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# THÈSE DE DOCTORAT

## Étude du rôle du nerf sinuso-carotidien dans l'inflammation

L'électrostimulation du nerf sinuso-carotidien chez la souris atténue l'inflammation en agissant sur l'axe hypothalamo-hypophyso-surrénalien et les cellules myéloïdes.

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**Présentée en vue de l'obtention  
du grade de docteur en discipline**

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**En français (obligatoire)**

Titre de la thèse	Étude du rôle du nerf sinuso-carotidien dans l'inflammation.
Sous-titre de la thèse	L'électrostimulation du nerf sinuso-carotidien chez la souris atténue l'inflammation en agissant sur l'axe hypothalamo-hypophysio-surrénalien et les cellules myéloïdes.
Mots-clés	Corps carotidien, inflammation, électrostimulation, souris, nerf sinuso-carotidien, endotoxémie, glucocorticoïdes, cellules myéloïdes, hypothalamus, médecine bioélectronique

Résumé (de 1700 à 4000 caractères espaces compris)

Les corps carotidiens sont des structures bilatérales situées au niveau de la bifurcation de l'artère carotide. Ils sont capables de détecter divers stimuli physiologiques tels que la concentration de gaz dans le sang et la pression sanguine. Suite à la variation de ces paramètres au-delà d'une valeur consigne, le corps carotidien, *via* un nerf afférent appelé nerf sinuso-carotidien (NSC), déclenche une réponse riposte par des voies nerveuses ou hormonales efférentes. De récentes études ont suggéré l'existence d'une relation entre le système immunitaire et le corps carotidien. Il a en particulier été démontré que le corps carotidien est capable de détecter des effecteurs de l'inflammation tels que les cytokines. En outre, des données *in vivo* démontrent que la résection bilatérale du NSC diminue la survie suite à un choc endotoxémique. Notre étude a porté sur les capacités immunomodulatrices du corps carotidien. Pour mener à bien cette étude, nous avons utilisé une approche d'électrostimulation du NSC plutôt qu'une approche pharmacologique moins spécifique. Nous avons en particulier développé une méthode chirurgicale pour isoler le NSC, implanter des électrodes puis stimuler le nerf. Nous avons pu constater que la stimulation électrique du NSC atténuait l'expression des cytokines pro-inflammatoires induites par injection de lipopolysaccharide (LPS), en particulier le Tumor Necrosis Factor (TNF). Par la suite nous avons pu montrer que l'effet de la stimulation du nerf sinusal de la carotide n'était médié ni par des nerfs sympathiques, ni par des nerfs parasympathiques efférents. En revanche, nous avons découvert que la stimulation du NSC augmentait le taux de corticostérone, une hormone anti-inflammatoire qui active le récepteur des glucocorticoïdes. L'antagoniste du récepteur des glucocorticoïdes, abolit les effets de l'atténuation des cytokines pro-inflammatoires par l'électrostimulation du NSC. De plus, l'utilisation de souris transgéniques ayant des cellules immunitaires myéloïdes dépourvues de récepteurs aux glucocorticoïdes, a montré que l'effet de la stimulation nerveuse du NSC était également perdu indiquant le rôle crucial joué par ces cellules et ce récepteur. Enfin, nous avons pu montrer que la stimulation du NSC conduisait à l'augmentation de l'activité de décharge spontanée des neurones hypothalamiques évoquant un lien entre le NSC et l'axe hypothalamo-hypophysaire. Au final,

ces résultats indiquent que l'électrostimulation du NSC atténue l'inflammation en agissant sur l'hypothalamus qui, en augmentant la sécrétion de glucocorticoïdes, conduit à une inhibition de la sécrétion de cytokines inflammatoires par les cellules myéloïdes. D'un point de vue thérapeutique, nous avons pu mettre en évidence que les souris qui avaient reçu une électrostimulation du NSC avaient un taux de survie plus important après injection d'une dose élevée de LPS. Ces résultats suggèrent que l'électrostimulation du NSC pourrait être un traitement potentiel considéré par la médecine bioélectronique pour les maladies inflammatoires immunomédiées en agissant sur l'axe hypothalamo-hypophysio-surrénalien.

### **In English (mandatory)**

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Thesis title	Study of the role of the carotid sinus nerve in inflammation
Thesis sub-title	Electrostimulation of the carotid sinus nerve in mice attenuates inflammation by acting on the hypothalamic-pituitary-adrenal axis and myeloid cells.
Keywords	carotid body, inflammation, electrostimulation, mouse, carotid sinus nerve, endotoxemia, glucocorticoids, myeloid cells, hypothalamus, bioelectronic medicine

### Abstract (from 1700 to 4000 prints including spaces)

The carotid bodies (CB) are located bilaterally at the carotid artery bifurcation. They are polymodal sensors capable of detecting various physiological stimuli – blood gas concentration and blood pressure. The carotid body, via its innervating nerve – the carotid sinus nerve (CSN), signals to the brain to modulate these physiological stimuli through efferent activity. Recent evidence has suggested that there is a relationship between the immune system and the carotid body. It has been shown that the carotid body detects inflammation and functionally responds. Additionally, there is *in vivo* data, demonstrating that bilateral removal of the CSN decreases survival to endotoxemic shock. We hypothesized that activation of the carotid body would attenuate inflammation. To carry out this study, we electrostimulated the CSN instead of using a non-specific pharmacological approach. A surgical method was developed to isolate the CSN, implant electrodes and then stimulate the nerve. We found that electrical stimulation of CSN attenuated the expression of pro-inflammatory cytokines induced by lipopolysaccharide (LPS) injection, including Tumor Necrosis Factor (TNF). The mechanism by which electrical activation of the CSN attenuated inflammation was unknown. It was investigated if the effect of CSN stimulation was mediated via efferent parasympathetic or sympathetic nerves - it was found that it was not mediated via these nerves. In contrast, it was discovered, that CSN stimulation increased corticosterone – an anti-inflammatory hormone, which activates the glucocorticoid receptor. An antagonist for the glucocorticoid receptor abolished the attenuation of pro-inflammatory cytokines by CSN electrostimulation. This indicated that corticosterone is the mediator of the effect of CSN stimulation. In addition, we found that the stimulation of the CSN led to the increase of the spontaneous discharge activity of the hypothalamic paraventricular nucleus likening the CSN activity to the hypothalamic-pituitary adrenal axis. Using transgenic mice with no glucocorticoid receptor on their myeloid immune cell, it was found that effect of CSN

stimulation was additionally prevented. Finally, we were able to show that the stimulation of the CSN led to the increase of the spontaneous discharge activity of the hypothalamic neurons suggesting a link between the CSN and the hypothalamus-pituitary axis. Overall, these results indicate that electrostimulation of CSN attenuates inflammation by acting on the hypothalamus which, by increasing the secretion of glucocorticoids, leads to an inhibition of the secretion of inflammatory cytokines by myeloid cells. From a clinical perspective, using a high dose of lipopolysaccharide, it was found that mice who had received electrostimulation of the CSN were more likely to survive than control mice. This result is particularly interesting as it demonstrates that electrostimulation of the CSN may be a potential therapeutic in bioelectronic medicine for immune-mediated inflammatory diseases.

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More than anything, here, I just want to say thank you to all the mice and congratulations to anytime you got lucky and bit me.

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Oh, actually, I will thank the tv show Doctor Who for getting me interested in doing a PhD

And yeah I will thank Signalife as well. Cheers.

Also one last time - Nice was nice.

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## Abbreviation list

Acetylcholine	ACh
Adrenocorticotrophic hormone	ACTH
Adrenergic receptor	AR
Adenosine tri-phosphate	ATP
Autonomic nervous system	ANS
Baroreceptor activation therapy	BAT
Bilateral carotid neurotomy	BCN
Blood brain barrier	BBB
Carotid body	CB
Carotid sinus nerve	CSN
Collagen-induced arthritis	CIA
Choline acetyltransferase expressing T-cells	CHAT
Central nervous system	CNS
Cortisol-releasing hormone	CRH
Dendritic cells	DC
Glucocorticoid-glucocorticoid receptor complex	GCR
Glucocorticoid response element	GRE
Glucocorticoid Receptor	GR
Hypothalamic-pituitary adrenal	HPA
Inflammatory bowel disease	IBD
Immune mediated inflammatory disease	IMID
Interferon	IFN
Interleukin	IL
Interleukin-1 receptor	IL-1R
Lipopolysaccharide	LPS
Muscarinic ACh receptor	mAChR
Natural Killer cells	NK
Nicotinic ACh receptor	nAChR
Nucleus tractus solarias	NTS
Pathogen-associated Molecular patterns	PAMPS
Protein Kinase A	PKA
Pro-opiomelanocortin	POMC
Peripheral nervous system	PNS
Parasympathetic nervous system	PSNS
Pattern recognition receptors	PRR
Paraventricular nucleus	PVN
Rostral ventrolateral medulla	RVLM
Suprachiasmatic nucleus	SCN
Suppressor of cytokines signalling	SOCS
Sympathetic nervous system	SNS
Transforming growth factor beta	TGF $\beta$
T-regulatory cell	Treg
Toll-like receptor	TLR
Tumour Necrosis Factor alpha	TNF
TNF receptor	TNFR
Thyroid stimulating hormone	TSH
White blood cell	WBC

## Guide to the introduction

My introduction consists of four primary sections:

1. The Immune System;
2. Regulation of the Immune System;
3. The Carotid Body;
4. And Bioelectronic Medicine.

Within these four primary sections, there are numerous sub-sections. When first viewed, these sections appear quite disparate and while they are linked within the text, I felt some clarification was required.

As seen from the abstract, my investigation focuses primarily on the role of the carotid body and its regulation of the immune system. This, however, is investigated through the mechanics of bioelectronic medicine, which in this context means the use of external electrodes placed onto the carotid sinus nerve (the carotid body nerve) and stimulating with electricity.

Therefore, it was necessary to explore bioelectronic medicine in the introduction to examine its impact on current research and within the clinical. Additionally, it was crucial to give an overview on the immune system – though innate immunity and pro-inflammatory cytokines are the focus – and the carotid body – focusing on its relationship to inflammation.

The section on the regulation of the immune response is divided into two large sub-sections – the hypothalamic-pituitary-adrenal axis and the inflammatory reflex. The reason for this is because they are both non-immune based endogenous mechanisms for regulating inflammation. The inflammatory reflex was the initial basis for how we believed the carotid body would regulate inflammation. We know now that it is actually dependent upon the hypothalamic-pituitary-adrenal axis, so it was necessary to talk about these two mechanisms for attenuating inflammation.

Overall, the integration of all sections becomes clear in the “hypothesis and aims” sections which follows the introduction.

# Introduction

## 1.A.0 – Overview

The immune system is one of the most diverse, complicated and fascinating systems within the body. The Immune system's functional components contain some of the smallest proteins to a body-wide vasculature system. Nearly every disease and disorder the body can be afflicted with has an immune system component to it and with that immune component the hope for a cure. New functional information comes out every day about the immune system and it is still surprising and delighting the people who research it.

The immune system is historically divided into two responses of the same system – the innate immune response and the adaptive immune response. In reality, these systems are very much interwoven and the activity of both is typically required for a healthy immune response. Within the innate and adaptive immune response there is four major roles of the immune system: Detecting a threat; responding to a threat; remembering that threat and regulation of activity. These roles of the immune system will be addressed in the coming sections.

## **1.B.0 – Innate Immune Response**

The components of the innate immune response are typically considered “the first responders” of the immune system because their primary role is the detection and initial response to a threat. To use the classic example: When we cut a finger, this gives external threats, such as bacteria, access to our bodies. There are resident components, cells, of the immune response that will detect this threat and signal to more components of the immune response to aid in the elimination of this threat and resolution of the immune response. These actions take place via the combined activity of cells and cytokines - which will be addressed in sections 1.B.i/ii respectively.

### **1.B.i – Innate Immune Response: Cells**

There are multiple immune cells, universally referred to as white blood cells (WBC) or leukocytes, within the innate immune response with varied roles. These cells can be found in tissues, as resident cells, within the vasculature or residing in organs of the immune system – lymph nodes and the spleen. To understand the cells of the immune system, understanding their lineage is crucial. All immune cells, innate or adaptive, arise initially from pluripotent haematopoietic stem cells found in the bone marrow. The stem cells first divide into common lymphoid and common myeloid progenitors. These progenitors further sub-divide into functional immune cells. Typically, common lymphoid progenitors are associated with cells of the adaptive immune response, except natural killer (NK) cells which are cells of the innate immune response. Cells of the myeloid lineage are all part of the innate immune response. Aside from NK cells, in the innate immune response there is also mast cells, eosinophils, basophils, neutrophils, dendritic cells (DC) and monocytes [82] [Illustration.1].

