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NEURAL CONTROL OF INFLAMMATION

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Neural Control of Inflammation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Pioneering research on neural control of inflammation has paved the way for new and exciting developments in the growing field of bioelectronic medicine. In the past couple of decades, pre-clinical research on the role of the vagus nerve in inflammation and immunity has brought electrical stimulation of select nerves into clinical trials for the treatment of chronic inflammatory diseases. Bioelectronic medicine continues to evolve and address challenges in optimizing interfaces and stimulation configurations for activation of specific neural circuits, and deciphering nerve signals that regulate inflammation and immunity with the goal of targeting specific nerve fibers for treatment of excessive inflammation. Ongoing basic, pre-clinical research strives to provide the insight necessary to develop therapeutic vagus nerve stimulation to mitigate inflammation in disease.

Inflammation is normally a protective process that defends from microbial invasion and promotes healing, provided that it is adequately resolved in a timely manner. Dysregulation of resolving mechanisms can result in chronic inflammation and thus, a better understanding of the mechanisms that regulate inflammation is important for improving diagnosis, prevention, and treatment of chronic diseases. Discoveries over three decades show that the central and peripheral nervous systems along with the immune system work together to regulate inflammation. The vagus nerve bridges communication between the central and peripheral nervous systems and other tissues, regulates homeostasis, and serves an immunoregulatory function. Work delineating vagus nerve-mediated regulation of inflammation in experimental models of disease has led to important breakthroughs toward enabling treatment methods using electronic interfaces and devices that activate homeostatic reflexes that regulate the immune system. Considering the speed of action potentials and the anatomical specificity of neurons, activation of nerves that regulate immune cell function and activity, potentially provides an anatomically and temporally precise method to deliver therapeutic interventions in excessive inflammation. Clinical trials aimed at investigating neural control of chronic inflammatory responses in conditions such as inflammatory bowel disease and rheumatoid arthritis have been launched and data is encouraging, however, not yet fully conclusive. Together, these studies show the potential that neural control of inflammation works as a strategy to control excessive inflammation. Accordingly, additional studies with improved design in terms of randomization and controls are needed to evaluate targeted neural stimulation for regulation of the molecular and cellular mechanisms that underlie regulation of inflammation and its resolution.

The work in this thesis sets forth to understand neural control mechanisms of inflammation by establishing methods and technology to study mechanisms of neural regulation of excessive

inflammation in experimental models. In Study I, we found that a minute-long electrical vagus nerve stimulation impacts the cytokine response to inflammatory stimuli for two days. Study II establishes an effective method for vagus nerve stimulation for studies of experimental inflammation. Study III provides evidence that the vagus nerve accelerates the active resolution phase of inflammation through a cholinergic mechanism that requires release of pro-resolving mediators. Because available methods for vagus nerve stimulation are not suitable for long-term experiments in mice, the understanding of mechanisms of vagus nerve regulation of inflammation in chronic diseases is yet incomplete. In Study IV, we developed technology that attempts to address this methodological shortcoming and enable studies of vagus nerve stimulation in genetic mouse models of chronic inflammatory diseases.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following studies:

I. Tarnawski L, Reardon C, Caravaca AS, Rosas-Ballina M, Tusche MW, Drake AR, Hudson LK, Hanes WM, Li JH, Parrish WR, Ojamaa K, Al-Abed Y, Faltys M, Pavlov VA, Andersson U, Chavan SS, Levine YA, Mak TW, Tracey KJ, Olofsson PS. **Adenylyl Cyclase 6 Mediates Inhibition of TNF in the Inflammatory Reflex.** Frontiers in Immunology. 2018 Nov 27;9:2648.

II. Caravaca AS, Gallina AL, Tarnawski L, Tracey KJ, Pavlov VA, Levine YA, Olofsson PS. **An Effective Method for Acute Vagus Nerve Stimulation in Experimental Inflammation.** Frontiers in Neuroscience. 2019 Aug 27;13:877.

III. Caravaca AS, Gallina AL, Tarnawski T, Shaava V, Colas RA, Dalli J, Malin SG, Arnardottir H, Olofsson PS. **Vagus nerve stimulation promotes resolution of inflammation by a mechanism that requires Alox15.** *Submitted.*

IV. Donahue M*, Caravaca AS*, Silverå-Ejneby M, Jakešová M, Gallina AL, Đerek V, Olofsson PS*, Głowacki ED*. **Wireless vagus nerve stimulation using organic electrolytic photocapacitors.** *Manuscript*.

**Co-authors with equal contribution*

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

V. Gallina GL, Rykaczewska U, Wirka RC, Caravaca AS, Shavva VS, Youness M, Karadimou G, Lengquist M, Razuvaev A, Paulsson-Berne G, Quertermous T, Gisterå A, Malin S, Tarnawski L, Matic L, Olofsson PS**. AMPA-type Glutamate Receptors Associate with Vascular Smooth Muscle Cell Subpopulations in Atherosclerosis and Vascular Injury.** Frontiers in Cardiovascular Medicine, *in press.*

VI. Brück E, Svennson-Raskh A, Larsson JW, Caravaca AS, Gallina AL, Eberhardson M, Sackey PV, Olofsson PS. **Plasma HMGB1 levels and physical performance in ICU survivors**. Acta Anaesthesiologica Scandinavica, *in press.*

VII. Karadimou G, Gisterå A, Gallina AL, Caravaca AS, Centa M, Salagianni M, Andreakos E, Hansson GK, Malin S, Olofsson PS, Paulsson-Berne G. **Treatment with a Toll-like Receptor 7 ligand evokes protective immunity against atherosclerosis in hypercholesterolaemic mice.** Journal of Internal Medicine. 2020 Sep;288(3):321-334.

VIII. Caravaca AS, Centa M, Gallina AL, Tarnawski L, Olofsson PS. **Neural reflex control of vascular inflammation.** Bioelectronic Medicine. 2020 Jan 31;6:3.

IX. Brück E, Lasselin J; (HICUS study group: Caravaca AS, Gallina AL, Bottai M, Eberhardson M, Sundman E), Andersson U, Sackey PV, Olofsson PS. **Prolonged elevation of plasma HMGB1 is associated with cognitive impairment in intensive care unit survivors.** Intensive Care Medicine. 2020 Apr;46(4):811-812.

X. Caravaca AS*, Tsaava T*, Goldman L*, Silverman H, Riggott G, Chavan SS, Bouton C, Tracey KJ, Desimone R, Boyden ES*, Sohal HS*, Olofsson PS*. **A novel flexible cuff-like microelectrode for dual purpose, acute and chronic electrical interfacing with the mouse cervical vagus nerve.** Journal of Neural Engineering. 2017 Dec;14(6):066005. **Co-authors with equal contribution*

XI. Söderström LÅ, Jin H, Caravaca AS, Klement ML, Li Y, Gisterå A, Hedin U, Maegdefessel L, Hansson GK, Olofsson PS. **Increased Carotid Artery Lesion Inflammation Upon Treatment With the CD137 Agonistic Antibody 2A.** Circulation Journal. 2017 Nov 24;81(12):1945-1952.

CONTENTS

LIST OF ABBREVIATIONS

α7nAChR	Alpha-7 nicotinic acetylcholine receptor subunit
AA	Arachidonic acid
ACh	Acetylcholine
AChR	Acetylcholine receptor
Ca^{2+}	Calcium ions
ChAT	Choline acetyl transferase
CNS	Central nervous system
CXCL1	C-X-C motif ligand 1
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ELISA	Enzyme-linked immunosorbent assay
HMGB1	High mobility group box 1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-17	Interleukin-17
KC	Keratinocytes-derived chemokine
KO	Knockout
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MaR	Maresin
MEA	Multi-electrode array

OEPC	Organic electrolytic photocapacitors
SPM	Specialized pro-resolving mediators
LM	Lipid mediators
NF-κB	Nuclear factor kappa B
PD	Protectin
Rv	Resolvin
TGF-β	Transforming growth factor beta
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
VNS	Vagus nerve stimulation
WT	Wild type

1 INTRODUCTION

The global burden of chronic inflammatory disease has increased over the last few decades, as seen in the number of cases of people suffering from conditions such as rheumatoid arthritis, cardiovascular disease, inflammatory bowel disease, chronic kidney disease and Crohn's disease (1–5). Inflammation is an immunological defense mechanism in response to harmful stimuli, serving an important mechanism to prevent further damage and facilitate healing, but it must be well-controlled. Excessive inflammation can in itself cause tissue damage and unresolved, persistent inflammation can significantly contribute to the pathogenesis of inflammatory diseases (6). Neural reflex circuits are now recognized as important regulators in inflammatory responses (7,8). This has generated considerable interest in investigating neural control of chronic inflammatory conditions and the prospect of developing novel therapeutic approaches for inflammatory-related diseases.

Bioelectronic medicine is an expanding field that investigates new diagnostic and treatment approaches to regulate inflammation and immunity by interfacing with neural circuits for therapeutic benefit. The field aims to bring about multidisciplinary collaborations that inspire future research efforts to create novel technology for neural modulation of peripheral nerves in order to treat disease. Substantial efforts have focused on optimization of peripheral nerve recording and stimulation, microfabrication techniques, optimizing electrodes and their integration to more chronic setups, and developing mathematical models and algorithms to better understand neural signals. This thesis contributes to the growing knowledge of mechanisms of neural regulation in inflammation and resolution and seeks to address the lack of chronic electrode applications of vagus nerve stimulation.

1.1 INFLAMMATION REGULATION AND RESOLUTION

In the 1st century AD, Celsus characterized inflammation by four cardinal signs: *rubor* (redness), *calor* (heat), *tumor* (swelling), and *dolor* (pain). Later, Galen added a fifth cardinal sign: *functio laesa* (loss of function). These five cardinal signs are the physiological result of the immunological responses to harmful external stimuli, such as pathogens or bacteria that has caused infection, injury, or irritation. Collectively, these responses involve immune cells, molecular mediators, and blood vessels. Acute inflammation begins shortly after insult and is usually a temporary response. Molecular mediators cause blood vessels to dilate and vascular permeability is increased allowing for immune cells to migrate into affected tissues through the

capillary wall (9). During infection, leukocytes progress from the blood migrating towards the site of inflammation with neutrophils as one of the predominant cells at early onset inflammation. This ensemble of mediators culminates into phagocytosing macrophages and degradation of cellular debris gearing towards resolution. Macrophages are involved in regulation of inflammation progression and the duration of the inflammatory response. In a process called efferocytosis, macrophages remove apoptotic neutrophils, triggering downstream intracellular signal transduction pathways, resulting in anti-inflammatory and pro-resolving effects (10).

Anti-inflammatory does not equate to pro-resolving. The former aims to limit or counter-regulate inflammatory responses, while the latter involves clearance of apoptotic cells, cellular debris and bacteria, and promotes tissue repair. The ideal outcome of acute inflammation is resolution. Resolution of inflammation is an active, orchestrated process mediated by lipid mediators from initiation to resolution (11). The success of resolution of inflammation rests upon the cessation of neutrophil influx and macrophage clearance of cellular debris at the site of inflammation (12), as well as lipid mediator class-switching from the production of pro-inflammatory mediators, classified as eicosanoids, towards pro-resolving mediators, termed specialized pro-resolving mediators (SPM) (13,14). SPM are enzymatically derived from essential fatty acids which include arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in a lipoxygenase (LOX)-dependent manner. Characteristic actions of SPM include limiting neutrophil recruitment, counter-regulating cytokine production and stimulating efferocytosis (13,14).

Eicosanoids, namely prostaglandins and leukotrienes, initiate the inflammatory response (i.e. cardinal signs of inflammation), by regulating vascular permeability and migration of neutrophils to the site of injury, respectively. Lipid mediator class switching from eicosanoids to lipoxins is a key process in initiating the termination of the acute inflammatory response. SPM, like the lipoxins and resolvins, stimulate recruitment of non-phlogistic monocytes, and stimulate efferocytosis, thereby promoting resolution of inflammation (11,14,15). Failure to resolve inflammation can lead to chronic conditions (16,17) (Figure 1). Unsuccessful damage control of the inflammatory stimulus results in the continued migration of immune cells to the site of insult, amplifying the inflammatory response and causing tissue damage. Persistent inflammation can give rise to diseases such as diabetes, rheumatoid arthritis, asthma, and atherosclerosis, for example (18). Therapeutics that trigger pro-resolving and anti-inflammatory processes could potentially be beneficial in changing the course of chronic conditions (Figure 1).

