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Catechol-O-Methyltransferase Loss Drives Cell-Specific Nociceptive Signaling via the Enteric COMT/miR-155/TNF-α Axis

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Abstract

BACKGROUND & AIMS: The etiology of abdominal pain in post-infectious, diarrhea-predominant IBS (PI-IBS-D) is unknown and few treatment options exist. Catechol-O-methyltransferase (COMT), an enzyme that inactivates and degrades biologically active catecholamines, plays an important role in numerous physiologic processes, including modulation of pain perception. Our objective was to determine the mechanism(s) of how decreased colonic COMT in PI-IBS-D patients contributes to the chronic abdominal pain phenotype following enteric infections.

METHODS: Colon neurons, epithelial cells, and macrophages were procured with LCM from PI-IBS-D patients to evaluate cell-specific colonic COMT, miR-155, and TNF-α expression levels compared to recovered subjects (infection cleared: did not develop PI-IBS-D) and controls. $COMT^{-/-}$, colon-specific $COMT^{-/-}$, $miR\text{-}155^{-/-}$ mice and human colonoids were used to model phenotypic expression of COMT in PI-IBS-D patients and to investigate signaling pathways linking abdominal pain. *C. rodentium* and TNBS animal models were used to model post-inflammatory changes seen in PI-IBS-D patients.

RESULTS: Colonic COMT levels were significantly decreased and correlated with increased VAS abdominal pain ratings in PI-IBS-D patients compared to recovered subjects and controls. Colonic miR-155 and TNF-α were increased in PI-IBS-D patients with diminished colonic

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COMT. COMT$^{-/-}$ mice had significantly increased expression of miR-155 and TNF-α in both colon tissues and DRGs. Introduction of cV1q antibody (anti-TNF-α) into mice reversed visceral hypersensitivity following C. rodentium and TNBS.

CONCLUSIONS: Decreased colonic COMT in PI-IBS-D patients drives abdominal pain phenotypes via the COMT/miR-155/TNF-α axis. These important findings will allow new treatment paradigms and more targeted and personalized medicine approaches for gastrointestinal disorders following enteric infections.

LAY SUMMARY

The authors identified molecules in colon tissues of irritable bowel syndrome patients that lead to abdominal pain. These molecules induced visceral pain in mice that was reversed with blocking agents.

Keywords

Irritable Bowel Syndrome; Catechol-O-Methyltransferase; Visceral Hypersensitivity; Abdominal Pain

Introduction

Irritable bowel syndrome (IBS) is characterized by persistent abdominal pain and alterations in bowel habits.[1] Available treatments are far from ideal as the pathophysiological mechanisms are still poorly understood.[2–3] Noxious environmental stimuli, tissue damage, and inflammation can all evoke enteric neuroplasticity and lead to chronic GI symptoms.[4–5] Following enteric infections, 15–20% of patients may develop chronic post-infectious, diarrhea-predominant, irritable bowel syndrome IBS (PI-IBS-D).[6] Similarly, transient bowel inflammation disrupts gut motility and increases visceral nociception, which persist long after resolution of the inflammation as demonstrated in animal models of colitis, intestinal hyperpermeability, inflammatory bowel disease, and bile acid diarrhea.[7–10]

microRNAs (miRNAs) are key regulators of gene expression in the gut and may mediate enteric neural damage that follows insults to the GI tract.[11–12] For example, miRNA-199 regulates enteric TRPV1 in patients with IBS leading to increased visceral pain.[11] miR-375 has also been shown to mediate palmite-induced enteric neuronal damage.[12] Catechol-O-methyltransferase (COMT), an enzyme that inactivates and degrades biologically-active catecholamines (dopamine, epinephrine, norepinephrine) has been shown to play an important role in numerous physiologic processes, including modulation of pain perception. Variation in the *COMT* gene, leads to altered perception of nociceptive stimuli, increased susceptibility to chronic pain conditions, and a need for escalating doses of opioids for pain.[13–14.] The COMT/Catecholamine pathway has been studied for decades, however, our current investigations focus on previously unidentified signaling pathways in an enteric infectious bioenvironment in addition to the COMT-Catecholamine pathway that leads to abdominal pain in PI-IBS-D patients.

Our *overall objective* was to determine the mechanism(s) of how decreased colonic COMT in PI-IBS-D patients contributes to chronic abdominal pain phenotypes following

enteric infections. Our *specific objectives* were to: (1) elucidate the mechanistic role of decreased colonic-COMT in chronic abdominal pain in PI-IBS-D patients; (2) use translational datasets (in vitro, in vivo, human ex vivo) to determine how the colonic COMT/miR-155/TNF-α axis drives visceral hypersensitivity and abdominal pain; (3) examine the mechanisms of COMT/miR-155/TNF-α axis regulated signaling in vitro using cell culture and colonoids; (4) develop a novel therapeutic approach for PI-IBS-D patients with elevated TNF-α to reduce visceral hypersensitivity and chronic abdominal pain. Achieving these goals should increase our understanding of the mechanisms involved in PI-IBS-D and lead the way to the development of more targeted therapies.

Materials and Methods

Recruitment:

Human studies were approved by the University Institutional Review Board. Informed consent was obtained from all patients. Patients (ages 18–72) who met the Rome IV criteria for IBS-D and had a documented case of enteric infection and were diagnosed with PI-IBS-D were included. In addition, “recovered subjects” were included who had a documented enteric infection that cleared, but they did not develop PI-IBS-D. Each patient had a comprehensive history, physical exam, hydrogen breath test (for bacterial overgrowth), and lab studies completed. Patients with a history or laboratory diagnosis of celiac sprue, inflammatory bowel disease, bacterial overgrowth, pancreatic disease, or GI surgery were excluded. Participants rated their abdominal pain once daily upon wakening on a continuous visual analog scale (VAS) from 0–10 for 7 days and the mean was calculated.

Mice:

Animal studies were approved by the Institutional Animal Care & Use Committee and were performed according to the accepted NIH guidelines. $COMT^{-/-}$, colon-specific $COMT^{-/-}$, and $miR\text{-}155^{-/-}$ mice were used along with the mouse model for Citrobacter rodentium (see supplementary methods).[15–18] Intracolonic infusion of 2,4,6-trinitrobenzene sulfonic acid (TNBS, 100 mg/kg in 50% ethanol) was used to produce colitis in $COMT^{-/-}$, colon-specific $COMT^{-/-}$, $miR\text{-}155^{-/-}$, and WT mice.[19–22] The mice were tested 4 weeks after TNBS or *C. rodentium*.

miR-155 Treatment:

mice received lentivirus containing GFP and control-miR expressing vector (control-lenti) or GFP and miR-155 expressing vector (Lenti-miR155-inhibitor). Lentivirus was introduced intraperitoneally (IP) with 100 μl lentivirus solution containing 1×10^8 TU (Transducing Units) lentivirus and 4 μg ml^{-1} polybrene in 1x PBS.

Anti-TNF-α Therapy:

Mice received daily intraperitoneal anti-TNF antibody (cV1q)(100 μg in 100 μl of PBS) or its isotype control antibody (cVam).[23]

Statistical Analysis:

All statistical analyses were done using GeneSpring GX software version 7.3 (Agilent Technologies, Santa Clara, CA), Prism version 6 (GraphPad Inc, San Diego, CA), and ASA software Version 9.1.3. One-way ANOVA analysis was done, followed by Tukey's comparison or by the Benjamini and Hochberg correction for false-positive reduction. T tests were also used.

Please refer to the online Supplementary Materials for additional detailed methods.