Each individual cell of the innate immune response can also have varying states of maturity which augment their physiological response, however in brief each cell will be described. Neutrophils, basophils and eosinophils are characterised as granulocytes, due to the presence of granules within their cell bodies. Basophils and eosinophils are believed to be crucial in defence against large parasites - it is also believed mast cells play a similar role. Neutrophils are incredibly abundant, particularly when the innate immune response has been activated and are crucial to the clearance of an infection through various means. NK cells are

particularly effective at clearing viral infections. DCs are capable of activating the adaptive immune response, which will be discussed further in section 1.D.0. [156]. Monocytes, and their derivative – macrophages, will be discussed in detail.

Macrophages migrate during development to tissues and become resident immune cells for that tissue. Additionally, monocytes when travelling to a source of inflamed tissue may differentiate into macrophages [158]. Macrophages are often described as sentinel immune cells, because they are supremely capable at detecting the presence of a threat, either from the environment such as bacteria or damaging tissue such as cancer cells. Macrophages are equipped with multiple sensory molecules known as pattern-recognition receptors (PRR). These PRR detect certain regions well conserved on pathogens – pathogen-associated molecular patterns (PAMP)s, instigating the innate immune response. Perhaps the best categorised of these PRR are the Toll-like receptors (TLR). There are multiple TLR's, some found on the macrophage cellular membrane and others within the cell [Illustration.2]. TLR4 can detect the PAMP lipopolysaccharide (LPS) a crucial component of gram-negative bacteria [2]. Synthetic components of LPS are often used experimentally to instigate the immune response. Once TLR4 recognises LPS, a complex network of signalling molecules on the cell surface and then internal signalling molecules start activating in a cascade, ultimately activating a pro-inflammatory transcription factor. In brief, LPS is bound by LPS-binding protein, which shuttles LPS to CD14 and facilitates the pairing of the two. CD14 performs a similar role for the TLR4 and MD2 receptor complex that recognises LPS and induces internal signalling. There are two internal signalling pathways – MyD88 dependent or independent pathway [Illustration.3]. The MyD88 dependent pathway uses TLR4/MD2 bound MyD88 to subsequently activate IRAK and TRAF signalling proteins to instigate transcription via NF- $\kappa$ B and AP-1. Whereas, the MyD88 independent pathway utilises TRIF and TRAF signalling proteins to activate pro-inflammatory transcription factor NF- $\kappa$ B and IRF3 [95]. NF- $\kappa$ B, as an example, will then translocate into the nucleus of the cell to generate the production of proteins that can recruit and activate other cells of the immune response [158]. These proteins are considered products of the immune response and are called cytokines and chemokines. LPS-induced production of the pro-inflammatory cytokines – TNF, IL-6 and IL-1 $\beta$  will be addressed in the following section (1.B.ii).

An additional response by macrophages upon activation is phagocytosis. The process of phagocytosis is whereby the macrophage, upon sensing a pathogen will encapsulate it within

itself and begin to degrade it. Interestingly, this process of pathogen degradation can augment the cytokine profile of macrophages, as macrophages display remarkable plasticity and can be activated in a pro-inflammatory state as described, but also into an anti-inflammatory state that promotes wound healing and resolution of the immune response [158]. Immune proteins, cytokines, will be discussed in this context of inflammatory states in the next section.

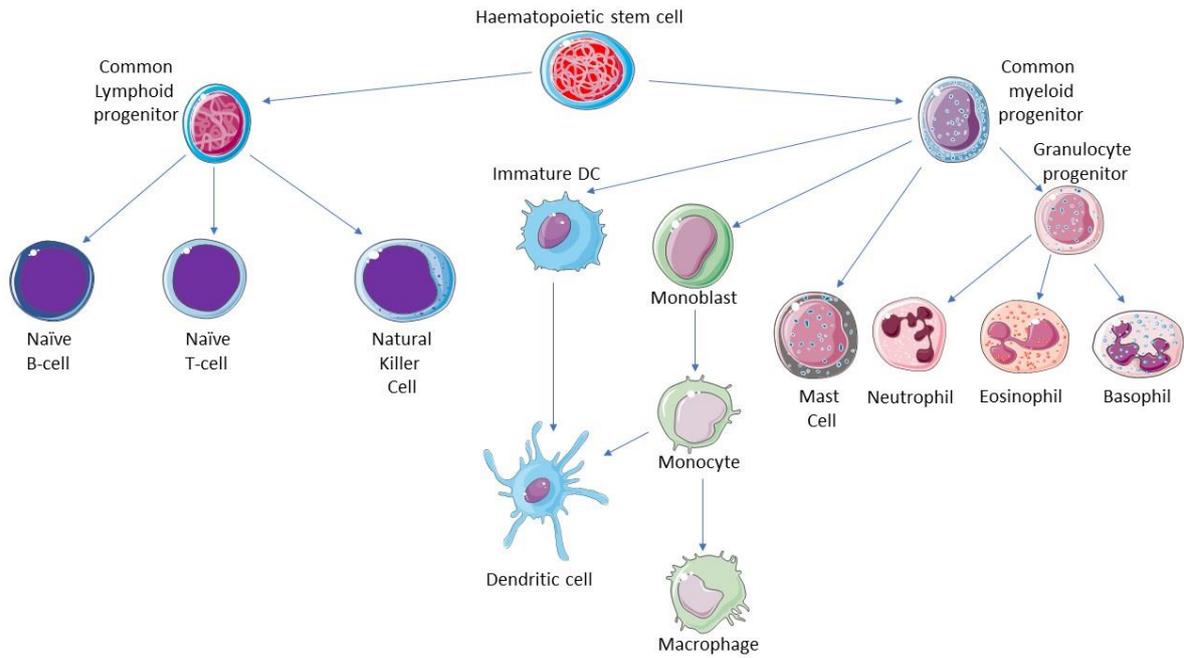
### **1.B.ii – Innate Immune Response: Cytokines**

Cytokines are typically very small proteins involved with the immune response and can signal in an autocrine (self-activation), paracrine (local cell activation) or hormonal fashion (travelling through the vasculature). There are many varieties of cytokines routinely divided into families called interferons (IFN), interleukins (IL) and the tumour-necrosis factor (TNF) family. Within these family groups, cytokines have a wide variety of functions that can be simplified to as pro-inflammatory, anti-inflammatory and chemotactic. When cytokines are considered to have a chemotactic role – the recruitment of additional immune cells – they are labelled as chemokines. One such chemokine is CXCL1, which is produced upon LPS-induced NF- $\kappa$ B activation and recruits' neutrophils to the infected tissue [155]. Anti-inflammatory cytokines, are essentially any cytokine that prevents further recruitment and/or activation of immune cells, occasionally aiding in wound healing. IL-10 is the primary anti-inflammatory cytokine as it is a potent inhibitor of macrophage function. Interestingly, this inhibition of macrophage function can be considered another activation state of macrophages as they still produce further anti-inflammatory cytokines [158]. Pro-inflammatory cytokines are broadly described as cytokines that promote further immune activity. IL-1 $\beta$  is a particularly potent pro-inflammatory cytokine: it is produced in a proto form by NF- $\kappa$ B transcription and requires cleavage by a separate PRR to be operational – NLRP3 [89]. IL-1 $\beta$  activates components of the adaptive immune response, macrophages and induces fever in the host to aid clearance of an infection – the sickness response [155]. Overall, however, cytokines are pleiotropic, meaning their function varies depending on the cell they activate and the presence of other cytokines in the vicinity [155]. IL-6 is an example of cytokine pleiotropy as it cannot be considered wholly a pro-inflammatory cytokine, as it has anti-inflammatory roles too [143]. IL-6 will induce the production of acute-phase proteins from

the liver that are involved in the immune response, but it also augments cells of the adaptive immune response towards an anti-inflammatory phenotype [143].

Another particularly important pro-inflammatory cytokine is TNF $\alpha$  (TNF) and it is routinely used as the gold-standard indicator of inflammation in immunological studies. TNF is produced during LPS-induced TLR4 activation of NF- $\kappa$ B, but it also produced by cells other than macrophages [21]. TNF was initially known as a protein capable of destroying tumours, however it is known now that this is not the sole role of TNF. Overall, TNF is a promotor of inflammation, however one key role is its ability to cause endothelial cells – found in blood vessels – to form looser junctions between themselves [155]. This enables the recruited cells, by chemokines, to leave the blood vessels and enter tissue in a process known as extravasation. It is known that a lethal dose of LPS will kill mice, however if you block TNF, the mice will survive [21]. This indicates that TNF is one of the primary mediators of lethality in an infection and egregious TNF can be dangerous, this will be addressed in section 1.D.0.

Signalling for these pro-inflammatory cytokines is crucial. Immune cells express their receptors – IL-1 $\beta$  binds to IL-1 receptor (IL-1R), IL-6 binds to IL-6 receptor (IL-6R) complexed with GP130 to be functional and TNF binds to TNF receptor (TNFR) 1 and 2. IL-1 $\beta$ -induced activation of IL-1R uses the TRAF signalling pathway to activate downstream NF- $\kappa$ B or other pro-inflammatory transcription factor AP-1. Whereas, IL-6-induced activation of IL-6R will mobilise the JAK-STAT signalling pathway to engender gene expression. Similarly, to IL-1 $\beta$  TNF via TNFR2 will mobilise the TRAF pathway to ultimately activate pro-inflammatory transcriptions factors – NF- $\kappa$ B and AP-1. Additionally, however, TNF can activate TNFR1 to induce apoptosis in the activated cell [155]. These cytokines, however, are not the only pro-inflammatory cytokines critical to the innate immune response - a list summarising these cytokines was adapted from Turner *et al* [155], [Illustration.4].



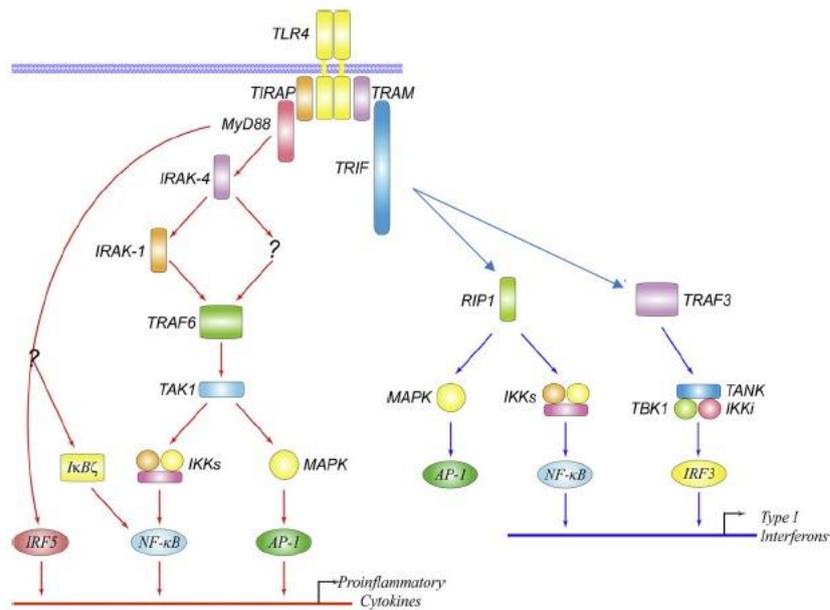
**Illustration 1: Immune cell lineage**

All immune cells are derived from the one haematopoietic stem cell, which can further subdivide into lymphoid or myeloid progenitors. The lymphoid progenitor will give rise to the lymphocytes – B-cells, T-cells and natural killer cells. The myeloid progenitor will give rise to multiple different proto-myeloid cells culminating with – DCs, monocytes, macrophages, mast cells, basophils, eosinophils and neutrophils. Image made using Servier Medical Art.

TLR	PAMP	Example pathogen
TLR1	Triacyl lipopeptides	Mycobacteria
TLR2	Zymosan	Fungus Saccharomyces cerevisiae
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative Bacteria
TLR5	Flagellin	Flagellated Bacteria
TLR6	Zymosan	Fungus Saccharomyces cerevisiae
TLR7	Single-stranded RNA	Viruses
TLR8	Single-stranded RNA	Viruses
TLR9	CpG-DNA	Bacteria

### Illustration 2: TLRs and their PAMPs

A representative table of the main TLR's and a sample of the pathogens and PAMPs that activate the receptors. It should be noted that all TLR's respond to more than one stimulus and the above table is representing primary stimuli for each TLR.