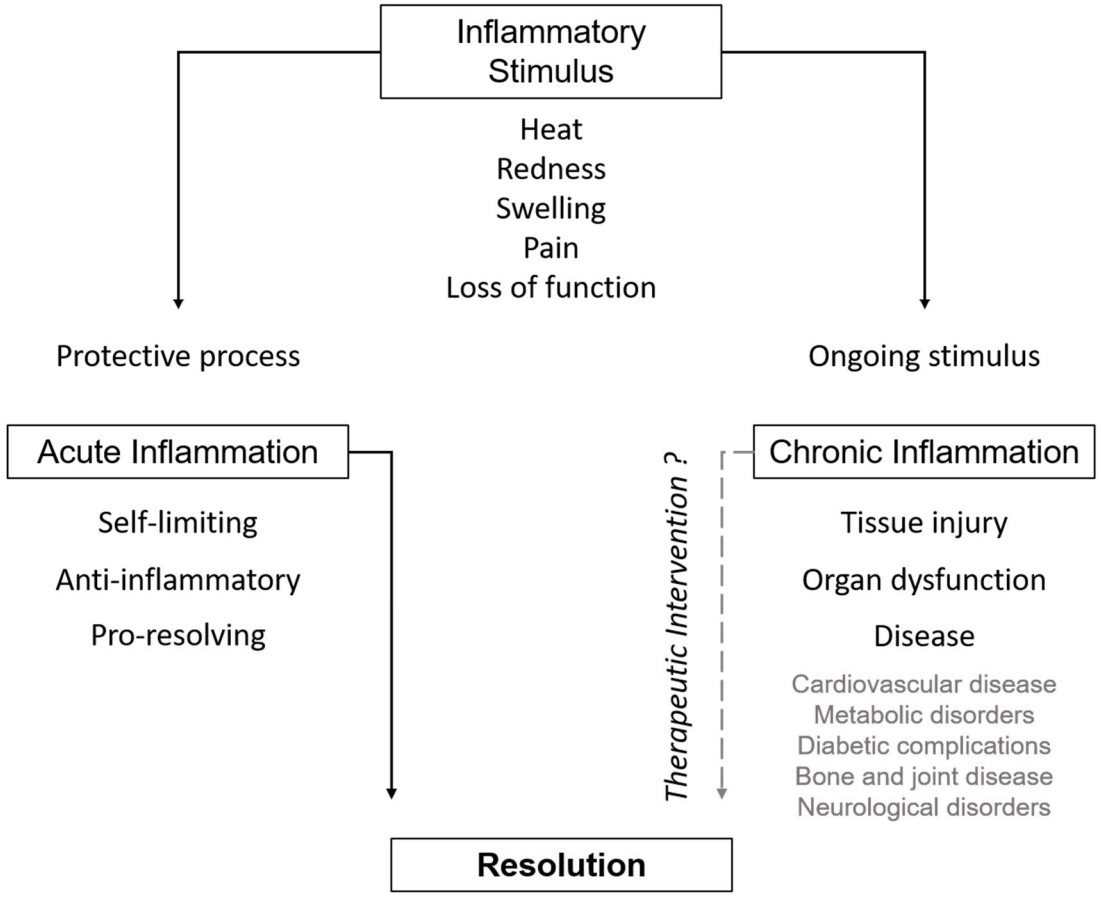

Figure 1. Persistent and excessive inflammation can lead to chronic disease.

1.2 CYTOKINE THEORY OF DISEASE

Cytokines are important mediators in maintaining immunological homeostasis and health (19) (Figure 2). Successful resolution of inflammation requires not only decreased leukocyte infiltration and increased phagocytosis of apoptotic cells and cellular debris, but also counter-regulation of cytokines. Interleukin 1 (IL-1), tumor necrosis factor (TNF) and its receptor families are archetypal pro-inflammatory cytokines that are released upon injury or infection. The overproduction of such cytokines can worsen inflammation. The development of cytokine targeting therapeutics such as TNF and IL-1 inhibitors for the treatment of rheumatoid arthritis or inflammatory bowel disease have revolutionized therapy for patients suffering from these conditions (20). However, cytokine inhibiting drugs have several drawbacks, are not effective for all patients, and can produce unwanted side effects, some of which can be lethal. The pleiotropic nature of cytokines makes them challenging targets and thus, additional and more selective treatment options are needed.

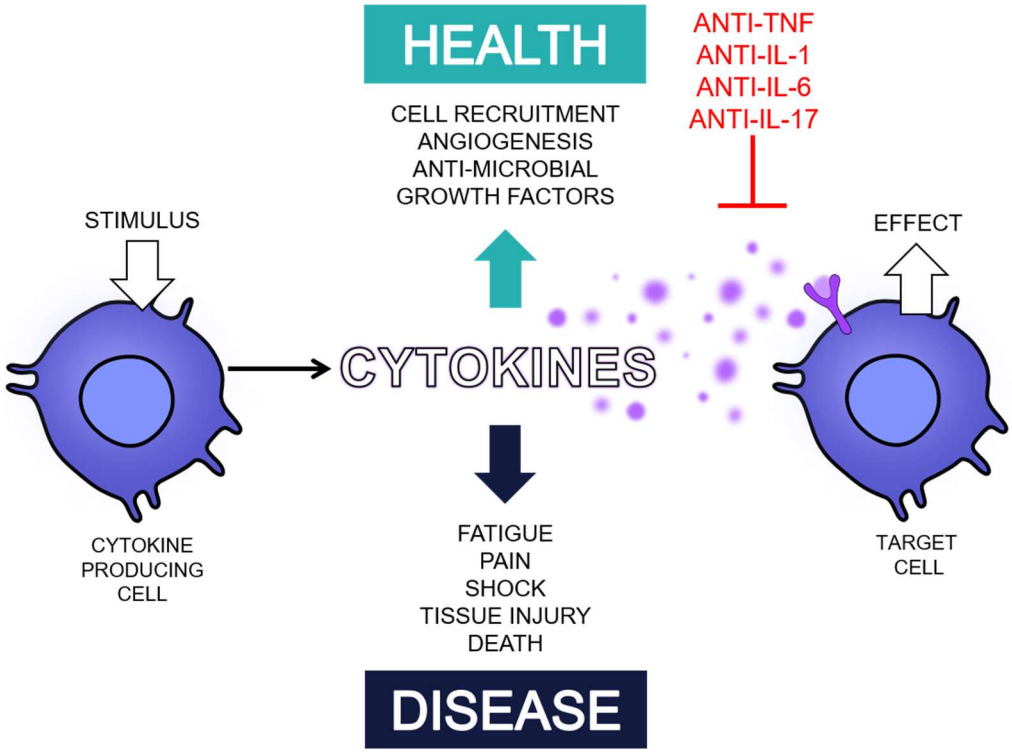

Figure 2. Cytokines can have protective or pathologic functions. During an immunological response, cytokines can have different effects on target cells (i.e. pleiotropic, synergic, antagonistic, cascade, redundant) which can recruit or activate immune cells. A dysregulated cytokine response can result in disease. The use of cytokine inhibiting drugs demonstrate how investigating these molecules can be utilized to understand how we may therapeutically target disease.

1.3 NEURAL REFLEX REGULATION OF IMMUNITY

Immune cells are not the only responders to the presence of pathogens. In fact, there are a multitude of mechanisms that defend against microbes and regulate inflammation. Importantly, neural reflexes are activated by pathogen invasion and injury (8,21). A neural reflex has a sensory receptor that responds to changes in the environment, a sensory arc that transmits action potentials to the central nervous system (CNS) and an efferent arc that sends action potentials from the CNS to the peripheral nervous system and impacts local physiology. For example, the gastrointestinal, respiratory, and cardiovascular organ systems are regulated by reflexes. The CNS transmits action potentials to control function of these organ systems in a matter of milliseconds.

This sensory mechanism can be traced back to one of the most simple organisms with a nervous system, the hermaphrodite soil nematode *Caenorhabditis elegans* (*C. elegans*), in which a neural reflex regulates immune responses, indicating that the presence of neural control of immunity is primordial (22,23). Neural signaling that regulates immune responses in *C. elegans* include neuronal secretion and both the insulin and TGF-β pathway in antifungal resistance (24,25). Additionally, the innate immune responses against the bacterial pathogen *Pseudomonas aeruginosa* by sensory neurons instead of specialized immune cells, were observed in *C.elegans* (22). Primitive neural circuits are initiated by sensory neurons to detect the presence of pathogens and to elicit a physiological response. Sensory information in *C. elegans* is relayed to a large somatic nervous system and small pharyngeal nervous system, whereas in humans, autonomic reflex circuits are composed of an afferent arc that reports to the CNS and an efferent arc that generates motor signals to elicit a response, such as regulatory signals, e.g. immune cell activation or release of cytokines. The nervous system and immune system work together to elicit a defense program against pathogens and to regulate inflammation (8).

Hence, in response to infection or tissue damage, nerves play an integral role. The nervous system receives alerting information to the presence of infection or damage from the immune system. In this bi-directional communication, mediators released through immune chemosensory cells detect the presence of threat and activates neural signaling in order to provide a defense response (26).

1.4 THE VAGUS NERVE

The vagus nerve is the longest cranial nerve traveling from the brainstem through the neck, thorax, and abdomen (27). The Latin word vagus translates to “wandering”, which appropriately describes its extensive and complex reach to multiple organ systems including the cardiovascular, gastrointestinal, respiratory, and immune system. The broad distribution of vagus nerve fibers to most inner organs, including those most in contact with pathogens, for example the lung and the gastrointestinal tract, allow for vagus nerve fibers to potentially detect pathogens. With its extensive reach, the vagus nerve serves as conduit for communication between the nervous system and immune system to work together to elicit a defense program against pathogens in order to regulate inflammation through cellular, humoral and neural mechanisms (8).

1.5 THE INFLAMMATORY REFLEX

The vagus nerve is an important component of the *inflammatory reflex*, a neural circuit that monitors and regulates inflammation (Figure 3) (28,29). Physiological changes in the body in response to inflammatory stimuli elicit action potentials in the vagus nerve and activate the neural reflex to suppress cytokine production and inhibit inflammation (28,30). These responses can occur in a matter of seconds to minutes (31).

The majority of vagus nerve fibers are afferent and can respond to chemical and mechanical stimuli, temperature, and even osmotic pressure (32). Afferent fibers are important for neuro-immune communication, as they sense inflammation in the periphery and convey signals to the brain. Afferent neurons associated with the vagus nerve reside in the nodose, petrosal and jugular ganglia, terminating in the dorsal vagal complex of the medulla oblongata (26).

Afferent neural signals can also be activated by cytokines. Our current understanding of the afferent arm of the inflammatory reflex stems from several important observations. CNI-1493 is a general inhibitor of inflammatory responses that was observed to inhibit TNF in the brain of animals subjected to cortical infarction (33). However, after these animals were vagotomized, the effect of CNI-1493 in the brain inhibiting TNF was no longer observed, suggesting signals transmitted by the vagus nerve regulate cytokine production and indicate a role for the vagus nerve in CNS regulation of TNF in the periphery, i.e. organ systems. In fact, the effect on cytokine release in inflammation by CNI-1493 administered in the CNS is several orders of magnitude stronger than CNI-1493 administered in the periphery. Furthermore,

subdiaphragmatically vagotomized rats fail to develop a fever response to intraperitoneal injection of the cytokine interleukin-1 beta (IL-1β) (34), indicating that signals in the vagus nerve are important in the physiological response to elevated cytokine levels in the peritoneal cavity. This suggests sensory signals in the vagus nerve are involved in the fever response. Moreover, injecting IL-1β into the portal vein of rats increases efferent splenic nerve activity, but not if the hepatic branch of the vagus nerve is ablated (35). This observation suggests that sensory vagus signals in response to IL-1β-injection intraperitoneally elicit a reflexive motor response.

Our present understanding of efferent signaling in the inflammatory reflex stems from investigation of mechanisms of vagus nerve regulation of cytokine release in the periphery. Vagotomized rats in an endotoxemia model of inflammation had increased serum TNF levels compared with sham-operated rats. When the vagus nerve was electrically stimulated, serum TNF was significantly decreased compared to both vagotomized and sham-operated rats (30). Vagus nerve stimulation (VNS)-treated animals have been shown to have reduced inflammation and cytokines compared with sham-treated animals in different experimental models of inflammation, such as inflammatory bowel disease, intestinal inflammation, rheumatoid arthritis, and kidney ischemia-reperfusion injury (36–39).

