed novel coding principles by primary sensory neurons.

2 Sensory Encoding Theories

Currently, there are two main competing theories about how sensory neurons encode and represent natural stimuli with different modalities: specificity theory versus combinatorial theory (Craig 2003; Ma 2010; Perl 2007; Prescott et al. 2014; Prescott and Ratte 2012). The specificity or labeled line theory postulates that different stimuli exclusively activate separated and distinct sensory pathways, and essentially, it is the activation of that specific sensory pathway that leads to the corresponding sensation (Craig 2003, Ma 2010, Perl 2007, Prescott et al. 2014, Prescott and Ratte 2012). The main pieces of evidence supporting the specificity theory came from behavioral studies using different transgenic mouse lines. Deleting certain genes or ablating specific subpopulations of sensory neurons often only affected one type of sensitivity, either mechano-sensation or thermo-sensation (Le Pichon and Chesler 2014). For example, deleting *Vglut3* selectively attenuated acute mechanical pain sensation to intense noxious stimuli, but did not affect thermal pain (Seal et al. 2009). On the contrary, knockout of FGF13 in sensory neurons only abolished heat pain but did not affect mechanical pain (Yang et al. 2017). Similarly, ablation of TRPV1-positive sensory afferents caused a complete and prolonged behavioral insensitivity to heat, but did not affect mechanical or cold pain (Cavanaugh et al. 2009), whereas mice that lost sensory neurons expressing sodium channel Nav1.8, displayed opposite phenotypes (Abrahamsen et al. 2008). These results support the

idea that specific subpopulations of sensory neurons mediate different sensory modalities.

The specificity theory posits that each neuron is activated by a specific type of stimulus. Oppositely, combinatorial coding holds that each stimulus may activate multiple types of neurons, but in a unique combination, and it is the activation pattern of the whole population that forms the code (Craig 2003; Ma 2010; Perl 2007; Prescott et al. 2014; Prescott and Ratte 2012). The polymodality of DRG neurons facilitates combinatorial coding, whereas it becomes a design flaw for the specificity theory (Wang et al. 2018). Thus, the existence and prevalence of polymodal sensory neurons are essential in the debate between specificity theory and combinatorial coding theory. Evidence of polymodality came from electrophysiological recordings in axons or soma of primary sensory neurons in rodents, and microneurography recording in humans as well. Variable proportions of sensory neurons have been identified as polymodal depending on the peripheral tissue and species studied (Bessou and Perl 1969; Hensel and Iggo 1971; Torebjork 1974; Dubner et al. 1975; Beitel and Dubner 1976; Lynn and Carpenter 1982; Torebjork 1985). In mice, polymodal afferents represent 80% of the tibial nerve, and 47% of the sural or saphenous nerves innervating glabrous and hairy skin, respectively (Koltzenburg et al. 1997; Cain et al. 2001). Although the existence of polymodal sensory neurons has been approved by hundreds of electrophysiological studies, most of them have used in vitro preparations, which can cause injury during preparation and might alter the physiology and sensitivity of sensory afferents (Emery et al. 2016; Perl 1996). And while a certain number of single unit electrophysiological recordings have been applied in vivo, one potential limitation of these experiments is that they often require extensive use of search stimuli, resulting in plastic changes over time (Woolf and Salter 2000). The emergence of the ability to simultaneously monitor activity from a large number of individually identified neurons in vivo opens new prospects to resolve these debates and decipher how information is encoded by sensory neurons: it provides unbiased large data sets, which is essential to study the coding properties at the population level as well as interactions at the circuit level. Below, we will argue that optical recording from large ensembles of cells offers even additional advantages over *blind* multi-unit electrophysiological recording.

3 Use of Optical Approach to Study Sensory Coding

The advent of fluorescence microscopy made molecular imaging possible in live cells and tissues and has transformed life sciences (Danial et al. 2016; Wang et al. 2016; Stephens and Allan 2003; Renz 2013). The introduction of clever beam shaping strategies and photo-switchable fluorophores has further pushed spatial resolution even beyond the diffraction limit (Betzig et al. 2006; Hell and Wichmann 1994; Rust et al. 2006; Wang et al. 2016; Godin et al. 2014).

Meanwhile, parallel developments in optical fibers for photometry and optrodes allow sending and collecting light into deep tissue in both anesthetized and freely

behaving animals (Dufour and De Koninck 2015; Kim et al. 2012; Vazquez-Guardado et al. 2020; Miyamoto and Murayama 2016). Furthermore, novel micro-optical components which combine light stimulation and collection with electrophysiology provide very deep tissue access with minimal damage (Dufour and De Koninck 2015; Lechasseur et al. 2011). This is achieved, however, at the cost of losing spatial information while nevertheless keeping genetic information.

Optical microscopy adds further spatial dimensions over photometry, which yields information on the cellular structure (identify cells based on their morphology) as well as spatio-temporal dynamics in neuronal circuits. And among microscopic approaches, those exploiting non-linear modalities such as two-photon microscopy have the advantage of superior resolution at increased depth and thinner optical sectioning (by eliminating out-of-focus fluorescence), yielding deep scanning and thus 3D spatial information (Denk et al. 1990; Helmchen and Denk 2005; Stosiek et al. 2003; Zipfel et al. 2003). Miniaturized microscopes, especially those based on micro-endoscopic lenses (e.g., gradient-index – GRIN – lenses), enable deep tissue imaging, up to centimeters into the tissue (Stamatakis et al. 2021; Laing et al. 2021; Ghosh et al. 2011), but it comes at the cost of greater tissue damage, lower resolution (GRIN lenses typically have poorer optical properties), and smaller field of view (the size of the lens is usually smaller than 1 mm to be less invasive).

Yet, it is the occurrence of genetically-encoded fluorescent sensors that have transformed multidimensional functional imaging capabilities. A growing array of sensors have been developed and optimized to monitor multiple signals, with ever-increasing brightness, larger dynamic range, faster temporal dynamics, and superior signal-to-noise ratio – all this, with an expanding palette of spectral ranges (Broussard et al. 2014; Kim et al. 2021; Palmer et al. 2011; Shemetov et al. 2021; Tian et al. 2012; Yang and St-Pierre 2016). Such signals include but are not limited to voltage and Ca^{2+} fluctuations, neurotransmitters, enzymatic reactions, protein interactions and trafficking, and organelle movement (Broussard et al. 2014; Kim et al. 2021; Tian et al. 2012; Sabatini and Tian 2020; Lindenburg and Merkx 2014; Bolbat and Schultz 2017; Okumoto 2010; Choe and Titov 2022; Gokerkucuk et al. 2020). As a recording modality, light offers the ability to probe multiple signals concurrently by taking advantage of a spectral palette of optical sensors. Combined with various transgenic mouse strategies and viral transduction approaches (Luo et al. 2008; Kasatkina and Verkhusha 2022; Luo et al. 2018), the resulting capabilities opened the possibility of simultaneously monitoring the activity of hundreds to thousands of cells, each identifiable based on their structure and their genetic makeup (Hamel et al. 2015; Kasatkina and Verkhusha 2022), which is a major advantage over electrophysiological approaches (including that performed with novel multi-electrode arrays). Finally, light also allows simultaneous control of neuronal events from a large population with genetic specificity, by taking advantage of light-gated actuators such as the growing array of opsins (De La Crompe et al. 2020; Deisseroth 2015; Zhang et al. 2011; Kim et al. 2017).

Figures 1 and 2 summarize the array of dimensional information that each technique offers for live cell and tissue functional interrogation. While each approach has its own advantage depending on the type of information sought, non-linear

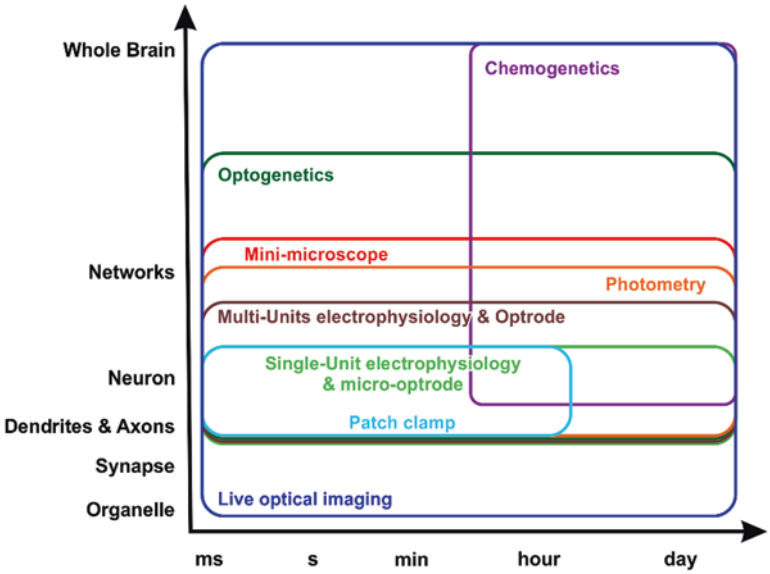

Fig. 1 Compared to other recording and manipulation methodologies for somatosensation research, live optical imaging has the best resolution and coverage in both spatial and temporal domains

optical microscopy emerges as the approach covering the broadest ranges in most dimensions to probe the activity of an ensemble of identified neurons. It follows that it is a technique of choice to study sensory information coding at both the individual and population levels.

Recording many neurons using functional imaging is also particularly useful when a certain type of stimulus only activates a small number of sensory neurons, which are hard to locate using electrophysiology. Compared to other sensory systems, such as the olfactory (Kauer and White 2001) or visual system (Ohki et al. 2005), however, applications of optical approaches in pain research are only beginning to emerge. One particular reason that pain research has lagged behind is that many of the techniques cannot be directly implemented to investigate key sensory relay sites within the pain pathways, such as the skin, DRG, and spinal cord, because they are difficult to access with light due to structural reasons (Wang et al. 2018). To illustrate the power of in vivo two-photon microscopy, below we will provide examples of the application of imaging from DRG neurons to decipher somatosensory coding strategies by primary afferent fibers.

	Temporal domain	2D-spatial domain	Volume	Depth	Structural Identity	Functionality
Non-Linear Optical Microscopy	milliseconds to days	μm2 to mm2	100s of μm3 to 10s of mm3	100s of μm to mm	Yes	Yes
Wide-field Microscopy	milliseconds to days	μm2 to mm2	100s of μm3	10s of μm	Yes	Yes
Endoscopy	seconds to hours	100s of μm2	100s of μm3	100s of μm	Yes	Yes
Super-resolution Microscopy	100s milliseconds	100 nm2 to 100s of μm2	10s of μm3	10s of μm	Yes	No
Mini-microscope	milliseconds to days	100s of μm2	100s of μm3	100s of μm to mm	Yes	Yes
Photometry	milliseconds to days	10s to 100s of μm2	100s of μm3	100s of μm to mm	Yes	No
Electrophysiology	microseconds to days	0	0	100s of μm to mm	No	Yes

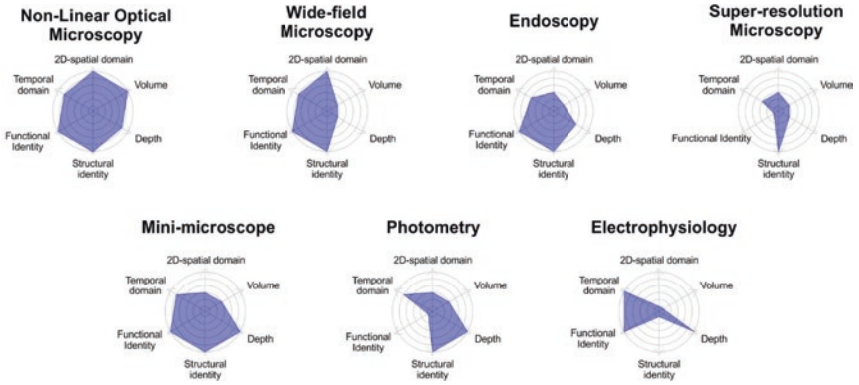

Fig. 2 The table (top) and star plot (below) summarize the multiple dimensionalities associated with various recording techniques for somatosensation studies. Among them, non-linear optical microscopy has the best coverage in all dimensions. Temporal domain: temporal resolution and feasible recording length. 2D-spatial domain: spatial resolution and feasible recording area. Volume: typical 3D volume which can be recorded. Depth: typical reachable depth. Structural identity: whether the subneuronal recording location and the genetic makeup of recorded neurons can be visualized. Functionality: whether the neuronal function can be revealed at a single neuron level

4 Polymodal and Combinatorial Coding in Primary Sensory Neurons

Recent developments in viral transduction approaches (Chisholm et al. 2018; Mason et al. 2010; Peterson et al. 2019; Vrontou et al. 2013; Wang et al. 2018) and transgenic mice (Kim et al. 2016) made it possible to express genetically-encoded sensors into sensory neurons. Meanwhile, adapting and refining experimental

preparations have enabled in vivo imaging from sensory relays which were previously difficult to reach (Johannssen and Helmchen 2013; Laffray et al. 2011; Sekiguchi et al. 2016; Steffens et al. 2012). Application of in vivo Ca^{2+} imaging opened new avenues to study the sensory coding and revealed an unanticipated complexity of sensory neurons – with multiple functionally distinct types of responses characterized by different activation thresholds and adaptation properties (Chisholm et al. 2018; Leijon et al. 2019; Wang et al. 2018; Yarmolinsky et al. 2016), which are in agreement with combinatorial coding theory.

Contradictory to the findings from electrophysiological studies, however, the polymodality of primary afferents was first called into question in mouse DRG by one of these early studies using optical in vivo Ca^{2+} imaging (Emery et al. 2016). It reported that most DRG neurons responded to only one type of noxious stimuli, suggesting that polymodality was an artifact caused by injury during tissue preparation (Emery et al. 2016). The study, however, only sampled a relatively small set of neurons (Emery et al. 2016), which can easily lead to a biased conclusion. Moreover, only noxious stimuli have been tested (Emery et al. 2016); therefore, the prevalence of polymodal neurons could have been underestimated. For instance, sensory neurons which respond to both innocuous and noxious mechanical stimuli, but not noxious thermal stimuli, would be wrongly categorized as single modal.

To target a large number of sensory neurons randomly, we used viral transduction to express genetically-encoded Ca^{2+} indicator, GCaMP6s, into all different types of primary sensory neurons without preferences (Wang et al. 2018). Then we used video-rate two-photon microscopy, instead of confocal microscopy, for in vivo imaging, which ensured high spatial and temporal resolutions. Furthermore, we delivered thermal and mechanical stimuli in both innocuous and noxious ranges to cover most modalities. Consistent with electrophysiological studies, our results revealed that approximately half of all DRG neurons are polymodal, being sensitive to more than one type of mechanical or thermal modalities. Moreover, more than 30% of DRG neurons responded to both mechanical and thermal stimuli (Wang et al. 2018). Our results were also consistent with another study using confocal microscopy for in vivo imaging (Chisholm et al. 2018), clearly demonstrated the prevalence of polymodality in primary sensory neurons, and supported combinatorial coding theory.

Meanwhile, novel behavioral studies using different activation approaches started to provide pieces of evidence corroborating combinatorial coding theory instead of specificity theory. For example, MrgprA3$^+$ DRG neurons are a subpopulation of chloroquine-responsive sensory afferents which have been suggested as a specific pathway for itch sensation (Liu et al. 2009). Indeed, activation of MrgprA3$^+$ DRG neurons can drive itch responses in mice when they are stimulated via the metabotropic pathway using chemogenetics (Sharif et al. 2020). However, activation of MrgprA3$^+$ DRG neurons can also drive pain-like behaviors when they are stimulated through fast ionotropic signaling pathways using optogenetics (Sharif et al. 2020). Consistently, in humans, stimulating a single polymodal C unit with microelectrodes induced distinct sensations at different stimulation intensities (Torebjork 1974). These behavioral and psychophysical studies indicated that the

same subpopulation of sensory neurons can engage in different sensory pathways depending on their activation pattern, which strongly supports combinatorial theory, but is against specificity theory.

5 Rich Population-Level Coding by Primary Sensory Neurons for Thermosensation

As pain sensations, thermosensation is one of the most ancient sensory processes (Vriens et al. 2014). It detects changes in environmental temperature and prompts adequate responses, which is crucial for survival and well-being. In mammals, temperature changes often induce both voluntary behaviors to seek a thermal-comfort environment and involuntary thermoregulations through autonomic responses, including sweating and vasodilatation to fight against heat, and vasoconstriction and thermogenesis to fight against cold (Insler and Sessler 2006; Tan and Knight 2018). Malfunction in thermosensation and thermoregulation, due to disease or extreme temperature conditions, can lead to harmful and even lethal hypo- or hyperthermia (Vriens et al. 2014).

Much progress has been made on the molecular, cellular, and circuit mechanisms of thermosensation in the past two decades (Dhaka et al. 2006; Julius 2013; Mckemy 2013); however, several fundamental questions remain unanswered. For instance, how does a population of primary sensory neurons encode different thermal stimuli? Does thermosensory system detect the absolute temperature or relative temperature changes? In addition to many years of research using electrophysiology, the recent application of in vivo non-linear optical microscopy on primary sensory neurons has revealed several coding principles for thermosensation.

5.1 Sensory Neurons Use Different Coding Strategies for Heat and Cold

Using single-cell recording, four main types of thermoreceptors with different activation thresholds and adaptation properties have been identified from different peripheral targets and among different species (Vriens et al. 2014). However, it was unclear how a population of sensory neurons represents different thermal stimuli. Using in vivo Ca^{2+} imaging of mice DRG, we revealed that in the warm to noxious heat temperature range, higher temperature activated more DRG neurons (Wang et al. 2018). More importantly, the neurons that responded to lower temperature formed nested sub-clusters to the neurons that responded to a higher temperature (Fig. 3a), demonstrating that as a population, DRG neurons use graded coding for warm to noxious heat (Wang et al. 2018).

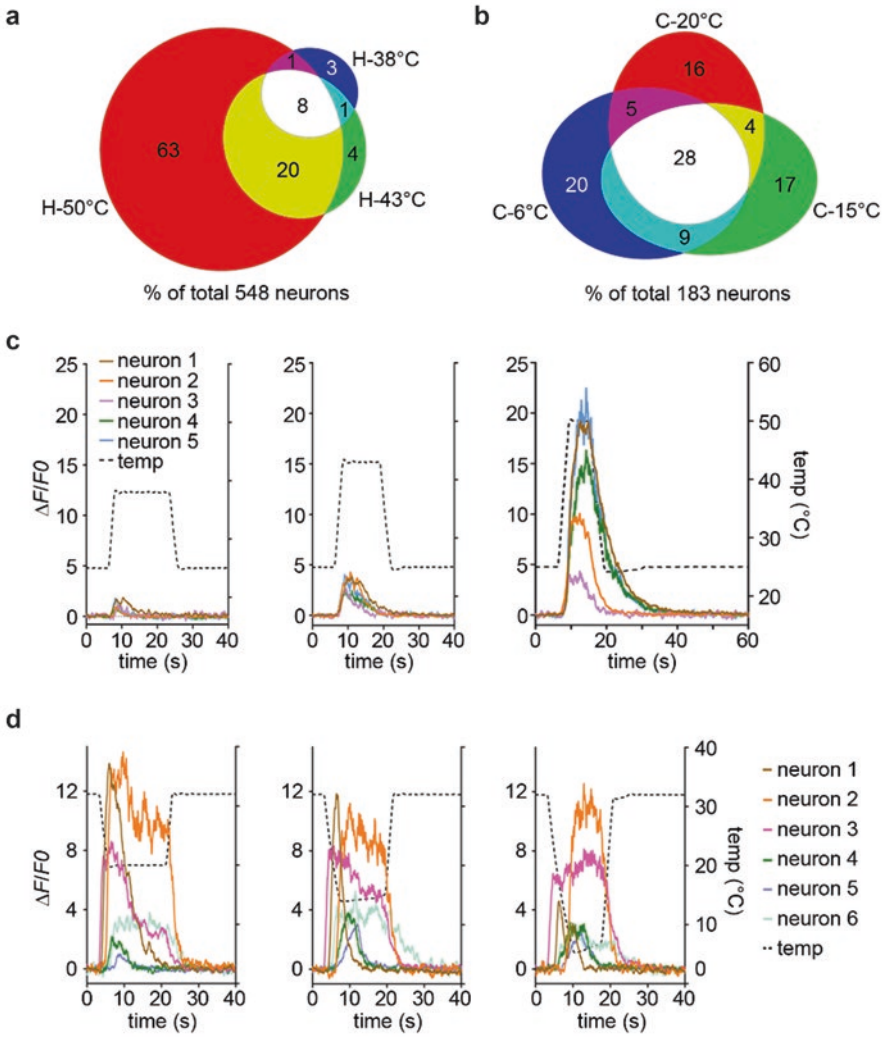

Fig. 3 Thermoreceptors encode heat and cold differently. The distribution of neurons that are responsive in the warm/heat (**a**) and cool/cold ranges (**b**). Numbers indicate the percentage of a total of the neurons responding to different thermal stimuli, as indicated. (**c**) Ca^{2+} traces of representative neurons that were sensitive to all heating stimuli from 38 °C to 50 °C (white overlap region in **a**). (**d**) Ca^{2+} traces of representative neurons that were sensitive to all cooling stimuli from 20 °C to 6 °C (white overlap region in **b**). (Adapted from Wang et al. (2018) with permission)

On the contrary, in the cool to the noxious cold range, lower temperatures did not recruit more DRG neurons; instead, they activated different combinations of DRG neurons (Fig. 3b) (Wang et al. 2018), indicating as a population, DRG neurons use combinatorial coding for cool to noxious cold.

Moreover, parametric analysis showed that higher temperature induced stronger activity in individual heating-sensitive DRG neurons (Fig. 3c), while lower temperature failed to induce stronger activity in individual cooling-sensitive DRG neurons (Fig. 3d), further demonstrating that at the individual neuron level, DRG neurons use graded coding for heat but not cold (Wang et al. 2018).

However, trigeminal ganglion (TG) neurons seem to adopt a different coding principle for cold. Different cooling stimuli activated all major groups of cooling-sensitive TG neurons, and lower temperature can induce stronger activity in the individual cooling-sensitive neuron (Yarmolinsky et al. 2016), suggesting that, contrary to DRG neurons, TG neurons use a graded coding strategy for cold. Thus, heating-sensitive neurons may use a graded coding strategy, while cooling-sensitive neurons utilize both graded and combinatorial strategies, depending on the peripheral target (Xiao and Xu 2021).

5.2 Absolute Versus Relative Temperature Sensing

There is a long debate about whether thermosensation relies on absolute temperature or relative temperature change (Vriens et al. 2014; Xiao and Xu 2021). Given the low occurrence of cooling-sensitive DRG neurons (Chisholm et al. 2018; Wang et al. 2018), the imaging approach with high throughput has a clear advantage over electrophysiology to tackle this question. Thus, in a large population of neurons, we compared the responses that started in the ramp phase of our thermal stimulation protocols with the responses that started in the returning phase of the opposite stimulation protocols (Wang et al. 2018). For heat coding, we analyzed responses when the temperature was ramped from room temperature to three different higher temperatures with responses when the temperature was ramped from different steady cold temperatures to the neutral temperature (Fig. 4a). A reverse protocol was used for cold coding (Fig. 4b). We observed that increasing numbers of heating-sensitive neurons responded to higher temperature in the active heating phase, while very few DRG neurons responded during the return phase from cold temperatures (Fig. 4a), indicating that heating-sensitive DRG neurons responded to absolute temperature (Wang et al. 2018).

In contrast, a significant proportion of cooling-sensitive neurons responded to a decrease of temperature during the return phase from steady heat temperature. The percentage of neurons that responded to the return phase from higher temperature was comparable to the percentage of neurons that responded to active cooling stimulation from neutral temperature (Fig. 4b), indicating that cooling-sensitive DRG neurons responded to relative changes in temperature (Wang et al. 2018). These imaging results have revealed that heating- and cooling-sensitive neurons are driven by different factors of temperature stimuli: heating-sensitive neurons are activated by absolute temperature, while cooling-sensitive neurons are more sensitive to relative changes in temperature.

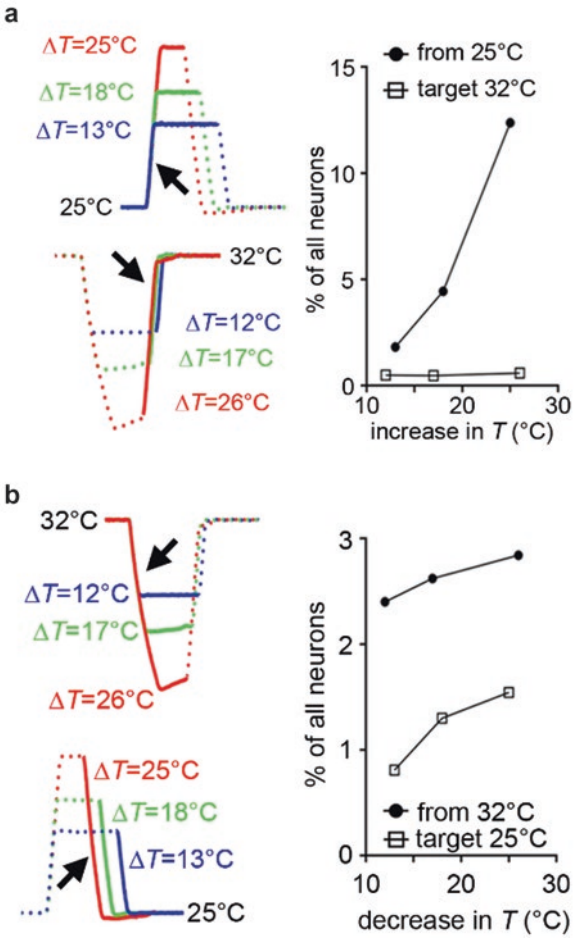

Fig. 4 Cooling-sensitive, but not heating-sensitive, neurons are sensitive to relative temperature change. (**a**) Heating-sensitive neurons primarily responded to absolute temperature. Left: the protocols used to test thermal responses. Responses were measured during the onset and plateau phases of heating stimuli, and the returning phase from cooling stimuli (arrows). Right: the proportion of neurons that responded to the onset and plateau phases of heating stimuli when starting from an adaptation temperature of 25 °C (solid circle) versus those that responded to an increasing temperature change with similar degrees but returning from different steady-state cool/cold temperatures toward a target temperature of 32 °C (empty square). (**b**) A significant proportion of cooling-sensitive neurons responded to a relative temperature change. Left: opposite protocol to that used in (**a**) to test for thermal responses during the onset and plateau phases of cooling stimuli, and the returning phase from heating stimuli (arrows). Right: the proportion of neurons that responded to the onset and plateau phases of cooling stimuli when starting from an adaptation temperature of 32 °C (solid circles) versus those that responded to a decreasing temperature change with similar degrees but returning from different steady-state warm/hot temperatures toward a target temperature of 25 °C (empty squares). (Adapted from Wang et al. (2018) with permission)

Interestingly, the same pattern has been observed in spinal cord neurons (Ran et al. 2016) and cortical neurons (Vestergaard et al. 2022) as well, indicating that these are essential characteristics of the thermal stimuli, so they have been preserved during the signal processing at different relays along the sensory axis.

6 Future Perspectives

The application of in vivo non-linear optical microscopy has allowed monitoring of activity from a large population of sensory neurons simultaneously and produced important new insights about sensory coding. It is a powerful tool to study the mechanism of sensation, including pain, at both cellular and circuitry levels. Combined with optogenetics, the optical approaches can open novel avenues for pain research and lead to potential therapeutic development for pain management.

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An Open Science Model to Accelerate the Generation, Implementation and Distribution of Optogenetics and Viral Tools

Marie-Eve Paquet and Yves De Koninck

Abstract Optogenetic techniques take advantage of the exquisite levels of cellular control that are enabled using the combination of light and genetic targeted constructs. They are becoming increasingly mainstream in neuroscience laboratories and show great promises in the therapeutic space. In the field of pain, there is hope that optogenetics might help achieve levels of control difficult to reach with available drug treatment which can cause disabling side effects.

However, these techniques are based on the combination of numerous advanced technologies (molecular biology, live microscopy, virology, tissue optics, etc.) which often exceed the expertise and capabilities of one single user team. Considering the complexity of the nervous system, the biological questions attempted to be answered are so broad that the validity of a given strategy established in a particular system might not hold in a different one. Testing of a tool in the proper experimental paradigm, as well as significant adaptation of that tool, is often required.

Here we present a model of BioFoundry developed in Canada, to accelerate the development, implementation and dissemination of optogenetics and viral delivery tools throughout the neuroscience community. We describe a few examples of newly developed genetically encoded sensors which have passed through the foundry cycle and have been deployed as well as several existing tools requiring specific validation. We also address the issue of customisation through the description of a few key tool components that may influence efficiency and reliability.

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1 Introduction

Genetically encoded light-sensitive proteins—optogenetic tools—have revolutionised neuroscience. The advent of light-activatable proteins, to generate novel sensors and actuators, has opened an immense array of possibilities to dramatically enhance the throughput of biological discoveries, enabling precise manipulations of the nervous system in an unprecedented manner to (i) decipher the neural code, (ii) test drug action in context-relevant manner, (iii) understand which components of neural circuits dysfunction in disease states and (iv) design next-generation, circuit-specific, closed-loop, smart neurostimulation approaches for neurotherapeutics and diagnostics.

However, the challenge of robustness remains significant in the design pipeline of these tools as "real world" applications are not tested at the time of development. Numerous strategies are reported each year that show promising features based on proof of principle experiments but end up being used very seldomly. This can be largely attributed to the difficulty for a laboratory specialised in protein engineering to perform a large array of tests in multiple experimental paradigms. Similarly, individual labs wanting to address research questions lack the time, expertise and resources to undertake the development of optogenetic-, associated gene transfer tools and ancillary hardware.

The optimisation required for these tools to be adopted by non-expert labs can only be achieved by building an active feedback loop between developers and testing teams. These teams must work with dedicated staff involved in tool validation in a range of models (from invertebrates to human tissue). This critical loop is the largest bottleneck which can only be accelerated with a stable collaboration between groups/cores dedicated to design/development and a series of groups/nodes involved in testing.

In the context of the national neurophotonics initiative (neurophotonics.ca), we developed a BioFoundry as the model structure to fill this gap. Borrowed from the engineering world and more recently conceived in the context of synthetic biology, biofoundries can be described as organisations that provide an integrated infrastructure to enable the rapid design, construction and testing of genetically reprogrammed organisms for biotechnology applications and research (Holowko et al. 2021). This model is based on a *Design-Build-Test* (DBT) cycle which we adopted and adapted to the niche of optogenetics and viral tools development (Fig. 1). The resulting platform was entitled the Canadian Optogenetics and Vectorology Foundry (COVF) ("Canadian Optogenetics & Vectorology Foundry" n.d.). The Foundry is based on three pillar elements: the design of new tools by the *optogenetic protein engineering*

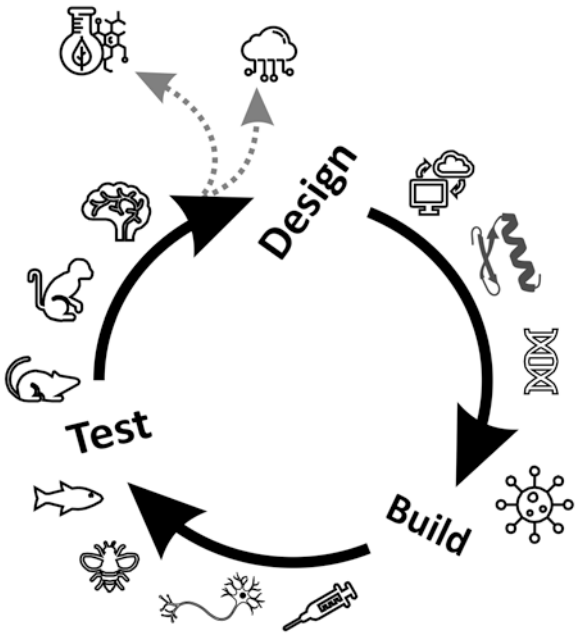

Fig. 1 The BioFoundry model is based on the *Design-Build-Test* (DBT) principle. In our implementation it involves the design of novel optogenetic tools, their testing into different experimental models, feeding back into the design step or graduating into distributable tools. Knowledge gained in the process is made available to the community through shared data repositories

core, which are then transferred to the *viral vector core* for assembly into a delivery vehicle that can be configured in different ways. Depending on user needs, *testing and validation* of the tools is performed in experimental models and paradigms within testing nodes (Fig. 2). The Design-Build-Test cycle is also applicable to existing tools which may require optimisation or adaptation to a particular type of work.

2 Development of New Tools

Concrete, powerful outcomes of this model are illustrated, first with the development of optogenetic sensors operating in the near-infrared (NIR) regime (Fig. 3). This development is critical to the field, to expand the application for deep tissue intervention—the main bottleneck in translating optogenetic technologies into clinical applications. The DBT cycle enabled the emergence of this entirely new class of sensors for the community (Qian et al. 2019) and, in turn, accelerated the development of improved generation of the tool (Qian et al. 2020) (Fig. 3). Another key class of tools under development are chemosensors (e.g. neurotransmitter or neuromodulator sensors). The most recent DBT task force was centred on the development of a lactate sensor. Lactate has been postulated as a fundamental signalling molecule linking metabolic demand with oxygen delivery in the brain (Pellerin and Magistretti 1994). The so-called lactate shuttle is postulated to serve as a regulation

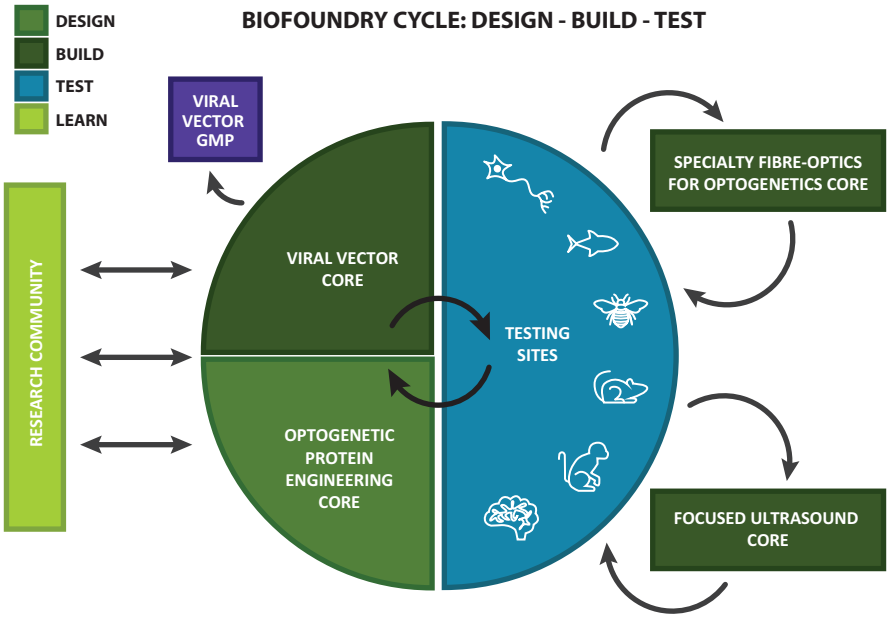

Fig. 2 Schematic representation of the different key pillars of the Canadian Optogenetics and Vectorology Foundry (COVF) supporting the Design-Build-Test cycle

sensor mechanism enabling the brain to control blood and metabolic energy flow to areas of activity. This mechanism essentially underpins the principle behind BOLD imaging, which has revolutionised the field of functional brain imaging (by MRI) to link neural activity with behaviour. For example, lactate has been shown to improve decision making in visceral hypersensitivity in rats (Wang et al. 2017). Yet, how lactate signalling operates remains elusive without the capacity to directly measure it in situ, both inside cells, where it is involved in the metabolic process, and outside cells, where it is postulated to serve as a signalling molecule and energetic currency. To fill this gap, the optogenetics protein engineering core embarked in developing a novel Lactate sensor. The active COVF Design-Build-Test cycle recently delivered a first-generation sensor (LACCO) (Nasu et al. 2021). Through feedback from the community, it became clear that the COVF needed to develop a much broader array of sensors, including intracellularly and extracellularly targeted, multicolour sensors coupled with other sensing capabilities linked to metabolic processes, such as pH. This yielded 15 new versions of prototype sensors (including inactive controls) with the potential to be configured into numerous combinations. To assess the needs of the community users and testing nodes for the validation of generation 2 of the LACCO sensor, the COVF conducted a survey allowing the teams to prioritise the work: e.g. initially, cassettes were configured with three promoters and AAV vectors packaged into two serotypes. Sixteen different configurations were engineered, packaged, purified (into batches of 500 μL to 2 mL), validated and distributed to 11 labs (testing nodes and other users) for testing in a wide array of paradigms. The

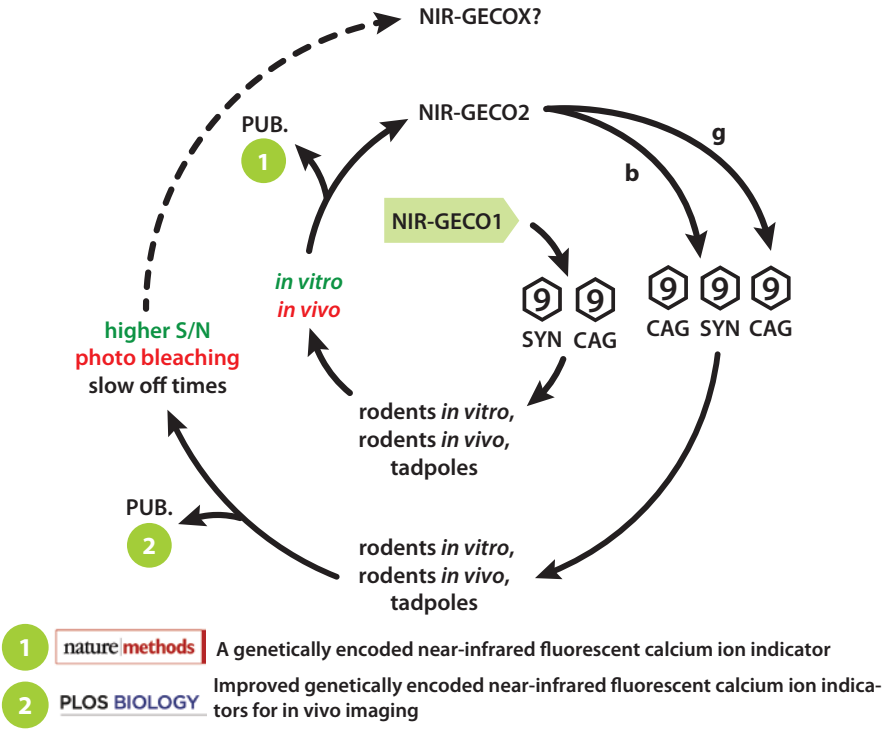

Fig. 3 Optimisation loop used for the Near Infrared calcium sensor NIR-GECO. The first iteration of the sensor was developed by Robert Campbell lab and packaged in AAV9 in two different configurations (CAG or Syn promoters) to allow for testing in vitro and in vivo in various models. The data showing good performance in vitro but relatively poor performance in vivo was reported in a first paper. Based on these results, sensor was optimised again through the same loop and its characteristics continue to be improved by further testing

centralisation of the process by one core team allows a systematic comparison of the tools (e.g. same vector lots, production process, quality control, etc.), dramatically improving and accelerating the development process and benchmarking of the tools. This systematic development enterprise can only be handled by an ecosystem such as the one supported by the COVF. To our knowledge, this is the only model of this kind at such a scale in the world.

3 Validation and Characterisation of Existing Tools

The democratisation of optogenetics also means that scientists are now applying the tools and associated strategies in a large array of models and for various applications which may be very different from what was initially designed. A few research laboratories are equipped with the proper staff and infrastructure to transfer a

technology from its initial application to another while also focusing on the scientific question at hand. However, for most groups, validation of existing tools can become tedious and time consuming and require a lot of resources. The DBT cycle we have implemented also applies to the validation of existing tools in different models as well as the sharing of associated results, positive or negative. Examples of tool validation that needs to be performed include the following.

Type of signal being probed (sensitivity and dynamics) A good example is the case of voltage and ion sensors. Many generations of these sensors have been implemented and continue to emerge at an accelerating rate. Each version comes optimised for different parameters, including response time/kinetics, dynamic range, sensitivity, maximum intensity, intensity in basal condition, response linearity across the dynamic range, ratiometric capabilities, etc. No single sensor can then be declared as the universal one for all applications because each application calls for specific parameters to be optimum. For example, when the dynamics of the response is the critical variable to measure, sensor kinetics is obviously the key deciding factor. In some cases, a certain level of basal fluorescence is key to be able to detect all positively labelled cells (in the absence of accompanying structural fluorescent reporter) including non-responders; if absolute, steady-state readings are needed, ratiometric indicators (e.g. FRET-based) can be preferred; a wide dynamic range may be preferred against maximal fluorescence in some cases to optimise signal to noise. The case of genetically encoded calcium sensors is a good illustrative example. Multiple generations are used and each now comes with different sub-versions optimised for different parameters. The GCaMP series became very mature and widely adopted with the advent of the GCaMP6 version, but in the slow (s), medium (m) and fast (f) series. GCaMP6s is often preferred for its overall sensitivity, yet the use of GCaMP6f is necessary when looking at short, high-frequency events. With the advent of versions 7 and 8 of the GCaMP, more sub-variants emerged with a wider set of parameters individually optimised. What is currently lacking is a thorough, quantitative assessment of the value of each of these sub-variants in specific functional settings, cell types and subcellular compartments. This requires the support of a DBT cycle based on a community of testing groups (testing nodes; Fig. 2), but also agreeing on a systematic set of quantitative assessment criteria to properly document the parameter sets in which each variant performs. The resulting dataset needs to be organised within an open data repository that individual labs can refer to make informed decisions on the choice for their respective application. This is the type of concerted effort that the COVF strives to support.

Cell type functionality The specific cell type delivery of markers, sensors and effectors can be obtained through different strategies which are unlikely to be universal. For example, viral vectors have been exploited and optimised for efficient retrograde access to projection neurons (Callaway and Luo 2015; Tervo et al. 2016; Wickersham et al. 2007; Hirano et al. 2013; Junyent and Kremer 2015). However, despite the simplicity of the concept, a given vector must be validated in the cells of interest to ensure its optimal functionality. For instance, *Rabies virus*-based vectors

have been used extensively for retrograde tracing and have shown enabling capabilities for neural circuit dissection (Xu et al. 2020). However, significant differences in direct retrograde infection capabilities of these viruses among cell types have been observed, which could have a critical impact on data interpretation. Some classes of unmyelinated sensory neurons are resistant to rabies infection in the spinal cord, for example. This demonstration was initiated by an observation of the unexpectedly low number of reports of efficient retrograde labelling of unmyelinated nonpeptidergic sensory neurons and confirmed by a systematic study involving rabies infections combined with the use of comprehensive sets or cell type markers (Albisetti et al. 2017). A similar systematic analysis comparing the efficiency of other viral retrograde tracers (e.g. *FugB2* Lentivirus, AAV2-retro, AAV9-retro, CAV2, AD5) should be conducted to ensure proper vector usage and interpretation of experimental results. Such an endeavour requires a community effort and an organisation such as the COVF, involving experts in viral vector packaging as well as a number of testing labs with expertise in different circuits and cell types of the nervous system.

Cellular identity also has an impact on promoter efficiency, including those typically known as "ubiquitous". For example, we have found that the EF1a promoter, which is widely used for central neurons, does not drive the expression of transgenes in primary afferents (unpublished). Despite repeated observations, this type of negative result can remain anecdotal as it is usually not reported, which often leads to a waste of time and resources for the global community. Organisations like the COVF are well positioned to perform these types of characterisation and establish data sharing initiatives to facilitate the exchange of useful results.

Species differences Although optogenetics has been pioneered and mostly developed in mice, the appeal of the technique as a therapeutic approach has recently brought the field to applications in species with more translational value. Optogenetics in non-human primates is now emerging as an essential validation step before the development of clinical applications. These developments have highlighted significant species differences in the efficiency of strategies such as viral vector-based delivery. For example, the engineering of new AAV serotypes able to cross the blood-brain barrier (BBB) for efficient targeting to the CNS from intravenous injections has been very successful but numerous iterations have been worked out to obtain positive results across species. The initial introduction of mutations into the capsid of AAV9 to generate BBB crossing serotypes such as PHP.B and PHP.S (Challis et al. 2019; Chan et al. 2017; Deverman et al. 2016) demonstrated that these vectors exhibit different targeting properties in rodent strains and in NHP and that toxicity is an issue (Hordeaux et al. 2018; Liguore et al. 2019). New versions of these vectors have now been developed to circumvent these issues but these results emphasise the importance of validating tools in various species (Chen et al. 2022; Goertsen et al. 2022). Similarly, gene targeting strategies involving promoters and enhancers developed for mice have also shown limited translatability in humans, whereas those developed in NHP are efficient at a much higher rate when tested in humans (Jüttner et al. 2019). These observations support

the approach brought forward by the COVF with the capability to validate tools in different model systems from invertebrates to non-human primates and post-mortem live human tissue.

4 Standard Requirements and Funding Model

A number of standards must be established and followed for the DBT cycle to function efficiently. For example, the production of viral vectors must be adapted to the large number of tools and iterations that need to be tested. Validation typically requires small amounts of high-quality material that can be reproduced under the same conditions if needed. Highly pure vector stocks are needed for testing in translational models such as non-human primates where low experimental replicates can be achieved. In these circumstances we want to avoid high variability in immune responses to impurities and contaminants. While production standards must be elevated, costs must remain compatible with research and validation in academic settings.

Support for this type of distributed and highly coordinated effort requires a national or international level funding scheme. The COVF has been made possible by the Brain Canada Foundation, through the Canada Brain Research Fund, with the financial support of the multiple regional partners and Health Canada. Only under such a global funding scheme, across many labs and many institutions, can an open science (open data) spirit be operational, which contrasts with the more traditional competitive funding models increasingly adopted in Western countries.

5 Conclusion

The monumental task of designing, optimising and validating optogenetics and viral delivery tools for multiple paradigms, tissue types and animal species far exceeds the capabilities of any one team and will succeed only if a proper structure of data sharing is put in place and the whole of the vested research community is compelled to contribute. A proposal to accelerate this effort is the rapid, unencumbered, dissemination of open-source constructs, combined with open communication of both positive findings and setbacks. Models like the COVF are well adapted to those needs and could be transposed to other areas of biological applications such as genome editing and testing of biologics. A comparable open science effort was developed to provide systematic, independent and unbiased characterisation and validation of antibody tools for the research community (“Home” n.d.; Laflamme et al. 2021).

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Vagal Neuroinflammation Accompanying Respiratory Viral Infection: An Overview of Mechanisms and Possible Clinical Significance

Nathalie A. J. Verzele, Kirsty R. Short, Stuart B. Mazzone, and Alice E. McGovern

Abstract Respiratory viral infections are pervasive in humans. For many, viral-induced pathogenesis is mild and largely an annoyance to daily life. However, for some, the severity of disease can be life-threatening, such as that seen with recent outbreaks of SARS-CoV-2 and influenza virus. This spectrum of disease presentation is underpinned by an inflammatory lung pathology, and many of the symptoms accompanying respiratory viral infections, including cough, an itchy or irritated throat, perceptions of difficulty breathing and excessive mucous and airflow limitations, suggest that viral-induced pulmonary inflammation alters the activity in neural pathways that ordinarily regulate bronchopulmonary function. In this chapter, we explore novel ways in which respiratory viral infections may impact bronchopulmonary nerves, focussing on the sensory fibres of the vagus nerves and their brain connections. We review work from our group and others that offers compelling evidence that vagal neuroinflammation may be an important and unrecognised component of respiratory viral pathogenesis and an important consideration for future advances in clinical management of patients with severe respiratory viral disease.

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Abbreviation

ACE2	Angiotensin-converting enzyme 2
ARDS	Acute respiratory disease syndrome
ATP	Adenosine 5′-triphosphate
CD4+ cells	Cluster of differentiation 4 (T helper cells)
CD8+ cells	Cluster of differentiation 8 (cytotoxic T cells)
CNS	Central nervous system
DAMPs	Damage-associated molecular patterns
hCoV	Human coronavirus
HMGB1	High mobility group box protein 1
hMPV	Human metapneumovirus
IAV	Influenza A virus
IFNAR1	Interferon alpha receptor, type 1
IFNAR2	Interferon alpha receptor, type 2
IFNb	Interferon beta
IFNγ	Interferon gamma
IL-12	Interleukin 12
IL-1b	Interleukin 1 beta
IL-23	Interleukin 23
IL-33	Interleukin 33
IL-5	Interleukin 5
RAGE	Receptor for advanced glycation end-products
RSV	Respiratory syncytial virus
SARS-CoV-2	Severe acute respiratory syndrome-coronavirus 2
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRPA1	Transient receptor potential, ankyrin 1
TRPV1	Transient receptor potential, vanilloid 1

1 An Overview of the Neurobiology of Airway Sensation

The respiratory tract is densely innervated by sensory neurons, many of which arise from the vagus nerves (Fig. 1) (Mazzone et al. 2020; Lee and Yu 2014; Mazzone and Undem 2016). The cell bodies of vagal sensory neurons reside in two distinct collections of cells known as the nodose (inferior) and jugular (superior) ganglia. These ganglia are located bilaterally, extrinsic to the respiratory system, at the cranial end of the vagus nerves (Mazzone and Undem 2016). The nodose and jugular ganglia differ in embryonic origin, and consequently the sensory neurons residing within

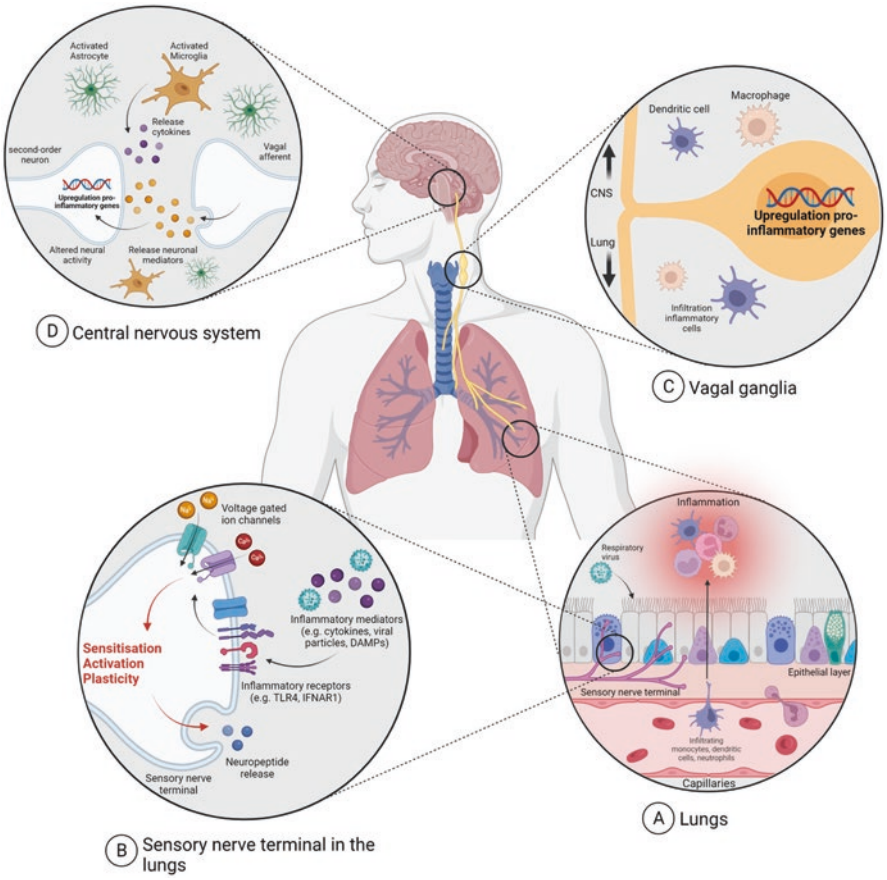

Fig. 1 Mechanisms involved in the pathogenesis of respiratory viral infections. (**a**) A viral infection of the respiratory epithelium initiates an inflammatory response which serves to limit the extent of viral propagation and promote viral clearance. (**b**) Respiratory epithelial cells are juxtaposed to vagal sensory nerve terminals, which may be activated or sensitised by respiratory viruses and/or the resultant products of inflammation. (**c**) Severe respiratory viral infections have been shown to cause inflammation within the vagal sensory ganglia, characterised by inflammatory cell infiltration and altered neuronal gene expression. (**d**) Neuroinflammation may extend to the brainstem and brain where alterations in glial cell activity, inflammatory gene expression and synaptic function have been reported. Collectively, alterations in normal function within these diverse sites of inflammatory pathology are thought to contribute to acute and long-term symptoms associated with respiratory viral infections. *Abbreviations*: CNS central nervous system; DAMPs damage-associated molecular patterns; IFNAR1 interferon alpha and beta receptor subunit 1; TLR4 toll-like receptor 4. (Created with BioRender.com)

each set of ganglia display differing patterns of gene and protein expression, resulting in distinct anatomical and functional airway sensory neuron phenotypes (Mazzone and Undem 2016; Wang et al. 2017; Carr and Undem 2003). Peripherally, although some overlap of the location of nodose and jugular neuron terminal fields

exists, nodose sensory neuron terminals are more prevalent within the intrapulmonary airways and lungs, whereas jugular neurons have terminations mostly in the large extrapulmonary airways and larynx. The central projections of nodose and jugular neurons are quite distinct. Nodose airway sensory fibres project mainly to the nucleus of the solitary tract, whereas jugular airway sensory fibres terminate in and around the paratrigeminal nucleus in the medulla. Collectively, these two broad classes of sensory neurons serve to detect a wide range of physical, chemical or thermal stimuli that can occur in the airways and lungs and send this information about the airway environment to the central nervous system to regulate a range of respiratory and autonomic processes important for normal homeostatic functioning and pulmonary defence (Lee and Yu 2014; Carr and Undem 2003).

Vagal sensory nerve fibres terminate in proximity to epithelial cells, mucosal glands, vasculature and/or airway smooth muscle in the conducting airways, respiratory airways and lung parenchyma (Lee and Yu 2014; Carr and Undem 2003; Brouns et al. 2021). A major functional subclass of airway sensory neurons serve as 'physiological' receptors, monitoring lung inflation, deflation and airway smooth muscle tone. These sensory neurons are typically myelinated fast-conducting A-fibres, derived from the nodose vagal ganglia, and play an important role in optimising breathing, airway patency and gas exchange (Mazzone and Undem 2016; Carr and Undem 2003; Kollarik et al. 2010; Mazzone 2004). This class of sensory neuron is not the focus of this chapter and has been described in detail elsewhere (Mazzone and Undem 2016; Wang et al. 2017; Carr and Undem 2003; Kollarik et al. 2010; Kupari et al. 2019). A second major functional class of airway sensory neurons, often called nociceptors, are derived from both the nodose and jugular ganglia and mainly consist of slower conducting C-fibres (although Aδ-fibre nociceptors exist in some species) (Mazzone and Undem 2016; Carr and Undem 2003). Nociceptors are so named as they are specialised to sense noxious and potentially harmful stimuli that could impact the airways and compromise normal pulmonary functions (Mazzone and Undem 2016; Carr and Undem 2003). They are especially important as the respiratory tract is an open system, exposed to the environment via the air that is inhaled which can contain pathogens and irritant stimuli (e.g. smoke or other chemicals) that can cause airway damage (Mazzone and Undem 2016; Carr and Undem 2003). Additionally, the initial anatomical conduits for foodstuffs and air are shared with potential for airway damage via the aspiration of consumed foods and liquids or refluxed gastric contents. Many nociceptors are positioned in or close to the airway epithelial barrier allowing for constant monitoring of the presence of these potentially damaging stimuli (Mazzone and Undem 2016; Carr and Undem 2003). Collectively, the two broad functional classes of vagal sensory neurons play an important role in pulmonary homeostasis and defence by providing feedback from the respiratory environment to the brainstem driving a variety of responses including the Hering-Breuer inflation and deflation reflexes, reflex changes in bronchomotor tone and cough (Lee and Yu 2014; Carr and Undem 2003; Brouns et al. 2021).

