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The cholinergic anti-inflammatory pathway revisited

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Abstract

Inflammatory bowel disease negatively affects the quality of life of millions of patients around the world. Although the precise etiology of the disease remains elusive, aberrant immune system activation is an underlying cause. As such, therapies that selectively inhibit immune cell activation without broad immunosuppression are desired. Inhibition of immune cell activation preventing pro-inflammatory cytokine production through neural stimulation has emerged as one such treatment. These therapeutics are based on the discovery of the cholinergic anti-inflammatory pathway, a reflex arc that induces efferent vagal nerve signaling to reduce immune cell activation and consequently mortality during septic shock. Despite the success of pre-clinical and clinical trials, the neural circuitry and mechanisms of action of these immune-regulatory circuits are controversial. At the heart of this controversy is the protective effect of vagal nerve stimulation despite an apparent lack of neuroanatomical connections between the vagus and target organs. Additional studies have further emphasized the importance of sympathetic innervation of these organs, and that alternative neural circuits could be involved in neural regulation of the immune system. Such controversies also extend to the regulation of intestinal inflammation, with the importance of efferent vagus nerve signals in question. Experiments that better characterize these pathways have now been performed by *Willemze et al.* in this issue of *Neurogastroenterology & Motility.* These continued efforts will be critical to the development of better neurostimulator based therapeutics for inflammatory bowel disease.

Graphical Abstract

This mini-review summarizes some of the key findings in the control of the immune system by the nervous system. In particular, we introduce the background that will be relevant for readers of the manuscript from the De Jonge lab.

Keywords

Colitis; vagal nerve stimulation; Cholinergic anti-inflammatory pathway; autonomic nervous system; inflammation

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1. Introduction

The nervous and immune systems communicate bidirectionally through an intricate network that work together to regulate and respond to local inflammation and infection[1, 2]. Over the past twenty years, a growing body of research has sought to define the precise mechanisms, and the role these “neuro-immune” interactions play during various disease states.

The immune system constantly monitors for perturbations to homeostasis, and reacts by releasing pro-inflammatory cytokines that can often exacerbate the response and lead to more damage[3]. Kevin Tracey and colleagues first demonstrated the ability of vagal nerve stimulation to inhibit over-exuberant immune cell activation, successfully reducing pro-inflammatory cytokine production in a murine model of sepsis[4]. This substantial finding offered the first insight into a reflex termed the “cholinergic anti-inflammatory pathway” (CAIP)[3]. This pathway highlights the intricacy of neuro-immune communication, utilizing efferent signaling from the vagus nerve to the lymphoid organs to reduce inflammation and damage. With clinical trials using neurostimulation devices to activate the efferent arm of the CAIP already reported in patients suffering from inflammatory bowel disease (IBD)[5–7], better characterization of this reflex is required. There are currently an estimated 4–4.5 million people in the United States and Europe[8] that are afflicted with IBD, characterized by chronic inflammation resulting in abdominal pain, fever, diarrhea, fatigue, and rectal bleeding[9]. Current therapeutic modalities generally seek to reduce inflammation and include non-steroidal anti-inflammatory drugs, biologicals targeted to specific cytokines, or surgical intervention[10, 11]. While monoclonal antibodies directed against TNFα have greatly advanced the treatment of IBD[10], these therapeutics increase the risk of infection and can lose efficacy overtime[9, 11, 12]. With this in mind novel therapeutic approaches targeting the molecular underpinnings of disease are in high demand[13].