### Illustration 3: TLR signalling

When TLR4 is activated by LPS it will induce a signalling cascade that is dependent or not on MyD88. Various signalling molecules are recruited: MyD88 signalling will predominantly induce the activation of pro-inflammatory transcription factor NF-κB to enhance transcription of pro-inflammatory cytokines; whereas, MyD88 independent signalling activates interferon regulatory factors to induce the expression of interferons – a specific type of pro-inflammatory cytokine. Image adapted from Lu *et al*, 2008.

Cytokines in the immune response				
	Cytokine	Main sources	Target cell	Major function
Interleukins	IL-1	Macrophages, B cells, DCs	B cells, NK cells, T-cells	Pyrogenic, pro-inflammatory, proliferation and differentiation, BM cell proliferation
	IL-2	T cells	Activated T and B cells, NK cells	Proliferation and activation
	IL-3	T cells, NK cells	Stem cells	Hematopoietic precursor proliferation and differentiation
	IL-4	Th cells	B cells, T cells, macrophages	Proliferation of B and cytotoxic T cells, enhances MHC class II expression, stimulates IgG and IgE production
	IL-5	Th cells	Eosinophils, B-cells	Proliferation and maturation, stimulates IgA and IgM production
	IL-6	Th cells, macrophages, fibroblasts	Activated B-cells, plasma cells	Differentiation into plasma cells, IgG production
	IL-7	BM stromal cells, epithelial cells	Stem cells	B and T cell growth factor
	IL-8	Macrophages	Neutrophils	Chemotaxis, pro-inflammatory
	IL-9	T cell	T cell	Growth and proliferation
	IL-10	T cell	B cells, macrophages	Inhibits cytokine production and mononuclear cell function, anti-inflammatory
	IL-11	BM stromal cells	B cells	Differentiation, induces acute phase proteins
	IL-12	T cells	NK cells	Activates NK cells
Tumour necrosis factors	TNF- $\alpha$	Macrophages	Macrophages	Phagocyte cell activation, endotoxic shock
	TNF- $\beta$	Monocytes T-cells	Tumour cells Phagocytes, tumour cells	Tumour cytotoxicity, cachexia Chemotactic, phagocytosis, oncostatic, induces other cytokines
Interferons	IFN- $\alpha$	Leukocytes	Various	Anti-viral
	IFN- $\beta$	Fibroblasts	Various	Anti-viral, anti-proliferative
	IFN- $\gamma$	T-cells	Various	Anti-viral, macrophage activation, increases neutrophil and monocyte function, MHC-I and -II expression on cells

#### Illustration 4: Cytokines

A table representing a sample of the cytokines crucial to the immune response. Representing both pro-inflammatory and anti-inflammatory cytokines. Chemokines are also present (IL-8). This table represents primarily information from humans, however most is transferable to rodents, except notably IL-8. This table is adapted from Turner *et al*, 2014.

## 1.C.0 – Adaptive Immune Response

The adaptive immune response can be considered the “memory” response of the immune system and is primarily concerned with remembering a threat and regulation of response. As outlined previously (section 1.B.i) the lineage of all cells in the adaptive immune response are lymphocytes. The common lymphoid progenitor becomes a B-cell, T-cell or natural killer cell in the bone marrow. B-cells mature in bone marrow, whereas for T-cells it is necessary to travel to the thymus to mature. Once mature, these lymphocytes (not including natural killer cells) reside in the organs of the immune system such as lymph nodes, the spleen and the lymphatic system. The lymphatic system primarily functions as a drainage system for extracellular fluid, however it is also as a vasculature system for WBCs with lymph nodes as the hubs for leukocytes. Mature B and T-cells are often described as naïve, because once activated they differentiate into their functional forms [20, 37].

DCs are at the interface of innate and adaptive immunity by playing a key role in the activation of T-cells. DCs, like macrophages, can phagocytose pathogens and degrade them internally. Once degraded, DCs present a small part of the pathogen on its cell surface – an antigen. Through chemotactic signals, DCs are signalled away from the infected area into the lymphatic system, where they present the antigen to any naïve T-cell they encounter, however only one T-cell will recognise this pathogen and mount the appropriate response [20, 37]. There are two primary subsets of T-cells – CD4+ and CD8+ T-cells. Both subsets of T-cells are activated in the same way by DCs, however, once activated they perform different roles. CD8+ T-cells, once activated, are referred to as cytotoxic T-cells as they seek the antigen that was presented and kill the pathogen infected cell once encountered. CD4+ T-cells are described as the T-helper-cells as they orchestrate and direct a successful response by the immune system to a pathogen. Upon activation, CD4+ T-cells differentiate further into specific types of functional T-cells – Th1, Th2 and Regulatory T-cells (Treg), which are defined by their activities. Th1 cells orchestrate the classically considered pro-inflammatory action of the immune response via production of TNF and IFN- $\gamma$ , which is a potent activator of macrophages. Th2 cells also activate the immune system but are more supportive to the function of B-cells via IL-4 [56]. Treg cells produce immunosuppressive proteins such as IL-10 and are a crucial component of the overall immune response as overt immune activity can lead to damage and immune disorders which will be addressed in the following section 1.D.0.

B-cells, similarly to T-cells, respond to one antigen and once activated undergo clonal expansion, differentiate and start producing antibodies. B-cell antigen activation, however, is a little more complex than T-cell antigen activation and generally requires the aid of specific CD4 T-cells, rather than DCs. The antibodies produced by plasma cells selectively bind to their antigen, perhaps a small section of a bacterial cell wall and once bound, it enables multiple components of the immune response to better recognise pathogens and ultimately remove them. A fraction of these activated B-cells will become memory B-cells and remember this antigen to produce a more rapid response, should the immune system re-encounter the pathogen [20, 37].

### 1.D.0 – Dysregulated Immune Response

The immune system is designed to respond to an infection, react strongly and then return to homeostasis. This design is evident by the short life-span of neutrophils [106], the plasticity of macrophages, switching to a state of near quiescence [158], and the role of Tregs [81]. Tregs are distinguished from other T-cell subsets by their transcription factor FOXP3 and their keen ability to suppress – pro-inflammatory cytokines, cytotoxicity, metabolic disruption and DC function [37]. Tregs produce copious quantities of anti-inflammatory cytokine IL-10 and transforming growth factor  $\beta$  (TGF $\beta$ ). TGF $\beta$  can induce cell differentiation and proliferation: together these cytokines are crucial to the role of Tregs and their loss can exacerbate already established pathologies [37]. Interestingly, CD4<sup>+</sup> T-cells can be induced to become a subset of Tregs and perform a similar role to natural Tregs [144]. Ultimately, the actions of Tregs and their production of anti-inflammatory cytokines induce quiescence in additional immune cells and encourage the resolution of inflammation.

It is crucial that the immune system can regulate its reactions, auto-immune disorders being a clear example of when the immune system fails. Auto-immune disorders are characterised by T and/or B-cells recognising a self-antigen and consequently mounting an immune response against that self-antigen. Myasthenia gravis is an example of an auto-immune disorder where antibodies are made against a component of neuro-muscular activity, which consequently, over time, prevents patients from moving their muscles and ultimately causes death. Alternatively, the immune system can fail by its inability to control and stop an immune reaction leading to chronic inflammation. Immune-mediated inflammatory diseases (IMID)s are defined by dysregulation of the immune response and frequently have continuously high levels of pro-inflammatory cytokines. Two examples of IMIDs are osteoarthritis and inflammatory bowel disease (IBD). Osteoarthritis is a disorder distinct of rheumatoid arthritis – which is an autoimmune disorder, however phenotypically they are similar with degradation of cartilage between joints. Mild symptoms of osteoarthritis are pain and swelling in joints, however this can become near insufferable and patients can have loss of movement in their joints. TNF is the crucial mediator of this disorder, due to chronically high levels. Blocking TNF can ameliorate osteoarthritis and improve symptoms; currently anti-TNF blocking drugs are used to treat osteoarthritis [21]. IBD, or more specifically, Chron's disease is an IMID affecting the intestines that is associated with abdominal pain and

diarrhoea. The exact pathogenesis of IBD is unknown, however, it has been shown that chronic inflammation can induce a Chron's disease-like disorder in mice. Furthermore, giving patients infliximab, an anti-TNF antibody treatment, can alleviate the symptoms of Chron's disease [21]. It is evident from these disorders that homeostasis is required – too much regulation of activity can result in the development of auto-immune disorders and too little regulation can result in the development of IMIDs. A final example of an immune system disorder due to dysregulation is sepsis, which is a catastrophic failure by the immune system at regulation. Sepsis is defined by a life-threatening infection coupled with organ dysfunction. During the early stage of sepsis, there is a massive pro-inflammatory response, while in the later stage of sepsis there is considerable immunosuppression. Early or late sepsis are both potentially deadly. The immune system tries to correct the overt inflammatory reaction but overcompensates and leaves the patient at risk of a rebound infection [68]. Sepsis is a complicated disorder and researchers are still searching for a reliable means of treatment. Overall, regulation of the immune response prevents chronic inflammation and the development of IMID's, which is crucial to maintain a healthy individual.

## **1.E.0 – Summary**

In the previous sections the primary roles of the immune system – detect, respond to, remember a pathogen and regulation – have been described. It is now evident that resident tissue macrophages will detect pathogens, release chemokines to draw in additional cells, such as DCs, of the innate immune system. DCs will then in turn, phagocytose a pathogen and present the antigen on its cell surface to activate T-cells and ultimately B-cells to aid in the clearance of the infection. Additionally, it is apparent now that the resolution of this inflammatory response is crucial. If the immune system remains unchecked, there can be chronic inflammation, which may lead to IMID's. These previous sections have described regulation of the immune system as if the immune system was entirely self-governing – this is not true. There are two main non-immune based endogenous mechanisms to regulate inflammation – the hypothalamic-pituitary adrenal (HPA)-axis and the inflammatory reflex, respectively acting via hormones and the nervous system. In the following section, these two mechanisms will be discussed and described in full.

## 2.A.0 – Hormones and nerves introduction

To pass information from one distant area of the body to another there needs to be communication. The two predominant means of distant communication in the body are via the endocrine system and the nervous system [Illustration.5].

The Endocrine system comprises a disparate conglomerate of hormone producing organs and glandular tissue. These hormones travel through the vasculature to distant endocrine organs, glandular tissue or act almost ubiquitously in the body. Hormones are predominantly either proteins or steroids – a derivative of cholesterol. Hormones can be further divided into hydrophobic and lipophilic groups which have their receptors respectively on the surface of a cell or internally. Typically, it can take minutes for hormones to reach their target area, and the desired effect can last up to days. The endocrine system as a whole is responsible for a myriad of functions within the body: Regulating reproduction; growth and development; sleep and the stress response. It can be said that the hypothalamus, an area of the central nervous system (CNS), is the primary controller of the endocrine system and this will be discussed further in sections 2.B.i/ii. In brief, the hypothalamus releases hormones typically in a circadian manner following a day-night cycle, however, separate hormones from the periphery can influence the release of their progenitor hormones. Typically, the neurons of the hypothalamus secrete synthesising hormones onto the pituitary gland – a peripheral zone of the hypothalamus responsible for hormone release into the periphery. An example of this is thyrotropin-releasing hormone released from hypothalamic neurons to activate the pituitary gland to secrete thyroid stimulating hormone (TSH). TSH will then activate the thyroid gland to produce thyroid hormones – thyroxine and triiodothyronine. These two thyroid hormones are crucial to the regulation of metabolism throughout the body, additionally they are essential during periods of development [84]. This is just one example of the crucial roles the endocrine system performs in the body.