Sensory neuron transduction of stimuli and conduction of action potentials is mediated by a suite of ionotropic receptors, G protein-coupled receptors and voltage-gated ion channels, expressed on the peripheral nerve terminals and axons (Fig. 1) (Lee and Yu 2014; Mazzone and Undem 2016; Wang et al. 2017; Kollarik et al. 2010). Some stimuli directly activate channels and receptors expressed by airway sensory neurons, whilst others activate sensory neurons via intermediary molecules produced from resident or infiltrating cells, including immune cells. The unique complement of these receptors and channels confers sensory neurons with their subtype functional specificity, allowing for the encoding of different modalities of stimulation (Lee and Yu 2014; Mazzone and Undem 2016; Wang et al. 2017; Kollarik et al. 2010). Nociceptors are often characterised by the expression of one or more of the transient receptor potential (TRP) family of ionotropic sensory transduction channels (Mazzone and Undem 2016; Kollarik et al. 2010; Mazzone 2004; De Logu et al. 2016). Of the TRP family, TRPV1 and TRPA1 channels are abundantly expressed on airway nociceptors and have been shown to play a key role in response to noxious thermal stimuli, exogenous chemicals (including components of smoke or natural products in plants such as capsaicin from chilli peppers) and endogenous inflammatory mediators (Mazzone and Undem 2016; De Logu et al. 2016; Caceres et al. 2009). Interestingly, the activation of these channels has been implicated as playing an important role in respiratory diseases. For example, the ablation of TRPA1 in a murine asthma model showed a reduction in bronchial hyper-responsiveness and inhibition of infiltrating eosinophils and levels of interleukin (IL)-5 (De Logu et al. 2016; Caceres et al. 2009).

Additional to these channels, nociceptors express a variety of receptors for inflammatory mediators and pathogen-associated molecular patterns including receptors for interferon alpha (IFNAR1, IFNAR2) (Wang et al. 2017; Patil et al. 2020), tumour necrosis factor (TNFR2) (Wang et al. 2017) and toll-like receptor 3 (Wang et al. 2017). Activation of each of these transduction receptors and channels results in the modification of membrane excitability and depolarisation. Altered membrane voltage leads to the activation of voltage-gated sodium channels (e.g. $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$), initiating centrally directed propagation of action potentials. Some nociceptors can also release neuropeptides including substance P, calcitonin gene-related peptide, neurokinin A and vasoactive intestinal peptide locally in the airway tissues (Lee and Yu 2014; Mazzone and Undem 2016; Carr and Undem 2003; Kollarik et al. 2010). Neuropeptides can interact directly and indirectly on structural cells to alter pulmonary physiology and on immune cells to modulate inflammation and are therefore important mediators regulating a variety of pulmonary pathologies. Calcitonin gene-related peptide, for example, is a potent vasodilator and has been shown to inhibit dendritic cell maturation by modulating the antigen presentation, negatively affecting T cell activation (Assas et al. 2014; Rochlitzer et al. 2011).

2 Pathogenesis of Respiratory Viruses

Pathogen-dependent respiratory diseases in humans are mostly caused by viruses, which notably include influenza A virus (IAV), human coronaviruses (hCoV), respiratory syncytial virus (RSV), human metapneumovirus (hMPV) and rhinovirus (Lee 2017; Schmidt and Varga 2018; Rey-Jurado et al. 2020; Allie and Randall 2017; Gillim-Ross and Subbarao 2006; Johansson and Kirsebom 2021). Viral respiratory tract infections are extremely frequent across the life span and can range in severity from subclinical presentation with no symptomatology, mild cold-like symptoms, to severe symptoms such as pneumonia leading to acute respiratory distress syndrome (ARDS) or even death (Lee 2017; Schmidt and Varga 2018; Rey-Jurado et al. 2020; Allie and Randall 2017; Gillim-Ross and Subbarao 2006; Johansson and Kirsebom 2021). The disease severity is dependent on the host immune response, patient age, any pre-existing patient comorbidities and type/strain of virus in question.

Respiratory viruses commonly infect epithelial cells in the upper (nose, pharynx and/or larynx) and lower (trachea, bronchi, bronchioles and alveoli) respiratory tracts (Fig. 1) (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019). Epithelial infection is an important mode of viral replication and a primary trigger for the initiation of the immune response. Viral-induced inflammation is also facilitated early in the infection by alveolar macrophages, whilst resident and infiltrating dendritic cells play an important role in both innate host defence and the coordination of innate and adaptive immune responses. Epithelial and resident immune cells in the respiratory tract can detect viral pathogens through a variety of host cell pathogen recognition receptors including toll-like receptors, retinoic acid-inducible gene 1-like receptors, cytosolic DNA sensors and nucleotide oligomerisation domain-like receptors (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019). These pathogen recognition receptors detect both pathogen-associated molecular patterns expressed by invading viruses and damage-associated molecular patterns released by infected, injured and necrotic/apoptotic cells (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019).

Early inflammation is orchestrated through the secretion of a first wave of tissue damage-associated molecular patterns and inflammatory cytokines including ATP, HMGB1, IL-33, IFNγ, IFNβ, IL-6, TNF, IL-12, IL-23 and IL-1β (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019; Troy and Bosco 2016; Yoo et al. 2013). In addition to effects on resident respiratory tract cells, these mediators can attract other innate immune cells such as cytotoxic T-cells, natural killer cells and innate lymphoid cells to the infected region. These cells release additional cytokines exerting cytotoxic effects on infected cells. The second wave of cytokines will also attract circulating neutrophils and monocytes, thereby further amplifying the immune response (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019; Troy and Bosco 2016; Yoo et al. 2013). In addition, cytokines promote dendritic cell maturation. Matured dendritic cells are antigen-presenting cells that migrate to local lymph nodes to prime the adaptive responses (Allie and Randall 2017; Braciale et al. 2012;

Wong et al. 2019). In the lymph nodes, naïve $CD4^+$ T-cells and $CD4^+$ effector cells are activated and proliferate and differentiate. $CD8^+$ T-cells differentiate to cytotoxic and memory $CD8^+$ T-cells releasing potent cytokines and aid in apoptosis and clearance of infected cells (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019; Troy and Bosco 2016; Yoo et al. 2013).

Host inflammatory responses following viral infection are ideally regulated to effectively clear virus and virally infected cells from the respiratory tract and promote tissue repair (Allie and Randall 2017; Braciale et al. 2012; Wong et al. 2019; Troy and Bosco 2016; Yoo et al. 2013). However, during some respiratory infections an uncontrolled immune response is observed with an exacerbated cytokine response, commonly referred to as a 'cytokine storm'. The uncontrolled immune response has cytotoxic properties, augmenting the tissue injury in the pulmonary system by increasing apoptosis and necrosis to the alveolar epithelium (Matthay and Zemans 2011; Matthay et al. 2019). A resultant excessive activation of neutrophils is central to this pathology, progressing affected patients towards the development of ARDS and the release of toxic mediators that increase paracellular epithelial permeability and promote severe vascular damage (Matthay and Zemans 2011; Matthay et al. 2019). This immunopathology contributes significantly to disease severity and patient morbidity typically includes symptoms such as excessive coughing and severe dyspnoea, suggestive of profound, yet poorly described impacts on pulmonary sensory nerves.

3 Neurotropism of Respiratory Viruses

Severe respiratory tract infections have been shown to result in systemic inflammation and cause extrapulmonary disorders, including neurological clinical manifestations such as encephalopathies characterised by seizures, memory loss, confusion, personality changes, depression, anxiety and 'brain fog', encephalitis and syndromes including Guillain-Barre syndrome. These neurological manifestations can be induced by neurotropic viruses with less severe symptoms usually caused by non-neurotropic viruses (Ruisanchez-Nieva et al. 2017; Ryabkova et al. 2021; Glaser et al. 2012; Mao et al. 2020; Zubair et al. 2020; Frankl et al. 2021; Bohmwald et al. 2018; Desforges et al. 2019).

Respiratory viruses including strains of RSV, hMPV, IAV and hCoV are known to be neurotropic and could conceivably invade the nervous system at the site of airway infection (Bohmwald et al. 2018; Desforges et al. 2014, 2019; Koyuncu et al. 2013; Dey et al. 2021). In support of this assertion, following intranasal inoculation of rodents with influenza virus, viral antigen has been recovered from trigeminal and vagal ganglia in the absence of any systemic infection (Shinya et al. 2000; Matsuda et al. 2004). hMPV has been shown to infect and persist in local neuronal processes in the airway wall following epithelial replication (Liu et al. 2009). These data suggest a capacity of sensory neurons to uptake and potentially transport some respiratory viral strains. As the nose is typically the first site of viral

infection, transport of virus to the brain via the olfactory nerves has been widely postulated (Bohmwald et al. 2018; Desforges et al. 2019; Koyuncu et al. 2013; Dey et al. 2021) and recent observations with SARS-CoV-2 suggest this is a possible mechanism for the neurological sequalae in patients with COVID-19 (Meinhardt et al. 2021; Douaud et al. 2022). However, little is known about the specific viral and neuronal entry factors that allow for neuronal infection. In the case of SARS-CoV-2, sensory neurons do not appreciably express the most common entry factor needed for viral uptake (ACE2) (Shiers et al. 2020), suggesting non-traditional factors may be important for viral interactions with neurons. Whether this extends to other neurotropic respiratory viruses is not entirely clear. After entry occurs at the nerve endings, the mobility of the virus along axons to the CNS is dependent on anterograde or retrograde transport using neuronal motor proteins, including dynein and kinesins (Bohmwald et al. 2018; Desforges et al. 2019; Koyuncu et al. 2013; Dey et al. 2021).

Viruses may impact the nervous system via means other than direct infection of neurons. Another possible route of entry is through the haematogenous route where the virus reaches peripheral ganglia and/or crosses either the blood-brain barrier or the blood-cerebrospinal fluid barrier in the choroid plexus, or by infection of leukocytes diffusing to the CNS (Bohmwald et al. 2018; Desforges et al. 2014, 2019; Koyuncu et al. 2013; Dey et al. 2021). This mode of action is not restricted to the intact native virus but can also occur for isolated viral proteins. For example, shed spike proteins from SARS-CoV-2 have been detected in the CNS driving inflammatory responses independent of any detectable replication competent virus (Rhea et al. 2021). Most IAV strains circulating seasonally in humans are non-neurotropic and cannot replicate in the CNS giving less severe neurological clinical manifestations (Bohmwald et al. 2018; Desforges et al. 2019; Koyuncu et al. 2013). However, through blood-borne routes viruses can still cause secondary effects on the CNS, including via systemic inflammation and the release of neurotropic factors produced by inflammation (Bohmwald et al. 2018; Desforges et al. 2019).

4 Evidence for Viral-Mediated Neuroinflammation

4.1 Impact of Respiratory Viral Infection on Vagal Sensory Neurons

Pulmonary vagal sensory neurons have not been studied extensively during respiratory viral infections. Kaelberer et al. (2020) showed lipopolysaccharide-induced pulmonary inflammation, mimicking a bacterial infection, resulting in transcriptional changes in neurons of vagal sensory ganglia innervating the pulmonary system. These transcriptional changes were characterised by an upregulation in gene expression associated with innate immune responses, more specifically genes *Lrg1* and *C3* that could indicate the maturing of resident or infiltrating ganglia immune

cells, or alternatively the changing of satellite cells towards an active immune cell type (Kaelberer et al. 2020). Transcriptomic changes in vagal sensory ganglia, including the pulmonary vagal sensory neurons themselves, were also observed during severe respiratory IAV infection in a murine model (Verzele et al. 2021). Notably in this study, the sensory neuron transcriptional alterations induced by active viral infection were mimicked by both the administration of cytokines and the inoculation of the lung of healthy mice with viral-inactivated lung homogenates from previously infected animals (Verzele et al. 2021). This suggests that the sensory neuron impacts of respiratory viral infection can occur independently of neuronal viral infection but instead are dependent on mediators of pulmonary inflammation. Consistent with this, the upregulated genes were associated with defence and pro-inflammatory responses, including signalling downstream of interferon production (Verzele et al. 2021).

Tissue injury is commonly associated with inflammatory cell influx and/or proliferation into the nerves that innervate the injured tissue. During respiratory IAV infection, inflammatory cells are increased in the vagal sensory ganglia (Fig. 2) (Verzele et al. 2021), a further sign of vagal neuroinflammation. The importance of inflammatory cell recruitment is still unknown; however, these immune cells could potentially play a role in inducing or facilitating molecular, structural and functional changes in sensory neurons. The ganglionic signalling mechanisms that lead to the

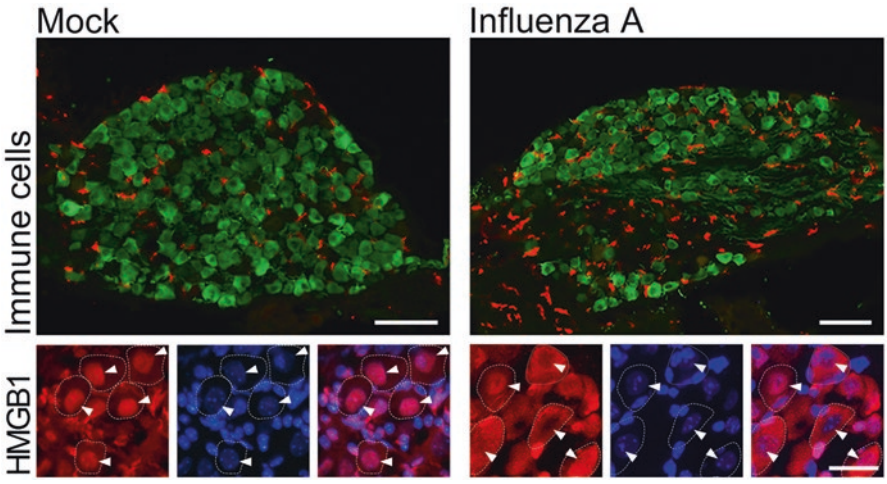

Fig. 2 Vagal neuroinflammation accompanying respiratory viral infection. Top panel micrographs show immune cell infiltration into the vagal sensory vagal ganglia following influenza A (IAV) infection. Immunohistochemistry for immune cells MHCII (major histocompatibility class II – red) and the pan-neuronal marker MAP2 (microtubule-associated protein 2 – green) following either Mock (saline) or IAV inoculation of the lungs. The bottom panel micrographs show inoculation of the lungs with IAV inducing translocation of the danger-associated molecular pattern, and HMGB1 (red) from the nucleus to the cytoplasm in vagal ganglia sensory neurons. Neurons are outlined by dotted lines and arrowheads point to nuclei, visualised by DAPI (blue). Scale bar in top and bottom panels represent 75 μm and 25 μm, respectively

increase in inflammatory cells are also unknown. One possible mediator is the tissue alarmin HMGB1, which can be released from injured or activated cells (Fig. 2) (Mazzone et al. 2021). We previously demonstrated the mobilisation of neuronal HMGB1 following respiratory infection with IAV or pneumovirus, and subsequent changes in sensory neuron structure and function dependent upon the HMGB1 receptor RAGE (Mazzone et al. 2021). Notably, respiratory infection resulted in the increase in neurite outgrowth in vagal sensory neurons, indicating neuronal sprouting and growth, a common feature in airway pathologies (Mazzone et al. 2021; Shapiro et al. 2021; Drake et al. 2021). Additionally, HMGB1 increased the excitability of the vagal sensory neurons, via a mechanism dependent on RAGE expression (Mazzone et al. 2021). HMGB1 has a well-known role in early initiation of inflammatory cell influx and activation (Yang et al. 2021; Magna and Pisetsky 2014) and thus neuronally released HMGB1 may be pivotal in establishing vagal neuroinflammation. How HMGB1 is mobilised from neurons is not clear, although increased action potential traffic along the axon may be involved (Yang et al. 2021).

Collectively, the sensory neuron impacts of respiratory viral infection may underpin the development of a sensory hypersensitivity, resulting in increased coughing and other defensive reflexes that are beneficial for the clearance of the airways and potentially for transmission of the virus to new hosts. Persistence of the hypersensitivity beyond the period of active viral infection, perhaps due to a sustained neuroinflammatory state, is hypothesised to be responsible for the long-lasting respiratory and neurological symptoms commonly associated with respiratory viral infection (Undem et al. 2015).

4.2 Impact of Respiratory Viral Infection on Vagal Sensory Pathways in the Brain

The impact of respiratory viral infections on the CNS is an underexplored area, although it has received more attention recently after increasing reports of long-term neurological complications seen in patients with SARS-CoV-2 infections. In general, viral-induced neurological manifestations have been noted after respiratory infections with both neurotropic and non-neurotropic viruses, perhaps indicating that the impacts on the CNS are not simply dependent on neuroinvasion (Fig. 1) (Hosseini et al. 2018).

Studies of asthma and allergic inflammation in the pulmonary system have offered insight into the impact of non-pathogenic lung diseases on vagal sensory processing pathways in the CNS (Bonham et al. 2006). At the level of the nucleus of the solitary tract, alterations in synaptic efficacy between primary vagal afferent and second-order neurons have been noted (Bonham et al. 2006; Chen et al. 2001; Klein et al. 2016), thought to reflect alterations in the release of neurotransmitters from primary afferent terminals and/or changes in the intrinsic excitability of nucleus of the solitary tract neurons (Bonham et al. 2006). For example, in allergen

sensitised rats, respiratory allergen challenge changed the membrane properties of the second-order neurons in the nucleus of the solitary tract, increasing membrane depolarisation and potentially modulating the output of vagal efferent nerves (Chen et al. 2001). Effects on nucleus of the solitary tract processing of vagal inputs have also been seen in models of cigarette smoke exposure and lung fibrosis (Litvin et al. 2018; Mutoh et al. 2000), suggesting that either an excessive level of vagal sensory neuronal activation in lung disease or accompanying systemic inflammation results in a centrally sensitised state that impacts vagal sensory processing.

Although peripheral inflammation is the likely initiator of CNS sensitisation, central processes also undoubtedly become involved which notably includes the recruitment and activation of CNS glial cells and the development of a central neuroinflammatory state. During respiratory viral infections or following exposure to either bleomycin or inhaled diesel exhaust particles, brainstem microglia cells and astrocytes have been shown to be activated, along with an upregulation of brainstem inflammatory gene expression (Undem et al. 2015; Litvin et al. 2018; Chen et al. 2021). These CNS support cells are important for the brain homeostasis and neuroprotective functions but can contribute to a CNS inflammatory state that alters the processing of incoming sensory inputs (Siracusa et al. 2019; Bachiller et al. 2018). For example, the activation of microglia leads to the production of pro-inflammatory cytokines that then affect neuronal morphology, synaptic structure and function leading to an imbalance in normal excitatory and inhibitory neurotransmission (Bohmwald et al. 2018; Hosseini et al. 2018). These inflammatory events are a hallmark feature of central sensitisation.

Similar events can occur beyond the brainstem, in higher brain regions that may contribute to complex sensory, motor or cognitive processing. For example, during severe respiratory viral infections, microglia and astrocyte activation and an accompanying pro-inflammatory state have been reported in a variety of brain regions including the hypothalamus, hippocampus and substantia nigra impacting the function of each region (Hosseini et al. 2018; Wang et al. 2018; Jang et al. 2009, 2012). Indeed, respiratory infection with highly pathogenic strains of IAV resulted in CNS viral infection of both neurons and microglia, induction of pro-inflammatory cytokines, prolonged microgliosis which coincided with a loss of dopaminergic neurons in the substantia nigra as well as aggregation of alpha-synuclein in the hippocampus, brainstem and cortex, suggesting that certain respiratory viruses could be important aetiological agents in the development of neurodegenerative diseases including Parkinson's and Alzheimer's disease (Bohmwald et al. 2018; Magna and Pisetsky 2014; Siracusa et al. 2019; Bachiller et al. 2018; Jang et al. 2009, 2012). How these higher brain CNS neuropathologies induced by respiratory viral infections impact vagal sensory processing circuits in the brain, important for pulmonary interoception, has not been investigated.

5 The Clinical Significance of Respiratory Viral-Induced Neuropathy

Respiratory viral infections represent a significant burden to the human health as they are extremely prevalent and can cause both acute and long-term clinical manifestations. The neurological impacts of respiratory viral infections are especially difficult to treat or manage. For example, cough is one of the most common symptoms associated with respiratory viral infection (Bohmwald et al. 2018), and whilst it may resolve spontaneously for many patients, soon after viral clearance and natural resolution of the disease, cough can persist for some people for many weeks or months. Post-viral cough syndrome is poorly understood, and there are no effective therapies for treatment, but may reflect the consequences of the neuroinflammatory state established during viral infection. Similarly, other persistent neurological symptoms have been reported following viral infections of the lungs. A significant population of patients infected with SARS-CoV-2 continue to experience chronic fatigue, cognitive impairment, pain and other symptoms as part of the long COVID syndrome, consistent with long-term changes in the functioning of the peripheral and central nervous system (Bohmwald et al. 2018; Song et al. 2021; Buckley et al. 2021). Similar symptomatology may occur following IAV infections. Again, the mechanism behind these long-lasting manifestations has yet to be determined and treatment options are limited. A deeper understanding of the neuroinflammatory events that accompany respiratory viral infections, including their persistence, responsiveness to standard therapies and the resultant changes that are imparted on neural function, may offer new avenues for therapeutic intervention and effective management of these challenging and debilitating symptoms.

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Stress-Induced Visceral Analgesia: Concept and Pathways

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Abstract Decades of research in the somatic pain field have established that stress has a major influence on pain modulation affecting it in two opposite ways: reduction (stress-induced analgesia) or enhancement (stress-induced hyperalgesia). Stress has long been implicated in the pathophysiology of visceral pain in both preclinical and clinical studies, with most reports supporting a proalgesic role of stress on visceral sensitivity, commonly referred to as stress-induced visceral hyperalgesia (SIVH). It is only recently that research attention has been brought to the phenomenon of stress-induced visceral analgesia (SIVA). In this mini-review, we will discuss current knowledge in regard to SIVA, how animals' preconditions play a key role in our ability to observe SIVA, what underlying neurochemical pathways are recruited for its expression, and what its relevance is to our understanding of bowel disorders of the gut-brain interaction (DGBI).

Keywords Stress-induced visceral analgesia · Preconditions · Manometry · Descending inhibitory pathways · Sex differences

1 Introduction

Pain is an intricate experience that encompasses both a physiological and a psychological response to a noxious stimulus. According to the 2011 updated International Association for the Study of Pain (IASP) pain terminology, pain is defined as "an unpleasant sensory and emotional experience associated with, or resembling that

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associated with, actual or potential tissue damage." Evolutionarily, the most important function of pain is to act as a warning mechanism that protects the organism by alerting it of the potential for injury or the threat of injury, leading to withdrawal from the source of injury. This adaptive response arises from the activation of sensory neurons (nociceptors) innervating most bodily tissues and entails the interaction of various neuroanatomic and neurochemical systems (Garland 2012). Pain can be classified into three major categories according to its duration: acute pain (less than 4 weeks) which is a normal, predicted, physiological response to a noxious stimulus, subacute pain (4 to 12 weeks), and chronic pain (more than 3 months) which is a persistent and debilitating condition associated with injury, disorders, or diseases (Dinakar and Stillman 2016). Chronic pain of somatic or visceral origin is considered to be a disease state affecting up to 20.7% of the adult US population, i.e., 1 in 5 adults (Yong et al. 2022).

The experience and expression of pain by an individual is influenced to varying degrees by biological, psychological, and social factors, and is the summation of the interaction of various agents: receptors, neurotransmitters involved in the regulation of pain perception, emotions, and memory related to pain (Swieboda et al. 2013). It is now widely accepted that both the context in which pain occurs and the emotional status (Wiech and Tracey 2009; Finan and Garland 2015) of the individual at the time can modulate the level of perceived pain following injury or inflammation both in rodents and in humans (Martin et al. 2019; Trask et al. 2022).

2 Stress and Pain Modulation: Analgesia Versus Hyperalgesia

Stress has a major impact on the perception of pain. In the somatic pain field, numerous studies (reviewed in (Butler and Finn 2009; Imbe et al. 2006; Jennings et al. 2014; Olango and Finn 2014)) have shown that depending on its nature, duration, intensity, controllability, but also on the nature of the pain induction itself, stress can exert potent and bidirectional modulatory effects on pain, either reducing (stress-induced analgesia, SIA) or exacerbating it (stress-induced hyperalgesia, SIH). The descending pain pathway, which includes both facilitatory and inhibitory systems (Vanegas and Schaible 2004; You et al. 2010), is a critical determinant of SIA and SIH (Butler and Finn 2009; Jennings et al. 2014; Olango and Finn 2014).

Decades of work on stress and somatic pain interaction indicate that exposure to a robust and intense acute stimulus typically induces SIA during and/or following stress. A component of the fight-or-flight response, SIA is considered as an innate adaptive response and a defense mechanism toward an imminent danger. From an evolutionary perspective, SIA therefore appears to have a protective, survival value (Bolles and Fanselow 1980). In contrast, prolonged or repeated exposure to physical or psychological stress generally results in maladaptive alterations and exacerbated

nociceptive responses in rodents and humans known as SIH (reviewed in Imbe et al. (2006), Jennings et al. (2014), Olango and Finn (2014)).

3 Is Stress-Induced Analgesia Occurring in Visceral Pain?

In contrast to the somatic pain field, investigations related to the influence of stress on visceral pain are relatively recent and the underlying mechanisms are not yet well understood (Moloney et al. 2015). The majority of reports available in the preclinical and clinical literature support an exacerbating influence of stress on visceral pain, or stress-induced visceral hyperalgesia (SIVH) (for review see Moloney et al. (2015), Meerveld and Johnson (2018), Larauche et al. (2011)). Nevertheless, although largely ignored by the research community up to now, stress-induced visceral analgesia (SIVA) has also been described in humans and rodents in a few studies (Larauche et al. 2011).

For instance, in the late 1990s to the beginning of 2000, research done in healthy men and women showed that sensory thresholds for rectal balloon distensions were higher during distraction (easy drawing task) (Mönnikes et al. 1995), mental stress (dichotomic listening) (Posserud et al. 2004; Métivier et al. 1996), or physical stress (hand in iced water) (Métivier et al. 1996).

Subsequently, a number of preclinical reports were generated that supported those earlier clinical findings. In naïve SD rats of both sexes, a combination of psychological and physical stress (water avoidance stress (WAS) combined with cold water (10 °C) swimming stress) induced an immediate SIVA to colorectal distension (CRD) measured 2 h after the end of the stress session (Gui et al. 2004). In the same study, the authors noted that the prolonged restraint (two consecutive 40-min sessions of CRD recording) associated with the visceral pain monitoring per se could also induce SIVA in male SD rats (Gui et al. 2004).

In line with those studies, we reported that psychological stress in the form of acute (1 h) or repeated WAS (rWAS, 10 days, 1 h/day) induced a visceral analgesic response to CRD immediately after the end of the stress in both male and female Wistar rats (Larauche et al. 2012a, b). Our findings were replicated and expanded by other groups in male and female Wistar rats (Lee et al. 2016) and male SD rats (Nozu et al. 2016).

Recently, naïve male CD-1 mice undergoing chemically induced visceral pain testing (intracolonic capsaicin or intraperitoneal acetic acid) were found to exhibit visceral analgesia when exposed to mild social stress (visual and olfactory exposure to unfamiliar conspecific mice) but to show no change or enhanced pain response in the presence of cage mates depending on the agent used to induce visceral pain (Pitcher et al. 2017).

Together, these data indicate that stress can affect visceral pain in a bidirectional manner as has been shown in the somatic pain field and lead to either an exacerbation of the pain response (SIVH) or a decrease (SIVA). If such is the case, one wonders why the number of reports on SIVA in the literature is so limited. Are there

impediments, whether technical (protocols, methods, or designs) or conceptual, in our research that prevent us from observing it?

4 Roles of Preconditions in the Expression of SIVA

One such impediment is what we have previously called preconditions (Mulak et al. 2012). Defined as experimental factors that change the background response of an otherwise healthy individual, preconditions play an important role in the expression of both somatic and visceral SIA in rodents and may potentiate or reduce the analgesic response to stress (Mulak et al. 2012).

A striking example of such preconditions is the study published by Schwetz et al. (Schwetz et al. 2005) where adult Long-Evans male rats were found to exhibit a differential visceral pain response to an acute session of WAS depending on the prior length and exposure to the same stressor in the form of maternal separation stress as neonates. While bred and raised in the same facility and tested using identical procedures, rats exposed to long maternal separation periods (180 min) demonstrated an immediate SIVH to WAS, non-handled rats showed no change in their visceral pain responses, and rats that had undergone short maternal separation periods (15 min) developed an immediate SIVA (Schwetz et al. 2005), emphasizing the importance of preconditioning on the visceral pain response to stress.

In another study on somatic pain, the authors demonstrated that the pain response to non-noxious environmental stress was dependent on the background of rats with naïve rats exhibiting an immediate SIA and rats previously exposed to inflammatory pain (carrageenan injection in hindpaw) or high dose of opioid showing an immediate SIH (Rivat et al. 2007). This study is particularly interesting in the context of visceral pain and the method used to monitor visceral sensitivity in rodents. There is indeed evidence of somato-visceral convergence of pain pathways, whereby skin incision in the hindpaw of rats is correlated with the development of long-lasting visceral hyperalgesia (Cameron et al. 2008). To date, the gold standard to monitor objectively visceral pain in rodents is done via EMG electrode implantation in the abdominal muscle (Ness and Gebhart 1988). This method requires surgery, abdominal skin incision, and in some cases laparotomy, chronic implantation of EMG electrodes, single housing, and analgesic postoperative treatment, all factors that have the potential to change the background or precondition of the animals being tested and affect its future response to a stressful event (Mulak et al. 2012).

To test this hypothesis, a decade ago, our group developed an alternative noninvasive solid-state manometric method to study visceral sensitivity to CRD in conscious rodents, using a commercially available miniaturized pressure catheter to record intraluminal colonic pressure (ICP) directly in the colonic lumen (Larauche et al. 2009, 2010). Using this technique, we were able to show in male C57Bl/6 mice that the visceral pain response to rWAS was affected differentially depending on the housing conditions (single housing vs. group housing) and the method used to monitor visceral pain (noninvasive manometry vs. EMG) (Larauche et al. 2010).

Mice that had undergone surgery for the placement of EMG electrodes and were subsequently single-housed to avoid deterioration of the implanted electrodes by cage mates developed visceral hyperalgesia to CRD in response to rWAS. By contrast, mice tested for visceral pain to CRD using the noninvasive solid-state ICP recording, which were naïve and kept group housed, developed a strong visceral analgesia under otherwise similar conditions of repeated intermittent WAS. Interestingly, singly housed naïve mice did not demonstrate SIVA (Larauche et al. 2010).

Likewise, in rats surgically equipped for EMG monitoring of the visceromotor response to CRD as classically performed, rWAS induced a visceral hyperalgesic response in 82–86% of the animals starting 24 h after the first exposure, which was maintained up to 40 days after the last stress session (Larauche et al. 2008; Bradesi et al. 2005). By contrast in naïve animals, with the use of a novel noninvasive manometric method of visceral sensitivity monitoring to CRD, the majority of rats (66.7–85.7%) exposed to rWAS exhibited a consistent visceral analgesic response (Larauche et al. 2012a). Expanding these findings, we have recently shown that SIVA induced by acute WAS in male SD rats was partially reduced by the prior surgery to implant an intracerebroventricular cannula (ICV) and concurrent single housing when compared to naïve group housed rats exposed to the same stressor (Larauche et al. 2019).

Collectively these data demonstrate that the state of the animal tested (naïve vs. exposed to surgery), its social environment (group housing vs. single housing, cage enrichment or not), the handling performed by the investigator, as well as the methods used to record VMRs (EMG requiring surgery, analgesic, and/or antibiotic post-surgery vs. manometry not requiring surgery/analgesic/antibiotic) can significantly affect the response to exteroceptive stressors (psychological, neurogenic).

5 Role of Descending Inhibitory Pathways in SIVA

The contribution of descending endogenous inhibitory pathways in somatic pain has been extensively studied (Butler and Finn 2009; Ferdousi and Finn 2018; Ford and Finn 2008), and both opiate-dependent and opiate-independent pathways have been found to be recruited differentially according to the modalities of stress procedures, the pain test used, and the genotype of the animal (Butler and Finn 2009; Lewis et al. 1980; Bodnar and Kest 2010). In contrast, the role of these pathways in stress-related visceral responses is not well known yet.

The contribution of opioids to the descending inhibition of visceral sensitivity following an acute stress was demonstrated after naloxone pretreatment unmasked WAS-induced hyperalgesia to CRD in naïve male Long-Evans rats and exacerbated the pain response to CRD in maternally separated male rats (Coutinho et al. 2002).

SIVA was also found to involve various opioid-independent pathways in rodents. Hence, neurotensin-dependent SIVA was demonstrated in male and female SD rats and C57Bl/6 mice following an acute session of WAS and cold-water swimming

stress (Gui et al. 2004). Nozu et al. (2016) showed that acute WAS-induced SIVA was naloxone-independent in male SD rats and mediated through peripheral corticotropin-releasing factor (CRF) receptor type 2 and central dopamine D2 receptor (Nozu et al. 2016). In our studies, acute and rWAS-induced SIVA was determined to be naloxone-independent in male Wistar rats but partially dependent on opioids in females (Larauche et al. 2012a), raising the potential for sex differences in the pathways mediating SIVA. The naloxone-independent WAS-induced SIVA in male SD rats was abolished by the ICV injection of the selective oxytocin receptor antagonist, tocinoic acid, indicating a role for brain oxytocin (Larauche et al. 2019). We found that central injection of astressinB, a non-selective antagonist of CRF receptors type 1 and 2 (CRF1/CRF2), before WAS abolished SIVA in male SD rats, indicating a contribution of brain CRF receptors in WAS-induced SIVA. To further dissect this pathway, we injected the selective CRF2 antagonist astressin2B ICV, which prevented SIVA all the while revealing a CRF1-mediated visceral hyperalgesic response at 60 mmHg (Larauche et al. 2019). We further investigated the role of CRF in SIVA and demonstrated a dose-dependent influence of brain CRF in the development of SIVA (Larauche et al. 2019) similar to what has been previously described in the somatic pain field (Vit et al. 2006).

As a whole, these data support the concomitant recruitment of analgesic and proalgesic pathways in response to stress and a phenotypic outcome (SIVA or SIVH) that depends on the summation of those responses.

Because descending endogenous inhibitory pathways are essential for the expression of SIVA in rodents, one important question is whether these can be enhanced. As indicated previously, a short period of handling during the neonatal period induced SIVA in male Long-Evans rats exposed to acute WAS (Schwetz et al. 2005). Similarly, a visceral hypoalgesic response to CRD was reported in adult male Wistar rats that were handled daily for 9 days, 7 days after the last handling (Winston et al. 2010). We also demonstrated that the analgesic response to rWAS could be enhanced by a prebiotic diet in male Wistar rats up to 24 h after the last session of stress (Larauche et al. 2012b). These data therefore suggest the possibility of promoting a visceral analgesic phenotype in rodents through interventions consisting of behavioral enrichment or change in diet, although to this date the underlying mechanisms involved in these enhanced visceral analgesic responses following those interventions are still unknown.

6 Influence of Sex on SIVA

Studies on the influence of sex on the expression of SIVA are scarce. Gui et al. (2004) were the first to show that the SIVA induced by combined psychological and physical stress (WAS and cold-water swimming stress) was more potent in males than in female SD rats. Interestingly, suppressing SIVA with a neurotensin antagonist unmasked a SIVH in both sexes that was significantly stronger in female than in male rats (Gui et al. 2004), indicating that there is a sex-dependent differential

recruitment of SIVA and SIVH pathways. We recently reported that male and female Wistar rats developed an immediate SIVA with similar levels of analgesic responses at noxious pressures of distensions 40 and 60 mmHg to acute WAS and rWAS, but only females develop a delayed SIVH 24 h after rWAS (Larauche et al. 2012a). More studies are warranted to evaluate sex differences in SIVA expression and underlying mechanisms.

7 Conclusion and Future Considerations

There is evidence that both analgesic and proalgesic pathways are being activated simultaneously in rodents in response to stress exposure. It has now become clearer that the expression of either SIVA or SIVH phenotype during or following an exposure to a stressor is the summation of those pathways modulated by the animals' preconditions, the nature of the stress, and the pain induction (Fig. 1). Going forward, it will be crucial when investigating and reporting on the influence of stress on visceral sensitivity in experimental animals that these preconditions be explicitly mentioned in the experimental details and taken into consideration in the design, conduct, and interpretations of data. While a great deal of knowledge has been acquired using traditional EMG monitoring for visceral pain assessment, the use of noninvasive methods represents a step forward for gaining insight into the neural substrates and neurochemistry of SIVA.

8 Clinical Relevance to DGBIs

Chronic visceral pain in patients with bowel disorders of gut-brain interaction (DGBI) such as irritable bowel syndrome (IBS) is the most important determinant of IBS severity, quality of life impairment, and healthcare utilization (Spiegel et al. 2010) and to this date remains the most significant challenge in IBS management (BouSaba et al. 2022). Visceral hypersensitivity to rectosigmoid distension is an important hallmark feature of IBS, believed to underlie abdominal pain in patients. It is estimated that 47–64% of IBS patients have lower rectal discomfort thresholds compared to controls (Chang et al. 2006).

Stress is well established to play a predominant role in the pathophysiology, symptom presentation, and treatment outcome in IBS (Pellissier and Bonaz 2017) and most of the investigations have been focused on the pathways leading to visceral hypersensitivity (Fukudo 2013). It is only recently that the role of alterations in descending pain modulatory pathways in IBS pathophysiology got attention. Studies indicate that patients with IBS and functional dyspepsia have deficient endogenous inhibitory descending pathways involving the descending noxious inhibitory control (DNIC) – as measured by conditioned pain modulation (CPM) – and other supraspinal modulatory pathways (Wilder-Smith et al. 2004;

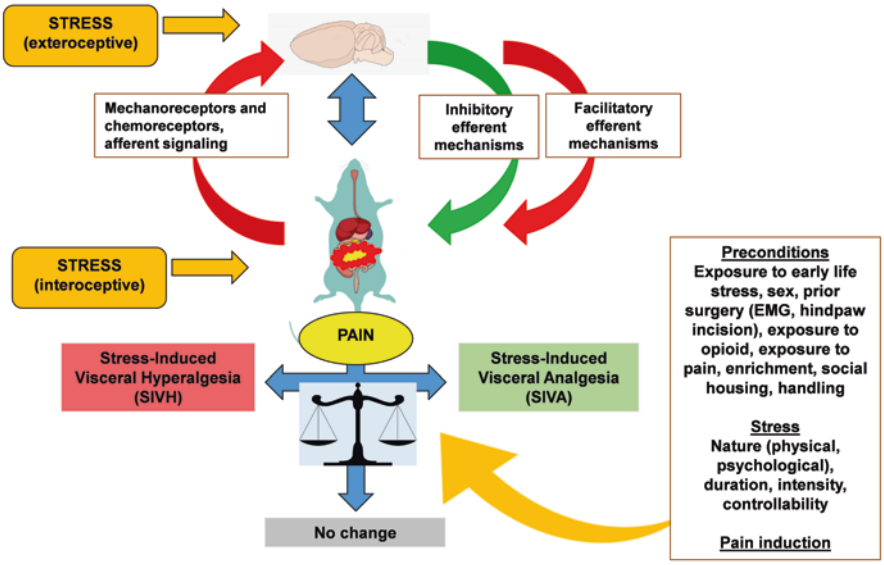

Fig. 1 Overview of the pathways involved in the modulation of visceral pain by stress. When a nociceptive input or injury occurs in the gut, sensory neurons are activated, sending a nociceptive input to the brain, which processes it and activates endogenous descending modulatory pathways. These pathways can be facilitatory or inhibitory in nature and modulate the pain response. Both interoceptive (physical, systemic) and exteroceptive (psychological, neurogenic) stressors can modulate those ascending and descending pain pathways and lead to an exacerbation of the pain response (stress-induced visceral hyperalgesia or SIVH) or a reduction (stress-induced visceral analgesia or SIVA). The analgesic pathways contributing to SIVA and the proalgesic pathways contributing to SIVH occur concurrently and can be affected by animals' preconditions, the nature of the stressor, and the type of pain induction. The summation of these analgesic and proalgesic pathways results in the expression of the final phenotype. In some cases, the visceral pain response is unaffected by stress because the analgesic and proalgesic pathways cancel each other out

Wilder-Smith and Robert-Yap 2007; Williams et al. 2013; Marcuzzi et al. 2019; Jarrett et al. 2014). These alterations in the endogenous descending inhibitory pathways may be at the origin of the visceral and somatic hypersensitivity reported in those patients and could explain the higher prevalence of chronic overlapping visceral and somatic pain syndromes observed in patients with DGBI (Whitehead et al. 2002).

Gaining a better understanding of the pathways recruited in the expression of SIVA and how these are affected in bowel DGBI may open new therapeutic avenues for patients. Several important questions pertaining to SIVA however remain to be answered. First, as highlighted in this mini-review, there is evidence that both SIVA and SIVH are taking place simultaneously in response to stress and that the expressed outcome is the result of the complex interplay between descending inhibitory and facilitatory pathways, the quality/quantity of stress, and the pain induction, among other factors (Fig. 1). In the case of patients with DGBI, it will be important to determine whether the pathways responsible for SIVA are deficient or

simply masked by an overpowering SIVH associated with an increased recruitment of pro-nociceptive pathways. Second, with the possibility of sex differences in the expression and pathways recruited by SIVA as noted previously, it will be key to assess whether the abilities of women and men to mount a SIVA response are comparable or differ and whether these contribute to the prevalence of DGBI in women. Lastly, determining whether SIVA can be rescued or enhanced, thereby decreasing the contribution of the SIVH response and restoring the balance, will certainly bring major therapeutic breakthroughs for DGBI patients.FundingSupport for this review was provided by NIH K01 DK088937, U01 DK57238, and P30 DK41301.

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Evidence of Early Life Stress Exposure and Epigenetic Modifications in Functional Chronic Pain Disorders

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Abstract Exposure to adversity or stress during early development increases the likelihood of developing chronic pain disorders, as well as comorbid mood disorders, later in life. Early life stress exposure can impact gray matter volume, functional connectivity, and neurochemical signals within central brain regions associated with pain and affect. In particular, the hippocampus is exquisitely sensitive to excess glucocorticoids during early development, which can result in glucocorticoid resistance that is largely driven by epigenetic modifications of the glucocorticoid receptor. In this review, the basic mechanisms of epigenetic modifications, particularly that of DNA methylation, are described, as well as exposures across the lifespan that promote these changes. Investigations of epigenetic signatures in patients with functional pain disorders that have a high incidence in biological females and are associated with early life stress exposure, including irritable bowel syndrome, fibromyalgia, and headache, report altered methylation patterns in stress-related and neuronal genes. These initial studies suggest that DNA methylation may be a useful tool to provide individualized insight into disease origin and progression and potentially guide therapeutic interventions for common chronic pain disorders.

Keywords Epigenetics · DNA methylation · Chronic pain · Early life stress

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1 Introduction

Chronic pain is defined as recurrent or persistent pain lasting for more than 3 months (Liossi and Howard 2016) and is one of the most common reasons why American adults seek medical care (McCaig and Nawar 2006). Chronic pain reduces overall quality of life and is also associated with opioid dependence and poor mental health (Smith et al. 2001; Mills et al. 2019). Women are more likely to experience chronic pain than men, with 21.7% and 19%, respectively, reporting chronic pain in 2019 (Zelaya et al. 2020). It is estimated that chronic pain generates more than $560 billion each year in direct medical costs, lost work days, and disability payments (Institute of Medicine Committee on Advancing Pain Research C, Education 2011). As the costs for treating chronic pain only continue to rise, many individuals struggle to find comfort and return to work as the currently available pharmacological, interventional, behavioral, and surgical therapies have limited effectiveness. This can be compounded by increased dependence on opioids to gain any relief and the development of opioid use disorder (Davis et al. 2020; Chakravarthy et al. 2018).

Although pediatric chronic pain is common, little is known about the global burden of chronic pain in children because it is often under-recognized and undertreated (Friedrichsdorf et al. 2015; Taylor et al. 2008). Studies estimate that 20–35% of children and adolescents are affected by chronic pain, although the prevalence rates vary depending on age, pain type and duration, and early life experience/adversity (King et al. 2011; Goodman and McGrath 1991). The number of children admitted to the hospital for chronic pain increased 831% between 2004 and 2010 with common comorbid pain and psychiatric diagnoses (Coffelt et al. 2013). Total annual costs of care for adolescents with moderate to severe chronic pain in the USA are estimated at $19.5 billion, which exceeds the costs of childhood asthma and obesity, presenting a significant economic burden on families (Soltani et al. 2019; Groenewald et al. 2020).

As children with chronic pain transition into adulthood, their pain and disability generally remain unresolved with an increased risk for worsening physical symptoms and psychiatric disorders (Kashikar-Zuck et al. 2019; Fearon and Hotopf 2001; Noel et al. 2016; Shelby et al. 2013; Walker et al. 2012). In fact, 17% of adults of with chronic pain report that their pain emerged in childhood or adolescence (Hassett et al. 2013). In one of the few studies that has investigated long-term outcomes of pediatric chronic pain, 35% of children with functional abdominal pain reported symptoms in adulthood. Of those patients with unresolved functional abdominal pain, over half (57.4%) reported comorbid headache, and two thirds (65.8%) of patients reported one or more sites of chronic non-abdominal pain (Walker et al. 2010). These outcomes suggest that a subgroup of pediatric chronic pain patients may mature into adults with more widespread chronic and comorbid pain disorders.

One important factor that increases the prevalence of chronic pain in both adult and pediatric populations is a history of early life stress (Fuentes and Christianson 2018; Nicol et al. 2016). Adversity early in life, which can take multiple forms

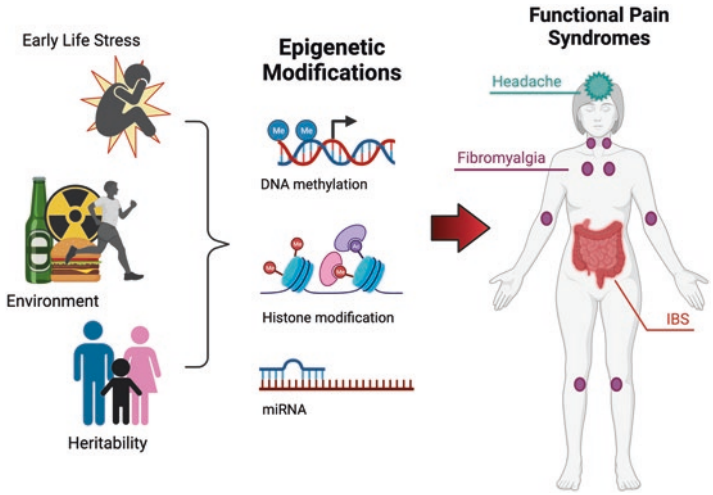

Fig. 1 Environmental exposures across the lifespan can influence epigenetic modifications that are associated with the development of functional chronic pain disorders

including abuse, neglect, premature birth, surgery, and extended stays in the neonatal intensive care unit, can permanently affect how pain is perceived and has been correlated with changes in gray matter volume, functional connectivity, and neurochemical concentrations in pain- and affect-related regions of the brain (Bhatt et al. 2020; Schrepf et al. 2018). Patients who report high levels of early life stress are more likely to have multiple overlapping pain conditions, be diagnosed with mood disorders, complain of fatigue and sleep disorders, and are also less likely to have meaningful improvements in symptoms over time (Bhatt et al. 2020; Schrepf et al. 2018). Recent studies have shown that epigenetic modifications resulting from early life stress exposure may drive many of these long-term chronic pain-related outcomes (Mahurkar-Joshi and Chang 2020; Romens et al. 2015; Schechter et al. 2015). Here, we will discuss the general function of epigenetic modifications, primarily DNA methylation, how they arise, and their implications in chronic pain syndromes (Fig. 1).

2 Epigenetic Modifications

Epigenetic modifications are heritable, reversible changes in genomic conformation that affect the regulation of gene expression and resulting protein levels without altering the primary DNA sequence (Inbar et al. 2013; Denk and McMahon 2012). Epigenetic modifications are highly influenced by environmental changes, can be tissue specific, and generally persist during cell replication (Gibney and Nolan 2010). Several different epigenetic mechanisms have been characterized, including

DNA methylation, histone modification, non-coding RNA (microRNAs and long non-coding RNAs), and higher-order chromatin structure, that regulate gene expression by modifying the accessibility of DNA to transcription and other regulatory factors (Zhang and Pradhan 2014). These epigenetic modifications can significantly influence an individual's behavior, alter their immune function, and modulate their stress response, demonstrating the impact the environment can have on regulation of gene expression and a variety of downstream outcomes, including health and disease risk (Portela and Esteller 2010; Bale et al. 2010; Ho et al. 2012).

DNA methylation is the addition of a methyl group to the fifth carbon position of a cytosine base to generate a methylcytosine (5mC) that is predominantly next to a guanine base or a CpG site. Regions of high CpG content are termed "CpG islands" and are where DNA can be covalently modified by the enzymatic reaction of DNA methyltransferase (DNMT) that catalyzes the transfer of a methyl group from the methyl donor S-adenosyl methionine (SAM). The DNMT enzyme family is responsible for CpG methylation maintenance and addition across the genome. DNMT1, the most abundant DNMT in cells, is responsible for preserving parental methyl marks in daughter cells during mitotic processes and conserving methylation patterns as cells divide (Xu 2019). In comparison, DNMT3A and DNMT3B are involved in de novo methylation or methylation of CpG sites that were previously unmethylated (Smith and Meissner 2013). These enzymes establish DNA methylation patterns by performing de novo methylation during development and in response to environmental stimuli (Xu 2019).

Although DNA methylation has been regarded as a relatively stable epigenetic mark, the loss of DNA methylation, or DNA demethylation, can occur, although the mechanism is not well understood. DNA demethylation can occur passively by the loss of 5mC through successive rounds of replication. By contrast, active demethylation occurs independently of DNA replication and instead removes or modifies the methyl group from 5mC by an enzymatic process. Active demethylation begins with DNA base modification by AID (activation-induced deaminase) catalyzed deamination, or TET (ten-eleven translocation) catalyzed oxidation and ends with the modified nucleotide being replaced (Bochtler et al. 2017).

3 Causes of Epigenetic Modifications

3.1 Early Life Stress

As mentioned above, painful and/or stressful events during early developmental periods can permanently impact central nervous system activity and architecture, resulting in amplification of the perception of pain as an individual ages (Kim et al. 2010; Burke et al. 2017). The hippocampus is a major negative regulator of the hypothalamic-pituitary-adrenal (HPA) axis (Herman et al. 2005), which mediates the stress response and is often altered in patients with centralized pain disorders

(Vierck Jr 2006). Hippocampal regulation of the HPA axis largely occurs through glucocorticoid signaling, which provides a negative feedback mechanism for maintenance of homeostasis (Herman et al. 2005). Excess glucocorticoid production, such as during stressful periods, can result in over-activation of the HPA axis. When this occurs during early development, excess glucocorticoids can lead to glucocorticoid resistance, impaired hippocampal integrity, and dysregulated stress response (Uno et al. 1994).

Glucocorticoid resistance resulting from excess glucocorticoids is largely mediated by epigenetic modifications (Szyf et al. 2005) and silencing by microRNAs (Vreugdenhil et al. 2009). The hippocampal-specific exon (Fearon and Hotopf 2001) of *NR3C1*, the gene that encodes glucocorticoid receptor (GR), is especially sensitive to early life stress and/or glucocorticoid exposure, resulting in increased methylation and reduced GR expression (Maccari et al. 2014). This has been observed in both animal models of early life stress (Szyf et al. 2005; Weaver et al. 2004; Crudo et al. 2012; Bockmuhl et al. 2015; Meaney and Szyf 2005) and in patients who report early adversity or trauma (Romens et al. 2015; McGowan et al. 2009; Weaver 2009; Tyrka et al. 2012; Perroud et al. 2011; Oberlander et al. 2008; Linnstaedt et al. 2018). The FK506 binding protein 51 (FKBP5/FKBP51) is encoded by *FKBP5* and is a co-chaperone that forms a complex with heat shock protein 90 (Hsp90) that binds to and inhibits GR activity (Zannas et al. 2016). Genetic variants of *FKBP5* have been identified as risk factors for developing stress-related psychiatric disorders and were shown to interact with severity of stress exposure in the NICU to generate a larger autonomic stress response in preterm infants (D'Agata et al. 2017). These polymorphisms have also been shown to interact with early life stress to predict post-traumatic stress disorder, major depression, and attempted suicide in adulthood (Mehta et al. 2011; Koenen and Uddin 2010; Koenen et al. 2005; Appel et al. 2011; Binder et al. 2008). Specific polymorphisms in *FKBP5* impact chromatin confirmation, which are further modified by early life stress exposure, resulting in diminished regulation of GR (Klengel et al. 2013).

3.2 Environmental

Environment and lifestyle factors, such as behavior, nutrition, and exposure to toxins or pollutants, also influence epigenetic changes (Feinberg, 2018). Epigenetic programming can begin during fetal development; therefore, maternal environmental exposures during gestation can cause the fetus to adapt epigenetically. Prenatal exposure to famine (Tobi et al. 2009, 2018; Shen et al. 2019), smoking (Chatterton et al. 2017; Fragou et al. 2019), and gestational diabetes mellitus (Haertle et al. 2017; El Hajj et al. 2013; Finer et al. 2015; Hjort et al. 2018) have been shown to cause widespread epigenetic changes in affected offspring. Additionally, a mother's nutritional status during early pregnancy can induce epigenetic changes in the fetus (Dominguez-Salas et al. 2014).

In addition to prenatal experiences, lifestyle factors and environment exposures can affect epigenetic modifications across the entire lifespan. Alcohol consumption, diet, body mass index, and physical activity are four modifiable lifestyle factors that cause epigenetic changes and alter gene expression. Chronic alcohol consumption results in global DNA hypomethylation due to a reduction in SAM levels (Zakhari 2013; Mahna et al. 2018). Furthermore, epigenetic modulations at the neurobiological level can result in tolerance and dependence (Ponomarev 2013; Berkel and Pandey 2017). Dietary nutrients are capable of altering epigenetic patterns by adding or removing epigenetic marks (Choi and Friso 2010; Dashwood and Ho 2007), and obesity has also been associated with negative epigenetic changes (Wang et al. 2010; Xu et al. 2013; Day et al. 2017). In comparison, individuals who engage in a high level of physical activity show positive epigenetic changes compared to those with a more sedentary lifestyle (Luttropp et al. 2013; Sailani et al. 2019). Environmental chemical exposure, such as arsenic (Pilsner et al. 2007, 2009; Chanda et al. 2006) and pesticides (Kim et al. 2010), can induce epigenetic changes and has been associated with numerous diseases (Baccarelli and Bollati 2009; Hou et al. 2011). Therefore, lifestyle factors and environmental exposures across the lifespan have the capability of eliciting epigenetic modifications with enduring positive and negative effects on human development and health.

3.3 Heritability

Epigenetic modifications can be inherited via genomic imprinting, which is a stable form of epigenetic regulation during mitosis. The maternal and paternal alleles are expressed in a parent-of-origin-specific manner by each germ line, providing distinct marks onto specific regions of the chromosome. These regions are then expressed at a specific time in development or in a particular cell type (Inbar et al. 2013; Ho et al. 2012; Bartolomei 2009). Intergenerational epigenetic inheritance is the transmission of epigenetic marks between two generations, whereas transgenerational epigenetic inheritance is the transmission of altered epigenetic marks across multiple generations (Lacal and Ventura 2018). For example, individuals persecuted during the Holocaust showed altered *FKBP5* methylation patterns that were passed down to their offspring, demonstrating the involvement of epigenetic mechanisms in intergenerational transmission (Yehuda et al. 2016). Direct proof of transgenerational epigenetic inheritance in humans is lacking, although multiple longitudinal studies and non-Mendelian forms of inheritance suggest its influence. For instance, increased obesity susceptibility, glucose intolerance, and coronary heart disease have been observed in the children and grandchildren of women that were prenatally exposed to famine (Painter et al. 2008; Veenendaal et al. 2013).

4 Evidence of Epigenetic Mechanisms in Functional Pain Syndromes

4.1 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is classified as a functional gastrointestinal disorder and is characterized by symptoms of chronic or recurrent abdominal pain that is associated with altered bowel habits (Schuster 2001). IBS is one of the most frequently diagnosed gastrointestinal disorders and affects approximately 10–15% of adults (Grundmann and Yoon 2010; Endo et al. 2015), 6–14% of children, and 22–25% of adolescents (Karabulut et al. 2013). Like other functional pain disorders, patients with IBS are more likely to be biologically female, have a history of early life stress, and report additional comorbid chronic pain and mood disorders (van Kessel et al. 2021; Peters et al. 2008; Chitkara et al. 2008; Fond et al. 2014). A role for dysregulation of the HPA axis has been implicated in IBS pathology, including altered GR mRNA expression and function in higher structures and augmented corticotropin-releasing factor (CRF) receptor mRNA expression both centrally and peripherally (Videlock et al. 2016; Taché et al. 2005).

Animal models of IBS often use stress-induced means of evoking visceral hypersensitivity and altered gastrointestinal motility. Exposure to water avoidance stress (WAS), which has been shown to produce visceral hypersensitivity in rodents, resulted in hypermethylation of GR and hypomethylation of CRF genes in the amygdala, resulting in decreased and increased mRNA levels of the two genes, respectively (Tran et al. 2013). Considering that the amygdala promotes activation of the HPA axis, in opposition to the hippocampus, these changes suggest increased positive feedback driving over-activation of the stress response system. Epigenetic studies in patients with IBS have only recently begun and a genome-wide DNA methylation screen of peripheral blood mononuclear cells (PBMCs) identified 133 differentially methylated positions between adults with IBS and healthy controls (Mahurkar et al. 2016) (Table 1). Analysis of the affected genes revealed associations with oxidative stress-related pathways and neuropeptide hormone activity with marked methylation differences between diarrhea- and constipation-predominant patient subgroups, as well as associations with patients who presented with comorbid depression (Mahurkar-Joshi and Chang 2020; Mahurkar et al. 2016).