Experimental modeling of IBD can be accomplished in a variety of well-characterized spontaneous or induced mouse models[14]. Although spontaneous disease models have yielded important insights into disease, variability of disease severity and penetrance can impose significant limitations[14]. Recognizing these limitations, induced models of colitis that each uniquely recapitulate certain aspects of pathology in patients have been developed. These include acute exposure to the chemical irritant dextran sodium sulfate (DSS), and adoptive transfer of colitogenic T-cells[14–16]. DSS is toxic to intestinal epithelial cells (IEC) resulting in a denuded epithelium and inflammation in the mid-distal colon due to recruitment and activation of monocytes/macrophages and neutrophils[17–19], without requiring T-cells[20]. Although this colitis model has provided detailed mechanisms of neutrophil recruitment, and mucosal wound healing, the T-cell independent nature of pathology is divergent from clinical presentation. Consequently, an induced T-cell dependent colitis model was developed using adoptive transfer of FACS sorted effector T-cells (T_E) into immunodeficient ($RAG1^{-/-}$) hosts[14]. It is important to consider that therapeutics that reduce disease severity or do not appear efficacious in one model may only reflect an inability to modulate a specific aspect of disease.

The study by *Willemze et al.* in the current issue of *Neurogastroenterology & Motility* significantly adds to the growing knowledge base on the regulation of inflammation by the

nervous system, demonstrating that the superior mesenteric, but not the vagus nerve, control the inflammatory response during acute DSS-induced colitis[21]. Identification of this specific functional neuroimmune circuit that controls inflammation in a pre-clinical model of IBD has the potential to allow for development of highly targeted and efficacious electroceuticals.

2. Cholinergic anti-inflammatory pathway vs. sympathetic regulation of immunity

The vagus nerve is the longest cranial nerve in the parasympathetic nervous system, composed of 70–80% afferent and 20–30% efferent signaling. The nerve innervates many peripheral organs, and maintains several physiological mechanisms, including digestion, heartbeat, immune responses, hormone secretion and respiratory function. Recently, the ability of efferent signaling from this nerve in controlling inflammation has been the focus of a growing body of research[22]. Electrical stimulation of the vagus nerve has now been demonstrated to significantly reduce inflammation in diverse conditions including rheumatoid arthritis[23], ischemia reperfusion injury[24], septic shock[25], and IBD[26–28]. Despite these advances, the precise neural circuitry and mechanisms of action responsible for immune inhibition remain contested.

The importance of the vagus nerve in regulating anti-inflammatory responses was demonstrated in experiments utilizing vagotomy and vagal nerve stimulation (VNS) techniques during acute inflammation. *Borovikova et al.* demonstrated that VNS significantly reduced TNFα production by splenic macrophages, and mortality in a mouse model of septic shock[4]. Subsequent experiments have suggested that the vagus directly interacts with the sympathetic neurons innervating the spleen in prevertebral ganglia including the celiac ganglia[29–31]. Activation of vagal efferent signaling by VNS therefore results in activation of the sympathetic innervation and release of norepinephrine in the spleen. Protection afforded during septic shock by VNS requires activation of a specialized subset of $CD4^+$ T-cells that express choline acetyltransferase (ChAT), synthesize acetylcholine (ACh) and serve as a non-neuronal source of ACh in the spleen[25]. The release of ACh from $ChAT^+$ T-cells is a critical step in regulating inflammation, and occurs following the activation of β2 adrenergic receptors (AR) on $ChAT^+$ $CD4^+$ T-cells by norepinephrine (NE). ACh released from these T-cells binds to nicotinic acetylcholine receptor alpha 7 receptors (α7R) expressed by macrophages, resulting in inhibition of activation and TNFα production. While the signaling cascade activated after ACh binding to α7R on immune cells remains unclear, a variety of mechanisms ranging from sequestration of NF-κB by STAT3, to the induction of a microRNA that reduces the production of bioactive TNFα[32] have been proposed.

These observations of neural control of aberrant immune cell activation have been further extended to intestinal inflammation. Corroborating studies confirmed this ability of VNS to reduce inflammation with vagotomy worsening colitis severity[33, 34]. Suggesting that there could be a similar α7R mediated mechanism in reducing colitis severity, peripheral administration of nicotine as a non-selective nicotinic receptor agonist ameliorated disease

in mice previously subjected to vagotomy[26, 35]. These data suggested the importance of the role of ACh receptors as targets that could reduce intestinal inflammation.