The nervous system comprises the CNS and a network of peripheral nerves throughout the body known as the peripheral nervous system (PNS). These peripheral nerves are divided into the somatic nervous system and autonomic nervous system (ANS). The somatic nervous system is the domain of voluntary movement and these nerves are used for controlling the musco-skeletal system. The ANS is subtler and ultimately more complex - these are the nerves that control non-voluntary actions, such as continuous lowering of the heart rate via the vagus

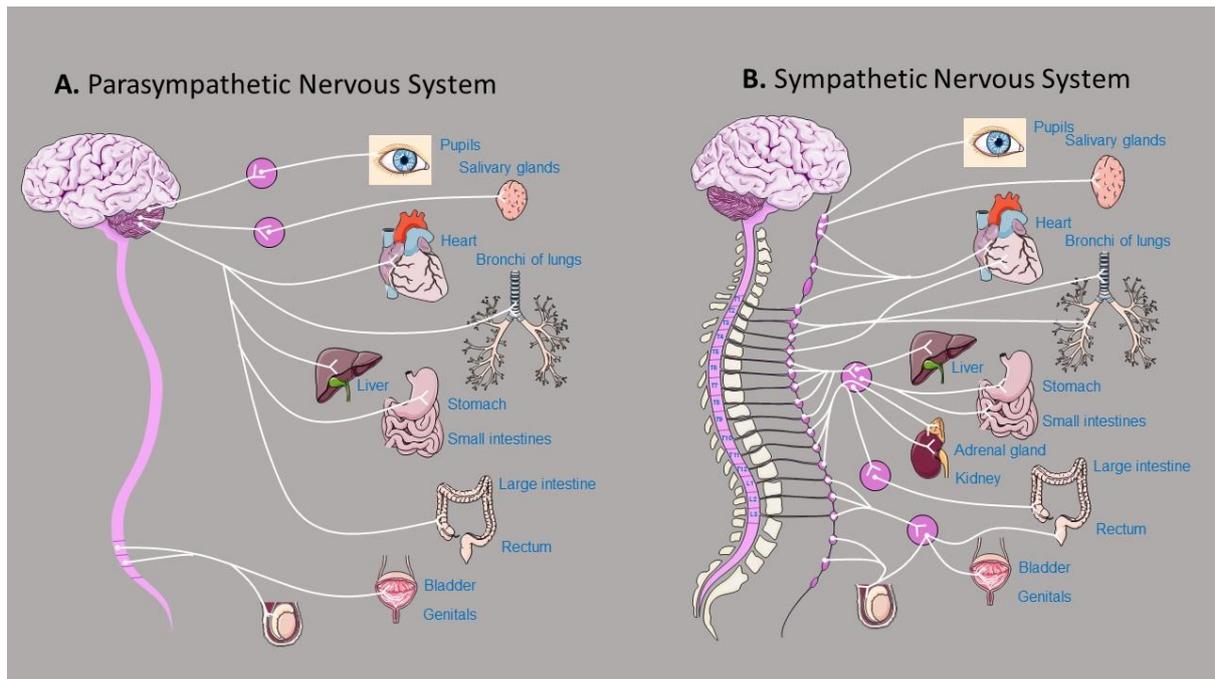
nerve, which will be discussed in detail in section 2.C.i. The ANS can be further sub-divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PSNS) [Illustration.6]. The SNS and PSNS are so named because respectively they rely on Norepinephrine or acetylcholine (ACh) as their primary. Frequently nerves of the SNS using norepinephrine are called catecholaminergic and nerves of the PSNS using ACh are called cholinergic. PSNS signalling via ACh generally activates either the nicotinic ACh receptor (nAChR) or the muscarinic ACh receptor (mAChR), whereas SNS signalling is generally via norepinephrine acting on the adrenergic receptors (AR) –  $\alpha$  or  $\beta$ AR. Traditionally, the PSNS and the SNS can be said to control “rest and digest” and “fight and flight” responses respectively [Illustration.6]. In recent years, however, the roles of the PSNS and SNS have been determined to be more diverse and that these old divisions may not be wholly accurate. In this investigation, nerves will be regarded individually based on their function and efferent nerves will be addressed in greater detail in section 2.C.iii. The nervous system is more rapid than the endocrine system and generally its effects will be induced within seconds and be partially transient.

It is worth noting, however, the nerves described here so far are efferent nerves – nerves that carry information to the periphery. Alternatively, afferent nerves carry information to the CNS and are crucial for processing our sense of the world. It is possible for afferent nerves to be distinct sources from efferent nerves or run in the same nerve bundle as efferent nerves, but information going in opposite directions. Afferent nerves in the context of the vagus nerve will be addressed in greater detail in section 2.C.ii.

	Endocrine System	Nervous System
Functional units	Hormone secreting cell -> Glandular tissue	Neurons -> Nerves -> CNS
Transmission - via	Hormones	Electrical impulse
Pathway - via	The cardiovascular system	Nervous tissue
Response time	Slow - Minutes	Fast - Seconds
Duration of effect	Long-term	Transient
Effect location	Disparate glandular tissue or whole body	Whole body, but contiguous to nerve end

**Illustration 5: Nervous and endocrine systems**

A table demonstrating the primary differences between the nervous and endocrine systems as a means of communication within the body.



**Illustration 6: PSNS and SNS**

**A.** The parasympathetic nervous system typically arises from cranial nerves and travels to numerous locations in the body to perform “rest and digest” responses. **B.** The sympathetic nervous system primarily arises from the spinal cord and synapses with one or more ganglions before arriving at its target location to induce “fight or flight” responses. Images taken from Servier Medical Art.

## **2.B.0 – The Hypothalamic-Pituitary Adrenal Axis Overview**

The Hypothalamic-Pituitary Adrenal (HPA) Axis is the name of the functional relationship between the hypothalamus, the key regulatory organ of the endocrine system, and the adrenal glands – hormone-producing tissue found bilaterally above the kidneys. One outcome of this system is the controlled production and release of glucocorticoids: a class of hormones that when activating their receptor, glucocorticoid receptor (GR), induce a myriad of effects in the body [74]. The HPA axis plays a major role in multiple systems: the cardiovascular system; metabolism; the nervous system and during neonatal development for lung maturation. Of particular interest to this investigation is the effect that the HPA axis has on the immune system and will be addressed in section 2.B.iii. Bioactive Endogenous Glucocorticoids – corticosterone in mice and cortisol in humans – acutely attenuate the immune response and versions of glucocorticoids have been used to treat inflammatory conditions for 70 years [74]. Considering the potent effect of glucocorticoids, it is no surprise that externally manufactured and exogenous glucocorticoids – prednisolone, dexamethasone and more – are widely used in modern medicine today [118]. This will be discussed at length in section 2.B.iv. The HPA-axis is a key regulator of immune function and will be discussed in full in the following sub-sections.

### **2.B.i – The HPA axis: Pathway**

As stated in the previous section, the HPA axis consists of the hypothalamus signalling to the adrenal glands to produce and/or release glucocorticoid. In reality, the process is more complicated with multiple other factors playing a crucial role in the HPA axis. To summarise: the hypothalamus releases cortisol releasing hormone (CRH), which acts on the anterior pituitary to release adrenocorticotrophic hormone (ACTH) and then goes through the vasculature to the adrenal glands and releases glucocorticoid [Illustration.7]. This pathway will be discussed in detail here.

The hypothalamus is a crucial component of the neuroendocrine system located at the base of the CNS and is only partially protected by the blood-brain barrier (BBB) [17], which is used to protect the CNS from peripheral stimuli. The overall architecture of the hypothalamus is the central out-growth of the CNS with a connecting stalk to the bulb of the pituitary gland –

anterior and posterior. The anterior and posterior pituitary glands are important neuroendocrine glands with a variety of roles and responsibilities for hormone production throughout the body. Within the hypothalamus, there is a topographically discrete region called the paraventricular nucleus (PVN) [154]. The PVN consists of neurosecretory regions – the magnocellular and parvocellular PVN. Both the magnocellular and parvocellular PVN have primary roles, however there is some overlap between their functions [154]. The magnocellular PVN primarily produces vasopressin, a hormone that can induce constriction in arterioles of the vasculature and encourages water reabsorption in the kidneys. Whereas, the parvocellular PVN is responsible for producing and releasing CRH. CRH is released at the end of the parvocellular PVN neurons into the hypophyseal portal vein system at the base of the median eminence. This portal vein system allows for release of CRH into capillaries and then direct transfer, via portal vessels, into the capillaries of the anterior pituitary [154]. Within the anterior pituitary, CRH will bind to its receptor on the surface of corticotropes, which will induce the synthesis and release of Pro-opiomelanocortin (POMC) – the precursor of ACTH – and release of ACTH [126].

The adrenal glands are located bilaterally above the kidneys and can be divided into adrenal medulla and the adrenal cortex. The adrenal medulla is a modified tissue of the SNS and can produce the typical neurotransmitters of the SNS – epinephrine and norepinephrine, which are released into the blood and not, strictly, used a neurotransmitter. Whereas, the adrenal cortex is glandular tissue and is divided into three functionally distinct zones: zona glomerulosa to produce mineralocorticoids; zona reticularis to produce sex hormones and zona fasciculata to synthesise glucocorticoids. ACTH will bind to its receptor, mineralocorticoid receptor type 2, in the cells of the zona fasciculata, which induces downstream signalling, leading to the activation of cAMP-dependent protein kinase A (PKA). PKA, once activated, can regulate free cholesterol to aid in the production of steroid hormones and ultimately aid in the release of glucocorticoids [52].

Glucocorticoid will then be released into the vasculature to search for the GR to exert its myriad of effects, which will be discussed in detail in section 2.B.iii. One particularly interesting aspect of the HPA axis is its self-regulation: It functions as a closed feedback loop. There is GR in the PVN and anterior pituitary, which, when activated by glucocorticoids, will block the synthesis and secretion of CRH and ACTH respectively [52]. This control of the CNS directly by glucocorticoids is partly because of their nature, but also because the

hypothalamus is partially unprotected by the BBB. This allows multiple types of peripheral stimuli to influence the activity of the hypothalamus and will be addressed in the following section.

### **2.B.ii – The HPA axis: Instigating stimulus for the hypothalamus**

The hypothalamus is in a somewhat unique position in the CNS, in that its activity can be regulated by the CNS, via nerves, or the periphery, via hormones and cytokines. The HPA axis, hypothalamic activity and ultimate glucocorticoid release, is regulated in a circadian manner. In humans, with a regular day-night routine, there is low glucocorticoid concentration in the blood by late evening and it rebounds after midnight to increase until peaking early morning [118]. The opposite is true in mice however, with their lowest levels during our morning. There is a region of the hypothalamus called the suprachiasmatic nucleus (SCN), which is responsible for the diurnal release of glucocorticoids. The SCN can track time autonomously but in order to function optimally it receives direct retinal innervation [62]. Additionally, the brainstem can send inputs to the hypothalamus to regulate activity [154]. These are the primary means by which the CNS regulates the hypothalamus, peripheral stimuli influencing the hypothalamus will now be discussed.

In the literature it has been well described that a variety of peripheral stimuli can affect the activity of the hypothalamus [154]. The focus will be the specific regulation of CRH and ACTH in response to cytokines. Moreover, as multiple cytokine receptors and cytokines can have an influencing effect on CRH/ACTH secretion, the focus will be on IL-6, TNF and IL-1 $\beta$ . Within the hypothalamus there is expression of the mRNA of IL-6 and IL-6R [51] and there are binding sites for IL-6 in the anterior pituitary [116]. Furthermore, IL-6 expression is enhanced in both the hypothalamus and the pituitary following stimulation by LPS [90]. It has been demonstrated that IL-6 will increase the release of ACTH *in vivo* in rats [97], this increase remaining evident up to 24 hours later with the concentration of glucocorticoid also enhanced compared to control stimuli [61]. Interestingly, however, it is not indicative that IL-6 will increase the mRNA of CRH or POMC [61]. This result, perhaps indicates that IL-6 acting on its receptor, does not induce the production of CRH or ACTH, but only affects the release of already stored ACTH. The receptor for TNF has been discovered in two cell lines representing the pituitary [80] and TNF expression is found in the hypothalamus and pituitary gland *in vivo*

after LPS injection [90]. In rats it has been demonstrated that TNF will increase the concentration of both ACTH and glucocorticoid in the blood. Furthermore, TNF will increase the secretion of CRH from primary hypothalamus cells tested *in vitro* and the increase of ACTH and glucocorticoid is blocked by CRH anti-serum [8]. This result indicates that TNF is acting primarily on the parvocellular PVN cells to release CRH, rather than directly on the pituitary. IL-1R was discovered in the murine pituitary gland [119] and LPS will increase the presence of IL-1 $\beta$  in the hypothalamus and pituitary [90]. Moreover, IL-1 $\beta$  can directly increase the mRNA of CRH and ACTH, as well as the secretion of ACTH and glucocorticoid even up to 24 hours after initial IL-1 $\beta$  injection into rats [61].

The relationship between IL-1 $\beta$  and the hypothalamus is particularly interesting, because IL-1 $\beta$  is one of the primary cytokines responsible for fever induction and the hypothalamus regulates temperature in the body. Researchers have investigated this relationship from multiple angles and this effect was crucial to the discovery of the inflammatory reflex, which will be discussed in section 2.C.ii. It is worth stating that there is a wealth of literature on cytokines affecting HPA activity and what is presented is only representative of the whole, while conflicting data can be found, it is well established that cytokines play a large role in the regulation of HPA activity [154].