The spleen plays a major role in systemic release of TNF in endotoxemia in murine models (40,41) and is a key component of the inflammatory reflex, despite not having any vagal innervation. The current understanding is that efferent signaling in the vagus nerve reaches the celiac ganglion where the splenic nerve arises. However, the details of the signal transmission in the ganglion are not completely understood. As the splenic nerve is activated, norepinephrine (NE) is subsequently released in the spleen, which activates target adrenergic receptors on splenic lymphocytes (42), promoting the release of acetylcholine (ACh) by choline acetyltransferase (ChAT)-expressing T cells (41,43–45) (Figure 3). The released acetylcholine, in turn, activates the α7 nicotinic acetylcholine receptor (α7nAChR) on macrophages in the spleen (Figure 3). This contributes to the vagus nerve regulation of the release of pro-inflammatory cytokines. Based on these findings, that electrical impulses in the efferent vagus nerve regulate cytokine release in the spleen, it became reasonable that electronic devices can be used to activate the vagus nerve and attenuate inflammation, including chronic inflammatory disease.

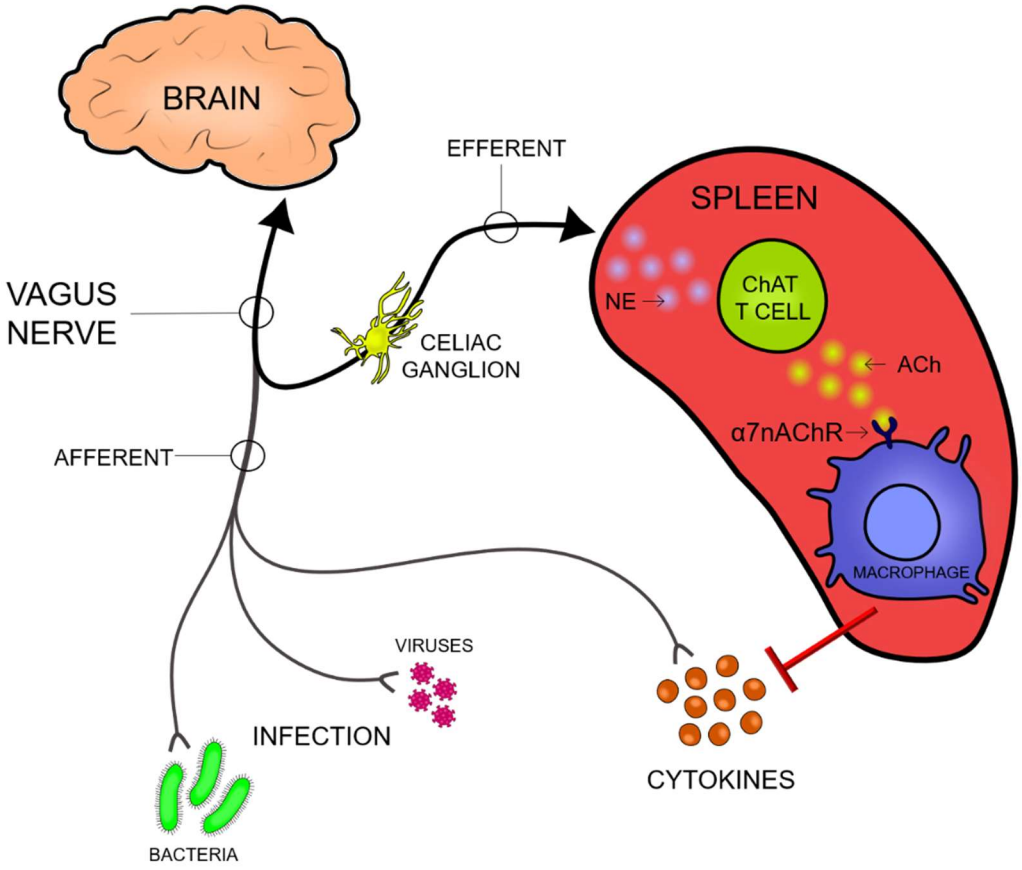

Figure 3. The vagus nerve transmits afferent signals to the central nervous system and efferent signals regulate cytokine release in a neural-immune reflexive arc called the *inflammatory reflex*. The molecular basis of cytokine inhibition in this reflex requires acetylcholine and the alpha-7 nicotinic receptor (α7nAChR) subunit expressed on immune cells such as macrophages.

1.6 ACETYLCHOLINE AND THE ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT

The chief neurotransmitter of the vagus nerve is acetylcholine (46). The α7nAChR is part of a ligand gated ion channel located in the central and peripheral nervous systems. Activation of the α7nAChR can mediate anti-inflammatory signaling in peripheral tissues (47,48). In contrast to wild type mice, mice deficient in the α7nAChR show blunted inhibition of LPS-induced TNF production (47). The α7nAChR is expressed on several cells including macrophages, monocytes, dendritic cells, endothelial cells, and T cells. ChAT is the enzyme that catalyzes acetylcholine biosynthesis (49). ACh is released from ChAT-expressing T-cells that are required to relay neural signals to α7nAChR expressing immune cells. α7nAChR has been found to suppress NF-κB activation through inhibition of intracellular Ca^{2+} release (50,51) and attenuates excessive production of pro-inflammatory cytokines downstream (7,43). Activation of components in the inflammatory reflex to attenuate release of pro-inflammatory cytokines

in inflammation can be achieved via α7nAChR agonists such as GTS-21 (52,53) or vagus nerve stimulation.

1.7 NEURAL REGULATION OF INFLAMMATION RESOLUTION

Resolution of inflammation is an important active process that terminates the response to infection and injury and ultimately promotes healing (13). In addition to its role in inhibition of cytokine release in inflammation, the vagus nerve may play an active role in promoting processes in resolution of inflammation. It has been shown that electrical stimulation of vagus nerve tissue increases production of pro-resolving mediators *ex vivo* and can regulate expression of netrin-1, an axonal guidance molecule, which is involved in dampening the inflammatory response and neutrophil infiltration (54,55). In contrast, vagotomized mice subjected to experimental peritonitis had a longer resolution time and increased pro-inflammatory mediators when compared to the sham group (55). Of note, the vagotomized group showed a stronger inflammatory response as measured by neutrophil infiltration in the peritoneum. Accordingly, it is difficult to conclude from this study whether activity in the vagus nerve specifically promotes the resolution phase of inflammation. Nevertheless, these are interesting observations since it provides evidence that neural signals may be involved in regulating resolution of inflammation. Considering the recent observations of prolonged resolution time in vagotomized mice, it is possible that stimulation of the vagus nerve could promote pro-resolving processes and reduce resolution time.

1.8 HARNESSING ELECTRICITY TO PROMOTE HEALING

The therapeutic use of electricity dates as far back to the 1st century AD. In Compositiones medicinae, Scribonius Largus describes using a live black torpedo fish (Torpedo nobiliana) to treat gout (56). This is not surprising to us today as we understand that nerve cells are electrically active, meaning that they generate a membrane potential, and that electrical stimulation can elicit action potentials. Action potentials are electrical impulses that send signals throughout the body and are propagated when the nerve cell's membrane potential shifts from negative to positive (i.e. depolarization) and voltage-gated sodium (Na^+) channels are activated (57). Voltage-gated Na^+ channels are widely expressed in excitable cells which include peripheral neurons, among many others. Many of these widely expressed ion channels can be activated by electrical, mechanical and chemical stimuli (58).

Action potentials transmitted in the vagus nerve regulate neuro-immune communication between the brain and the periphery. Accordingly, fluxes of ions are fundamental in facilitating physiological responses and with increased mechanistic understanding of the involvement of neural signals in the physiology of inflammation, it is possible that electricity can potentially be used to promote healing.

1.9 THERAPEUTIC MONITORING AND STIMULATION OF PERIPHERAL NERVES

In response to physiological changes in the body, the sensory arc of the vagus nerve transmits action potentials to the CNS to provide information of the body's condition. Decoding neural activity related to the motor and sensory fibers of the vagus nerve could be beneficial to understanding the 'language' in which the immune system and nervous system communicate. Neural decoding has been successful in motor decoding for devices and application in paralysis (59). Neural interfaces have the potential to advance our mechanistic understanding of how the CNS communicates with the peripheral nervous system in ways that are not otherwise possible, such as the development of vagus nerve recording methodology which have provided us with some insights on how levels of anesthesia, nutritional status, and administration of cytokines can affect baseline vagus nerve activity (60). Analysis of compound action potentials recorded in the cervical vagus nerve of mice indicate that the vagus nerve transmits distinct neural signatures in response to specific cytokines (61). Furthermore, hypoglycemia-specific neural signals decoded from vagus nerve activity of mice reveal a potentially new way to measure blood glucose levels (62). Ideally, a closed-looped device that could record sensory information or vagal tone, interpret the signals, and then respond accordingly to therapeutically stimulate and restore normal vagal tone would be a major advancement in the field – and likely in medicine. Expansion of this knowledge and technological advances in miniaturized interfaces attempts to better understand the neural code in inflammation regulation.

1.10 PERIPHERAL NERVE INTERFACE TECHNOLOGY

Development of peripheral nerve interfaces are challenging due to the physiological and anatomical conditions of nerve size and shape depending on the location (i.e. near organs or muscles) and the species. Also, certain nerves contain either afferent or efferent fibers or a mix of both, like the vagus nerve. There is a wide range of different commercially available

electrodes to choose from (e.g. cuff, spiral, needle or hook electrodes), and many laboratories even construct their own electrodes. For electrode fabrication, biocompatible materials are suitable for nerve interfacing which include metallic biomaterials (e.g. platinum iridium), synthetic and naturally occurring polymeric biomaterials (e.g. Parylene) and composite biomaterials (e.g. bone cement). Acute and chronic experiments require different electrode designs, for instance, hook electrodes are not used for chronic stimulation settings while cuff electrodes may be suitable for both. Chronic electrodes need to be biocompatible, thin, and flexible but strong enough to withstand compressive strain and stress cracks, maintain good adhesion without constriction or damage to the nerve, and perform reliably over time (63). Availability of chronic electrodes for mice are limited and implementation of current designs have been challenging, in this context, for small peripheral nerves such as the cervical vagus nerve. Electrode specifications for rats and mice differ for obvious reasons of size, and it has been suggested that the inner diameter for cuff electrodes for example, should be 1.4 times the outer diameter of the nerve (64,65).

There have been some recent advancements to interfacing with the mouse cervical vagus nerve (66,67), though these devices operate using wires which do not allow the animals to move freely and thus recording and stimulation of peripheral nerves are still performed under anesthesia. Furthermore, external electrode contacts and connecting wires can cause entanglement with intervening wires, distress to the mouse, affect normal locomotion and grooming, alter the group housing situation and dynamic, as well as their habitual behavior. Recently it was shown that it may be possible to perform long-term vagus nerve stimulation for up to 4 weeks in mice using commercially available bipolar cuff electrodes (68). However, the study did report cases of lead wire breakage in the connection between the lead wire and electrode, suggesting wireless stimulation would likely be a potentially better solution to technical challenges with wired devices.

Ultrasound technology (e.g. StimDust, Neural Dust) has been utilized for peripheral nerve stimulation and recording, but regarding rodents, this has been largely studied in rats (69–71). In mice, ultrasound technology has been investigated in the sciatic nerve or in sub-organ stimulation (72,73), however, published studies exploring ultrasound vagus nerve activation are lacking. Additionally, the mechanisms of neural activation by ultrasound are not yet known, though some proposed mechanisms include thermal modulation (74), intramembrane cavitation (75), or mechanical effects of radiation force (76).

More recently, an innovative discovery in wireless technology has come to light. Organic electrolytic photocapacitors (OEPC) are photostimulation devices capable of generating an electric current sufficient for nerve stimulation when illuminated with deep-red light. Capacitive coupling has been shown in an *in vitro* model using *Xenopus laevis* oocytes to be the mechanism in which specific ion channels respond to OEPC illumination (77). Development of such devices with minimal invasiveness can open for potential replacement of traditional wired electrodes (77). Deep-red light (wavelengths above ≈ 600 nm) penetrates tissue and therefore has the potential to reach and activate implanted OEPCs to stimulate peripheral nerves wirelessly. Recently it has been shown that OEPCs can be chronically implanted and wirelessly stimulate the rat sciatic nerve (78), suggesting the possibility for application in other peripheral nerves. OEPCs can be manufactured to fit stringent constraints for size and shape and thus avoid excessive rigidity and bulkiness. Such devices may allow *in vivo* chronic application, for example expanding the use of vagus nerve stimulation in investigating molecular mechanism in chronic inflammatory conditions and thus finally enable the long-awaited long-term studies of well-characterized genetic models of inflammatory diseases (Figure 4).