4.2 Fibromyalgia

Fibromyalgia is characterized by chronic widespread musculoskeletal pain and is associated with disrupted sleep, memory problems, and depression (Bair and Krebs 2020). Fibromyalgia is diagnosed in about 2% of adults and juvenile fibromyalgia syndrome is diagnosed in 1–6% of pediatric patients (De Sanctis et al. 2019). Similar to IBS, fibromyalgia is more commonly diagnosed in biologically female

patients and is associated with exposure to early life stress (Nicol et al. 2016). A genome-wide methylation study that compared peripheral blood samples from female fibromyalgia patients and healthy controls reported 1610 differentially methylated positions, of which 65% were hypomethylated and 35% were hypermethylated in patients compared to controls (Ciampi de Andrade et al. 2017) (Table 1). A separate genome-wide methylation pattern assessment detected 69 differentially methylated (DM) sites between women with fibromyalgia and controls, with 91% of the DM sites showing increased methylation in patients (Menzies et al. 2013) (Table 1). The identified DM sites were largely involved in neuronal differentiation and development of nervous, skeletal, and organ systems. An additional study used targeted bisulfite sequencing to investigate differential methylation in 112 genes associated with fibromyalgia in leukocytes from 8 women with fibromyalgia and their healthy biological sisters as controls (Gerra et al. 2021) (Table 1). They observed an increase in methylation on the *GRM2* gene that encodes glutamate metabotropic receptor 2, and the methylation levels of depression- and inflammation-related genes were significantly associated with the risk of fibromyalgia.

4.3 Headache

Severe headache and migraine affect approximately 15% of adults in the USA, with a higher prevalence among women (21%) than men (10%) (Burch et al. 2018). High levels of early life stress are correlated with headache frequency, a younger age at headache onset, and peripheral markers of inflammation (Tietjen et al. 2012). A genome-wide study in peripheral blood samples was used to determine methylation changes related to the transition from episodic headache to chronic headache. In a combined meta-analysis of baseline and follow-up measures, 11.4 years later, the two most significant CpG sites were related to the *SH2D5* (SH2 domain-containing 5) and *NPTX2* (neuronal pentraxin II) genes, both of which are expressed throughout the brain and are involved in regulating neuronal and synaptic activity (Winsvold et al. 2018) (Table 1). These two genes were further analyzed in healthy controls and chronic and episodic migraine patients using specific methylation primers for two representative CpG sites within these genes, although neither *NPTX2* nor *SHRD5* showed significant differences in methylation related to headache (Pérez Pereda et al. 2020) (Table 1). Methylation status of receptor activity-modifying protein 1 (*RAMP1*), which is a key receptor subunit for calcitonin gene-related peptide (CGPR) that is implicated in migraine pathology, was investigated in peripheral blood samples of patients with migraine and healthy controls (Wan et al. 2015). Although there was no significant difference in methylation of *RAMP1* gene promoter, there were several CpG units that had higher methylation levels in individuals with migraine family history compared to those without. Further, there was an increase in methylation in CpG units for female migraine patients compared to

Table 1 DNA methylation analysis in functional pain disorders

Functional pain disorder	# of subjects (patients, controls)	% of participants female (patients, controls)	Mean age ± SD, years (patients, controls)	Tissue/cell	Target gene	Reference
Irritable bowel syndrome	27, 23	59.3, 60.9%	40.22 ± 9.12, 36.48 ± 8.63	PBMCs	Genome-wide	Mahurkar et al. (2016)
Fibromyalgia	24, 23	100%	54 ± 9.9, NR	Whole blood	Genome-wide	Ciampi de Andrade et al. (2017)
Fibromyalgia	10, 8	100%	48.2 ± 6.7, 52.0 ± 9.8	Whole blood	Genome-wide	Menzies et al. (2013)
Fibromyalgia	8, 8	100%	51 ± 7.87, 52.6 ± 12.3	Leukocytes	Relevant targeted sequences	Gerra et al. (2021)
Headache	36, 35	100%	26.1 ± 4.2, 26.3 ± 4.6[a]	Leukocytes	Genome-wide	Winsvold et al. (2018)
Headache	109 (CM), 98 (EM), 98	89 (CM), 91 (EM), 90%	42.2 ± 10.6 (CM), 41.6 ± 10.9 (EM), 41.6 ± 10.6	Whole blood	NPTX2, SH2D5	Pérez Pereda et al. (2020)
Headache	26, 25	65.4, 56%	35.0 ± 6.9, 31.8 ± 7.0	Whole blood	RAMP1	Wan et al. (2015)

Published studies evaluating changes in DNA methylation in patients diagnosed with irritable bowel syndrome, fibromyalgia, or headache. *NR* not reported; *CM* chronic migraine; *EM* episodic migraine; *NPTX2* neuronal pentraxin II protein; *SH2D5* SH2 domain-containing 5 protein; *RAMP1* receptor activity-modifying protein 1

[a]age at baseline

healthy females, and when these specific CpG sites decreased in methylation, the risk for migraine increased in females but not in males (Table 1).

5 Conclusion and Future Directions

Disease outcomes are influenced by a combination of genetic factors, environmental exposures, and the interactions between the two (Hunter 2005). Epigenetic modifications, driven by hereditary or environmental influences, provide an opportunity to identify non-genomic drivers of disability and disease, as well as a potential understanding of underlying mechanisms. Currently, DNA methylation is the best characterized and easily identified epigenetic marker in the study of human disease,

due to its stability over time and simplicity of measurement (Bommarito and Fry 2019; Feinberg 2018; Feng et al. 2010). The use of methylation could become a primary tool in precision medicine to identify individualized mechanisms of disease origin and progression, as well as to personalize treatment plans for improved health.

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Epigenetic Regulation of Stress-Induced Visceral Pain

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Abstract Persistent abdominal pain is the most important reason for patients with irritable bowel syndrome (IBS) to seek medical care. However, current therapies fail to adequately treat abdominal pain. In part, this is due to a poor understanding of the mechanisms underlying abdominal pain in IBS. Chronic stress can contribute to the development or exacerbation of IBS symptoms. For instance, early life adversity has been associated with the development of IBS later in life. In addition, chronic stress in adulthood can trigger or exacerbate IBS symptoms. In rodent models, early life adversity and chronic stress in adulthood lead to long-term changes in gene expression in key regions of the (abdominal) pain pathway, which results in abdominal hypersensitivity. It is now becoming clear that changes in the epigenome are underlying these stress-induced changes in gene expression. Exposure to chronic stress can alter the epigenetic mechanisms (histone (tail) modifications, DNA methylation, and/or RNA interference), regulating the expression of pro- and anti-nociceptive genes in key regions of the pain pathways. Since epigenetic mechanisms are reversible, studies in rodents have shown that epigenetic interventions can attenuate stress-induced abdominal hypersensitivity. This research may lead to the development of new strategies to treat abdominal pain in IBS patients.

Keywords Irritable bowel syndrome · Abdominal pain · Stress · Amygdala · Epigenetic Mechanisms · Pain pathways

Abbreviations

CeA	Central nucleus of the amygdala
CNR1	Cannabinoid receptor 1
CORT	Cortisol/corticosterone

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CRH	Corticotrophin-releasing hormone
DNMT	DNA methyltransferase
DRG	Dorsal root ganglion
ELS	Early life stress
GR	Glucocorticoid receptor
H3K9	Histone 3 lysine 9
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPA	Hypothalamic-pituitary-adrenal
IBS	Irritable bowel syndrome
mGluR2	Type II metabotropic glutamate receptor
MR	Mineralocorticoid receptor
SAHA	Suberoylanilide hydroxamic acid
TRPV1	Transient receptor potential cation channel subfamily V member 1
TSA	Trichostatin A
WAS	Water avoidance stress

1 Introduction

The International Association for the Study of Pain defines chronic pain as pain lasting longer than 3 months after the resolution of or in absence of an injury. Chronic pain is characterized by a marked reduction in the threshold required to induce pain. As a result, patients can experience allodynia (and experience innocuous stimuli as painful) and/or hyperalgesia (experience a noxious stimulus as more painful). In this chapter, the primary focus will be on chronic abdominal pain, as experienced by patients with irritable bowel syndrome (IBS). However, other visceral disorders, such as inflammatory bowel disease, pancreatitis, bladder pain syndrome/interstitial cystitis, functional dyspepsia, functional chest pain, functional heartburn, functional dysphagia, centrally mediated abdominal pain syndrome, narcotic bowel syndrome, and functional anorectal pain, are also characterized by chronic pain. In these disorders, chronic pain emanates from a thoracic, pelvic, or abdominal origin that is poorly localized with regard to the specific organ affected. Although in some of these visceral disorders, chronic pain is associated with mucosal inflammation, other disorders lack distinct structural or histological abnormalities that could explain the origin of chronic visceral pain.

With a prevalence of up to 10%, IBS is one of the most prevalent disorders of the brain-gut axis (Oka et al. 2020). The majority of these patients are female, since the female/male ratio of IBS is 2:1 (Talley 1999). IBS patients are classified into three categories, based on their predominant stool type: diarrhea (IBS-D), constipation (IBS-C), or mixed (IBS-M, when stool type alters between diarrhea and constipation) (Lacy and Patel 2017). However, chronic abdominal pain is one feature that all IBS patient groups have in common. Furthermore, this recurrent pain has a severe negative impact on the quality of life of IBS patients and drives their

healthcare-seeking behavior (Enck et al. 2016). Unfortunately, the currently available pain therapies fall short in providing adequate and long-term pain relief for IBS patients. In part, this is due to our incomplete understanding of the etiology of IBS.

Although the etiology of IBS is likely multifactorial, we will focus on the role of stress as a predisposing or exacerbating factor for IBS. Epidemiological research has indicated that exposure to poverty, physical or emotional abuse, or trauma in the early life (early life stress, ELS) is a risk factor for developing IBS later in life (Bradford et al. 2012). In addition, patients with gastroenteritis who experience chronic stress are more likely to develop IBS afterwards than non-stressed gastroenteritis patients (Spence and Moss-Morris 2007). Furthermore, IBS patients often report that stress can trigger or exacerbate their symptoms (Hertig et al. 2007; Surdea-Blaga et al. 2012). The causal links between ELS or chronic stress in adulthood and the development of abdominal hypersensitivity have been confirmed in multiple animal studies. Therefore, we will discuss the results from these studies in the chapter below, since these results may provide useful insights in the pathophysiology of stress-induced abdominal pain in IBS patients.

2 Stress and Chronic Pain in IBS

The body's neuroendocrine response to stress is mediated by the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is activated when stress signals are integrated into the paraventricular nucleus of the hypothalamus (PVN), which will secrete corticotrophin-releasing hormone (CRH) into the hypophyseal portal circulation. When CRH binds the CRH1 receptor on the pituitary gland, adrenocorticotropic hormone (ACTH) will be released in the systemic circulation. When ACTH reaches the adrenal cortex, it will trigger the de novo synthesis and release of the main stress hormone cortisol (in humans) or corticosterone (in rodents) (CORT) in the bloodstream. The receptors for CORT, glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) are expressed throughout the body, and these receptors are involved in regulation of HPA axis activity. The interactions between CORT and GR in the PVN and hippocampus will provide negative feedback and inhibit HPA axis activation. The opposite happens when CORT binds GR on the amygdala, which will lead to the facilitation of the HPA axis. The balance between inhibitory and facilitatory signals will determine overall HPA axis activity.

The HPA axis of IBS patients is often disturbed, leading to abnormal circulating CORT levels during the day or an abnormal response to CRH (Kano et al. 2017; Patacchioli et al. 2001). Interestingly, certain brain regions involved in HPA axis regulation are also involved in abdominal pain processing. For instance, during colorectal distension, the amygdala of IBS patients becomes abnormally active when compared to healthy controls (Bonaz et al. 2002). Other brain imaging studies have revealed an abnormal connectivity between brain areas processing pain and emotions in IBS patients (Yu et al. 2022). Therefore, it is possible that a prolonged exposure to stress and HPA axis activity, whether in early life or adulthood, can

disturb the normal functionality or connectivity between brain regions involved in abdominal pain processing. In this way, stress-induced disturbances in neuronal networks contribute to sensitization and chronic pain, as prolonged exposure to stress can change the balance of pro- and anti-nociceptive neurotransmitters, receptors, other molecules involved in pain signaling.

Sensitization of neurons can occur due to changes in the gene expression patterns of the neurons involved in pain transmission/processing. One potential mechanism, linking external factors such as stress and changes in gene expression, is epigenetic mechanism. Histone tail modifications, DNA methylation, and RNA interference are important cellular mechanisms that regulate cellular gene expression. These epigenetic mechanisms can be influenced by environmental stimuli. In addition, epigenetic modifications are long-lasting and can remain in place, even in the absence of the initial trigger. Taken together, stress-induced changes in the epigenome could explain sensitization and the development of chronic abdominal pain. Furthermore, epigenetic changes could explain the long-lasting effects on pro-nociceptive gene expression after exposure to ELS or chronic stress in adulthood.

3 Epigenetic Changes in the CeA of Animals Exposed to ELS and WAS

ELS is a risk factor to develop IBS later in life. Therefore, several animal models of ELS have been used to study the development of abdominal hypersensitivity that resembles chronic abdominal pain in IBS patients. The most-used animal models of ELS are maternal separation and limited nesting, which mimic neglect and poverty, respectively. These models rely on removing the pups for a prolonged period from the dam or removing the all the nesting material from the cage. Both models will severely impact maternal behavior, which ultimately causes a premature activation of the HPA axis in the neonates. In adulthood, HPA axis activity remains abnormal, and the animals display abdominal hypersensitivity. Interestingly, only male animals, previously exposed to maternal separation or limited nesting, develop abdominal hypersensitivity (Prusator and Greenwood-Van Meerveld 2016). In order to recapitulate the female predominance of IBS, the odor-attachment learning model could be used, which mimics attachment to an abusive caregiver. In this model, the amount of time the pups are separated from the dam does not induce changes in maternal behavior. As a result, the HPA axis is not prematurely activated and shows normal reactivity to stress throughout life (Prusator and Greenwood-Van Meerveld 2016). However, in the odor-attachment learning model, neonates are exposed to predictable or unpredictable electric shocks, which will lead to a premature activation of the amygdala (Sullivan et al. 2000). The predictability of the shock determines which female rats will develop abdominal hypersensitivity: only female rats, previously exposed to unpredictable ELS, develop abdominal hypersensitivity, whereas female rats, previously exposed to predictable ELS, and male rats,

regardless of ELS exposure, remain normosensitive (Chaloner and Greenwood-Van Meerveld 2013).

In recent years, we have studied the gene expression alterations that underlie ELS-induced abdominal hypersensitivity in the central nucleus of the amygdala (CeA) of female rats. The CeA is also known as the nociceptive amygdala, since it is involved in the processing of (the emotional component) pain. Neurons in the CeA express CRH, and the release of this neuropeptide is pivotal in ELS-induced abdominal hypersensitivity (Prusator and Greenwood-Van Meerveld 2017). In the CeA of female rats previously exposed to unpredictable ELS, CRH expression is increased when compared to control animals (Prusator and Greenwood-Van Meerveld 2017). Interestingly, although GR expression is also increased, GR fails to bind the CRH promoter and negatively regulate CRH expression in the CeA of these females (Prusator and Greenwood-Van Meerveld 2017; Louwies and Greenwood-Van Meerveld 2020). Epigenetically, unpredictable ELS leads to a global increase in histone 3 lysine 9 (H3K9) acetylation in the CeA of adult female rats. These increases in H3K9 acetylation are also observed at the GR and CRH promoters (Louwies and Greenwood-Van Meerveld 2020) and may explain the increase in GR and CRH expression, since increases in H3K9 acetylation are associated with increased gene expression. Although epigenetic mechanisms are stable, they need to be maintained by epigenetic enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), enzymes that mediate the addition or removal of acetyl residues at the histone tails, respectively. In order to counter the abnormal, ELS-induced increase in global H3K9 acetylation, we showed that the administration of an HAT inhibitor directly in the CeA of adult female rats, previously exposed to unpredictable ELS, decreased H3K9 acetylation at the CRH promoter and attenuated ELS-induced abdominal hypersensitivity (Louwies and Greenwood-Van Meerveld 2020) (Fig. 1a).

Adult IBS patients often report that chronic stress can trigger or exacerbate their symptoms. Repeated water avoidance stress (WAS) is often used as a rodent model for chronic psychological stress in adulthood. In contrast to the odor-attachment learning model, WAS does activate the HPA axis and increases circulating CORT levels. Repeated activation of the HPA axis will lead to remodeling of stress-sensitive brain areas and abdominal hypersensitivity in rodents (Bradesi et al. 2005). We have shown that chronic WAS leads to a downregulation of GR expression in the CeA. Consequently, the decreased inhibitory actions of GR will lead to an increase in CRH expression in the CeA, which drives stress-induced abdominal hypersensitivity in rodents (Johnson et al. 2015; Tran et al. 2013). In order to investigate the role of epigenetic changes in the CeA underlying abdominal hypersensitivity, we chronically exposed the CeA to elevated levels of CORT. This localized increased CORT exposure resulted in a global decrease in H3K9 acetylation in the CeA. In addition, we observed a higher recruitment of HDACs to the GR promoter, H3K9 deacetylation, and a decrease in GR expression in the CeA, mimicking the effects observed in the WAS model. Concomitantly, at the CRH promoter, GR binding was reduced, and the transcription factor complex AP-1 was formed instead, leading to higher CRH gene expression (Tran et al. 2015). Administration of the HDAC

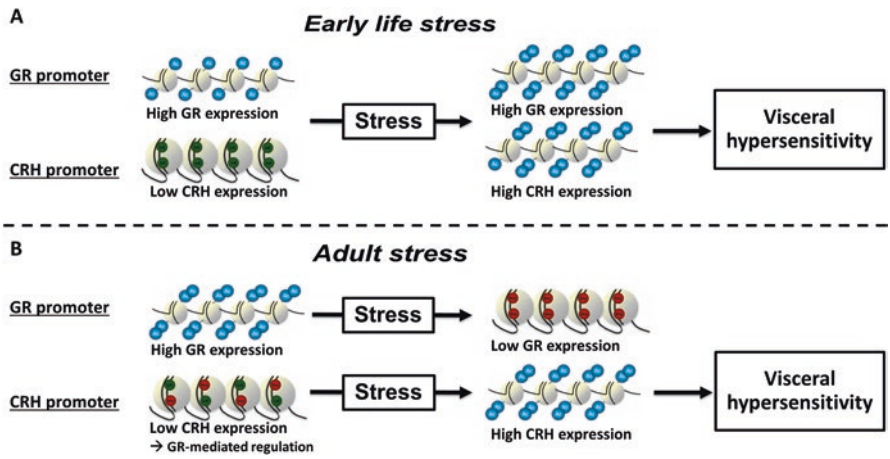

Fig. 1 Epigenetic mechanisms in the CeA underlying ELS and WAS-induced abdominal hypersensitivity. (**a**) WAS induces abdominal hypersensitivity through epigenetic mechanisms that involve removal of histone 3 lysine 9 residues at the GR promoter and addition of methyl groups at the DNA of the GR promoter region. These epigenetic changes lead to a decrease in GR expression in the CeA. Since GR is an important regulator of CRH expression, the loss of this regulator, in combination with an increase in histone 3 lysine 9 acetylation, leads to an increase in CRH expression in the CeA. High CRH expression in the CeA is responsible for abdominal hypersensitivity. (**b**) The epigenetic mechanism underlying ELS-induced abdominal hypersensitivity involves a global histone 3 lysine 9 acetylation at both the GR and CRH promoter regions and a loss of GR-mediated negative regulation of CRH expression. As a result, CRH is upregulated in the CeA, leading to abdominal hypersensitivity

inhibitors TSA or SAHA prevented the H3K9 deacetylation at the GR promoter and the downstream effects at the CRH promoter, ultimately preventing CORT-induced abdominal hypersensitivity (Tran et al. 2015). In a subsequent study, we showed that chronic WAS changes the DNA methylome in the CeA of male animals. After WAS, DNA methylation was increased at the GR promoter and decreased at the CRH promoter. These epigenetic modifications were associated with, respectively, decreased GR and increased CRH expression in the CeA (Tran et al. 2013). In a recent study, we showed that chronic WAS also changed H3K9 acetylation at the GR and CRH promoters in the CeA of female rats (Louwies et al. 2021). In addition, chronic WAS also increased DNA methylation at the GR promoter (Louwies and Greenwood-Van Meerveld 2022). Interestingly, when we administered TSA directly in the CeA of female rats exposed to chronic WAS, we prevented the decrease in H3K9 acetylation and the increase in DNA methylation at the GR promoter, which attenuated WAS-induced abdominal hypersensitivity. Our results indicate that stress-induced histone deacetylation (at the GR promoter) is an important first step in the development of stress-induced abdominal hypersensitivity. Following histone deacetylation, increases in DNA methylation at the promoter region ensure that GR gene expression is further silenced. The opposite process takes place at the CRH promoter, which requires DNA demethylation and histone acetylation in order to increase CRH gene expression (Fig. 1b).

4 Two Epigenetic Mechanisms, One Outcome?

As discussed above, both ELS and repeated exposure to WAS in adulthood lead to abdominal hypersensitivity. However, the epigenetic mechanisms in the CeA leading to abdominal hypersensitivity are different. The mechanism behind ELS is driven by a global increase in H3K9 acetylation, whereas prolonged CORT exposure leads to a global decrease in H3K9 acetylation (Louwies and Greenwood-Van Meerveld 2020; Louwies et al. 2021). The only similarity is the increase in H3K9 acetylation at the CRH promoter. This would imply that both ELS and WAS-induced abdominal hypersensitivity could be attenuated by using HAT inhibitors in the CeA, if they can prevent or reverse H3K9 acetylation at the CRH promoter. Conversely, this would also imply that HDAC inhibitors might not be effective to attenuate ELS-induced visceral hypersensitivity, since histone deacetylation (of the GR promoter) appears not to be involved in the mechanism underlying ELS-induced visceral hypersensitivity (Fig. 1).

5 Stress-Induced Epigenetic Mechanisms Outside the CeA and Abdominal Hypersensitivity

The stress-induced epigenetic changes underlying abdominal hypersensitivity are not limited to the CeA, but occur throughout the abdominal pain pathway. For instance, maternal separation-induced abdominal hypersensitivity can be attenuated by intraperitoneal administration of the HDAC inhibitor SAHA (Moloney et al. 2015). However, whether the HDAC inhibitor acted peripherally or centrally to attenuate maternal separation-induced abdominal hypersensitivity, and which genes were affected by HDAC inhibition, remains to be elucidated. It is possible that similar epigenetic interventions may prove successful in ameliorating limited nesting-induced abdominal hypersensitivity, but studies on this topic remain to be conducted.

The epigenetic mechanisms underlying chronic adult stress-induced abdominal hypersensitivity are better characterized. For instance, epigenetic remodeling after WAS has been observed in the colonic epithelium and lumbosacral DRGs (Hong et al. 2015; Wiley et al. 2020). Repeated CORT-GR interactions in colonic epithelial cells lead to the epigenetic remodeling of the promoter regions of tight junction proteins, resulting in downregulation of occludin and increases in paracellular permeability (Wiley et al. 2020). In this way, luminal contents can translocate into the gut wall and activate visceral afferents. Prolonged activation could lead to neural sensitization and increased transmission of pain signals. In a different study, WAS caused an increase in transient receptor potential cation channel subfamily V member 1 (TRPV1) expression in the L6-S2 DRGs. The repeated exposure to high circulating CORT levels caused an increase in DNA methylation of the GR and cannabinoid receptor 1 (CNR1) promoter regions in the L6-S2 DRG. As a result, GR and CNR1 expressions were decreased, preventing CNR1 to act as an inhibitor

of TRPV1 expression (Hong et al. 2015). At the same time, GR activated the HAT ep300, which increased acetylation at the TRPV1 promoter region, facilitating an increase in TRPV1 expression. The detrimental effects of WAS were attenuated, knocking down DNMT1 or ep300 in the L6-S2 DRGs (Hong et al. 2015). In another study, it was shown that by using a GR antagonist, WAS-induced changes in CNR1 and TRPV1 expression and the consequent WAS-induced abdominal hypersensitivity could be prevented (Hong et al. 2011). Additionally, GR antagonists also proved effective in preventing WAS-induced changes in colonic permeability, illustrating the importance of peripheral GR signaling in stress-induced abdominal hypersensitivity (Zheng et al. 2013). In the forced swim test model, it was shown that repeated forced swim-induced abdominal hypersensitivity could be attenuated through intrathecal injections of the HDAC inhibitor SAHA. HDAC inhibition increased H3K9 and H3K18 acetylation and expression of type II metabotropic glutamate receptor (mGluR2) in the lumbosacral spinal cord. Blocking mGluR2 signaling with an antagonist prevented the anti-nociceptive effects of intrathecal SAHA administration on forced-swim-induced abdominal hypersensitivity, showing that the SAHA-induced upregulation of mGluR2 was necessary to attenuate abdominal hypersensitivity (Cao et al. 2016).

6 Conclusion

The role of different epigenetic mechanisms in stress-induced abdominal pain is slowly being uncovered. In its essence, epigenetic changes are cellular adaptations to environmental changes. Therefore, beneficially changing the environment could potentially reverse detrimental epigenetic changes. In animal models of environmental enrichment, epigenetic mechanisms are involved in the attenuation of stress-induced abdominal hypersensitivity (Orock et al. 2021). Additionally, the development of new epigenetically active drugs, with higher specificity and selectivity, may make it possible to intervene at specific locations in the pain pathway to treat chronic abdominal pain.

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The Biomechanics of Distal Colon and Rectum and Its Relevance to Visceral Pain

Bin Feng and David M. Pierce

Abstract Visceral pain differs significantly from cutaneous pain in both its clinical and psychophysical manifestations. There is a prominent "mechanical" component associated with visceral pain, which is usually elicited from mechanical distension/stretch of hollow visceral organs like the gastrointestinal (GI) tract. In contrast, stimuli like heating, inflammatory, cutting, pinching, or piercing, which usually evoke pain from the skin, do not reliably cause visceral pain. Since GI-related visceral pain usually arises from the distal colon and rectum (colorectum), encoding of colorectal mechanical stimuli by sensory afferents is crucial to visceral nociception. The neural encoding part of this colorectal mechanotransduction, i.e., afferent spiking in response to mechanical stimulations of their receptive endings within the organ wall, has been extensively discussed in the previous chapters. In this chapter we focus on the macro- and microscopic biomechanics of the colorectum, which is critical in determining the local mechanical stress and strain states around individual mechanosensitive endings that directly generate afferent spikes.

Keywords Biomechanics · Collagen · Submucosa · Colon · Rectum

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1 Psychophysical and Neurophysiological Evidence Suggesting the "Mechanical" Nature of Visceral Pain

1.1 The Unique Clinical Characteristics of Visceral Pain

Pain is protective as it alerts us of threats and hazards in the environment through nociceptive sensing and signaling of tissue-injurious stimuli, including hot, cold, acidic, inflammatory, chemical, stabbing, rubbing, tearing/stretching stimuli, etc. The outer skin directly interfaces with the outside environment and contains sensory nerve endings dedicated to reliable and robust encoding of stimuli, e.g., above, to inform the brain (see Gold and Gebhart (2010) for review). In contrast, solid visceral organs inside the body like the pancreas, lung, liver, and spleen usually lack prominent sensory innervations to cause pain (Cervero and Laird 1999). The absence of this protective mechanism associates with unnoticed tissue injury and damage to those solid visceral organs until at the late stage of severe diseases, e.g., pancreatic cancer (Greenwald et al. 1987; Kelsen et al. 1995) and liver cirrhosis (Marotta et al. 2000). Similar to skin, the mucosa of hollow visceral organs also faces an "outside" environment of the body, the GI lumen of the colorectum. Like the cutaneous counterpart, visceral pain from the GI tract is also protective as it alerts us to abnormalities inside the lumen.

Although sharing the common protective function, cutaneous and GI-related visceral pain differ significantly in the following clinical manifestations. *First*, organ inflammation drives nociception from the skin but not from the GI tract. Unlike the protective role of cutaneous pain to alert tissue injuries and inflammation, GI-related visceral pain can be dissociated from gut injuries and inflammation. Visceral pain is the major complaint of patients with irritable bowel syndrome (IBS) whose colons appear "normal" relative to healthy controls, i.e., in the absence of apparent organ damage or inflammation (Feng et al. 2012). In contrast, patients with inflammatory bowel disease (IBD) in relapse show normo- or even hypo-sensitivity to noxious colorectal distension despite the presence of overt gut inflammation (Bernstein et al. 1996; Chang et al. 2000; Annahazi et al. 2009). *Second*, GI-related visceral pain does not accompany immediate motor withdrawal response prominently associated with cutaneous pain, i.e., moving affected body parts away from harmful stimuli (Cervero and Laird 2004). GI reflexes like vomiting and peristalsis are likely medium- and long-term coping mechanisms and do not immediately follow the onset of visceral pain. To meet the requirement of a fast motor withdrawal, cutaneous pain is often "sharp," acuate in the origin of pain, and rapid in transmission. As the exact opposite, visceral pain is "dull," diffuse in localization and often with characteristic referral, and slow in transmission (Pasricha et al. 2006). *Third*, visceral pain usually associates with stronger emotional and psychological components than cutaneous pain, likely caused by the significant overlap between the visceral pain circuits and the autonomic nervous system that plays major roles in the body's emotional responses (Cueva et al. 2007).

1.2 *Mechanical Distension of Distal Colon and Rectum (Colorectum) Drives Visceral Pain*

The environment inside the colorectum differs significantly from outside the skin in thermal, chemical, biological, and mechanical features. This likely causes limited perception modality of visceral pain as compared to a vast array of cutaneous pain modalities. *First*, the temperature inside the colorectum does not fluctuate as with outside the skin. Thus, hot or cold stimuli applied to the colorectum usually do not evoke visceral pain (Falt et al. 2013). *Second*, chemical signaling in the GI tract is complicated by the presence of commensal microbiomes, which regularly secrete chemicals like bile acids, fatty acids, peptides, and carbohydrate molecules (Kau et al. 2011). Thus, chemicals and acids that adequately trigger cutaneous pain are less effective at evoking visceral pain and vice versa. Inflammatory stimuli, for example, effective at causing pain from the skin, do not lead to heightened visceral pain in relapsed IBD patients (Bernstein et al. 1996; Chang et al. 2000; Annahazi et al. 2009). An opposite example is glycerol, which causes severe visceral pain when infused into the colon and rectum (Louvel et al. 1996; Bouin et al. 2001) but is not an effective skin irritant. *Third*, not all mechanical stimuli evoke visceral pain. In-plane normal stresses from colorectal wall distension (Ness and Gebhart 1988; Ness et al. 1990), not shear stresses (from cutting or pinching), or out-of-plane normal stresses (from stabbing) reliably evoke GI-related visceral pain (Lewis 1942; Ness and Gebhart 1990; Brierley et al. 2018).

1.3 *The Colorectum Is Innervated Predominantly by Mechanosensitive Afferents*

Consistent with clinical observations on the importance of colorectal mechanotransduction in GI-related visceral pain, the majority of the extrinsic afferents innervating the colorectum are mechanosensitive (as summarized in the previous chapters). Functional characterization of colorectal neural encoding using rodent models indicates that 67% of colorectal afferents in the lumbar splanchnic nerve (LSN) pathway and 77% in the pelvic nerve (PN) pathway are mechanosensitive and respond to at least one of three mechanical stimuli within their receptive fields (in ascending order of stimulus intensity): mucosal shearing by gentle stroking (10–20 milligrams force), colorectal stretch/distension (equivalent to approximately 15–60 mmHg luminal distending pressure), and punctate probing with a von Frey-like monofilament (1–1.4 grams force) (Feng and Gebhart 2011). Here, we need to emphasize the following features of mechanosensitive colorectal afferents that closely correlate with the heterogeneous biomechanical properties of the colorectum.

Along the longitudinal direction, the LSN and PN pathways dominate extrinsic sensory innervation of the distal colon and rectum, respectively (Fig. 1a). The LSN

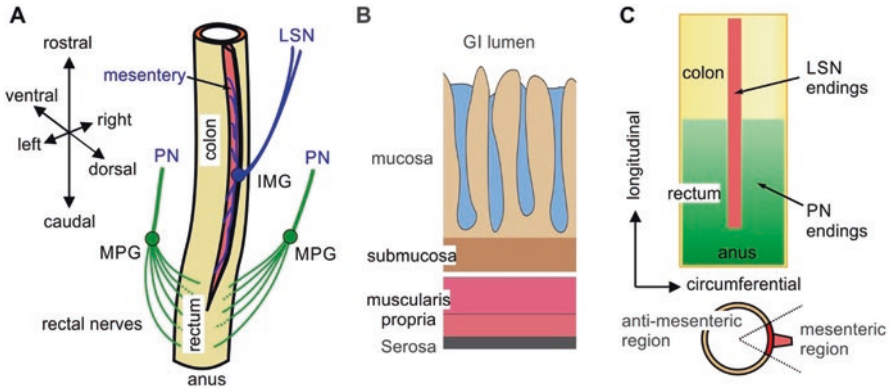

Fig. 1 The extrinsic sensory innervation of distal colon and rectum (colorectum). (**a**) Afferents in the lumbar splanchnic nerve (LSN) and pelvic nerve (PN) predominantly innervate the distal colon and rectum, respectively. (**b**) The colorectal wall consists of four major layers. (**c**) Inhomogeneous circumferential distribution of LSN afferents concentrated in the mesenteric region. The colorectum is displayed both as a flat sheet by cutting along the anti-mesenteric region (upper) and at a cross section (lower). MPG major pelvic ganglion, IMG inferior mesenteric ganglion. (Adapted from Feng and Guo (2020) with permission)

and PN afferents differ significantly in mechanical neural encoding. Compared with the PN afferents, the LSN afferents consist of a larger proportion of high-threshold afferents that only respond to punctate probing, the most intense of the three mechanical stimuli above. Consistently, a larger proportion of PN afferents respond to mucosal shearing, the least intensive stimuli of the three, versus LSN afferents. This differential mechanical neural encoding between the LSN and PN colorectal afferents also correlates with the longitudinal biomechanical heterogeneity between distal colon and rectum.

Through the thickness of the wall, the colorectum is a composite of multiple layers with distinct biomechanical properties and anatomical features, including the mucosal, submucosal, muscularis propria, and serosal layers. Colorectal afferent endings reside in all layers of the colorectum except in the serosa (Fig. 1b). Thus, biomechanical heterogeneity through the wall thickness likely impacts the mechanical neural encoding of individual colorectal afferents with their ending locations in specific layers.

The circumferential distribution of afferent endings in the colorectum is heterogeneous. LSN afferent endings concentrate along the regions next to the mesenteric attachment (i.e., the mesenteric region) and remain virtually absent in regions away from the mesenteric (i.e., the anti-mesenteric region) (Fig. 1c). Our recent findings of differential biomechanical properties between the mesenteric and anti-mesenteric region closely correlate with this unique spatial distribution of LSN afferent endings.

2 Methods for Characterizing Colorectal Biomechanics

The psychophysical and neurophysiological evidence above strongly indicates the critical roles of colorectal tissue biomechanics in GI-related visceral nociception and pain. Biomechanical heterogeneity of the colorectum follows from observations regarding the anatomical features. First, the distal colon and rectum differ greatly in thickness. Second, in the presence of mesentery, the colorectum is not axisymmetric like other tubular organs (e.g., blood vessels, ureter, and vas deferens). Further, the colorectal wall comprises a composite of multiple layers with distinct mechanical properties. Thus, to systematically characterize colorectal biomechanics we require a comprehensive approach leveraging macroscopic mechanical tests on whole organ or tissue patches, microscopic imaging of the load-bearing structure of collagen, and computational modeling with nonlinear and anisotropic constitutive equations.

2.1 Macroscopic Mechanical Tests

Many methods for characterizing macroscopic colorectal biomechanics are adopted from conventional testing methods on nonbiological materials, including uniaxial and biaxial extension and luminal inflation and, to a lesser extent compression, shear, and indentation. Biaxial tensile stretch and luminal inflation best match the mechanical conditions of tubular organs like the large intestine in vivo and have been used extensively in studying the upper GI tract like the esophagus and small intestine. In contrast, the body of literature characterizing the macroscopic biomechanics of the colon and rectum is comparably small and is thus completely summarized in Table 1. Most in vivo tests were conducted on human tissues by luminal inflation of the distal colon and rectum. Uniaxial tensile tests were most widely used in in vitro tests on excised tissues. Most studies considered the intestinal wall as a homogeneous "thick-walled" membrane and applied mechanical tests on intact tissue from the bulk wall. In contrast, only two studies considered the layered wall structure and conducted mechanical tests on separated layers (Egorov et al. 2002, Siri et al. 2019a, b).

2.2 Microscopic Imaging of Collagen Fibers

As with most soft tissues, networked collagen fibers at the microscale constitute the major load-bearing structures and they drive the macroscale mechanical properties of the colorectum. The relationship between microscopic collagen structures and macroscopic mechanical properties of soft tissue is reported in many other biological tissues like the skin (Reihsner and Menzel 1998), tendon and cartilage (Ker 1999; Maier et al. 2019; Szarek et al. 2020), and blood vessels (Hariton et al. 2007).

Table 1 Macroscopic mechanical tests on the colon and rectum

Specimen	Condition	Test methods	References
Mechanical tests on intact intestinal wall			
Human	In vivo	Pressure-volume	Parks (1970), Smith et al. (1981), Bharucha et al. (2001)
		Pressure-CSA (cross-sectional area)	Arhan et al. (1976), Dall et al. (1993), Drewes et al. (2001), Petersen et al. (2001), Drewes et al. (2006)
Human	In vitro	Uniaxial stretch	Watters et al. (1985b), Glavind et al. (1993), Massalou et al. (2016, 2019a, b)
		Biaxial stretch	Howes and Hardy (2012)
Porcine	In vitro	Inflation	Carniel et al. (2015), Patel et al. (2018)
		Compression and shear	Qiao et al. (2005)
		Uniaxial stretch	Qiao et al. (2005), Ciarletta et al. (2009), Carniel et al. (2014), Christensen et al. (2015)
		Biaxial extension	Puértolas et al. (2020)
Goat	In vitro	Compression	Higa et al. (2007)
Rat	In vitro	Pressure-diameter	Gao and Gregersen (2000), Sokolis et al. (2011), Sokolis and Sassani (2013)
		Uniaxial stretch	Watters et al. (1985a, b)
		Indentation	Stewart et al. (2016)
Mice	In vitro	Biaxial stretch	Siri et al. (2019a, b)
Mechanical tests on separated intestinal layers			
Mice	In vitro	Biaxial stretch	Siri et al. (2019a, b)
Human	In vitro	Uniaxial stretch	Egorov et al. (2002)

Assembled from thread-like collagen fibrils, collagen fibers are 0.5–2 microns thick and form a cross-linked network structure (Hulmes 2002). Determining the morphology of collagen fibers in the colon and rectum was classically conducted on sectioned tissue slices by chromatic and immunological staining and scanning electron microscopy (Orberg et al. 1982, 1983; Gabella 1983; Storkholm et al. 1998; Zeng et al. 2003; Yu et al. 2004). In the past decade, second-harmonic generation (SHG) microscopy has emerged as a powerful method for imaging collagen fibers with submicron resolution in a diverse range of tissues. SHG is highly selective for the collagen fibril/fiber structure and facilitates visualization of collagen fibers several hundred microns deep into the tissue using excitation light in the infra-red range (800–1200 nm). SHG imaging on large intestinal tissues was reported in several recent studies (Jiang et al. 2011; Zhuo et al. 2011, 2012; Liu et al. 2013; Schürmann et al. 2013; Bianchi et al. 2014; Birk et al. 2014; Mao et al. 2016; He et al. 2019; Sarri et al. 2019; Despotović et al. 2020a, b). Compared with conventional staining methods on thin tissue slides (~10 microns thick), SHG microscopy can visualize collagen fibers through the thickness of intact mouse colon (~200 microns thick) and most of the rectum (300–400 microns) (Maier et al. 2021). SHG allows systematic characterization of the collagen fiber density, distribution, alignment, and orientation at different layers throughout the colorectum (Maier et al.

Table 2 Quantification of collagen fibers in the small and large intestine

Specimens	Tissue	Layers	References
Chromatic and fluorescent staining			
Human	Large intestine	Mucosa	Zonios et al. (1996)
Rats	Large intestine	Mucosa	Sokolis and Sassani (2013)
		Submucosa	Sokolis and Sassani (2013)
		Muscular layers	Sokolis and Sassani (2013)
Porcine	Small intestine	Submucosa	Abraham et al. (2000)
Small-angle light scattering			
Porcine	Small intestine	Submucosa	Sacks and Gloeckner (1999)
Polarized light microscopy			
Rats	Small intestine	Submucosa	Orberg et al. (1983), Zeng et al. (2003), Yu et al. (2004)
Electron microscopy			
Human	Large intestine	Mucosa	Shamsuddin et al. (1982)
		Submucosa	Thomson et al. (1987)
Rats	Small intestine	Submucosa	Orberg et al. (1982, 1983), Gabella (1983)
Porcine	Small intestine	Submucosa	Gabella (1983)
Second-harmonic generation microscopy			
Human	Large intestine	Mucosa	Zhuo et al. (2011, 2012), Liu et al. (2013), Schürmann et al. (2013), Bianchi et al. (2014), Birk et al. (2014), Mao et al. (2016), He et al. (2019), Sarri et al. (2019), Despotović et al. (2020a, b)
		Submucosa	Jiang et al. (2011), Bianchi et al. (2014)
Mice	Large intestine	Mucosa	Xu et al. (2013), Prieto et al. (2019), Maier et al. (2021)
		Submucosa	Maier et al. (2021)
		Muscular layers	Maier et al. (2021)
		Serosa	Maier et al. (2021)

2021). Overall, there are relatively few studies reporting the collagen content, morphology, and orientation in the large intestine, and these are summarized in Table 2. Reports on the small intestine are also included due to the small body of literature on the large intestine.

2.3 Computational Modeling with Nonlinear and Anisotropic Constitutive Equations

The aforementioned studies in combination provide a significant amount of experimental data on the macro- and microscopic biomechanical properties of the colon and rectum. Such experiments often sought to capture specific aspects of the biomechanics, e.g., either the bottom-up collagen fiber properties or the top-down whole-organ responses to distension. However, systematic characterization of colorectal biomechanics including its heterogeneous and composite (layered) nature demands an unreasonable amount of additional experimental studies. Alternatively, high-fidelity computational modeling can incorporate the anatomic, geometric, and material heterogeneity into a comprehensive simulation to recapitulate existing experimental results (calibration and validation) and predict colorectal organ/tissue biomechanics (prediction). In addition, computational simulation is the only means to extract important information relevant to colorectal mechanotransduction that cannot be directly measured experimentally with existing technologies, i.e., the mechanical coupling between microns-thick sensory nerve endings and surrounding extracellular tissue matrix and local mechanical stress and strain profiles that directly drive action potential generation. The development of suitable constitutive relations that characterize the tissue mechanics is the cornerstone of such computational models, i.e., a mathematical formulation for the stress-strain relations. The assumed constitutive relations must then be fit to experimental data under loading conditions of interest and can be validated by successfully predicting results from independent experiments.

The colorectum undergoes large deformations in vivo, distending up to 30% radially to accommodate variable quantities of fecal matter (Patel et al. 2018). Accordingly, mechanical analyses of the colon employ large-strain and materially nonlinear mechanics, usually adopting the theoretical framework of hyperelasticity. There are two main categories of constitutive models for the colorectum: (1) phenomenological models with equations developed only to provide best fits to experimental data and (2) structure-based models that aim to incorporate the morphological and mechanical properties of fiber constituents. Classical phenomenological constitutive models include a coupled Mooney-Rivlin model with a convolution integral (Higa et al. 2007a, b) and an orthotropic Fung-type exponential model (Bellini et al. 2011; Sokolis et al. 2011). Relying on empirical curve fitting to experimental data, phenomenological models do not leverage empirical data on the microstructure of the tissue and are gradually shifting out of favor. Microstructure-based models include our understanding of the underlying microstructure of the colon and rectum. Thus, parameters in structure-based models usually have clear physical interpretations.

Structure-based constitutive models derive stress/strain relations from an assumed strain-energy function generally divided into an isotropic contribution simulating the ground matrix and an anisotropic contribution simulating the embedded collagen fibers. The first microstructure-based constitutive model of large

intestine was reported by Ciarletta et al. in which the strain-energy function represents an isotropic ground matrix and an anisotropic component including four families of aligned fibers (Ciarletta et al. 2009). Similar modeling strategies were used in subsequent studies from other groups with slight variations in the representations of collagen fibers or muscle fibers in the anisotropic components of the strain-energy functions (Sokolis et al. 2011; Sokolis and Sassani 2013; Carniel et al. 2014). In addition to families of directional fibers, recent models introduced additional families of dispersed collagen fibers in their constitutive equation to capture the statistical distributions of orientations of collagen fibers measured experimentally in colonic tissues (Patel et al. 2018; Puértolas et al. 2020). In all of the models above, the inclusion of directional or dispersed fibers accounted for in-plane biomechanical anisotropy, but material heterogeneity through the thickness of the intestinal wall, i.e., the layered, through-thickness constitution, was not considered.

Recently, we reported a constitutive model to capture the different mechanical properties of the sublayers of the colorectum based upon second-harmonic generation (SHG) imaging of collagen fibers through the wall thickness (Zhao et al. 2021). We adopted the orientation distribution functions (ODF) reported previously for cartilage (Pierce et al. 2016) and proposed a set of constitutive models to simulate sublayers of the colorectum at three different longitudinal regions, i.e., the colonic, intermediate, and rectal regions. Briefly, we modeled the individual mechanical responses of the inner and outer composites using an additive decomposition of the isochoric strain energy as

$$\bar{\Psi} = \bar{\Psi}_{\mathrm{IM}} + \bar{\Psi}_{\mathrm{FN}}, \tag{1}$$

with contributions from an isotropic neo-Hookean matrix $\bar{\Psi}_{\mathrm{IM}}\left(\bar{I}_1\right) = \mu\left(\bar{I}_1 - 3\right)/2$ (wherein $\mu > 0$ is the shear modulus of the underlying matrix), and from a network of collagen fibers (Holzapfel et al. 2014; Pierce et al. 2016) as

$$\bar{\Psi}_{\mathrm{FN}} = \int_{\Omega} \rho(\mathbf{M}) \frac{k_1}{2k_2} \left(\exp\left[k_2 \left(\bar{I}_4 - 1 \right)^2 \right] - 1 \right) \mathcal{H}\left(\bar{I}_4 - 1 \right) d\Omega,$$

where $k_1 > 0$ is a stress-like material parameter, $k_2 > 0$ is a dimensionless parameter, $\bar{I}_4 = \mathbf{M} \cdot \mathbf{C}\mathbf{M}$ is the isochoric fourth pseudo-invariant of $\mathbf{M}$ (the reference angular orientation of a single fiber), and $\mathcal{H}$ is a Heaviside function evaluated at $\left(\bar{I}_4 - 1\right)$, i.e., the collagen fibers only support tension. Here $\rho(\mathbf{M})$ is an orientation distribution function (ODF) characterizing the local angular orientation density of the fiber network (Pierce et al. 2016) as

$$\rho\left(\mathbf{M},\mathbf{D}\right) = \frac{\sin\theta}{\left|\mathbf{D}\right|^{1/2}\left(\mathbf{M}^{\mathrm{T}}\mathbf{D}^{-1}\mathbf{M}\right)^{3/2}},$$

where $\mathbf{D}$ is a second-order, symmetric, positive-definite tensor quantifying the local architecture of the collagen network and with $1/4 \int_{\Omega} \rho(\mathbf{M})\, d\Omega = 1$, where

$\Omega = \mathbf{M} \in R^3 \ : |\mathbf{M}| = 1$ is the unit sphere. We determined four model parameters (μ, k_1, k_2, α; with α the in-plane angle between the principal orientation of fibers and the circumferential direction) from fitting our experiments in biaxial extension (Siri et al. 2019a, b). Specifically, we determined the model parameters for individual layers of the colorectum using nonlinear optimization to fit our experimental stress-strain relations on layer-separated colonic, intermediate, and rectal segments.

The state-of-the-art constitutive models above can predict a comprehensive range of mechanical responses of the colon tissue using a coupled experimental-computational approach. The combination of experiments and computational modeling has synergistically advanced our understanding of the heterogeneous, biomechanical properties of the colorectum as summarized below.

3 The Biomechanical Heterogeneity of the Colorectum

As shown in Fig. 2, we consider the large intestine as a thick-walled cylindrical tube and assign a cylindrical coordinate system with longitudinal, radial (through-thickness), and circumferential directions. Anatomical heterogeneity of the colorectal tissue presents in all three directions. In the *longitudinal* direction, the colorectum changes its geometry progressively, significantly increasing in thickness from the proximal to distal regions. *Circumferentially*, the colorectum is not completely axisymmetric like other tubular organs (e.g., blood vessels) but has a mesenteric attachment aligned along the mesenteric region to vascularize the colorectum. Also, the longitudinal muscular layers in human and porcine colorectum are not distributed homogenously in the circumferential direction but are concentrated in three bands, i.e., the taenia coli. The mesenteric attachment aligns longitudinally with one of the

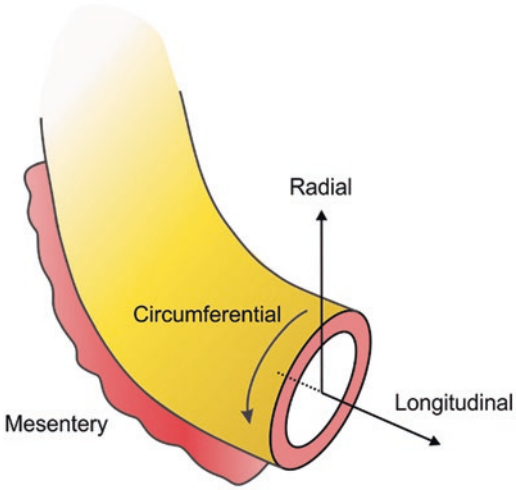

Fig. 2 Cylindrical coordinates of the colorectum. (Adapted from Siri et al. (2020) with permission)

three taenia coli. *Radially*, the colorectum is heterogeneous through the wall thickness which consists of four major layers (Fig. 1b). We will first summarize the anatomy and function of those four layers and then systematically discuss the biomechanical heterogeneity of the colorectum along the three cylindrical coordinates.

3.1 The Anatomy and Function of Layers of the Colorectum

The main functions of the colon are to push waste content down the GI tract by coordinated wave-like mechanical movements (peristalsis), absorb water to form solid feces, and send contents to the rectum. The rectum's main functions are continence and defecation, i.e., temporarily storing feces and expelling feces out of the body, respectively. To fulfill those functions, the colorectum consists of four main layers through the thickness of the wall: mucosa, submucosa, muscularis propria (longitudinal and circumferential muscle layers), and serosa. Each layer has distinct anatomical structures and neural tissue contents and consequently serves distinct biomechanical roles in GI physiology and pathophysiology.

3.1.1 Mucosa

The mucosa is the inner lining of the colon and rectum, consisting of a thin layer of epithelium, a connective tissue layer (i.e., lamina propria), and a thin layer of muscle (muscularis mucosa). Unlike in the small intestine, the colorectum lacks the villi structure, i.e., small, folded components that greatly enhance the intraluminal surface area to enable efficient nutrient absorption. Accordingly, the colorectum does not play a prominent role in nutrient absorption. The secretary and absorptive processes (mostly water) do take place in the colorectum in crypts (Robert et al. 2001), i.e., cylindrical structures "sinking" down from the lamina propria to the muscularis mucosa to increase the surface area (Shamsuddin et al. 1982). Sensory nerve endings extend to the lamina propria between the crypts and act as "taste buds" of the gut to survey the mucosal contents (Holzer et al. 2001). As shown in SHG images in Fig. 3, the collagen fibers in the mucosal layers appear to wrap around individual colonic crypts. Collagen fibers there do not seem to form an in-plane network like their counterparts in the submucosa and thus are unlikely to play a significant load-bearing role in resisting colorectal distension. However, mucosal collagen fibers likely provide structural support maintaining the crypt structures which are themselves innervated by afferent endings (Spencer et al. 2014). The biomechanical role of collagen fibers in the mucosa remains unclear and awaits further experimental studies. It may contribute to colorectal mechanotransduction by translating mucosal shearing into specialized, local mechanical stresses/strains around the crypts.

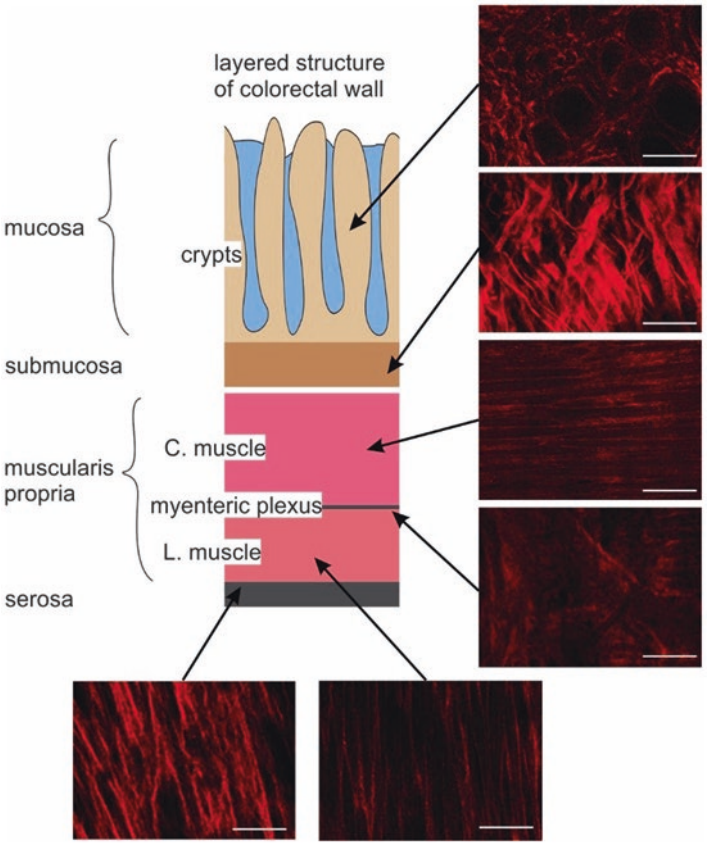

Fig. 3 Second-harmonic generation (SHG) imaging of collagen fibers through the wall thickness of the colorectum. Scale bars: 50 μm. (Adapted from Siri et al. (2019a, b) with permission. C. muscle: circular muscle layer; L. muscle: longitudinal muscle layer

3.1.2 Submucosa

The submucosa is a fibrous connective tissue layer that surrounds the mucosa. It contains major branches of blood and lymph vessels supplying the large intestine. It has a high concentration of lymphocytes, fibroblasts, and mast cells. In addition, the submucosa hosts one of the two major enteric neural plexuses, i.e., the submucosa plexus that regulates the configuration of the luminal surface, controls glandular secretions, alters electrolyte and water transport, and regulates local blood flow. We recently reported the concentrated presence of thick collagen fibers within the submucosa via second-harmonic generation imaging (Maier et al. 2021), thus providing anatomic support for its load-bearing function as established from layer-separated tensile tests (Egorov et al. 2002, Siri et al. 2019a, b). In addition, among all four layers of the colon, the submucosa has the highest proportion (32%) of extrinsic

sensory neural endings (Spencer et al. 2014; Guo et al. 2021). Most sensory endings in the submucosa are free endings, small in diameter (~1 micron), and meandering, features which correspond to the anatomic features of typical nociceptors that encode tissue-injurious mechanical stimuli (Guo et al. 2021).

Collagen fibers concentrate in the submucosa of the colorectum (Fig. 3). More significantly, collagen fibers in the submucosa are wavy when the colorectum is in a load-free condition and gradually straighten with increasing distension of the colorectum (Maier et al. 2021). This progressive recruitment of collagen fibers agrees with the nonlinear tension-stretch relations recorded from the mucosal/submucosal composite showing increased mechanical stiffness with deformation (Siri et al. 2019a, b). Under noxious distension, the collagen fibers in the submucosa straighten to reveal two principal families of fibers that orient approximately ±30° from the longitudinal direction, respectively (Orberg et al. 1982, 1983; Siri et al. 2019a, b; Maier et al. 2021). The two principal families of fibers appear not to lay in parallel planes but interweave with one another to form a reinforced network of collagen fibers. In addition, the network of collagen fibers in the submucosa does not seem to vary significantly in thickness, fiber density, or fiber diameter from proximal to distal colorectum (Siri et al. 2019a, b, Maier et al. 2021). This likely contributes to the consistent longitudinal stiffness in tension despite the significant increase in colorectal wall thickness from colonic to rectal regions. Consistent observations between the microscopic collagen fiber network and bulk mechanical properties from biaxial tensile tests strongly indicate that the submucosa is the load-bearing "skeleton" for the colorectum and protects it from excessive distension.