### **2.B.iii – Glucocorticoids**

Glucocorticoid is a steroid hormone that binds to the nuclear GR. The GR is almost ubiquitously expressed in the body; however, the receptor has multiple isoforms – GR $\alpha$ , GR $\beta$ , GR $\gamma$ , GR-A and GR-P. Furthermore, within these isoforms of GR there are further subdivisions. Each isoform plays a functionally different role, but in this investigation, unless otherwise stated, GR will be considered as a whole and weaker or negative regulation of glucocorticoids by different GR isoforms will not be focused upon [74]. When describing the functional signalling of glucocorticoids GR $\alpha$  is used as the prime example as it is the main mediator of glucocorticoids effects [74]. How glucocorticoid via GR regulates inflammation will be the focus of this investigation with a brief examination of general GR activity.

The importance of GR signalling is particularly evident in the two glucocorticoid based disorders – Cushing's syndrome and Addison's disease. Cushing's syndrome is marked by increased cortisol levels in the blood, potentially because of continuous increased production

of CRH or ACTH. The phenotypical symptoms of Cushing's syndrome are obesity, high blood pressure, impaired immunological function and moon face [74]. Whereas, Addison's disease is characterised by chronically low levels of cortisol, generally because the adrenal glands are functioning sub-optimally. Addison's disease is characterised by weight loss, low blood pressure, nausea, diarrhoea and occasionally the development of auto-immune disorders [74]. It is evident that glucocorticoid signalling requires balance in the body and its dysregulation can cause damage beyond the immune system.

Glucocorticoids and the immune system have a complicated relationship that cannot be stated as just a suppressor of immune activity. The impact of GR activation varies from cell to cell and even cell maturity [32]. The focus in this investigation will be to examine the anti-inflammatory role glucocorticoids impart on macrophages, however in brief additional effects will be described. T-cells require GR to undergo successful clonal expansion and survival [23] and, additionally, there is a controversial role, as yet not wholly elucidated, for GR in T-cell maturation in the thymus [32].

Glucocorticoids activating the GR, found internally in the cytoplasm of a cell, can attenuate inflammation in a genomic and non-genomic fashion. The genomic method for attenuation of inflammation is via transcription of anti-inflammatory proteins. Whereas, the non-genomic methods are mRNA destabilisation, post-transcriptional modifications to proteins or interference with other transcriptional factors binding to DNA [112, 7]. The glucocorticoid-GR complex (GCR) can bind to AP-1, a transcription factor involved in the pro-inflammatory immune response and prevent it from initiating transcription [24]. Additionally, the GCR can block the activity of NF- $\kappa$ B by binding to it [131]. This blocking of typical transcription factors for pro-inflammatory cytokines is interesting because of its speed which highlights the potent anti-inflammatory effect of GCR, which in-turn is further compounded by its genomic activity. Glucocorticoid once bound to the GR can translocate to the nucleus and depending on the glucocorticoid response element (GRE) available different genomic activity will take place. GRE's can have a positive or negative role. Typically, positive GRE's will perform gene transcription, whereas negative GRE's will block it [112]. Positive GRE signalling can be further sub-divided into composite, tethering and simple. Tethering and composite GRE responses can either enhance or block the activity of additional transcription factors [74]. Whereas positive GRE response can increase the expression of powerful anti-inflammatory cytokine IL-10, I $\kappa$ B $\alpha$  – an inhibitor of NF- $\kappa$ B and suppressor of cytokine signalling (SOCS) proteins which

can block the signalling molecules of multiple pro-inflammatory cytokines - as well as additional anti-inflammatory proteins [7].

The anti-inflammatory effect of the GCR cannot be overstated, one key experiment to demonstrate its physiological effect on myeloid cells was seen using Lysm-Cre:GR<sup>fl/fl</sup> mice – mice with no GR in their myeloid cells. A lethal dose of LPS was administered to Lysm-Cre:GR<sup>fl/fl</sup> mice and their GR<sup>fl/fl</sup> negative littermates which demonstrated that the mice lacking GR had dramatically reduced survival in comparison to their littermate controls. Furthermore, the Lysm-Cre:GR<sup>fl/fl</sup> mice had significantly higher levels of pro-inflammatory cytokines such as TNF [13]. The varied and potent anti-inflammatory role of endogenous glucocorticoids has now been outlined: exogenous glucocorticoids produce similar results, though in diverse capacities. In the following section exogenous glucocorticoid use in medicine will be discussed.

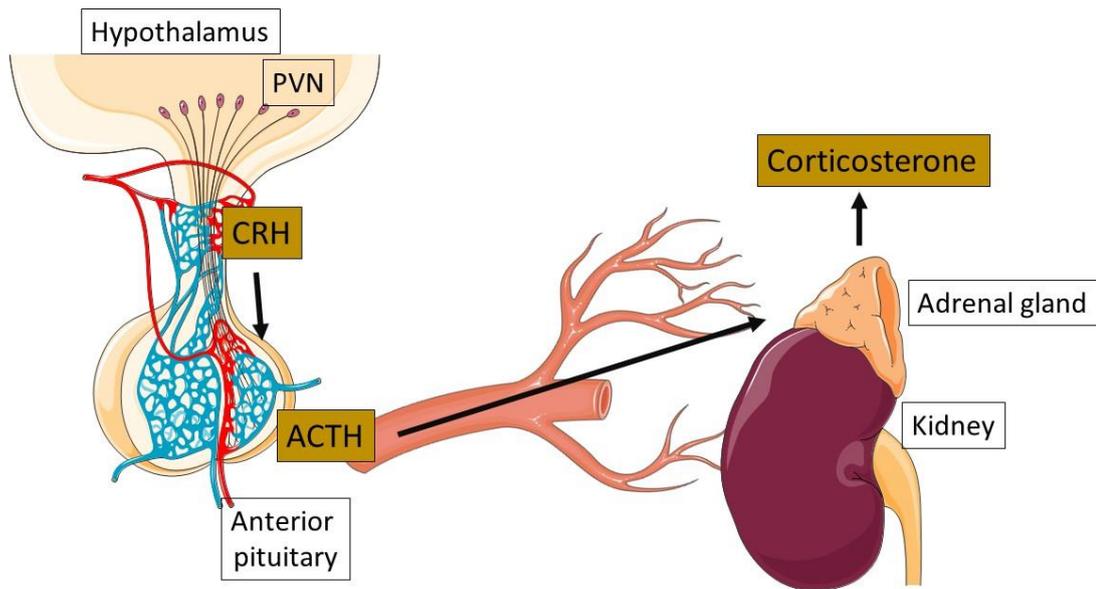
#### **2.B.iv – Glucocorticoids in Medicine**

Glucocorticoids have been in use since the late 1940's in medicine: Today the use of glucocorticoids is one of the most widely prescribed drugs. Depending on the desired treatment, different forms of glucocorticoids can be used – hydrocortisone, prednisolone and dexamethasone. Hydrocortisone is the closest synthetic to human cortisol in activation potency and is considered low potency with 20 mg and with a half-life of 8-12 hours. Prednisolone is considered medium strength as its potency is 4 times greater than that of hydrocortisone at a 5 mg dose and a half-life of 12-36 hours. Whereas, dexamethasone is considered high potency as its anti-inflammatory activity is 30 times stronger than that of hydrocortisone at a dose of 0.75 mg and has a long half-life of 36-72 hours [118]. These are just examples of the many forms of synthetic glucocorticoids which can be for short- or long-term use. Additionally, glucocorticoids can be administered orally, topically, inhaled or injected intra-articularly. While, synthetic glucocorticoids are best known as medication against the immune response, there are some strictly non-immune related disorders treated by glucocorticoids. Sufferers of Addison's disease generally require continuous administration of synthetic glucocorticoids such as hydrocortisone. Ambulatory surgery, or day surgery, is frequently complicated by post-operative and post-discharge nausea and vomiting, for which orally administered dexamethasone can give a sense of well-being, stimulate appetite and

reduce the occurrence of nausea or vomiting [147]. Routinely, glucocorticoids are used for their anti-inflammatory properties after surgeries and particularly for solid-organ transplants [96]. Furthermore, glucocorticoids are used for a large range of inflammatory disorders – sudden severe auto-immune disorder, respiratory infections and disorders, inflammatory skin conditions, but also IMID's such as non-infectious colitis and arthritis [150].

The benefit of glucocorticoids is undoubtable, however there are risks associated with long-term use. There is considerable evidence for adverse effects due to long-term high-dose use of glucocorticoids [118], but even low-dose medium-term (>60 days) usage of glucocorticoids can cause adverse effects. In a study based in the United States with 2 446 active participants, 90% of the participants reported an adverse effect, with 55% stating it was bothersome. The predominant adverse effect was weight gain – 70% of all participants reported this outcome, but skin bruising and acne were also commonly reported. More serious conditions such as cataracts and fractures were also reported in a minority of the participants [35]. Long-term, high dose glucocorticoid use is a risk-factor for osteoporosis, T2D, hypertension, infection and iatrogenic Cushing's syndrome [118]. Iatrogenic Cushing's syndrome is an artificial or induced form of Cushing's syndrome due to high-dose glucocorticoid treatment, the symptoms of which are like that of the physiological Cushing's syndrome, but without the underlying pathology. Alternatively, there is also a risk of the opposite – iatrogenic adrenal insufficiency. Within the hypothalamus, as discussed, there is GR which glucocorticoids can bind to with the same efficiency as their target area. This activation in the hypothalamus performs the role of activating the negative feedback loop and prevents the production of CRH and ACTH, ultimately not producing natural glucocorticoids. This is a concern for patients who are going off long-term high dose usage of synthetic glucocorticoids, as it is possible a reset in the basal levels of cortisol has occurred [118]. Glucocorticoid administration can additionally be a risk factor for the immune system [59], even generating apoptosis in nearly all cells of the immune system in certain conditions [59, 173].

Overall the HPA axis and glucocorticoids are invaluable suppressors of the immune response, both physiologically and pharmacologically.



**Illustration 7: The HPA axis**

The HPA-axis consists of neurons of the PVN, found in the hypothalamus will release CRH into the hypophyseal portal vein system. There, released CRH will immediately arrive at the anterior pituitary to activate ACTH-secreting cells. Subsequently, ACTH is released into the blood and travels to the adrenal glands to induce the release of corticosterone. Corticosterone will then induce its myriad of effects on the body and potentially feedback on the hypothalamus and control its own regulation.

## **2.C.0 – The Inflammatory reflex overview**

It is widely known that there is some relationship between the immune system and the nervous system, other than the activity of the hypothalamus, as immune cells have functional receptors for neurotransmitters. Sympathetic activity, via NE, in particular has been known for decades to regulate immune functions – generating a shift from Th1 to Th2 T-cells and discouraging pro-inflammatory activities [43]. The true control the PNS wields over the immune system with the inflammatory reflex was not yet known. It was less than 20 years ago that Kevin Tracey published the initial result that demonstrated the existence of the inflammatory reflex [152] and his team has been involved in practically every major result in this field since [3]. Prior to Tracey's work on the inflammatory reflex, there was evidence of this connection between nerves and the immune system but it had not been amalgamated. In this investigation, due to the relatively recent nature of these discoveries, the inflammatory reflex will be outlined in a historical fashion in sections 2.C.ii/iii.

In brief the inflammatory reflex, or the cholinergic anti-inflammatory reflex, is characterised by peripheral vagal afferent nerves detecting inflammation and sending this information to the CNS. Consequently, the CNS activates vagal efferent nerves, which synapse at the celiac ganglion to activate adrenergic nerves to the spleen, which in turn releases norepinephrine and ultimately attenuates inflammation [3]. The truly interesting aspect of the inflammatory reflex is that while it is a physiological reflex, it can be induced by electrical activation. In this section of the investigation, the inflammatory reflex will be regarded as a pure physiological reflex and, though electrostimulation, will be mentioned as means of activating the reflex. The evoked potential of electrical activation in the vagus will not be discussed until the bioelectronic medicine section 4.B.i-ii.