Results

1. Down-regulated colonic-COMT expression drives abdominal pain in PI-IBS-D patients.

To identify underlying mechanisms of abdominal pain in IBS patients, we carried out real-time PCR-based gene profiling super-array analysis. The Human Neurotransmitter Receptors and Regulators PCR Array was selected because it contained 84 key genes associated with pain-related biological pathways and other neuronal functions. The expressions of COMT, NPY1R, and NPY2R were decreased in IBS patients along with markedly increased GRIN1, GRIA1 and DRD2 expression (Figure 1A). Real-time PCR analysis revealed decreased COMT expression from colon tissues in all IBS patients (n = 61)(PI-IBS-D [n=39], IBS-A [n=14], IBS-C [n=8]) compared to controls (n =24) (**$p<0.01$, Figure 1B, Supplementary Figure 1, Supplementary Table 1). Real-time PCR showed a 3-fold reduction in COMT expression in PI-IBS-D patients compared to controls (***$p<0.001$), however, a much smaller decrease in COMT expression was seen in IBS-A and IBS-C patients compared to controls respectively (Figure 1C). Additional PCR Arrays (Neurotransmitter Receptors and Regulators) done in IBS-A and IBS-C patients' colon tissue (Supplementary Figures 2 and 3) confirmed no changes in COMT suggesting that IBS-A & IBS-C may have different pathways. Therefore, we focused our studies on the PI-IBS-D group that had decreased colonic-COMT and abdominal pain that inversely correlated with VAS abdominal pain ratings (Figure 1D, $r= -0.77$; $p<0.001$).

The Human Neurotransmitter Receptors and Regulators PCR Array was used to identify the COMT target neurotransmitter receptor genes (Figure 1E) in colon tissues from PI-IBS-D patients. Among 5 α and β adrenergic receptors included in the array, 4 were significantly decreased in PI-IBS-D patients compared to controls and correlated with the inhibited COMT signaling pathway. Meanwhile, GABRA6, HCRTR2, GABRA, and BRS3 were the most up-regulated genes in the colon tissues from PI-IBS-D patients. Altered catecholamine expression (epinephrine, norepinephrine and dopamine) was also observed in colon tissues (Supplementary Figure 4) and plasma (Supplementary Figure 5) in PI-IBS-D patients.

Next, $COMT^{-/-}$ and colon-specific $COMT^{-/-}$ mice were used to investigate *new* COMT signaling pathways linking abdominal pain in PI-IBS-D patients.[15]

2. Enhanced miR-155/TNF-α expression in $COMT^{-/-}$ mice drives visceral hypersensitivity

2.1. Up-regulation of TNF-α in $COMT^{-/-}$ mice: $COMT^{-/-}$ mice were used to model phenotypic expression of COMT in PI-IBS-D patients. To determine if decreased colonic-

COMT expression drives afferent input to DRGs in PI-IBS-D patients, DRGs isolated from six COMT$^{-/-}$ and six WT mice were analyzed with the Neuropathic & Inflammatory PCR arrays. TNF-α was among the most up-regulated genes ($p<0.001$) in DRGs from COMT$^{-/-}$ mice compared to WT mice (Supplemental Figure 6). Interestingly, the Ingenuity Pathway Analysis revealed that TNF-α expression was linked with COMT expression (Supplemental Figure 7). Furthermore, Figure 2A shows that TNF-α is significantly increased in DRGs from COMT$^{-/-}$ mice. Real-time PCR analysis confirmed that TNF-α was significantly up-regulated not only in DRGs, but also, in colonic tissues in COMT$^{-/-}$ mice compared to WT mice (Figure 2B).

To validate the COMT$^{-/-}$ mice data, we used a COMT inhibitor (OR486) in post-TNBS mice and found similar results (Supplemental Figure 8). We examined epinephrine, norepinephrine, and dopamine in the colon, DRGs, spinal cord, and plasma of mice post-TNBS, as well as following COMT inhibitor (OR486) treatment (Supplementary Figures 9 and 10).

There was significantly enhanced TNF-α expression following COMT inhibitor (OR486) treatment; however, colon-TNF-α was significantly blocked by both a β2AR and a β3AR antagonist (SR59320A) (Figure 2C, left panel). This is similar to previous studies where peripheral TNF-α production was blocked following a COMT inhibitor combined with β2 and β3 adrenergic receptor antagonists.[24–25] Interestingly, when the same experiments were done using β2 & β3 antagonists in mice that received *C. rodentium*, there was no significant inhibition of TNF-α production (Figure 2C, right panel). These interesting data suggest that there may be an alternative pathway operating in the enteric COMT/miR-155/TNF-α axis following infection which is different from a non-infectious state. Could miR-155 be the additional factor driving TNF-α production in this infectious bioenvironment? To evaluate this alternative pathway, a miR-155 inhibitor was given in addition to β2 & β3 antagonists. In this condition, TNF-α production was significantly inhibited (Figure 2C, right panel: E&G). Thus, the COMT/miR-155/TNF-α axis may be an important signaling pathway in the GI tract that mediates chronic abdominal pain following enteric infections in PI-IBS-D patients. The β2 & β3 adrenergic system may also be involved in COMT-mediated abdominal pain; however, miR-155 modulation factor is an important component that leads to TNF-α production under diminished COMT conditions.

2.2. Enhancement of miR-155 expression in COMT$^{-/-}$ mice: TNF-α has been shown to be regulated by specific miRNAs such as miR-155 in IBD patients.[19] Thus, we hypothesized that COMT may affect TNF- α production in PI-IBS-D patients via regulation of specific miRNAs. miRNA profiling microarrays compared miRNA expression in DRGs from COMT$^{-/-}$ mice to WT mice to detect in vivo miRNA expression in DRGs under COMT knockout conditions. Of the ~1900 mmu-miRNAs represented in this microarray; aberrant expression of some miRNAs was observed in DRGs from COMT$^{-/-}$ mice. This cluster analysis identified expression of specific DRG miRNAs that were either significantly decreased or significantly increased in DRGs from COMT$^{-/-}$ mice (Figure 2D, upper panel) compared to DRGs from WT mice. Expression of miR-155 was significantly up-regulated. The upper panel in Figure 2D illustrates miRNA expression analysis for miR-155. Real-time PCR confirmed a significant increase in colonic miR-155 and DRG miR-155 levels in

COMT$^{-/-}$ mice compared to WT mice (Figure 2D, lower panel). To validate the COMT$^{-/-}$ mice data, we used a COMT inhibitor (OR486) in post-TNBS mice and found similar results (Supplemental Figure 11). Enhanced colonic-miR-155 expression was also observed in post-TNBS mice treated with β2AR antagonist (ICI-118,551) plus a COMT inhibitor (OR486) compared to post-TNBS mice with β2AR antagonist-treated mice (Supplementary Figure 12).

2.3. Co-localization of TNF-α and miR-155 expression under COMT knockout conditions.—Recent studies have shown that inhibition of miR-155 expression decreased TNF-α mRNA half-life, whereas miR-155 over-expression, increased TNF-α.[20] FISH analysis was done and revealed a substantial increase in miR-155 expression in the colon of COMT$^{-/-}$ mice, which was associated with enhanced TNF-α expression by fluorescence immunocytochemistry (Figure 2E-1) compared to WT mice (Figure 2E-2). Similarly, there was increased TNF-α expression in DRGs of COMT$^{-/-}$ mice accompanied by up-regulation of miR-155 (Figure 2F-1, upper panel) compared to WT mice (Figure 2F-1, lower panel). Even more interesting, decreased COMT was associated with increased TNF-α expression in DRGs of COMT$^{-/-}$ compared to WT mouse (Figure 2F-2). These results suggest that inhibition of COMT enhances TNF-α expression through miR-155 induced signaling pathways.

2.4. Evidence for altered COMT-miR-155-TNF-α signaling in a post-TNBS & Citrobacter rodentium mouse model.—Mouse inflammatory cytokines and receptors RT^2 profiler PCR array was used to study inflammation-associated genes in post-TNBS mice. TNF-α was up-regulated in post-TNBS mouse colon with or without COMT depletion (Supplementary, Figure 13). There was significantly increased visceral hypersensitivity 4 weeks post-TNBS in COMT$^{-/-}$ mice (Figure 2G-1, lower panel). Higher external oblique abdominal muscle EMG activity was present in COMT$^{-/-}$ mice 4 weeks post-TNBS mice compared to WT mice during colonic distension (Figure 2G-1, upper panel). To mimic colon-dependent COMT depletion in PI-IBS-D patients, colon-specific COMT$^{-/-}$ mice were also used to test visceral hypersensitivity in post-TNBS. There was significantly enhanced visceral hypersensitivity in colon-specific COMT$^{-/-}$ mice after TNBS (Figure 2G-1, lower panel) compared to colon-specific COMT$^{-/-}$ naïve mice. miR-155$^{-/-}$ mice were used to test visceral hypersensitivity post-TNBS. There was slightly increased visceral hypersensitivity in miR-155$^{-/-}$ mice after TNBS (Figure 2G-1, lower panel) compared to naïve miR-155$^{-/-}$ mice.