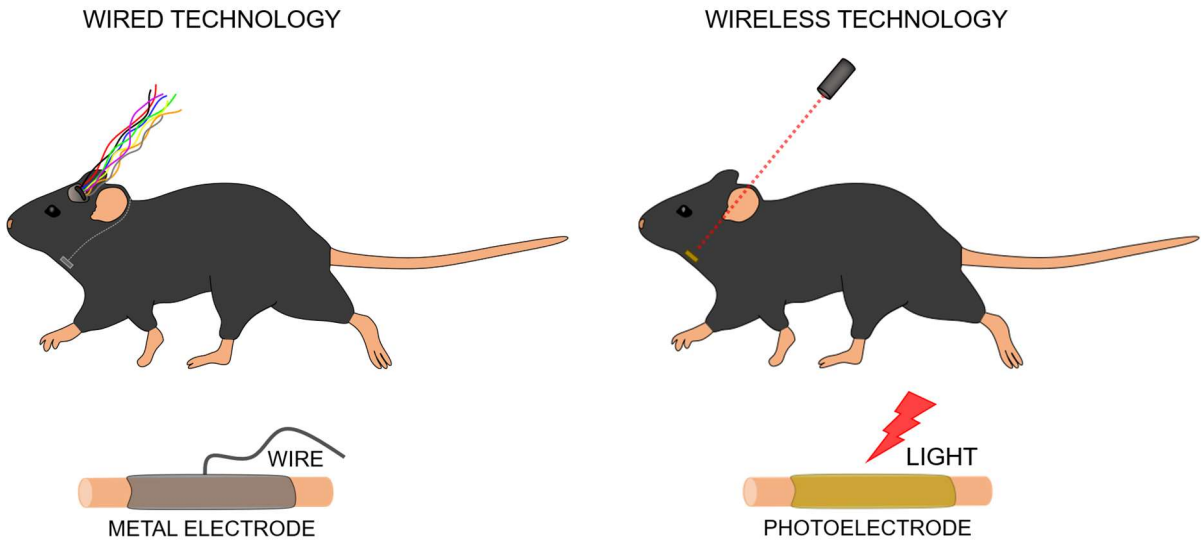

Figure 4. Wireless organic electrolytic photocapacitor technology could potentially replace current vagus nerve stimulation setups. Conventional metal electrodes (*left*) require connectors, bond pads, wires, and are often attached to a head stage and then a stimulator. The electrode is implanted on the vagus nerve and the integrated cable is tunneled subcutaneously to the connector. Photoelectrodes (*right*) can be implanted on the vagus nerve. The electrode could then be activated by deep-red light aimed at the area of implantation.

1.11 TRANSLATIONAL DEVELOPMENTS

Discoveries in pre-clinical disease models support that the use of electrodes to stimulate the vagus nerve might be suitable for therapeutic stimulation to decrease inflammation, providing an alternative to drugs in a variety of conditions and diseases characterized by excessive inflammation such as sepsis, ischemia/reperfusion injury, rheumatoid arthritis, and inflammatory bowel disease (30,33,36,38,79,80). Insights from these studies have brought forth clinical trials that utilize VNS devices for treatment of inflammatory diseases. A relatively recent pioneering study implanted a vagus nerve stimulator (commercially available device used in an open-label study) in humans for treatment of rheumatoid arthritis (81). This study showed that VNS decreased serum TNF and C-reactive protein in the included patients, and improved the 28-joint disease activity score (DAS score) over a period of 3 months (81). Recently, miniaturized devices designed to simplify implantation surgery, follow-up, programming and monitoring are under development. One study reported findings using a novel microregulator device for VNS-treatment in patients with multi-drug refractory rheumatoid arthritis, indicating that half of the patients had significant clinical improvement and decreased inflammation comparable to the previous study (82). Currently, we are involved in a multi-center study of VNS in therapy-resistant Crohn's disease led by SetPoint Medical Inc. A preliminary report shows that Crohn's symptoms were reduced in six of the eight patients, with three of the patients in remission (83). In another Crohn's disease study, an implantable vagus nerve stimulator was reported to restore vagal tone and clinical, biological, and endoscopic remission in patients over a 6 month period (84). While results from these clinical trials are encouraging, it is important to note that more well-designed and much larger studies are needed. To date, the use of randomization and placebo has been insufficient for definite conclusions. In addition, more experiments are needed to define optimal stimulation parameters and protocols. For the future, it would be beneficial, likely even crucial, to better define the neural circuits that regulate inflammation. With this information it might be feasible to develop technology that is more selective for more precise molecular targets by pinpointing specific nerve fibers – or perhaps bundles of fibers – when such technology becomes available. The vision is to optimize treatment paradigms and enable the use of electrons for specific anatomical and functional targeting of key pathogenic mechanisms of inflammatory diseases.

2 RESEARCH AIMS

This thesis aims to investigate neural control of inflammation and develop technology to enable long-term mechanistic studies in experimental animal models.

In particular, the specific aims were to:

I. Uncover mechanisms underlying sustained reduction of cytokine production in response to vagus nerve stimulation (Paper I).
II. Standardize a practical and feasible method to isolate and stimulate the vagus nerve in mice (Paper II).
III. Investigate whether electrical stimulation of the vagus nerve promotes resolution of inflammation (Paper III).
IV. Develop technology for chronic vagus nerve stimulation (Paper IV).

3 EXPERIMENTAL METHODOLOGY

3.1 *IN VIVO* MOUSE MODELS

Prior to our animal studies presented here, ethical permits (N104/16 and 20818-2020) were approved by the Regional Ethical Committee on Animal Experiments.

Animal research has made tremendous contributions to our understanding of various chronic diseases, particularly in terms of mechanistic understanding of biological processes and for development of treatment. Genetic mouse models are important tools to study mechanism of physiology and disease.

3.2 ENDOTOXEMIA

Experimental murine endotoxemia is a well-established and commonly used model of systemic inflammation (85,86). Lipopolysaccharide (LPS) binds to toll-like receptor 4 (TLR4) which is expressed on a range of immune cells and other cells. TLR4 activation commonly promotes the secretion of TNF. Since it is known that VNS reduces the release of pro-inflammatory cytokines like TNF in endotoxemia, the use of this model allowed us to observe differences in cytokine levels of sham- and VNS-treated mice. After VNS- or sham-treatment, mice received a single intraperitoneal dose of LPS and were euthanized 90 minutes after injection. Blood was collected for later analysis of serum TNF and other mediators of interest.

3.3 ZYMOSAN-INDUCED PERITONITIS

Zymosan is a toll-like receptor 2 (TLR2) agonist. The zymosan-induced peritonitis model is a self-resolving inflammation model that peaks within several hours and is cleared within 48 to 72 hours (87). Mice received a single intraperitoneal dose of zymosan and were euthanized at different time points. Peritoneal exudate was collected for leukocyte, cytokine and lipid mediator analysis.

3.4 IMPLANTATION

Development of biocompatible electrodes for vagus nerve stimulation was studied *in vivo*, as it necessary to observe inflammation in response to electrical activation of the inflammatory reflex. The physiology of neural regulation of inflammation involves interactions between multiple cell types and tissues at several anatomical locations. It would be exceedingly challenging – currently impossible – to investigate electrode biocompatibility, electrical activation of neural circuits and their interactions with the immune system and other tissues *in vitro*. Our studies involve both acute and chronic implantation of electrodes which include custom-built bipolar hook electrodes (66), multi-electrode arrays (MEA) and OEPC devices.

3.5 VAGUS NERVE STIMULATION

VNS is a procedure that involves the delivery of electrical impulses to the vagus nerve and is studied *in vivo*. In our studies, the cervical vagus nerve was isolated and stimulated in anesthetized mice. Isolating the vagus nerve and suspending the nerve on an electrode without electrical stimulation does not necessarily elicit activation of the vagus nerve (88). We found in a number of method development and verification experiments over several years that there was no significant difference between careful vagus nerve isolation and electrode placement versus a simplified sham surgery. Thus, for sham-treatment, mice were subjected to surgery without vagus nerve isolation or stimulation. However, for experiments validating the use of new electrodes that utilize OEPC technology, VNS-treated mice were implanted with OEPC devices while sham mice were implanted with sham devices (Parylene-C substrate with gold, without the photoactive PN pixel). Both groups were subjected to deep-red light-mediated photovoltaic stimulation. Sham devices yield no response to illumination, as they lack the photoactive PN pixel which responds to light to generate electrical current, as previously shown in acute sciatic nerve photostimulation (78).

4 RESULTS & DISCUSSION

4.1 SUSTAINED INHIBITION OF ENDOTOXIN-INDUCED CYTOKINE RELEASE FOR ≥ 24 H AFTER VAGUS NERVE STIMULATION REQUIRES THE ALPHA-7 NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNIT (PAPER I)

VNS reduces release of pro-inflammatory cytokines in inflammation in experimental and clinical studies (36,81). However, how long this effect is sustained and the underlying mechanism for this effect, are not well understood. To investigate this, VNS- and sham-treated animals were subjected to endotoxemia at 0, 2, 24 and 48 h after surgery. Blood was then collected, and serum TNF was analyzed. VNS-treated animals had significantly lower levels of serum TNF for up to 48 h compared to sham-treated animals (Figure 5A).

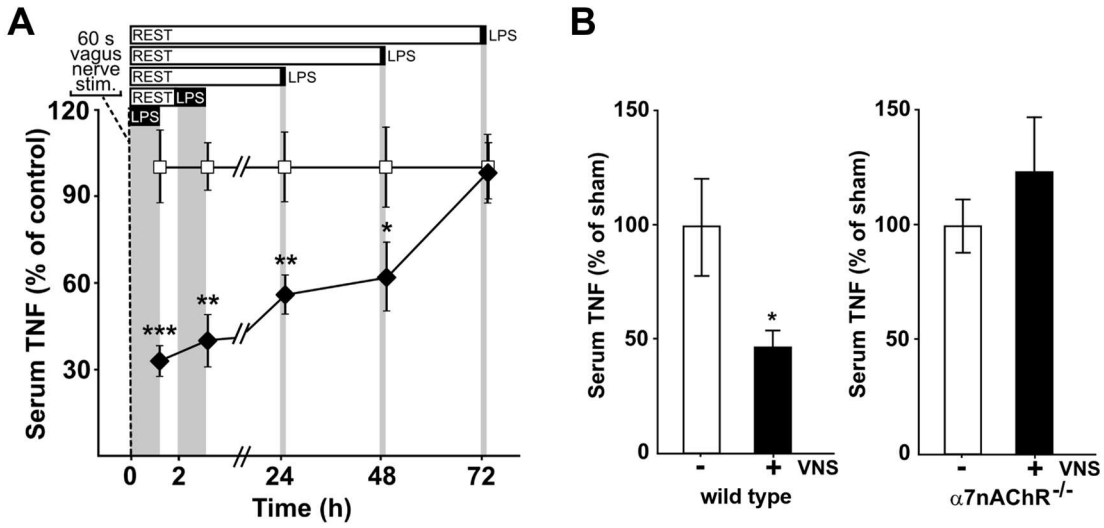

Figure 5. Sustained inhibition of endotoxin-induced cytokine-release for ≥ 24 h after vagus nerve stimulation requires the alpha-7 nicotinic acetylcholine receptor subunit. (A) Animals were subjected to VNS- or sham-treatment and after recovery were injected with endotoxin at different time points after surgery. White squares represent mean TNF ± SEM in sham-treated animals, black diamonds represent mean TNF ± SEM in VNS-treated animals. (B) Wild type (*left*) and α7nAChR-deficient (*right*) mice were subjected to VNS- or sham-treatment and after 24 h recovery were injected with endotoxin. Serum TNF levels relative to sham mice are shown as mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05.

Vagus nerve signals in the inflammatory reflex are relayed by $ChAT^+$ T cells that release ACh which then binds to the α7nAChR on cytokine producing cells, such as macrophages. To study whether VNS-induced prolonged suppression over time requires the α7nAChR, VNS-treated wild type and α7nAChR-deficient mice were subjected to endotoxemia. After 24 h, blood was collected, and serum TNF was analyzed. The sustained effect on endotoxin-induced TNF by VNS was absent in α7nAChR-deficient mice, suggesting that persistent inhibition of TNF by VNS requires the α7nAChR (Figure 5B).