### **2.C.i – The Inflammatory Reflex: Vagus**

The vagus nerve is the tenth of twelve cranial nerves, a group of ANS nerves that arise directly from the brainstem. It has an important role in multiple systems aside from the inflammatory reflex. Anatomically, the vagus can be found bilaterally running parallel to the trachea at the cervical level of intervention and additionally, sub-diaphragmatically, it runs parallel to the oesophagus. Furthermore, the vagus nerve splits and separates into various smaller nerves

and innervates most major organs – the heart, lungs, liver, stomach, intestines, pancreas [11], non-organ ganglions – like the coeliac ganglion [125] and potentially, via the coeliac ganglion, the spleen [3]. Continuous vagal efferent stimulation inhibits the beat rate of the heart, which physiologically runs around 100 beats per minute. The efferent parasympathetic tone from the vagus keeps the heart rate closer to 60 beats per minute and this is perhaps the best characterised activity of vagal efferent fibres. It should be noted however that up to 90% of fibres within the vagus are afferent [11]. As widely dispersed as the vagus is throughout the body, at most nerve endings there is a sensor for the local environment, which will detect stimuli and induce afferent activity. In the lungs, there are neuroepithelial bodies which can function as mechanosensors for the CNS, which signal through vagal afferent fibres [91]. These mechanosensors can inform the CNS on breath rate and potentially induce an adjustment in the response. Additionally, there is a specific form of mechanoreceptors – baroreceptors, for measuring blood pressure – located in the aortic arch, which signal via afferent vagal fibres. Furthermore, there is also some evidence of chemoreceptors located in the rat aortic arch, though there is no evidence of what they are specifically detecting [26]. These diffuse vagal afferents re-combine with the core vagus nerves sub-diaphragmatically or at the cervical level, before the vagus arrives at the brainstem of the CNS and more specifically the nucleus tractus solariis (NTS) [47].

Interestingly, at the terminal of vagal afferents there are occasionally small tissues known as paraganglia. Paraganglia are non-neuronal cells derived from neural tissue during development. Vagal paraganglia are located densely at the cervical level, near the liver and comparatively sparsely near the pancreas. Paraganglia act as sensors of their environment and induce activity in the vagal afferent fibres [11]. Their specific role in regard to the inflammatory reflex will be outlined in the following section.

### **2.C.ii – The Inflammatory Reflex – Afferent sensors**

In this section, afferent vagal paraganglia will be discussed from a historical perspective. During the mid-1990's, researchers were particularly interested in how IL-1 $\beta$  could induce the fever response – there were two hypotheses. The first was that IL-1 $\beta$  travelled to the CNS and acted on receptors there – particularly in the hypothalamus [12], in a similar manner to as outlined in section 2.B.ii. The second hypothesis was that IL-1 $\beta$  was being detected in the

periphery by vagal nerves. In one study, it was shown that IL-1 $\beta$  will induce the fever response. The fever response, however, could be abolished by previous sub-diaphragmatic vagotomy (bilateral removal of the vagus nerve) in rats. More specifically, this result was replicable with hepatic branch vagotomy [165] – an area rich with vagal paraganglia. Therefore, these results indicate that IL-1 $\beta$  is acting on peripheral nerves to induce fever. This study launched the idea of peripheral nerves communicating inflammation to the brain.

Continuing their research, the group next assessed if IL-1 $\beta$  administered to the hepatic portal vein, a major blood vessel in the liver, induced activity in the afferent hepatic branch of the vagus. It was discovered, in a dose-dependent manner, that IL-1 $\beta$  will generate increased impulses per second in the hepatic branch nerve up to 60 minutes after initial injection [113]. This study provides strong evidence that the immune system can activate peripheral nerves, however it was still uncertain how this was happening. In a follow up study, the group assessed cryostat sections of the vagus nerve – whole, abdominal and liver. A staining was performed for IL-1R and it was discovered in multiple sections of the vagus nerve, particularly clustered in vagal paraganglia. This discovery lent further credence to their hypothesis that peripheral nerves sense IL-1 $\beta$  [55].

External groups have challenged their assertions [6] due to new evidence demonstrating that sub-diaphragmatic vagotomy cannot prevent the fever response in rats to IL-1 $\beta$  or LPS [36]. A separate study investigated an important paraganglion of the vagus nerve – the nodose ganglion, which is located at the cervical level and is believed to coalesce sensory information from the thoracic cavity [91]. They discovered that the nodose ganglion has functional IL-1R, this was determined by a cFos study, activation of cFos is indicative of nerve activation. Moreover, this study investigated the gastric vagal nerve and detected increased activity in response to intravenous injection of IL-1 $\beta$  [42]. Additionally, in a separate study it was demonstrated that the nodose ganglion has TLR4 mRNA and protein expression [67] however it was not investigated if the TLR4 was functional.

The original authors [165, 113, 55] expanded their investigation of how vagal afferents respond to IL-1 $\beta$ . It was discovered that immune-like cells located in tissues contiguous with vagal afferent nerves display positive staining for IL-1 $\beta$  in response to LPS. The authors proposed that these immune-like cells were adjoining vagal afferents to rapidly release IL-1 $\beta$  in the vicinity of the nerves and subsequently induce nervous activity [54].

The superior laryngeal nerve is a cervical branch of the vagus nerve: one group assessed ex

*vivo* and then *in vivo* if this section of vagal nerve will respond to IL-1 $\beta$ , IL-6, TNF or LPS. They demonstrated that the nerve was functional but found no impact on nerve activity in response to inflammation [58, 30]. Interestingly, a recent paper recorded the *in vivo* response of the afferent cervical vagus to IL-1 $\beta$  and TNF in mice. Peripheral injection of IL-1 $\beta$  and TNF individually enhanced the amount of action potentials in the vagus nerve. Indicating that the vagus has some mechanism of sensing inflammatory cytokines and the authors hypothesised it would be in the various murine vagal paraganglia [171].

Overall, it is clear that there needs to be more research into vagal afferent paraganglia to determine their exact role in the inflammatory reflex. Recent results are encouraging [171], however more attention on the sensing aspect of the inflammatory reflex should be investigated. Moreover, investigations into the afferent sensing component of the vagus nerve may lead to new functional targets for electrostimulation [5]. Ultimately, the efferent aspect of the inflammatory reflex has received more focused investigation, and this will be explored in the following section.

### **2.C.iii – The Inflammatory Reflex – Efferent Response**

Having outlined the hypotheses behind afferent activation of the inflammatory reflex in section 2.C.ii, the efferent response will be expounded upon, again using a historical perspective. The elucidation of the cholinergic anti-inflammatory reflex is in a large part thanks to Dr. Kevin Tracey and his team who have been involved in nearly all major discoveries for the last 20 years. Originally, Dr. Tracey was searching for a cure for septic shock and that is a focus in his early discussions. Since, however, the inflammatory reflex has expanded past the idea of septic shock. The original paper demonstrated that, in rats, isolation and electrical activation of the vagus nerve would attenuate LPS-mediated production of serum TNF, liver homogenate TNF and prevented the endotoxin-mediated decrease in mean arterial blood pressure – indicative of shock. Additionally, the authors likened the decrease of pro-inflammatory cytokines by ACh to ACh produced by the parasympathetic vagus nerve [152]. Overall this was an interesting study that launched additional research on the cholinergic anti-inflammatory reflex.

The follow up paper from Dr. Tracey's lab provided additional information on the cholinergic anti-inflammatory reflex: it established that electrical stimulation of the vagus nerve would

mitigate LPS-induced TNF production in the heart, but not the lungs. Additionally, differences between left and right vagus were examined, interestingly left vagal stimulation (Left side – when animal is on stomach) was more effective than right vagal stimulation in managing mean arterial blood pressure in response to endotoxin challenge [9]. Currently, when the vagus is stimulated, the left nerve is typically used. In their next experiment, mice were used to test the inflammatory reflex. Wildtype mice receiving vagal electrostimulation had attenuated concentration of serum TNF, however  $\alpha 7$ nAChR (a sub-unit of the nAChR) knockout mice were incapable of replicating this response. In the study the result is likened to the fact that macrophages not expressing  $\alpha 7$ nAChR are incapable of responding to ACh and do not have decreased inflammation compared to control macrophages [162]. It was suggested, but not confirmed, that macrophages expressing  $\alpha 7$ nAChR mediate this effect. Overall, this study adds to the developing mechanism of action associated with the cholinergic anti-inflammatory reflex. A study by a separate group, in the same year, demonstrated the importance of the nAChR to the cholinergic anti-inflammatory reflex using agonists. Chlorisondamine, an antagonist of nAChR, reversed the decrease of liver TNF mRNA, serum TNF and NF- $\kappa$ B expression by rat vagus electrostimulation [60].

While it is known now that the spleen is a crucial component of the inflammatory reflex, it was not known in 2003, when an independent group were investigating splenic nerve stimulation. An *ex vivo* perfusion preparation of the spleen was performed: The spleen was kept intact, as well as the surrounding tissue and particularly the splenic nerve – which was electrostimulated. It was shown that LPS-mediated production of TNF was attenuated due to the electrostimulation and it was wholly mediated by  $\beta$ AR [77]. This result indicates that there is sympathetic regulation of inflammation via nerves, in addition to parasympathetic. Interestingly, this paper gives a hint as to what the cholinergic anti-inflammatory reflex would become, but it also is the basis of an alternative theory on the inflammatory reflex, which will be discussed in the following section 2.C.iv.

Dr. Tracey's group determined that nicotine administration could enhance survival outcome in mice that underwent cecal ligation and puncture - a technique for inducing septic shock. This result was likened to the activity of the inflammatory reflex [161]. The molecular mechanisms of how nicotinic activation of its receptor on macrophages were explored by an additional group. It was shown that jak2 and STAT3 were the mediators necessary to attenuate inflammation via nicotine in mice. Interestingly, it was determined that vagal

stimulation decreased the production and release of pro-inflammatory cytokines due to intestinal manipulation. Additionally, it was demonstrated that intestinal macrophages expressed activated STAT3 in response to vagal stimulation [73]. This study suggested at the far-reaching potentiality of the effects of the cholinergic anti-inflammatory reflex. Dr. Tracey's group further explored the mechanism of action of the cholinergic anti-inflammatory reflex in their next paper. It was determined that the spleen manufactures large quantities of TNF in response to endotoxin and that this was prevented by vagal nerve stimulation. Additionally, removal of the spleen prevents the increase in pro-inflammatory cytokines via LPS. Furthermore, lesioning of the celiac branch of the vagus nerve abolishes the decrease in splenic and serum TNF via vagus nerve stimulation [71]. This study was crucial to the development of the inflammatory reflex, as it determined the necessary nerve and central end-point of impact for the reflex. Their follow-up study examined the effect of vagal nerve stimulation in survival. Mice who received vagus electrostimulation and undergone cecal ligation and puncture had a greater chance of survival compared to sham-stimulated mice. Additionally, the stimulated mice had a reduced clinical sickness score compared to the sham mice [70]. This paper confirms the physiological relevance of vagal nerve stimulation and its therapeutic potential, which will be discussed further in section 4.B.i/ii.

In another study from Dr. Tracey's lab, it was determined via confocal microscopy and immunostaining that macrophages in the spleen are largely responsible for the production of TNF due to endotoxin and that vagal nerve stimulation decreases this response. Nerves in the vicinity of these splenic macrophages were discovered, however, they were determined to be sympathetic nerves and not parasympathetic. Additionally, it was shown that abolishment of the splenic nerve reversed the vagus electrostimulation-mediated decrease in serum TNF [10]. This discovery complicated the cholinergic anti-inflammatory reflex as it was mostly accepted that it was due to ACh acting on the  $\alpha 7$ nAChR on macrophages. Sympathetic nerves do not use ACh as their neurotransmitter, except in one rare instance. Additional research was required to solve this conundrum.

A series of papers in 2011 were published that describe the inflammatory reflex as it is known now [3], the first of which presented that  $\alpha 7$ nAChR is only crucial in the celiac ganglion and not the spleen, however, this result was mostly overturned by the next two papers. Interestingly, though, this paper demonstrates *in vivo* electrical activation of the splenic nerve will decrease serum TNF concentration [159]. This paper will be further discussed in the

following section 2.C.iv. The second paper discovered choline acetyltransferase expressing T-cells (CHAT cells) in the spleen, which are crucial to the inflammatory reflex and are found next to the terminals of sympathetic nerves. Utilising nude mice, which lack an adaptive immune system, it was found that vagal nerve stimulation was not successful [136]. In the last paper, the previous result was expanded upon and it was discovered that  $\beta_2$ AR signalling was crucial to the response of the inflammatory reflex. This result was shown pharmacologically, but additionally tested *in vivo* in  $\beta_2$ AR knockout mice, who failed to replicate the decrease in LPS-induced TNF production via vagal nerve stimulation. Interestingly, it was also assessed whether it was possible in the case of nude mice to rescue the positive response of vagal nerve stimulation. It was determined that transferred T-cells from a healthy mouse could rescue the vagal nerve stimulation response in nude mice, but not T-cells from  $\beta_2$ AR knockout mice. Furthermore, in  $\beta_2$ AR knockout mice who do not have a successful response to vagal nerve stimulation, the response was recuperated when wild type T-cells were transferred to the  $\beta_2$ AR knockout mice [160]. These final results confirm the cholinergic anti-inflammatory reflex as it is now known [Illustration.8]. More correctly, the reflex should be described as the inflammatory reflex as sympathetic action and parasympathetic action work in conjunction together [25]. The capacities of the inflammatory reflex were assessed by Dr. Tracey's group in a further experiment. It was assessed whether afferent unilateral and bilateral block via lidocaine would prevent the mitigation of TNF by vagal nerve stimulation – it did not in both cases. This result confirms that efferent stimulation of the vagus is the key component of the inflammatory reflex. Alternatively, however, it was gauged if efferent block of the vagus unilaterally and bilaterally, via lidocaine, would affect the effect of the inflammatory reflex – surprisingly, it did not [117]. The interpretation of this result can only be that there are alternative methods in the body to regulate inflammation than what is currently known: this thought will be explored further in section 3.C.iv. Additionally, within the same study an assessment was undertaken to establish whether rats and mice would respond differently to vagal nerve stimulation, no difference was found and furthermore it was demonstrated that a lower volume of stimulation was just as effective as a higher volume [117].