Despite these seminal findings and ongoing clinical trials, there are still large gaps in our knowledge concerning the functional circuitry and mechanisms of action in the inhibition of colonic inflammation by the nervous system. Several studies have questioned the proposed neuro-immune circuit; with conflicting evidence of a connection between the efferent vagus and celiac ganglia. Neuroanatomical tracing studies using injection of DiI in the dorsal motor nucleus and fast blue in the spleen have failed to identify co-labelled cell bodies in the celiac ganglia[36]. Electrical stimulation of the vagus in these studies was also found to have no stimulatory effect on splenic nerve activity[36]. Adding to this controversy, viral tracing using injection of pseudorabies virus has generated evidence in support of a vagus-to-spleen[37], or a sympathetic ganglia-central nervous system pathway[38]. As further evidence of a sympathetic pathway to inhibit macrophage activation, LPS-induced TNFα production was significantly increased in mice with bilateral ablation of the splanchnic sympathetic nerves but not vagotomy[39, 40]. Despite the questions raised over the functional circuitry raised by these experiments in the spleen, neuro-modulation of inflammation has been demonstrated in models of colitis. As evidence for the importance of vagal nerve signaling in intestinal inflammation, vagotomy increases pro-inflammatory cytokine production, histological damage, and weight loss in the acute[27] and chronic DSS-induced colitis[41].

With these continued controversies in the field, and the limited innervation of the distal colon by the vagus, *Willemze et al.* have undertaken a series of elegant experiments to better characterize the contributions of the vagus or superior mesenteric nerve (SMN) in the regulation of DSS-induced colonic inflammation[21]. Using a surgical approach, SMN sympathectomy, but not vagotomy, significantly increased the severity of an induced colitis. As further supporting evidence of the importance of the SMN, electrical stimulation of this nerve attenuated the clinical severity of DSS-induced colitis[21]. These results support the contention that acute colonic inflammation can be regulated by tonic and elicited neuronal signals conducted by the sympathetic innervation. At this point, it is unknown if this protective effect is unique to the acute DSS colitis model, or if SMN can regulate colonic inflammation in general by reducing activation of various immune cells. The ability of SMN neurostimulation or vagotomy during chronic DSS administration or use of the $CD45RB^{hi}$ T-cell transfer model could provide invaluable data as to the target cells of this pathway. With respect to the T-cell transfer model, it is unknown what effect vagotomy, sympathectomy, or SMN stimulation would have on the expansion or effector function of colitogenic T-cells. Perhaps equally important and currently unknown is the effect of SMN stimulation on secondary lymphoid organs. Highlighting the potential importance of such studies, activation of the sympathetic innervation of the spleen is critical to protection against ischemia-reperfusion injury in the kidney[24, 42].

Based on the observations that VNS reduced dendritic cell activation, and vagotomy increased DSS-induced DC activation[28]; it would seem reasonable to investigate how stimulation or removal of basal sympathetic signaling would affect the secondary lymphoid organs that drain the small intestine and colon. As these tissues are critical for the induction of oral tolerance, host defense, and generation of immunopathological responses[43],

understanding how the nervous system affects these organs will be crucial to applying neuromodulation for intestinal immunopathology. It should be noted that the lymph nodes draining the small intestine and colon are discrete tissues that are anatomically separate with unique functions[43]. Not only are the mesenteric lymph nodes divided into small intestinal or colonic draining, the iliac LN function to drain the distal colon[44]. In addition, each of these LN receive unique phenotypes of DC homing from these different regions of the intestine, imparting different T-cells responses[43, 44]. While these experiments describe unique roles for the MLN and iliac lymph nodes in health, the role of the innervation in immune responses in these specific LN is not clear. The presence of sympathetic innervation in a variety of LN has been long reported in rats, mice, cats, and humans [45–47]. It is however unclear from these studies if the anatomically different MLN or iliac LN have unique patterns of innervation, or if these fibers originate from different prevertebral ganglia. These data could then conceivably allow for more precise modulation of the mucosal immune responses while reducing unintending side effects such as disruption of tolerance to the commensal microbiota or susceptibility to pathogens.