Several mechanisms have been proposed for cholinergic control of immune cell release of pro-inflammatory cytokines, including signals interfering with NF-κB and JAK2/STAT3 (50,79,89). The ionotropic activity of α7nAChRs is likely not sufficient to elicit currents in immune cells to mediate inhibition of TNF release (89), which suggests there are other molecular components involved. α7nAChR in neurons have been shown to interact with adenylyl cyclase 6 (AC6) (90), thus we investigated whether an analogous interaction occurs in macrophages. RAW 264.7 cells were exposed to the adenylyl cyclase-inhibitor MDL 12,330A, and the selective α7nAChR agonist, choline. Subsequently, cells were exposed to endotoxin and TNF mRNA was measured in cell lysates by qPCR. Exposure to MDL 12,330 suppressed choline-mediated reduction of TNF mRNA, suggesting cholinergic activation of adenylyl cyclase mediates endotoxin induced-TNF release (Figure 6A). In another experiment, RAW 264.7 cells were transfected with siRNA targeting AC6 or scrambled siRNA. Subsequently, cells were exposed to choline and endotoxin. Knock-down of AC6 cells abolished cholinergic attenuation of TNF mRNA, indicating that cholinergic suppression of endotoxin-induced TNF release requires AC6 (Figure 6B).

Taken together, results from this study identify that the α7nAChR and adenylyl cyclase are involved in sustained reduction of TNF release in endotoxemia after VNS, thus improving our understanding of the inflammatory reflex with insights that may have implications for development of therapeutic stimulation strategies, in particular the required frequency of VNS for inhibition of TNF release.

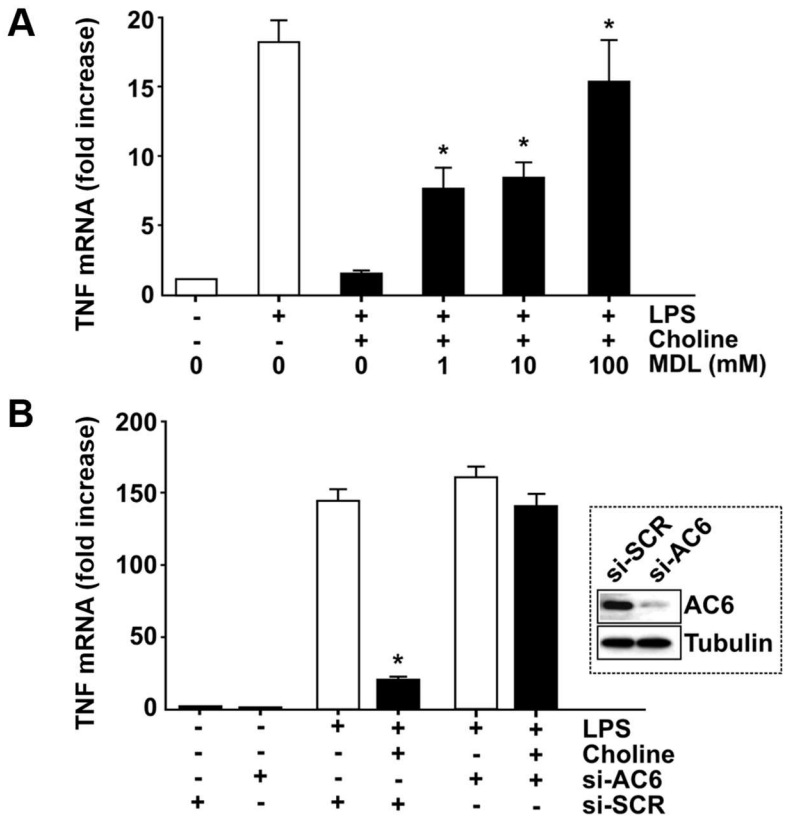

Figure 6. Adenylyl cyclase 6 mediates inhibition of endotoxin-induced TNF. (A) RAW 264.7 cells were incubated with the adenylyl cyclase inhibitor MDL 12,330A, then exposed to choline and endotoxin. Bar graphs represent mean fold increase of TNF mRNA ± SEM relative to cells exposed to endotoxin and choline in the absence of MDL 12,330A. (B) Adenylyl cyclase 6 (AC6) was knocked down using siRNA in RAW 264.7 cells. Subsequently, cells were exposed to the α7nAChR selective agonist, choline. (*Inset*) Western blot shows cells treated with siRNA targeting AC6 (si-AC6) or scrambled siRNA (si-SCR). Bar graphs represent fold increase ± SEM of TNF mRNA compared to cells not challenged with endotoxin. *p < 0.05.

4.2 A SIMPLE, CONSISTENT AND REPRODUCIBLE METHOD FOR PERFORMING VAGUS NERVE STIMULATION FOR THE STUDY OF EXPERIMENTAL INFLAMMATION (PAPER II)

Recent results from clinical trials using electrical VNS to treat rheumatoid arthritis and Crohn's disease support VNS as a prospective treatment of diseases characterized by excessive inflammation (81–84). This has created increasing interest across numerous labs in exploring VNS in a wide range of experimental models of inflammation. However, there is a lack of comprehensive description of how these experiments should be performed to promote simplicity of implementation and consistency between sites. Accordingly, we sought to describe an effective and practical method to perform VNS in acute inflammation studies involving mice intended to be readily introduced and reproduced in other laboratories with consistent results.

Lot-to-lot variation exists in commercially available biochemicals and reagents, therefore it is necessary to evaluate each new batch of biochemical or reagent when inducing inflammation (91). In our previous studies, as much as 8 mg/kg of endotoxin was used to study effects of VNS intervention on serum TNF levels, however as low as 0.1 mg/kg has also been used depending on the batch of LPS used, regardless whether the purchase was made from the same manufacturer (88,92). Therefore, titration must be performed during the experimental setup to ensure suitable dosing to induce inflammation within physiological limits based on the animal strain and species (93). Based on our observations and the current batch of endotoxin titrated, the dose-response curve plateaus at doses ≥ 2.5 mg/kg and thus concentrations below this threshold are suitable for studying VNS in endotoxemia for the experiments in our setup (Figure 7).

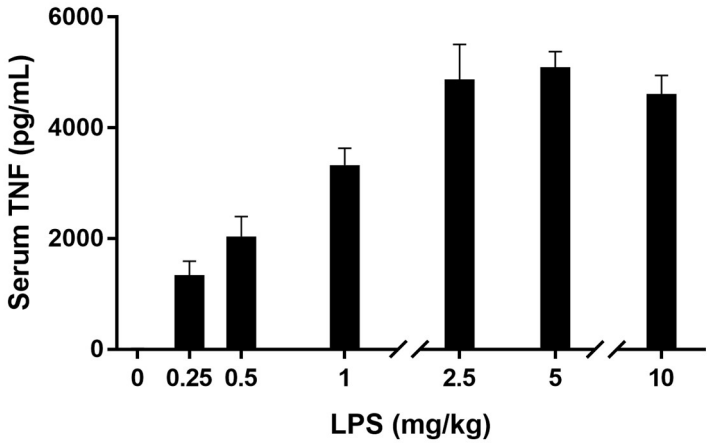

Figure 7. TNF dose response in endotoxemia. Mice were injected intraperitoneally with endotoxin and after 90 min blood was collected. Serum TNF levels were measured by ELISA. Bar graphs represent mean TNF ± SEM.

To perform VNS, the basic components required include a computer, a pulse generator, stimulator, electrode and a microscope. An oscilloscope can be used to visualize voltage output and is recommended to monitor impedance changes (Figure 8A-B) and other issues that may for example result from inadequate electrode or stimulator integrity, leaking current, or poor nerve-electrode contact. It is recommended to use a current-controlled stimulator instead of a voltage-controlled stimulator to consistently deliver the desired charge for nerve stimulation. Current-controlled stimulation compensates for variations in electrode impedance and promotes consistency in current and charge delivery. In contrast, charge delivery in voltage-

controlled stimulation is sensitive to variations in impedance and may fail to deliver adequate charge for depolarization if impedance increases.

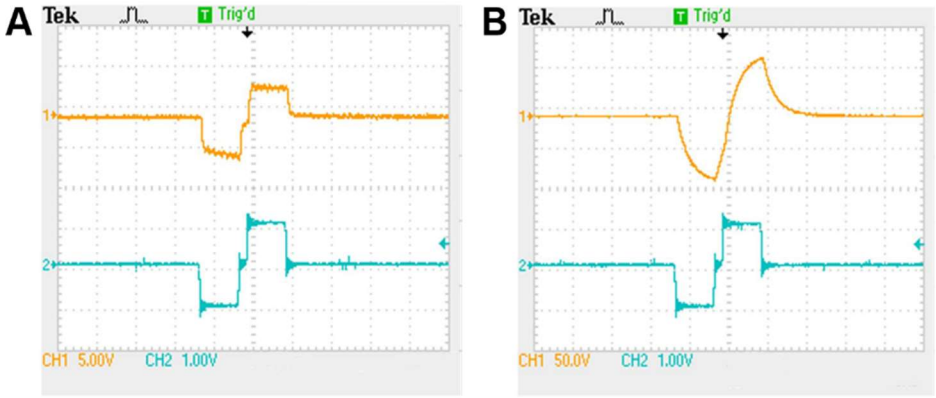

Figure 8. Maintaining constant current. (A) Oscilloscope tracing depicting voltage output from the digital-to-analog interface (bottom blue tracing, scale 1 V/square) at the desired impedance and voltage measured over the electrode leads (top orange tracing, scale 5 V/square). (B) Oscilloscope tracing depicting electrode-nerve interface with a high impedance level (top orange tracing, scale 50 V/square).

Biphasic waveforms are preferred over monophasic waveforms for VNS because of safety reasons – reducing the risk of nerve and tissue damage. Biphasic cathodic leading waveforms are less likely to lead to faradaic reactions which may cause tissue damage (94,95). Optimal stimulation parameters to activate the inflammatory reflex are not known, however we have previously investigated suitable settings in experimental endotoxemia, i.e. current-controlled stimulation, biphasic waveform, 250 µs pulse width, 10 Hz, that yield reproducible results with consistent reduction of serum TNF concentration in endotoxemia. Recently however, specific waveform combinations for electrical stimulation of the vagus nerve in the absence of inflammation have been found to alter cytokine levels differently (96). These findings suggest that depending on the waveform and stimulation pattern used, electrical vagus nerve stimulation may not only reduce release of pro-inflammatory cytokines, but also enhance it. Different stimulation parameters can elicit different physiological effects such as anti-inflammatory (perhaps attributed to A- and B- fiber activation) or cardioinhibitory (perhaps attributed to B-fibers) (65,97). It is therefore important moving forward to determine optimal stimulation combinations of current, pulse width, and frequency that are closer to optimal for specific and different fiber recruitments.

4.3 VAGUS NERVE STIMULATION INCREASES SPECIALIZED PRO-RESOLVING MEDIATORS AND ACCELERATES RESOLUTION OF INFLAMMATION BY A MECHANISM THAT REQUIRES ALOX15 AND CHOLINERGIC SIGNALING (PAPER III)

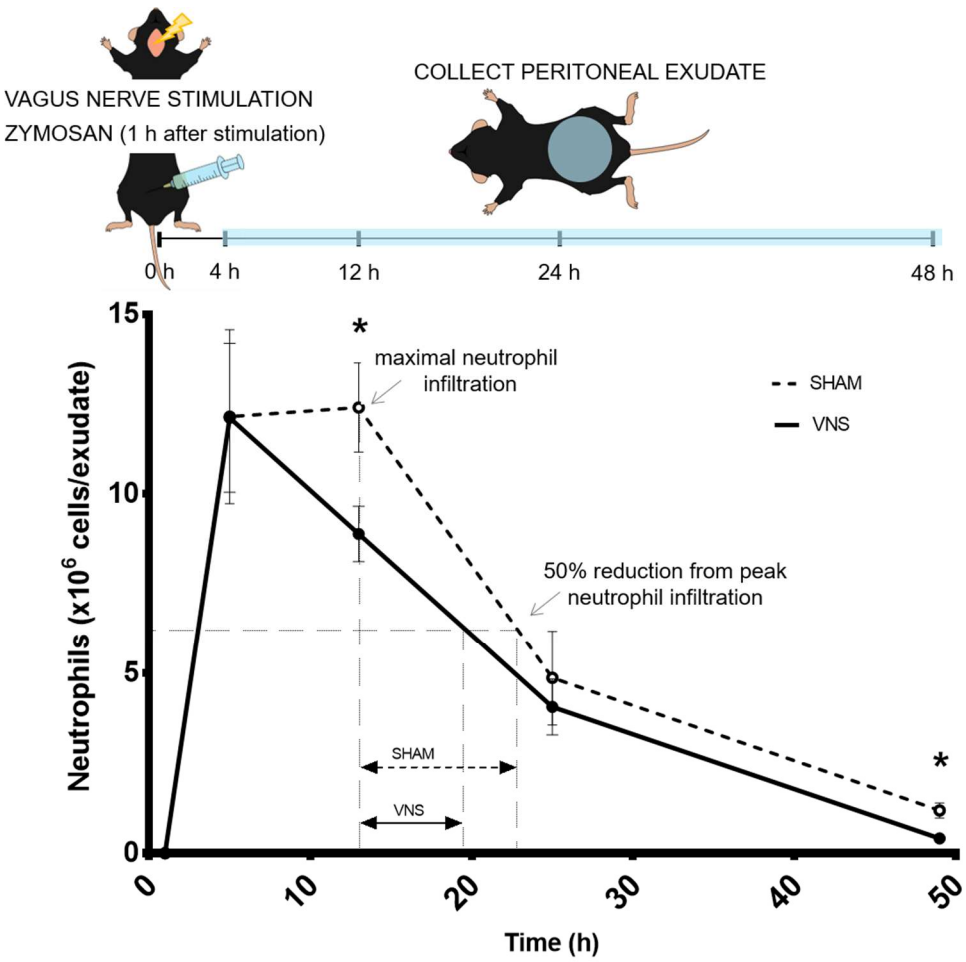

Figure 9. Electrical vagus nerve stimulation shortens resolution time. Mice were subjected to vagus nerve stimulation and zymosan-induced peritonitis. Peritoneal exudates were collected 4, 12, 24 and 48 h later. Peritoneal exudates were analyzed by flow cytometry. Results are plotted as mean ± SEM. *p < 0.05.