It is evident that the Dr. Tracey group developed a considerable amount of information on the inflammatory reflex. Perhaps, unsurprisingly, there are alternative hypotheses to what the inflammatory reflex actually is and while, there has not been many new mechanistic

studies on the inflammatory reflex in recent years, that does not mean there is not more to discover – as will be discussed in the following section.

#### **2.C.iv – The Inflammatory reflex – an Evolving theory**

It is natural with any new theory that additional labs replicate the original work and expand upon it – that is science. It is also natural, however, for the development of alternative hypotheses on any evolving theory. The inflammatory reflex is no exception to this and there are major concerns from an experimental and a physiological viewpoint. One concern is the lack of information on vagal afferent sensing, as outlined in section 2.C.ii. An experimental concern was raised by one group, as anaesthetic/analgesia are commonly known to induce an anti-inflammatory response [101]. Much of the *in vivo* studies produced by Dr. Tracey's group were conducted acutely [152], meaning animals were stimulated and then injected with LPS; there was no recovery period and it could be that the anaesthesia/analgesia were influencing the results. To avoid this problem, it is necessary to work with conscious animals. Electrostimulation in conscious animals is considerably more difficult and requires the implantation of electrodes securely and safely long-term. The concern, while valid, was adequately refuted by results from further studies [83].

There is a major physiological concern, however, that is not as easily refuted. There is no strong evidence for a connection between the vagus and the spleen anatomically [19, 11, 22, 31]. Moreover, this concern is further emphasised by the fact that parasympathetic and sympathetic nerves are working in conjunction, an event which is mostly undescribed in the literature. In a review, the Dr. Tracey group counter argued this point [121], questioning the methods used in the study [22] and citing literature which demonstrated that there was efferent catecholaminergic innervation of the spleen from the celiac-mesenteric plexus ganglia [110], an area known to receive vagal input [103]. Ultimately, this issue remains mostly unresolved with both groups concerned, seemingly content in their respective results. Interestingly, this split led to the development of an alternative hypothesis to explain the inflammatory reflex – the splanchnic anti-inflammatory reflex [101].

The first study investigating the splanchnic anti-inflammatory reflex demonstrated, similarly to work done by the Dr. Tracey group, that sectioning of the splenic nerve will increase TNF serum, splenic mRNA and liver mRNA in response to LPS [101]. This result became the basis

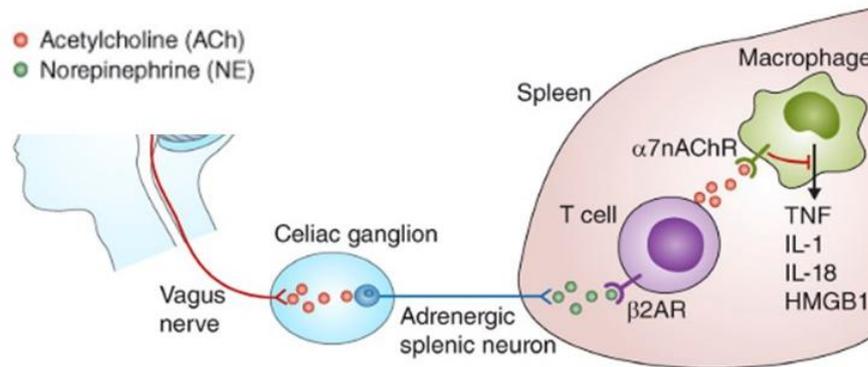
of the splanchnic anti-inflammatory reflex. In the group's follow-up paper, the results of the previous paper were further expounded upon. It was discovered that splenic sympathetic nerve activity was enhanced by LPS, however, the activity was unchanged by bilateral cervical vagotomy. Splenic sympathetic nerve activity was, however, reduced by splanchnic nerve cut – a sympathetic nerve entirely independent of the vagus. Furthermore, it was demonstrated that the splanchnic nerve had increased activity following LPS and bilateral cervical vagotomy had no impact on this activity. Additionally, splanchnic nerve cut increased LPS-induced plasma TNF concentration [100]. Overall, the alternative hypothesis of the inflammatory reflex is summarised by the splanchnic nerve integrating with a sympathetic ganglion, contiguous with the splenic nerve. Curiously, the group has not since published new original studies on the splanchnic anti-inflammatory reflex, though they have published reviews on the topic [103, 99].

Furthermore, an additional external study proposed a semi-alternative inflammatory reflex [159] to the classic inflammatory reflex [3]. The authors [159] demonstrated in  $\alpha 7nAChR$  knockout mice that vagus nerve stimulation did not attenuate inflammation – similarly to previous work [162], however splenic nerve stimulation could still mediate the reduced inflammation. The authors proposed that  $\alpha 7nAChR$  was crucial on the celiac ganglion and not on macrophages in the spleen [159]. Overall, these alternative hypotheses on the inflammatory reflex are interesting, however there has been no reconciliation of opinions on what the inflammatory reflex is, though Dr. Tracey's group have explored the concept of a broader definition on immune-brain-immune communication and regulation [25].

Interestingly, recent unpublished work from Dr. Philippe Blancou's may be able to resolve what the inflammatory reflex is. The group's work discovered that there are three functional nerves going into the spleen of mice, two of which are associated with arteries and a third at the apex of the spleen – the apical nerve. Potentially, some of the confusion over splenic retrograde tracing experiments could be due to the splenic nerve that was examined. Interestingly, it was discovered that the arterial nerves are purely catecholaminergic in origin, whereas the apical nerve is catecholaminergic and cholinergic. It was demonstrated that both the arterial nerves and apical nerve could inhibit the LPS-induced production of TNF via electrostimulation. The arterial nerves, however required lymphocytes to induce their anti-inflammatory response, whereas the apical nerve did not. Apical nerve electrostimulation, tested using antagonists, required the combined action of catecholaminergic and cholinergic

antagonist to abolish the decrease in LPS-induced TNF via electrostimulation. A similar result was obtained using genetic animal models of  $\beta_2$ AR knockout and  $\alpha_7$ nAChR knockout where the combination of both these models was required to reverse the positive effect of apical nerve stimulation [unpublished data].

The results from Dr. Blancou's group offer further enlightenment on the mechanics of the inflammatory reflex. Whether these results produce an accord between the functional hypotheses on the inflammatory reflex is yet unknown. Moreover, the concept of the inflammatory reflex is expanding: Dr. Blancou's research is a clear example of this, however, there are additional studies stretching the original concept of the inflammatory reflex. It was discovered that stimulation of the aortic depressor nerve, which is an afferent vagus source can attenuate inflammation [5]. Additionally, it has been shown that a vagal afferent source activates the locus coeruleus and that deep brain stimulation of this brain region can also attenuate inflammation [6]. Furthermore, the Dr. Tracey group has even demonstrated in one experiment that bilateral block of the efferent vagus nerve does not prevent the positive effect of vagus nerve stimulation [117]. This result can only be interpreted singularly - there is alternative means for decreasing inflammation in the body, via nerves, then the classic inflammatory reflex [3]. Excitingly, these studies expand upon the original inflammatory reflex and further demonstrate its potent capabilities.



### Illustration 8: The Inflammatory reflex

Afferent vagal paraganglia (unshown) will detect inflammation and signal to the CNS. Efferent vagus nerve activity will be induced. Signals from the vagus nerve will instigate activity in the celiac ganglion via ACh, which activates adrenergic signalling to the spleen. The sympathetic nerve will propagate norepinephrine into the spleen to activate ACh producing T-cells via the  $\beta_2$ AR. These T-cell, in turn, release ACh which prevents macrophages from releasing pro-inflammatory cytokines. Image adapted from Andersson and Tracey 2012.

## **2.D.0 – Regulation of the Immune System Summary**

The HPA axis and the inflammatory reflex have been described in detail and the means by which they regulate inflammation. There are distinct positives and negatives for these two regulatory pathways. The HPA axis induces a long-lasting anti-inflammatory response whole body wide but is slow to respond to immediate inflammation. Whereas, the inflammatory reflex responds rapidly to inflammation and continuously ensures some attenuation of pro-inflammatory cytokine production, but its location of action is limited to the spleen. Overall, both mechanisms have their role and perform it, otherwise there would be rampant inflammation. It is worth mentioning that these two mechanisms are not the sole means, separate to the immune system, for regulating inflammation. As research continues, there is likely to be discoveries of additional systems and mechanisms for regulating inflammation [25]. In this regard, one intriguing area of study is the Carotid Body and its role in the immune response.

### **3.A.0 – Carotid Body: Overview**

The Carotid Body (CB) is a paraganglia and the largest in the body. It is a polymodal afferent sensor involved in a fascinating array of activities. Historically, the CB is most closely associated with oxygen sensing, as it is the primary sensor for oxygen concentration in the blood. This detection of low or high oxygen concentration leads to physiological change, in this case increasing or decreasing breath rate respectively. Furthermore, the CB can make similar changes in response to CO<sub>2</sub>, blood pressure and metabolism, amongst many potential other physiological variables. Interestingly, there is a role that has yet to be fully elucidated between the CB and the immune system. In section 3.C.iv this topic will be addressed in full, as it is the primary basis for this investigation, however first the CB will be introduced in the following sections.

### 3.B.0 – Carotid Body Anatomy

The CB is located bilaterally at the bifurcation of the common carotid artery, between the internal carotid artery and the external carotid artery. The innervating nerve of the CB, the carotid sinus nerve (CSN), is a divergence from the glossopharyngeal nerve – the ninth cranial nerve. The glossopharyngeal lies superior, but parallel to the pharyngeal nerve, however it is dorsal to the hypoglossal nerve, the twelfth cranial nerve [86, 151, 145]. Additionally, there is some evidence that there is a connection between the CB/CSN and the vagus [151, 145]. Interestingly, there is also efferent fibres to the CB in the CSN which are primarily sympathetic [86, 98]. Whereas the afferent fibres in the CSN are partially catecholaminergic, but also partially purinergic [86]. The CSN is also the innervating nerve of the carotid sinus - a region of the internal carotid artery for sensing blood pressure via baroreceptors [169]. The baroreceptors respond to distension in the wall of the internal carotid artery and this induces CSN activity. Afferent signals generated from the CB synapse with the CSN to the ascending glossopharyngeal nerve which reaches the brainstem at the NTS [39]. Further projecting to the rostral ventrolateral medulla (RVLM) which is responsible for efferent sympathetic outflow [Illustration.9] [34].

Efferent signalling to the CB modulates the function of the CB primarily by altering its blood flow [86]. Anatomically, the CB varies between animals, in some it is a distinct capsule, while in others it is encased in the artery that supplies it with blood. While the CB is the largest paraganglia in the body, it is still an incredibly small tissue. Humans have a CB of maximal diameter 3.3 mm [64] and an average CSN length of 29 mm [151]. Generally, it is the larger the animal, the more mass in the CB [86]. Anecdotally, our observational results on the size of the mouse CSN determine it to be 0.8 – 1 mm in length with an average diameter of 35 µm, however a rigorous measuring procedure was not performed.

The CB is a highly vascularised tissue and receives an inordinate amount of blood flow for its size. Considered in the context of 100 gram of tissue, the CB receives 2000 ml/100 g per minute, which is a larger volume than that of any organ in the body at a comparable weight [146]. The CB can receive this quantity of blood flow from multiple vessels – internal/external carotid artery and the occipital artery, however there are some apparent differences between species [86]. The high-vascularisation of the CB is due to the tissue-components, chemoreceptors, of the paraganglia itself, which are responsible for sensing their

environment. The various functions of the chemoreceptors will be addressed in the following sections 3.C.i-iv.