3.1.3 Muscularis Propria

The muscularis propria comprises two layers of smooth muscle: an inner circular muscle layer and an outer longitudinal muscle layer. In some species including humans, the longitudinal muscle layer concentrates into three discrete bands known as taenia coli. The myenteric plexus is a thin layer of neural network "sandwiched" between the two muscular layers. It is mainly responsible for colorectal peristalsis, which are coordinated wave-like propulsive movements generated and controlled largely by a "hardwired" polarized polysynaptic circuit intrinsic within the GI tract (Wood 2008). In addition, the myenteric plexus receives extrinsic projections from preganglionic parasympathetic fibers as well as postganglionic sympathetic fibers and is thus also modulated by neural signals from outside the GI tract (Furness 2012). Although relatively thin in dimension (~2% of total wall thickness of mouse colon from the authors' unpublished observations), the myenteric plexus hosts a large proportion of extrinsic sensory nerve endings, ~22% in mouse colon (Spencer et al. 2014). Only the circular muscle layer, which is significantly thicker than the myenteric plexus, has a slightly greater amount of sensory nerve endings (~25%) (Spencer et al. 2014). In contrast, there are virtually no extrinsic, sensory innervations in the longitudinal muscle layer (Spencer et al. 2014).

Compared with the submucosa and serosa, the circular and longitudinal muscle layers contain only minor collagen fiber content (cf. Fig. 3) (Maier et al. 2021). However, the outer muscular/serosal composite shows comparable longitudinal and circumferential stiffness to the inner mucosal/submucosal composite (Siri et al. 2019a, b), indicating the contribution of muscle fibers to the mechanical stiffness in the outer composite. The collagen fibers in the two muscular layers are well aligned with the orientations of muscle fibers, i.e., longitudinal and circumferential, respectively. Those two families of collagen fibers, perpendicular to one another, collectively lead to the reduced in-plane tissue anisotropy in the outer muscular/serosal composite as compared with more pronounced tissue anisotropy in the inner mucosal/submucosal composite (Siri et al. 2019a, b).

3.1.4 Serosa

The serosa is the outermost layer of the colorectum, representing an extension of the visceral peritoneum and mesentery, and consists of a continuous sheet of squamous epithelia cells, i.e., the mesothelium (Rao and Wang 2010). Although the serosa has significant collagen fiber content (Fig. 3) (Maier et al. 2021), it is unlikely to have a major load-bearing role due to its thin radial dimension compared to the total intestinal wall thickness (Egorov et al. 2002). The thickness of the serosa is usually no more than 50 μm in human colon (Egorov et al. 2002). Similar to the longitudinal muscle layer, extrinsic sensory innervation is absent in the serosa (Spencer et al. 2014; Patel et al. 2019).

3.2 Biomechanical Heterogeneity in the Axial Direction

Studies leveraging macroscopic tissue testing and microscopic collagen fiber imaging recently characterized the biomechanical properties of the colorectum along the axial direction (Siri et al. 2019a, b; Maier et al. 2021). Particular focus was given to the differential properties between the distal colon and rectum, which are predominately innervated by sensory afferents from the lumbar splanchnic nerve (LSN) and the pelvic nerve (PN), respectively (Feng and Gebhart 2011). From the distal colon to the rectum, tissue compliance increases progressively, and so do the residual strains as measured by increased opening angles. Collectively these findings indicate significantly higher level of strain in the rectum than in the colon. This correlates nicely with the presence of most, if not all, stretch-sensitive afferents in the PN pathway, whereas most LSN afferents do not respond to colorectal stretch (strain). In addition, stretch-sensitive afferents with endings in the rectum showed significantly higher firing rates under circumferential colorectal stretch than endings in the colon (Feng et al. 2010), likely reflecting the higher level of strain in the rectum. Colorectal tissue is viscoelastic and dissipates more energy under deformation in

the circumferential direction than in the longitudinal direction (Siri et al. 2019a, b), which could explain the adaptation of afferent activities to circumferential intestinal stretch (Feng et al. 2010). Finally, the rectum is significantly thicker than the distal colon (Feng et al. 2010; Maier et al. 2021), especially in the circular muscular layers, which implies that PN sensory endings are more affected by smooth muscle activities during normal GI functions than their LSN counterparts.

3.3 Biomechanical Heterogeneity in the Radial (Through-Thickness) Direction

The layered structure of the intestinal wall confirms heterogeneous biomechanical properties through the wall thickness, i.e., in the radial direction. Although further indicated by the distinct anatomic differences, the differential biomechanical properties of the layers of the colorectum were not characterized in the literature until recently. Egorov et al. (2002) conducted studies on layer-separated large intestinal tissues harvested from human cadavers and reported that the mechanical strength of the bowel wall is determined by the submucosa and muscular layers, while the serosa and mucosa have no significant strength. However, the authors provide no technical details regarding how they separated and tested the different intestinal layers. We recently reported a layer-separated biomechanical study on mouse distal colon and rectum. In mouse large intestine, there is an apparent interstitial space between the submucosa and circular muscle layers. This unique anatomic feature allowed us to conduct fine dissections to gently separate the intestinal wall into inner and outer composites, the inner consisting of the mucosa and submucosa, and the outer of the muscularis propria and serosa (Siri et al. 2019a, b). We established that the inner mucosal/submucosal composite has slightly higher longitudinal stiffness than the outer composite, while the outer muscularis/serosal composite has higher circumferential stiffness. This macroscopic tissue biomechanics is consistent with the contents and orientation of collagen fibers (Fig. 3), which concentrate in the submucosa with two symmetric families of fibers more aligned towards the longitudinal than the circumferential direction. Hence, the wall tension resulting from colorectal distension deforms both composites, the inner composite taking slightly more longitudinal tension and the outer composite more circumferential tension. Within the inner composite, mechanical stress (force per area) likely concentrates in the submucosa due to its high content of collagen. In contrast, stress should be more evenly distributed in the outer composite between the circular and longitudinal muscle layers which have comparable muscle/collagen contents.

3.4 Biomechanical Heterogeneity in the Circumferential Direction

Unlike tubular organs such as the blood vessels that are usually axisymmetric along the central axis, the colorectum is not homogeneous circumferentially due to the mesenteric attachment along one side of the colorectum. Moreover, the distribution of LSN sensory endings in the circumferential direction is heterogeneous and is only concentrated close to the mesentery (Feng and Gebhart 2011; Feng and Guo 2020). In human and porcine colons, the longitudinal muscle layers are not continuously distributed along the circumference but concentrated in three bands, i.e., the taeniae coli. One of the taeniae coli connects with the mesentery and is termed the mesenteric taeniae, whereas the other two are termed anti-mesenteric taeniae (Matrana and Margolin 2009). In combination this anatomic evidence strongly suggests differential biomechanical properties along the circumferential direction between the mesenteric and anti-mesenteric zones. In colons from patients with diverticular disease, small sacs or pockets predominantly develop in regions next to the mesenteric taeniae (Hughes 1969; Matrana and Margolin 2009), providing indirect evidence suggesting weaker mechanical stiffness at mesenteric regions versus anti-mesenteric regions in the large intestine. In support of this working hypothesis, our preliminary unpublished observations indicate that mouse colorectum is indeed more compliant in the mesenteric region than in the anti-mesenteric region, and under extremely high intraluminal pressure (approximately 200 mmHg) the colorectal wall unanimously ruptures at the mesenteric region. However, there appears to be no apparent difference in collagen fiber contents and orientations between mesenteric and anti-mesenteric regions (unpublished observations). The microstructural origins of this difference in circumferential compliances in the colorectum await further studies.

3.5 In-Plane Biomechanical Heterogeneity

When considering the colorectum in ex vivo tissue testing, a longitudinal-circumferential patch of tissue can be stretched to form a flat specimen with the longitudinal and circumferential directions forming a planar surface. The in-plane mechanical properties of the colorectum in those principal directions are quantitatively anisotropic, i.e., compliance in the circumferential direction is greater than that in the longitudinal direction. Greater circumferential compliance facilitates the physiological functions of the large intestine (fecal storage and propagation), while lower longitudinal compliance reduces longitudinal deformation to keep the large intestine in position during distension (Sokolis et al. 2011). Overall, the distal colon is significantly stiffer circumferentially than the rectum, but only modestly stiffer longitudinally than the rectum (Siri et al. 2019a, b). This collectively results in significantly greater in-plane anisotropy in the rectum than in the distal colon. The

increased circumferential compliance in the rectum likely supports its physiological role of fecal storage.

4 Altered Colorectal Biomechanics in Lower GI Disorders

Many lower GI disorders associate with changes in microscopic colorectal biomechanics. Colon inflammation generally corresponds with increased stiffness of the colon as reported in most inflammatory bowel diseases (Pucilowska et al. 2000), with a few exceptions when the inflammation is ongoing and more localized in the mucosa (e.g., active ulcerative colitis (Drewes et al. 2006)). Colons from patients with diverticular diseases often present reduced mechanical strength, reduced distensibility, and premature relaxation to distension (Parks 1970; Smith et al. 1981), along with prominent structural changes such as thickened circular muscular layer, shortened colon length, and narrowed lumen (Parks 1970; Smith et al. 1981). Hirschsprung's disease, for example, features loss of ganglionic neurons in the colon and rectum, increased circular muscle thickness, and increased intraluminal pressure during colonic inflation (Hillemeier and Biancani 1990). In contrast, no apparent change of colonic or rectal biomechanics associates with patients with irritable bowel syndrome (Drewes et al. 2001). In summary, the change in the macroscopic biomechanics of the large intestine is evident in most lower GI disorders except for functional disorders like the irritable bowel syndrome.

In the clinic, many lower GI disorders also correlate with changes in the microscopic mechanical properties in the colorectum and particularly by collagen content, usually quantified only in the mucosal layer. Crohn's disease (CD), ulcerative colitis (UC), intestinal tuberculosis (ITB), and colonic cancer/dysplasia all include altered collagen fiber density and orientation in the colorectal mucosa. Compared with healthy controls, UC patients have more frequent defects in the collagen stroma and fibroblasts of the colonic mucosa, significantly decreased mucosal blood flow, and more severe multiple platelet agglutinations within the small mucosal vessels (Donnellan and Beal 1966). It also remains challenging to diagnose patients for CD versus ITB, which may show significantly different collagen contents in their colonic mucosa (Mao et al. 2016). Mucosal collagen content is significantly higher in ITB colons than in CD ones. Moreover, CD affects the distribution of collagen by forming clusters, while ITB affects collagen around the perimeter of caseating granulomata. Finally, the collagen fiber density and direction underneath the intestinal epithelium helps clinicians discriminate between normal and dysplastic colonic mucosa. In normal colon and rectum, there is a dense matrix of collagen fibers almost parallel to the interface of the epithelium and stroma, whereas in colon with dysplasia the collagen matrix is loosened with an angle tilted to the interface (Zhuo et al. 2011; Birk et al. 2014). In addition, a malignant tumor may alter the organization of collagen fibers in the colonic mucosa even 10–20 cm away from its location (Despotović et al. 2020a).

5 Summary and Conclusions

Visceral pain arising from the colorectum has psychophysical characteristics distinct from pain arising from the skin, which reflects the vastly different environment inside the intestinal lumen versus outside the body. Stimuli like heat, cold, or inflammation that are noxious to the skin are not similarly noxious to the colorectum. It is mechanical stimuli, especially luminal distension, that are noxious and reliably evoke visceral pain from the colorectum. Thus, mechanotransduction plays critical roles in evoking visceral pain, a process that takes place at the microns-thick afferent endings in the colorectal wall and is significantly affected by the biomechanics of colorectal tissue. The large intestine varies in diameter and thickness longitudinally, consists of four anatomically distinct sublayers leading to through-thickness mechanical heterogeneity (axially), and is circumferentially unsymmetrical due to the mesenteric attachment along one side of the intestine. These anatomical features correlate with heterogeneous biomechanical properties along the longitudinal, radial, and circumferential directions. Longitudinally, compliance increases from the distal colon to rectum, facilitating the function of the rectum, i.e., fecal storage. Through the wall thickness, the submucosa and the muscularis propria are the major load-bearing structures, whereas the mucosa and serosa have no significant stiffness. Along the circumferential direction, regions close to the mesentery are mechanically more compliant than regions away from the mesentery as suggested by the concentrated distribution of sacs or pockets in the mesenteric region of the colon present in patients with diverticular diseases. Overall, the large intestine is stiffer longitudinally than circumferentially, reducing elongation during physiological distension and peristalsis and allowing the intestine to maintain its position.

The microscale, intra-tissue biomechanics of the large intestine is driven by the contents, morphology, and orientation of collagen fibers within different layers of the large intestine. Collagen concentrates in the submucosa and serosa, but not in the muscularis propria or mucosa. In addition, collagen fibers in the submucosa consist of two families of fibers oriented approximately ±30° along the longitudinal direction to form a tight-knit, helical network. This architecture of the collagen fibers in the submucosa indicates its load-bearing role. The muscularis propria, despite relatively low collagen fiber content, shows a mechanical stiffness comparable to the submucosal/mucosal composite, and its load-bearing role is likely provided by the thick bundles of muscle in the circular and longitudinal muscle layers. Extrinsic and intrinsic neural innervations concentrate in the submucosa and myenteric plexus in the muscularis propria, which are regions of high mechanical stresses during physiological distension and peristalsis. The nociceptor-like nerve endings in the submucosa strongly indicate their critical roles in detecting tissue-injurious mechanical stimuli by evoking pain from the colon and rectum. In addition, changes in the content and morphology of collagen in intestinal mucosa can potentially be a marker for many lower GI disorders.

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Visceral Nociception in Gastrointestinal Disease

James Higham, Rohit Gupta, and David C. Bulmer

Abstract Abdominal pain is common across organic and functional gastrointestinal disorders. For example, most patients with inflammatory bowel disease (IBD) report experiencing abdominal pain related to their condition, and many functional disorders are diagnosed by the presence of pain. Although great strides have been made in the treatment of IBD and functional disorders such as irritable bowel syndrome (IBS), pain continues to be a challenge for a significant number of patients refractory to treatment or experience pain during disease remission. This chapter describes our recent work which demonstrates how the use of human tissue from carefully phenotyped patients in combination with omics technology and assays of nociceptor signalling has enabled the identification of mediators (MMP-12) and mechanisms (PAR1, $Na_V1.9$) responsible for the activation of colonic nociceptors in GI disease states, validating their utility as therapeutic targets for novel analgesic therapies. This process has been accelerated with the advent of next-generation sequencing which has facilitated detailed interactome analysis of putative signalling pathways in disease states as exemplified for prokineticin-2, highlighting the future opportunities for hypothesis-driven translational studies of visceral nociception in GI diseases, the findings from which should accelerate the development of visceral analgesics.

Keywords Visceral pain · Nociception · Human biopsies · NaV1.9 · Inflammatory bowel disease

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1 Visceral Nociception in Gastrointestinal Disease

Abdominal pain is common across organic and functional gastrointestinal disorders, with a significant proportion of inflammatory bowel disease (IBD) patients reporting abdominal pain related to their condition, and many functional disorders utilising pain as a diagnostic criterion (Bielefeldt et al. 2009; Longstreth et al. 2006). Great strides have been made in the treatment of IBD and functional disorders such as irritable bowel syndrome (IBS); however, the management of pain continues to be a challenge for a significant number of patients refractory to treatment or during disease remission.

> An unmet clinical need therefore exists for the development of new visceral analgesics to treat abdominal pain in gastrointestinal diseases such as IBD and IBD

2 Pain Signalling from the Gut

Relevant stimuli for the generation of pain from the gastrointestinal tract consist of noxious distension or contraction of the gut, often in the presence of inflammation which promotes the production of algogenic inflammatory mediators (Bentley and Smithwick Reginald 1940; Kuiken et al. 2005). These noxious, painful and tissue damaging stimuli are detected by a subset of sensory nerves called nociceptors which project via nerve fibres with cell bodies located within the dorsal root ganglia (DRG) to the spinal cord and from there to pain processing regions of the central nervous system (CNS) leading to the conscious perception of pain (Gold and Gebhart 2010). In addition, many of the mediators released from the damaged or inflamed gut also sensitise nociceptors enhancing both responses to painful stimuli and the activation of nociceptors by previously innocuous levels of stimuli, a state frequently referred to as visceral hypersensitivity (Bueno and Fioramonti 2002). The colorectum, a common site of disease in IBD patients and region of hypersensitivity in IBS patients (Ritchie 1973; Rao et al. 1987), is innervated by nociceptors from two anatomically distinct sensory nerve pathways: one, responsible for the relay of pain from the rectum, projects to the lumbosacral (LS) spinal cord via the pelvic nerve, and the second responsible for relaying pain from the sigmoid colon and descending colon travels with lumbar splanchnic nerves (LSN) to terminate within thoracolumbar (TL) divisions of the spinal cord (Ray and Neill 1947).

This chapter focuses on studies that have examined colonic nociceptors within the LSN pathway. Experimentally these fibres can be characterised as nociceptors by their higher threshold to activation by luminal distension, which for mouse colonic (LSN) nociceptors is typically between 30 and 40 mmHg, a pressure comparable with the pain threshold to barostat balloon distension of the sigmoid or descending colon in humans (Hughes et al. 2009; Klooker et al. 2010; Buhner et al. 2014; Peiris et al. 2011). These observations indicate that the mechanisms underpinning transduction of noxious mechanical stimuli may be preserved between mouse and human colonic nociceptors.

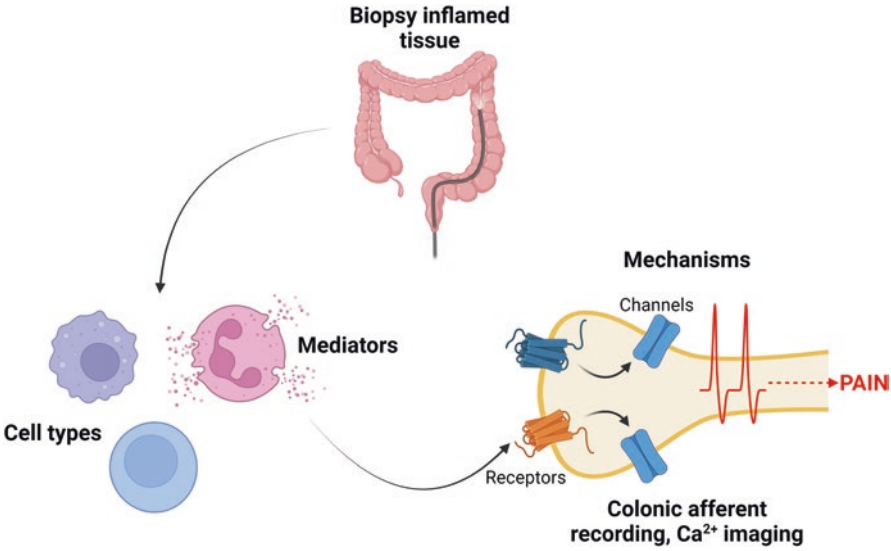

Fig. 1 Tissue biopsies from the inflamed bowel of clinically phenotyped patients provide information on the cell types and mediators present during inflammation. The impact of these mediators and the mechanisms utilised to elicit nociceptor signalling can then be determined using in vitro and ex vivo experimental approaches such as live cell imaging and electrophysiological studies in cultured sensory neurons and colonic afferent nerves

Preventing the activation and sensitisation of colonic nociceptors is one strategy to treat abdominal pain in gastrointestinal diseases such as IBS and IBD, and the subject of this chapter describes recent work from our lab presented at the FNM 2020 visceral pain satellite meeting which has used tissue from patients to identify novel mediators and mechanisms of disease nociception (Fig. 1).

2.1 IBS Biopsy Supernatants as a Disease-Relevant Noxious Stimulus

Experiments from my group and other researchers, including seminal work from Giovani Barabara (Barbara et al. 2007) and Nicolas Cenac (Bautzova et al. 2018; Cenac et al. 2007; Cenac et al. 2015), have repeatedly shown that supernatants generated from the bowel of IBS patients stimulate pain signalling pathways (Bautzova et al. 2018; Cenac et al. 2007; Cenac et al. 2015; Balemans et al. 2017). These studies have demonstrated increases in intracellular Ca^{2+} in sensory DRG neurons following pre-treatment with IBS biopsy supernatants, alongside increased ongoing afferent nerve discharge and behavioural responses (visceromotor response, VMR) to colorectal distension or mechanical probing (Table 1). Collectively these findings provide robust evidence for receptor engagement, activation and sensitisation of

Table 1 Summary of findings from studies utilising supernatants generated from mucosal biopsy of the colorectum in IBS patients which have repeatedly demonstrated the presence of a pronociceptive bowel environment in IBS patients

Disease type	Endpoints	Summary	References
IBS	Intestinal mesenteric afferent fibre activity Ca^{2+} mobilisation in sensory dorsal root ganglia (DRG) neurons	Supernatants generated from IBS patient biopsies markedly enhanced the firing of mesenteric nerves and Ca^{2+} mobilisation in rat DRGs IBS-dependent excitation of DRG neurons was inhibited by histamine H_1 receptor blockade and serine protease inactivation consistent with elevated biopsy supernatant histamine levels and protease activity	Barbara et al. (2007)
IBS	Ca^{2+} mobilisation in sensory DRG neurons Somatic nociception and visceromotor response (VMR) to colorectal distension	Increased trypsin and tryptase expression and release from IBS biopsies compared with control subjects Biopsy supernatant from IBS patients sensitised mouse DRG neurons and caused somatic and visceral hyperalgesia and allodynia in mice (in vivo) Sensitisation and pronociceptive effects were inhibited by serine protease inhibitors, a PAR2 antagonist, and were absent in PAR2-deficient mice	Cenac et al. (2007)
IBS	Visceromotor response (VMR) to colorectal distension Ca^{2+} mobilisation in sensory DRG neurons	Supernatants from IBS biopsies, but not from controls, induced visceral hypersensitivity in mice Small interfering RNA knockdown of TRPV4 in mouse DRG neurons inhibited the hypersensitivity caused by supernatants from IBS biopsies	Cenac et al. (2015)
PI-IBS (post-infectious IBS)	Colonic afferent activation Ca^{2+} mobilisation in sensory DRG neurons Ca^{2+} mobilisation in human enteric neurons	Supernatants generated from PI-IBS patient biopsies stimulate colonic afferent activity and evoke mechanosensitivity Supernatants sensitise capsaicin-mediated Ca^{2+} flux in DRG neurons. Effect attenuated by histamine H_1 receptor antagonist treatment or abolished in tissue from histamine H_1 receptor knock-out mice Ca^{2+} flux to capsaicin is enhanced in enteric neurons from PI-IBS patients compared to controls	Balemans et al. (2017)

(continued)

Table 1 (continued)

Disease type	Endpoints	Summary	References
IBS-D (diarrhoea-predominant IBS)	Colonic afferent activity and evoked mechanosensitivity Symptom scores for pain, urgency, frequency and consistency of stool, anxiety and depression	Significant correlation found between biopsy supernatant-mediated colonic afferent activation and patient pain severity scores. No correlation observed between biopsy-mediated colonic afferent activity and symptom scores for bowel habit and psychology factors Biopsy-mediated colonic afferent activity abolished in tissue from $Na_V1.9$ channel knock-out mice	Cibert-Goton et al. (2021)

mouse nociceptors by supernatants from IBS patients confirming the presence of a pronociceptive environment in the bowel of IBS patients. Building on this work findings presented at FNM 2020 illustrated our most recent study conducted with Prof Robin Spiller (University of Nottingham), in which we examined the effect of applying individual supernatants generated from sigmoid colon biopsies obtained from patients (n = 42) with diarrhoea-predominant IBS (IBS-D) directly onto the mucosa containing the receptive field of colonic nociceptors using electrophysiological recordings of LSN activity in a flat sheet preparation of mouse colon (Cibert-Goton et al. 2021). This study demonstrated a significant correlation between supernatant-mediated nociceptor activation and the average daily pain score reported by respective patients using a 14-day bowel symptom diary prior to endoscopy (see Table 1). Importantly no correlation was found between supernatant-mediated nociceptor firing and symptom scores for bowel frequency or stool consistency, in addition to psychological assessments of anxiety or depression. These data indicate that the correlation with pain scores was specific for pain and not related to a more general increase in disease activity and suggest that studies of biopsy supernatant-mediated nociceptor activation provide mechanistically relevant insight into the generation of pain symptomology in patients.

Having established a robust link between supernatant-mediated nociceptor activation and pain, we proceeded to use biopsy supernatants from both IBS and IBD patients to (a) identify the mediators and mechanisms driving visceral nociceptor activation in these diseases and (b) provide a disease-relevant stimulus to validate the therapeutic utility of drug targets for the treatment of abdominal pain.

3 $Na_V1.9$ as a Target for the Treatment of Pain in IBS-D

Stimulus transduction in sensory nerves occurs in response to the production of generator potential within afferent nerve terminals, which if sufficient in magnitude trigger the formation of action potentials that are relayed along the axon from the periphery to the spinal cord and subsequently the central nervous system (Blackshaw

and Gebhart 2002). Within nociceptors the process of stimulus transduction is mediated through ion channels gated by noxious stimuli such as heat, cold, acidity and mechanical stimuli, with TRPV1 and TRPA1 channels from the transient receptor potential receptor family representing important examples of such channels expressed on visceral nociceptors innervating the gut (Caterina et al. 2000; Brierley et al. 2009; Brierley et al. 2005). In addition, algogenic and inflammatory mediators such as bradykinin or ATP can also elicit generator potentials directly by promoting the closure of potassium channels responsible for determining the resting membrane potential of nociceptors such as the K_V7 subtype voltage-gated potassium channels, or by activating ligand-gated ion channels such as the $P2X_{2/3}$ purinoreceptor (Liu et al. 2010; Wynn et al. 2003; Shinoda et al. 2010). In addition, mediators may also facilitate the activity of stimulus-transducing ion channels such as TRPV1 or TRPA1 via signalling pathways downstream to the activation of their cognate receptors (Bautista et al. 2006; Moriyama et al. 2005; Chung et al. 2011) (Fig. 2).

Alongside these stimulus- or mediator-transducing events, voltage-gated ion channels with low activation thresholds (close to the resting membrane potential) such as the voltage-gated sodium channel $Na_V1.9$, expressed in nociceptors, act to further amplify generator potentials, and by doing so $Na_V1.9$ plays an important role in determining the nociceptor sensitivity (Baker et al. 2003). The contribution of $Na_V1.9$ to nociceptor firing is of particular importance during inflammation where a plethora of inflammatory mediators greatly enhance the persistent sodium current elicited by $Na_V1.9$ sufficient to trigger action potential firing (Baker 2005; Maingret et al. 2008) (Fig. 2). $Na_V1.9$ is therefore well placed to facilitate visceral nociception

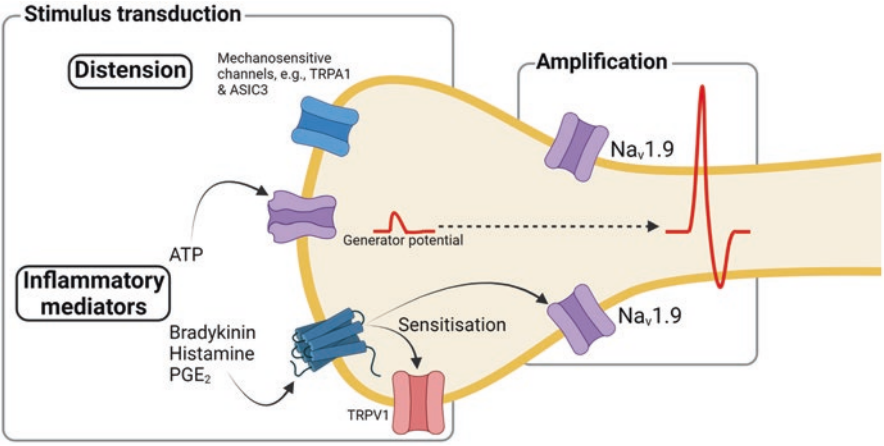

Fig. 2 Stimulus transduction at the afferent terminal leads to membrane depolarisation due to the activation of ion channels such as TRPA1 and TRPV1 gated by external stimuli for example distension or acidity. These responses can be amplified by voltage-gated ion channels such as $Na_v1.9$ which have a low activation voltage. During inflammation, released mediators enhance the activity of both stimulus-transducing and amplifying channels to cause nociceptor sensitisation. In addition, some mediators such as ATP may also directly contribute to nociceptor depolarisation through the activation of ligand-gated channels such as the $P2X_{2/3}$ purinoreceptor

in response to a wide range of noxious inflammatory mediators and stimuli as demonstrated by the abdominal pain phenotype displayed by patients that have gain-of-function mutations in $Na_V1.9$ (Woods et al. 2015; Hockley et al. 2014). Work from my group has explored this utility further and we presented data highlighting the markedly attenuation of colonic afferent response to IBS-D patient supernatants in tissue from $Na_V1.9$ −/− mice (courtesy of Prof John Wood, UCL) (Hockley et al. 2014). This data is confirming the high value of $Na_V1.9$ as a drug target for the treatment of pain in IBS.

4 Matrix Metalloproteinase 12 (MMP12) as a Mediator of Visceral Nociception in IBD Patients

In addition to supernatants from IBS patients, work from my lab performed in collaboration with Prof Nick Croft (QMUL) was presented confirming the ability of biopsy supernatants from paediatric patients with Crohn's disease (CD), ulcerative colitis (UC) and functional abdominal pain (FAP) to simulate colonic nociceptors (Tranter et al. 2017). Nociceptor responses were comparable across these three patient groups, demonstrating that the pronociceptive potential of the bowel was comparable between IBD and FAP patients in marked contrast to the inflammatory status of the bowel in these patients but consistent with the presence of pain in each patient group. Supernatant cytokine and biopsy transcript expression confirmed the presence of inflammation in tissue from IBD but not FAP patients. Further comparison of transcripts for genes whose expression was significantly elevated in IBD tissue with respective supernatant-evoked responses revealed a significant correlation with matrix metalloproteinase 12 (MMP-12) expression, suggesting a novel role for MMP-12 in colonic nociception. This was explored further, and data presented demonstrated the ability of MMP-12 to stimulate colonic nociceptors and MMP-12 inhibitors to block the activation of colonic nociceptors by supernatants generated from colitis mouse tissue. MMP-12 has previously been shown to activate protease-activated receptor 1 through cleavage of the PAR1 tethered ligand (Jacenik et al. 2021; Heuberger and Schuepbach 2019). Given PAR1 is markedly expressed in mouse and human sensory DRG neurons and has recently been shown to mediate Ca^{2+} flux in human sensory in response to PAR1 agonist activation by thrombin and biopsy supernatants from IBS patients (Desormeaux et al. 2018), we investigated the role of PAR1 in MMP-12 mediated sensory nerve stimulation. Data was presented demonstrating that MMP-12 increases intracellular Ca^{2+} in capsaicin-sensitive mouse DRG nociceptors, a response significantly attenuated by pre-treatment with the selective PAR1 antagonist SCH79797 (Fig. 3). These data collectively demonstrate how interrogation of IBD biopsy tissue and its effect on nociceptor signalling has led to the identification of a novel mediator (MMP-12) of visceral nociception and the mechanism (PAR1) through which this occurs.

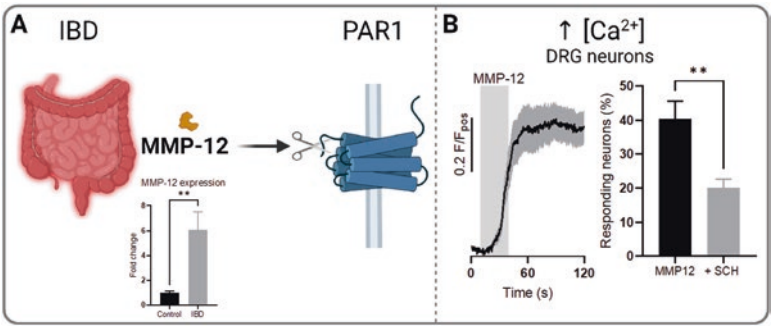

Fig. 3 (**a**) MMP-12 is elevated in biopsies from the inflamed colon (inset graph, $n = 14$ controls, $n = 17$ IBD, $p = 0.0031$), which has been suggested to cleave PAR1. (**b**) MMP-12 (100 pM) elicited an increase in intracellular Ca^{2+} (left panel, $n = 20$ exemplar responsive neurons) in 40.3 ± 5.3% of DRG neurons studied which was significantly attenuated in DRG neurons pre-treated with the PAR-1 antagonist SCH79797 (10 μM) ($p = 0.0088$; control, $n = 7$ experiments from 10 independent cultures; SCH79797, $n = 7$ experiments from 3 independent cultures)

5 Interactome Analysis of Transcriptomic Data from Sensory Neurons and IBD Biopsies

Having confirmed the utility of our patient biopsy-based approach to identify mediators and mechanisms of visceral nociception in gastrointestinal disease, the talk was concluded by a presentation of preliminary findings from our interactome analysis transcripts elevated in IBD patient biopsy samples with expression of their cognate receptors in mouse colonic DRG neurons using a publicly available database (https://hockley.shinyapps.io/ColonicRNAseq/) generated in collaboration with Dr. James Hockley and Prof Ewan St John Smith (University of Cambridge) (Hockley et al. 2019). This study identified the presence of seven populations of colonic DRG neurons based on discrete clusters of gene expression, two of whom were exclusive to LS DRG neurons and five combining TL and LS neurons. This powerful dataset allows whole transcriptome gene expression to be examined within each DRG cluster, thereby revealing previously unrecognised patterns of expression for receptors and ion channels which can then be targeted for the treatment of visceral pain, in addition to providing insight into novel mechanisms of signalling in sensory neurons based on co-expression of different receptors with specific transducer channels.

For example, comparison of transcript expression for the 5-HT_3 receptor targeted by 5-HT_3 antagonist treatments of IBS-D such as alosetron, ondansetron and ramosetron with the expression of the 5-HT_4 receptor targeted by treatments of IBS-C such as tegaserod revealed a mutually exclusive pattern of gene expression, with 5-HT_4 receptors being expressed in a population of colonic DRGs delineated by their expression of the MRGPRD receptor and the 5-HT_3 receptor being expressed across all populations of colonic DRG neurons with the notable exception of the MRGPRD positive population (Fig. 4). These findings highlight the potential

importance of targeting the MRGPRD expressing sensory neurons for the relief of pain in IBS-C patients and a need to target a broad range of colonic afferent populations to provide pain relief in IBS-D. These findings are supported by a recent study by Dr. Nicolas Cenac (Inserm Toulouse) that demonstrated a marked increase in the arachidonic acid metabolite 5-oxo-eicosatetraenoic acid (5-oxo-ETE) which selectively stimulates MRGPRD expressing DRG neurons in biopsy samples from IBS-C but not IBS-D patients (Bautzova et al. 2018).

Finally, we also presented interactome data based on the marked increase in prokineticin-2 transcripts in biopsy samples from IBD patients and expression of the prokineticin receptor in colonic DRG neurons (Hockley et al. 2019). These findings also revealed the differential expression of transcripts for prokineticin receptors within different colonic DRG populations. These differed in their expression of TRPA1 and ASIC3 ion channels which have previously been implicated in the activation of colonic nociceptors by colorectal distension (Brierley et al. 2009; Jones 3rd et al. 2005). For example, the prokineticin 1 receptor (PKR_1) is largely expressed within the MRDPRD population of colonic DRG neurons which express TRPA1 but not ASIC3 transcripts. By contrast the prokineticin 2 receptor (PKR_2) is markedly expressed within colonic DRGs that express ASIC3 but not TRPA1. This analysis predicts that prokineticin-2 may contribute to visceral nociception in IBD through two distinct colonic nociceptor populations, one of which mechanosensitivity is mediated via TRPA1 and a second of which mechanosensitivity is mediated by ASIC3 (Fig. 4). In addition, sensitisation of colorectal distension to prokineticin-2 (e.g. in IBD patients) would therefore be predicted to occur in one subset of nociceptors through the modulation of TRPA1 activity downstream to PKR_1 activation and a second subset of nociceptors via the modulation of ASIC3 activity downstream to PKR_2 activation. These populations selectively express 5-HT_4 and 5-HT_3 receptors, respectively.

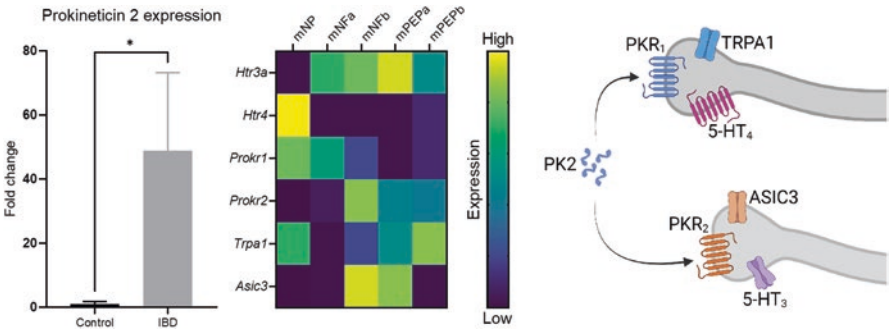

Fig. 4 (**a**) Prokineticin 2 (PK2) expression is elevated in inflammatory bowel disease ($p = 0.02$, non-inflamed control $n = 14$, IBD $n = 9$). (**b**) Expression pattern of receptors for serotonin (*Htr3a* and *Htr4*), PK2 (*Prokr1* and *Prokr2*) and mechanical stimuli (*Trpa1* and *Asic3*). (**c**) A model for the interaction of PK2 with its receptors (PKR_1 and PKR_2) highlighting the likely modulation of two distinct populations of sensory neurons by PK2

6 Summary

In this presentation we demonstrate how the use of human tissue from carefully phenotyped patients in combination with omics technology and assays of nociceptor signalling has enabled the identification of mediators (MMP-12) and mechanisms (PAR1, $Na_V1.9$) responsible for the activation of colonic nociceptors in GI disease states, validating their utility as therapeutic targets for novel analgesic therapies. This process has been accelerated with the advent of next-generation sequencing technologies that has facilitated detailed interactome analysis of putative signalling pathways in disease states as exemplified for prokineticin-2, highlighting the future opportunities for hypothesis-driven translational studies of visceral nociception in GI diseases, the findings from which should accelerate the development of visceral analgesics.

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Epithelial-Neuronal Communication in Visceral Pain

Sarah A. Najjar

Abstract Visceral hypersensitivity and pain are common symptoms of irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD). Pain results, at least in part, from sensitization of spinal afferent neurons that transmit nociceptive information from the gut to the central nervous system. Recent evidence from studies of the colon suggests that the epithelial cells surrounding these extrinsic primary afferent neurons (ExPANs) may also have a substantial role in pain signaling and visceral hypersensitivity. Anatomical studies have revealed synapse-like connections between specialized epithelial cells and surrounding nerve fibers. Optogenetic and pharmacological study experiments have demonstrated communication between colonic epithelial cells and ExPANs and have confirmed that colonic epithelial cells can initiate nociceptive responses. There are numerous possible mechanisms by which epithelial signaling contributes to visceral pain, which are outlined in this review. Understanding colonic epithelial-neuronal communication in normal and pathological conditions may lead to better treatments for visceral pain.

Keywords Sensory neurons · Colon epithelium · Gut-brain axis · Visceral hypersensitivity · Abdominal pain

1 Introduction

Visceral pain is a common, debilitating symptom of gastrointestinal disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) which, combined, affect up to 20% of the population (Abdul Rani et al. 2016). Pain is

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associated with active inflammation of the colon (Akbar et al. 2009), in which immune cell responses impact sensory signaling (Sharkey and Kroese 2001). Visceral pain is also reported in patients lacking signs of inflammation (Bielefeldt et al. 2009), suggesting that there are other factors driving persistent pain. Pathophysiological changes occurring in IBS and IBD (e.g., compromised epithelial permeability, immune signaling, and stress) may result in sensitization of spinal afferent neurons that innervate the colon, contributing to visceral pain in these conditions (Azpiroz et al. 2007; Gold and Gebhart 2010). Recent studies have suggested that epithelial regulation of these extrinsic primary afferent neurons (ExPANs) has a significant role in pain sensation. This review explores the colonic epithelial cell types and epithelial-released transmitters that may be involved in pain signaling, as well as the recent studies demonstrating how experimentally induced changes in epithelial cell activity impact visceral pain.

2 Heterogeneity of Colonic Epithelial Cells

The colonic epithelium is a simple columnar structure whose major functions are to absorb nutrients and water and to form a protective barrier. The vast majority of this epithelial layer is made up of absorptive enterocytes, and the remaining cell types have secretory functions, including goblet cells that secrete mucus, tuft cells that secrete opioids and immune mediators, and enteroendocrine cells (EECs) that secrete hormones and peptides (Gerbe and Jay 2016; Leushacke and Barker 2014). There are at least ten types of EECs, but the most prevalent in the human and mouse colon are enterochromaffin (EC) cells that release serotonin (5-HT) and L-cells that release peptide YY and glucagon-like peptides 1 and 2 (Gunawardene et al. 2011). The mouse colon also contains I-cells that release cholecystokinin (CCK) and the human colon contains a small amount of D-cells which express somatostatin (Egerod et al. 2012; Sjolund et al. 1983). The intestinal epithelium synthesizes and releases neurotransmitters such as ATP (Burnstock 2001), glutamate (Uehara et al. 2006), serotonin (Gershon 2013), and acetylcholine (Klapproth et al. 1997), all of which can act on surrounding ExPAN fibers.

3 The Role of Enteroendocrine Cells in Visceral Pain

Although EECs make up a small proportion (1%) of the colonic epithelium, they are most likely to participate in sensory signaling. They express sensory receptors involved in mechanosensation and nociception, such as Piezo2 and transient receptor potential ankyrin 1 (TRPA1) (Doihara et al. 2009; Nozawa et al. 2009; Wang et al. 2017). EECs are electrically excitable, and several studies have shown that they can form synaptic connections with neurons. Using EEC-specific fluorescent

reporter mouse lines, researchers identified axon-like processes on the basal end of EECs, named neuropods (Bohorquez and Liddle 2011; Bohorquez et al. 2014). Multiple studies have identified pre- and post-synaptic proteins within EECs using molecular profiling (Bellono et al. 2017; Bohorquez et al. 2015; Treichel et al. 2022). In studies where rabies virus was targeted to EECs in the colon, synaptic connectivity was established with surrounding neurons (Bohorquez et al. 2015), including ExPANs with cell bodies in lumbar dorsal root ganglia (Kaelberer et al. 2018). Enterochromaffin (EC) cells, which produce over 90% of the body's 5-HT, are an EEC of particular interest because they make up the vast majority of Piezo2+ mechanosensitive cells in the colon (Alcaino et al. 2018; Treichel et al. 2022). EC cells also have high expression of TRPA1, a chemical irritant receptor shown to have a role in visceral pain (Mitrovic et al. 2010; Nozawa et al. 2009; Yang et al. 2008). EC cells may communicate with ExPANs via paracrine 5-HT release or direct synaptic connectivity (Bellono et al. 2017).

4 Chemical Mediators of Colonic Epithelial-Neuronal Communication

4.1 Adenosine Triphosphate (ATP)

Earlier studies have shown that the colonic epithelium likely contributes to mechanosensory transduction via release of ATP (Burnstock 2001). Colonic distension evokes ATP release from the epithelium, which then acts on purinergic receptors on ExPAN fibers (Wynn et al. 2003). Vesicular nucleotide transporter (VNUT) is ubiquitously expressed in the colonic epithelium, suggesting all epithelial cell types are capable of ATP release (Mihara et al. 2018). Additionally, ATP can be released from EECs in combination with other neurotransmitters or hormones (Winkler and Westhead 1980). The mechanosensory transient receptor potential vanilloid-type 4 (TRPV4), present throughout the colonic epithelium, mediates the distension-evoked release of ATP (Cenac et al. 2008; D'Aldebert et al. 2011; Mihara et al. 2018).

ExPAN fibers express several types of purinergic receptors including the ionotropic $P2X_3$ and $P2X_{2/3}$ receptors (Shinoda et al. 2009; Wynn et al. 2003) and the metabotropic $P2Y_1$ and $P2Y_2$ receptors (Hockley et al. 2016). ATP has also been shown to activate and sensitize transient receptor potential vanilloid-type 1 (TRPV1) channels on ExPANs (Lakshmi and Joshi 2005), which are heavily involved in the initiation and maintenance of visceral pain (Defaye et al. 2021; Lapointe et al. 2015). Rodent models of IBS and IBD have shown that ATP signaling has a significant role in visceral hypersensitivity. Inflammation of the colon results in increased levels of ATP in the colon lumen, likely released from the epithelium, and an upregulation of ExPAN $P2X_3$ receptors, correlated with increased sensitivity to ATP application (Wynn et al. 2004). In a model of post-infectious IBS, $P2X_3$ knockout mice failed to develop colonic hypersensitivity (Shinoda et al. 2009). In human

studies, researchers have found that $P2X_3$ protein level is higher in colon biopsies from IBD patients compared to healthy controls (Yiangou et al. 2001). Together, these data suggest that inflammation-induced increases in ATP release from epithelial cells and ExPAN purinergic receptor activity may contribute to visceral hypersensitivity.

4.2 Serotonin (5-HT)

Over 95% of the body's 5-HT is synthesized in the gut, mostly by EC cells of the intestinal epithelium (Gershon 2013). EC cells form close contacts with surrounding nerve fibers and the 5-HT release machinery is located at the basal surface, suggesting that EC cells can communicate directly with ExPANs that express 5-HT receptors (Bellono et al. 2017; Mawe and Hoffman 2013). Additionally, nearly all gut epithelial cells express the serotonin reuptake transporter (SERT) and therefore have a major role in modulating mucosal 5-HT levels (Wade et al. 1996).

The ionotropic $5\text{-}HT_3$ receptor ($5\text{-}HT_3R$) is widely expressed on ExPANs that innervate the colon and is involved in visceral pain signaling (Hockley et al. 2019). Studies have shown that experimental colitis increases $5\text{-}HT_3R$ expression in the mucosa of the mouse colon (Matsumoto et al. 2012) and that administration of alosetron, a $5\text{-}HT_3R$ antagonist, reduces visceral sensitivity (Kozlowski et al. 2000). Alosetron has been widely used for the treatment of diarrhea-predominant IBS and clinical trials have confirmed its pain-relieving effects (Fayyaz and Lackner 2008).

Studies have revealed abnormalities in mucosal 5-HT in IBD and IBS patients. One study of inflamed colon biopsies from IBS patients showed decreased 5-HT levels, decreased SERT expression, and fewer EC cells per crypt in the mucosa, compared to healthy controls (Coates et al. 2004). This contrasts with mouse studies, in which researchers have found that experimental colitis results in a greater amount of EC cells and increased mucosal 5-HT levels (Bertrand et al. 2010; Linden et al. 2005). In studies of IBS patients, both diarrhea- and constipation-predominant patients displayed decreased mucosal SERT expression (Coates et al. 2004). Studies of post-infectious IBS patients showed increased EC cell density in colon biopsies compared to healthy controls (Lee et al. 2008). In a study of IBS patients of all phenotypes, there was no correlation between EC count and visceral hypersensitivity (Kerckhoffs et al. 2012). Together these data show that mucosal 5-HT is altered in IBD and IBS but it is unclear whether the visceral pain associated with these conditions is mediated by the EC cells themselves or the 5-HT receptors on colonic ExPANs.

4.3 Proteases

Proteases released from colonic epithelial cells, such as trypsin-3, likely have a role in visceral hypersensitivity. The proteases present in supernatants collected from IBS patient colon tissue have been shown to increase the excitability of ExPAN neurons in culture (Valdez-Morales et al. 2013). These supernatants can also induce visceral hypersensitivity in mice, an effect that is blocked by protease-activated receptor-2 (PAR_2) antagonists (Cenac et al. 2007). Studies have confirmed that activation of PAR_2 receptors can sensitize colonic ExPANs and induce visceral hyperalgesia (Coelho et al. 2002; Sipe et al. 2008). Trypsin-3 is increased in the epithelium of colon biopsies of human IBS patients and rat IBS models and has been shown to induce visceral hypersensitivity in a PAR_2-dependent manner (Rolland-Fourcade et al. 2017). Efforts to identify other proteases active in IBS and IBD patients are ongoing; functional proteomic assays of IBD patient colon samples have shown increased activity of trypsin, cathepsin G, and thrombin (Denadai-Souza et al. 2018). As in mice, PAR_2 is widely expressed in human ExPAN neurons, but studies show that human neuronal responses to supernatants from IBS patients are mediated by PAR_1 (Desormeaux et al. 2018).

4.4 Cyclic Guanosine-3′,5′-Monophosphate (cGMP)

In contrast to the epithelial-secreted mediators described above, the molecule cGMP enables inhibition of neuronal activity. The activation of the guanylate cyclase C (GC-C) receptor on the luminal aspect of epithelial cells induces the release of cGMP (Hannig et al. 2014). Linaclotide is a GC-C agonist that is commonly prescribed for constipation-predominant IBS and provides relief of both abdominal pain and constipation (Brierley et al. 2022). Linaclotide stimulates the synthesis and release of cGMP from epithelial cells which facilitates fluid production in the intestinal lumen and also inhibits colonic ExPAN activity (Busby et al. 2010). Studies have shown that cGMP inhibits ExPAN firing via action on a membrane receptor target, but little is known about the identity of this target (Grundy et al. 2018). The analgesic effects of cGMP are robust in rodent models of inflammation (Eutamene et al. 2010; Grundy et al. 2018). Although the inhibitory effects of cGMP are not yet fully understood, linaclotide has proven to be effective in patients with chronic constipation (Fukudo et al. 2018; Lembo et al. 2010) and the molecular targets of cGMP continue to be investigated.

5 Optogenetic Investigation of Colonic Epithelial-Neuronal Communication

Although the transmitters and sensory receptor profiles of colonic epithelial cells have been well-studied (Najjar et al. 2020), it has been difficult to distinguish their contribution to sensory signaling because of the close physical association between epithelial cells and ExPANs. To overcome this limitation, researchers have implemented optogenetic techniques to selectively activate or inhibit colonic epithelial cells. Cre/Lox recombination technology has been used to drive expression of the excitatory opsin channelrhodopsin (ChR2) and the inhibitory opsin archaerhodopsin (Arch) in the colonic epithelium. To achieve this, mice containing either the ChR2-YFP or Arch-EGFP protein were crossed with mice expressing Cre recombinase under control of the villin gene, which is specific to the intestinal epithelium (Najjar and Albers 2021).

To determine the effects of specific activation of the colonic epithelium, ExPAN activity was measured in an ex vivo colon-nerve preparation from mice expressing ChR2 in colonic epithelial cells (Villin-ChR2 mice). Individual ExPAN fibers were recorded extracellularly and blue light (473 nm) was shone on the mucosa of the receptive fields. Blue light stimulation of the colonic epithelium initiated high-frequency trains of action potentials in nearly half of ExPAN fibers, and these firing patterns were similar to those evoked by naturalistic mechanical stimuli (Makadia et al. 2018). The epithelial-evoked action potential firing was diminished after application of purinergic receptor (P2X and P2Y) antagonists, supporting the idea that ATP and UTP mediate epithelial-neuronal signaling in the colon.

The effect of optogenetic activation of the colonic epithelium was also assessed in vivo by measurement of visceromotor responses, which are a surrogate for pain-like behavior. When blue light stimulation was applied to the colon lumen of villin-ChR2 mice, they displayed nociceptive responses. Although the visceromotor responses to blue light stimulation had a longer latency, they closely mimicked responses to balloon distension (stretching) of the colon (Makadia et al. 2018). In all, these data indicate that optogenetic activation of colonic epithelial cells alone is sufficient to evoke action potential firing in ExPANs and engage the neural circuitry involved in visceromotor responses.

Studies of ChR2-mediated epithelial activation showed evidence that colonic epithelial cells are drivers of visceral sensation and pain, suggesting that inhibition of these cells may diminish visceral hypersensitivity. Using mice expressing the Arch inhibitory opsin specifically in the intestinal epithelium (Villin-Arch), visceromotor responses to colonic distension were measured while applying yellow laser (589 nm) to the colon lumen. Yellow light-mediated inhibition of colonic epithelial cells reduced visceromotor responses to colonic distension by 76%, comparable to light-mediated inhibition of the ExPANs themselves (in TRPV1-Arch mice), which diminished visceromotor responses by 77% (Najjar et al. 2021). Inflammation was induced in these mice via dextran sulfate sodium treatment, resulting in hypersensitivity to colonic distension. Yellow light-mediated inhibition of the epithelium

reduced visceromotor responses by 55% in inflamed mice, thus relieving their hypersensitivity. These optogenetic studies confirm that the colonic epithelium has a substantial role in visceral pain signaling.

6 Conclusions and Future Directions

As recent optogenetic studies have confirmed (Makadia et al. 2018; Najjar et al. 2021), colonic epithelial cells have a major role in mediating ExPAN activity and thus contribute to visceral pain. The main neurotransmitters involved in colonic epithelial-neuronal communication include ATP and 5-HT, but epithelial-released proteases and molecules such as cGMP also have an impact on ExPAN signaling. Receptors for chemical and mechanical stimuli are found throughout the colonic epithelium, indicating that they have a role in monitoring the gut lumen environment and initiating sensory signaling. Neurotransmitter release from epithelial cells may be diffuse and have slow or indirect actions on ExPAN nerve terminals; however, communication between EECs and ExPANs can occur through direct synaptic transmission.

The studies reviewed here support the hypothesis that a combination of all epithelial cell types is necessary for normal sensation in the colon. However, two sensory receptors linked to pain, Piezo2 and TRPA1, are mostly present in 5-HT producing EC cells in the colon, indicating that this cell type may be particularly important in visceral pain signaling. More cell type-specific manipulations (e.g., via optogenetics or chemogenetics) are necessary to determine the contribution of EC cells, as well as other epithelial cell types, to sensory signaling. Molecular profiling studies are required to identify additional sensory receptors expressed in each colonic epithelial cell type, as in the single-cell RNAseq analysis of small intestinal epithelium (Haber et al. 2017). Additional epithelial-released molecules, such as neuropeptides, should also be investigated for their impact on ExPAN signaling. Functional analysis of colonic ExPAN receptors is also needed to determine which are critical in epithelial-neuronal signaling.

To gain insight on visceral pain associated with IBS and IBD, colonic epithelial-neuronal communication should be investigated in disease models. It is well-known that ExPANs can become hypersensitive in response to inflammatory insult (Gold and Gebhart 2010), but future studies using experimental colitis should examine the activity of colonic epithelial cells. Electrically excitable EECs, for example, may be subject to inflammation-induced changes in voltage-sensitive ion channels. Several types of animal models could be used to study gastrointestinal disorders of different etiology (e.g., dextran sulfate sodium to mimic IBD, early life stress to mimic IBS, and parasite infection to mimic post-infectious IBS). Future studies will reveal how epithelial-neuronal communication in the colon becomes altered in pathological conditions, which will inform how to target specific epithelial cell types and epithelial-released molecules for treatment of pain conditions (Fig. 1).