Interestingly, 16 weeks post-TNBS, visceral hypersensitivity resolved in WT mice compared to colon specific COMT$^{-/-}$ mice which still had persistent visceral hypersensitivity (Supplementary Figure 14). These findings suggest that the reduction of COMT may be a key factor for development of chronic visceral hypersensitivity following an inflammatory event. Mechanical sensitivity using Von Frey filaments was used to verify increased excitability of mechanonociceptor neurons in visceral hypersensitivity in both WT and colon-specific COMT$^{-/-}$ mice following TNBS (Supplementary Figure 15). Then, to investigate neuronal excitability of the DRG neurons projecting to the terminal colon, patch clamp recordings were used to determine if the excitability of DRG neurons is altered in

both colon-specific COMT$^{-/-}$ and wild type mice following TNBS treatment compared to naive control mice (Supplementary Figures 16-1 and 16-2). Comparing results from WT control DRGs to WT DRGs following TNBS to COMT−/− DRGs following TNBS, we found a progressive decrease in resting membrane potential and a progressive increase in the number of action potentials elicited by 2X rheobase current. These results indicate an increase in several indices of DRG neuronal excitibity following TNBS, an effect further increased in colon specific COMT$^{-/-}$ plus TNBS group.

There was significantly reduced TNF-α expression in miR-155$^{-/-}$ mouse DRGs/colonic tissues 4 weeks post-TNBS treatment compared to WT mice (Figure 2G-2, lower panel). Immunofluorescence analysis revealed diminished TNF-α expression post-TNBS in DRGs of miR-155$^{-/-}$ mice compared to WT mice (Figure 2G-2, upper panels). Additional analysis revealed that post-TNBS treated mice compared to TNBS+β1AR, TNBS+ β2AR, and TNBS+ β3AR show no significant alteration in both colon and DRGs for TNF-α and miR-155 expression (Supplementary Figure 17 & 18).

To mimic a post-infectious bioenvironment, experiments were repeated using the validated *C. rodentium* mouse model (Supplementary Figure 19).[17] Both post-TNBS and *C. rodentium* are validated and well-accepted post-inflammatory/infectious animal models for IBS.[16,–18,22] Enhanced miR-155 and TNF-α in the DRGs and colon tissue were confirmed following infection with *C. rodentium* in COMT$^{-/-}$ and colon-specific COMT$^{-/-}$ mice (Supplementary Figure 20, upper panels). miR-155 and TNF-α were also examined in spinal lumbosacral dorsal horn to investigate the miR-155/TNF-α expression in colon tissue derived COMT. There was significantly enhanced miR-155 and TNF-α expression in the lumbosacral dorsal horn in COMT$^{-/-}$ post-*C. rodentium* mice compared to COMT$^{-/-}$ naïve controls (Supplementary Figure 20, lower panels). Thus, the COMT/miR-155/TNF-α axis may be involved in the development of both the post-TNBS & post-*C. rodentium* animal models suggesting that knockout of miR-155 prevents visceral hypersensitivity in post-TNBS and post-*C. rodentium* mice.

We also measured stool pellet production to quantify changes in transit time. There was a slightly decreased transit time in COMT$^{-/-}$ mice treated with *C. rodentium* compared to WT mice post-*C. rodentium* treatment (Supplementary Figure 21). However, we did not observe a significant change in transit time in naïve COMT$^{-/-}$ compared to naïve WT mice.

2.5. Effect of COMT depletion on the NF-κB/miR-155 signaling pathway.—

The interaction between NF-κB and COMT, which contributes to the pathogenesis of inflammatory pain states, has been previously established. NF-κB activates miR-155 expression in various types of tissues and cells. Therefore, COMT may regulate miR-155 through a NF-κB associated signaling mechanism. To further advance insights into the role/mechanisms of COMT in innate immunity, including its effects on cytokines and the NF-κB signaling pathway[26–28], we assessed NF-κB activation and gene expression profiles of colon tissues from post-TNBS mice. NF-κB p65 Transcription Factor Assays revealed that TNBS treatment of both WT and COMT$^{-/-}$ mice significantly activated NF-κB (Figure 2H, upper panel), and enhanced expression of miR-155 (Figure 2H, lower panel). Altered expression of key NF-κB signaling mediators, including the NF-κB p65 unit, IκBα, and

phosphor-p65, were observed in COMT$^{-/-}$ mouse tissues with and without TNBS using immunohistochemistry and real-time PCR assays (Supplementary Figures 22 & 23).

3. Enhanced miR-155/TNF-α signaling by COMT down-regulation drives abdominal pain through the COMT/miR-155/TNF-α axis in PI-IBS-D patients

Upregulated miR-155/TNF-α axis signaling was found in COMT$^{-/-}$ mice which prompted us to determine how colonic-COMT depletion drives chronic abdominal pain in PI-IBS-D patients.

3.1. Enhanced miR-155/TNF-α signaling by COMT down-regulation induces abdominal pain in PI-IBS-D patients.—

Proinflammatory cytokines (i.e., IFN-γ and TNF-α) are elevated in some IBS patients.[11] Our studies revealed mRNA colonic TNF-α expression (not IFN-γ) was higher ($p<0.05$) in PI-IBS-D patients compared to controls (Figure 3A, left panel). ELISA shows increased colonic TNF-α expression in PI-IBS-D patients (Figure 3A, right panel). miR-155 was also increased in PI-IBS-D patients and positively correlated with TNF-α expression ($r=0.81$, $p<0.001$) (Supplementary Figure 24). Interestingly, TNF-α expression was also positively correlated with VAS abdominal pain scores in PI-IBS-D patients ($r=0.74$, $p<0.01$) (Figure 3B). FISH analysis was then done which revealed colocalization of colonic miR-155 with TNF-α expression in PI-IBS-D patients (Figure 3C).

3.2. Cell-specific alterations of the COMT/miR-155/TNF-a axis in human colonic neurons, epithelial cells, and macrophages

3.2.1. Colonic Neurons: LCM was used to procure colonic neurons from PI-IBS-D patients, recovered subjects, and controls using a neuronal marker, Pan-neuron, under direct microscopy (Supplementary Figure 25, left panel) and the right panel Supplementary Figure 25 indicates the genes and targets (COMT, TNF-α, miR-155) that were examined. COMT expression was decreased; TNF-α expression was increased; and there were no significant changes in miR-155 expression (Figure 3D-1, left panel). Recovered subjects showed no significant changes in COMT, miR-155, and TNF-α in colon-neuronal cells compared to controls (Figure 3D-1, right panel).

3.2.2. Colonic Epithelial Cells and Macrophages: LCM was also used to procure colonic epithelial cells from PI-IBS-D patients, recovered subjects, and controls using an epithelial marker, CK8 (Supplemental Figure 26). COMT expression was decreased and TNF-α and miR-155 expression were increased in PI-IBS-D patients (Figure 3D-2, left panel). Recovered subjects showed no significant changes in COMT, miR-155, and TNF-α compared to controls (Figure 3D-2, right panel). Similar findings were also verified in LCM isolated colonic macrophages from PI-IBS-D patients (Figure 3D-3 and Supplemental Figure 27) using CD68 as the specific marker. Thus, these interesting data suggests there may be cell-specific signaling pathways in these 3 colonic cell types that are mediators of chronic abdominal pain in PI-IBS-D patients.