The mechanisms that regulate resolution of inflammation are not fully understood, however it is known that neural reflexes regulate the intensity of inflammation, for example through signals in the vagus nerve, suggesting that activation of the vagus nerve may play a role in resolution of inflammation. To address this hypothesis experimentally, mice were subjected to VNS- or sham-treatment prior to the induction of peritonitis. Neutrophil numbers in peritoneal exudates of VNS-treated mice compared with sham-treated mice subjected to zymosan were significantly reduced 12 h after zymosan challenge (Figure 9). VNS-treated mice had a shortened resolution interval and increased efferocytosis compared with sham-treated mice (Figure 9). Furthermore, VNS-treatment shifted the peritoneal exudate lipid mediator content

in peritonitis toward a more pro-resolving profile through promotion of the Alox15 pathway. This was evident in the enhanced production of specific DHA- and DPA-derived SPM (Figure 10A) – in particular, VNS-treatment enhanced biosynthesis of the protectin (PD) and maresin (MaR) families (Figure 10B). This was further demonstrated through identification of 17R-PD1 and MaR2 as well as their pathway markers 10S,17S-diHDHA (PDX) and 7S,14S-diHDHA.

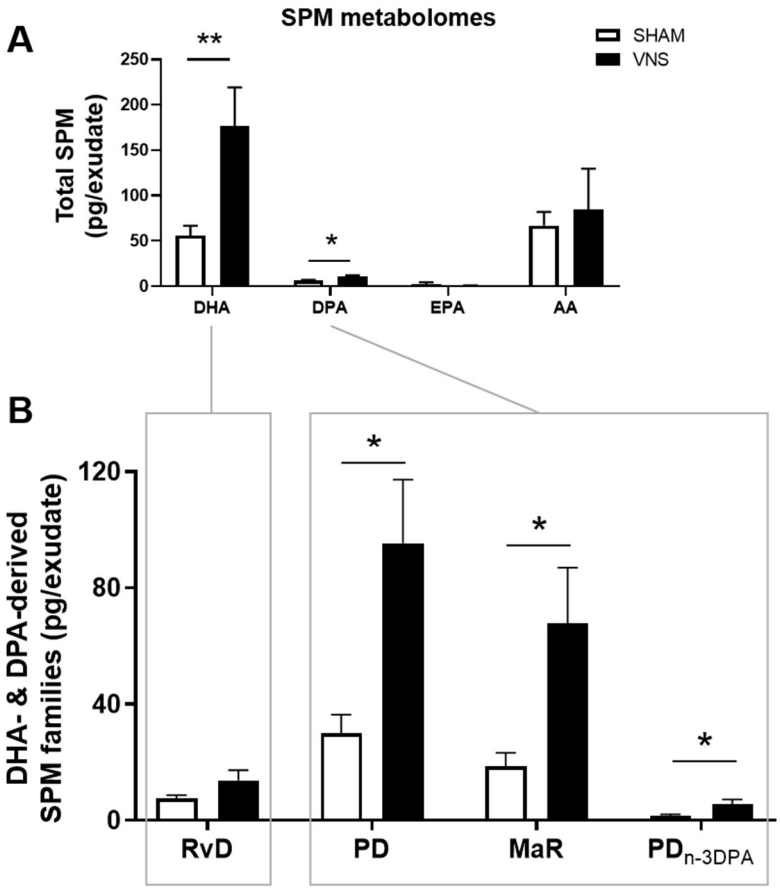

Figure 10. VNS increases levels of lipoxygenase-derived SPM. Peritoneal exudates from VNS- and sham-treated mice were collected 12 h after intraperitoneal zymosan injection. Levels of lipid mediators in peritoneal exudates were measured using LC-MS/MS. (A) Levels of total SPM identified from the metabolome families DHA, EPA, EPA, and AA. (B) Levels of identified DHA- and DPA-derived SPM. Results are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

These observations infer that VNS-treatment accelerated inflammation resolution by activating the Alox15 biosynthetic pathways, which was further supported in observations that effects of VNS-treatment on neutrophil numbers and efferocytosis were lost in mice deficient of Alox15 (Figure 11). Furthermore, this effect was also lost in α7nAChR-deficient mice (Figure 11), suggesting the α7nAChR was required for the VNS-mediated effects on resolution of inflammation in peritonitis. Findings from this study indicate electrical activation of the inflammatory reflex promoted resolution of inflammation.

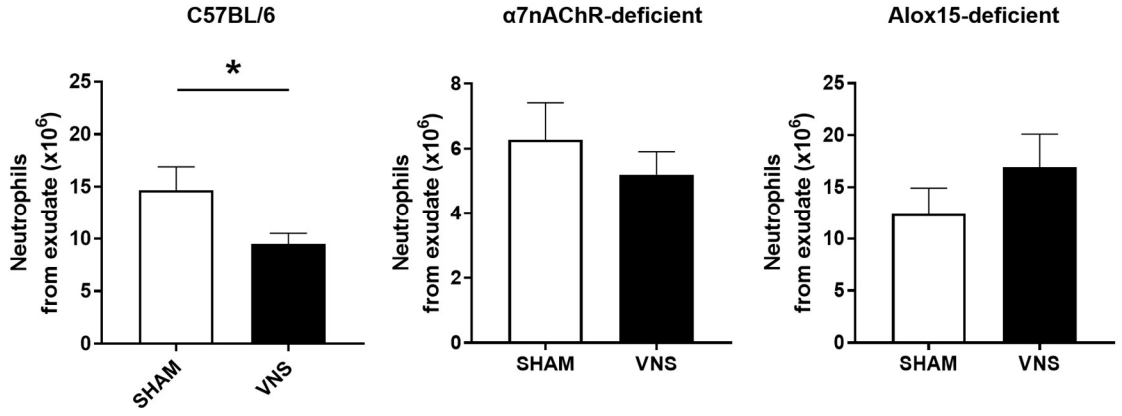

Figure 11. Electrical vagus nerve stimulation reduces neutrophil numbers *in vivo* through a mechanism that requires cholinergic signaling and Alox15. Mice were subjected to left cervical vagus nerve stimulation and zymosan-induced peritonitis. Peritoneal exudates were collected 12 h after zymosan challenge and analyzed by flow cytometry. Neutrophil numbers in (*Left)* C57BL/6 sham- compared with VNS-treated mice, *(Middle)* α7nAChR-deficient sham- compared with VNS-treated mice, and *(Right)* Alox15-deficient sham- compared with VNS-treated mice. Results are plotted as mean ± SEM. *p < 0.05.

4.4 ORGANIC ELECTROLYTIC PHOTOCAPACITORS CAN POTENTIALLY ENABLE WIRELESS VAGUS NERVE STIMULATION IN MICE (PAPER IV)

Electrode technology for chronic nerve activation in mice are lacking. With the availability of genetic mouse models to study disease mechanisms, electrodes for chronic nerve activation would enable improved mechanistic studies of peripheral nerve regulation of inflammation in health and disease. Recent advances in OEPC technology have facilitated development of suitable alternatives to wired interfaces (77,78), and could potentially be utilized for development of a wireless electrode for peripheral nerve stimulation in mice. Accordingly, we set out to investigate OEPC technology for stimulation of the mouse vagus nerve and activation of the inflammatory reflex (Figure 12).

Under anesthesia, mice were implanted with an OEPC or sham device on their cervical vagus nerve and the electrode was illuminated using deep-red light. The device was removed, the wound closed and after recovery the mice were injected intraperitoneally with endotoxin. Serum TNF levels of sham- and VNS-treated mice were analyzed by ELISA. While the mean TNF level of VNS-treated mice were lower compared with the sham-treated mice, the difference did not reach statistical significance. We postulated that this photocapacitor technology was promising but needed to be developed further for stimulation of mouse

peripheral nerves and proceeded to develop a wired MEA with variable stimulation contacts to investigate the effectiveness in nerve stimulation using different configuration arrangements.

Since activation of the vagus nerve is known to slow heart rate, heart rate reduction can be used to validate activation of vagal fibers. Stimulation-induced heart rate reduction is an appealing method for these experimental purposes because it can be monitored in real-time using pulse oximetry with relative simplicity, thus it is reasonable to verify effective electrode stimulation of the vagus nerve using this setup. We investigated MEA stimulation-evoked reduction of heart rate to determine suitable stimulation parameters and electrode layout for OEPC device fabrication. Indeed, this experiment investigates vagus nerve activation and heart rate, not primarily vagus nerve stimulation and regulation of inflammation, yet it serves as a reasonable proxy to investigate functionality of the electrode technology *in vivo.* We observed that a longitudinal configuration required a relatively limited current to activate the vagus nerve and thus appears suitable for design of the OEPC device (Figure 12A). Since light intensity will be reduced as it traverses the skin and tissues on its way to an implanted OEPC device, it is beneficial to find an electrode configuration that does not need to produce a high current to depolarize the nerve sufficiently. Accordingly, a longitudinal configuration was chosen and the configuration of an OEPC device adapted to approximate this design.

Mice were implanted with the OEPC device and exposed to illumination of deep-red light. Stimulation-induced heart rate reduction was observed by pulse oximetry. As the electrode was illuminated by the deep-red light, we observed a reduction in heart rate. Thus, we conclude that OEPC devices with this configuration placed on the vagus nerve can induce heart rate reduction in mice (Figure 12B). In other words, these OEPC devices are capable of electrically activating the cervical vagus nerve as they are exposed to the deep-red light.

Initial findings from this study lay the foundation for potentially implementing light-activated photocapacitors as small peripheral nerve stimulators with low energy requirements. However,

further work is required and underway to improve OEPC layouts and illumination parameters for a more optimal design and setup.

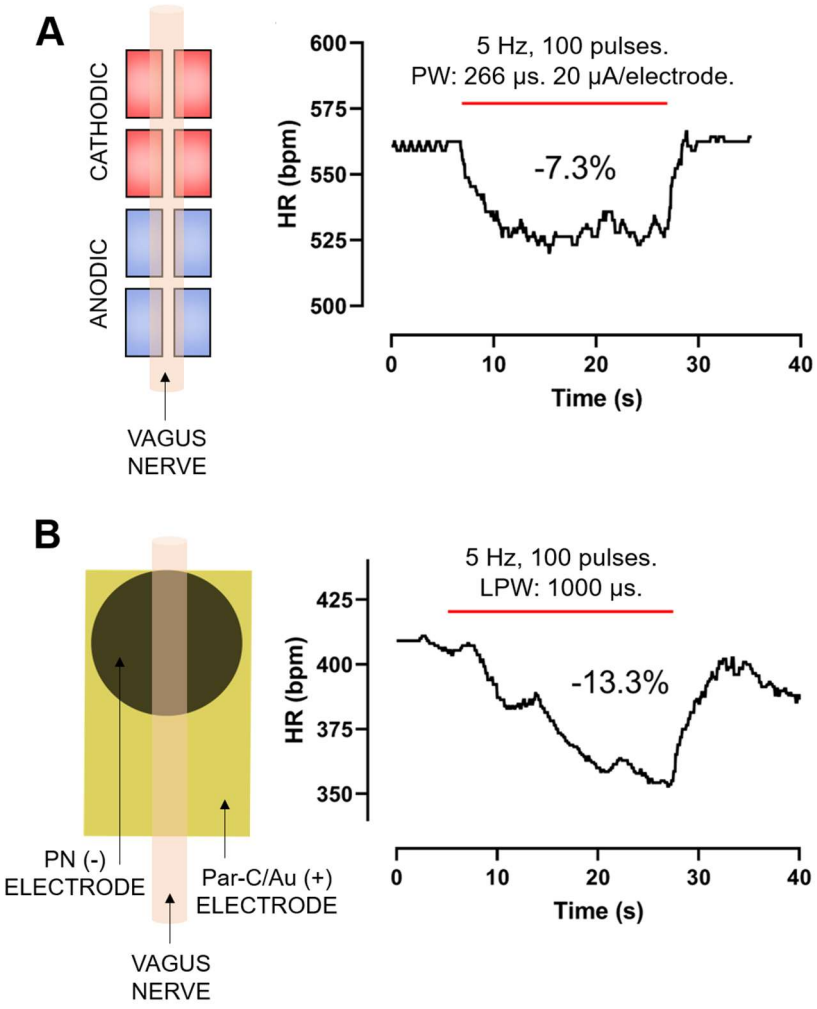

Figure 12. Stimulation-induced heart rate reduction with a longitudinal electrode arrangement. (A) Stimulation-induced heart rate reduction while stimulating with the longitudinal bipolar MEA configuration at 5 Hz, 100 pulses, 266 µs pulse width (PW), 20 µA/electrode. (B) Longitudinal OEPC-device induced heart rate reduction upon OEPC stimulation of the right cervical vagus nerve. Light illumination parameters: 5 Hz, 100 light pulses, with a light pulse width (LPW) of 1000 µs.