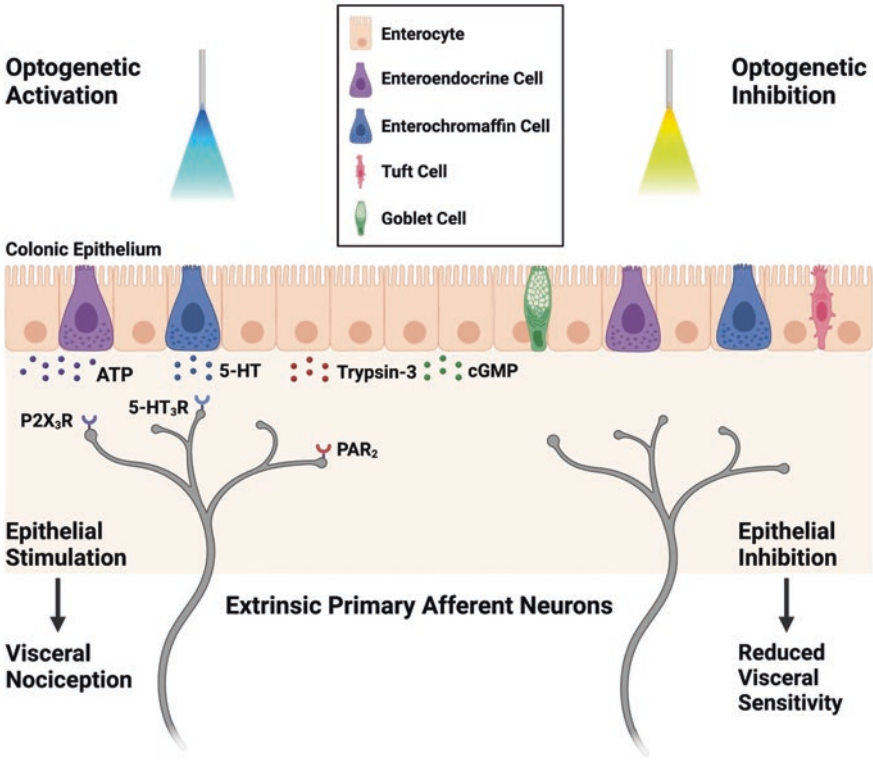

Fig. 1 Colonic epithelial regulation of visceral pain signaling. The colonic epithelium is comprised of a heterogeneous cell population including enterocytes, enteroendocrine cells (including enterochromaffin cells), tuft cells, and goblet cells. Epithelial cells release several neurotransmitters that are thought to be involved in regulating the activity of extrinsic primary afferent neurons (ExPANs), which convey nociceptive information to the central nervous system. The main transmitters involved are adenosine triphosphate (ATP), released from both enterocytes and enteroendocrine cells, serotonin (5-HT) released from enterochromaffin cells, and proteases such as trypsin-3, which are released from enterocytes. Receptors for these transmitters are present on ExPANs innervating the colon. ExPAN receptors involved in pain signaling include $P2X_3$ (receptor for ATP), 5-HT_3 (receptor for 5-HT), and PAR_2 (receptor for proteases such as trypsin-3). The colonic epithelium also releases cGMP, which has an inhibitory effect on ExPANs via an unknown membrane receptor target. Optogenetic studies show that activation of the epithelium initiates ExPAN activity and visceral nociception, likely through the outlined mechanisms. Conversely, optogenetic inhibition of the epithelium reduces sensitivity to colorectal distension, indicating that the colonic epithelium has a major role in pain signaling

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Physiological Mechanisms Underpinning Heightened Perception of Visceral Afferent Signalling in Irritable Bowel Syndrome

Dervla O'Malley

Abstract Interoceptive information relating to intestinal activity and environmental changes in the gut lumen is continuously and, generally, subconsciously being relayed to the central nervous system (CNS). Vagal and spinal afferents are likely to act as the primary interrogators of peripheral intestinal signals, and activation of intestinal nociceptors is associated with the central interpretation of visceral pain. For most of us, gut-to-brain communication is not consciously perceived; however, some are acutely aware of these signals. Individuals with irritable bowel syndrome (IBS), a prevalent and heterogeneous functional bowel disorder, exhibit abnormal sensory perception of signals originating in the gut, which presents as abdominal pain. Although central activity is likely to contribute to visceral hypersensitivity, peripheral factors, including immune, paracrine and endocrine molecules, are also likely to play a role in the sensitisation of gut-to-brain signalling in IBS. We have reviewed the evidence of the contributory mechanisms in IBS-associated visceral hypersensitivity and found that stress, sex and enteroendocrine hormones, in addition to immune molecules, are important contributory factors in intestinal afferent sensitisation. Comorbid psychological disorders also appear to influence the likelihood of individuals with IBS exhibiting symptoms consistent with visceral pain hypersensitivity.

Keywords Visceral hypersensitivity · Vagal afferents · Functional bowel disorder · Cytokines · Enteroendocrine

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1 Context

1.1 Heightened Interoceptive Awareness

A continuous flow of interoceptive information subconsciously advises the central nervous system (CNS) of intestinal activity and environmental changes in the gut lumen (Mayer 2011). Functional regulation of the gut is mediated by extrinsic and intrinsic nerves and also by paracrine, endocrine and immune factors (Stasi et al. 2012). Stimulation of visceral nociceptors is associated with activation of several regions of the cerebral cortex (Derbyshire 2003), including the perigenual anterior cingulate cortex (Vogt 2005), which differentiates it from brain regions activated in response to somatic pain. Vagal afferents are likely to act as the primary interrogators of peripheral intestinal signals, with more than 80% of vagal fibres, which terminate in the nucleus solitary tract, identified as being sensory (Mayer et al. 2006). However, both branches of the autonomic nervous system, which are anatomically and functionally integrated within the bidirectional gut-brain communication axis, contribute to neural regulation of intestinal function (Aggarwal et al. 1994). Indeed, spinal sympathetic nociceptive afferents mainly signal to the sensory cortex and pain matrix (Fukudo 2013). For most of us, gut-to-brain communication is not consciously perceived; however, some individuals are acutely aware of these signals, which are interpreted as visceral pain. Irritable bowel syndrome (IBS) is a common functional bowel disorder where abdominal pain represents one of the most debilitating symptoms (Tillisch et al. 2011).

1.2 Functional Bowel Disorders

IBS is a heterogeneous disorder (Lovell and Ford 2012; Oka et al. 2020) characterised by episodic flares of abdominal pain, bloating and altered bowel habit (Enck et al. 2016). Subtypes include diarrhoea- (IBS-D) and constipation-predominant (IBS-C) IBS and also mixed phenotypes (IBS-M), which is the most prevalent subtype, when individuals were diagnosed using Rome III criteria (Oka et al. 2020). The biopsychosocial model of IBS, which incorporates genetic, immunological, psychological, neuroendocrine, dietary and environmental factors, in addition to dysfunctional gut-brain signalling (Holtmann et al. 2016), denotes the complexity and heterogeneity of this disorder. Manifestation of IBS symptoms has been linked to autonomic dysfunction (Adeyemi et al. 1999; Spaziani et al. 2008; Spetalen et al. 2008; van Orshoven et al. 2006), with a suggestion that the balance between sympathetic and parasympathetic gut regulation is irregular in individuals with IBS (Pellissier et al. 2010), with increased sympathetic activity and decreased parasympathetic activity (Adeyemi et al. 1999). Females with IBS-C who report severe abdominal pain exhibited decreased vagal regulation (Cain et al. 2007). In contrast, adrenergic sympathetic neuronal activity was associated with IBS-D (Aggarwal et al. 1994). Modulatory mechanisms, which sensitise primary afferent pathways,

increase activity of endogenous pain facilitation or suppress endogenous pain inhibition (Mayer and Tillisch 2011) and may be further influenced by environmental stressors and mood. Indeed, psychological disorders are highly comorbid in individuals with IBS, with a three-fold increase in the likelihood of having either anxiety or depression in addition to IBS (Zamani et al. 2019). IBS patients exhibit anxiety, interpersonal sensitivity, depression, hostility and somatisation of effect (Whitehead et al. 1980), emotions which may influence pain perception and in particular visceral hypersensitivity (Fig. 1) and the exaggerated sensory interpretation of a stimulus such as non-noxious distension of the colorectal region (Whitehead et al. 2002).

1.3 Central Pain Amplification

The subjective experience of pain deviates from the objective intensity of the noxious stimulus and is influenced by the expectation of the severity and controllability of the painful stimulus in addition to the individual's current mood and psychological wellbeing (Wiech and Tracey 2009). Individuals with IBS report lower pain

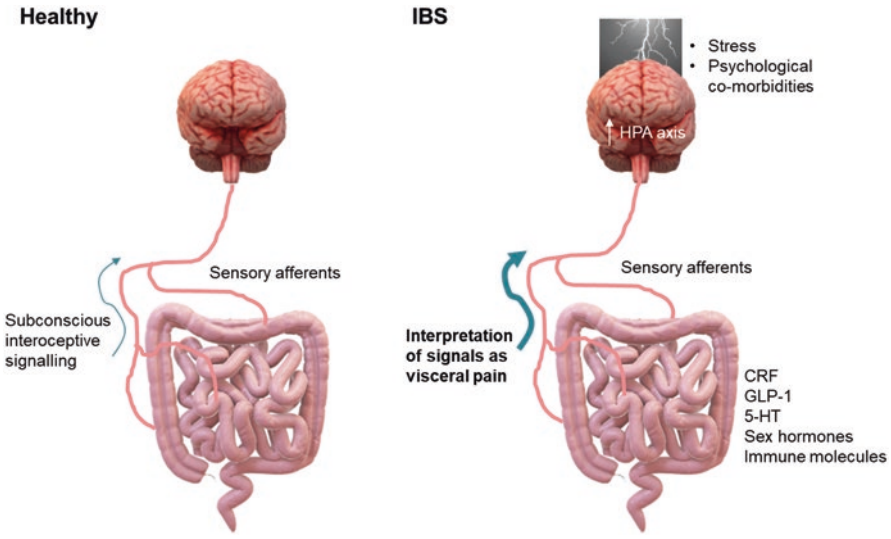

Fig. 1 Central and peripheral factors important in visceral hypersensitivity in irritable bowel syndrome. The image illustrates some of the contributory factors that may underpin visceral hypersensitivity, a prevalent and debilitating symptom of the functional bowel disorder, irritable bowel syndrome (IBS). The central interpretation of interoceptive intestinal signals is altered in individuals with IBS, who exhibit chronic activation of the hypothalamic-pituitary-adrenal (HPA) stress axis and alterations in brain regions associated with pain. Peripherally, paracrine factors such as serotonin (5-HT) and glucagon-like peptide 1 (GLP-1), in addition to stress hormones, such as corticotropin-releasing factor (CRF), sex hormones and a host of immune molecules, contribute to altered gut-to-brain sensory signalling

thresholds and increased sensitivity to rectal distension, accompanied by enhanced activity of the CNS pain matrix, which includes the thalamus, insula and anterior cingulate cortex (Price et al. 2009; Chen et al. 2011; Mertz et al. 2000). Centrally, altered neural processing of visceral stimuli was detected in individuals with IBS, observations that were further influenced by emotional factors (Elsenbruch 2011). Reduced volume in brain regions associated with perception of the internal state, such as the anterior mid-cingulate and insular cortices, was associated with an IBS diagnosis (Blankstein et al. 2010), as was reduced grey matter in the medial and ventrolateral prefrontal cortex, thalamus and periaqueductal grey (Seminowicz et al. 2010). These region-specific changes may impinge on the appropriate functionality of the descending pain inhibition system (Hall et al. 2010) and thereby contribute to visceral hypersensitivity. Indeed, reduction in grey matter in the ventrolateral prefrontal cortex was only evident in individuals with high levels of IBS-related pain. In contrast, brain regions which process the affective component of pain, such as the pregenual anterior cingulate cortex and the orbital frontal cortex, had increased grey matter volume in IBS patients, a finding that was dependent upon comorbid anxiety and depression (Seminowicz et al. 2010). The regions involved reflect the important emotional input to the perception of visceral pain. Sensitivity to colon and rectal distension was increased in individuals with IBS-D (Prior et al. 1990; Simren et al. 2001), while IBS-C subtypes showed conflicting results (Harraf et al. 1998; Slater et al. 1997). However, no significant difference in pain threshold was noted between IBS subtypes (Steens et al. 2002). Interestingly, females with IBS exhibit enhanced engagement of the emotional arousal system during expectation of visceral pain (Labus et al. 2008), highlighting the contributory nature of the individual's emotional status to the perception of pain. Moreover, the female predominance in IBS manifestation may be in part due to gender-related differences in the activation of circuits involved in stress and arousal (Mayer and Tillisch 2011).

1.4 Activation of the Stress Axis

In addition to directly modulating gut activity, vagal activation influences the hypothalamic-pituitary-adrenal (HPA) stress axis (Pavlov et al. 2003), a signalling pathway that is reported to be chronically activated in individuals with IBS (Fond et al. 2014). A maladaptive stress response, involving biological, psychological and social factors, contributes to the initiation, exacerbation and persistence of symptom flares (Dinan et al. 2006; Stengel and Tache 2009; O'Malley 2015; O'Malley et al. 2011b). However, studies investigating acute psychological stressors, such as public speaking, show mixed results in IBS patients (Schaper and Stengel 2022). Some studies detected elevated cortisol in individuals with IBS (Heitkemper et al. 2012), but others did not detect any divergence from the healthy control group (Braak et al. 2012; Fournier et al. 2018). That said, interpretation of study findings may be

confounded by the high comorbidity of psychiatric disorders in this patient group. Indeed, anticipatory behaviours and evidence of increased vigilance to visceral sensations in individuals with IBS appear to be exacerbated by emotional stress (Fukudo et al. 1993). Brain activity was increased in regions associated with fear learning in response to rectal distension in individuals with IBS (Claassen et al. 2017; Icenhour et al. 2015). Thus, the stress response is altered in individuals with IBS, but it is difficult to discern if this is part of the pathophysiology of the disorder or due to the high comorbidity of psychological dysfunction in this group (Zamani et al. 2019).

2 Sensitisation of Primary Afferents by Peripheral Factors

2.1 Corticotropin-Releasing Factor (CRF)

CRF is secreted in response to perceived stressors and activates the HPA axis. This hormone evokes its biological effects through activation of CRF1 and CRF2 receptors, which are expressed centrally in the hypothalamus and other brain regions. Exposure of the central nucleus of the amygdala, which is important in the integration of emotional and sensory information, to CRF, results in sensitisation of visceral nociception, an effect that is inhibited using a CRF1 receptor antagonist (Su et al. 2015). Despite the importance of central modulation of gut function in the manifestation of IBS symptoms, changes in peripheral signalling also contribute to bowel dysfunction (Larauche 2012). Indeed, rectally applied analgesics suppress visceral pain hypersensitivity (Price et al. 2009).

The initiation point of perceived visceral pain is evoked by stimulation of peripheral sensory afferents (Feng et al. 2012), which are sensitive to a variety of peripheral factors. Receptors for CRF are expressed on human intestinal mucosa (Saruta et al. 2004), enterochromaffin cells (Kawahito et al. 1994) and immune cells (Baker et al. 2003). Rodent studies have also detected CRF receptors on enteric neurons (Liu et al. 2010), where they are ideally placed to mediate the effects of stress on gastric emptying, transit and gut motility (Tache and Million 2015). Activation of peripheral CRF1 receptors also mediates stress-induced defecation and visceral hypersensitivity (Buckley et al. 2014; Greenwood-Van Meerveld et al. 2005; Million et al. 2013). Moreover, vagal afferent signalling is critical to stress-induced activation of the HPA axis and is involved in the modulation of colonic motility in response to peripherally applied CRF (Tsukamoto et al. 2006). The efficacy of CRF1 receptor antagonists as potential therapeutics in animal models of bowel dysfunction has been encouraging (Buckley et al. 2014; Greenwood-Van Meerveld et al. 2005; Million et al. 2013); however, clinical studies using CRFR1 antagonists have thus far been disappointing in the context of restoring normal gut homeostasis (Hubbard et al. 2011; Labus et al. 2013; Sweetser et al. 2009).

2.2 Glucagon-like Peptide-1 (GLP-1)

An enteroendocrine hormone with a possible role in the pathophysiology of IBS is GLP-1, an incretin factor found to have anti-spasmodic and pain-relieving benefits in individuals with IBS, particularly females with IBS-C or IBS-M (Mosinska et al. 2016; Hellstrom et al. 2009; Li et al. 2017; Touny et al. 2022). Individuals with IBS-C have decreased circulating GLP-1 and reduced mucosal expression of GLP-1 receptors (Li et al. 2017). Circulating GLP-1 was also decreased in a rat model of visceral hypersensitivity (Yang et al. 2014). GLP-1 is secreted basolaterally from L-cells (Bohorquez et al. 2015), found embedded in the gut epithelium throughout the small and large intestine (Hansen et al. 2013). They are electrically excitable and may be stimulated by a variety of intrinsic and extrinsic stimuli in a region-specific manner (Chimerel et al. 2014). Both paracrine and endocrine targets for this signalling factor have been detected, with GLP-1 receptor-expressing neurons present in the enteric nervous system, nucleus tractus solitarius and the ventrolateral medulla (Lim et al. 2009). GLP-1 receptors are also found in the area postrema and hypothalamus (Richards et al. 2014) and other areas associated with mood regulation such as the amygdala and the hippocampus (Merchenthaler et al. 1999). However, the short half-life of circulating GLP-1 may imply that local activation of neural afferents is the most efficient method of gut-brain signalling. Indeed, exposure of vagal afferents and dorsal root ganglia to GLP-1 resulted in increased afferent firing rates (McKee and Quigley 1993). Furthermore, L-cells can form synaptic connections with extrinsic afferent fibres, providing a direct neural pathway to the CNS (Kaelberer et al. 2018). Thus, GLP-1 released from L-cells may be important signalling molecules in mediating gut-brain nociceptive pain signals. Further complexity lies in the reported stimulatory effect of GLP-1 on CRF neurons (Nakade et al. 2007a). Moreover, participants with diabetes administered a GLP-1 analogue reported decreased anxiety and depression, which was distinct from those receiving insulin (Grant et al. 2011), thus revealing interactions between this incretin hormone and the HPA stress axis. This further underscores the complex physiological relationships involved in gut-brain communication.

2.3 Serotonin

Serotonin (5-HT)-secreting enterochromaffin cells can also act as chemosensory transducers signalling environmental, metabolic and homeostatic changes from the gut lumen to the nervous system. This is observed when chemical irritants, volatile fatty acid fermentation products and catecholamines stimulate 5-HT biosynthesis (Yano et al. 2015). Serotonin acts as a neurotransmitter and paracrine molecule that has, when released in excess, been linked to visceral hyperalgesia in IBS (Barbara et al. 2011; Cremon et al. 2011). Meta-analysis of several studies detected increased 5-HT in blood samples from individuals with IBS (Luo et al. 2021), which may

contribute to the pathogenesis of the disorder or could be linked with altered microbial signatures in IBS (Clarke et al. 2013). In rodent models, 5-HT activates vagal afferents, particularly in the small intestine (Hillsley and Grundy 1998; Kreis et al. 2002), and has a stimulatory effect on spinal afferents in the colon (Hicks et al. 2002). Like L-cells, enterochromaffin cells directly synapse with extrinsic sensory neurons (Bellono et al. 2017), providing a direct neural pathway to the brain (Kaelberer et al. 2018). Activation of neurons within the spinal cord was inhibited by a 5-HT_3 receptor antagonist (Kozlowski et al. 2000), a finding that fits with the clinical efficacy of 5-HT_3 receptor antagonists in supressing IBS symptoms, including abdominal pain (Qi et al. 2018).

2.4 Menstrual Cycle Hormones

A systematic review and meta-analysis of published data suggests that females with IBS are more likely to report abdominal pain and constipation-related symptoms than their male counterparts; however, the prevalence of IBS in females, although present, is relatively modest. Nonetheless, symptom presentation in females with IBS was exacerbated during menses (Adeyemo et al. 2010), a conclusion that is consistent with reports that rectal sensitivity to distension was increased during menses in women with IBS but not in controls (Houghton et al. 2002). At menses progesterone and oestrogen levels fall from high to low, and this may be important in this cyclical symptom exacerbation.

3 Inflammatory Factors

Nociceptors may be activated by physical distension of the intestinal tract, but they may also be activated by inflammatory mediators. Immune activation is characteristic of IBS pathophysiology (O'Malley 2015; Feng et al. 2012; Ishihara et al. 2013), including mucosal infiltration of immune cells in IBS biopsies (Chadwick et al. 2002; Goral et al. 2010) and detection of mast cells, which release a range of inflammatory mediators, in closer proximity to colonic nerve endings in individuals with IBS, a finding which correlated with sensitivity to visceral pain (Barbara et al. 2004). Prior experience of bacterial or viral gastroenteritis is a strong predictor of developing post-infective IBS (Spiller and Garsed 2009; Halvorson et al. 2006) and is associated with prolonged visceral hypersensitivity which may be related to neuroplastic remodelling (Vergnolle 2008) and modified response properties in primary afferents (Mayer and Tillisch 2011; Moore et al. 2002). Indeed, the density of TRPV-1-expressing nerve fibres was increased in IBS samples of colonic mucosa and this was correlated with reported abdominal pain (Akbar et al. 2008). Altered immune signatures have also been linked to functional bowel disorders, with elevated levels of pro-inflammatory cytokines detected in plasma from individuals

with IBS (Dinan et al. 2006, 2008; Liebregts et al. 2007). Circulating immune factors in IBS plasma similarly exhibited neurostimulatory properties in rat submucosal (O'Malley et al. 2015) and myenteric (Buckley et al. 2014) neurons. The neurostimulatory actions of some of these cytokines, including interleukin (IL)-6 (O'Malley et al. 2011a), IL-1β (Xia et al. 1999) and tumour necrosis factor α (Rehn et al. 2004), result in modified regulation of intestinal motility (Zhang et al. 2013) and absorption-secretory function (O'Malley et al. 2011a; Natale et al. 2003). Pro-inflammatory cytokines also activate sensory vagal fibres with cytokine-selective signals (Steinberg et al. 2016). Furthermore, the secretory products from IBS but not healthy biopsies had neurostimulatory actions on enteric neurons, and this was found to be dependent on mast cell-derived inflammatory mediators (Buhner et al. 2009).

Stimulation of the HPA axis generally promotes an anti-inflammatory response (Saper et al. 2012) but activation of CRF receptors has been linked to decreased colonic barrier integrity and subsequent inflammation (Larauche et al. 2009). Moreover, stress induces degranulation of mast cells, resulting in the release of pro-inflammatory mediators (Nakade et al. 2007b; Konturek et al. 2008). In IBS the HPA stress axis is chronically activated (Dinan et al. 2006) and immune activation is also evident (O'Mahony et al. 2005). In response to an acute psychological stressor, individuals with IBS displayed greater state anxiety than controls. However, despite elevation of circulating leukocytes and lymphocytes, no differences were detected between individuals with and without IBS (Elsenbruch et al. 2006). Increased epithelial permeability and an associated immune response may contribute to altered pain sensitivity (Zhou et al. 2009). Indeed, in an animal model of IBS, inhibition of cytokine signalling was beneficial in alleviating visceral pain (Buckley et al. 2014). While immune dysregulation may contribute to visceral pain sensitivity in IBS, further human studies are needed to confirm this connection.

4 Summary and Conclusions

Abdominal pain and visceral hypersensitivity are prevalent in individuals with IBS. Comorbidity with psychological disorders and changes in central processing of peripheral signals are likely to be exacerbating influences in the abnormal sensory perception of gut function experienced by individuals with IBS. However, local endocrine, paracrine and immune factors are also recognised as contributory features in the pathophysiology of this disorder and understanding the relationships between these factors may be critical in developing effective therapeutic strategies for this common and debilitating bowel disorder.

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A Fentanyl Analogue That Activates u-Opioid Receptors in Acidified Tissues Inhibits Colitis Pain without Opioid Side Effects

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Abstract Opioid agonists are effective analgesics for treating visceral pain but their use is limited by their on-target side effects, such as constipation, respiratory depression and sedation. The development of a fentanyl analogue, (±)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide (NFEPP), which preferentially activates μ opioid receptors (MOPr) in acidified microenvironments of diseased tissues, represents a novel alternative for inhibiting pain in inflamed tissues. Here we review the pharmacokinetic properties of this compound, study the somatic inflammatory and cancer pain models and describe in more detail a series of experiments in a preclinical model of inflammatory bowel disease (IBD) that assess its analgesic efficacy and potential to mitigate on-target side effects in uninflamed tissues. Using a combination of patch-clamp recordings, ex vivo colonic afferent nerve recordings and in vivo visceromotor reflex recordings, as well as monitoring of cardiorespiratory function, colonic motility and locomotion in the DSS colitis mouse model, we showed that NFEPP has similar analgesic efficacy compared to the conventional opioid fentanyl but lacked the common side effects observed with fentanyl. Taken together, these studies show that NFEPP has considerable promise as a highly effective analgesic agent to treat pain in inflamed visceral

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tissues without opioid side effects, similar to its profile in inflammatory somatic and cancer pain.

Keywords NFEPP · pH-sensitive opioid agonists · Inflammation pain related · Acidified tissue · Inflammation selective agonists · Opioids · Fentanyl · Opioid side effects

Abbreviations

BRET	Bioluminescence-resonance energy transfer
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CRD	Colorectal distension
DRG	Dorsal root ganglia
DSS	Dextran sodium sulphate
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
FRET	Fluorescence-resonance energy transfer
GI	Gastrointestinal
GPCR	G-protein-coupled receptors
IBD	Inflammatory bowel disease
MOPr, μ; DOPr, *∂*; KOPr, κ; NOPr	Nociceptin/orphanin receptors
MPO	Myeloperoxidase
NEFPP	(±)-*N*-(3-fluoro-1-phenethylpiperidine-4-yl)-*N*-phenyl propionamide
NF-H	Heavy neurofilament protein
NK1R	Neurokinin receptor 1
OPr	Opioid receptors
PKA	Protein kinase A
TRPV1	Transient receptor potential V1
VMR	Visceromotor response

1 Introduction

Opioid drugs are the most effective analgesics for treating acute pain but the morbidity and potential mortality that results from their unfavourable side effect profile has created a powerful impetus to develop new classes of analgesics. One major thrust has been to develop biased agonists that are designed to activate G-protein signalling preferentially over the recruitment of β-arrestins, thereby limiting the

development of tolerance and the resulting dose escalation that leads to inhibition of gastrointestinal function, cognitive changes and respiratory depression (Lambert and Calo 2020; Schmid et al. 2017). Other strategies include dual-action agonists binding to, e.g. μ opioid receptors (MOPr) and nociceptin/orphanin receptors (NOPr) (e.g. BU08028 and BU08070), and the allosteric modulation of MOPr (i.e. BMS) (Camilleri 2018; Kandasamy et al. 2021). While promising, to date none of these strategies have adequately negated the potential for opioid side effects.

A recent approach has been to design opioids that are active in acidic microenvironments but not in normal tissues at physiological pH (Spahn et al. 2017). Stein and colleagues created a pH-sensitive opioid derivate of fentanyl, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), that activates MOPr at acidic conditions. In this review we examine the pharmacokinetic properties of this compound, study the somatic inflammatory and cancer pain models and describe in more detail a series of experiments in a preclinical model of inflammatory bowel disease (IBD) that assess its analgesic efficacy and potential to mitigate on-target side effects in uninflamed tissues.

2 Pain Mechanisms and Visceral Sensory Pathways

Abdominal pain is a major cause of morbidity in patients suffering from inflammatory disorders such as IBD, acute pancreatitis and cancers of the digestive system as well as disorders of gut-brain interaction (e.g. irritable bowel syndrome). The visceral sensory pathways in the autonomic nervous system and enteric nervous system (ENS) have been well described in several recent reviews (Brierley and Linden 2014; Furness et al. 2013; Hockley et al. 2018). Inflammatory mediators initiate the pain signal by activating and sensitizing peripheral nerve terminals of spinal sensory afferents (Grundy et al. 2019; Gottesman-Katz et al. 2021), which have their cell bodies in the dorsal root ganglia (DRG). The distal axons of these pseudo-unipolar neurons innervate the intestine and other viscera and their proximal axons synapse with second-order neurons in the dorsal horn of the spinal cord. These nociceptive DRG neurons are mostly peptidergic C-fibres that express calcitonin gene-related peptide (CGRP) and heavy neurofilament protein (NF-H), as well as pro- (e.g. transient receptor potential V1 (TRPV1), neurokinin receptor 1 (NK1R)) and anti-nociceptive receptors. The axons innervating the inflamed intestine express opioid receptors making them an ideal target for suppressing pain.

Opioid receptors (OPr) are seven-transmembrane alpha-helix proteins, members of the family of G-protein-coupled receptors (GPCR). There are three major classes: μ (MOPr), *∂* (DOPr) and κ (KOPr) and the non-classical nociceptin/orphanin (NOPr) receptors, widely expressed in the central and peripheral nervous system (Corder et al. 2018). Recent studies have characterized the distribution of functional opioid receptors in human and murine sensory DRG neurons (Guerrero-Alba et al. 2018; Moy et al. 2020). Activating OPr on the axon terminals of these nociceptors initiates the classical G*i*/G*o* signalling pathways. After the agonist binds, both

MOPr and DOPr link to the Gαi-subunit to cause inhibition of adenylate cyclase, which decreases the activity of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA). On the other hand, linking to the Gβγ subunits causes inhibition of Ca^{2+} channels, which will decrease neurotransmitter release from nerve endings. In addition, Gβγ subunits cause activation of K^+ channels, which results in hyperpolarization of the membrane potential and a reduced probability of action potential firing by the affected neurons (Galligan and Akbarali 2014; Grim et al. 2020).

Agonists binding to MOPr trigger a cascade of intracellular events that promote phosphorylation of OPr amino acid residues and recruitment of the β-arrestin/clathrin complex. These latter events lead to receptor endocytosis and can result in receptor desensitization. Studies using biased agonists and genetically modified rodents suggest that this receptor internalization is a key mechanism underlying the development of opioid tolerance and, with resulting dose escalation, the risk of respiratory depression and other common side effects (Grim et al. 2020; Raehal et al. 2005; Allouche et al. 2014). For example, in the gastrointestinal (GI) tract, MOPr have been localized in enteric neurons of both myenteric and submucosal ganglia (DiCello et al. 2020). MOPr activation in this tissue inhibits enteric neuronal activity, thereby suppressing peristaltic smooth muscle contraction and electrolyte and fluid secretion.

3 Challenges of Conventional Opioid Drugs

Opioids are among the most effective analgesics to treat acute pain and chronic cancer-related pain syndromes (Volkow and McLellan 2016; Bateman et al. 2021). However, their serious side effect profile continues to fuel the debate around their clinical use and overall benefit (Ballantyne and Mao 2003; Schofferman 1993). Abdominal pain is a leading cause of morbidity in patients suffering from IBD, and pain relief often relies on the administration of opioids, with 21% of patients taking opioids at home and 62% during hospitalizations (Niccum et al. 2021). The side effects of acute and chronic opioid use occur through an on-target activation of ubiquitously expressed OPr throughout the entire body. The most frequently reported adverse effects of opioid treatment affect the intestinal tract where it causes impaired gastric, biliary and intestinal motility and secretion, which leads to severe constipation, nausea and bloating (Wood and Galligan 2004; Swegle and Logemann 2006). The central adverse effects of opioids cause sedation and respiratory depression and these can result in life-threatening events (Bateman et al. 2021; Urman et al. 2021). Sedation, cognitive decline and delirium are yet other central side effects reported in hospitalized patients (Gaudreau et al. 2005). Finally, addictive properties and reduced analgesic efficacy over time (i.e. tolerance) are additional adverse effects that complicate treatment with opioids (Khan and Mehan 2021; Volkow et al. 2019). The use of opioids in IBD patients has been associated with more severe disease activity, increased mortality and ultimately higher health-care

use (Niccum et al. 2021). The imbalance of severe side effects and analgesic potency underscores the need for new analgesic drugs that provide similar analgesic potency but lack the side effects.

4 Pharmacodynamic Properties of NFEPP

Inflammation results in tissue acidosis and this pH change can affect the protonation of ligands and thereby their binding to their receptor. Following this hypothesis, Spahn and colleagues (2017) created a pH-sensitive opioid derivate of fentanyl, (±)-N-(3-fluoro-1-phenethylpiperidine-4-yl)-N-phenyl propionamide (NFEPP), that activates MOPr in acidic conditions. The computational simulations of MOPr and fentanyl structures reveal that the dissociation constant (p*K*a) for fentanyl is ~8, thus enabling binding MOPr at both physiological (pH 7.4) and inflamed (~ pH 6) conditions (Spahn et al. 2017; Lesnik et al. 2020). Studies that simulated the binding energy of ligands showed that when fentanyl was deprotonated it lost its robust interaction with MOPr. Quantum mechanical calculations predicted the optimum p*K*a (6.73–6.93) and sites of hydrogen substitution for the fentanyl molecule. Based on this data, NFEPP was synthesized by replacing hydrogen with fluorine to achieve an experimental p*K*a of 6.8. This change results in a significant decay in the binding affinity to MOPr at physiological pH (7.4) compared to an acidic condition (pH 5.5 and 6.5). To validate the binding affinity, an inhibition constant (*Ki*) of NFEPP was demonstrated in human embryonic kidney 293 (HEK293) cells expressing MOPr (Spahn et al. 2017). These studies showed that the fentanyl *Ki* was similar in different pH environments (0.9–1.4 nM), whereas the NFEPP *Ki* dropped to 3.7 nM in acidic pH (6.5) compared to physiological pH (*Ki* = 17.9 nM). As a result, the NFEPP half-maximal inhibitory concentration (IC50) shifted one order of magnitude from pH 6.5 (~ 9.6 nM) to pH 7.4 (~60.9–88.1 nM) when assessed in rodent brain membranes (Baamonde et al. 2020; Rodriguez-Gaztelumendi et al. 2018). Therefore, the low p*K*a of NFEPP compared to its parent compound fentanyl increases its activity in acidic tissues compared to uninflamed tissues where the pH is 7.4.

5 In Vitro Studies of NFEPP in Acidified and Physiologic Conditions

To study the actions of NFEPP in acidic conditions to mimic what occurs in inflamed tissues, we conducted electrophysiological studies in single DRG neurons using patch-clamp recordings and in ex vivo colons using extracellular recordings from colonic afferent nerves (Jimenez-Vargas et al. 2022). NFEPP in acidic conditions (pH 6.5, 6.8) inhibited neuronal excitability but had no effect at physiological pH

(pH 7.4) in patch-clamp recordings of DRG neurons or afferent nerve recordings from mouse colons.

In parallel experiments, Bunnett and colleagues (Jimenez-Vargas et al. 2022) evaluated NFEPP-initiated MOPr signalling and trafficking using fluorescence-resonance energy transfer (FRET) and bioluminescence-resonance energy transfer (BRET). In HEK cells transfected with MOPr, NFEPP dissociates the G-protein heterotrimer (βγ-α complex) (Spahn et al. 2017) and inhibits the cAMP formation (Jimenez-Vargas et al. 2022) in acidic conditions but not at physiological pH (7.4). These effects were blocked by naloxone, a non-selective antagonist of opioid receptors. Additional studies showed that NFEPP in acidic (pH 6.5 and 6.8) but not physiological conditions (pH 7.4) promotes the recruitment of β-arrestin2 and Rab5 and activates nuclear extracellular signal-regulated kinase (ERK) (Jimenez-Vargas et al. 2022), events that are associated with desensitization, endocytosis and endosomal signalling. These findings suggest that NFEPP can activate MOPr to cause endocytosis and induce compartmentalized signalling in HEK cells under acidic conditions. Further studies are needed, however, as in the electrophysiological studies of DRG neurons the actions of NFEPP were not blocked by inhibitors of clathrin-dependent endocytosis. It is unknown whether NFEPP exhibits the same level of tolerance found with fentanyl.

6 NFEPP Inhibits Nociception in Preclinical Inflammatory Pain and Cancer Models

The pH-sensitive property of NFEPP affords tremendous opportunity to treat pain in GI disorders, such as IBD and cancer, as well as many somatic inflammatory disorders. To assess this possibility, NFEPP and its parent compound fentanyl have been studied in preclinical somatic and visceral inflammatory pain models and cancer pain models.

Somatic Inflammatory Pain Studies conducted in a unilateral inflammation paw model in rats induced by intraplantar incision or intraplantar injection of complete Freund's adjuvant (CFA) showed the pH-acidic-dependent and dose-dependent actions of NFEPP in response to mechanical and noxious heat in the inflamed but not in the contralateral paw (Spahn et al. 2017). A separate study tested this hypothesis in the neuropathic pain model of chronic constriction injury (CCI) of the sciatic nerve of rats (Spahn et al. 2017). In these studies NFEPP and fentanyl had similar analgesic action in the ipsilateral (injured) hindlimb in response to noxious stimuli, whereas NFEPP had no effect in the contralateral (noninjured) hindlimb. Acidification of tissue surrounding the sciatic nerve was detected with a pH microelectrode.

Visceral Inflammatory Pain Using a preclinical IBD mouse model of colitis induced with dextran sodium sulphate (DSS), Jimenez-Vargas and colleagues

(2022) evaluated the analgesic actions of NFEPP to reduce the visceromotor response (VMR, electromyographic activity) to noxious colorectal distension (CRD). Subcutaneous injections of fentanyl reduced the nociceptive response in both DSS colitis and healthy mice. In contrast, an equivalent dose of NFEPP reduced VMR response to CRD in the DSS colitis mice to the same degree as fentanyl but had no effect in the healthy mice. Colonic inflammation was demonstrated by measuring myeloperoxidase (MPO) and histological scoring, and acidification of inflamed colonic tissue (pH 6.7 ± 0.09) was detected with a pH indicator. Similar results were found in a rat model of visceral inflammation following intraperitoneal injection of acetic acid (Baamonde et al. 2020).

Cancer Pain Baamonde and colleagues (2020) evaluated the effectiveness of NFEPP to activate MOPr in a bone cancer-induced pain model in mice. NFEPP and fentanyl increased the withdrawal latency response to noxious heat in the hindlimb injected with live B16-F10 melanoma cells. However, unlike fentanyl, NFEPP did not affect the contralateral hindlimb that lacks tumour. In addition, the NFEPP analgesic actions were reversed by naloxone methiodide, a peripheral-selective OPr antagonist. Tissue acidification in the tumour was not measured in this study but many studies have demonstrated the acidic pH environment in tumours (Anderson et al. 2016; Rohani et al. 2019).

7 On-Target Side Effects of NFEPP and Fentanyl

Activation of MOPr in the intestinal tract and the central nervous system commonly results in opioid on-target side effects, including constipation, nausea, sedation, respiratory depression and addiction. NFEPP, in contrast, was suggested to lack these severe side effects in several animal pain models as its activation of MOPr is confined to compromised tissues exhibiting acidic conditions (Spahn et al. 2017; Baamonde et al. 2020; Rodriguez-Gaztelumendi et al. 2018; Jimenez-Vargas et al. 2022). Indeed, in acute and persistent inflammatory somatic pain rat models, aforementioned, NFEPP did not affect defecation, locomotor behaviour and the cardiorespiratory system (i.e. heart rate and blood oxygen saturation) nor exhibit an addictive potential (Spahn et al. 2017). In contrast, fentanyl and morphine, which are widely used in clinical practice, negatively impacted all of these endpoints (Spahn et al. 2017, 2018; Jimenez-Vargas et al. 2022; Massaly et al. 2020). In vitro measurements of colon motility measuring colonic migrating motor complexes by Margolis and colleagues (Jimenez-Vargas et al. 2022) revealed that acidification of the surrounding bath solution led to a decrease of contractile activity when NFEPP was applied. This contrasts with the in vivo DSS colitis studies where NFEPP had no effect on pelleting. This difference may reflect different levels of acidification within the layers of the inflamed colon wall, as the DSS colitis model predominantly causes inflammation in the mucosa and submucosa (Chassaing et al. 2014; Okayasu et al. 1990).

8 Repeated Applications of NFEPP During the Evolution of Inflammation

The aforementioned studies demonstrated that acute administration of NFEPP (single dose) in several pain models (Spahn et al. 2017, 2018; Rodriguez-Gaztelumendi et al. 2018; Jimenez-Vargas et al. 2022; Massaly et al. 2020) had a potent antinociceptive effect without causing typical opioid side effects. In clinical practice, management of pain typically requires repeated administration of analgesic drugs to induce stable analgesia, which led to the following question: Does NFEPP retain its analgesic potency and lack of on-target side effects with repeated administrations per day during the evolution of inflammation? In a preliminary study (Degro et al. 2022) addressing this question, NFEPP or fentanyl was administered twice daily over nearly 1 week during the evolution of acute DSS colitis. NFEPP provided analgesia throughout the period of acute colitis, assessed by visceromotor responses to stepwise colorectal distensions. Importantly, the greatest antinociceptive effect was observed at the peak of colonic inflammation when colon pH was at the lowest level. Moreover, repeated NFEPP administrations did not lead to an impaired gastrointestinal transit nor reduced defecation in vivo. In sharp contrast, fentanyl caused marked delays in transit and almost complete cessation of pelleting. Lastly, NFEPP also did not affect blood oxygen saturation during repeated administrations, whereas fentanyl induced significant hypoxemia. A transient decrease in heart rate was detected with NFEPP but was smaller than that observed with fentanyl and had a shorter duration of effect.

9 Conclusion and Future Directions

The studies outlined in this review suggest NFEPP is a very promising pH-sensitive opioid analogue that provides highly effective analgesia and lacks the common side effects seen with conventional opioids. This evidence from these preclinical models suggests that it would be highly beneficial for treating acute pain in patients with somatic and visceral inflammatory pain disorders such as rheumatoid arthritis, IBD, and pancreatitis, as well as many cancers. The opioid crisis has generated justifiable concern regarding the use of opioids but these drugs remain necessary analgesics for many patients with acute pain and are thus used widely in the acute clinical care setting. Providing a similar level of analgesia without the morbidity and potential mortality associated with conventional opioids would be a very important step forward in patient care. Phase I clinical trials are needed to demonstrate the promising analgesic properties and lack of on-target side effects in humans.

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Signalling in the Gut

Friederike Uhlig and David Grundy

Abstract Signalling via the gut-brain axis has gained a lot of attention because of the growing interest in modulating brain activity through microbiome-targeting strategies. The sensory nerve fibres which arise from neurons in the nodose ganglion and dorsal root ganglia and innervate the entire gastrointestinal tract constitute the neuronal component of this gut-brain axis. The activation patterns of these sensory nerves have been studied in electrophysiological experiments using either flat sheet or tube preparations in the context of intestinal sensing and visceral pain. A recent study from our lab, in line with other studies of somatic pain, has now demonstrated that these nerves can sense metabolites released by the opportunistic pathogen *Staphylococcus aureus*. Given that the secretion of these neuromodulatory mediators was dependent on bacterial density, these findings suggest that sensory nerve fibres also constitute an important signalling mechanism for microbiota-gut-brain communication.

Keywords Gut-brain axis · Pathogenic bacteria · Microbiota-gut-brain communication · Sensory afferents

Sensory afferent nerves are ubiquitous throughout the periphery and provide important information to the central nervous system regarding the physical and chemical status of organs and tissues. Sensory nerve terminals can be activated by a variety of sensory signals such as temperature, mechanical forces and a variety of chemicals derived from the host and from the environment.

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1 General Considerations on Extrinsic Intestinal Sensing

The gastrointestinal (GI) tract is innervated by sensory afferents originating from the nodose ganglion (vagal afferents) and dorsal root ganglia in the spinal cord (spinal afferents). Our laboratory and others have studied intestinal afferents using extracellular recordings from rodent, ferret and human tissue. These studies have demonstrated that afferent nerves comprise fibres with distinct activation patterns corresponding to their terminal distribution of their endings within the gut wall and mesenteric connections (Fig. 1) (Blackshaw et al. 2007). (1) Mucosal fibre endings are located most closely to the intestinal lumen sitting below the mucosal epithelium and are acutely sensitive to mechanical deformation, achieved experimentally by mucosal stroking or by chemicals either crossing the mucosa or released from specialised enteroendocrine calls within the epithelial lining. (2) Muscular fibres in contrast do not respond to low-intensity mechanical stimulation (stroking) but can be activated during distension or by contraction. (3) Serosal fibres are most profoundly activated by high-threshold mechanical stimulation caused by distortion and torsion on the mesentery and the receptive fields can be identified by blunt

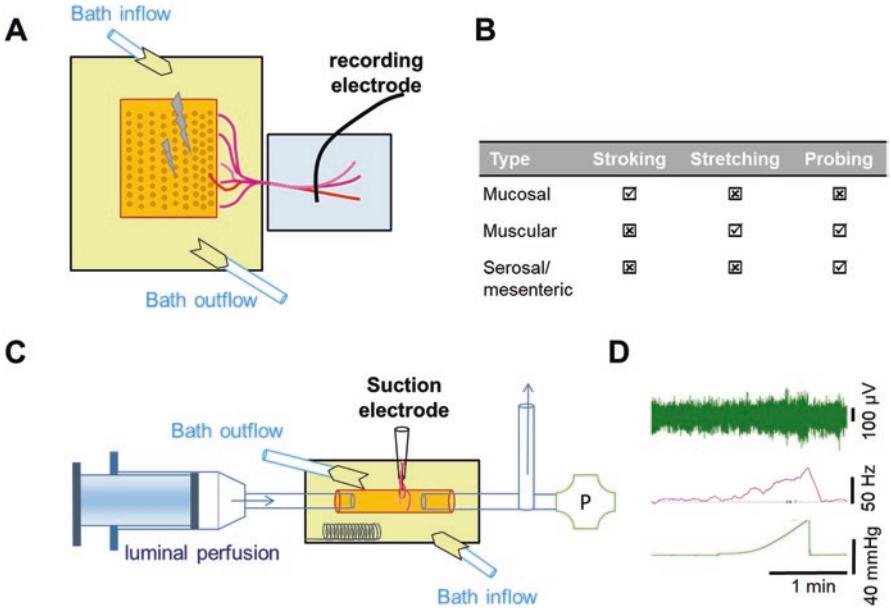

Fig. 1 Different techniques to record extrinsic intestinal sensory nerves (depicted in red-pink). Flat-sheet preparations (**a**) are used to distinguish different populations of mechanosensitive afferent. Mucosal, muscular and serosal fibres respond to stimuli of different intensity (**b**). Tube preparations (**c**) allow the quantification of nerve activity in response to distension (**d**) and during luminal perfusion with various chemical agents including supernatants derived from *S. aureus* (see later). Top trace (neurogram) shows nerve firing which is quantified in the second channel (pink). The bottom trace shows the pressure profile during distension

probing. In addition to these three major classes there are mixed mucosal-muscular afferents and mechanically insensitive afferents which are thought to be silent in the healthy gut. To segregate these populations in the course of an electrophysiological recording, the intestinal tissue is pinned out flat in an organ bath chamber whilst nerve activity in response to the different stimuli (stroking, stretching, probing) is determined. This technique has provided important insights into the contribution of mucosal, muscular and serosal fibres to intestinal (hyper-) sensitivity. In order to investigate the effect of luminal signals or distension on afferent discharge, we have also developed a tube preparation which keeps the gut wall intact and allows to measure pressure simultaneously with nerve activity.

Intestinal sensory afferent firing can also be induced by a range of chemicals applied either directly to the nerve terminals or intraluminally in tube preparations. Electrophysiological recordings from nerve fibres have demonstrated sensitivity to the TRPV1 agonist capsaicin, TRPA1 agonist AITC and bile acids, among others. Calcium imaging and gene expression data obtained from retrogradely traced dorsal root ganglia neurons have confirmed the presence and relevance of these receptors in gut-brain communication.

Afferent signalling pathways are important to induce reflex behaviours and changes in brain activity in a healthy state, but exaggerated responsiveness (hypersensitivity) of afferent signalling is implicated in chronic pain conditions. Using either the flat sheet or tube preparation, hypersensitivity of intestinal afferents to mechanical and chemical stimulation has been demonstrated in animal models of IBS or after incubation of intestinal tissue with inflammatory cytokines (Brierley and Linden 2014). These studies have provided enormous insights into the contribution of inflammatory mediators ("inflammatory soup") such as serotonin, cytokines, histamine and proteases to the activation, sensitisation and recruitment of afferent signals.

In addition to inflammatory mediators, supernatants from intestinal mucosal biopsies or faecal extracts from IBS patients have also been shown to induce hypersensitivity, indicating a potential role of bacteria-derived substances. This is in line with changes of the microbiota during visceral hypersensitivity and a link between gastrointestinal infection and visceral hypersensitivity. Evidence from other sensory systems also suggests that bacteria are able to induce hypersensitivity and pain through the secretion of soluble mediators.

2 Intestinal Sensing of Pathogenic Bacteria

We investigated the possibility of bacteria-mediated afferent signalling in a recent study and identified a key role of quorum-sensing-regulated proteins in gut-brain signalling (Uhlig et al. 2020). Ex vivo nerve recordings were performed on tissue from healthy C57Bl/6 mice and their response to supernatants from the *S. aureus* strain JE2 was assessed. Application of supernatants induced a biphasic response pattern in small intestinal afferent nerves. After an initial phase of excitation (up to

45 min after application), we observed a profound inhibition of afferent nerve activity. The response to distension (mechanosensitivity) was also attenuated during longer incubation periods. To determine which combination of mediators in *S. aureus* is responsible for these changes of nerve activity, we applied microbiological and genetic techniques to eliminate the secretion of specific exoproteins from the bacterial supernatant. Using the Nebraska Library of *S. aureus* mutants, we observed that the transcription factor AgrA which is activated at high bacteria density (quorum sensing) regulated the transcription of both excitatory and inhibitory mediators. Supernatants from mutants lacking AgrA neither excited nor inhibited intestinal afferents. Using different genetic mutants (Fey et al. 2013; Wang et al. 2007), we were also able to identify which AgrA-regulated mediators cause excitation and inhibition. Because the excitatory response was ablated in supernatants from *S. aureus* mutants lacking α-haemolysin and the inhibitory response was reduced in supernatants from *S. aureus* mutants lacking phenol-soluble modulins (Psms), we concluded that the pore-forming toxin (PFT) α-haemolysin mediated excitation and phenol-soluble modulins contributed to the inhibition of intestinal afferents. We further evaluated these findings in primary cultures of dorsal root and nodose ganglia neurons. Supernatants from wild type but not AgrA- or Psm-deficient *S. aureus* increase cell membrane permeability, suggesting that this mechanism is linked with inhibition of intestinal afferents. Hla-deficient supernatants continued to increase membrane permeability which suggests that Hla-induced excitation is mediated through different mechanisms. Because the intestine harbours intrinsic sensory neurons within the enteric nervous system in addition to the extrinsic sensory afferents, we tested whether *S. aureus* supernatants modulated enteric neuronally regulated functions (secretion and motility). In Ussing chamber experiments, we found that wild type supernatants induced a secretory response which was absent when supernatants from AgrA-deficient *S. aureus* were applied. Finally, wild type but not AgrA-deficient supernatants profoundly inhibited intestinal motility. The latter experiments indicate that in addition to extrinsic sensory neurons, intrinsic neurons of the enteric nervous system also respond to bacterial mediators that are under the influence of quorum sensing.

These findings highlight that environmental cues such as quorum sensing profoundly modify bacterial secretion and that our understanding how these mediators affect host physiology is extremely limited. Bacteria in the intestine interact with host-, food- and bacteria-derived substances that can all exert marked effects on gene expression. For example, it is known that in addition to quorum sensing, metabolic stress induced by immune mediators or nutrient depletion causes the transcription of phenol-soluble modulins in *S. aureus* (Harper et al. 2018; Kavanaugh and Horswill 2016). On the contrary, the presence of quorum-sensing molecules from other *Staphylococcus* species may be able to inhibit the production of Psms. Recent studies have introduced software tools such as MASI (Microbiota-Active Substance Interactions database), gutSMASH and Metage2Metabo to shed light into the complex interaction between environment, changes of microbiota composition and host status (Belcour et al. 2020; Pascal Andreu et al. 2021; Zeng et al. 2021). While these are powerful tools for hypothesis generation, in vivo experiments are required in

order to assess the validity of these predictions and investigate which metabolic pathways are active in a flow-system gut environment and whether/how those signals affect the production of specific mediators including virulence factors.

In addition to *S. aureus*, other bacteria are also able to produce virulence factors, some of which have structures similar to the pore-forming toxins that we have demonstrated to act as neuromodulators in the intestine. *Candida albicans* has been found to produce a cytolytic toxin named candidalysin during infection of mucosal surfaces and *Streptococcus pyogenes* secretes streptolysin during acute infection which activates sensory neurons and causes pain (Moyes et al. 2016; Pinho-Ribeiro et al. 2018). Further, important gut pathogens such as *Escherichia coli*, *Salmonella typhimurium* and *Campylobacter jejuni* are also known to produce similar toxins which can, in the context of infection, cause acute and long-term gastrointestinal symptoms such as nausea, vomiting and visceral pain that are indicative of neuronal dysfunction. Some of these symptoms can be alleviated by preventing gut-brain communication through vagotomy (Goehler et al. 2005, 2007). These studies demonstrate the importance of investigating virulence factors in addition to other microbiome metabolites such as short-chain fatty acids and amino acid derivatives, particularly under pathophysiological conditions where the intestinal barrier may be impaired and beneficial bacteria may be depleted.

Examining these mechanisms and particularly neuronal involvement in vivo remains challenging because of the immense cross-talk between the nervous system, the immune system, the microbiome and pathogenic bacteria. Some interesting studies have demonstrated an important role for virulence factor-related pathways in the intestine. Stacy et al. have demonstrated that transient infection with *Yersinia pseudotuberculosis* induces hydrogen sulphide production by taurine-metabolising bacteria. This subsequently inhibits pathogen expansion and thus contributes to colonisation resistance, a mechanism by which resident bacteria prevent infection by pathogenic bacteria (Stacy et al. 2021). Previously, others have found that pathogenic but not avirulent *Salmonella enterica* serovar Typhimurium modulated the microbiota in such a way that favoured its own survival and replication (Stecher et al. 2007). Unfortunately, the involved virulence factor(s) remains to be identified and neither study examined the potential involvement of the intestinal epithelium or intestinal neurons. However, hydrogen sulphide is known to activate sensory neurons and modulate secretion in the intestine (Krueger et al. 2010), and activation of nociceptors in the periphery induces the release of CGRP which inhibits parts of the immune response to *Streptococcus pyogenes* (Pinho-Ribeiro et al. 2017). These studies demonstrate that with the availability of novel mouse genetic tools and advances in microbiological techniques, we will be able to better understand host-microbe interaction in health and disease.

Such investigations will also be essential to understand the significance of supernatant-induced excitation versus inhibition of afferent nerve activity (Fig. 2). Traditionally, an increase in intestinal nerve activity is associated with satiety signals that ultimately will lead to the termination of food intake. Further increases of nerve activity will then cause the sensation of pain. In the context of infection, the mediators that increased nerve activity in our preparation (pore-forming toxin,

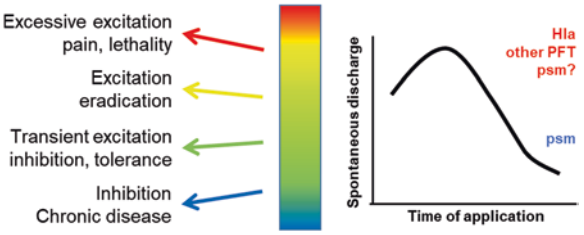

Fig. 2 Modulation of extrinsic intestinal sensory neurons by supernatants from *S. aureus* and potential physiological role of excitation and inhibition of afferent nerve activity

α-haemolysin) have been shown to also correlate with mechanical hypersensitivity and pain (Blake et al. 2018; Chiu et al. 2013), indicating that this activation might be part of the inflammatory response raised to eradicate pathogens. On the other hand, the physiological role of inhibition of nerve activity is less intuitive. Given that neuronal activation leads to the release of neurotransmitters that influence the activity of the immune system (Sun et al. 2007), it can be hypothesised that inhibition of these nerve fibres could constitute a means for the bacteria to limit the extent of the local immune response to establish themselves in a particular niche and induce tolerance in the host (Pinho-Ribeiro et al. 2017). Extensive inhibition in this sense could provide an environment favouring chronic disease. The precise mechanisms linking nerve activity and immune modulation remain to be investigated.

3 Conclusion

Numerous chronic diseases such as inflammatory bowel disease, irritable bowel disease, obesity and neurodegenerative diseases have become major socio-economic burdens. They have been linked to a persistent low-grade inflammation but are also associated with neuronal dysfunction and documented increases in the abundance of potentially pathogenic bacteria in the microbiomes of patients compared to healthy controls (Bennet et al. 2017; Duvallet et al. 2017; Rinttilä et al. 2011). This suggests that neuronal dysfunction and visceral pain could be a direct consequence of pathogen-neuron interaction and this could also lead to subsequent low-grade inflammation. Neuronal activation causes the release of neurotransmitter in the periphery which can modulate the immune system (Pinho-Ribeiro et al. 2017; Talbot et al. 2015). Future studies are needed to establish whether such mechanisms contribute to the above-mentioned pathophysiology. More importantly, it will be most interesting to investigate the effect of modulating these pathways for disease outcome.

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Translating Colonic Sensory Afferent Peripheral Mechanosensitivity into the Spinal Cord Dorsal Horn

Andrea M. Harrington

Abstract Sensory afferent signalling from the colon and rectum is relayed into the spinal cord. The spinal cord contains distinct neurocircuits that control in what manner incoming sensory input is conveyed into the brain. The spinal cord is therefore a key relay point shaping how colonic nociceptive stimuli are ultimately perceived. This minireview summarises what is currently known of the spinal cord circuits that receive sensory input from the colon and how they shape colonic nociceptive relay into the brain.

Keywords Colon · Rectum · Spinal cord Dorsal Horn · Visceral pain · Nociception and neurocircuits

Abbreviations

CRD	Colorectal distension
DGC	Dorsal grey commissure
DH	Dorsal horn
DRG	Dorsal root ganglia
ERK1/2	Extracellular signal-regulated kinase
IML	Intermediolateral cell column
L	Laminae
LI	Laminae 1
L-L neurons	Long-latency neurons

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LS	Lumbosacral
pERK	Phosphorylated MAP kinase
SPN	Sacral parasympathetic nuclei
SL-A	Short-latency abrupt neurons
SL-S	Short-latency sustained neurons
SDH	Superficial dorsal horn
TL	Thoracolumbar

1 Introduction

Sensory information is transmitted into the spinal dorsal horn by primary afferent nerves, which extend from peripheral tissue into the spinal cord and synapse onto neurons within the dorsal horn. The spinal cord dorsal horn functions as an intermediary processing centre shaping how peripheral sensory input is ultimately conveyed into supraspinal targets for sensory discrimination and integration with effector motor circuits. Our knowledge of the dorsal horn circuits processing modality-specific peripheral input is largely based on the somatosensory pathways (Basbaum et al. 2009; Harding et al. 2020).