3.3. The effect of COMT depletion on the NF-κB/miR-155 signaling pathway in PI-IBS-D patients.—

We explored the interaction between NF-κB and COMT in PI-

IBS-D patients, recovered subjects, and controls to determine if NF-κB activates miR-155 expression. Thus, COMT may regulate miR-155 through NF-κB. NF-κB activation and gene expression profiles of colon tissues were assessed from PI-IBS-D patients. NF-κB p65 Transcription Factor Assays revealed significantly activated NF-κB (Figure 3E, left panel) and enhanced expression of miR-155 (Figure 3E, right panel) in colonic tissues of PI-IBS-D patients. We examined the key NF-κB signaling mediators (NF-κB-50, NF-κB-p65, NF-κB1-p105 and IκB-alpha) expression in colon tissues of PI-IBS-D patients, recovered subjects and controls (Figure 3F). There was significantly enhanced NF-κB p65 expression accompanied by significantly diminished IκB-alpha in colonic tissues of PI-IBS-D patients (*$p<0.05$). Further analysis in colonic-epithelial, -macrophage, and -neuronal cells, showed that there was diminished IκB-α in colonic neuronal cells accompanied by diminished IκB-α. There was decreased IκB-α in colon epithelial cells and enhanced NF-κB expression in macrophages in PI-IBS-D patients compared to controls and recovered subjects (Figure 3G). These data may help to explain why there was no significant increase of miR-155 in PI-IBS-D patients' colonic neuronal cells due to significant upregulation of NF-κB, superseding the miR-155 pathway. Cell culture studies were also done in which FHs 74 Int cells were transfected with si-COMT and control siRNA with and without co-incubation of the α-adrenergic receptor antagonist doxazosin or ß2-adrenergic receptor antagonist ICI 118,551 or ß3-adrenergic receptor antagonist SR59320A for 24 hours. No major changes of α and ß adrenergic receptor antagonists on si-COMT induced NF-κB activation were observed (Supplementary Figures 28–29).

4. COMT regulated miR-155 /TNF-α signaling in vitro via cell culture & human colonoid study.

The next series of experiments verified our findings for humans and mouse models by using various cell culture systems to determine the functional and mechanistic role of different cell types in the COMT/miR-155/TNF-α axis, and whether a mechanistic relationship exists between COMT silencing and increases in miR-155 and TNF-α expression.

4.1. Cell culture study of human colonic epithelial cells, intestinal epithelial cells and macrophage-like cells:

To study mechanisms under decreased COMT conditions, intestinal epithelial cells (FHs 74 Int) and colonic epithelial cells (FHC CRL-1831) were transfected with si-COMT (siRNA) co-transfected with miRzip-155 (anti-miR-155) or Lenti-miR-155 (Pre-miR-155) with or without LPS stimulation. Transfection with a miR-155-specific inhibitor reduced si-COMT-associated TNF-α production in FHs 74 Int and FHC-CRL cells (Figure 4A, left panels). A more important finding was that inhibition of miR-155 expression not only reduced LPS-induced TNF-α production, but also, decreased TNF-α production in response to treatment with si-COMT plus LPS. These results indicated that up-regulation of TNF-α can be prevented by anti-miR-155 in FHs 74 Int and FHC CRL cells treated with si-COMT, LPS, or their combination. Intestinal (FHs 74 Int) epithelial cells and colonic (FHC-CRL) epithelial cells were also transfected with pre-miR-155 or anti-miR-155 or controls and further incubated with or without 100 ng/ml LPS for 6 hours and were analyzed for TNF-α production by ELISA (Figure 4A, right panels). The results showed that FHs-74 Int and FHC-CRL cells with anti-COMT treatment led to increases in TNF-α production and miR-155 expression. The similar findings were

also confirmed in human macrophage-like U937 cells (Figure 4B). However, real-time PCR and ELISA demonstrated that there was no increase in TNF-α production in cells transfected with pre-miR-155 alone when compared with pre-miR-155 and treated with si-COMT plus LPS (Figure 4B). These data suggest that macrophages may reach a maximum threshold for the miR-155 effect on TNF-α after transfection of pre-miR-155, whereas the miR-155 effect is saturated and cannot result in further increases in TNF-α production. See Supplemental Figure 30 middle and left panel for additional culture experiments with anti-COMT regulated catecholamines.

4.2. Neuron isolation and cell culture studies—To study neuronal mechanisms under conditions of decreased COMT, DRGs from $COMT^{-/-}$ mice were isolated, cultured, and: (i) transfected with miRzip-155 (anti-miR-155) or Lenti-miR-155 (pre-miR-155) into neurons to examine TNF-α expression; (ii) transfection of miRzip-155 (anti-miR-155) or Lenti-miR-155 (pre-miR-155) plus LPS into neuronal cells to simulate an infectious bioenvironment. Over-expression of miR-155 resulted in increased TNF-α production after COMT silencing in neuronal cells compared to cells transfected with pre-miR-controls. This suggests that miR-155 can augment the effect of LPS on TNF-α production (Figure 4C). Similarly, inhibition of miR-155 expression not only reduced LPS-induced TNF-α production, but also, decreased TNF-α production in response to COMT silencing conditions in neurons with LPS. See Supplemental Figure 30 right panel for additional culture neuron experiments with anti-COMT regulated catecholamines.

4.3. Effect of COMT modulation on NF-κB-miR-155 signaling and miR-155-SHIP1 signaling in cultured human intestinal epithelial cells—NF-κB activity and miR-155 expression was performed in COMT modulated human intestinal epithelial cells with/without LPS stimulation. Over-expression of COMT decreased NF-κB p65 transcriptional activity with/without LPS treatment, whereas si-COMT treatment greatly enhanced miR-155 expression in FHs 74 Int cells (Figure 4D). To assess whether si-COMT increases miR-155 expression through enhanced NF-κB binding to the 5'-promoter regions of miR-155, si-COMT or control siRNA treated human FHs 74 Int cells were co-transfected with either wild-type or NF-κB mutant BIC/miR-155 reporter plasmids, and dual-luciferase reporter assays were done. The NF-κB mutant co-transfected with si-COMT was ~41% less active than the wild-type promoter with si-COMT in this system, suggesting a possible contribution of COMT-regulated NF-κB signaling to the BIC/miR-155 promoter activity in human intestine epithelial cells (Figure 4E). Previous studies have suggested that miR-155 increased TNF-α expression through its target genes SHIP-1 and SOCS1.[29–30] Through real-time PCR assays, we demonstrated that only SHIP1, but not SOCS1, was significantly down-regulated in colon tissues from PI-IBS-D patients compared to controls (Supplementary Figure 31, left panel). To address this target relationship, luciferase reporter assays were carried out and confirmed that SHIP-1 is the direct target of miR-155 in colon epithelial cells (Supplementary Figure 31, right panel). Additionally, overexpression of SHIP1 significantly reduced TNF-α expression in Pre-miR-155 transfected colon epithelial cells (Supplementary Figure 32).

4.4. Defining COMT/miR-155/TNF-α axis associated cell-cell communications in human colonoids—A primary human neuron/macrophage-colonoid co-culture model was developed for in depth studies of epithelial, neuron, and macrophage interactions. Normal human colon tissues (from 3 controls) were collected and prepared for colonoid production (see Supplementary Methods). Lentivirus-COMT was used to knockdown COMT in human neuronal cells (LUHMES) and human macrophage like cells (U937). They were co-cultured with individual human colonoids (Figure 4F-1) and miR-155, TNF-α, NF-κB p65, and IκB-α expression was examined. Figure 4F-2 shows colonoid cultured with macrophages in a diminished COMT condition. Significantly enhanced miR-155 and TNF-α expression were linked with diminished IκB-α compared to naïve colonoids and colonoids with naïve macrophages, but not NF-κB-p65 expression. Figure 4F-3 demonstrates colonoid co-cultured human neuronal cells in a knockdown COMT condition. There was significantly enhanced TNF-α and NF-kB p65 expression linked with decreased IκB-α compared to naïve colonoids and colonoids with naïve neuronal cells, however, the small increase in miR-155 didn't reach significance in the neuronal cells under diminished COMT conditions. This finding may explain how neuronal cells may be modulated directly through the COMT/NF-κB/TNF-α pathway. Figure 4G shows the colocalization of colonoids co-cultured with macrophages and Figure 4H shows colonoids co-cultured with neuronal cells. Moderately increased catecholamine levels were found in mouse colonoids co-cultured with mouse macrophages or mouse-DRG neuron cells under decreased COMT conditions compared to colonoid only controls (Supplementary Figures 33 & 34).