5 CONCLUSIONS

This thesis reveals several previously unknown mechanisms in neural control of inflammation, including cholinergic activation of adenylyl cyclase in regulation of TNF release and cholinergic control of SPM release and inflammation resolution time. The work in this thesis also provides evidence that implanted, wirelessly controlled electrodes can activate the cervical vagus nerve. This novel approach promises to enable greatly needed mechanistic studies of peripheral nerve activation in a wide range of experimental models of disease.

Bioelectronic medicine is a rapidly evolving field with many opportunities for new discoveries in therapeutic monitoring and stimulation to treat and detect inflammation. The inflammatory reflex is one of many reflex circuits and there are still gaps in our understanding of neural reflexes and regulation of immunity. However, observations described in this thesis provide a glimpse of the future direction of possible therapeutic modalities through which vagus nerve stimulation can regulate excessive inflammation and its resolution.

With the growing interest in neural reflex control of inflammation, it is important that implementation of VNS for activation of the inflammatory reflex is consistent. With the next generation of neural interfaces for bioelectronic medicine it is crucial to update and provide clear methodology to perform VNS for the study of experimental inflammation in order to yield reproducible results across laboratories. While we have shared methodology that in our experience yields reproducibility, recent progress in neural interfaces and development in more sophisticated technology call for updated methodology and design of integrated research workflows. Even in the time this thesis was being written, we have continually improved our experimental setup and physiological monitoring of mice (e.g. upgraded how temperature sensors and warming devices, pulse oximeters, and electrodes are used) Improved consistency and reproducibility of results are achieved with better control of experimental variables.

Our findings that electrical vagus nerve stimulation regulates resolution of inflammation *in vivo* demonstrate for the first time that signals in the inflammatory reflex not only regulate inflammation but also promotes resolution of inflammation. This opens a new avenue of exploration in therapeutic stimulation, and further experiments are ongoing on mechanism to determine if other components of the inflammatory reflex are involved in resolution of inflammation.

Observations that inflammation was sustained for over 24 h after vagus nerve stimulation in endotoxemia shed light on an important time frame of intervention in acute experimental inflammation (92). Although a single electrical vagus nerve stimulation has effects on

inflammation that persist for over 24 h, there is a critical need for persistent and long-term electrodes for repeated stimulations to study chronic inflammatory disease. Thus, to contribute to solving this technological barrier, we sought to investigate new, wireless technology for chronic nerve stimulation. We adapted organic electrolytic photocapacitor technology for replacement of wired electrodes for vagus nerve stimulation. Further work is still needed to confirm observations that a longitudinal configuration is suitable for activation of the inflammatory reflex. We have yet to exploit the numerous configurations the MEA electrode is capable of testing and may find even more suitable configurations to activate the vagus nerve. We are currently continuing to explore this, as well as assessing various OEPC device layouts and illumination parameters. Results from our pilot experiments are promising, and findings from this thesis already demonstrate the potential for photocapacitors to wirelessly stimulate the vagus nerve and activate the inflammatory reflex, preceding future research and development of wireless neural interfaces for peripheral nerve applications for therapeutic intervention.

The findings here improve our understanding of how activation of the vagus nerve regulates inflammation. Hopefully, the advancements in experimental procedures and techniques developed here will enable and expand the study of electrical activation of the vagus nerve – and perhaps other peripheral nerves – in experimental models of chronic diseases. Perhaps these efforts will improve our mechanistic understanding of the regulation of inflammation and provide important elements missing in our knowledge of immune system function that can contribute to both improving health and treating disease.

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7 REFERENCES

1. Cross M, Smith E, Hoy D, Carmona L, Wolfe F, Vos T, et al. The global burden of rheumatoid arthritis: Estimates from the Global Burden of Disease 2010 study. Ann Rheum Dis. 2014 Jul 1;73(7):1316–22.

2. Lim GB. Public health: Global burden of cardiovascular disease. Vol. 10, Nature Reviews Cardiology. Nature Publishing Group; 2013. p. 59.

3. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet. 2017 Dec 23;390(10114):2769–78.

4. Kaplan GG. The global burden of IBD: From 2015 to 2025. Vol. 12, Nature Reviews Gastroenterology and Hepatology. Nature Publishing Group; 2015. p. 720–7.

5. Glassock RJ, Warnock DG, Delanaye P. The global burden of chronic kidney disease: Estimates, variability and pitfalls. Vol. 13, Nature Reviews Nephrology. 2017. p. 104–14.

6. Nathan C, Ding A. Nonresolving Inflammation. Cell. 2010;140(6):871–82.

7. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. Rethinking inflammation: neural circuits in the regulation of immunity. Immunol Rev. 2012;248(1):188–204.

8. Andersson U, Tracey KJ. Neural reflexes in inflammation and immunity. Vol. 209, Journal of Experimental Medicine. 2012. p. 1057–68.

9. Pober JS, Sessa WC. Inflammation and the blood microvascular system. Cold Spring Harb Perspect Biol. 2015 Jan 1;7(1).

10. Ortega-Gómez A, Perretti M, Soehnlein O. Resolution of inflammation: An integrated view. Vol. 5, EMBO Molecular Medicine. John Wiley & Sons, Ltd; 2013. p. 661–74.

11. Serhan CN, Chiang N, Dalli J, Levy BD. Lipid mediators in the resolution of inflammation. Cold Spring Harb Perspect Biol. 2015;7(2).

12. Serhan CN, Chiang N, Dalli J. The resolution code of acute inflammation: Novel pro-resolving lipid mediators in resolution. Semin Immunol. 2015 May;27(3):200–15.

13. Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol. 2005 Dec;6(12):1191–7.

14. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. Nature. 2014;510(7503):92–101.

15. Buckley CD, Gilroy DW, Serhan CN. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. Vol. 40, Immunity. Cell Press; 2014. p. 315–27.

16. Lawrence T, Gilroy DW. Chronic inflammation: A failure of resolution? Vol. 88, International Journal of Experimental Pathology. Wiley-Blackwell; 2007. p. 85–94.

17. Serhan CN. Novel lipid mediators and resolution mechanisms in acute inflammation: To resolve or not? Vol. 177, American Journal of Pathology. 2010. p. 1576–91.

18. Furman D, Campisi J, Verdin E, Carrera-Bastos P, Targ S, Franceschi C, et al. Chronic

inflammation in the etiology of disease across the life span. Nat Med. 2019 Dec 1;25(12):1822–32.

19. Tracey KJ. Physiology and immunology of the cholinergic antiinflammatory pathway. Vol. 117, Journal of Clinical Investigation. American Society for Clinical Investigation; 2007. p. 289–96.

20. Scheinecker C, Redlich K, Smolen JS. Cytokines as Therapeutic Targets: Advances and Limitations. Vol. 28, Immunity. Elsevier; 2008. p. 440–4.

21. Sundman E, Olofsson PS. Neural control of the immune system. Adv Physiol Educ. 2015;38(2):135–9.

22. Sun J, Singh V, Kajino-Sakamoto R, Aballay A. Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. Science. 2011;332(6030):729–32.

23. Tracey KJ. Ancient neurons regulate immunity. Vol. 332, Science. American Association for the Advancement of Science; 2011. p. 673–4.

24. Kawli T, Tan MW. Neuroendocrine signals modulate the innate immunity of Caenorhabditis elegans through insulin signaling. Nat Immunol. 2008;9(12):1415–24.

25. Anyanful A, Easley KA, Benian GM, Kalman D. Conditioning Protects C. elegans from Lethal Effects of Enteropathogenic E. coli by Activating Genes that Regulate Lifespan and Innate Immunity. Cell Host Microbe. 2009;5(5):450–62.

26. Goehler LE, Gaykema RPA, Hansen MK, Anderson K, Maier SF, Watkins LR. Vagal immune-to-brain communication: A visceral chemosensory pathway. In: Autonomic Neuroscience: Basic and Clinical. 2000. p. 49–59.

27. Gardner WD, Osburn WA. Anatomy of the human body. 1978;

28. Tracey KJ. The inflammatory reflex. Vol. 420, Nature. 2002. p. 853–9.

29. Andersson J. The inflammatory reflex - Introduction. J Intern Med. 2005;257(2):122–5.

30. Borovikova L V., Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature. 2000 May;405(6785):458–62.

31. Fairchild KD, Srinivasan V, Randall Moorman J, Gaykema RPA, Goehler LE. Pathogen-induced heart rate changes associated with cholinergic nervous system activation. Am J Physiol Integr Comp Physiol. 2011 Feb;300(2):R330–9.

32. Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. In: Autonomic Neuroscience: Basic and Clinical. 2000. p. 1–17.

33. Bernik TR, Friedman SG, Ochani M, DiRaimo R, Susarla S, Czura CJ, et al. Cholinergic antiinflammatory pathway inhibition of tumor necrosis factor during ischemia reperfusion. J Vasc Surg. 2002;36(6):1231–6.

34. Watkins LR, Goehler LE, Relton JK, Tartaglia N, Silbert L, Martin D, et al. Blockade of interleukin-1 induced hyperthermia by subdiaphragmatic vagotomy: evidence for vagal mediation of immune-brain communication. Neurosci Lett. 1995;183(1–2):27–31.

35. Niijima A. The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat. J Auton Nerv Syst. 1996;61(3):287–91.

36. Levine YA, Koopman FA, Faltys M, Caravaca A, Bendele A, Zitnik R, et al. Neurostimulation of the cholinergic anti-inflammatory pathway ameliorates disease in rat collagen-induced arthritis. PLoS One. 2014;9(8):e104530.

37. Meregnani J, Clarençon D, Vivier M, Peinnequin A, Mouret C, Sinniger V, et al. Anti-inflammatory effect of vagus nerve stimulation in a rat model of inflammatory bowel disease. Auton Neurosci. 2011 Feb 24;160(1–2):82–9.

38. Inoue T, Abe C, Sung SSJ, Moscalu S, Jankowski J, Huang L, et al. Vagus nerve stimulation mediates protection from kidney ischemia-reperfusion injury through α7nAChR+ splenocytes. J Clin Invest. 2016;126(5):1939–52.

39. Bosmans G, Appeltans I, Stakenborg N, Gomez-Pinilla PJ, Florens M V., Aguilera-Lizarraga J, et al. Vagus nerve stimulation dampens intestinal inflammation in a murine model of experimental food allergy. Allergy Eur J Allergy Clin Immunol. 2019 Sep 15;74(9):1748–59.

40. Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA, et al. Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. 2006;203(7):1623–8.

41. Rosas-Ballina M, Ochani M, Parrish WR, Ochani K, Harris YT, Huston JM, et al. Splenic nerve is required for cholinergic antiinflammatory pathway control of TNF in endotoxemia. 2008;105(31).

42. Murray K, Rude KM, Sladek J, Reardon C. Divergence of neuroimmune circuits activated by afferent and efferent vagal nerve stimulation in the regulation of inflammation. J Physiol. 2021 Feb 4;JP281189.

43. Rosas-Ballina M, Olofsson PS, Ochani M, Valdes-Ferrer SI, Levine YA, Reardon C, et al. Acetylcholine-Synthesizing T Cells Relay Neural Signals in a Vagus Nerve Circuit. Science. 2011;334(6052):98–101.