Somatic sensory afferent input in the dorsal horn is highly organised, based on fibre size and sensory modality (Basbaum et al. 2009; Harding et al. 2020). Briefly, the four main types of somatic afferent fibres (Aα, Aβ, Aδ, C) show selectivity in sensory modality transmitted and synapse into different layers (laminae) of the dorsal horn (Harding et al. 2020). Thus how somatosensory afferent input is organised within the dorsal horn has shaped our knowledge of the sensory modality processed within each lamina of the dorsal horn. Moreover, it has given functional relevance to the types of dorsal horn neurons involved. Sensory afferent input synapses onto populations of interneurons and projection neurons. The patterns by which different primary afferent fibres synapse onto which type of dorsal horn neuron or whether a defined pattern exists is not definitively known. Dorsal horn projection neurons relaying into supraspinal targets are dispersed across all laminae, except LII, which is populated solely by interneurons (Wercberger and Basbaum 2019). Populations of projection neurons within LI primarily relay nociceptive information into anterolateral tracts (Cameron et al. 2015; Todd 2002). Whilst projection neurons within LIII-V consist of more heterogeneous populations, consisting of wide dynamic range neurons responding to nociceptive and non-nociceptive input and relaying into multiple ascending tracts targeting the medulla, midbrain and forebrain (Wercberger and Basbaum 2019; Todd 2010), interneurons account for 99% of all neurons in the spinal cord dorsal horn (Graham and Hughes 2020). They form complex microcircuits within the dorsal horn that modulate afferent input and projection neuron output (Graham and Hughes 2020; Polgar et al. 2013). Thus, interneurons are just as critical as projection neurons to determining how peripheral-encoded sensitivity is maintained within the spinal cord and relayed into the brain.

Nociresponsive behaviours, avoidance and abdominal guarding, evoked by noxious circumferential stretch of the colon and rectum (colorectal distension, CRD), are shaped by spinal circuits in which sensory discrimination is integrated with autonomic motor functions. Specifically, spinal cord circuits relaying within dorsal column and ventrolateral ascending tracts to the brain mediate CRD-evoked nociresponsive behaviours (Palecek and Willis 2003; Ness 2000; Al-Chaer et al. 1999).

The predominance of signalling within the dorsal column is unique to visceral nociception, as it has a relatively minor role in somatosensory nociception (Willis et al. 1999; Sikandar and Dickenson 2012; Kang et al. 2013; Al-Chaer et al. 1998). We know that the spinal afferent neurons innervating the colon and rectum detect a wide range of physiological, innocuous and noxious mechanical and chemical events which they relay into the spinal cord dorsal horn (Fig. 1). This minireview summarises what is known of how the input from colonic sensory afferent nerves is organised within the dorsal horn, relative to somatic afferent input, and how such organisation shapes the translation of peripheral-encoded colorectal mechanosensitivity into specific dorsal horn circuits relaying into the brain.

2 Peripheral-Encoded Colonic Mechanosensitivity

Sensory signalling from the colon and rectum is relayed by spinal afferent nerves within the lumbar splanchnic and sacral pelvic pathways into the spinal cord (Brierley et al. 2018) (Fig. 1). Colonic afferent neurons that relay via the splanchnic nerve reside within the thoracolumbar (TL) dorsal root ganglia (DRG; T10-L1 DRG), whilst those of the pelvic nerve are present in the lumbosacral (LS, L5-S1) DRG (1–6). Colonic afferent signalling is relayed into the respective TL (splanchnic) and LS (pelvic) spinal cord levels (Fig. 1a). Unlike their somatic counterparts, colonic spinal afferent fibres are largely unmyelinated C-fibres, the vast majority (~80%) being peptidergic, having small diameter cell bodies and conduction velocities in the C-fibre range, with only a minor population classed as Aδ-fibres (Lu et al. 2005; Robinson et al. 2004; Christianson et al. 2006). Despite this degree of homogeneity, endings of colonic afferent nerves differ in their peripheral sensitivity to mechanical stimuli due to the unique molecular expression of mechanosensitive ion channels and locality within the colon wall (Brierley et al. 2018; Brierley 2011). The peripheral endings of colonic afferents reside within multiple layers (Brookes et al. 2013; Kyloh and Spencer 2014) and have been characterised into subtypes based on their mechanosensitive properties (Brierley et al. 2004; Hughes et al. 2009a; Feng and Gebhart 2011) (Fig. 1b). Collectively, based on these peripheral mechanosensitivity properties and their relative proportions, colonic afferents in the pelvic pathway transmit low- and high-intensity mechanical and tactile events into the LS spinal cord, whereas those in the splanchnic pathway largely transmit noxious mechanosensory events into the TL spinal cord (Brierley et al. 2004; Hibberd et al. 2016; Harrington et al. 2018) (Fig. 1b). The question remains: how is the

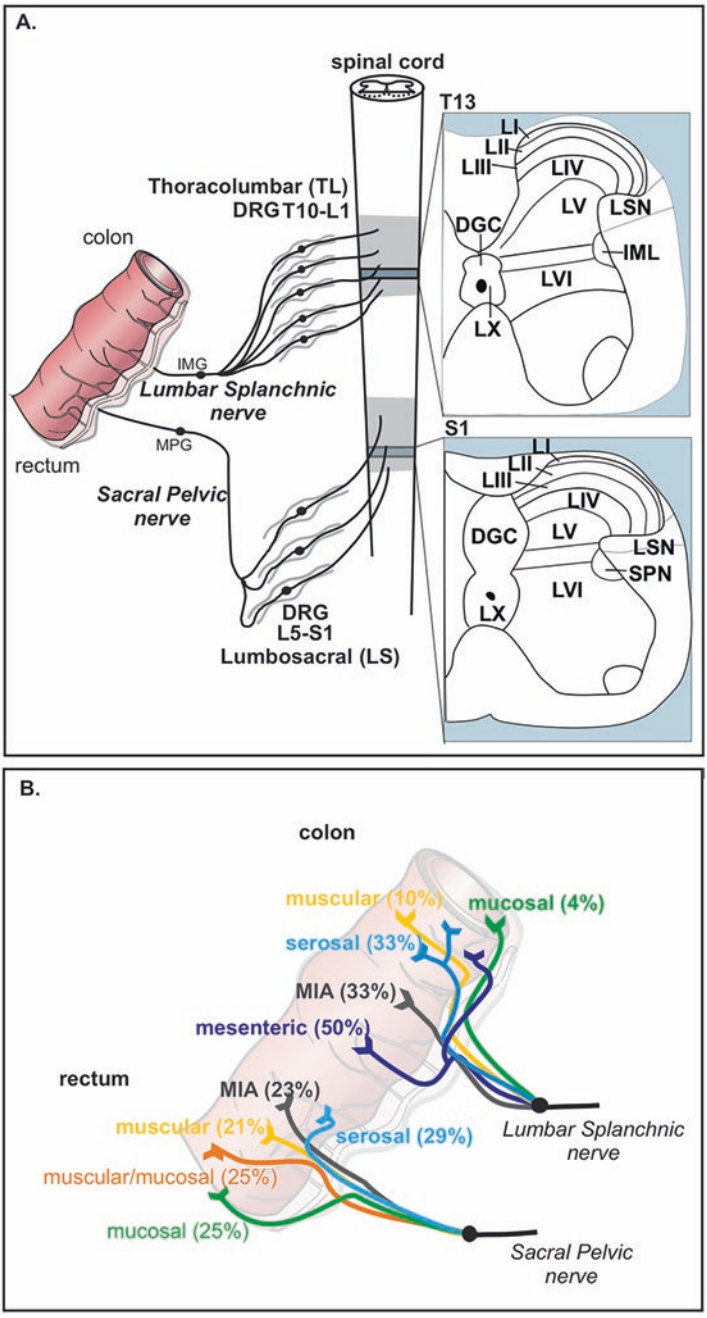

Fig. 1 Spinal afferent innervation of the colon and rectum. (**a**) Sensory afferents innervating the colon and rectum travel through two distinct anatomical pathways en route to the spinal cord. Afferents innervating the length of the colon travel via the lumbar splanchnic nerve and have cell

peripheral mechanosensitivity encoded by splanchnic and pelvic colonic afferent endings relayed into and maintained within the spinal cord circuits such that it leads to an appropriate sensation?

3 Organisation of Colonic Afferent Input in the Spinal Cord

To identify how visceral afferent input is organised within the spinal cord, relative to somatic afferent input, various neural tract-tracing approaches have been used. These have given insight into how colonic afferent input may be organised in a way that reflects peripheral-encoded mechanosensitivity.

Fig. 1 (continued) bodies located in the dorsal root ganglia (DRG) and central projections across a wide distribution pattern in the thoracolumbar (T10-L1) spinal cord levels. Afferents innervating the distal colon and rectum travel via the sacral pelvic nerves and have cell bodies located in the DRG and central projections across a relatively narrow distribution pattern in the lumbosacral (L5-S1) spinal cord levels. Schematic representations of the various layers and contrasting topology of the thoracolumbar (TL; shown here for simplicity as T13) and lumbosacral (LS; S1 shown here for simplicity) spinal cord. DGC dorsal grey commissure, LT lateral tract, LI lamina I, LII lamina II, LIII lamina III, LIV lamina IV, LV lamina V, LX lamina X, LSN lateral spinal nuclei, CC central canal, SPN sacral parasympathetic nuclei, IML intermediolateral cell column. (**b**) Colonic afferent fibres signal a wide range of physiological, innocuous and noxious mechanical and chemical events into the spinal cord. Colonic afferent subtypes have been characterised based on the location of their endings in the colorectal wall and their activation properties to mechanical stimuli (Brierley et al. 2004). Subtypes are found to be differentially distributed between the splanchnic and pelvic nerve, with relative proportions of each subtype of the total mechanosensitive afferents per pathway found in the mouse noted in brackets (Brierley et al. 2004). *Serosal afferents (light blue)* associated with blood vessels in the gut wall are generally unresponsive to low-threshold stimuli and respond to much higher intensities of distension (>40 mmHg; or stretch (>9 g) (Brierley et al. 2004; Hughes et al. 2009a). *Mesenteric afferents (navy)* have similar properties but are located on blood vessels within the mesenteric attachment and are specific to the splanchnic innervation. As such serosal and mesenteric afferents are also referred to as 'vascular afferents' (Brookes et al. 2013). *Mucosal afferents (green)* respond to very fine mucosal stroking and are insensitive to stretch. They are prevalent in the pelvic pathway, but are very rare in the splanchnic pathway (Brierley et al. 2004). *Muscular afferents* respond to low distension pressures (e.g. <20 mmHg) or low-intensity stretch stimuli (<3 g) and are prevalent in the pelvic pathway, but rare in the splanchnic pathway. *Muscular/mucosal (orange)* afferents are exclusive to the pelvic pathway and respond to both fine mucosal stroking and low-intensity stretch. Separate *mechanically insensitive afferent (MIA or silent afferent, grey)* does not initially display mechanosensitivity, but subsequently either responds only to chemical stimuli (Brierley et al. 2005a, b) or becomes mechanically sensitive following sensitisation with inflammatory mediators (Feng and Gebhart 2011)

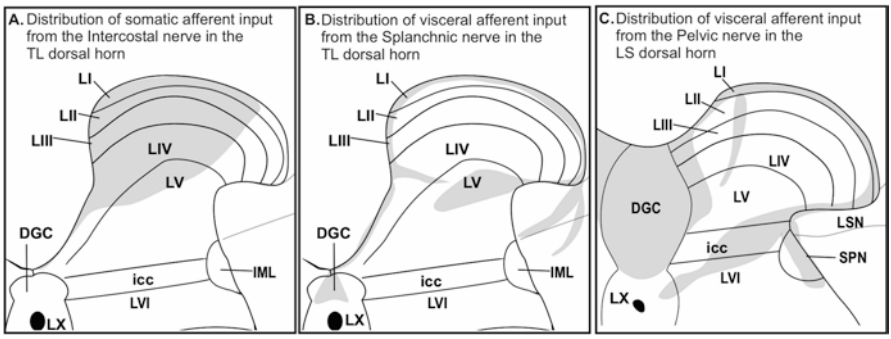

Fig. 2 Distribution of intercostal nerve (somatic afferent), splanchnic and pelvic nerve (visceral afferent) input into the spinal cord identified by HRP retrograde tracing in the rat. Schematic representations of the (**a** and **b**) thoracolumbar (TL) and (**c**) lumbosacral (LS) dorsal horn summarising the typical distribution patterns (grey shading) of central afferent projections in the rat of the (**a**) intercostal nerve (somatic afferents), (**b**) lumbar splanchnic nerve (visceral afferents) as described in (Neuhuber et al. 1986) and (**c**) sacral pelvic nerve (visceral afferents) as described in (Morgan et al. 1981; Nadelhaft and Booth 1984) using horseradish peroxidase (HRP) retrograde tracer incubations of severed nerve trunks. (**a**) HRP labelling of the intercostal nerve identified in the projection pattern of somatic afferents in the TL spinal cord (shown here for simplicity as T12), with terminals (presumptive) located throughout laminae I-LV (maximal density within LII-IV) projected from the medial tracts of Lissauer. (**b**) HRP labelling of the splanchnic nerve identified in the projection patterns of visceral afferents in the TL spinal cord (shown here for simplicity as T12), located within the lateral and medial tracts of Lissauer to terminate (presumptive) in LI and LV and X. (**c**) HRP labelling of the pelvic nerve identified in the projection patterns of visceral afferents in the LS spinal cord (shown here for simplicity as S1). Predominantly localised within LI and lateral collateral tracts of Lissauer that extend into LV-VI and the sacral parasympathetic nuclei (SPN). Dense labelling was also evident within the medial collateral tracts of Lissauer extending into the dorsal grey commissure (DGC) where they formed large terminal (presumptive) fields. DGC dorsal grey commissure, LT lateral tract, MT medial tract, LI lamina I, LII lamina II, LIII lamina III, LIV lamina IV, LV lamina V, LX lamina X, LSN lateral spinal nuclei, SPN sacral parasympathetic nuclei, IML intermediolateral cell column

3.1 Horseradish Peroxidase (HRP) Retrograde Tracing from Splanchnic and Pelvic Afferent Nerve Trunks

Initial studies used retrogradely transported horseradish peroxidase (HRP) applied directly to severed intercostal (somatic), splanchnic (visceral) and pelvic (visceral) nerve trunks to directly compare how somatic afferent (Fig. 2a) and visceral afferent (Fig. 2b, c) terminals are differentially organised within the spinal cord (Nadelhaft et al. 1983; Morgan et al. 1981; Cervero and Connell 1984a, b; Nadelhaft and Booth 1984; Neuhuber et al. 1986). Importantly these studies provided direct evidence that the organisation of the visceral afferent input (Fig. 2b, c) differs vastly from that of the somatic afferent input (Fig. 2a). Specifically, somatic afferents project within the medial collateral tracts and terminate throughout in LI-V (Fig. 2a), whereas visceral afferents project within the lateral and medial tracts terminating in more discrete laminae (Fig. 2b, c). Organisational differences are also evident between splanchnic

(Fig. 2b) and pelvic (Fig. 2c) nerve inputs, with regard to the discrete laminae they target.

3.2 *Cholera Toxin Subunit B (CTB) Retrograde Tracing from the Colon Wall*

Retrograde tracing from the distal colon wall in mouse using fluorescent-conjugated cholera toxin subunit B (CTB) identifies splanchnic and pelvic afferent input specifically from the colon and rectum (Hou et al. 2009; Harrington et al. 2012a, 2019; Grundy et al. 2018) (Fig. 3). Furthermore, to assess if different subtypes of colonic afferents project into the spinal cord in a sensory modality-specific pattern, as somatic afferents, we have recently used dual fluorescence CTB retrograde tracing from the distal colon wall and lumen (Harrington et al. 2019) (Fig. 3a). This dual tracing approach aimed to label spinal inputs from colonic afferent subtypes that differ in their peripheral mechanosensitivity, utilising the fact that they are differentially distributed between the outer and inner layers of the colonic wall (Fig. 1b) (Kyloh and Spencer 2014; Brierley et al. 2004). Consequently, this tracing method labelled distinct populations of neurons in the DRG (Fig. 3b) and fibres in the spinal cord dorsal horn (Fig. 3c, d). Strikingly, the differential labelling pattern observed between the TL (Fig. 3c) and LS (Fig. 3d) spinal cord levels aligned with that from the splanchnic and pelvic HRP studies. In the TL spinal cord (Fig. 3c), projections labelled from wall-directed injections are observed in discrete laminae (Fig. 3c-i), whilst very few fibres, if any, were labelled from the lumen directed CTB (Fig. 3c-ii) or dual labelled (Fig. 3c-iii). In contrast, in the LS dorsal horn (Fig. 3d) projections labelled from wall- (Fig. 3d-i) and lumen-directed (Fig. 3d-ii) tracing were abundant in addition to dual-labelled fibres (Fig. 3d-iii). This difference in labelling between the TL and LS DRG and dorsal horn correlates with the relative proportions of colonic afferent endings within the inner and outer layers of the colon wall that differentially relay within the splanchnic and pelvic pathways (Brierley et al. 2004). This includes mucosal and muscular afferents being abundant in the pelvic pathway and rare in the splanchnic pathway, whilst muscular/mucosal colonic afferents are exclusive to the pelvic pathway. In contrast, mesenteric and serosal 'vascular' afferents are present in both pathways yet predominate within the splanchnic nerve.

The results from these tracing studies suggest that colonic afferents may project into the spinal cord in a modality-specific pattern. In the TL spinal cord, the organisation of afferent input aligns with the majority relaying nociceptive input. As based on somatic characterisation, LI and LV primarily receive and process nociceptive afferent input. In the LS spinal cord, discrete laminae organisation of modality-specific inputs is less clear. Colon wall-traced and lumen-traced projections were both localised to LI. This aligns with input from high-threshold pelvic afferent fibres in the outer layers. Conversely, mucosal endings are largely

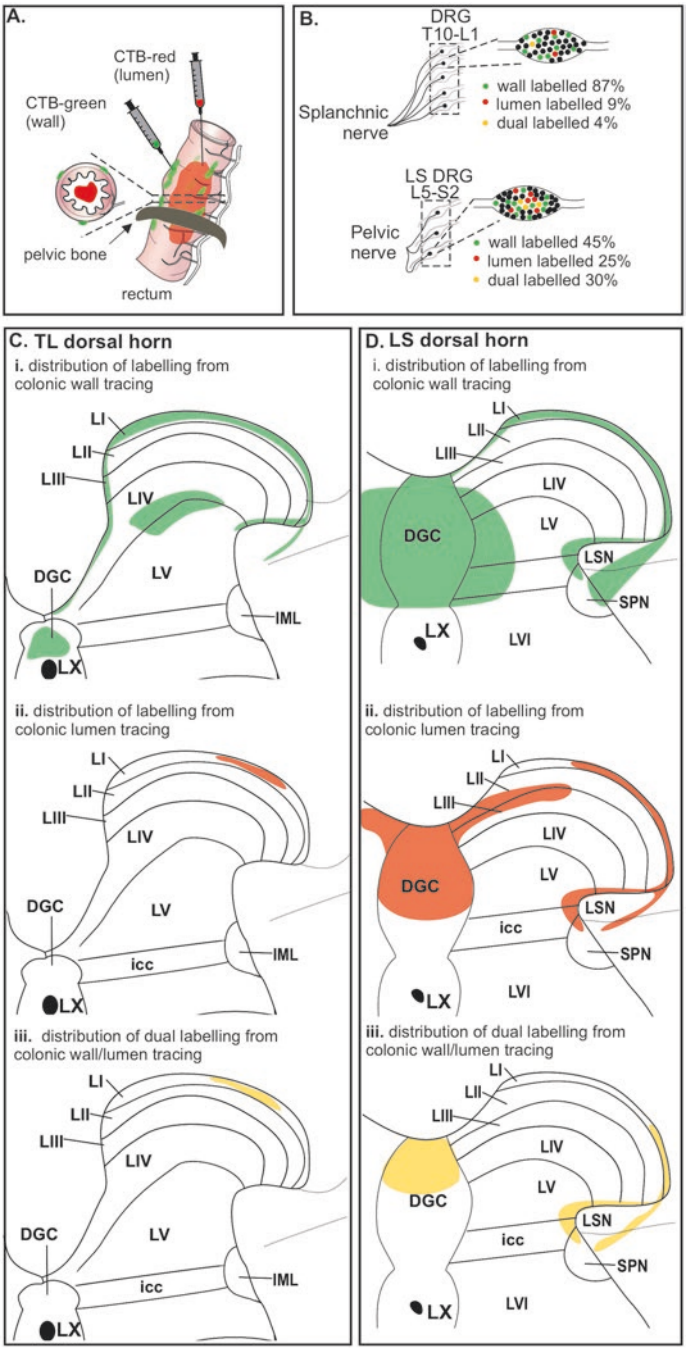

Fig. 3 Distribution of colonic afferent input in the dorsal horn identified by cholera toxin subunit B (CTB) retrograde tracing from the distal colon wall and lumen in the mouse. (**a**) Schematic

associated with relaying innocuous, tactile information based on their peripheral sensitivity. However, recent studies show that the activation of the colon epithelium can initiate pain-like responses (Makadia et al. 2018). The central mechanisms mediating this remain to be established. Given the common locality of projections labelled from the colon wall and the lumen in LI, it is of interest to determine if this identifies a potential mechanism by which low-threshold afferent input influences nociceptive transmission. Further to this, both wall-traced and lumen-traced projections were abundant in the dorsal grey commissure (DGC), which based on somatic studies contains neurons processing wide dynamic ranges of stimuli that are fundamental to pain discrimination.

The abundant labelling from the lumen extending from LIII into the medial edges of the DGC is a novel finding and may reflect mucosal afferents having a role in relaying innocuous, fine tactile information into specific dorsal horn circuits.

Fig. 3 (continued) representation of the cholera toxin subunit B (CTB) injection sites targeting the wall (green) and lumen (red) of the distal colon in the mouse as described in (Harrington et al. 2019) to identify colonic afferent input in the spinal cord dorsal horn. CTB conjugated to a red Alexa Fluor was applied into the lumen of the colon, whilst CTB conjugated to a green Alexa Fluor was injected into the colonic sub-serosa (referred to as wall injections for simplicity). Injections covered the region of the distal colon 0.5 cm distal and 2 cm proximal to the pelvic bone, targeting regions of the colorectum known to be supplied by the splanchnic and pelvic nerves. (**b**) Schematic representation of the proportions of neurons labelled in the thoracolumbar (TL) and lumbosacral (LS) DRG from CTB wall (green) and lumen (red) colonic injections, adapted from (Harrington et al. 2019). In the TL DRG, the vast majority of the total number of neurons labelled from the colon were from wall injections, with very few labelled from the lumen or dual directed injections, whereas in the LS DRG, three distinct populations of neurons in relatively equal proportions were labelled: (1) those that were labelled from the wall (green), (2) those that were labelled from the lumen (red) and (3) those that were dual labelled (yellow). (**c**) Schematic representation of the differential distribution of afferent projections (**i**) wall or (**ii**) lumen or (**iii**) dual labelled in the TL dorsal horn (T10-L1; T13 depicted for simplicity) from the distal colon as reported in (Harrington et al. 2019). The regions with observed labelled are highlighted. Projections labelled from (**i**) wall-directed injections are observed in the medial and lateral collateral tracts and presumed to terminate within LI, ventral edges of IV and dorsal edges of LV, the DGC and within LX medial to the central canal (Harrington et al. 2012a). Projections labelled from the (**ii**) lumen or those (**iii**) dual labelled were rare in the TL dorsal horn; however, if found they were only in LI. (**d**) Schematic representation of the differential distribution of afferent projections (**i**) wall or (**ii**) lumen or (**iii**) dual labelled in the LS dorsal horn (L5-S2, S1 depicted for simplicity) from the distal colon as reported in (Harrington et al. 2019). The regions with observed labelling are highlighted. Projections labelled from (**i**) wall-directed injections are observed in the medial and lateral collateral tracts and presumed to terminate within LI, medial edges of LIV and LV, in the DGC and within the SPN (Harrington et al. 2012a). Projections labelled from the (**ii**) lumen were observed in the lateral edges of LI and lateral collateral tracts that extended towards the SPN. Dense lumen labelling is also evident in medial collateral tracts extending from the medial edges of LIII into the DGC. (**iii**) Dual traced projections were less abundant than those wall or lumen traced; however, they were present in the lateral edges of LI and extending into the lateral tracts in the SPN and within the dorsal medial edges of the DGC. DGC dorsal grey commissure, LT lateral tract, MT medial tract, LI lamina I, LII lamina II, LIII lamina III, LIV lamina IV, LV lamina V, LX lamina X, LSN lateral spinal nuclei, SPN sacral parasympathetic nuclei, IML intermediolateral cell column

However, to assign functional relevance to the differentially labelled colonic input to shaping colonic spinal processing, identifying the dorsal horn circuits the relay into and their activation profiles to various modalities of colonic stimuli is required.

3.3 Trans-neuronal Viral Tracing from the Colon Wall

Trans-neuronal viral tracing from the colon wall, using pseudorabies virus (PRV), identifies dorsal horn neurons which are synaptically connected, directly or indirectly, to colonic afferent input (Vizzard et al. 2000; Valentino et al. 2000). Trans-neuronal PRV-labelled cells at early time points after colonic infection identify cells receiving direct afferent input. As such in the TL (Fig. 4a-i) and LS (Fig. 4a-ii) spinal cord, the distribution of colonic labelled PRV cells corresponds well with that of colonic afferent projections retrogradely labelled from wall injections (Fig. 3). Following longer infection times, the distribution of PRV-IR cells increases within the same locations but also spread into deep dorsal horn laminae (Vizzard et al. 2000; Valentino et al. 2000). This is indicative of labelling dorsal horn neurons synaptically linked to those receiving direct input, i.e. third- and fourth-order neurons. Given the development of viral tracing constructs that express various fluorescent reporters, it would be valuable to use these in a similar context as the wall/lumen dual retrograde tracing discussed above to identify the dorsal horn circuits synaptically connected to the differential colonic afferent inputs.

4 Spinal Cord Dorsal Horn Circuits Receiving and Processing Colonic Afferent Input

To identify the dorsal horn circuits processing colonic afferent input, noxious pressures of CRD has primarily been used as the activating stimulus. Immunolabelling for markers of neuronal activation or in vivo extracellular recordings have been used to identify CRD-activated neurons and classify them based on laminae distribution or response profiles. Comparisons of these properties between the TL and LS spinal cord provide insight into how colonic mechanosensitivity encoded in the periphery

Fig. 4 (continued) input. (**ii**) In the LS spinal cord, non-noxious CRD evokes neuronal activation (c-Fos neurons) throughout the dorsal horn, with noxious CRD evoking more neuronal activation specifically within the superficial dorsal horn (LI-II) and within the lateral deep dorsal horn (LV-LVI and the SPN) and within the DGC. The wide distribution pattern of CRD activated in the LS dorsal horn only partially corresponds to the pattern of colonic afferent input. DGC dorsal grey commissure, LT lateral tract, MT medial tract, LI lamina I, LII lamina II, LIII lamina III, LIV lamina IV, LV lamina V, LX lamina X, LSN lateral spinal nuclei, SPN sacral parasympathetic nuclei, IML intermediolateral cell column

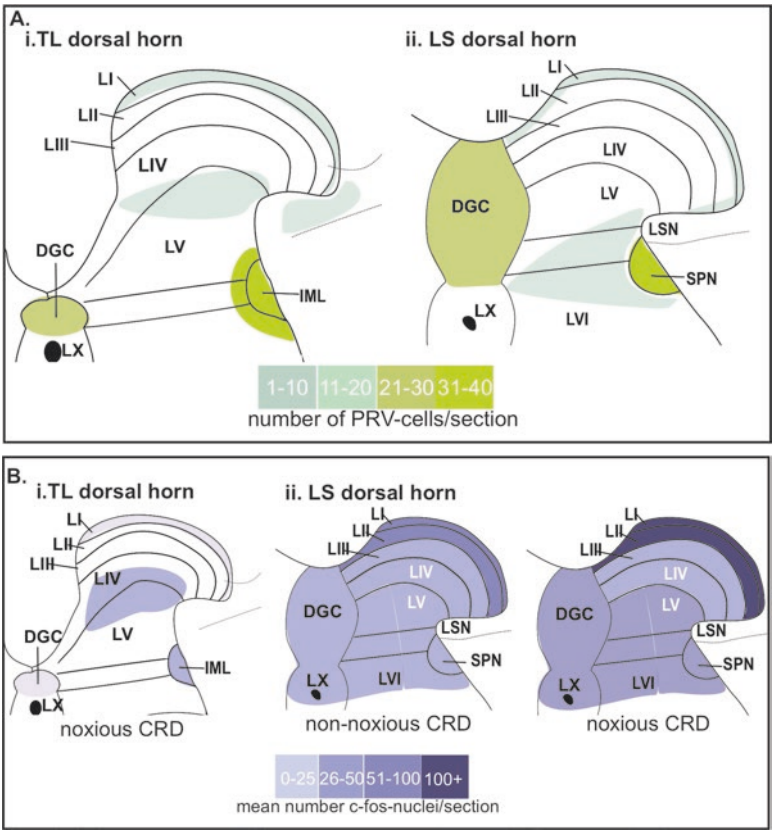

Fig. 4 Distribution and density of cells in the dorsal horn connected to and activated by colonic afferent input in the rat. (**a**) Schematic representations of the (**i**) thoracolumbar (TL) and (**ii**) lumbosacral (LS) dorsal horn summarising the distribution (grey shaded areas) and density (indicated by intensity of shading mean number of cells/section) of PRV-labelled cells 72 h after PRV inoculation in the distal colon wall as reported in (Vizzard et al. 2000; Valentino et al. 2000).(**i**) Within the TL spinal cord, 72 h after inoculation PRV cells are primarily localised to the DGC medial to the central canal and in the IML, with a small number of PRV cells in the medial and lateral regions of the superficial DH, primarily within LI and LV (Vizzard et al. 2000). (**ii**) Within the LS spinal cord, 72 h after inoculation PRV cells are primarily localised to the DGC and in the SPN, with a small number of PRV cells scattered in the medial and lateral regions of the superficial DH, primarily within LI and LV. Located within the medial and lateral superficial DH, around the DCM and the sacral parasympathetic nuclei (SPN) (Vizzard et al. 2000; Valentino et al. 2000). (**b**) Schematic representations of the (**i**) thoracolumbar (TL) and (**ii**) lumbosacral (LS) dorsal horn summarising the distribution (purple shaded areas) and density (indicated by intensity of shading mean number of c-Fos nuclei/section) of activated neurons following in vivo CRD as reported in (Traub and Murphy 2002; Traub et al. 1992, 1993). Reflecting peripheral sensitivity of splanchnic and pelvic colonic afferent nerves, CRD evokes increasing neuronal activation in the LS dorsal horn with ascending CRD pressure, whilst neuronal activation in the TL dorsal horn is primarily evoked by noxious CRD. (**i**) In the TL spinal cord, following noxious CRD, c-Fos-positive neurons are most abundant within the deeper laminae (lateral LIV-V and IML), followed by the superficial dorsal horn (LI) and the DGC. This distribution pattern corresponds to the distribution of colonic afferent

is transferred in the spinal cord, and how it is shaped by afferent input. Various approaches have then been used to identify if CRD-responsive neurons are types of supraspinal projection neurons or interneurons.

4.1 Insights into Dorsal Horn Circuits Processing CRD from Neuronal Activation Distribution Mapping

Numerous studies in rats and mice have used immunolabelling for markers of neuronal activation, c-Fos and phosphorylated MAP kinase ERK1/2 (pERK), to identify the general distribution of dorsal horn neurons activated by CRD (Palecek and Willis 2003; Harrington et al. 2012a, 2019; Palecek et al. 2003a; Traub et al. 1992, 1993, 1995; Traub and Murphy 2002; Zhang et al. 2009). Collectively, these studies show that the amount of neuronal activation evoked by CRD in the TL and LS spinal levels directly reflects the peripheral mechanosensitivity thresholds of splanchnic (high-threshold) and pelvic (mixed low- and high-threshold) afferent endings. Very few neurons, if any, in the TL dorsal horn are activated by non-noxious CRD yet substantial activation is observed upon noxious CRD (Fig. 4b-i) (Harrington et al. 2019; Traub et al. 1993), whereas neurons in the LS dorsal horn are activated in increasing numbers by ascending pressures of distension (Fig. 4b-ii) (Harrington et al. 2019; Traub et al. 1992).

These studies also highlight how the organisation of colonic afferent input shapes where colonic processing occurs in the dorsal horn and thus recruiting the functionally relevant dorsal horn circuits (Harrington et al. 2019; Traub and Murphy 2002). This is more specific in the TL dorsal horn, with the distribution of noxious CRD-activated neurons (Fig. 4b-i) aligning with distribution of retrogradely labelled colonic projections (Fig. 3c) and colonic trans-neuronal PRV cells after short infection times (Fig. 4a-i) (Vizzard et al. 2000). Correspondingly, retrogradely labelled colonic projections have been shown in close proximity to CRD-activated neurons within these laminae (Harrington et al. 2019).

In the LS dorsal horn, CRD-activated neurons are widely distributed across laminae (Fig. 4b-ii) and localised within laminae, in LII-III, in which retrograde labelled input is not evident. However, colonic afferent input directly onto neurons in LI, DGC and the SPN is evident from retrograde tracing and trans-neuronal studies (Fig. 4b-ii) (Harrington et al. 2019; Vizzard et al. 2000; Valentino et al. 2000). Moreover, colonic afferent projections retrogradely labelled from wall/lumen (dual labelled) were shown to be in close association with CRD-activated neurons in the lateral edges of LI, in the mid-dorsal regions of the DGC and in the sacral parasympathetic nuclei (SPN) (Harrington et al. 2019). As such, potentially identifying the input from muscular and muscular/mucosal afferents (stretch sensitive in non-noxious and noxious ranges) that specifically targets spinal circuits in which sensory discrimination and autonomic motor reflexes occur (Brierley et al. 2004; Hughes et al. 2009a; Feng and Gebhart 2011). Lumen-labelled projections are not

found to be closely associated with CRD-activated neurons (Harrington et al. 2019), reflecting the stretch-insensitive nature of mucosal afferent endings, or potentially that these labelled projections could be from mechanically insensitive colonic subtypes. Further studies utilising non-mechanical stimuli will provide more insight into links between specific subtypes of colonic afferents and dorsal horn circuits.

4.2 Insights into Dorsal Horn Circuits Processing CRD from In Vivo Neuron Response Profiling

Studies using in vivo extracellular single-unit electrophysiology have subclassified four dorsal horn neurons involved in processing colonic mechanosensitivity based on their distinct supra-threshold response profiles evoked by noxious CRD (Ness 1999, 2000; Ness and Gebhart 1987, 1988, 1989, 2000, 2001; Wang et al. 2005). Neurons are classified by the length of their excitatory responses following distension termination, with most responses elicited by noxious CRD having short latency and abruptly terminate upon releasing distension (categorised as short-latency abrupt neurons; SL-A). The second type of neurons also responds with short latency; however, responses are sustained after distension termination (categorised as short-latency sustained; SL-S) (Ness and Gebhart 1988). The third type of response is differentiated by their long latency of excitation following the onset of distension categorised as long latency (LL) and is rare in both spinal regions. The fourth class are those inhibited by the onset of distension (Inhib neurons), whereby their spontaneous activity is inhibited by CRD and is more common in the TL spinal cord (Ness and Gebhart 1987, 1988, 1989; Wang et al. 2005). A striking feature in both spinal regions is the amount of descending inhibitory influences shaping every type of neuronal response evoked by noxious CRD. This in context of this review would substantially influence if peripheral input elicits dorsal horn responses.

The abundance of responses with short latency is thought to reflect direct inputs from colonic afferents. This is most evident in the TL spinal cord, with neurons responding to noxious CRD localised in LI, LV and the DGC aligning with input from splanchnic colonic afferents (Ness and Gebhart 1988, 1989). No subclass of neurons can be claimed to be specifically linked to nociceptive input, with all subclasses of neurons responding to CRD throughout a range of distending pressures (Ness and Gebhart 1987, 1988). However, neurons inhibited by CRD have relatively higher thresholds to CRD (30–50 mmHg) than other subclasses of CRD-responsive neurons in the TL dorsal horn. Studies that classify neurons prior to CRD as low-threshold, wide dynamic range (WDR) or high-threshold neurons based on cutaneous responses (Katter et al. 1996) show that LS neurons most responsive to CRD are WDR neurons. This aligns with the LS deep dorsal horn receiving input from functionally diverse colonic afferents.

Comparisons between the TL and LS spinal cord, focusing on the deep dorsal horn, show that response thresholds and maximum responses differ between spinal

levels that reflect peripheral thresholds (Wang et al. 2005). Specifically, neurons in the LS spinal cord have lower activation thresholds and greater response magnitudes than those in the TL spinal cord (Ness and Gebhart 1988; Wang et al. 2005). These studies also highlight that different types of neurons process colonic afferent input between spinal levels, with more SL-S-responsive neurons evident in the LS spinal cord (Ness and Gebhart 1987, 1988; Wang et al. 2005), which may reflect the difference in afferent input or difference in the functional types of neuron-activated between the spinal regions (Wang et al. 2005).

4.3 Distribution and Types of Projection Neurons Relaying Colonic Nociceptive Signalling into the Brain

Neuronal activation marker immunolabelling combined with retrograde tracing from brainstem, midbrain and forebrain nuclei has been used to identify populations of dorsal horn projection neurons activated by visceral stimuli (Palecek et al. 2003a; Menetrey and De Pommery 1991; Menetrey et al. 1989; Clement et al. 2000). However, very few studies have used CRD as the visceral stimuli (Traub and Murphy 2002; Murphy et al. 2009). These CRD studies have predominantly used retrograde labelling from the pontine parabrachial nuclei, which are important relay sites involved in the discrimination and modulation of nociception, and identify the vast majority of LI projection neurons relaying within antero-lateral tracts (Todd 2002, 2010). These studies show a greater percentage of parabrachial projecting neurons are activated by noxious CRD in the LS than the TL spinal cord. In the TL spinal cord these are primarily localised within LI, yet in the LS spinal cord they are more widely dispersed localised within the DGC and in the SPN (Traub and Murphy 2002; Murphy et al. 2009).

Combining in vivo dorsal horn recordings with electrical stimulation of specific supraspinal sites has been used to classify populations of CRD-responsive neurons as projection neurons (Al-Chaer et al. 1999; Ness and Gebhart 1987, 1988, 1989). Supraspinal stimulation sites include upper cervical spinal cord (to identify neurons relaying within the dorsal column), thalamic, midbrain and/or medulla (caudal ventrolateral medulla or the ventromedial medulla). In T13-L2 spinal segments, the majority (66–88%) of CRD-responsive neurons are classed as projection neurons as they are stimulated from the caudal ventrolateral medulla (Ness and Gebhart 1988, 1989). However, other potential projection sites of CRD-responsive TL dorsal horn neurons remain to be assessed. In LS spinal cord, just over half of CRD-responsive neurons in the deep dorsal horn (DGC) are activated by supraspinal stimuli and relay within dorsal column tracts, and in the minority via spinothalamic and spinoreticular tracts (Al-Chaer et al. 1999; Ness and Gebhart 1987).

4.4 Interneuronal Circuits Influencing Colonic Nociceptive Signalling into the Brain

The presence of CRD-activated neurons in the LS dorsal horn LII-IV (Harrington et al. 2019) suggests the recruitment of interneuronal circuits. This is supported by neurochemical labelling for calbindin (LI projection neurons and LI-III excitatory interneurons) and GABA (LI-II inhibitory interneurons) of CRD-activated neurons (Harrington et al. 2019). In the TL spinal cord, a third of CRD-activated neurons within LI contain calbindin and are rarely GABAergic, whereas in the LS spinal cord, CRD-activated neurons that contain calbindin or GABA are distributed throughout LI-III and LV, indicative of interneuron labelling. In the LS dorsal horn, the proportion of CRD-activated neurons co-labelled for calbindin is modestly increased upon noxious CRD when compared to non-noxious CRD. This can be attributed to the recruitment of LI neurons, presumptive projection neurons, which are shown to be in close apposition to colonic afferent projections. Contrastingly, the distribution and proportions of CRD-activated neurons labelled with GABA are unchanged between distension pressures. This indicates that such interneural processing may not be nociceptive specific. Utilisation of more functionally relevant neurochemical markers, molecular profiling and genetic driven manipulation of interneuron circuits is required to distinguish the types of dorsal horn interneurons activated by CRD and what their role is in the spinal processing of visceral nociceptive signalling (Harding et al. 2020; Graham and Hughes 2020; Peirs et al. 2020).

5 Relevance of Characterising Spinal Cord Processing to the Management of Chronic Visceral Pain

Peripheral hypersensitivity evokes adaptative and maladaptive neuroplasticity within the spinal cord dorsal horn and is a fundamental mechanism mediating the development and maintenance of chronic pain (D'Mello and Dickenson 2008). Peripheral sensitisation of colonic afferent endings and the associated mechanisms have been extensively studied in various animal models of acute colitis and post-colitis chronic visceral hypersensitivity (CVH) (Hughes et al. 2009b; Brierley and Linden 2014). Comparatively, colitis-induced remodelling within the spinal cord, specifically those altering the spinal outputs to the brain, is not as well understood. This is despite spinal remodelling proposed as being a key mechanism in facilitating the chronicity and referred pain to non-inflamed visceral organs observed in functional bowel disorders such as irritable bowel syndrome (Verne and Price 2002; Price et al. 2006).

5.1 Spinal Mechanisms of Colitis-Induced Visceral Pain

The approaches outlined above have been used to evaluate how colitis-induced peripheral hypersensitivity translates to activity within the spinal cord dorsal horn. CRD-evoked dorsal horn neuron responses are heightened upon colitis, which persists in models of CVH, primarily of neurons within the superficial laminae and in the DGC (Harrington et al. 2012a; Grundy et al. 2018; Traub and Murphy 2002; Ness and Gebhart 2000, 2001; Castro et al. 2017; de Araujo et al. 2014; Al-Chaer et al. 1997; Olivar et al. 2000). Molecular changes are also evident in the spinal cord upon colitis showing evidence of enhanced excitatory tachykinergic and glutamatergic mechanisms, thus central sensitisation, which in part persist post-colitis (Palecek et al. 2003b; Zhou et al. 2009; Qiao et al. 2008). Opposingly, enhanced inhibitory synaptic properties of CRD-responsive dorsal horn neurons are also evident post-colitis, indicative to neuroplasticity adapting to increased peripheral input (Harrington et al. 2012a; Farrell et al. 2017).

Overall, the summation of pro- and anti-nociceptive plasticity is believed to permit the un-filtered relay of peripheral hypersensitivity into the brain, where it is perceived as hyperalgesia and allodynia. Enhanced output from the TL spinal cord into the antero-lateral ascending tracts and from the LS spinal cord into the dorsal column is evident post-colitis (Traub and Murphy 2002; Al-Chaer et al. 1997). As such altered brain processing of CRD is evident upon colitis and in models of persistent colonic inflammation (Lyubashina et al. 2018, 2019, 2022; Huang et al. 2019; Brenner et al. 2021), replicating alterations observed in ulcerative colitis and irritable bowel syndrome (Mayer et al. 2005). It is however unclear how much of this enhanced spinal relay into the brain is linked directly to hypersensitive afferent input and how much is shaped by alterations within the spinal circuitry. Altered organisation of colonic afferent input is evident in mouse models of chronic recurring and remitting colitis and post-colitis chronic visceral hypersensitivity (Grundy et al. 2018; Benson et al. 2014; Harrington et al. 2012b). Specifically, increased input within superficial LI and ectopic labelling within deeper laminae is evident (Benson et al. 2014; Harrington et al. 2012b). Such anatomical re-organisation of colonic afferent input may have a role in recruiting novel signalling observed within ascending pathways to the brain post-colitis.

6 Conclusion

Collectively, the studies reviewed highlight how the organisation of colonic afferent central terminals within the dorsal horn contributes to translating peripheral-encoded mechanosensitivity within the spinal cord. However, further work is required to establish direct links between subtypes of colonic afferent nerves to modality-specific dorsal horn processing. This will be aided by advances in circuit mapping and optogenetic tools (Harding et al. 2020; Peirs et al. 2020) and the

ever-expanding knowledge on genetic expression profiles of colonic afferent subtypes (Meerschaert et al. 2020; Hockley et al. 2019; Castro et al. 2019). This information will significantly advance how we interpret peripheral hypersensitivity as leading to altered brain processing of visceral pain and aid in the evaluation of peripheral-directed targets for chronic visceral pain management.

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Mechanisms of Spinal Cord Plasticity in Rodent Models of Acute and Post-Colitis Visceral Hypersensitivity

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Abstract Inflammation of the colon is well-established as an initiating factor in the development of chronic visceral pain. Colitis induces sensitisation at peripheral and central sites of the sensory afferent pathways that relay nociceptive signals into the brain. Sensitisation within the spinal cord is an important mechanism maintaining chronic visceral pain beyond colitis resolution and facilitates the development of cross-organ sensitization. This minireview summarises what is currently known from animal acute and post-colitis models on spinal sensitisation and the plasticity involved. These mechanisms have relevance to human inflammatory and functional bowel disorders such as inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). These prevalent conditions afflict millions of people globally and yet adequate treatments for the chronic visceral pain associated with these disorders are lacking.

Keywords Visceral afferent pathways · Colon · Rectum · Colitis · Spinal cord · Visceral pain · Nociception · Dorsal horn and chronic visceral pain

Abbreviations

CRD	Colorectal distension
CVH	Chronic visceral hypersensitivity
DCA	Deoxycholic acid
DH	Dorsal horn
DNBS	Dinitrobenzene sulfonic acid

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DRG	Dorsal root ganglia
DSS	Dextran sulphate sodium
L	Laminae
LS	Lumbosacral
TL	Thoracolumbar
TNBS	2,4,6-Trinitrobenzene sulfonic acid
VMR	Visceromotor response

1 Introduction

Irritable bowel syndrome (IBS) is a functional bowel disorder reported to affect more than 10% of the global population (Oka et al. 2020; Aziz and Simren 2021; Palsson et al. 2020). Recurrent abdominal pain, associated with changes in stool frequency and form, is a key symptom for IBS diagnosis (Aziz and Simren 2021; Schmulson and Drossman 2017). As such pain relief is a major stimulus to seek clinical care (Tornkvist et al. 2021). Despite this, recurrent abdominal pain persists as the most allusive symptom of IBS to clinically manage and is largely due to its multifactorial aetiology that it is incompletely understood (BouSaba et al. 2022). Post-infectious IBS (PI-IBS) develops in more than 10% of patients with acute gastroenteritis and symptomatic abdominal pain can persists for at least 5 years after initiating events (Neal et al. 1997, 2002; Klem et al. 2017; Barbara et al. 2019). The mechanisms underlying chronic pain post-colitis remain to be completely established (Barbara et al. 2019). However it is thought to involve ongoing low-grade mucosal inflammation, abnormal immune activation, microbial dysbiosis and altered intestinal permeability that lead to sensitisation of the sensory afferent nerves innervating the bowel and altering nociceptive signalling to the brain (Price et al. 2006; Brierley and Linden 2014; Balemans et al. 2017).

The colon and rectum are innervated by spinal afferent pathways via the splanchnic and pelvic nerves, which travel up to and synapse onto dorsal horn neurons within the thoracolumbar and lumbosacral spinal cord. Via these nerves, sensory signals from the colon and rectum are transmitted to and processed within the spinal cord before being relayed to the brain. As the spinal cord is the interface between the periphery and the brain, neural circuits within the spinal cord are highly plastic. Therefore, in response to peripheral hypersensitivity spinal cord circuitry can rewire to support protective (adaptive) changes that prevent the relay of harmful nociceptive signalling into the brain. However, plasticity can also be maladaptive and lead to sensitisation of spinal circuits that further amplifies nociceptive output to the brain. Spinal sensitisation enhancing nociceptive output facilitates the development and maintenance of chronic pain in the absence of peripheral drive (Woolf and Salter 2000). Sensitisation occurring at both peripheral and central sites contributes to chronic abdominal pain in IBS (Price et al. 2006). Consistent with sensitisation occurring at the level of the spinal cord, IBS patients in general experience

secondary referred pain and enhanced spinal reflexes from somatic and other viscera organs (Coffin et al. 2004; Verne et al. 2001; Verne 2003; Rodrigues et al. 2005; Moshiree et al. 2007; Grundy and Brierley 2018; Verne and Price 2002). The plasticity supporting spinal sensitisation, specifically induced by inflammatory events in PI-IBS cohorts, remains to be characterised. Therefore, rodent models have largely been instrumental to our understanding of colonic inflammation-induced spinal sensitisation and the plasticity involved.

Replicating PI-IBS, enhanced nociresponsive behaviours to noxious (hyperalgesia) and non-noxious (allodynia) pressures of colorectal distension (CRD) are accompanied by somatic and bladder hypersensitivity in rodent models of post-colitis chronic visceral hypersensitivity (CVH) (Zhou et al. 2008a, b; Grundy et al. 2018; Traub et al. 2008). It is evident from these studies that hypersensitivity is facilitated by spinal sensitisation maintained by ongoing peripheral sensitisation (Feng et al. 2012; Hughes et al. 2009a; Zhou et al. 2008c) and also by plasticity within the spinal cord (Zhou et al. 2009; Harrington et al. 2012). This minireview summarises findings from studies using rodent models of acute and post-colitis CVH on spinal sensitisation and the spinal plasticity shaping altered signalling into the brain.

2 Colitis Induces Peripheral and Spinal Hypersensitivity

2.1 Rodent Models of Colitis

For the benefit of clarification, in this review "acute colitis models" are defined as studies using chemically induced colitis models with experimental end points whilst there is active colitis. These end points range from immediately after inflammation initiating chemical instillation in the colon to 3 h to 3–7 days, depending on the initiating chemical. Colonic instillation of chemicals (such as capsaicin, acetic acid, zymosan or mustard oil) causes immediate neurogenic or short-term colonic inflammation (Lu and Westlund 2001), whereas agents (TNBS, DNBS and DCA) cause T-cell-mediated transmural inflammatory responses that can take a number of days to peak and can persist at low levels for a number of weeks (Traub et al. 2008; Hughes et al. 2009a; Antoniou et al. 2016; Barone et al. 2018). "Post-colitis models" are defined as those that have experimental end points (ranging from 2 weeks to 16 weeks) when active colitis is no longer evident, yet visceral hypersensitivity remains (Zhou et al. 2008a; Traub et al. 2008; Hughes et al. 2009a; Eijkelkamp et al. 2007). Models using DSS to induce colitis, administered via drinking water, are also included in this review despite being referred to as transient chronic colitis models that replicate inflammatory bowel disease, as low-grade colonic inflammation can persist for up to 5 weeks (Eijkelkamp et al. 2007; Dieleman et al. 1998).

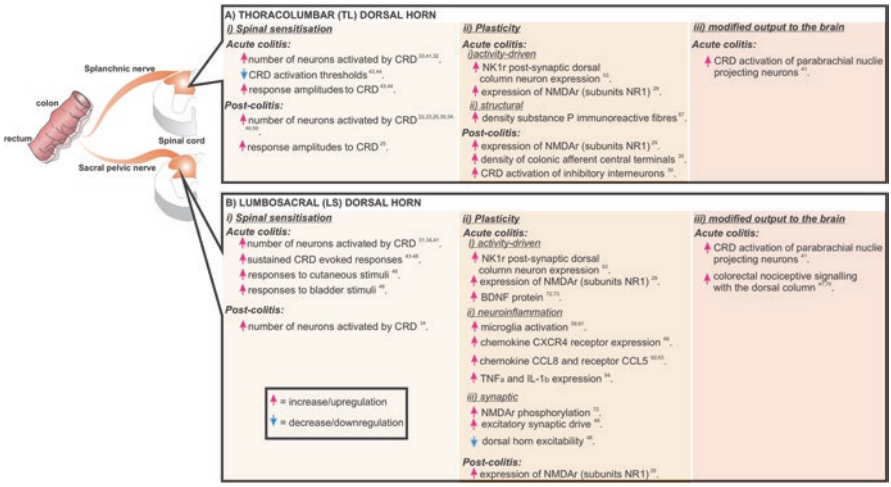

Fig. 1 Summary of the changes within the spinal cord observed in acute colitis and post-colitis rodent models. Schematic representation of the sensory afferent pathways innervating the colon and rectum that travel through two distinct anatomical pathways to the spinal cord. Afferents innervating the length of the colon travel via the lumbar splanchnic nerve and project into the (**a**) thoracolumbar (T10-L1) spinal cord. Afferents innervating the distal colon and rectum travel via the sacral pelvic nerves and project into the (**b**) lumbosacral (L5-S1) spinal cord. In rodent models of acute neurogenic or inflammatory colitis and post-colitis visceral hypersensitivity, enhanced neuronal activation suggestive of ***(i)*** spinal sensitization is evident at both thoracolumbar and lumbosacral spinal levels. Spinal sensitisation is driven in part by the ***(ii)*** plasticity evident within dorsal horn circuits, as a consequence of enhanced afferent input (activity-driven plasticity), and neuroinflammation. Overall, spinal sensitisation following colitis ***(iii)*** modifies nociceptive output from the spinal cord to the brain. Abbreviations: BDNF brain-derived neurotrophic factor, CCL5 chemokine ligand 5, CCL8 chemokine ligand 8, CRD colorectal distension, CXCR4 chemokine receptor type 4, IL-1β interleukin 1 β, MCP-2 monocyte chemotactic protein-2, NMDAr N-methyl-D-aspartate receptors, NK1r substance P neurokinin 1 receptor, TNFα tumour necrosis factor α

2.2 *Peripheral Hypersensitivity*

The colon and rectum are innervated by spinal afferent sensory nerves that relay within the lumbar splanchnic and sacral pelvic afferent pathways (Fig. 1) into the thoracolumbar (TL) (Fig. 1a) and lumbosacral (LS) (Fig. 1b) spinal cord, respectively. Acute colitis rodent models, during active colonic inflammation, show that peripheral endings of both splanchnic and pelvic colonic afferent sensory nerves are hypersensitive to mechanical and chemical stimuli (Feng et al. 2012; Hughes et al. 2009a; Deiteren et al. 2015; Feng and Gebhart 2011). This peripheral hypersensitivity persists in post-colitis models, evident at time points when colitis has resolved, and thus they are referred to as models of chronic visceral hypersensitivity (CVH) (Zhou et al. 2008a; Traub et al. 2008; Feng et al. 2012; Hughes et al. 2009a; Keating et al. 2008). In both acute and post-colitis CVH models, mechanical thresholds of high-threshold colonic afferent endings (nociceptors) are reduced, and conversely

low-threshold or mechanically insensitive afferent endings acquire mechanosensitivity properties (Feng et al. 2012; Hughes et al. 2009b). The neuroplastic changes occurring in colonic afferent neuronal cell bodies contribute to colitis-induced peripheral sensitisation and have been the focus of decades of research; for in-depth review see (Brierley and Linden 2014). Importantly, rodent colitis models have proven clinical translation capacity, having aided the discovery of peripherally restricted agents for the effective management of IBS chronic pain (Castro et al. 2013).

2.3 Spinal Hypersensitivity

Increased neuronal activation is evident in both the thoracolumbar and lumbosacral spinal cord following colitis, which is summarised in Fig. 1a-i, b-i, respectively. Studies using immunolabelling for markers of neuronal activation, such as cFos, show that the number of dorsal horn neurons activated by noxious CRD is increased by acute neurogenic and inflammatory colitis in both thoracolumbar and lumbosacral spinal cord regions (Lu and Westlund 2001; Traub and Murphy 2002; Traub 2000). Of the two spinal regions, however, dorsal horn activation is most significantly enhanced in the thoracolumbar spinal cord (Traub and Murphy 2002; Traub 2000). Supporting this are studies using in vivo electrophysiology approaches that show that during neurogenic and inflammatory colitis dorsal neurons in the thoracolumbar spinal cord are generally more excitable, with a reduction in activation thresholds and greater response magnitudes to CRD (Ness and Gebhart 2000; Wang et al. 2005). This is matched by a marked decrease in the proportion of neurons inhibited by CRD (Wang et al. 2005). In the lumbosacral spinal cord, CRD-evoked responses are similarly potentiated by neurogenic and inflammatory colitis; in particular sustained neuronal responses evoked by CRD are enhanced (Ness and Gebhart 2000, 2001; Wang et al. 2005; Olivar et al. 2000; Al-Chaer et al. 1997; Farrell et al. 2017). Using the same approach, lumbosacral dorsal horn responses evoked by bladder stimulation are shown to be enhanced by colitis (Qin et al. 2005). Collectively these studies show that colitis induces dorsal horn-wide changes in neuronal excitability, indicative of spinal sensitisation, that facilitates increased dorsal horn activation to colonic input and cross-organ sensitisation. Heightened dorsal horn neuron responses at both spinal levels to noxious colorectal stimuli are also evident in models of post-colitis CVH, indicating that dorsal horn hyperexcitability persists chronically in the absence of overt peripheral inflammation (Grundy et al. 2018; Traub et al. 2008; Harrington et al. 2012; Eijkelkamp et al. 2007; de Araujo et al. 2014).

