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Investigating epithelial-neuronal signaling contribution in visceral pain through colon organoid-dorsal root ganglion neuron co-cultures

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Abdominal pain is a common symptom associated with irritable bowel syndrome and inflammatory bowel diseases (IBDs), affecting about 20% of the global population (Grundy et al., 2019). Current pain therapies are poorly effective on visceral pain of intestinal origin and present several side effects, hence the need to identify novel molecular and cellular targets for drug development. The pathophysiology of visceral-abdominal pain, which often originates from the colorectal region, involves several actors, including the gut microbiome, intestinal epithelium, immune system, and nervous system at different levels through the gut-brain axis (Lucarini et al., 2020, 2022; Najjar et al., 2020). Nociceptive stimuli from the bowel to the central nervous system are mainly encoded by spinal afferents, whose cell bodies reside within the thoracolumbar and lumbosacral dorsal root ganglia (DRGs; Grundy et al., 2019). Visceral pain results from an altered neuronal transduction and transmission of stimuli generated within the gut. Current evidence attests that visceral hypersensitivity is a complex phenomenon, consisting of multiple mechanisms, with immune cells playing a role in hypersensitizing colon afferents through the release of different mediators (Grundy et al., 2019). However, pain is also reported without any inflammatory status, suggesting factors other than the inflammatory/immune signaling in driving pain transmission/persistence.

Colon epithelial cells have been recently described as direct regulators of the spinal afferents. Optogenetic studies revealed that the light-activation of channel rhodopsin 2-expressing colon epithelial cells was sufficient to activate nociceptive neurons and induce pain-like behaviors in mice. In contrast, the light-induced inhibition of archaerhodopsin-expressing colon epithelial cells reduced hypersensitivity in a mouse model of IBD (Najjar and Albers, 2021). Among the different colon epithelial cells, enteroendocrine cells play a central role in epithelial-neuronal signaling because of their sensing function, electrical excitability, and ability to form synaptic structures with neurons (Bayrer et al., 2023). However, mechanisms underlying epithelial-neuronal communication still need to be elucidated. The first studies using optogenetic approaches in animal models have been fundamental for demonstrating the role of the epithelium in pain signaling, but they display some limitations as animal models can differ from human diseases, are subjected to many variables, and do not allow to dissect molecular signals between the different actors mentioned above.

Colon organoid cultures, deriving from human stem cells and exclusively reflecting epithelial components, could represent a highly promising tool to specifically investigate the role of the intestinal epithelial cells in the epithelial-neuronal interplay during abdominal pain perception. Several studies reported that intestinal organoids established from IBD patients differ from healthy controls and can preserve some disease-specific epigenetic and transcriptional modifications (d'Aldebert et al., 2020), though pro-inflammatory stimuli are needed to recreate *in vitro* the phenotype of the epithelium subjected to a long term-inflammatory status in IBD patients (Noben et al., 2017). Furthermore, growing colon organoids with fecal supernatants from irritable bowel syndrome/IBD patients (Holst et al., 2022) or gut bacteria allowed to investigate the relationship between microbiota alterations and epithelial dysfunction associated with painful gastrointestinal diseases. Although organoids alone do not reproduce all *in vivo* cellular partners and thus are unable to reflect the complex network of interactions occurring in the whole intestine, they can be co-cultured with different gut components, such as immune cells (Hentschel et al., 2021), myofibroblasts, and enteric neurons among others. Furthermore, co-cultures offer different degrees of complexity, which allow either the investigation of singular cell-to-cell interactions or to recreate multicellular networks. Lastly, co-cultures can be almost entirely recreated by human cells.