3. Mechanisms of SMN-mediated immune modulation

Sympathetic neurotransmitters such as NE have been well established to increase or reduce immune cell activation. Although it is beyond the scope of this article to detail these studies, there are several comprehensive reviews that describe these receptors expressed by different immune cell populations and the functional consequences signaling activated through these pathways[48, 49]. Here we will focus on the role of NE in the context of inflammation and immune function. While the reduced inflammation following stimulation of the SMN was proposed to occur through the evoked release of norepinephrine, this has not been formerly demonstrated. This presents an opportunity to determine the receptors and cellular targets required for immune suppression during SMN. Functional α-AR and β-AR are well established to be expressed by myeloid cells, with stimulation through these receptors resulting in opposing outcomes. For example, stimulation of β-AR resulting in increased intracellular cAMP inhibits TNFα production by monocytes[50] and splenic macrophages[51] *in vitro.* Inhibition of immune cell activation and pro-inflammatory cytokine production is therefore dependent on the activated receptor and expression by myeloid cells. For example, while β-AR agonists can inhibit LPS induced TNFα production, selective agonists of α-AR receptors augment TNFα production *in vitro*[52] and *in vivo*[53]. In the context of intestinal inflammation, blockade of α2-AR significantly reduced weight loss, pro-inflammatory cytokine production and histological damage in TNBS induced colitis[54]. In keeping with the presumed anti-inflammatory role of the β-AR, administration of a β3-AR agonist significantly reduced the severity of an induced colitis in rats[55]. Despite these results that generally corroborate the pro- and anti-inflammatory role of α-AR and β-AR respectively, the role of catecholamines during intestinal inflammation does not appear to be simple. Although chemically induced sympathectomy by administration of 6-hydroxydopamine reduces the severity of acute DSS-induced colitis, this treatment significantly enhances inflammation in chronic DSS colitis[56]. These studies highlight the importance of defining how chronic inflammation effects expression of αAR and βAR. In addition to this question of what adrenergic receptors are required, the possibility that mechanisms common to the

CAIP could apply during intestinal inflammation remains to be determined. Although a small number of $ChAT^+$ $CD4^+$ T-cells are present in the small intestine, and T-cell derived ACh is important in host-commensal microbiota interactions[57], the role of this specialized T-cell subset in intestinal inflammation is remains unknown.

4. Clinical Trials; clinical challenges and opportunities

With these generally positive results in pre-clinical models of septic shock[58], intestinal inflammation[26, 27], there has been a substantial effort to translate these findings into therapeutics[59]. In the clinical setting, VNS typically refers to a helical or cuff electrode surgically placed on the left cervical vagus nerve that is then connected to a stimulus unit. In general, several clinical trials have produced promising albeit mixed results, conceivably due to the gaps in knowledge surrounding the critical stimulation parameters and mechanisms behind which VNS exerts an effect.

In a study with seven patients with Crohn's disease, VNS was applied by a spiral electrode wrapped around the left cervical vagus, with initial stimulation at 0.25 mA increasing to 1.25 mA for 6 months. Once the trial had ended, five patients saw improvements in symptoms and four of those five had entered remission. It should be noted that two of the seven patients were withdrawn from the study, due to a worsening of the disease, though these individuals had worse disease scores prior to treatment than the other patients[5]. As the electrode was well tolerate and no significant adverse events were observed, these results highlight the promise of a safe and efficacious new therapy. Curiously, the effect of pre-existing and often chronic inflammation on the success of VNS in treatment of inflammation does not appear to have been addressed a variable. Acute colonic inflammation in DSS-induced colitis significantly reduces the evoked neurotransmitter release from sympathetic neurons in the superior mesenteric ganglia, and in the intestine[60, 61]. These results would suggest that efficacy of therapeutic VNS for inflammatory disease such as IBD could vary between patients depending on the severity and state of disease at treatment initiation. Despite the potential importance of this variable, this aspect does not appear to have been assessed in either pre-clinical or clinical studies.