3 Plasticity in the Spinal Cord Mediating Colitis-Induced Spinal Sensitisation

The increase in dorsal horn neuron activation and recruitment of dorsal horn neurons to colonic nociceptive signalling upon colitis may be a direct reflection of peripheral hypersensitivity recruiting low-threshold and mechanical insensitive afferents to nociceptive signalling. However, plasticity within the dorsal horn circuits is also evident upon colitis, summarised in Fig. 1a-ii, b-ii, that would contribute to spinal sensitisation. Studies identifying colitis-induced plasticity within the spinal cord have focused on two main mechanisms, activity-driven plasticity and neuroinflammation, and have primarily targeted the lumbosacral spinal cord.

3.1 Activity-Driven Plasticity

Common mechanisms by which peripheral inflammation leads to spinal sensitisation involve increased release of transmitters substance P (SP), glutamate, calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor (BDNF) from sensitised afferent input. The subsequent activation of their post-synaptic receptors is key to producing neuronal sensitisation (Latremoliere and Woolf 2009). Substance P has been linked to the generation, but not the maintenance, of spinal hyperalgesia induced by colitis (Farrell et al. 2017). This is thought to be mediated via de novo expression of substance P neurokinin 1 receptors (NK1rs) by post-synaptic dorsal column neurons in the thoracic and lumbosacral spinal cord levels (Laird et al. 2000; Palecek et al. 2003), whilst spinal ionotropic glutamate receptors, N-methyl-D-aspartate (NMDAr) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAr) have been shown to mediate enhanced colorectal nociresponsive behaviours during acute colitis and post-colitis (Zhou et al. 2009; Coutinho et al. 1996). Specifically, intrathecal administration of NMDAr antagonists, at the lumbosacral spinal level, attenuates colitis-enhanced nociresponsive behaviours by 60%, whereas AMPAr receptor antagonist reduced responses by only 20% (Coutinho et al. 1996). Correspondingly, increased expression of NMDrs in both thoracolumbar and lumbosacral spinal regions is evident during acute colitis, which persists for up to 16 weeks post-colitis (Zhou et al. 2009). Importantly, these studies showed that increased NMDAr expression only occurred in rodents that developed chronic hypersensitivity post-colitis.

3.2 Neuroinflammation

Increased signalling from sensitised afferent input can also promote resident spinal glia, microglia and astrocytes, to release pro-inflammatory cytokines, chemokines and growth factors that can then cause further structural and synaptic plasticity of

afferent input and dorsal horn neurons. For more in-depth review on neuroinflammatory mechanisms of dorsal horn structural and synaptic plasticity in inflammatory chronic pain, see (Hiraga et al. 2022; Dodds et al. 2016; Long et al. 2022). The activation of spinal cord microglia has a significant role in the development of visceral hyperalgesia and cross-organ sensitisation induced by colitis (Lu 2014; Kannampalli et al. 2014; Saab et al. 2006). This is made evident by studies showing that intrathecal administration of minocycline, a tetracyclic antibiotic that blocks microglial activation, into the lumbosacral spinal cord attenuates colitis-induced microglia proliferation within the spinal cord (Majima et al. 2018). And by doing so it reduces the nociresponsive behaviours and dorsal horn responses enhanced by colitis (Kannampalli et al. 2014), as well as colitis-induced bladder overactivity (Majima et al. 2018). Numerous studies have since shown that neuronal expression of pro-inflammatory mediators and chemokines (Majima et al. 2018; Lu et al. 2017, 2018, 2019; Basso et al. 2017) and their receptors (Zhang et al. 2022) plays a role in the development of colorectal hyperalgesia and bladder dysfunction following colitis. The influence of such neuroinflammation on structural and synaptic plasticity within the dorsal horn post-colitis remains to be assessed.

3.3 Structural Plasticity

Structural plasticity in the spinal cord is evident in chronic colitis and post-colitis models; however, more in relation to how visceral afferent input is organised rather than in the dorsal horn circuits themselves. In a mouse model of chronic recurring and remitting DSS-induced colitis, the density of substance P-immunoreactive fibres, indicative of afferent input, is increased within the dorsal horn (Benson et al. 2014). More specific to colonic afferent organisation, in a model of post-colitis CVH, the density of afferent projections retrogradely labelled from the colon wall is increased in the superficial layers of the dorsal horn of the thoracolumbar spinal cord (Grundy et al. 2018; Harrington et al. 2012). This increase occurs without changes to the number of colonic labelled afferent neuronal cell bodies labelled within the dorsal root ganglia, suggesting re-organisation of colonic input is local to the dorsal horn. Both studies show evidence of increased colonic afferent input within deep dorsal horn regions not observed in healthy mice that may contribute to the recruitment of novel dorsal horn circuits to colorectal nociceptive processing (Harrington et al. 2012; Benson et al. 2014).

3.4 Synaptic Plasticity

Modulation of excitatory and inhibitory synaptic strength within the dorsal horn is a common mechanism facilitating chronic pain resulting from inflammation (Bardoni et al. 2013). This involves a strengthening of excitatory controls and a

reduction in inhibitory controls, referred to as disinhibition (Zeilhofer et al. 2021; Harvey et al. 2004), the summation of which facilitates increased high- and low-threshold afferent input onto projection neurons altering output to the brain (Torsney and MacDermott 2006). Up-regulation of NMDAr NR1 phosphorylation, indicative of enhanced excitatory synaptic strength and efficacy, is evident upon colitis (Liu et al. 2015). This has shown to be mediated by BDNF (Liu et al. 2015). As BDNF protein, but not mRNA, is increased in both thoracolumbar and lumbosacral spinal levels following colitis (Qiao et al. 2008), the source of BDNF is proposed to be sensitised afferent input rather than activated glia. In vivo patch-clamp recordings of dorsal horn responses to CRD show functionally that excitatory synaptic drive within the superficial layers of the dorsal horn is enhanced during acute colitis (Farrell et al. 2017). However, this was shown to be matched by an overall decrease in the excitability of dorsal horn networks (Farrell et al. 2017). Such findings were proposed to indicate that increased afferent signalling into the dorsal horn is accompanied by a degree of adaptative plasticity within dorsal horn circuits (Hughes and Todd 2020). In accordance with this, inhibitory interneurons have shown to be more responsive to CRD in a model of post-colitis CVH (Harrington et al. 2012).

4 Spinal Sensitisation Contribution to Altered Nociceptive Signalling to the Brain

There have been few studies directly assessing how enhanced activity and plasticity evident in the dorsal horn upon colitis modify spinal cord output to the brain. These are summarised in Fig. 1a-iii, b-iii. Dorsal horn projection neurons terminating in the pontine lateral parabrachial nuclei are more responsive to noxious CRD during colitis, most significantly in the thoracolumbar spinal cord (Traub and Murphy 2002), whilst colorectal nociceptive signalling within the ascending dorsal column from the lumbosacral spinal cord is enhanced upon colitis (Al-Chaer et al. 1997; Palecek and Willis 2003). However, it remains to be determined if these specific changes continue post-colitis, if they are also recruited by non-noxious input, facilitating allodynia, and/or if other populations of projection neurons with different supraspinal targets may also be recruited to colonic nociceptive signalling.

The parabrachial nuclei are involved in conveying nociceptive information to higher structures; thus, the increased activity within the thoracolumbar spinal cord may contribute to an overall net increase in colorectal nociception upon colitis, manifesting as hyperalgesia, whereas sensitisation within dorsal column in the lumbosacral spinal cord is suggested to contribute more towards changes in pain discrimination, manifesting as allodynia. However, the parabrachial nuclei and the dorsal column are also involved in engaging descending pain modulation mechanisms that either facilitate or inhibit nociceptive transmission within the spinal cord. Correspondingly, signalling within such spino-bulbo-spinal loops has shown to be enhanced in various models of inflammatory chronic pain (Chen and Heinricher 2019a, b; Heinricher et al. 2009; Porreca et al. 2002). A number of studies show

changes within medullary and brainstem regions that suggest descending input into the spinal cord is altered upon colonic inflammation (Lu and Westlund 2001; Lyubashina et al. 2018, 2019; Coutinho et al. 1998; Friedrich and Gebhart 2003; Sanoja et al. 2010). However, studies exploring spinal mechanisms are limited, with these showing potential enhanced opioidergic signalling but no change to inhibitory adrenergic influences upon colitis (Traub et al. 2008; Pertovaara and Kalmari 2003). This is despite colorectal nociceptive signalling in the spinal cord shown to be strongly influenced by descending input (Sikandar et al. 2012; Liu et al. 2008; Gebhart 1993; Sikandar and Dickenson 2012) and that the impairment of descending pain modulation controls are evident in IBS cohorts (Wilder-Smith et al. 2004; Heymen et al. 2010).

5 Concluding Remarks

Overall, it is evident that colitis-induced peripheral hypersensitivity translates to enhanced activation within spinal cord dorsal horn circuits and that this facilitates spinal sensitisation and altered spinal signalling to the brain. As outlined, activity-driven plasticity and neuroinflammation are evident in the spinal cord upon colitis. Furthermore, such mechanisms may initiate structural and synaptic plasticity. However, many of the studies exploring spinal changes induced by colitis have used models at acute stages, during periods at which inflammation would still be significantly driving peripheral hypersensitivity. Given this, it remains to be determined how the plasticity evident during acute colitis phase develops in a post-colitis setting and contributes to long-term remodelling of the dorsal horn circuits projecting into the brain. As such there is a knowledge gap on the spinal plasticity maintaining hyperalgesia, allodynia and cross-organ sensitisation post-colitis, which is most relevant to PI-IBS.

Identifying the plasticity relevant to chronic pain is important when evaluating novel therapeutic targets and how effective they are at rewiring peripheral and central plasticity shaping chronic pain and cross-organ sensitisation associated with PI-IBS. Spinal remodelling can maintain abnormal pain signalling in the absence of peripheral hypersensitivity, which can be targeted by peripheral-restricted therapies. Therefore, evaluating the capacity of such peripherally restricted agents to also correct changes within spinal circuits long term is vital to assess rodent models of post-colitis CVH, as are the effects of therapies directly targeting the spinal cord, such as spinal cord stimulation, or those that target cross-sensitised somatic afferents, such as acupuncture.(Kim et al. 2017)

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Neuron-Microglia Dynamic Duo in Chronic Abdominal Pain

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Abstract Abdominal pain is one of the main symptoms of chronic gastrointestinal disorders including inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). The pathological mechanisms of chronic visceral pain have not been fully resolved. Pain management remains challenging and a source of frustration for health-care providers as current treatments are limited and lead to many side effects. Growing evidence indicates that functional changes in neuron-glia communication, at different levels of the gut-brain axis, contribute to visceral sensitization. In fact, a key role of spinal glial cells in the development and maintenance of hypersensitivity has been reported in several preclinical models of visceral pain. Here we provide an overview of the current knowledge on the factors, receptors, and transduction signaling pathways responsible for spinal neuron-glia interaction in chronic visceral pain.

Keywords Colitis · Sensitization · Microglia · Astrocytes · Nociceptors

1 Introduction

Chronic abdominal pain is one of the most common causes of disability and impaired quality of life for patients with bowel diseases such as inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). IBD, including Crohn's disease and ulcerative colitis, are chronic relapsing and remitting inflammatory diseases associated with abdominal pain (Norton et al. 2017). Pain is reported by over 90%

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of patients with active IBD, with the expectation that in the majority of patients, symptoms will resolve if the inflammation is under control (Schirbel et al. 2010; Wagtmans 1998). However, many IBD patients (20–60%) continue to experience visceral hypersensitivity (VHS), despite being in endoscopic remission (Farrokhyar et al. 2006; Schirbel et al. 2010). Accordingly, chronic abdominal pain in IBD has often been related to co-existing IBS where abdominal pain is considered a cardinal feature (Schmulson and Drossman 2017). IBS is a functional bowel disorder characterized by abdominal pain, stool irregularities, and bloating (Enck et al. 2016). In comparison with active IBD, IBS is not associated with structural or biochemical abnormalities that are detectable with the current routine diagnostic tools (Enck et al. 2016). Thus, the overlap between quiescent IBD and IBS has proven difficult to tease apart. Some of the chronic abdominal pain in IBD has been reported to be due to IBS-type symptom patterns, with up to 35% of quiescent IBD patients meeting IBS diagnostic criteria (Ozer et al. 2020; Teruel et al. 2016).

Given the complexity of the pathogenesis of chronic abdominal pain under these disease conditions, current pharmacotherapies are quite ineffective and rely mostly on NSAIDs or narcotics that can lead to serious side effects (Zielińska et al. 2019). The establishment of chronic visceral pain results from neuroplasticity in colonic nociceptors first and then along the entire neural axis. Providing functional and metabolic support to neurons, glial cells sense neural activity and respond to fine-tune excitability in the peripheral and central nervous system. Recent work has shown that glial cells in the CNS can contribute to visceral sensitization. Identifying the role of neuronal and non-neuronal cells, particularly glial cells, in the development and persistence of pain, may advance the development of novel and safer treatment modalities to manage chronic abdominal pain.

2 Visceral Pain Circuits

The GI tract is innervated by spinal sensory neurons or nociceptors that have their cell bodies in the dorsal root ganglia (DRGs) along the spinal cord. Primary afferents that innervate the upper GI tract originate from the thoracolumbar region (TL; T10-L2) of the spinal cord (via the splanchnic nerve), while those that innervate the distal colon and rectum originate from the lumbosacral region (LS; L5-S1) (via the pelvic nerve) (Abdullah et al. 2020; Gebhart and Bielefeldt 2016; Grundy et al. 2019). In the dorsal horn of the spinal cord, gut-innervating afferents synapse with interneurons both excitatory and inhibitory and second-order neurons which convey nociceptive signals to supraspinal sites by two ascending spinal pathways: the spinothalamic tract and spinoparabrachial pathway (Gebhart and Bielefeldt 2016; Grundy et al. 2019). The spinoparabrachial pathway is made of superficial dorsal horn neurons (laminae I and II), projecting to affective areas of the brain, including the amygdala, hypothalamus, and periaqueductal gray (PAG). In contrast, the spinothalamic tract originates from the deep dorsal horn (lamina X), relaying visceral

sensory input to the thalamus and cortical areas for sensory discrimination and localization (Grundy et al. 2019).

3 Visceral Sensitization

The establishment and maintenance of persistent visceral pain is caused by long-lasting neuroplastic changes that occur in both the peripheral and central nervous system (Brierley and Linden 2014). Under inflammatory conditions, pro-inflammatory lipids, peptides, cytokines, and chemokines not only stimulate immune, epithelial, and stromal cells of the GI tract but also act on gut-innervating primary afferent neurons (Abdullah et al. 2020). Mechanistically, activation of transduction signaling pathways upon inflammation leads to ion channel modulation that in turn induces changes in the electrophysiological properties of colonic sensory neurons, characterized by reduced threshold of activation and enhanced excitability, a process described as sensitization (Basbaum et al. 2009). Among ion channels found in visceral afferent neurons, the transient receptor potential vanilloid 1 (TRPV1) has been implicated in sensing and transducing inflammatory signals (Bourinet et al. 2014; Lapointe et al. 2015). Several studies have demonstrated that peripheral sensitization of TRPV1 mediates VHS in both animal model and IBD/IBS patients (Akbar et al. 2010; Lapointe et al. 2015; Perna et al. 2020; Wouters et al. 2016). Although we have a good understanding of the mechanisms of sensitization at the periphery during intestinal inflammation, little is known about the cells and mediators that drive central sensitization in the spinal cord and the brain (Brierley and Linden 2014). Recent evidence indicates that activation of glial cells could play a central role. Eight major cell types have been identified in the mouse spinal cord (Sathyamurthy et al. 2018). Among them, microglia and astrocytes are activated following nerve injury or inflammation (Gu et al. 2016; Peng et al. 2016; Zhang et al. 2005), highlighting the importance of dorsal root ganglia (DRG) neuron-glial cell interactions in pathological pain.

4 Spinal Glial Cells in VHS

4.1 Microglia Activation in VHS

Microglia, the resident immune-like macrophages of the central nervous system (CNS), make up 10% of the total CNS glial population (Lu 2014). During neurodevelopment, microglia play a central role in the elimination and consolidation of synapses, shaping neural circuit connectivity and regulating subsequent functional activity (Schafer and Stevens 2015). Moreover, microglia control neuronal homeostasis and their activation is associated with a wide range of neurological disorders

(Szepesi et al. 2018). Notably, there is a long list of publications on microglia and pain. Over 70% of the publications focused on spinal microglia, showing spinal microglia-neuron cross-talk as a main contributor to the development of chronic pain (Ho et al. 2020). Once activated, they congregate in the dorsal horn of the spinal cord (microgliosis) where they initiate a panel of innate defense mechanisms, including the production of cytokines that contribute to the activation and sensitization of colonic nociceptors (Marinelli et al. 2019). Additionally, microglia are known to express receptors for neuropeptides and neurotransmitters which, upon activation, modulate microglia reactivity (Marinelli et al. 2019). With regard to visceral sensitivity, the role of spinal microglia and the spinal neuro-immune mechanisms underlying visceral sensitization have been barely addressed.

Colitis-induced pain models In active 2,4,6-trinitrobenzenesulfonic acid solution (TNBS)-induced colitis, Kannampalli et al. first demonstrated that mice with VHS exhibited microglial activation in the lumbosacral (LS) spinal dorsal horn receiving sensory input from the colon (Kannampalli et al. 2014). Notably, Majima et al. showed that microglial reactivity in TNBS-induced colitis model participates to cross-sensitization of bladder afferents (Majima et al. 2018). Using a dextran sodium sulfate (DSS) model of ulcerative colitis, we reported an activation of microglia in the spinal dorsal horn (Basso et al. 2017; Defaye et al. 2021; Huang et al. 2020). This was denoted by an increase in Iba1-labeled cells and a change from ramified to ameboid shape. Interestingly, microgliosis was associated with VHS acutely and post-resolution of inflammation (Defaye et al. 2021; Lucarini et al. 2020), suggesting a putative role of microglia activation in the maintenance of visceral hypersensitivity following disease remission.

During active colitis, microglial activation is characterized by both microgliosis and increased levels of inflammatory cytokines such as interleukin (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1 α (MIP-1α or CCL3), brain-derived neurotrophic factor (BDNF), and granulocyte-colony-stimulating factor (G-CSF) (Basso et al. 2017; Huang et al. 2020; Majima et al. 2018). Local administration of the anti-mitotic agent, AraC, that stops microgliosis in the spinal dorsal horn reduced colitis-induced VHS (Kannampalli et al. 2014). Along these lines, inhibiting microglial activation with intrathecal injection of the tetracycline antibiotic minocycline could prevent VHS in TNBS colitis (Kannampalli et al. 2014; Majima et al. 2018). Importantly, intrathecal minocycline was shown to normalize the colitis-induced expression of pro-inflammatory IL-1β and CCL3, as well as the neurogenic and pronociceptive BDNF, in the spinal cord (Majima et al. 2018). Overall, cumulative evidence suggests that colitis-induced activation of microglia leads to the production and release of pro-inflammatory cytokines such as IL-1β and TNF-α, at proximity of synapses that relay nociceptive information from the GI tract. Facilitating synaptic transmission in the spinal dorsal horn by these mediators and neurogenic factors will play a central role in sensitizing nociceptive input and triggering pain-specific neuroplastic changes in the ascending pain pathway (Kawasaki et al. 2008). Accordingly, glutamatergic synapses contribute to the process of central sensitization associated with all forms of pathological pain

conditions, and phosphorylation of N-methyl-D-aspartate (NMDA) receptor-NR2B subunit was found to be a surrogate marker of central sensitization in the spinal cord (Huang et al. 2020). Notably, the increase in NR2B phosphorylation was found to persist after resolution of intestinal inflammation and to correlate with hyperalgesia. Whether NMDAR phosphorylation dictates the activation state of microglia in visceral pain models remains to be explored. In the 2,4-dinitrobenzenesulfonic acid (DNBS) model of colitis, inflammation peaks at day 3 and resolves progressively from day 7. Both mice and rat models develop VHS up to 21 days post-administration, and Lucarini et al. reported microglial activation in both dorsal and ventral horn of the lumbar spinal cord during the recovery phase (Lucarini et al. 2020). Interestingly, microglia did not change in density but underwent well-defined morphological alterations (loss of the processes under activation). Using the DSS-induced colitis model, we demonstrated that pro-inflammatory G-CSF activates microglia through G-CSF receptor. We showed that G-CSF drives post-inflammatory pain via cathepsin S-fractalkine-NO signaling axis from spinal microglia and acts on colonic nociceptors that express TRPV1 (Fig. 1) (Basso et al. 2017). Several studies have demonstrated that sensitization of TRPV1-expressing colonic neurons mediates VHS during resolution of colitis, a process that promotes abdominal pain in preclinical models and likely patients with quiescent IBD and IBS (Akbar et al. 2010; Lapointe et al. 2015; Perna et al. 2020; Wouters et al. 2016). We recently investigated whether VHS results from the direct communication between sensitized

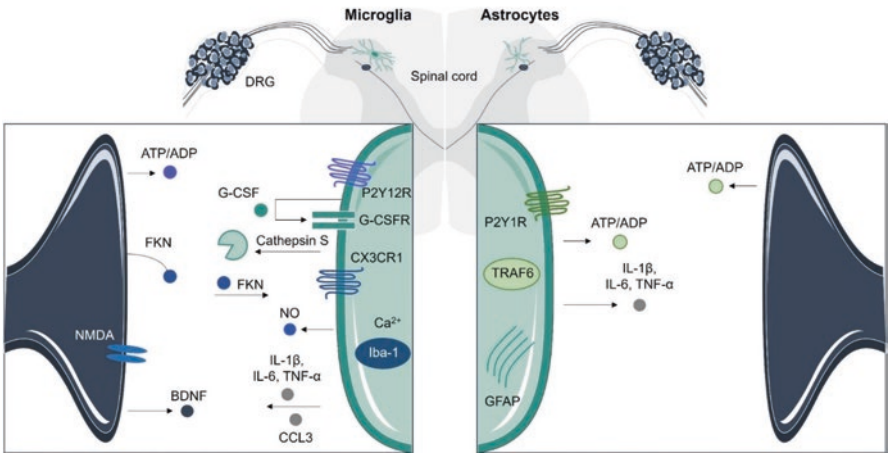

Fig. 1 Spinal neuron-glia communication in visceral hypersensitivity. The schematic figure represents the main molecular signaling pathways involved in neuron-glial cell communication. ATP adenosine triphosphate; ADP adenosine diphosphate; G-CSF granulocyte colony-stimulating factor; CX3CL1/FKN fractalkine; CX3CR1 C-X3-C motif chemokine receptor 1; NO nitric oxide; NK1R neurokinin 1 receptor; NMDA receptor N-methyl-D-aspartate receptor; IL-1β interleukin-1β; IL-6 interleukin-6; TNF-α tumor necrosis factor-α; CCL3 C-C motif chemokine ligand 3; BDNF brain-derived neurotrophic factor; TRAF6 TNF receptor-associated factor 6; GFAP glial fibrillary acidic protein; DRG dorsal root ganglion

TRPV1 nociceptors and spinal microglia. Using designer receptors exclusively activated by designer drugs (DREADD) expressed in TRPV1+ nociceptors, we tested whether neuronal activity was indispensable to control spinal microglia activation and VHS. We found that chemogenetic inhibition of TRPV1+ nociceptors could prevent microglial activation in the spinal dorsal horn and subsequent VHS in colitis mice. In contrast, in naïve condition, chemogenetic activation of gut-innervating TRPV1+ nociceptors enhanced microglial activation and associated VHS, in the absence of colitis. We then identified a purinergic signaling mechanism mediated by neuronal ATP and microglial P2Y12 receptor, triggering VHS in colitis. Importantly, inhibition of P2RY12 prevented microglial reactivity and chronic VHS post-colitis. Altogether, these results demonstrated that ATP-releasing TRPV1+ visceral afferents and P2RY12 signaling in the spinal microglia orchestrate the establishment of VHS that persists following remission of colitis (Defaye et al. 2021). Our work strongly suggests that preventing microglial activation or inhibiting P2Y12 receptors centrally could provide therapeutic value to treat both acute and post-inflammatory visceral pain (Fig. 1).

It will be important to determine whether distinct microglia subpopulations or activation states contribute to different stages of visceral sensitization. This could be done using single-cell transcriptomic analysis in the lumbosacral spinal cord at different stages of colitis. Lastly, while we did not notice any sexual dimorphism in microgliosis under colitis, the activation states and microglia subtypes may be different between males and females (Tansley et al. 2022). Future work will address how transcriptional and functional changes in microglia subpopulations drive VHS. Identifying a VHS-related transcriptional signature of microglia will provide insights into the central mechanisms and potential targets to stop the development and maintenance of VHS in colitis.

Non-inflammatory pain models IBS-like symptoms, including pain, are often experienced in IBD patients in remission (Enck et al. 2016). Yet, whether similar mechanisms of neuron-microglia interactions contribute to VHS in IBS has been poorly investigated. Using various models of stress-induced IBS, several studies highlighted the role of microglia in non-inflammatory VHS. Saab et al. first showed that stress induced by neonatal irritation (CCI) induced VHS associated with microglial reactivity in the lumbosacral spinal dorsal horn at adulthood (Saab et al. 2006). In addition, chronic psychological stress induced by repetitive water avoidance leads to microglia activation in the lumbar dorsal horn (Bradesi et al. 2009). Both of these studies demonstrated that intrathecal injection of fractalkine (FKN) in naïve animals induces VHS, supporting the hypothesis that FKN could trigger microglial activation and visceral sensitization (Bradesi et al. 2009; Saab et al. 2006). Accordingly, both water avoidance and fractalkine-induced hyperalgesia were blocked by minocycline. Recently, Zhan et al. reported an increase of Iba-1-positive cells in the spinal dorsal horn of acetic acid-treated mice. They found that ulinastatin, a broad-spectrum serine protease inhibitor, reduced acetic acid-induced writhing and microglial activation in the spinal dorsal horn (Zhan et al. 2021).

Importantly, similar to stress, drugs can also trigger neuroinflammation leading to VHS. One example is the use of opioids for functional and chronic gastrointestinal pain, which is not as beneficial as previously assumed. Indeed, opioids can cause unexpected abdominal pain (Farmer et al. 2017; Kong and Burns 2021). When pain is the major symptom of opioid use, this condition is called narcotic bowel syndrome (NBS). NBS is defined by an increase in abdominal pain despite maintaining or increasing doses of narcotics. Agostini et al. showed that chronic morphine-induced VHS is associated with spinal microglia activation in rats (Agostini et al. 2010). Using minocycline, they reduced narcotic-induced hypersensitivity responses to CRD. While it is clear that the mu-opioid receptor is not responsible for morphine-induced microglia activation (Corder et al. 2017), how microglia sense and respond to morphine is still a matter of debate.

4.2 Astrocyte Activation in VHS

Astrocytes make up 20–40% of all of the glial cells in the CNS. Under homeostasis conditions, astrocytes regulate functions, such as synapse formation and CNS homeostasis, by providing neurons with metabolites and growth factors and regulating the balance of ions, fluids, and neurotransmitters (Sofroniew and Vinters 2010). Evidence supports a role of astrocytes in persistent neuropathic pain. After injury, the phenotype, functions, and gene expression profile of spinal astrocytes can undergo significant changes, known as reactive astrogliosis (Li et al. 2019). Only few studies have described the involvement of astrocytes in VHS.

Colitis-induced pain models Intestinal inflammation induced by TNBS leads to the activation of astrocytes in the lumbosacral spinal dorsal horn as measured by glial fibrillary acidic protein (GFAP) expression, an astrocyte gliosis marker (Sun et al. 2005). The density of $GFAP^+$ astrocytes in the spinal cord was found to decrease comparable to control level at day 28 post-TNBS, when the animals recovered from intestinal inflammation. This indicated that astrocyte activation is inflammation dependent and returns to homeostatic state after remission of colitis. Accordingly, Lucarini et al. reported an activation of astrocytes in both dorsal and ventral horn of the spinal cord in a rat model of DNBS-induced VHS. Astrocytes increased significantly in density (about 30%) and exhibited activated states characterized by expansions of cellular bodies and processes in the dorsal spinal cord (Lucarini et al. 2020) (Fig. 1).

However, the mechanisms underlying astrocyte activation are still unclear. Future works will address whether neuronal subsets and mediators contribute to VHS-associated astrocyte reactivity. Also, as mentioned previously for microglia, different astrocyte subpopulations may contribute to visceral sensitization (Li et al. 2019). It will be important to investigate whether distinct astrocyte subpopulations contribute to different disease stages.

Non-inflammatory pain models In preclinical models of IBS, spinal astrocyte activation is still controversial. Using the maternal separation model of IBS, Gosselin et al. did not find a global change in spinal astrocytic phenotype (Gosselin et al. 2010). However, an increase of GFAP positive cells in the spinal dorsal horn was reported using neonatal intracolonic acetic acid (AA) model of IBS (Weng et al. 2020; Zhao et al. 2020). In addition, an upregulation of TRAF6 in spinal astrocytes was observed in adult mice in response to neonatal irritation (Weng et al. 2020). Depletion of TRAF6 using small interfering RNA (siRNA) alleviated VHS and reduced the amplitude of spontaneous excitatory postsynaptic currents in the spinal dorsal horn (Weng et al. 2020). Zhao et al. provided insights into the interactions that occur between astrocytes and nociceptors in the spinal cord (Zhao et al. 2020). Previous studies had shown a role of P2Y receptors in stimulating visceral hypersensitivity. P2YR is expressed by 56% to 80% of retrogradely labeled colonic neurons, indicating a P2Y-dependent mechanism of VHS (Hockley et al. 2016). Using the AA model of visceral pain, Zhao et al. found a reduction in spinal expression of IL-6, IL-1β, TNF-α, GFAP, and P2Y1 in response to electroacupuncture. These molecular changes were associated with a decrease in VHS (Zhao et al. 2020). Importantly, intrathecal administration of the astrocyte inhibitor, fluorocitrate, or the P2Y1 receptor antagonist, MRS2179, reduced AA-induced visceral hypersensitivity, raising the hypothesis that electroacupuncture negatively regulates P2Y1 receptor in astrocytes. Finally, as previously described with microglial activation, ulinastatin treatment blocked astrocyte activation (Zhan et al. 2021).

5 Conclusion and Future Perspectives

Children and adults living with chronic visceral pain tend to develop sleep disorder, anxiety, and depression. While efforts have been made to understand the mechanisms and neural circuits of visceral sensation, the pathophysiology of gut pain is very complex and involves many cell types and signaling pathways at the peripheral and central level. Our group and others have demonstrated the importance of both spinal microglia and astrocytes in the transition from acute to chronic visceral pain. Collective results strongly suggest that modulating spinal glial cells via the targeting of purinergic signaling necessary for glial reactivity may provide therapeutic value to treat chronic visceral pain. Although many purinergic blockers are clinically available, it will be important to address their analgesic properties in both IBD and IBS conditions. Lastly, given the growing importance of the role of the microbiome in the gut-brain axis, future work should identify microbial-based molecules as regulators of neuron-glial communication and treatments for visceral pain.FundingThis work was supported by operating grants from the Crohn's and Colitis Canada (CCC) and the Canadian Institutes of Health Research (CIHR). MD holds a fellowship from the Alberta Children's Hospital Research Institute (ACHRI). CA holds a Canada Research Chair in inflammatory pain.

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Pre-clinical Models of Endometriosis: A Focus on Chronic Pain

Jessica Maddern, Stuart M. Brierley, and Joel Castro

Abstract Endometriosis is a multifaceted chronic disease with a complex and variable display of clinical symptoms. Chronic pelvic pain (CPP) affects most women with endometriosis, leaving patients frequently suffering from widespread pain, often associated with painful comorbidities. Despite this significant disease burden, limited progress has been made in elucidating the mechanisms contributing to endometriosis-associated CPP development. Accordingly, therapies that adequately target and treat the debilitating pain remain lacking. Small animal models of endometriosis show considerable promise as pre-clinical tools to investigate the mechanisms of CPP; however, the majority of these studies focus on the pathogenesis and development of endometriosis, which leaves the mechanisms underlying CPP elusive. When established behavioural techniques are incorporated into these pre-clinical animal models of endometriosis, it is apparent that they recapitulate key pain attributes that are observed clinically in women with endometriosis. However, studies often employ only one or two behavioural techniques focused on an individual symptom, rather than utilising multiple techniques to uncover the mechanisms underlying diverse and comorbid pain. As many women with endometriosis suffer from widespread pain that frequently affecting multiple organs, a paradigm shift aimed at encompassing a multifaceted approach is essential for the future study of endometriosis-associated pain.

Keywords Endometriosis · Chronic pelvic pain · Pre-clinical models · Pain mechanism · Evoked behaviours · Spontaneous behaviours

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Abbreviations

CD3	Cluster of differentiation 3
CPP	Chronic pelvic pain
HLA-DR	Human leukocyte antigen – DR isotope
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IC/BPS	Interstitial cystitis/bladder pain syndrome
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor
VEGF	Vascular endothelial growth factor

1 Introduction

Endometriosis is a chronic inflammatory disorder, characterised by the growth and development of endometrial cells outside of the uterus. Endometriosis imparts a financial and socioeconomic burden similar to major chronic diseases such as diabetes, inflammatory bowel disease (IBD) and rheumatoid arthritis (Simoens et al. 2007). Chronic pain is the most prevalent symptom associated with endometriosis. The financial burden associated with direct healthcare costs and lost productivity, as well as the social and psychological strains, continues to reduce the quality of life for millions of women worldwide (Simoens et al. 2007; Nnoaham et al. 2011; Adamson et al. 2010; Maddern et al. 2020). Pain associated with endometriosis can encompass disorders such as chronic pelvic pain (CPP) as well as comorbidities associated with chronic pain, including irritable bowel syndrome (IBS) and interstitial cystitis/bladder pain syndrome (IC/BPS) (Schomacker et al. 2018; Jess et al. 2012). Current treatments for the crippling pain affecting women with endometriosis, including invasive surgical interventions, hormonal suppression of ovarian function and pharmacological-based analgesics, achieve limited success. Moreover, they are often incompatible with a woman's reproductive plans and impart major side effects (Maddern et al. 2020; Howard 2000; Guo 2009). In many respects, the present limitation in treatment options relates to an underlying lack of knowledge of both the aetiology and mechanisms contributing to endometriosis-induced pain. With endometriosis affecting over 10% of women worldwide, unmatched with effective treatment strategies, there remains a clear need for relevant and translatable models of endometriosis to support research progression and to broaden the development of targeted and effective treatment options (Rogers et al. 2017).

Pre-clinical models have advanced scientific knowledge into the development and progression of endometriosis and are paramount to testing viable treatment options (Table 1) (Rogers et al. 2017; Quereda et al. 2008; Ingelmo et al. 1999). Unfortunately, despite a rise of over 150% in published endometriosis research over the last decade, pre-clinical studies have failed to translate into effective clinical

Table 1 Animal models of endometriosis

Species	Advantages	Disadvantages	Examples of endometriosis research
Non-human primates	Genetically similar to humans with spontaneous endometriosis development (Dick Jr. et al. 2003; D'Hooghe et al. 2009) Physiologically relevant to human disease	Sensitive to captivity Expensive Limited genetic modifications	Development (Braundmeier and Fazleabas 2009) Inflammation (Gashaw et al. 2006; D'Hooghe et al. 2001)
Rodents (rats and mice)	Easily bred/sources Affordable Genetic modifications readily available Advanced knowledge of physiological body systems due to popular use in research Have been successfully used to study endometriosis	Do not naturally menstruate Intervention always required for endometriosis development Clinical translation has proven difficult	Development (Quereda et al. 2008; Sharpe et al. 1990, 1991; Becker et al. 2008; Pelch et al. 2012; Alali et al. 2020; Nothnick et al. 2011; Birt et al. 2013) Inflammation (Pelch et al. 2010) Infertility (Birt et al. 2013; Stilley et al. 2009, 2010) Treatments (Sharpe et al. 1990; Xu et al. 2011; Ge et al. 2019; Hull et al. 2003; Horne et al. 2019a) Painful phenotype (Greaves et al. 2014b; McAllister et al. 2009, 2012; McKinnon et al. 2012; Alvarez et al. 2014)

outcomes once they reach clinical trials (Malvezzi et al. 2020). A major factor suggested to contribute to the translational block may come down to the consistency between animal models and their ability to replicate the spectrum of disease to that which is spontaneously developed in humans (Nunez-Badinez et al. 2021). It is also important to note that women with endometriosis represent a heterogenous patient group, whereby the number, size and location of lesion and overall pain severity can greatly vary. Therefore, a key factor in the development of any pre-clinical model of chronic disease is to encompass its complex features, from disease aetiology to mechanisms of disease progression and symptom development (Nunez-Badinez et al. 2021). This is no different when considering a model for endometriosis. To enable the successful translation of pre-clinical endometriosis models to clinically relevant outcomes, in such a way that we can quantify targeted treatment of widespread CPP, we need to consider concurrent development of interrelated mechanisms leading to disease symptomology.

Clinically, the development of endometriosis is multifactorial in nature, with complex mechanisms of chronic inflammation and chronic pain development contributing to disease pathogenesis (Maddern et al. 2020). As such, whole body systems with an intact nervous system are of fundamental benefit to pre-clinical models. Fortunately, the development of pre-clinical endometriosis models has improved in

recent years, building on the utilisation of human tissue as well as animal models, sometimes in combination (Malvezzi et al. 2020; Nunez-Badinez et al. 2021; Greaves et al. 2017a). Forming the basis of endometriosis development in animal models, translocation of eutopic endometrial tissue to ectopic locations has led to the growth of endometrial-like lesions and the successful production of animal models of endometriosis. Of these, non-human primates and murine models are discussed below.

2 Animal Models of Endometriosis

2.1 Non-human Primates

As the most genetically alike to humans, non-human primates, such as the baboon and rhesus monkey, have been used in the study of endometriosis (Table 1) (Braundmeier and Fazleabas 2009). As menstruating primates, they share physiological similarities to humans, with similar menstrual cycles and instances of spontaneous endometriosis (Dick Jr. et al. 2003; D'Hooghe et al. 2009). Following inoculation of menstrual endometrial tissue into the peritoneal cavity, baboons develop lesions like those seen in humans, retaining structural similarities such as endometrial glands and stroma as early as 1 month after inoculation (Langoi et al. 2013; Gashaw et al. 2006). Similarities are also apparent in the inflammatory parameters of the peritoneal fluid (PF), with elevated cytokines including tumour necrosis factor (TNFα), transforming growth factor β (TGFβ), cluster of differentiation 3 (CD3) and human leukocyte antigen – DR isotope (HLA-DR) (D'Hooghe et al. 2001). The angiogenic factor vascular endothelial growth factor (VEGF) is also elevated in both human and baboon ectopic endometrium following endometriosis development (Gashaw et al. 2006).

With clear similarities in disease, the non-human primate models of endometriosis have provided useful insights into mechanisms of endometriosis development (Braundmeier and Fazleabas 2009), although it has been noted that research has thus far failed to translate to clinically relevant treatments (Malvezzi et al. 2020). Although physiologically relevant to human disease, non-human primates are sensitive to captivity and expensive, which makes high-throughput experiments difficult and ultimately limiting to research output.

2.2 Rodent

More commonly, rodent models of disease have been important in many areas of scientific discovery and development. Rats and mice are a popular choice for many pre-clinical models as they are easily bred and affordable and offer an array of

genetic modification options, and advanced physiological knowledge of their body systems exists due to their extensive use in many fields (Table 1) (Saunders 2020). Despite distinct advantages, laboratory mice and rats do not naturally menstruate or develop spontaneous endometriosis; therefore, model development requires intervention.

The earliest small animal model of endometriosis was developed by Vernon and Wilson in 1985, employing surgical transplantation of uterine tissue onto the mesenteric arteries of rats (Vernon and Wilson 1985). This model was the first to demonstrate the attachment and growth of endometriosis lesions within the peritoneal cavity and successfully aided in the development of treatments utilised today (Sharpe et al. 1990). In an extension of this surgical attachment model, a model reducing the invasive nature of induction by instead injecting fragments of uterine tissue into the peritoneal cavity to induce endometriosis has also been developed (Fattori et al. 2020). Due to the success of this model and more recent technological innovations, variations have been incorporated to progress key areas of endometriosis research. Modifications include injecting fluorescently tagged uterine tissue to enable fluorescent imaging of lesion growth (Becker et al. 2008; Greaves et al. 2014a), inoculating human tissue into immunocompromised mice for the establishment of human endometrial endometriosis (Hull et al. 2012; Xu et al. 2011) and developing a menstruating mouse model to more closely mimic the human phenotype (Greaves et al. 2014a). All variations of these models successfully report ectopic lesion development, resembling what is seen in women with endometriosis.

Thanks to the development of these small animal models of endometriosis, research has progressed in many areas including endometriosis growth and development (Quereda et al. 2008; Sharpe et al. 1990, 1991; Becker et al. 2008; Pelch et al. 2012; Alali et al. 2020; Nothnick et al. 2011; Birt et al. 2013), inflammation (Pelch et al. 2010), infertility (Birt et al. 2013; Stilley et al. 2009, 2010), treatment (Sharpe et al. 1990; Xu et al. 2011; Ge et al. 2019; Hull et al. 2003; Horne et al. 2019a) and to a certain extent characterisation of a painful phenotype (Greaves et al. 2014b; McAllister et al. 2009, 2012; McKinnon et al. 2012; Alvarez et al. 2014), to name a few. Despite these contributions, it is recognised that research into pain mechanisms remains lacking (Greaves et al. 2020), and further refining these models to understand widespread pain development is necessary to advance research into endometriosis-associated pain (Saunders 2020).

3 Pain Assessment in Animal Models

Rodent models are the most commonly used species for pain assessment studies that more closely resemble clinical pain states (Mogil 2009). Animals cannot self-report pain like humans; however, measuring inferred pain from both spontaneous and evoked interactions provides insight into ‘pain-like’ behaviours (Mogil 2009).

3.1 Evoked Responses

Evoked behavioural studies allow researchers to assess changes in the response of an animal to an external factor, such as thermal or mechanical stimuli, initiating pain (Fig. 1). These techniques allow measurement of hypersensitivity to a set stimulus (i.e. mechanical allodynia and hyperalgesia) (Mogil 2009) and have been employed in a variety of studies to confirm pain development in pre-clinical models of chronic pain, including rodent models of endometriosis (Ge et al. 2019; McAllister et al. 2009, 2012; Nagabukuro and Berkley 2007; Dmitrieva et al. 2012; Liu et al. 2012, 2018; Forster et al. 2019; Greaves et al. 2017b; Chen et al. 2015; Li et al. 2018; Zheng et al. 2012).

As one of the most frequently measured responses, mechanical allodynia and hyperalgesia have been demonstrated using a variety of techniques in both rat and mouse models of endometriosis. For example, mechanical allodynia has been demonstrated in endometriosis mice by measuring abdominal and hind paw withdrawal thresholds to von Frey hair filaments (Liu et al. 2018; Forster et al. 2019; Greaves et al. 2017b), with an earlier withdrawal reflex indicating the development of hypersensitivity to a mechanical stimulus.

Endometriosis-induced cutaneous thermal hypersensitivity has also been demonstrated using both the hot plate and tail flick test, with reduced reaction latency

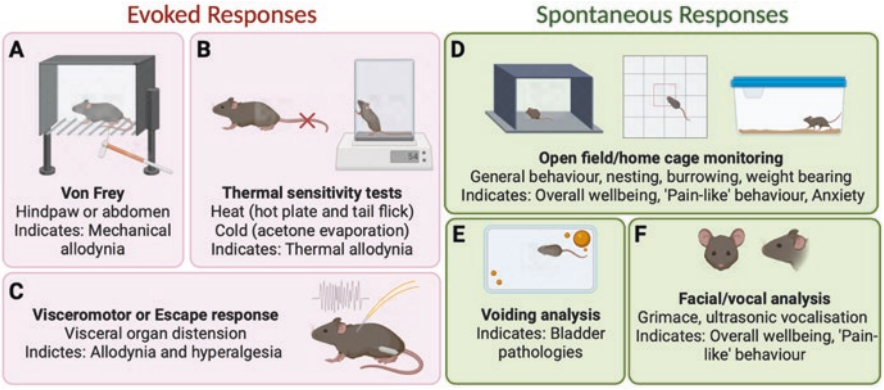

Fig. 1 Experimental techniques used to determine alterations in evoked and spontaneous behavioural responses. Techniques to measure evoked behavioural responses: (**a**) von Frey analysis (to the abdomen or paws) to determine mechanical allodynia**.** (**b**) Thermal sensitivity tests include exposing the paws or the tail to a hot (heat plate or tail flick test, respectively) or cold (acetone evaporation test) stimulus, to determine thermal allodynia. (**c**) Visceromotor responses or escape measurements in response to visceral organ distension (i.e. the vagina, colon or bladder), to determine allodynia and hyperalgesia associated with visceral hypersensitivity. Spontaneous behavioural response techniques: (**d**) open field/home cage behavioural analysis (measuring general behaviours such as nesting, burrowing and weight bearing) to determine overall wellbeing and the development of psychological conditions such as anxiety. (**e**) Voiding pattern analysis to determine the development of comorbid bladder pathologies. (**f**) Facial and vocal analysis to determine overall wellbeing and the development of 'pain-like' behaviours. (Figure created with BioRender.com)

demonstrating a developed sensitivity to noxious thermal stimuli (Li et al. 2018; Liu et al. 2012; Zheng et al. 2012). In a similar way, the development of cold sensitivity has also been demonstrated using the acetone evaporation test, with a heightened response to acetone administered to the hind paw demonstrating the development of altered thermal sensitivity (Liu et al. 2018).

In addition, various groups have also demonstrated the development of vaginal mechanical hypersensitivity (both allodynia and hyperalgesia) in rodent models of endometriosis by measuring the escape response or visceromotor response (VMR) to vaginal distension (Ge et al. 2019; McAllister et al. 2009, 2012; Nagabukuro and Berkley 2007; Dmitrieva et al. 2012). The development of widespread visceral pain in rodent models of endometriosis has also been demonstrated by an increased abdominal withdrawal reflex to colonic distension compared to sham animals (Chen et al. 2015).

Importantly, these evoked response measurements have served to successfully indicate changes in visceral and cutaneous sensitivity to nociceptive stimuli, allowing researchers to test potential therapeutics to target these individual 'pain-like' responses associated with endometriosis (Dmitrieva et al. 2012; Liu et al. 2012, 2018; Forster et al. 2019; Greaves et al. 2017b; Zheng et al. 2012).

3.2 Spontaneous Responses

Spontaneous behavioural responses are assessed by monitoring spontaneous behaviours to a set of non-invasive parameters (Fig. 1). Whilst measuring differences in evoked behavioural responses allows researchers to uncover changes in pain sensitivity, changes in spontaneous behaviours can give an indication of pathophysiological changes and quality of life, which differ from those that are uncovered when considering evoked changes (Mogil and Crager 2004). Unfortunately, evoked pain responses form the majority of pain-related testing in endometriosis models, leaving non-evoked spontaneous methods (which are suggested to be a better predictor of overall pain) lacking (Nunez-Badinez et al. 2021). Spontaneous behavioural analysis in rodents can range from non-reflexive behavioural monitoring, including analysis of vocalisation and facial expression, nesting and burrowing, or voiding and behavioural patterns within the home cage/open field (Fig. 1), to free-choice behaviours, including temperature preference and escape/avoidance testing (Tappe-Theodor and Kuner 2014). This range of measurements allows researchers to unearth altered overall wellbeing and changes to emotional states, which are often developed with pain-related conditions, including anxiety, depression and altered social interactions (Tappe-Theodor and Kuner 2014).

Although more scarcely measured, endometriosis mice have demonstrated altered spontaneous behavioural responses to some of these non-evoked techniques. For example, increased abdominal licking and reduced tunnel entries have been observed in a mouse model of endometriosis (Forster et al. 2019) together with the development of anxiety-related behaviour by 6 weeks of endometriosis

development (Li et al. 2018). In these experiments, open field testing was used to measure general activity and indications of anxiety, recognising reduced time and distance spent within a central area, known as an aversive place, in endometriosis mice (Li et al. 2018). Similarly, an increase in anxiety like behaviours developed in endometriosis mice has also been reported with a reduction in free-choice exploration of an elevated maze by 4 weeks after endometriosis induction (Escudero-Lara et al. 2020, 2021).

When considering alterations in spontaneous behaviours which suggest the development of painful comorbidities, analysis of home cage activity has revealed that mice with endometriosis spend significantly less time drinking or climbing compared to control mice, behaviours which are suggested to identify non-mechanical associated pain in mice (Tejada et al. 2022). Moreover, spontaneous voiding pattern analysis, a technique previously described to indicate the development of comorbidities associated with bladder pathologies, such as overactive bladder (OAB) and IC/PBS (Grundy et al. 2018; Hill et al. 2018), has recently been utilised in pre-clinical models of endometriosis to demonstrate the concurrent development of comorbid bladder symptoms (Maddern et al. 2022; Castro et al. 2021).

4 Shifting the Paradigm of Isolated Behavioural Analysis to Study Widespread Pain Development in Endometriosis

Women with endometriosis are 13 times more likely to experience abdominal pain than healthy women (Ballard et al. 2008), enhanced by a high comorbidity rate with other visceral pain syndromes including IBS, inflammatory bowel disease (IBD), IC/BPS and dyspareunia (Schomacker et al. 2018; Jess et al. 2012; Signorile et al. 2022; Chung et al. 2005; Surrey et al. 2018; Tirlapur et al. 2013). With this in mind, when considering the possible mechanisms of pain development and maintenance in endometriosis, it is crucial to consider the complexity of disease progression with disease establishment. Unfortunately, developing and characterising pre-clinical models that recapitulate multiple pain symptoms associated with endometriosis is not currently evident at an extensive level. Whilst behavioural changes are often reported using individual techniques, studies don't frequently assess multiple parameters. Using a combination of both evoked and spontaneous behavioural experiments would therefore allow researchers to illustrate a more comprehensive characterisation of endometriosis-related pain and comorbidities. For example, mechanical hypersensitivity uncovered using von Frey testing was described to occur together with anxiety-like behaviour within an elevated maze at 27 days of endometriosis development (Escudero-Lara et al. 2021). Similarly, a recent study by Tejada et al. (Tejada et al. 2022) successfully demonstrated endometriosis-induced development of mechanical hypersensitivity using von Frey testing as well as concurrent alteration in spontaneous home cage activities such as drinking and climbing, suggesting the development of non-mechanical pain (Tejada et al. 2022).

These studies demonstrate that combining individual techniques to uncover evoked hypersensitivity, as well as spontaneous signs of anxiety and pain-like behaviours over a longer period of time, supports the broader characterisation of an endometriosis-induced painful phenotype, where chronic development leads to a variety of symptoms, often affecting multiple organs.

Taking a combination of techniques a step further, our group has explored the widespread development of painful comorbidities by building upon two clinically relevant mouse models of endometriosis (Maddern et al. 2022; Castro et al. 2021). By utilising surgical attachment of uterine horn tissue amongst visceral organs or inoculation of minced uterine horn tissue into the peritoneal cavity in combination with multiple in vivo techniques addressing widespread evoked and spontaneous behaviours, we were able to demonstrate the concurrent development of vaginal, colonic, cutaneous and bladder sensory comorbidities at 8–10 weeks of development (Maddern et al. 2022; Castro et al. 2021). Moreover, we were also able to assess the spontaneous behaviours of endometriosis mice as an indicator of anxiety (Maddern et al. 2022; Castro et al. 2021). This advanced characterisation not only provides a platform whereby we can examine a variety of mechanisms contributing to widespread CPP, but it also provides a pre-clinical model for testing novel treatment options. Importantly, by measuring changes in multiple clinically relevant comorbidities in these mouse models, we can determine whether individual therapeutic treatments successfully alleviate comorbidities which are also experienced by women clinically.

5 Clinically Relevant Animal Models of Endometriosis for the Study of CPP: A Present Challenge

Endometriosis is a chronic disease that is often diagnosed after years of development facilitating complex disease progression (Nnoaham et al. 2011; Hadfield et al. 1996; Husby et al. 2003; Arruda et al. 2003). Currently, the timing of many behavioural studies performed in animal models is limiting, with major part of the studies completed during the first 4 weeks of endometriosis induction, preferentially reflecting early disease development. Although this is helpful to uncover early mechanisms, as a chronic disease with years of development preceding diagnosis in most cases, the mechanisms by which endometriosis can induce CPP may reflect a different phase of disease than during early development. As such, it is important to look at later time points, as they may provide a more relevant indication of chronic pain mechanisms (Saunders 2020). The importance of timing is apparent in studies which utilise a longitudinal approach to behavioural monitoring. For example, another strength of the study mentioned above by Tejada et al. (Tejada et al. 2022) was that the reported development of mechanical allodynia and non-mechanical pain like behaviour was evident due to their extension of endometriosis progression to 8 weeks of development. The longitudinal behavioural measurements allowed researchers to unmask ‘pain-like’ behaviours which would not have been apparent

if only measured prior to 6 weeks of disease progression (Tejada et al. 2022). With an extensive list of possible mechanisms involved in chronic pain development (Maddern et al. 2020), a reproducible pre-clinical model of endometriosis that exhibits multiple altered pain-like behaviours reflective of chronic human disease development is fundamental to the complex progression of CPP in endometriosis.

In addition to this chronic progression, variations in model induction may also play a key role in various mechanisms which may be responsible for CPP development in endometriosis. Ectopic endometrial cell growth outside of the uterus defines endometriosis, with peritoneal endometriosis found most commonly in and around the peritoneum and visceral organs (Saunders 2020; Horne et al. 2019b). However, it is important to consider that women with endometriosis rarely develop a single lesion subtype and a combination of both superficial peritoneal lesions and deep infiltrating lesions often develops (Zondervan et al. 2020). This is significant, as both deep infiltrating and superficial peritoneal lesions are suggested to contribute to pelvic pain via different mechanisms (Anaf et al. 2000; Khan et al. 2013). With this in mind, it has recently been suggested that a single standardised model is not likely to provide complete translational benefit, but rather, research using multiple models may support more robust pre-clinical data and ultimately lead to stronger clinical outcomes (Dorning et al. 2021). Supporting this, a recent study by Dorning et al. illustrated that even slight variations in donor uterine tissue type inoculated into the peritoneal cavity of mice resulted in clear differences in both disease progression and pain phenotype (Dorning et al. 2021). As the development of endometriosis in women is complex, the importance and availability of pre-clinical models that reflect different subgroups of endometriosis observed in women may offer more robust and translatable findings relevant to heterogenous disease mechanisms.

6 Closing Remarks

The substantial impact that endometriosis has on women contributes to the continued research priority appeal, urging researchers to advance understanding of this multifaceted disease and ultimately enable vital progress towards improving the quality of life for millions of patients worldwide. There is a clear need for relevant and translatable models of endometriosis to research mechanisms responsible for the development and maintenance of CPP and to broaden the development of targeted and effective treatment strategies. The traditionally narrow approach to study pain in animal models of endometriosis limits translatability when considering the widespread and complex nature of this disorder. Combining a comprehensive range of behavioural techniques, at advanced stages of this disease, would provide a well-rounded platform to drive progress in CPP mechanisms associated with endometriosis. Importantly, utilising animal models which display a spectrum of clinically relevant symptomology may ultimately uncover potential therapeutic avenues for much-needed treatment of CPP in endometriosis.

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Spinal Afferent Innervation of the Uterus

Kelsi N. Dodds and Nick J. Spencer

Abstract The extrinsic neural innervation of the uterus plays an important role in modulating uterine functions that are critical to reproductive success. Its sensory division serves dual afferent and efferent roles: relaying information on innocuous and noxious stimuli from the uterus to the central nervous system and regulating uterine smooth muscle activity via local release of neuropeptides. Such sensory innervation is primarily supplied by spinal afferents with nerve cell bodies in thoracolumbar and lumbosacral dorsal root ganglia (DRG). Here, we summarise the neuroanatomy and physiology of spinal afferents innervating the rodent uterus. Findings arising from techniques pioneered in our laboratory are highlighted, which target select DRG for labelling and manipulation of specific spinal afferent populations. Future insights in this field are anticipated to expose new mechanisms related to disorders of uterine sensation, such as dysmenorrhoea.