Despite their potential usefulness to explore both the epithelial-neuronal signaling and the neuro-epithelial feedback effects, no reports exist on co-cultures of colon organoids and DRG neurons. Most of the knowledge about the role of DRG neurons in visceral pain comes mainly from studies using monoculture of rodent neurons besides *in vivo*/*ex vivo* approaches. Regarding humans, the establishment of DRG neurons cultures has been limited by the inaccessibility of human nervous tissues until it emerged the possibility of obtaining sensory cells from human embryonic or pluripotent stem cells to generate 2D cultures or even 3D DRG organoids, that better replicate DRG spatial architecture and cellular diversity (Mazzara et al., 2020). By keeping cultures physically separated, human sensory neurons or organoids could be treated with conditioned media from patient-derived colon organoids, reproducing a one-way signaling flow. Evaluating the effects of conditioned media and their proteo-metabolic characterization could help identify which epithelium-derived signal drives visceral hypersensitivity. Only colon organoids-DRG neuron co-cultures could recreate the native bidirectional cross-talk between colon epithelium and sensory neurons, allowing to study how they mutually affect each other's functionality. Growing colon organoids as polarized monolayer cultures on transwells (Jelinsky et al., 2023) ensures a luminal and basal side that can be exposed to DRG neurons. The two-chamber transwell system allows to re-separate the two components anytime, thus enabling parted experimental analyses on both. In this context, electrophysiological recordings or calcium imaging of neurons are promising approaches to evaluate the modulatory effects of intestinal epithelial cells on neuronal excitability. Moreover, combining colon and DRG sensory organoids could generate assembloids, intriguing 3D cellular systems that could resemble with higher complexity the bidirectional sensory neuron-to-epithelium signaling circuitries (Kanton and Pasça, 2022). In this regard, recent evidence demonstrated the ability of DRG organoids to independently develop intricate connections with specific peripheral cells (i.e. muscle fibers and keratinocytes), leading to the establishment of proprioceptive or neurosensory-epithelial networks, providing substantial evidence for the *in vitro* capability of DRG organoids to generate specialized connections with epithelial structures (Mazzara et al., 2020, 2022). An improved integration of cellular components might be achieved by "gut-on-a-chip" platforms that enable different cell types to grow and interact under peristaltic-like deformations, fluid flows, and chemical gradients (Bein et al., 2018), thereby allowing to explore any modulatory effects of microbiota as well as immune cells, myofibroblasts, enteric neurons or other cell populations on the binary epithelial-neuronal interplay. All the described co-culture systems (**Figure 1**) are suitable to investigate different experimental conditions, including gut microbiome alterations and exposure to drugs or other compounds. Of course, in interpreting the results, it is always important to consider that *in vitro* studies represent a useful but simplified way for modeling pain compared to *in vivo* models, where the pain processing extends from the periphery to the central nervous system, involving several partners along the path. On the other hand, the *in vitro* simplification of the system allows for the dissection and the study of specific cell-to-cell signaling occurring *in vivo* and might be exploited for developing targeted interventions for pain. In this regard, several painful scenarios might be explored, such as dysbiosis, inflammation or ischemic conditions, gut exposure to dietary components or toxic agents, as well as their combination. Indeed, elucidating the complex changes occurring in the bidirectional interaction between epithelium and neurons contributing to pain among the different scenarios is expected to help in the development of tailored therapies.

On this base, colon organoids-DRG neurons co-cultures represent a simplified yet suggestive *in vitro* model that might help in unraveling the intricate mechanisms behind visceral sensitivity regulation and offer fresh perspectives on pain physiology across different gastrointestinal diseases, revealing innovative targets and facilitating the validation of newly developed therapeutic interventions.

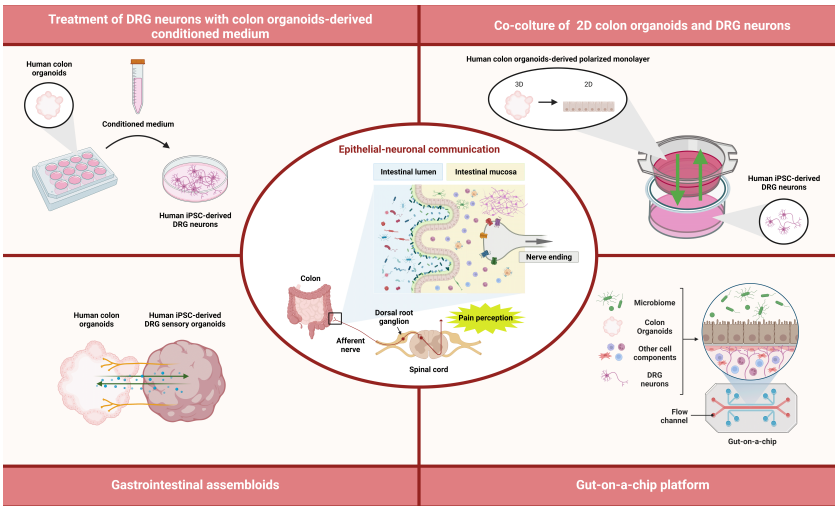

Figure 1 | **Illustrative image of colon organoid-DRG neuron co-colture systems.**
In vitro models based on colon organoids and DRG neurons to explore the bidirectional epithelial-neuronal interplay in visceral pain regulation. Created with BioRender.com. DRG: Dorsal root ganglion; iPSC: induced pluripotent stem cell.

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