5. Reversal of visceral hypersensitivity via modulation of miR-155 and TNF-α

5.1. Lenti-miR-155 inhibitor reverses visceral hypersensitivity via miR-155/TNF-α signaling: There was significantly diminished COMT after TNBS and *C. rodentium* treatment compared to naïve mice (Supplementary Figure 35). Following IP injections of miRNA (lenti-miR-155 inhibitor) into post-TNBS mice (Figure 5A, upper panel) or post-*C. rodentium* mice (Figure 5A, lower panel), there was a significant decrease in visceral hypersensitivity at 8 days. Figure 5B illustrates significantly decreased colon-TNF-α expression 8 days after lenti-miR-155 inhibitor IP injections in both the post-TNBS and post-C. rodentium mouse models. TNF-α was also tested in mouse DRGs following Lenti-miR-155 treatment (Figure 5C).

5.2. Reversal of visceral hypersensitivity by anti-TNF-α therapy in post-TNBS and post-C. rodentium mouse models: Anti-TNF-α antibody (cV1q) or its isotype antibody control were given IP to post-TNBS and post-*C. rodentium* mice.[23] We tested visceral hypersensitivity at 3 and 7 days following anti-TNF-α treatment compared to isotype control and there was a significant reduction in visceral hypersensitivity (Figure 5D). ELISA (Figure 5E) shows diminished TNF-α in the colon staring at 3 days after anti-TNF-α administration in both animal models. Interestingly, TNF-α in DRGs was also significantly reduced after anti-TNF-α (Figure 5F). This data suggests a mechanistic link between abdominal pain and the COMT/miR-155/TNF-α axis and indicates the possible therapeutic potential of anti-miR-155 or anti TNF-α therapy in PI-IBS-D patients (Figure 5G, right panel). Additional studies were done and are included in the Supplementary

Section evaluating the linkage of the COMT/miR-155/TNF-α axis with catecholamine signaling (Figure 5G, left panel).

Discussion

Irritable Bowel Syndrome (IBS), is a common GI disorder with persistent abdominal pain and alterations in bowel habits. Available IBS treatments are not ideal as the pathophysiologic mechanisms are not fully understood. There is a great need to develop safer, more effective treatment options especially for the chronic abdominal pain. One subset of IBS-D patients occurs following an enteric infection with enteropathogenic bacteria leading to post-infectious IBS-D (PI-IBS-D). To identify therapeutically targetable mechanisms in PI-IBS-D, we set out to determine the factors that modulate or mediate nociceptive signaling in the gut in PI-IBS-D patients. A promising candidate and one that is central to our current study is Catechol-O-Methyl-Transferase (COMT). The COMT protein is encoded by the *COMT* gene and variation in the expression of the *COMT* gene has been shown to be associated with increased perception of nociceptive stimuli.[14] COMT inhibitors can extend the effectiveness of carbidopa-levodopa therapy to treat the symptoms of Parkinson's disease. They also allow for lower doses of carbidopa-levodopa. However, the most common side effects of COMT inhibitors include abdominal pain and diarrhea. Here, we hypothesize that COMT down-regulation is critical to the chronic abdominal pain in PI-IBS-D pathophysiology.

To our knowledge, our current findings are the first translational datasets (in vitro, in vivo, and human ex vivo) to demonstrate that down-regulated colonic COMT expression drives abdominal pain in PI-IBS-D patients. It is well established that COMT metabolizes biologically-active catecholamines, including the important neurotransmitters dopamine, epinephrine, and norepinephrine, which are involved in numerous physiological processes, including modulation of pain.[13–14] Although the COMT-catecholamine pathway has been extensively studied, our current investigations focused on discovering novel signaling pathways not previously identified in the enteric post-infectious bioenvironment that lead to persistent abdominal pain in PI-IBS-D patients. We have found colon-dependent-COMT depletion is present in PI-IBS-D patients who have chronic abdominal pain. This important and novel finding in our current study indicates that the COMT/miR-155/TNF-α axis may be an important signaling pathway in the GI track that mediates chronic abdominal pain following an enteric infection. The mechanism may involve a unique signaling pathway that involves the COMT/miR-155/TNF-α axis following an insult from an enteric infection.

Noxious environmental stimuli, tissue damage, and inflammatory disease all evoke pain. Neuropathic and inflammatory pain both result from activation of damage-sensing neurons (via nociceptors) that innervate the viscera.[11,29] Chronic abdominal pain may be modulated through the COMT/miR-155/TNF-α axis that affects both colonic and dorsal root ganglion neurons. The transduction of pain by nociceptors can be modulated by mediators of inflammation such as TNF-α, which is released by infiltrating immune cells and by damaged neurons.[27] Thus, prior exposure to pathogenic enteric bacteria in PI-IBS-D patients could lead to release of cytokines and chemokines by intestinal epithelial cells.[31] Interestingly, it has been reported that COMT expression is inhibited by NF-kB

activation.[32–33] Our results here show that anti-TNF therapy can silence miR-155 signaling and reverse visceral hypersensitivity in post-TNBS and post-*C. rodentium* mice. This suggests that anti-TNF therapy may reverse abdominal pain in PI-IBS-D patients and could lead to innovative targets and treatments for patients with post-inflammatory gastrointestinal disorders.

Our current study also advances the understanding of cell-specific regulation of colonic neurons, epithelial cells, and macrophages in the pathophysiology of postinfectious gastrointestinal disorders. Cell-specific phenotypic expression along the COMT/miR-155/TNF-α axis signaling maintains chronic GI symptoms in PI-IBS-D patients. In our current study, we focused on the peripheral neural input due to the primary afferent colonic stimulus (enteric infection) that subsequently produced chronic visceral pain due to enhanced TNF-α through miR-155 signaling via diminished colonic COMT. Our patch clamp data also suggested that diminished colon-COMT may be one of the factors to enhance DRG or terminal firing and lead to visceral pain. This persistent decrease in colonic COMT is the most likely cause that leads to chronic visceral pain in PI-IBS-D patients.

In conclusion, we believe that we achieved our stated objectives. Through the present study we acquired a better understanding of how decreased colonic COMT activity interacts with TNF-α via specific miRNAs to contribute to chronic abdominal pain in PI-IBS-D patients. Achievement of this objective increases our understanding of the mechanisms involved in the development of visceral pain in PI-IBS-D patients. Achieving our objective should also lead to a novel translational benefit: it should suggest strategies for the development of more effective and novel therapeutic agents such as ncRNA that can reduce and/or prevent abdominal pain and symptoms in postinfectious gastrointestinal disorders.[34–37] These important pathophysiologic findings may also lead to new treatment paradigms and personalized medicine approaches in patients with gastrointestinal disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

IBS-D	diarrhea-predominant irritable bowel syndrome
IBS-C	constipation-predominant IBS
PI-IBS-D	post-infectious IBS-D
Recovered subjects	had enteric infection but did not develop PI-IBS-D

DRG	dorsal root ganglion
COMT	Catechol-O-Methyltransferase
FISH	Fluorescence in Situ Hybridization
miR-155	microRNA-155
SHIP1	Scr homology 2-containing-inositol-phosphatase-1
TNF-α	tumor necrosis factor alpha
VAS	visual analog scale
TNBS	trinitrobenzene sulfonic acid
GI	gastrointestinal
LCM	laser capture microdissection
WT mice	Wild-type mice
C. rodentium	*Citrobacter rodentium*

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WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

The underlying etiology of chronic gastrointestinal symptoms in post-infectious, diarrhea-predominant IBS (PI-IBS-D) remains unknown.