Keywords Uterus · Female reproductive tract · Pelvic pain · Calcitonin gene-related peptide · Spinal ganglia

1 Introduction

The uterus is a major visceral organ that forms part of the female reproductive tract. It is richly supplied by extrinsic sensory, parasympathetic, and sympathetic nerve fibres that modulate uterine contractions, blood flow, and other regulatory processes critical to reproductive function and behaviour. Intriguingly, nerves of the uterus are highly plastic, undergoing major episodes of reorganisation throughout the lifespan

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to accommodate changes in reproductive need. The primary source of uterine sensory innervation is supplied by spinal afferent neurons, whose cell bodies lie adjacent to the spinal cord in dorsal root ganglia (DRG). These afferents detect a wide range of sensory information in the uterus, including mechanical stimuli, hypoxic conditions, and inflammatory mediators, providing important feedback for neuroendocrine and sensorimotor reflexes related to reproduction, as well as pain (Houdeau et al. 2002; Berkley et al. 1993).

Most, if not all, spinal afferent neurons innervating the uterus are peptidergic, distinguished by their immunoreactivity to calcitonin gene-related peptide (CGRP) (Dodds et al. 2021). Other co-transmitters may be present, such as substance P, neurokinin A, cholecystokinin, galanin, and secretoneurin (Traurig et al. 1991; Collins et al. 2000; Shew et al. 1992). In addition to their afferent role, uterine sensory nerves commonly demonstrate efferent functions by releasing these peptides from their endings into the uterine wall. CGRP, for example, is well known to cause relaxation of spontaneous and evoked contractions of the uterine muscle (Anouar et al. 1998; Naghashpour et al. 1997; Klukovits et al. 2004) and vasculature (Gangula et al. 2003), while substance P potently stimulates uterine contractility (Shew et al. 1991).

This minireview principally draws from results obtained in rodents, where uterine spinal afferents are currently best characterised. We first provide an overview of the central origins of spinal afferent neurons and their anatomical distribution within the uterus. Detailed morphological characteristics are then described, comparing several unique features of uterine spinal afferents to those observed in other visceral organs, such as the colon. Recent findings revealed by techniques developed in our laboratory that allow for selective labelling and manipulation of specific spinal afferent populations are highlighted. We conclude by summarising known uterine afferent adaptations, and their proposed functional contributions, to changes and processes involved in major reproductive stages. For brevity, the term ‘uterus’ herein refers to the main body of the uterine horn.

2 Central Origins and Distribution of Spinal Afferents Innervating the Uterus

The cell bodies and central axons of uterine spinal afferents are bimodally distributed along the spinal column, forming peak populations in the thoracolumbar (T12-L2) and lumbosacral (L6-S1) regions. The peripheral axons of thoracolumbar afferents predominantly travel from their uterine targets to the spinal cord via the hypogastric and splanchnic nerves, while lumbosacral axons travel via the pelvic nerves (Berkley et al. 1988; Nance et al. 1988; Herweijer et al. 2014). Sympathetic and parasympathetic efferent fibres accompany spinal afferents within the hypogastric and pelvic pathways, respectively (Nance et al. 1988), and some afferent axon collaterals synapse with cholinergic parasympathetic fibres in uterine-associated

autonomic ganglia (Papka and Traurig 1989; Papka and Mcneill 1993; Houdeau et al. 2002). In addition to spinal afferents, few sensory nerve fibres originate from nodose ganglia, which innervate the uterus via the vagal nerves (Ortega-Villalobos et al. 1990; Collins et al. 1999; Dodds et al. 2021) (Fig. 1).

The distribution of spinal afferent innervation within the uterus follows a topographical arrangement, such that fibres projecting from thoracolumbar DRG largely occupy the cranial (ovarian-to-mid) region of the uterine horn, as well as the adjacent ovaries and oviducts. Conversely, lumbosacral DRG preferentially innervate the caudal (mid-to-cervical) uterine horn, extending distally to include the uterine cervix and vagina (Nance et al. 1988; Berkley et al. 1988). The anatomical area(s) occupied by uterine-projecting vagal afferent fibres is less well established, but may include the entire uterus (Ortega-Villalobos et al. 1990).

Marked regional division of spinal afferent innervation has been demonstrated in the rat (Nance et al. 1988) and mouse (Kyloh et al. 2022) uterus following the unilateral removal of thoracolumbar DRG, in vivo. Subsequent loss of substance P or CGRP immunoreactivity, respectively, due to deafferentation in the uterine horn, revealed the spatial territories occupied by thoracolumbar neurons. Since rodent uteri display a predominantly ipsilateral spinal afferent input to each half of the female reproductive tract, nerve fibre density along the uterine horn of unilateral DRG-removed animals can be compared against an internal control of the unaffected, contralateral side. Using this method in mice, for example, we showed mean CGRP-immunoreactivity depletion of ~60% in the cranial region of the uterine horn following thoracolumbar DRG removal, versus ~45% in the caudal uterus (Kyloh et al. 2022) (Fig. 2).

In addition to the different regional projections of spinal afferent pathways, the uterus displays local variations in the density of nerve fibres. Total innervation (afferent and efferent) is highest in the caudal region of the uterine horn and denser in the cranial region than the mid-uterus (Traurig et al. 1991; Zoubina et al. 1998), while CGRP-containing afferent fibres are most concentrated within the cranial region, compared to the middle and caudal portions (Zoubina et al. 1998; Kyloh et al. 2022).

It remains undetermined whether the dual central distribution, and/or regional innervation of the uterus, simply reflects the anatomical segregation of the ovarian and uterine arteries, along which spinal afferents primarily enter the uterine horn (Nance et al. 1988). Alternatively, these distinct afferent pathways could serve functionally different roles between the cranial and caudal ends of the uterus. Indeed, it has been proposed that afferent activity of the hypogastric nerves primarily plays a role in pregnancy and nociception, whereas pelvic innervation may facilitate processes of mating and conception, with both nerves serving functions during parturition (Berkley et al. 1993). There is also an interesting possibility that the roles of each spinal pathway may shift under pathological conditions or during pregnancy (Temple et al. 1999; Kirby et al. 2010).

Research on other pelvic visceral organs, like the colon, supports the concept that different stimulus modalities can be conducted via the two neural pathways. For instance, colonic spinal afferents appear to relay non-noxious mechanosensory

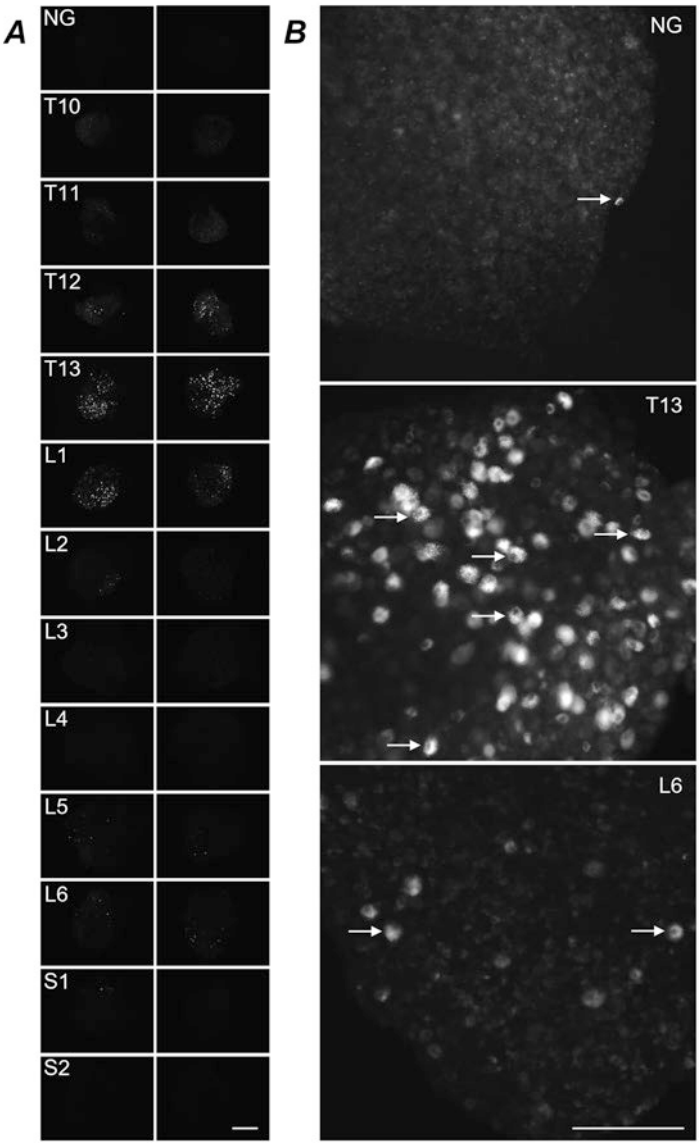

Fig. 1 Central origins of uterine-projecting spinal afferent neurons. (**a**) Pairs of thoracic (T10–13), lumbar (L1–6), and sacral (S1–2) dorsal root ganglia (DRG) and nodose ganglia (NG) retrogradely labelled with DiI neuronal tracer from the mouse uterus, in vivo. The greatest number of positively labelled neuronal cell bodies, indicating the degree of afferent innervation, occurs in thoracolumbar T12-L2 (via the hypogastric nerves), followed by lumbosacral L5-S1 (pelvic nerves) and NG (vagal nerves). (**b**) Higher magnification images of NG, T13, and S1, highlighting the extent of afferent innervation supplied to the uterus by vagal, hypogastric, and pelvic nerves, respectively. Examples of positively labelled neuronal cell bodies are indicated by the arrows. Scale bar in (**a**) = 500 μm for all images in the same panel; scale bar in (**b**) = 200 μm for all images in the same panel. (Some images presented in this figure are adapted from Dodds et al. (2021))

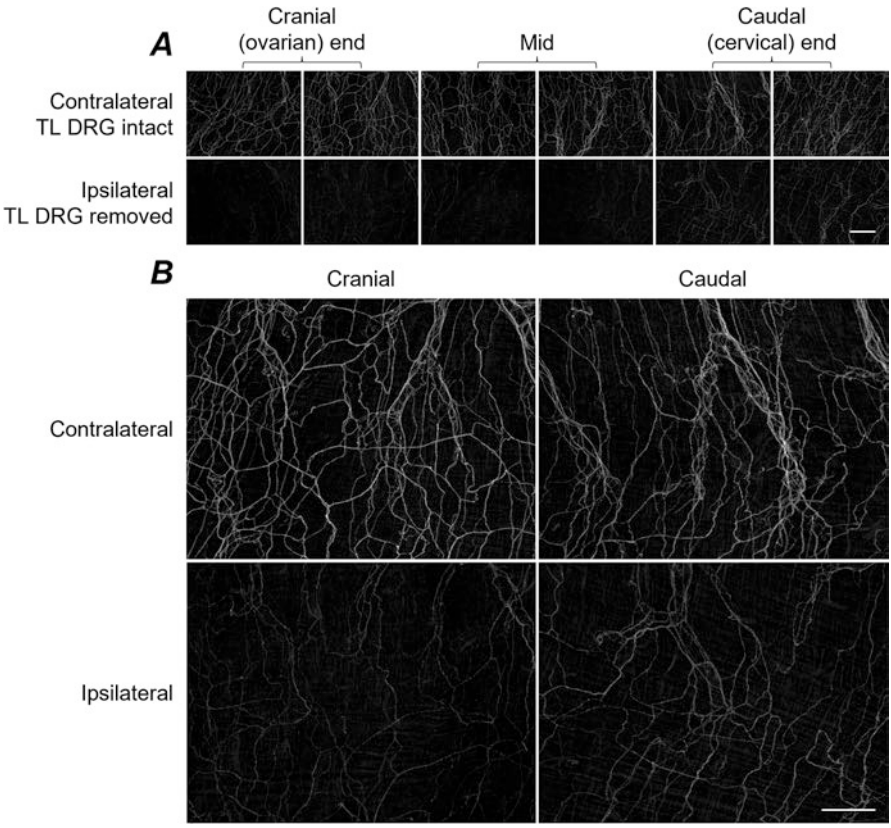

Fig. 2 Distribution of thoracolumbar spinal afferents in the uterus. (**a**) Representative images from the mouse uterine horn following in vivo unilateral removal of thoracolumbar (TL) T13-L2 dorsal root ganglia (DRG). The contralateral uterine horn remains densely innervated across all regions with calcitonin gene-related peptide (CGRP)-containing spinal afferent nerve fibres. However, there is a marked reduction in CGRP immunoreactivity of the ipsilateral uterine horn, which is most pronounced at the cranial (ovarian) end. This indicates that thoracolumbar spinal afferents (via the hypogastric nerves) provide major sensory innervation to the mouse uterus that distribute primarily within the cranial region. (**b**) Enlarged images from cranial and caudal regions of the uterine horn highlighting the extent of CGRP-immunoreactivity depletion on the ipsilateral side. Scale bar in (**a**) = 200 μm for all images in the same panel; scale bar in (**b**) = 200 μm for all images in the same panel. (Some images presented in this figure are adapted from Kyloh et al. (2022))

information through the lumbosacral spinal cord alone, whereas both thoracolumbar and lumbosacral spinal pathways become active following noxious mechanosensitive stimulation (Kyloh et al. 2022; Harrington et al. 2019). Selective DRG removal may be particularly useful in future studies to further distinguish these roles, in both the uterus and adjacent viscera (Kyloh et al. 2022). Subsequent loss of function associated with the targeted deafferentation would therefore indicate processes normally regulated by certain populations of spinal afferent neurons.

3 Morphological Characteristics of Uterine-Projecting Spinal Afferent Neurons

Spinal afferents enter the uterus via the mesometrium, with most axons traversing alongside terminal branches of the ovarian and uterine arteries. Few fibres have also been observed independent of the uterine vasculature, travelling free within the mesometrial space (Dodds et al. 2021; Shew et al. 1991). Upon entry into the uterus, spinal afferent axons distribute within all layers of the uterine wall: the longitudinal and circular smooth muscle (myometrium), the intermuscular vascular plexus, and the inner mucosal lining (endometrium) (Zoubina et al. 1998; Gnanamanickam and Llewellyn-Smith 2011; Dodds et al. 2021). The greatest density of CGRP-immunoreactive neurons occurs within the vascular plexus, the blood vessels of which are thought to serve as a conduit for these nerves travelling to other uterine layers (Zoubina et al. 1998; Haase et al. 1997).

3.1 Spinal Afferent Axons

To determine the projections of single spinal afferent neurons within the uterine wall, we recently employed an in vivo anterograde tracing technique in mice, where neuronal tracer was injected into thoracolumbar DRG (Dodds et al. 2021). In this procedure, the injected tracer is taken up by nerve cell bodies and axons in DRG and transported to the peripheral terminals of spinal afferents, allowing detailed morphological analysis of their axons and endings (Kyloh and Spencer 2014; Spencer et al. 2014). The specific uptake and labelling of spinal afferent neurons represents a major advantage of this approach for identification of afferents, over neuroanatomical tracing from visceral nerve trunks that contain multiple populations of afferent and efferent nerve fibres. Previously, this has been a key technical challenge, as the major known neurochemical markers expressed in DRG, including CGRP and vanilloid receptor 1 (TRPV1), are also expressed in vagal afferents (Zhang et al. 2004; Zhong et al. 2008). Mass labelling of axons using alternative approaches, such as transgenic reporter mice, has also precluded the identification and discrimination of individual spinal afferent neurons.

Anterograde tracing from DRG revealed no consistent trajectories of spinal afferent axons in the uterus. Groups of uterine spinal afferent axons were found to branch similar distances in the circumferential and longitudinal axes and with little cranial-caudal polarisation from their entry points into the uterine wall. These ramifications spanned approximately 10% of the length and 50% of the width of the uterine horn. Such features differ from other viscera, such as the colon, where subsets of spinal afferent axons can preferentially ramify along circumferential or longitudinal trajectories (Kyloh and Spencer 2014; Spencer et al. 2014).

Spinal afferent axons typically branched soon after entering the uterine wall, approximately one-third of which displayed varicosities prior to the first

bifurcation. Another unique feature of uterine spinal afferents was that, at each bifurcation, axon diameter (~1 μm) remained constant. This contrasts with colonic and bladder spinal afferents whose axon diameters typically decrease at each branching point (Spencer et al. 2018, 2020b). The implications of this are currently unclear but are likely to influence the conduction velocities of sensory information across different visceral organs.

3.2 Spinal Afferent Endings

In the same study, we reported the first thorough characterisation of the major morphological types of uterine spinal afferent endings and their sites of innervation (Dodds et al. 2021). Anterogradely labelled endings were mostly varicose, colocalised with CGRP immunoreactivity, and only occurred from axonal branches, where multiple endings arose from single axons. Three primary morphological classes of uterine spinal afferent endings were identified – simple, branching, and complex type (Herweijer et al. 2014) – which were further subclassified based on finer terminal details (Fig. 3). Most endings were of simple-type morphology, displaying considerably less structural diversity and complexity compared to those observed in the colon and bladder (Spencer et al. 2018). The reasons for this are not yet known but are likely to underlie inter-organ differences in the sensitivities and responses of spinal afferents to sensory stimuli. Regardless, some or all morphological types of spinal afferent endings identified must contribute to visceral pain arising from the uterus, such as that which occurs in dysmenorrhoea. The next major challenge will be therefore to determine exactly which classes (or subclasses) of uterine spinal afferent endings specifically underlie the detection of innocuous and noxious stimuli, and to compare those with findings from adjacent viscera.

Further, we found that spinal afferent endings terminated within multiple layers of the uterine wall. Overall, most endings occurred in the circular muscle, although there appeared to be some layer specificity for each morphological class: branching-type endings in the vascular plexus, simple-type in the circular muscle, and complex-type evenly divided between these two layers. While endometrial spinal afferents were not directly studied, CGRP expression has been described in the rat endometrium (Gnanamanickam and Llewellyn-Smith 2011; Zoubina et al. 1998; Shew et al. 1990), warranting further investigation. In addition, it is unknown whether the individual spinal afferent axons that have multiple endings in the uterus terminate across several layers of the uterine wall. In the colon, different types of morphological endings arising from single spinal afferent axons terminate across various wall layers, indicating that such neurons can detect and integrate a variety of sensory information (Spencer et al. 2020a, b).

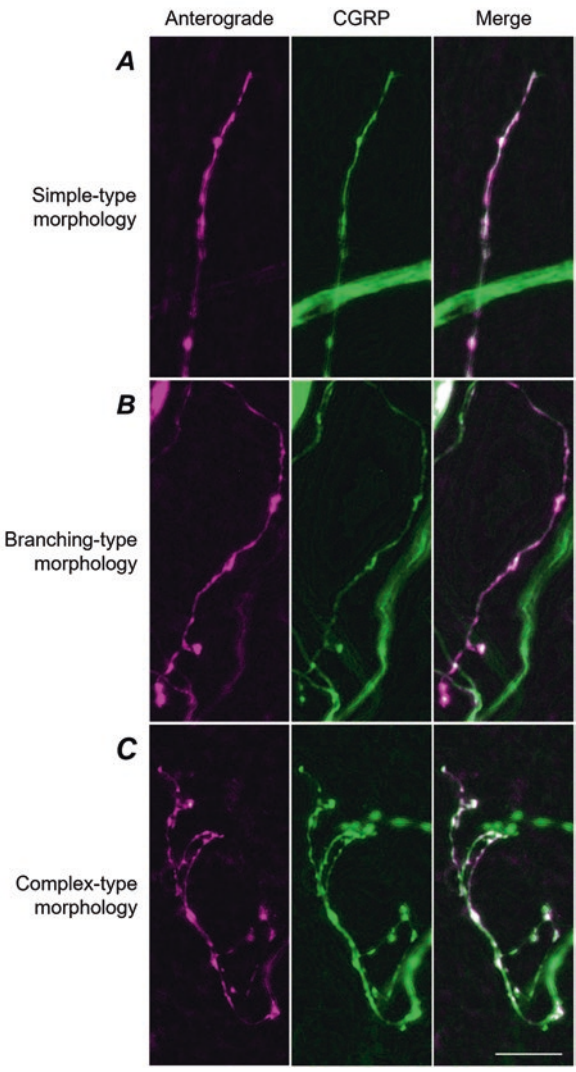

Fig. 3 Major morphological classes of uterine spinal afferent endings. (**a**) Example of a 'simple-type' spinal afferent nerve ending in the mouse uterus. This morphological class was most commonly identified by in vivo anterograde tracing from thoracolumbar (T13-L3) dorsal root ganglia (DRG), often terminating within circular smooth muscle. (**b**) Example of a 'branching-type' spinal afferent nerve ending, typically found within the uterine vascular plexus. (**c**) Example of a claw-like 'complex-type' uterine spinal afferent nerve ending. These endings displayed a wide range of sub-morphologies that also include looped and entangled variations. All morphological types of spinal afferent nerve endings colocalised with calcitonin gene-related peptide (CGRP) immunoreactivity. Scale bar in (**c**) = 20 μm applies across all images. (Some images presented in this figure are adapted from Dodds et al. (2021))

4 Adaptations of Uterine Afferents to Changes in Reproductive Status

The innervation of the female reproductive tract is highly sensitive to changes in sex hormones, undergoing profound metabolic, functional, and structural changes associated with maturation, the reproductive cycle, pregnancy, parturition, and the postpartum period (Brauer and Smith 2015). Sympathetic nerve fibres in the uterine myometrium appear to be most susceptible, displaying marked axonal degeneration under oestrogen-dominant conditions, such as the periovulatory phase and during pregnancy (Zoubina et al. 1998; Haase et al. 1997; Latini et al. 2008). The extent of

such alterations in uterine sensory fibres is less well established, although they are of key interest given their increasingly recognised importance in various reproductive processes. Specific adaptations in uterine spinal afferent neurons are yet to be determined, and, as such, general findings for sensory fibres are presented below.

4.1 Puberty and the Reproductive Cycle

During puberty, there is a significant increase in the weight of the uterine horn. Afferent nerve fibres in the uterus containing substance P and CGRP increase relative to this growth, resulting in the maintenance of nerve density and peptide concentration (Brauer et al. 1994). CGRP immunoreactivity remains comparatively unchanged across the ensuing reproductive cycle, despite an overall decrease in total nerve fibres throughout all layers of the rat uterine horn around ovulation (Zoubina et al. 1998). Since myometrial thickness fluctuates in response to sex hormone levels that define the different cycle stages, this finding indicates that afferent nerves are highly dynamic and, akin to puberty, continually adjust their density in parallel to uterine size.

Both the afferent and efferent functions of uterine sensory nerves are also modulated by cycle stage. Heightened sensitivity of the rat hypogastric nerves to uterine distension has been observed during proestrus (pre-ovulation) and oestrus (peri-ovulation) as opposed to metestrus and diestrus (post-ovulation) (Robbins et al. 1990, 1992), suggesting that these afferents are more likely to detect potentially damaging stimuli at a time of reproductive benefit (i.e. during conception). Yet, behavioural outcomes appear to contradict this idea, where a higher percentage of escape responses to noxious uterine stimulation have been reported during the post-ovulatory phases (Bradshaw et al. 1999), perhaps indicating differential central processing of such stimuli across the reproductive cycle phases. Regardless, this still confers reproductive advantage, in that there would be enhanced receptivity to uterine stimulation during the fertile period. The efferent function of uterine sensory nerves is also subject to cyclic variation. CGRP-mediated inhibition of uterine contractions is lowest at oestrus compared to metestrus and diestrus, likely due to changes in CGRP receptor expression and signalling (Naghashpour and Dahl 2000). This may explain why propagating uterine contractions are strongest during oestrus (Dodds et al. 2015), a mechanism that may facilitate gamete transport.

4.2 Pregnancy and Parturition

Throughout pregnancy, there is a gradual decline in the density of CGRP-containing uterine nerves until term, where profound denervation occurs in all layers of the uterine wall (Haase et al. 1997; Anouar et al. 1998). Corresponding levels of myometrial CGRP receptor expression and binding are elevated in pregnancy, followed by a sharp decline at the onset of labour (Yallampalli et al. 1999). Due to the

relaxant effect of CGRP on myometrial contractility and vascular tone, it is thought that this upregulated activity during gestation may be important for maintaining uterine quiescence and regulating uteroplacental blood flow to sustain normal foetal development (Gangula et al. 2002). At term, there is a dramatic loss of CGRP-mediated relaxation (Anouar et al. 1998; Naghashpour et al. 1997) accompanied by increased uterine content of substance P (Amira et al. 1995) and other myometrial stimulatory factors, such as oxytocin. Thus, the concentration of different afferent-associated peptides during labour may collectively assist in generating strong, rhythmic uterine contractions to help bring about normal delivery.

5 Concluding Remarks

Sensory innervation to the rodent uterus is predominantly supplied by CGRP-containing spinal afferent nerves that arise from thoracolumbar and lumbosacral DRG. Using techniques that selectively target these DRG, such as in vivo anterograde tracing and ganglion removal, has allowed significant progress to be made in defining their morphological characteristics and distribution within the uterus, as well as adjacent visceral organs. While we now have a greater understanding of the anatomy of uterine spinal afferents, there is still much to discover about their functional properties: both afferent, in their specificity and sensitivity to various sensory stimuli, and efferent, in their ability to modulate autonomic input to the uterus and directly alter uterine muscular and vascular tone. Taken together, uterine spinal afferent neurons are considered to have a promising role in many different regulatory and reproductive processes and, with further research, may reveal important mechanisms contributing to uterine-associated sensory disorders, such as dysmenorrhoea.

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Post-Infectious Bladder Hypersensitivity in the Development of Interstitial Cystitis/ Bladder Pain Syndrome (IC/BPS)

Harman Sharma, Georgia Bourlotous, and Luke Grundy

Abstract The bladder is innervated by a complex network of afferent nerves that project into the spinal cord, providing sensory input to the central nervous system to regulate normal bladder function. Patients with interstitial cystitis/bladder pain syndrome (IC/BPS) experience painful bladder sensations during normal bladder filling, which leads to the development of chronic pelvic pain and bladder dysfunction. Increased urothelial permeability and inflammation are key contributing factors in the sensitisation of bladder-innervating afferents and development of chronic pelvic pain in IC/BPS patients. However, the mechanisms that underlie the pathogenesis of IC/BPS have yet to be determined. Urinary tract infections (UTIs) are by far the most common pathological insult that occurs in the bladder, and accumulating clinical and pre-clinical studies support a role for UTI in the etiopathogenesis of IC/BPS. This mini-review summarises the epidemiological studies identifying UTI as a significant risk factor for the development of IC/BPS and discusses the complex interactions underlying UTI-induced urothelial permeability, inflammation, and neuroplasticity that may contribute to the development of post-infectious bladder hypersensitivity and IC/BPS.

Keywords Urinary tract infection · Pelvic pain · Neuroplasticity

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Non-standard Abbreviations

DRG	Dorsal root ganglia
GAG	Glycosaminoglycan
IC/BPS	Interstitial cystitis/bladder pain syndrome
MCP1	Monocyte chemoattractant protein-1
pERK	Phosphorylated MAP kinase
TRPV1	Transient receptor potential cation channel subfamily V member 1
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection

1 Introduction

Interstitial cystitis/bladder pain syndrome (IC/BPS) is a chronic pelvic disorder that affects approximately 4% of the population in Western countries (Berry et al. 2011). IC/BPS is characterised by chronic pelvic pain and the presence of other urinary symptoms such as persistent urgency and/or urinary frequency that drastically diminishes quality of life (Grundy et al. 2018a). Many therapeutic options have been trialled for IC/BPS; however, due to the limited knowledge on the etiopathogenesis of this disease, they show limited efficacy. As a consequence, IC/BPS is associated with a significant ongoing health burden and a corresponding social and economic cost of greater than $20 billion per annum in the United States (Pierce and Christianson 2015).

The prevailing theory underlying the development of chronic pain and bladder dysfunction in IC/BPS is that bladder-innervating sensory afferent nerves become sensitised. This neuronal hypersensitivity results in exaggerated sensory signals being sent to the central nervous system during normal bladder filling that gives rise to painful bladder sensations (Grundy et al. 2018a, 2019a; Pierce and Christianson 2015; de Groat and Yoshimura 2009). A variety of factors have been shown to contribute to bladder afferent hypersensitivity in IC/BPS, including increased urothelial permeability, inflammation, and dysregulation of spinal and/or cortical networks (Grundy et al. 2018a; de Groat et al. 2015); however, the events contributing to the genesis of IC/BPS have yet to be determined.

Accumulating clinical and pre-clinical evidence suggests that a history of recurrent or severe urinary tract infection (UTI) represents a significant risk factor for the development of IC/BPS (Rosen and Klumpp 2014; Peters et al. 2009; Rosen et al. 2015; Warren et al. 2008). UTIs are by far the most common insult to the bladder, inducing urothelial permeability and inflammation that initiates bladder hypersensitivity and bladder symptoms characterised by urinary urgency, frequency, and pain (Flores-Mireles et al. 2015). This chapter provides a comprehensive summary of the literature supporting a role for UTI in the development of IC/BPS and delivers insights into the mechanisms that may underlie this phenomenon.

2 Epidemiology and Clinical Significance of Interstitial Cystitis/Bladder Pain Syndrome (IC/BPS)

IC/BPS affects approximately 4% of the Western population with a significant female predominance (Berry et al. 2011; Pierce and Christianson 2015; Jones and Nyberg 1997). Patients with IC/BPS exhibit sensations of bladder fullness, urge to void, and pain at lower bladder distension volumes compared to healthy controls (Grundy et al. 2019a; Kim et al. 2009). These symptoms drastically diminish quality of life, incessantly impacting personal, psychological, sexual, social, and professional life (Berry et al. 2011; Vasudevan and Moldwin 2017). As a result, psychosocial comorbidities are common in IC/BPS patients, with increased severity and prevalence of anxiety and depression compounding to sustain a gradual deterioration of the patient's mental and physical health (Clemens et al. 2008; Chung et al. 2014). Despite this health burden, there are no effective long-term treatments or cures for IC/BPS, with patients often prescribed a variety of non-specific medications including sildenafil, antihistamines, tricyclic antidepressants, and pentosan polysulfate that exhibit limited clinical efficacy (Garzon et al. 2020). Due to this lack of effective treatments, IC/BPS patients in the United States alone carry an economic burden of ~$17 k/person/year, which is equivalent to ~$20–40 billion per annum (Pierce and Christianson 2015). There is an urgent need to understand the pathogenesis and pathophysiology of this disease in order to develop effective treatments for IC/BPS patients.

3 Bladder Sensation in Health and IC/BPS

Bladder sensation is initiated via the activation of primary afferent neurons embedded throughout the bladder wall that innervate both the detrusor smooth muscle and urothelium (Fig. 1) (de Groat and Yoshimura 2009; Spencer et al. 2018). These bladder afferent nerves express a variety of mechanosensitive ion channels that allow the detection of bladder stretch (Marshall et al. 2020; Meerschaert et al. 2020). Bladder afferents also express an array of pro- and anti-nociceptive receptors and ion channels that allow the detection of various neurotransmitters and neuromodulators released from neighbouring urothelial, immune, and interstitial cells (de Groat and Yoshimura 2009; Meerschaert et al. 2020; Grundy et al. 2020a, 2021). Bladder afferents project via the pelvic and hypogastric/splanchnic nerves into the spinal cord where sensory signals are relayed via synapses within the dorsal horn to the brainstem or thalamus, feeding into central circuits responsible for generating bladder sensations and maintaining continence (Fowler et al. 2008). In health, the intensity of the sensory signal carried by bladder afferents is related to the degree of bladder stretch as it fills with urine (Grundy et al. 2019b), leading to the gradual progression of bladder sensations from fullness, to urge, discomfort, and finally pain (Fowler et al. 2008; Grundy et al. 2018b). Patients with IC/BPS perceive

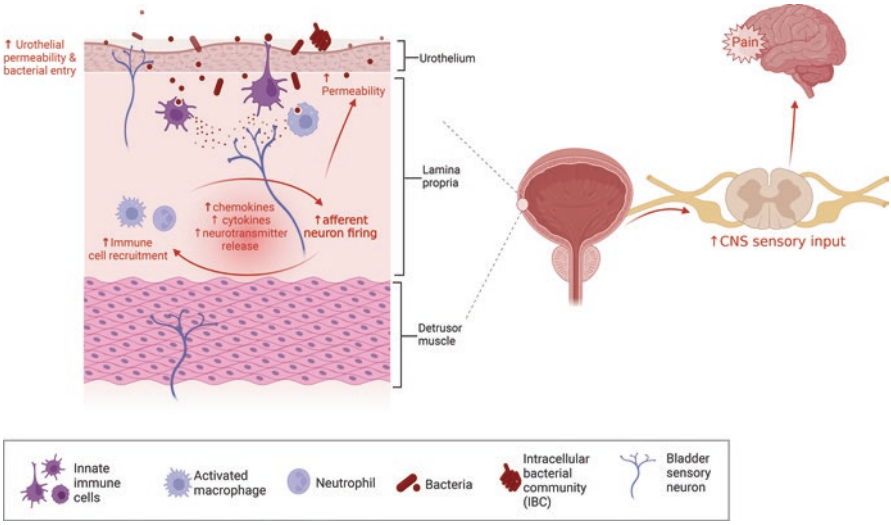

Fig. 1 Mechanisms underlying urinary tract infection (UTI)-induced peripheral afferent hypersensitivity. Pathogenic bacteria such as uropathogenic *E. coli* (UPEC) invade superficial umbrella cells to colonise the urothelium and establish an infection. In response to bacterial colonisation, and to aid bacterial clearance, superficial umbrella cells exfoliate, rapidly removing large quantities of intracellular bacteria (IBCs) that can then be excreted in the urine. An increase in urothelial permeability increases the penetration of urine into the bladder interstitium that can sensitise the peripheral endings of bladder afferents. Bacterial invasion of the bladder wall also initiates a substantial innate immune response that is characterised by the release of numerous cytokines and chemokines, as well as the recruitment of neutrophils, monocyte-derived cells, dendritic cells, and eosinophils. Inflammatory mediators, bacteria, and their soluble release factors can sensitise bladder afferents to physiological stimuli such as bladder distension. Bladder afferent hypersensitivity during UTI increases sensory outflow to the central nervous system (CNS) leading to bladder pain that stimulates urinary frequency and increases expulsion of the bacterial reservoir from the bladder lumen

sensations of bladder fullness, urge to void, and pain at lower cystometric volumes than healthy subjects (Kim et al. 2009), implicating hypersensitivity of bladder afferents to bladder stretch as a key component in IC/BPS pathophysiology (Grundy et al. 2018a; de Groat and Yoshimura 2009).

4 Mechanisms Underlying Afferent Hypersensitivity in IC/BPS

Whilst it is well established that sensitisation of bladder afferents is a crucial component underlying the hypersensitivity symptoms of IC/BPS, the mechanisms contributing to neuronal sensitisation have not been fully elucidated. Evidence for multiple potential mechanisms underlying the development of IC/BPS have been comprehensively described in a number of excellent reviews (Grundy et al. 2018a;

Pierce and Christianson 2015; Akiyama et al. 2020; Karamali et al. 2019). Irrespective of the aetiology, however, a consensus exists to support disruption of mucosal homeostasis, characterised by urothelial permeability and inflammation, in the development of bladder afferent hypersensitivity in IC/BPS (Grundy et al. 2018a, 2019a; Pierce and Christianson 2015; de Groat et al. 2015; Karamali et al. 2019; Parsons 2007).

4.1 Urothelial Permeability

In a healthy bladder, the urothelium forms an impermeable epithelial barrier, preventing the toxic waste metabolites excreted in urine from accessing the bladder interstitium and underlying afferent nerves (Hurst et al. 2015; Dalghi et al. 2020). However, clinical studies commonly identify IC/BPS patients as having a diminished or damaged urothelium (Parsons 2007; Hurst et al. 2015; Elbadawi and Light 1996; Lai et al. 2013; Liu et al. 2012; Tomaszewski et al. 2001), allowing urinary contents to penetrate into the underlying layers of the bladder wall to sensitise sensory afferents. This increased urothelial permeability is assumed to underlie the allodynia and hyperalgesia experienced by IC/BPS patients but not healthy controls undergoing the potassium sensitivity test, in which potassium chloride is infused into the bladder (Hurst et al. 2015; Jiang et al. 2016; Parsons et al. 1998; Montalbetti et al. 2019). In pre-clinical animal models, increased urothelial permeability correlates with sensitisation of bladder afferents to bladder filling, pelvic hypersensitivity to von Frey probing, increased excitability of the cell bodies of bladder afferents within the dorsal root ganglion, and increased c-fos and pERK immunoreactivity in the spinal cord during bladder distension (Montalbetti et al. 2017; Grundy et al. 2020b; Towner et al. 2021). However, where reported, bladder afferent sensitivity to distension following experimentally induced urothelial permeability is transient, returning to control levels by 7 days (Grundy et al. 2020b). This normalisation of bladder sensitivity corresponds with the reported time course of urothelial barrier recovery as a result of injury-induced proliferation of urothelial cells (Dalghi et al. 2020; Shin et al. 2011; Wang et al. 2017; Lavelle et al. 2002; Greenwood-Van Meerveld et al. 2015). As such, it is not clear if increased urothelial permeability is part of the underlying pathogenesis of bladder hypersensitivity in IC/PBS patients or a downstream consequence of additional pathology, such as inflammation, that acts to further embed the chronicity of the condition.

4.2 Bladder Inflammation

Only a small population of IC/PBS patients exhibit significant inflammation, which is characterised by the presence of Hunner's ulcers (Whitmore et al. 2019; Leiby et al. 2007). However, it is widely reported that there are increases in both the

amount of pro-inflammatory mediators, including histamine, nerve growth factor, cytokines, and chemokines, and the number of immune cells, including mast cell, macrophages, and eosinophils within the bladder of IC/BPS patients compared to controls (Liu et al. 2012; Jhang and Kuo 2016; Peters et al. 1999; Liu and Kuo 2012; Furuta et al. 2018; Jacobs et al. 2010; El-Mansoury et al. 1994; Kastrup et al. 1983; Abernethy et al. 2017; Hauser et al. 2008; Sant et al. 2007; Grover et al. 2011). Importantly, these and other pro-inflammatory mediators known to be released from immune cells can directly activate afferent nerve terminals and sensitise bladder afferents to distension (de Groat and Yoshimura 2009; Grundy et al. 2020a, 2021; Davidson et al. 2014; Hughes et al. 2013). Inflammatory mediator-facilitated sensitisation of sensory afferent is a pivotal component of the healing process, providing awareness of an injury to alter behaviour and promote tissue regeneration. However, if inflammation becomes uncontrolled, this can be detrimental to tissue repair (Eming et al. 2007; Landén et al. 2016; Leoni et al. 2015) and can trigger long-term changes in sensory afferent function to induce a persistent hypersensitive state. Inflammatory animal models of IC/BPS have been shown to induce both acute and longer lasting bladder hyperactivity and inflammation, as well as increased voiding frequency and enhanced pain responses during bladder distension. As such, experimentally induced inflammation replicates the IC/BPS phenotype observed in humans (Mills et al. 2020; Takezawa et al. 2014; Hughes Jr. et al. 2016; Lai et al. 2011; DeBerry et al. 2007, 2014, 2015) leading to the suggestion that inflammation provokes peripheral neuroplasticity and may represent a key mechanism in the development of IC/BPS symptoms.

It seems increasingly likely that multiple pathological mechanisms exist concurrently and interact to maintain a protracted state of bladder hypersensitivity in IC/BPS patients. A common theory is that localised inflammation and changes in bladder permeability can combine to allow continuous access of urine from the bladder into the bladder wall, establishing a positive feedback cycle that further promotes an inflammatory state (Grundy et al. 2018a; Sant et al. 2007; Grover et al. 2011). In this scenario, increased inflammatory mediator release combined with greater access of urine into the bladder wall induces sensitisation of peripheral afferent endings leading to chronic bladder hypersensitivity. In this way, it is possible that in susceptible individuals, a physiological response to a bladder insult initiates a feedback cycle that perpetuates bladder inflammation and urothelial permeability to sensitise bladder afferents. If this hypersensitivity persists for long enough, structural, synaptic, and/or intrinsic changes of peripheral or central sensory structures can occur that may drive the persistence of IC/BPS even after the resolution of inflammation and bladder permeability.

Inflammatory models of cystitis, specifically rodent studies that have investigated the consequences of neonatal inflammation on bladder function later in life support this potential mechanism, identifying chronic changes in both bladder sensitivity and bladder function as adults. Bladder inflammation induced by intravesical zymosan in a neonatal rat enhanced the visceromotor response (VMR) to urinary bladder distension, increased micturition frequency, and reduced micturition volume thresholds when tested as adults (Randich et al. 2006). Follow-up

studies revealed hallmarks of peripheral neuroplasticity, including increased bladder content of neuropeptides calcitonin gene-related peptide and substance P and reductions in endogenous opioid inhibitory mechanisms (DeBerry et al. 2007, 2010; Shaffer et al. 2011). As such, early in life bladder inflammation can cause long-lasting changes to the sensory innervation of the bladder that may underlie a susceptibility to the development of painful bladder disorders as adults.

5 Urinary Tract Infections

A UTI is an infection in any part of the upper or lower urinary tract, including kidneys (e.g. pyelonephritis), ureters, bladder, and urethra (Smelov et al. 2016). UTIs are by far the most common pathological insult that occurs in the bladder, affecting more than 150 million people annually (Öztürk and Murt 2020). Like IC/BPS, women are significantly more likely to experience UTIs than men (Öztürk and Murt 2020; Foxman 2002). Recurrent UTIs, whereby a symptomatic UTI returns within 3–6 months following successful resolution of an earlier infection, are also common amongst healthy women, but not men, with around 40% of women experiencing recurrence of UTI (Smelov et al. 2016; Tandogdu and Wagenlehner 2016).

Despite the very high prevalence of UTIs, around 80–90% of all infections are caused by *Escherichia coli (E. coli)* adapted to colonise the bladder (uropathogenic *E. coli,* UPEC) (Smelov et al. 2016). UTIs typically occur most frequently in the lower urinary tract and present with some or all of the following symptoms: dysuria (painful urination), urinary urgency, urinary frequency, and pelvic pain (Chu and Lowder 2018). As these symptoms overlap significantly with the symptoms of IC/BPS, a genuine IC/BPS diagnosis is reliant on a negative urine culture for UTI.

The bladder has evolved a number of mechanisms to prevent UTIs. These include the physical barrier of the urothelium, constitutively expressed proteins and peptides within the urine and mucus layers, and resident immune cells (Abraham and Miao 2015; Lacerda Mariano and Ingersoll 2020). The glycosaminoglycan (GAG) layer, a thick mucus layer of glycoproteins and proteoglycans on the luminal surface of urothelial cells, hinders bacterial adhesion to underlying urothelial cells (Lacerda Mariano and Ingersoll 2020), providing the first line of defence against UTI. If bacteria penetrate the mucus layer, they can invade superficial umbrella cells to colonise the urothelium and establish an infection. In response to bacterial colonisation, and to aid bacterial clearance, superficial umbrella cells exfoliate, rapidly removing large quantities of intracellular bacteria that can then be excreted in the urine (Shin et al. 2011; Klumpp et al. 2006; Mysorekar and Hultgren 2006). Bacterial invasion of urothelial cells also initiates signalling cascades that mobilise a substantial immune response (Lacerda Mariano and Ingersoll 2020). Resident bladder immune cells dominated by macrophages and dendritic cells raise an initial innate immune response to infection that is characterised by the release of numerous cytokines and chemokines, as well as the recruitment of neutrophils, monocyte-derived cells, dendritic cells, and eosinophils (Abraham and Miao 2015; Lacerda Mariano and

Ingersoll 2020). This inflammatory response plays a direct role in clearing the infection. Additionally, evidence is now accumulating that inflammation during UTI is also important in sensitising bladder afferents, which subsequently increases urinary frequency to aid the rapid expulsion of the bacterial reservoir from the bladder lumen (Grundy et al. 2020a; Shin et al. 2011; Klumpp et al. 2006; Mysorekar and Hultgren 2006; Brierley et al. 2020).

5.1 Evidence for UTIs in the Pathogenesis of IC/BPS

There is accumulating evidence that a history of UTI is a significant risk factor for developing IC/BPS and, in some cases, may be the defining event that leads to long-term remodelling of bladder physiology and sensory pathways to induce chronic bladder hypersensitivity (Brierley et al. 2020; Moore et al. 2000; Walsh et al. 2011; Driscoll and Teichman 2001).

Clinical studies supported by anecdotal reporting from patients with IC/BPS provided the first evidence that UTI may be an initiating factor in the development of bladder hypersensitivity (Driscoll and Teichman 2001; Porru et al. 2004; Warren et al. 2006). These studies were conducted by a retrospective review of medical records and telephone interviews of patients with IC/BPS. In 60% of a single patient cohort, recurrent UTIs, diagnosed by positive urine culture, were found to foreshadow the development of IC/BPS (Porru et al. 2004). In these patients, negative urine cultures indicated that infection had successfully cleared following a period of recurrent UTI, yet bladder hypersensitivity symptoms persisted and intensified over the following 12 months in the absence of further infection (Porru et al. 2004). The average lag time from infection through to the development of a complete set of IC/BPS symptoms, combining urinary urgency, frequency, and pelvic pain, was 10 months, supporting a number of other reports that IC/BPS presents as a progressive disease that often starts with a single symptom (Jones and Nyberg 1997; Driscoll and Teichman 2001). Taking a similar approach, but on a larger scale, 314 women with recent-onset IC/BPS had medical records reviewed to identify the presence of UTI at the start of symptoms (Warren et al. 2006). Based on the analysis of retrospective urine cultures and urinalyses, the prevalence of UTI at the onset of IC/PBS, at a minimum, was 16% to 33% (Warren et al. 2006).

A number of studies have also identified that patients with IC/BPS are significantly more likely to have a history of UTIs compared to patients without IC/BPS (Peters et al. 2009; Rosen et al. 2015; Driscoll and Teichman 2001; Ito et al. 2000; Li et al. 2010). In a nationwide cohort of male and female Japanese IC/BPS patients, almost 30% of IC/BPS patients had a medical history of recurrent UTI (Ito et al. 2000), compared to around 5–10% of the general population (Medina and Castillo-Pino 2019). Similar results were obtained from a multicentre study of IC/BPS patients from urology departments across China, which found female IC/BPS patients were significantly more likely than control women without IC/PBS to report a history of UTI, with 32% of IC/BPS patients having a history of infections,

compared to 12% of control patients (Li et al. 2010). In an American cohort, a history of UTI is twice as common amongst IC/BPS patients as controls without bladder pain (Shaffer et al. 2011).

Epidemiology studies have also now identified an increased susceptibility or risk of developing IC/BPS in later life following UTIs in childhood. Recurrent UTI in childhood was found to occur in 18.6% of patients later diagnosed with IC/BPS versus just 2% for asymptomatic control patients (Peters et al. 2009). Controlling for differences in economic circumstances, social status, and race, IC/BPS patients were still significantly more likely to have had recurrent UTIs as children and develop urinary urgency as adolescents, yet no significant differences were seen between patients and the symptomatic control group for any other predictors (Peters et al. 2009). A separate study found 30% of IC/BPS patients had a history of childhood UTIs (Peters et al. 2008), considerably higher than the 10% rate reported in the general population (Shaikh et al. 2008). A more recent study found 18% of children aged 10 that had a UTI diagnosis within the first year of life went on to develop chronic pelvic pain that was not associated with a functional gastrointestinal disorder versus just 3.4% of sibling controls (Rosen et al. 2015). Whether these children will go on to develop a diagnosis of IC/BPS in adulthood remains to be seen.

5.2 Potential Mechanisms Underlying IC/BPS Following UTI

5.2.1 UTI Initiates a Cycle of Bladder Permeability and Inflammation

As described in detail above, clinical and pre-clinical studies have highlighted a crucial role for increased bladder permeability and inflammation in the pathophysiology of IC/BPS. UTIs are by far the most common reason for bladder inflammation and urothelial permeability to occur in a previously healthy bladder. UTIs induce a cascade of events that causes damage to the GAG layer and eventually leads to urothelial apoptosis in an attempt to rapidly excrete urothelial cells that contain invading bacteria (Lacerda Mariano and Ingersoll 2020). Whilst this physiological response hastens the excretion of pathogenic bacteria, there is a corresponding increase in urothelial permeability (Shin et al. 2011). Additionally, UTI induces an immune response characterised by the recruitment of immune cells and the release of numerous pro-inflammatory cytokines and chemokines. Under normal circumstances, when the urothelial barrier is disrupted, there is rapid proliferation and differentiation of organ-specific stem and progenitor cells to regenerate the urothelium (Shin et al. 2011; Mysorekar et al. 2009; O'Brien et al. 2016). However, it is possible that in susceptible individuals, a threshold of urothelial permeability and inflammation is breached that provokes a self-perpetuating feedback cycle leading to persistent inflammation and repetitive injury of the urothelium (Figs. 1 and 2) even after the resolution of the initial infection. Similar mechanisms are thought to contribute to the pathogenesis of inflammatory bowel diseases (Luissint et al. 2016)

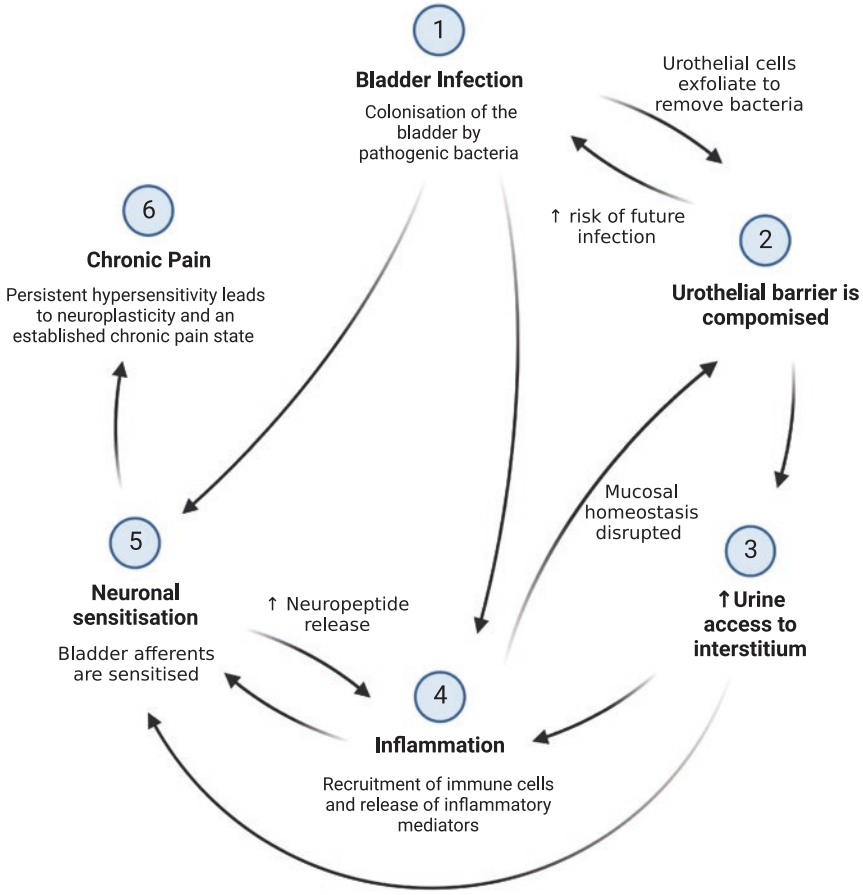

Fig. 2 Proposed mechanisms and feedback cycles underlying post-infectious bladder hypersensitivity: Colonisation of the bladder with pathogenic bacteria such as uropathogenic *E. coli* (UPEC) (1) leads to urothelial cell exfoliation and an increase in urothelial barrier permeability (2). Greater bladder permeability increases susceptibility to future urinary tract infections (UTIs) and increases urine access into the bladder interstitium (3). The myriad solutes within the urine can sensitise peripheral afferent endings within the bladder wall, increasing excitability and lowering activation thresholds of sensory nerves (5). Bacterial invasion of the bladder also initiates a substantial immune response characterised by immune cell recruitment and the release of numerous inflammatory mediators including cytokines and chemokines (4). Bladder inflammation disrupts mucosal homeostasis which perpetuates urothelial barrier permeability (2) and urine access into the interstitium (3). Inflammatory mediators released during inflammation can sensitise bladder afferents to physiological stimuli, increasing excitability and reducing firing thresholds (5). Sensitised bladder afferents release greater amounts of neuropeptides, including substance P and CGRP, that can perpetuate inflammation. Chronic hypersensitivity of peripheral sensory afferents can lead to structural, synaptic, and/or intrinsic changes to neuronal function that encourage the development of chronic neuronal hypersensitivity even after the resolution of the initial sensitising stimuli (6). Persistent neuronal hypersensitivity leads to the development of a chronic pain state and bladder dysfunction characteristic of IC/BPS

and chronic skin wounds such as ulcers (Eming et al. 2007; Landén et al. 2016; Leoni et al. 2015). In the gut, an inflammatory microenvironment disrupts mucosal homeostasis by altering the structure and function of epithelial barrier function, promoting excessive immune responses to the commensal bacteria that comprise the gut microbiota (Luissint et al. 2016; Ordás et al. 2012). Similarly, in the skin, uncontrolled inflammation is detrimental to tissue regeneration and repair of the epithelial barrier during wound healing, leading to the development of chronic skin disease (Frykberg and Banks 2015). Whilst significant challenges remain in the treatment of chronic wounds, opportunities exist to improve healing through protection of the site of injury and/or topical administration of anti-inflammatory agents to try and restore mucosal homeostasis. However, the critical function of the bladder to excrete waste continues in perpetuity, irrespective of the mucosal environment. As such, once a persistent inflammatory state is established, there is limited opportunity for the bladder to heal. Consequently, in patients that experience recurrent UTI the bladder does not have enough time to completely heal between infections, providing the ideal conditions for persistent and damaging inflammation to occur.

5.2.2 UTI-Induced Neuroplasticity of Bladder Afferent Pathways

The nervous system has the ability to modify its activity in response to extrinsic stimuli by reorganising its structure, functions, or connections (neuroplasticity). This is an absolutely critical component in neuronal development and function, allowing the nervous system to modify its physiology to suit unique and evolving environments. However, neuroplasticity can also be the cause of significant morbidity, with neuronal hypersensitivity and structural changes that occur during pathophysiology or injury persisting long after the resolution of the initial sensitising stimuli (Chapman and Vierck 2017; Brierley and Linden 2014; Arendt-Nielsen et al. 2018). This is an established concept in sensory neurobiology and is considered a key contributing factor in the development of other common chronic visceral hypersensitivity disorders such as irritable bowel syndrome and airway hypersensitivity syndromes such as chronic cough (Brierley and Linden 2014; Mazzone and Undem 2016).

Evidence from pre-clinical mouse models shows that single or repeated UTI can induce long-term pelvic hypersensitivity following infection resolution, indicative of neuronal hypersensitivity associated with neuroplasticity (Rosen et al. 2018; Rudick et al. 2012). Intriguingly, the degree of hypersensitivity, as characterised by the % increase in allodynia, increased with subsequent infections (Rudick et al. 2012), suggesting neuronal hypersensitivity could be enhanced by persistent and repetitive insults that recapitulate a recurrent UTI phenotype. The development of chronic pelvic hypersensitivity following UTI in mice has been shown to be reliant on both peripheral and central neuroplasticity. The induction and maintenance of chronic hypersensitivity was shown to be regulated by both TRPV1 and MCP1, respectively, implicating both peripheral afferent signalling and neuroinflammation in the development of post-UTI pain (Rosen et al. 2018). Furthermore, UTI enhanced

spontaneous firing of neurons within regions of the sacral spinal cord where bladder afferents terminate (Rudick et al. 2012), suggestive of central sensitisation. As described in detail above, inflammation and urothelial barrier breakdown have both been shown to sensitise bladder afferent neurons in adult mice (Grundy et al. 2020b; Brierley et al. 2020; Rosen et al. 2018; Rudick et al. 2012). Furthermore, bacteria themselves have also recently been shown to directly act on sensory nerves to evoke hypersensitivity (Montalbetti et al. 2022), providing an additional mechanism through which bacteria can modulate bladder sensory signalling. As such, experimental evidence exists to support a pathological mechanism whereby bladder infection contributes to the development of peripheral afferent hypersensitivity. If this peripheral hypersensitivity persists, long-term changes in central bladder signalling can occur to establish a protracted state of hypersensitivity after the resolution of infection.

The myriad physiological mechanisms that are initiated during a UTI suggest that multiple mechanisms likely coordinate to induce changes in sensory function that induces a chronic hypersensitive state and the clinical symptoms of chronic pelvic pain.

6 Conclusions

Accumulating epidemiological and experimental evidence supports the hypothesis that UTI is a significant risk factor in the development of chronic pelvic pain in IC/BPS. The physiological responses essential to clearing UTIs, including urothelial sloughing, inflammation, and neuronal hypersensitivity, are likely also key mechanisms in the development and maintenance of IC/BPS. Despite this, many people who develop UTIs and even recurrent UTIs do not go on to develop IC/BPS, suggesting that additional environmental and/or physiological risk factors that have yet to be identified are key to the pathogenesis of post-infectious UTI in susceptible populations. The time between initial bladder infection and the development of a complete set of IC/BPS symptoms represents a key window of opportunity for therapeutic intervention to prevent the development of post-infectious hypersensitivity. However, unravelling the underlying cause of neuronal hypersensitivity to develop a targeted and appropriate therapeutic approach remains a significant challenge.

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