The invasiveness of electrode implantation has also led to the design and implementation of a non-invasive methodology for VNS. Preclinical studies have demonstrated the efficacy of transcutaneous vagal nerve stimulation (tcVNS), where the electrode is placed externally on the side of the neck during stimulation, in the control of inflammation[62]. This approach also appears to be effective clinically, as peripheral blood mononuclear cells from health volunteers stimulated with tcVNS produced significantly less pro-inflammatory cytokines compared to non-stimulated when challenged *ex vivo* with LPS[6]. As another alternative to wrapping of the electrode, transvenous stimulation has also been attempted. In this procedure the electrode is placed using a catheter on the left internal jugular vein near the vagus. While this method of VNS is less invasive than implantation of a cuff electrode and was well tolerated by the subjects; VNS did not reduce pro-inflammatory cytokine production or prevent LPS (2 ng/kg i.v.) induced fever, flu-like symptoms and increased heart rate. Additionally, when whole blood samples taken from the subjects were challenged with LPS *ex vivo*, VNS failed to reduce inflammatory cytokine release[7]. Despite the non-

classical electrode placement, the authors indicate that ineffective stimulation does not seem to be the underlying reason for failure in this study as VNS induced laryngeal vibration was apparent. In addition, the authors note that different amplitudes and frequency of stimulation were used, based on the comfort level of the individual patient. This seemingly trial and error approach to electrode placement and stimulation parameters highlight the need for robust models that integrates the type and site of electrode placement coupled with electrophysiological properties of the targeted neurons. This type of modelling has recently been applied to understanding the failure of kilohertz frequency blockade to achieve primary clinical endpoints as a therapy in obesity[63]. Such analysis indicated that the selected parameters based on prior assumptions of electrophysiological properties of the nerve would appear to be ineffective, or could result in stimulation rather than blockade[63]. With the use of unique electrodes, electrode placement, and stimulation parameters perhaps the variability in clinical outcomes should not be surprising. These issues remain unique challenges and opportunities for the continued development of therapeutic electroceutical device for practical application in the clinic.

5. Conclusion

The interaction and complex communication between the nervous and immune systems has the potential to be translated into novel and effective therapeutic in the treatment of IBD. With largely positive results accumulating from pre-clinical, and select clinical trials, this is a truly exciting time in the field of electroceuticals. Despite these successes, there remains several challenges and opportunities in the field. These include rectifying seemingly inconsistent findings, functional circuits in different organ systems and animal species, and a thorough understanding of how electrode design and stimulation parameters can be best suited to stimulate specific nerves. Finally, as these therapies are designed for treatment of patients with chronic inflammation, the effect of disease on neuronal signaling should be addressed. The future and potential promise of neural stimulation as a novel therapeutic for these diseases depends on this research.

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Abbreviations

α7R	nicotinic acetylcholine receptor alpha 7 receptors
AR	adrenergic receptors
CAIP	cholinergic anti-inflammatory pathway
ChAT	choline acetyltransferase
DSS	dextran sodium sulfate
IBD	inflammatory bowel disease

IEC	intestinal epithelial cells
tcVNS	transcutaneous vagal nerve stimulation
SMN	superior mesenteric nerve
VNS	vagal nerve stimulation

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Key Points

- Intestinal inflammation can be inhibited following electrical neural stimulation.
- There are multiple neural immune inhibitory circuits, with some appearing to be unique to the targeted organ.
- The promise of neural stimulation as a therapeutic will be enhanced by better characterization of these functional circuits.