NEW FINDINGS

Reduced colonic Catechol-O-methyltransferase (COMT) expression drives increased TNF-α/miR-155 axis signaling in PI-IBS-D patients. Silencing of the TNF-α/miR-155 axis reverses visceral hypersensitivity in $COMT^{-/-}$ mice following TNBS and *C. rodentium* colitis.

LIMITATIONS

Translational clinical studies aimed at silencing the TNF-α/miR-155 axis in PI-IBS-D patients to reduce chronic gastrointestinal symptoms are needed.

IMPACT

Alterations in the COMT/TNF-α/miR-155 axis provide a new perspective in post-infectious visceral nociception that might be exploited as a biomarker and therapy in PI-IBS-D patients.

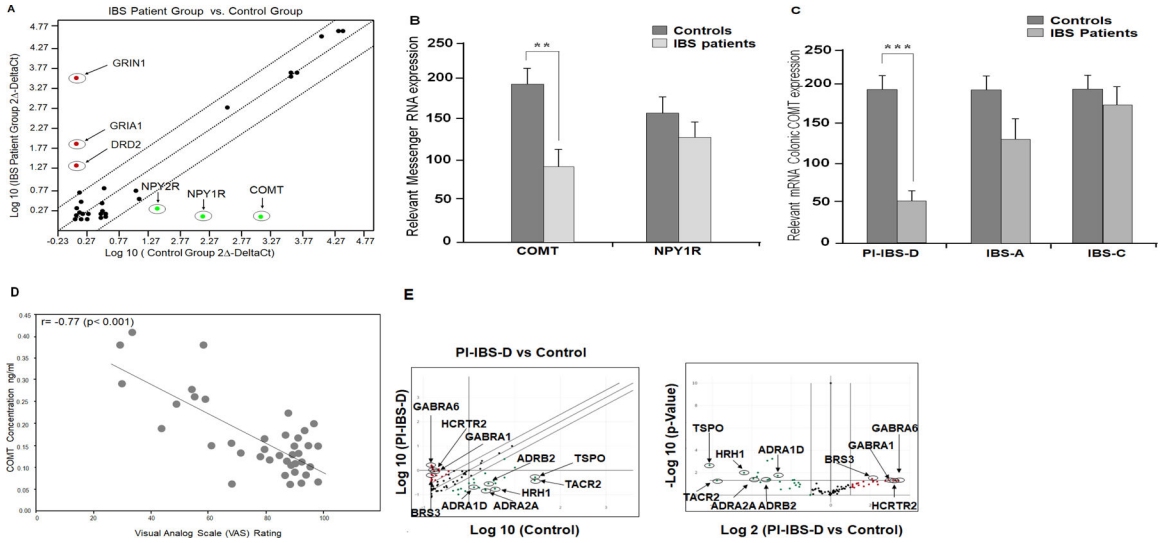

Figure 1. Down-regulated colonic-COMT expression drives abdominal pain in PI-IBS-D patients.

(A) Colon biopsies from 6 IBS patients and 6 controls show COMT, NPY1 and NPY2 genes with the greatest decrease (Quantitative PCR Arrays, Neurotransmitter Receptors and Regulators, cat. #PAHS-060, SA Biosciences). **(B)** Real-time PCR performed on colon biopsies from 61 IBS patients and 24 controls show a significant decrease in COMT but not NPY1R expression (**p<0.01). **(C)** Analyses by IBS subtype, using real-time PCR: (i) 3-fold reduction in COMT expression in PI-IBS-D patients(n=39,***p<0.001); (ii) non-significant decrease in COMT in IBS-A patients (n=14) and (iii) non-significant decrease in COMT expression in IBS-C patients(n = 8). **(D)** There was a significant (r = − 0.77) (p<0.001) inverse correlation between VAS pain scores and colonic COMT expression (ELISA assay). **(E)** Human Neurotransmitter Receptors and Regulators PCR Array identifying COMT target neurotransmitter receptor genes in colon tissues from PI-IBS-D patients. Values are means ± standard error of the mean.

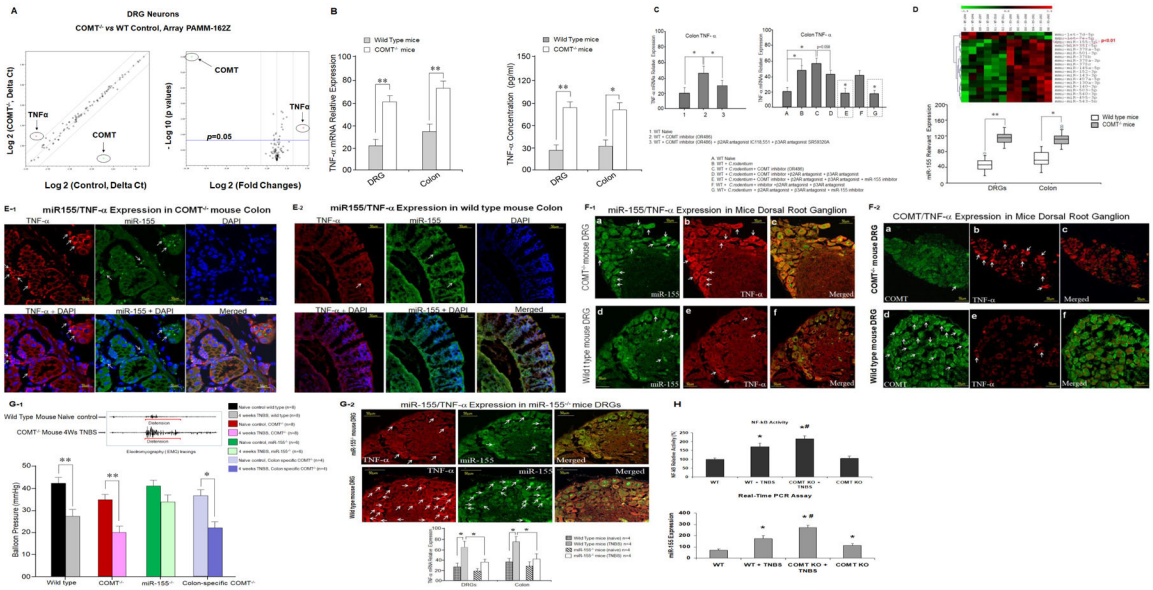

Figure 2. Enhanced miR-155/TNF-α expression in COMT$^{-/-}$ mice drives visceral hypersensitivity.

(A) TNF-α was significantly increased ($p<0.05$) in DRGs from COMT$^{-/-}$ mice (n=6) vs. WT mice (n=6). **(B)** TNF-α mRNA was significantly up-regulated in DRGs and colon tissue of COMT$^{-/-}$ mice (n=10) vs. WT mice (n=10) (**$p<0.01$) (PCR-left panel). TNF-α expression in COMT$^{-/-}$ mice DRGs (**$p<0.01$) and colon tissue (*$p<0.05$) was increased vs. WT mice (n=10) (ELISA-right panel). **(C)** Significantly enhanced TNF-α expression was seen following COMT inhibitor (OR486) treatment. Colon-TNF-α was significantly blocked by β2AR antagonist (IC118,551) and β3AR antagonist (SR59320A)(left panel). Same experiments done using β2 & β3 antagonists in mice that received *C. rodentium*, there was no significant block of TNF-α production (right panel). **(D)** miR-155 expression in DRGs was significantly upregulated($p<0.01$) in COMT$^{-/-}$ mice(n=6) vs. WT mice(n=6) (microarray-upper panel). miR-155 levels in COMT$^{-/-}$ mouse (n=10) DRGs (**$p<0.01$) and colon tissues(*$p<0.05$) were significantly elevated vs. WT mice (n=10) (qPCR-lower panel). **(E-1 & E-2)** White arrows indicate increased colonic TNF-α and miR-155 expression in the colon of COMT$^{-/-}$ mouse compared to WT mouse colon. **(F-1)** White arrows in panels a & d show miR-155 expression; panels b & e show TNF-α expression; panels c & f show co-localization of miR-155 and TNF-α, and indicate TNF-α and miR-155 expression in COMT$^{-/-}$ mouse DRGs (upper panel) vs. WT mouse DRGs (lower panel). **(F-2)** White arrows in panels a & d show COMT expression; panels b & e show TNF-α expression; and panels c & f show co-localization of COMT and TNF-α. There was increased TNF-α expression in DRGs of COMT$^{-/-}$ mice (upper panel) vs. WT mice (lower panel). **(G-1)** Upper panel illustrates EMG activity following colonic distension. Higher EMG activity was present in COMT$^{-/-}$ mice 4 weeks post-TNBS compared to WT mice during colonic distension (upper panel). Lower panel compares threshold balloon distension pressures in WT, COMT$^{-/-}$, miR-155$^{-/-}$, and colon-specific COMT$^{-/-}$ mice. There was significantly increased visceral hypersensitivity 4 weeks post-TNBS in COMT$^{-/-}$ mice. Significantly enhanced visceral hypersensitivity in colon-specific COMT$^{-/-}$ mice was present after TNBS (lower panel) compared to colon-specific COMT$^{-/-}$ naïve mice. There was slightly