44. Olofsson PS, Steinberg BE, Sobbi R, Cox MA, Ahmed MN, Oswald M, et al. Blood pressure regulation by CD4+ lymphocytes expressing choline acetyltransferase. 2016;34(10):1066–71.

45. Cox MA, Duncan GS, Lin GHY, Steinberg BE, Yu LX, Brenner D, et al. Choline acetyltransferase–expressing T cells are required to control chronic viral infection. Science. 2019;

46. Sourkes TL. Acetylcholine - From Vagusstoff to cerebral neurotransmitter. Vol. 18, Journal of the History of the Neurosciences. 2009. p. 47–58.

47. Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S, et al. Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. Nature. 2002;421(6921):384–8.

48. Olofsson PS, Katz DA, Rosas-Ballina M, Levine YA, Ochani M, Valdés-Ferrer SI, et al. α7 nicotinic acetylcholine receptor (α7nAChR) expression in bone marrow-derived non-T cells is required for the inflammatory reflex. Mol Med. 2012;18(1):539–43.

49. Fujii T, Takada-Takatori Y, Kawashima K. Basic and clinical aspects of non-neuronal

acetylcholine: Expression of an independent, non-neuronal cholinergic system in lymphocytes and its clinical significance in immunotherapy. Vol. 106, Journal of Pharmacological Sciences. 2008. p. 186–92.

50. de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, et al. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. Nat Immunol. 2005;6(8):844–51.

51. Lu B, Kwan K, Levine YA, Olofsson PS, Yang H, Li J, et al. α7 nicotinic acetylcholine receptor signaling inhibits inflammasome activation by preventing mitochondrial DNA release. Mol Med. 2014;20(1):350–8.

52. van Westerloo DJ, Giebelen IA, Florquin S, Bruno MJ, LaRosa GJ, Ulloa L, et al. The Vagus Nerve and Nicotinic Receptors Modulate Experimental Pancreatitis Severity in Mice. Gastroenterology. 2006;130(6):1822–30.

53. Pavlov VA, Ochani M, Yang LH, Gallowitsch-Puerta M, Ochani K, Lin X, et al. Selective α7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. Crit Care Med. 2007;35(4):1139–44.

54. Mirakaj V, Thix CA, Laucher S, Mielke C, Morote-Garcia JC, Schmit MA, et al. Netrin-1 dampens pulmonary inflammation during acute lung injury. Am J Respir Crit Care Med. 2010;181(8):815–24.

55. Mirakaj V, Dalli J, Granja T, Rosenberger P, Serhan CN. Vagus nerve controls resolution and pro-resolving mediators of inflammation. J Exp Med. 2014;211(6):1037–48.

56. Tsoucalas G, Karamanou M, Lymperi M, Gennimata V, Androutsos G. The “torpedo” effect in medicine. Int Marit Health. 2014;65(2):65–7.

57. Hodgkin AL, Huxley AF. Propagation of electrical signals along giant nerve fibers. Proc R Soc Lond B Biol Sci. 1952 Oct 16;140(899):177–83.

58. Alberts B, Johnson A, Lewis J. Ion channels and the Electrical Properties of Membrane. Mol Biol Cell. 2002;1–18.

59. Bouton CE, Shaikhouni A, Annetta N V., Bockbrader MA, Friedenberg DA, Nielson DM, et al. Restoring cortical control of functional movement in a human with quadriplegia. Nature. 2016;533(7602):247–50.

60. Silverman HA, Stiegler A, Tsaava T, Newman J, Steinberg BE, Masi EB, et al. Standardization of methods to record Vagus nerve activity in mice. Bioelectron Med. 2018 Dec 15;4(1):3.

61. Steinberg BE, Silverman HA, Robbiati S, Gunasekaran MK, Tsaava T, Battinelli E, et al. Cytokine-specific Neurograms in the Sensory Vagus Nerve. Bioelectron Med. 2016;3(1):7–17.

62. Masi EB, Levy T, Tsaava T, Bouton CE, Tracey KJ, Chavan SS, et al. Identification of hypoglycemia-specific neural signals by decoding murine vagus nerve activity. Bioelectron Med. 2019 Dec 11;5(1):9.

63. Loeb GEE, Peck RAA. Cuff electrodes for chronic stimulation and recording of peripheral nerve activity. J Neurosci Methods. 1996;64(1):95–103.

64. Noller CM, Levine YA, Urakov TM, Aronson JP, Nash MS. Vagus Nerve Stimulation in Rodent Models: An Overview of Technical Considerations. Front Neurosci. 2019 Sep 4;13.

65. Olofsson PS, Levine YA, Caravaca A, Chavan SS, Pavlov VA, Faltys M, et al. Single-pulse and unidirectional electrical activation of the cervical vagus nerve reduces TNF in endotoxemia. Bioelectron Med. 2015;(June):37–42.

66. Caravaca AS, Tsaava T, Goldman L, Silverman H, Riggott G, Chavan SS, et al. A novel flexible cuff-like microelectrode for dual purpose, acute and chronic electrical interfacing with the mouse cervical vagus nerve. J Neural Eng. 2017 Dec 1;14(6):066005.

67. Falcone JD, Liu T, Goldman L, Pogue DD, Straka M, Rieth L, et al. A wrappable microwire electrode for awake, chronic interfacing with small diameter autonomic peripheral nerves. bioRxiv. 2018.

68. Mughrabi I, Hickman J, Jayaprakash N, Papadoyannis E, Abbas A, Chang Y-C, et al. An implant for long-term cervical vagus nerve stimulation in mice. bioRxiv. 2020 Jun 20;2020.06.20.160473.

69. Seo D, Neely RM, Shen K, Singhal U, Alon E, Rabaey JM, et al. Wireless Recording in the Peripheral Nervous System with Ultrasonic Neural Dust. Neuron. 2016 Aug 3;91(3):529–39.

70. Johnson BC, Shen K, Piech D, Ghanbari MM, Li KY, Neely R, et al. StimDust: A 6.5mm3, wireless ultrasonic peripheral nerve stimulator with 82% peak chip efficiency. In: 2018 IEEE Custom Integrated Circuits Conference, CICC 2018. Institute of Electrical and Electronics Engineers Inc.; 2018. p. 1–4.

71. Piech DK, Johnson BC, Shen K, Ghanbari MM, Li KY, Neely RM, et al. A wireless millimetre-scale implantable neural stimulator with ultrasonically powered bidirectional communication. Nat Biomed Eng. 2020 Feb 1;4(2):207–22.

72. Downs ME, Lee SA, Yang G, Kim S, Wang Q, Konofagou EE. Non-invasive peripheral nerve stimulation via focused ultrasound in vivo. Phys Med Biol. 2018 Feb 1;63(3):35011.

73. Cotero V, Fan Y, Tsaava T, Kressel AM, Hancu I, Fitzgerald P, et al. Noninvasive sub-organ ultrasound stimulation for targeted neuromodulation. Nat Commun. 2019 Dec 12;10(1):952.

74. Zhu L, Altman MB, Laszlo A, Straube W, Zoberi I, Hallahan DE, et al. Ultrasound Hyperthermia Technology for Radiosensitization. Vol. 45, Ultrasound in Medicine and Biology. Elsevier USA; 2019. p. 1025–43.

75. Krasovitski B, Frenkel V, Shoham S, Kimmel E. Intramembrane cavitation as a unifying mechanism for ultrasound-induced bioeffects. Proc Natl Acad Sci U S A. 2011 Feb 22;108(8):3258–63.

76. Sarvazyan AP, Rudenko O V., Nyborg WL. Biomedical applications of radiation force of ultrasound: Historical roots and physical basis. Vol. 36, Ultrasound in Medicine and Biology. Ultrasound Med Biol; 2010. p. 1379–94.

77. Jakešová M, Silverå Ejneby M, Đerek V, Schmidt T, Gryszel M, Brask J, et al. Optoelectronic control of single cells using organic photocapacitors. Sci Adv. 2019

Apr 1;5(4):eaav5265.

78. Silverå-Ejneby M, Jakešová M, Ferrero J, Migliaccio L, Zhao Z, Berggren M, et al. A chronic photocapacitor implant for noninvasive neurostimulation with deep red light. bioRxiv. 2020;2020.07.01.182113.

79. Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, et al. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med. 2004;10(11):1216–21.

80. Bonaz B, Sinniger V, Pellissier S. Vagus nerve stimulation: a new promising therapeutic tool in inflammatory bowel disease. J Intern Med. 2017;282(1):46–63.

81. Koopman FA, Chavan SS, Miljko S, Grazio S, Sokolovic S, Schuurman PR, et al. Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis. Proc Natl Acad Sci U S A. 2016;113(29):8284–9.

82. Genovese MC, Gaylis NB, Sikes D, Kivitz A, Lewis Horowitz D, Peterfy C, et al. Safety and efficacy of neurostimulation with a miniaturised vagus nerve stimulation device in patients with multidrug-refractory rheumatoid arthritis: a two-stage multicentre, randomised pilot study. Lancet Rheumatol. 2020;2(9):e527–38.

83. D'Haens G, Cabrijan Z, Eberhardson M, van den Bergh R, Löwenberg M, Fiorino G, et al. P574 The effects of vagus nerve stimulation in biologic-refractory Crohn's disease: A prospective clinical trial. J Crohn's Colitis. 2018;12:S397–8.

84. Bonaz B, Sinniger V, Hoffmann D, Clarençon D, Mathieu N, Dantzer C, et al. Chronic vagus nerve stimulation in Crohn's disease: a 6-month follow-up pilot study. Neurogastroenterol Motil. 2016 Jun;28(6):948–53.

85. Remick DG, Ward PA. Evaluation of endotoxin models for the study of sepsis. Vol. 24, Shock. 2005. p. 7–11.

86. Lewis AJ, Seymour CW, Rosengart MR. Current Murine Models of Sepsis. Surg Infect (Larchmt). 2016;17(4):385–93.

87. Cash JL, White GE, Greaves DR. Chapter 17 Zymosan-Induced Peritonitis as a Simple Experimental System for the Study of Inflammation. Vol. 461, Methods in Enzymology. Academic Press; 2009. p. 379–96.

88. Caravaca AS, Gallina AL, Tarnawski L, Tracey KJ, Pavlov VA, Levine YA, et al. An Effective Method for Acute Vagus Nerve Stimulation in Experimental Inflammation. Front Neurosci. 2019;13.

89. Báez-Pagán CA, Delgado-Vélez M, Lasalde-Dominicci JA. Activation of the Macrophage α7 Nicotinic Acetylcholine Receptor and Control of Inflammation. J Neuroimmune Pharmacol. 2015 Sep 14;10(3):468–76.

90. Oshikawa J, Toya Y, Fujita T, Egawa M, Kawabe J, Umemura S, et al. Nicotinic acetylcholine receptor α7 regulates cAMP signal within lipid rafts. Am J Physiol - Cell Physiol. 2003 Sep 1;285(3 54-3):C567–74.

91. Thompson S, Chesher D. Lot-to-lot variation. Vol. 39, Clinical Biochemist Reviews. 2018. p. 51–60.

92. Tarnawski L, Reardon C, Caravaca AS, Rosas-Ballina M, Tusche MW, Drake AR, et

al. Adenylyl Cyclase 6 Mediates Inhibition of TNF in the Inflammatory Reflex. Front Immunol. 2018;9:2648.

93. Thomas RC, Bath MF, Stover CM, Lambert DG, Thompson JP. Exploring LPS-induced sepsis in rats and mice as a model to study potential protective effects of the nociceptin/orphanin FQ system. Peptides. 2014 Nov;61:56–60.

94. Merrill DR, Bikson M, Jefferys JGR. Electrical stimulation of excitable tissue: Design of efficacious and safe protocols. Journal of Neuroscience Methods Elsevier; Feb 15, 2005 p. 171–98.

95. Piallat B, Chabardès S, Devergnas A, Torres N, Allain M, Barrat E, et al. Monophasic but not biphasic pulses induce brain tissue damage during monopolar high-frequency deep brain stimulation. Neurosurgery. 2009;64(1):156–62.

96. Tsaava T, Datta-Chaudhuri T, Addorisio ME, Masi EB, Silverman HA, Newman JE, et al. Specific vagus nerve stimulation parameters alter serum cytokine levels in the absence of inflammation. Bioelectron Med. 2020;6(1):8.

97. Yoo PB, Liu H, Hincapie JG, Ruble SB, Hamann JJ, Grill WM. Modulation of heart rate by temporally patterned vagus nerve stimulation in the anesthetized dog. Physiol Rep. 2016 Feb 25;4(2):e12689.