increased (nonsignificant) visceral hypersensitivity in miR-155$^{-/-}$ mice after TNBS (lower panel) compared to naïve miR-155$^{-/-}$ mice (**p<0.01; *p<0.05). **(G-2)** White arrows show greater TNF-α and miR-155 expression in WT mouse DRGs (lower panel) vs. miR-155$^{-/-}$ mouse DRGs (upper panel). There was decreased TNF-α mRNA expression in DRGs 28 days post-TNBS in miR-155$^{-/-}$ mouse DRGs and colon vs. WT (*p<0.05)(lower graph). **(H)** NF-κB p65 Transcription Factor Assays revealed that TNBS treatment of both WT and COMT$^{-/-}$ mice significantly activated NF-κB (upper panel), and enhanced expression of miR-155 (lower panel) (*$^{\#}$ p<0.01; *p<0.05)

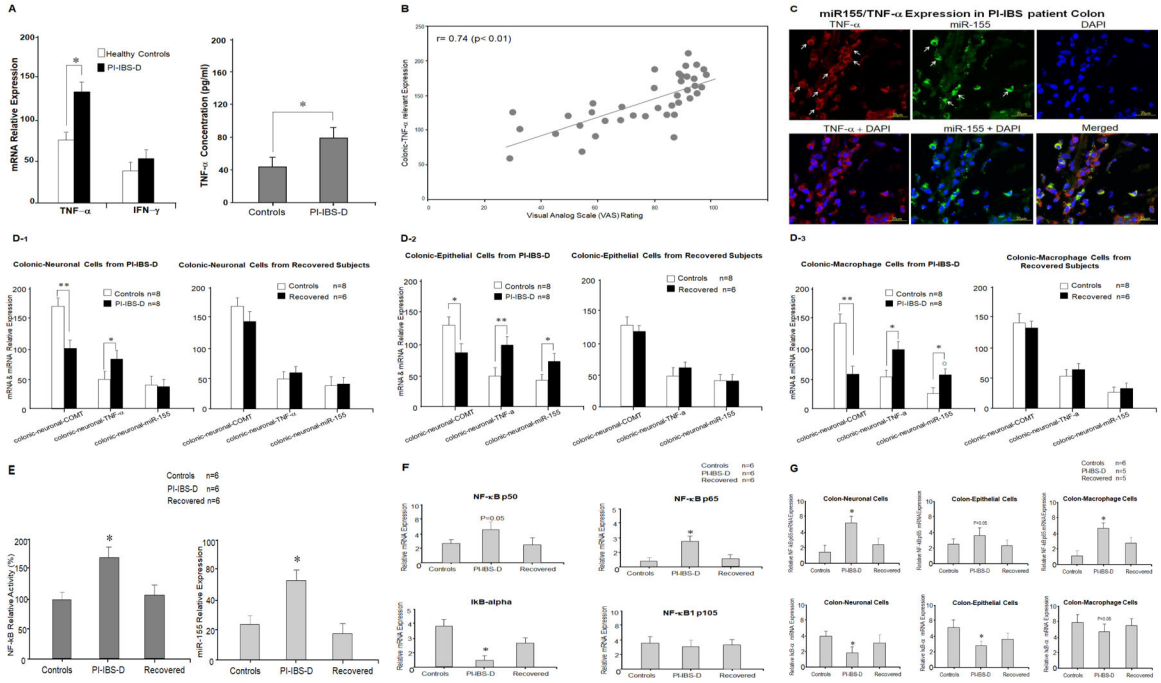

Figure 3. Enhanced miR-155/TNF-α signaling by COMT down-regulation drives abdominal pain through the COMT/miR-155/TNF-α axis in PI-IBS-D patients.

(A) Left panel shows mRNA expression of TNF-α and IFN-γ expression and TNF-α protein concentration (right panel) in PI-IBS-D patients vs. controls. TNF-α mRNA expression was increased in PI-IBS-D patients vs. controls (*p<0.05). TNF-α protein concentration was significantly increased in PI-IBS-D patients. (**B) TNF-α** expression was also positively correlated with VAS abdominal pain scores in PI-IBS-D patients (r=0.74, p<0.01). **(C)** White arrows indicate colocalization of miR-155 and TNF−α expression in PI-IBS-D colon tissues. **(D-1)** LCM captured colonic neuronal cells (identified by neuronal marker-PanNeuron) show enhanced TNF-α expression in PI-IBS-D patients' colonic-neuronal-cells (left panel) accompanied by decreased COMT expression compared to controls (*p<0.05, **p<0.01), but not colonic-neuronal-miR-155. Recovered subjects (right panel) showed no significant changes compared to controls. **(D-2)** LCM captured colonic epithelial cells show enhanced TNF-α and miR-155 expression in PI-IBS-D patients' colonic epithelial cells with decreased COMT expression vs. controls (left panel) (*p<0.05,**p<0.01). No significant decrease in COMT, or increase in TNF-α or miR-155 in recovered subjects compared to controls (right panel). **(D-3)** LCM captured colonic macrophages from PI-IBS-D patients (left panel) show a significant decrease in COMT expression with increased TNF-α and miR-155 (*p<0.05;**p<0.01). No significant decrease in COMT, or increase in TNF-α or miR-155 was present in recovered subjects compared to controls (right panel). **(E)** NF-κB activation and gene expression profiles of colon tissues were assessed from PI-IBS-D patients. NF-κB p65 Transcription Factor Assays revealed that PI-IBS-D patients' colon tissue had significantly activated NF-κB (Figure 3E, left panel) and enhanced expression of miR-155 (Figure 3E, right panel) compared to recovered subjects and controls. **(F)** The key NF-κB signaling mediators were tested in PI-IBS-D patients, and recovered subjects, and controls. There is significantly enhanced NF-κB p65 (*p<0.05) and

significant diminished IκB-α (*p<0.05) in PI-IBS-D patients. **(G)** LCM captured colonic neuronal cells, epithelial cells and macrophages and single cell PCR was done, there was diminished IκB-α in colonic neuronal and epithelial cells and significantly increased NF-κB expression in colonic neuronal cells and macrophages in PI-IBS-D patients compared to controls and recovered subjects.

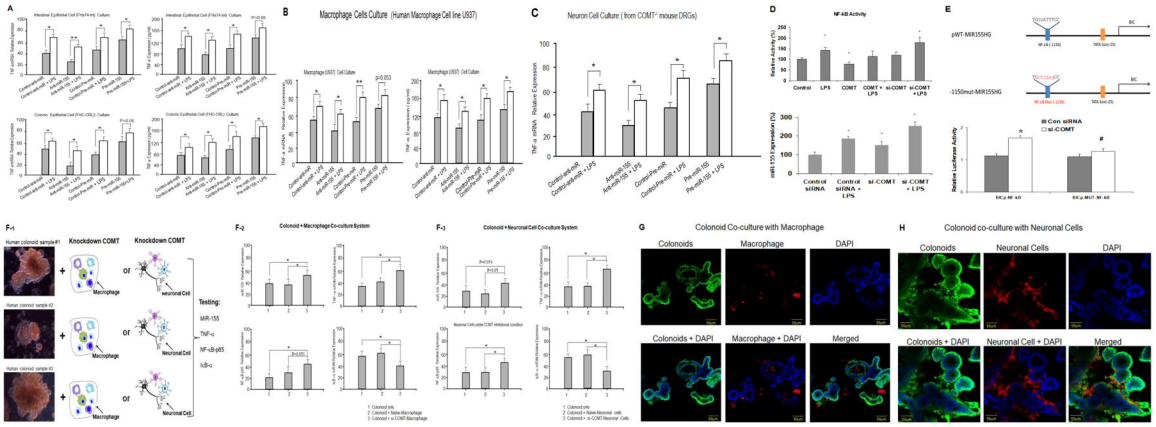

Figure 4. COMT regulated miR-155/TNF-αsignaling in vitro via cell culture & human colonoid study.

(A) Up-regulation of TNF-α expression prevented by anti-miR-155 in intestinal (FHs 74 Int, FHC-CRL) epithelial cells treated with anti-COMT, LPS, or their combination (left panels). Right panels indicate changes in TNF-α production with anti-COMT treatment (*p<0.05; **p<0.01). **(B)** Depicts macrophage-like cells (U937 cells) after transfection with an miR-155-specific inhibitor (left panel): Reduced anti-COMT-associated TNF-α production. No significant increase in TNF-α production was found after transfection with pre-miR-155 vs.pre-miR-155 and treated with anti-COMT plus LPS (p=0.053). ELISA assay is shown in right panel indicating TNF-α expression. **(C**) Depicts overexpression of miR-155 with increased TNF-α production in $COMT^{-/-}$ neuronal cells vs. cells transfected with pre-miR-controls indicating miR-155 augmented the effect of LPS on TNF-α production. Inhibition of miR-155 expression reduced LPS-induced TNF-α production and decreased TNF-α production in response to COMT knock down of neuronal cells with LPS(*p<0.05). **(D**) Overexpression of COMT in intestinal epithelial cells (FHS 74 Int cells) significantly reduced NF-κB activity. Silencing COMT enhanced miR-155 expression with or without LPS treatment relative to respective controls. **(E)** Bic promoter of miR-155 encoding Bic gene contains NF-κB binding site at −1150, the TATA box at −25(top panel). Silencing COMT significantly increased reporter activity of NF-κB binding site in FHS 74 Int cells (lower panel). Reporter constructs with random mutations in the recognition sequence of the NF-κB binding site at −1150, the effects of reporter deactivation by COMT were abolished(*p<0.05) vs WT or siRNA controls (#p< 0.05) vs. TNBS or LPS treatment groups. **(F-1**) This diagram summarizes our strategy for human colonoid co-cultured with primary human neuron cells or macrophages with knockdown COMT condition, then testing miR-155, TNF-α, NF-κB and IκB-α. **(F-2**) Enhanced miR-155 and TNF-α expression was linked with diminished IκB-α after colonoids were co-cultured with macrophages in a diminished COMT condition, but not NF-κBp65. **(F-3)** Enhanced TNF-α and NF-kB p65 expression linked with decreased IκB-α was found after colonoid was co-cultured with human neuronal cells in a knockdown COMT condition, but not miR-155. **(G)** Illustrates the colocalization of colonoids (Green) and macrophages (Red). **(H)** Illustrates the colocalization of colonoids (Green) and neuronal cells (Red).

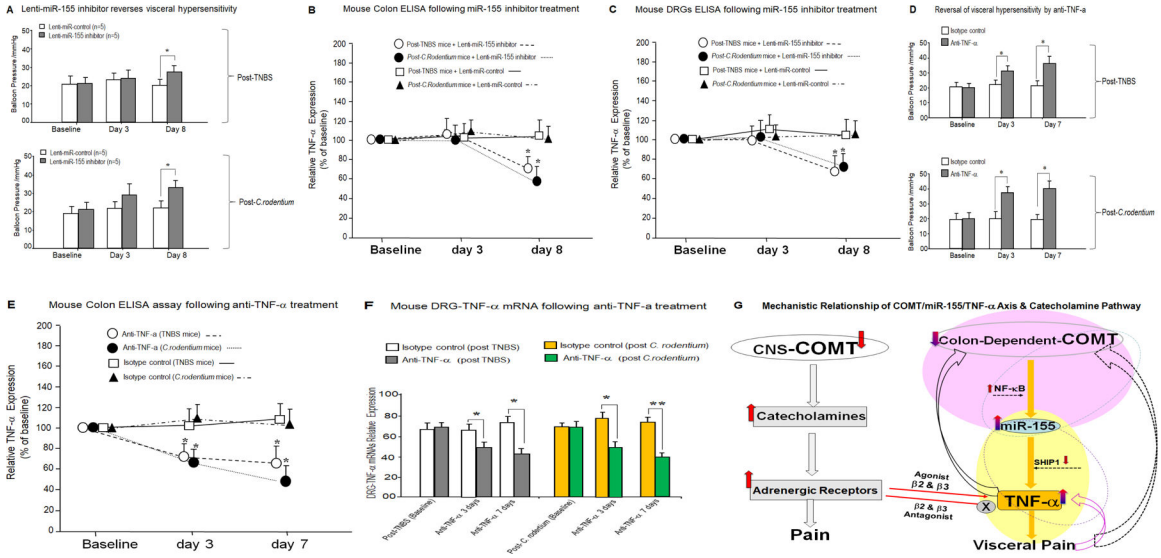

Figure 5. Reversal of visceral hypersensitivity via modulation of miR-155 and TNF-α

. **(A)** Following IP injections of miRNA (lenti-miR-155 inhibitor) into post-TNBS mice (upper panel) or post-*C. rodentium* mice (lower panel), there was a significant decrease in visceral hypersensitivity at 8 days in both post-TNBS and post-*C. rodentium* mice (*p<0.05). **(B)** TNF-α was tested after lenti-miR155 inhibitor injection. ELISA assay showed a significantly diminished TNF-α at 8 days following miR-155 inhibitor treatment in both post-TNBS and post-*C. rodentium* mice (*p<0.05). **(C)** There was a significant reduction in visceral hypersensitivity at 8 days following anti-TNF-α treatment compared to isotype control in both post-TNBS and post-*C.rodentium* mice (*p<0.05). **(D)** There was significantly diminished visceral hypersensitivity starting at 3 days after anti-TNF-α injection in both post-TNBS and post-*C. rodentium* mice (*p<0.05). **(E)** Colonic-TNF-α expression was significantly reduced after anti-TNF-α injections starting at 3 days in both post-TNBS and post- *C. rodentium* mice (*p<0.05). **(F)** DRG-TNF-α mRNA expression was also significantly reduced after anti-TNF-α injections starting at 3 days in both post-TNBS and post- *C. rodentium* mice (*p<0.05). **(G)** This figure illustrates the mechanistic relationship(s) between the COMT/miR-155/TNF-α axis and the catecholamine pathway. Left: Previous investigations have focused on decreased CNS COMT that leads to increased circulating catecholamines with activation of adrenergic receptors that drive chronic pain conditions. Right: The current study evaluated new colon-specific COMT signaling pathways that act through peripheral mechanisms. In this model, enteric infections lead to colonic-COMT depletion which is augmented by increased miR-155 coupled with NF-kB. miR-155 targets SHIP1 to promote TNF-α productions during IBS development and progression. This leads to enhanced TNF-α expression in colon & DRGs that drives chronic visceral pain. CNS COMT TNF-α production is blocked with a COMT inhibitor combined with β2 and β3 adrenergic receptor antagonists. However, this blockade is not effective following enteric infections. Thus, the COMT/miR-155/TNF-α axis is an important signaling pathway in the GI track that mediates chronic abdominal pain following enteric infections in PI-IBS-D patients.