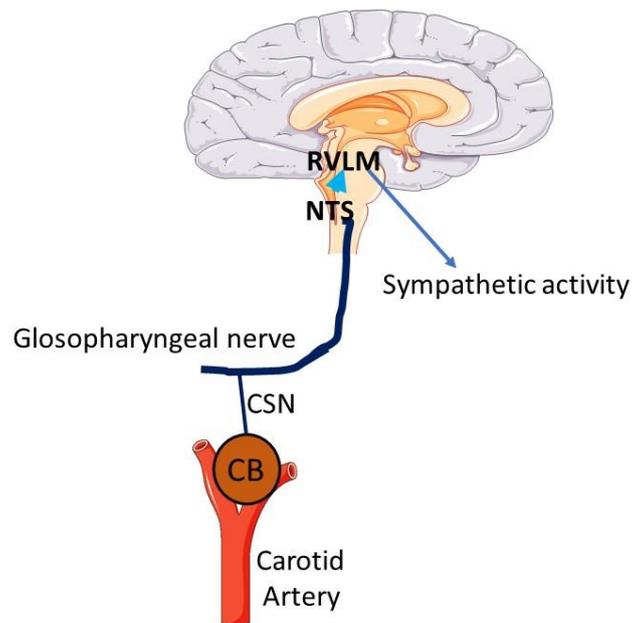
### **3.B.i – Carotid Body Anatomy: Molecular and Cellular**

The CB is a true polymodal sensor due to its detection of a vast array of physiological stimuli. The tissue of the CB is a rich matrix of blood vessels and nerves mixed with CB-specific cells—the type 1 cells and the type 2 cells are the most important. Typically, in the CB there are several times more type 1 than type 2 cells. A small group of type 1 cells will be covered by a thin sheath of type 2 cells forming a functional unit, together with blood vessels and nerves, called the glomerulus [137, 2]. Type 1 cells, or glomus cells, are the chemoreceptors of the CB and are the cells responsible for detecting stimuli and instigating afferent nervous activity. Whereas, type 2 cells are generally considered to be the supporting cells for type 1 cells. Frequently, type 2 cells are compared to astroglia in the brain, which support neurons, and can be labelled glia-like cells [115].

The chemoreceptors of the CB detect a fascinating array of stimuli in a physiologically relevant fashion [86]. The physiological detection and subsequent functions of chemoreceptors detecting O<sub>2</sub>, blood pressure, indicators of metabolic and immune activity will be discussed in the following section. Here, the relatively controversial topic of signal transduction to afferent nerve fibres will be discussed. There is considerable evidence for different neurotransmitters with the CB. This is in part due to the high variety of potential neurotransmitters residing in the chemoreceptors – dopamine, norepinephrine, ACh, adenosine and adenosine triphosphate (ATP) [114, 57]. It may be that different neurotransmitters are used by the CB depending on the detected stimuli. As this is uncertain, however, a general overview of signal transduction will be presented, which skews toward hypoxic activation of the chemoreceptors reflecting the focus of most research.

Regardless of the neurotransmitter used, chemoreceptor activation seems to be associated with an inhibition of potassium efflux and an increase of intracellular calcium, engendering membrane depolarisation and neurotransmitter release [57]. A compelling case for purinergic signalling, via ATP, can be presented for the primary neurotransmitter from chemoreceptors to afferent terminals of the CSN. On the CSN fibres that colocalise with the chemoreceptors

there are P2X2 receptors and an ATP receptor. When this ATP receptor is pharmacologically blocked there is decreased sensory discharge from an *ex vivo* recording of CSN activity [175]. Knockout of P2X2 in mice results in an attenuated ventilatory response to hypoxia [134], confirming the relevance of ATP signalling in signal transduction. Interestingly, however, it has been suggested that ACh acts as a co-neurotransmitter for ATP signalling to instigate afferent activity [86, 57, 175]. Furthermore, it is likely that all the above-mentioned neurotransmitters play a role in signal transduction, whether it is excitatory, inhibitory or even modulatory [86, 114]. Additionally, in the literature there is potential evidence that even the type 2 cells may play some role in signal transduction [115].



**Illustration 9: Carotid body anatomical connections**

The CB is located at the apex of the carotid artery resting between the internal and external carotid artery. It is innervated by the CSN which connects to the glossopharyngeal nerve – a cranial nerve which arrives to the medulla and synapses with the NTS. There is a further connection to the RVLM which can then induce sympathetic outflow. Images adapted from Servier Medical Art.

### **3.C.0 – Physiological functions**

The role of the CB is not limited to detection of various stimuli – O<sub>2</sub>, blood pressure, metabolites and inflammation – but extends also to the functional response to these stimuli [Illustration.10]. The CB, through the signals it generates in the CSN which synapses in the brainstem, which instigates efferent nervous activity to fix a breach in homeostasis. One particularly useful tool for investigating the physiological functions of the CB is the concept of bilateral carotid sinus nerve resection (BCN). This surgical intervention prevents the CB from transmitting signals to the brain and ultimately the loss of function is seen – determining the role the CB typically performs. For each of the mentioned physiological functions above, it will be explored in brief how the signal is detected, before describing the function of the CB in relation to these physiological functions. O<sub>2</sub>, metabolic activity and blood pressure will also be described in brief, followed by a thorough examination of the immune system and the CB.

#### **3.C.i – Physiological Functions: O<sub>2</sub> Sensing**

The CB can detect high, hyperoxia, and low, hypoxia, levels of oxygen. Throughout history a huge amount of research has focused on how the CB detects hypoxia. Currently, there is no one accepted hypothesis on the true mechanism for oxygen detection in the CB. There are, however, five main hypotheses for oxygen detection in the CB, each with reasoning for and against that mechanism. To list the hypotheses: First, the lactate/olfr78 mechanism for oxygen chemotransduction; Second, Mitochondrial ATP's activity in chemostransduction; Third, the AMPK mechanism for oxygen sensing; Fourth, Reactive oxygen sensing as the mediators for oxygen sensing; Fifth, and final, the integrated activity of hydrogen sulphide, reactive oxygen species and heme oxygenase [130]. Whichever hypothesis proves to be true, there is likely to be alternative oxygen sensing mechanisms in the CB.

Regardless of the mechanism used to induce the physiological response, whenever the CB detects hypoxia there is subsequent increase in the frequency of breathing [102]. Whereas with hyperoxia, there is a decrease in the frequency of breathing [88]. In animals that have undergone BCN of some variation (nerve section or CB excision), typically they cannot respond as well to hypoxia [138, 16] and their breath rate does not increase, this is particularly true for animals under anaesthesia [102]. A simple experiment performed to determine a

functional CB or CSN is to test for hypoxia or hyperoxia and measure the subsequent breath rate. The CB is believed to be crucial for mountain climbers and their adjustment to decreasing oxygen concentrations as they ascend. Moreover, humans who have undergone carotid surgery cannot adapt as easily to high altitude [86]. In addition to hyperoxia and hypoxia the CB can respond to hypercapnia [86]. Within the literature there is extensive information on the role of the CB in the physiological response to hypoxia, as it is the most frequently studied aspect of CB activity. This is just a brief summary on a broad field of study.

### **3.C.ii – Physiological functions: Metabolism**

There is consensus in the field of the CB, that the CB detects products of metabolism, however there is some controversy on whether it is a glucose or insulin sensor. Insulin is a hormone released primarily by the pancreas and acts on its receptor – insulin receptor – to increase glucose uptake into cells. As glucose and insulin are intimately related it has led to some difficulty accurately interpreting experiments [29]. Additionally, as there is no receptor for glucose, it is left to examining the function of the CB in response to glucose. It has been investigated both *in vivo* and *in vitro* the response of the CB to glucose in a variety of concentrations. Some studies suggest that the CB can respond to glucose [174], whereas others do not [28]. There is no real consensus of results and it appears methodological considerations may have prevented an accord. Perhaps a little easier to ascertain is the role of chemoreceptors as insulin sensors. Chemoreceptors express insulin receptor, which is shown to be activated via phosphorylation in the presence of insulin. Additionally, insulin administration to the CB increases neurotransmitter release *in vitro* and *in vivo* increases respiratory frequency [133].

Regardless of the metabolite affecting the CB, there is a clear physiological role for the CB in glucose homeostasis and metabolic syndrome. Dogs which had undergone BCN demonstrated lower levels of glucagon – a hormone for releasing glucose into the blood – basally and in response to hypoglycaemia. Additionally, they had decreased endogenous glucose appearance in the BCN group [85]. Overall this indicates the role of the CB in glucose homeostasis and that there is reduced glucose in the blood following BCN. Additionally, it was shown in rats that BCN restores insulin sensitivity to control levels in high fat diet rats [133],

via normalising, and in some cases enhancing, the activity of the functional components of insulin receptor signalling [140].

Due to these results and others, it has been suggested that BCN could be a viable therapeutic option for metabolic syndromes, but also hypertension and heart failure – as will be discussed in the following section. The reason for this connection is due to the evidence that CB hyperactivity engenders increased sympathetic activity in the brainstem specifically via the RVLM [72]. This increased sympathetic activity plays a negative role in the regulation of metabolic syndromes via enhanced insulin resistance and hypertension [29]. How sympathetic activity induces this effect is mostly unknown and an additional project of mine is currently investigating this problem.

To describe briefly, as the focus of this investigation, is the relationship between the immune system and the CB. It is known that rats fed a high-fat diet will become obese and develop an insulin resistance-like phenotype and that BCN will prevent the overt development of these metabolic issues via decreased sympathetic activity. The mechanism, however, is unknown and we are currently investigating the relationship  $\beta_2$ AR has in this effect.

### **3.C.iii – physiological functions: Hypertension**

The CB and blood pressure have an interesting relationship. Activated chemoreceptors augment blood pressure, via increased efferent sympathetic output, engendering reflex hypertension potentially via adrenal-mediated increase of epinephrine and norepinephrine [98, 94]. Alternatively, the baroreceptors of the carotid sinus, an area of the internal carotid artery, respond to stretch of the artery and signal via the CSN to attenuate hypertension [94]. The primary mechanism for this attenuation is the inhibition of sympathetic activity normally influencing cardiovascular responses [65, 104]. BCN is an effective means for decreasing blood pressure and it has even been proposed for treatment of resistant or neurogenic hypertension [104]. One alternative to a full BCN for resistant hypertension – is a unilateral removal of the CSN. Unfortunately, the experiments did not prove to be successful [120]. There is, however, a subtler means for treating hypertension via the CSN - bioelectronic medicine [168, 142]. Targeting of the Carotid sinus/CSN has received attention in recent years

as a means for treating resistant hypertension and will be discussed in full in the section 4.C.i/ii.

### **3.C.iv – Physiological Functions – Immune system**

In recent years it is becoming increasingly apparent that the immune system can influence the CB and that the CB can influence the immune system [124]. Immune cells can be found within the CB in a basal setting, mast cells were discovered to be in human CB, though not contiguous to the glomus cells. The mast cells in the CB were next to blood vessels: the author's hypothesised that they may influence blood flow to the glomus cells [63]. Additionally, in the rat CB abundant numbers of macrophages have been detected, as well as mast cells [41]. Furthermore, there is evidence of lymphocytes, potentially due to aging, in the human CB – predominantly T-cells [78]. The CB may degenerate with age and this enables further immune infiltration of the CB [124]. Overall, it is unclear what exactly these immune cells are doing in the CB, it can be hypothesised that they modulate signalling of the chemoreceptors in some fashion considering the impact of pro-inflammatory cytokines on the glomus cells. The roles of IL-1 $\beta$ , IL-6, TNF, LPS and zymosan in the CB have been reasonably explored in the literature and will be discussed in detail below.

One study examined the CB transcriptome by microarray and PCR analysis in humans and mice. The mRNA for IL-1R was found in the human CB, but not in two strains of mice. IL-1A, a decoy receptor for IL-1 $\beta$ , was absent from the human and mouse CB [109]. In rats, however, using fluorescent microscopy IL-1R was discovered to be colocalised to tyrosine hydroxylase – a common histological marker for the CB, as it is representative of catecholaminergic cells. Additionally, this result was further confirmed using western immunoblotting [163]. Furthermore, intraperitoneal (IP) injection of IL-1 $\beta$  enhances the expression of IL-1R on the CB, confirmed via western immunoblotting and confocal microscopy [163]. Zymosan, a PAMP found on the surface of fungi, can enhance the expression of IL-1 $\beta$  in rat glomus cells detected by confocal microscopy. NLRP1 and NLRP3, the inflammasome responsible for cleaving IL-1 $\beta$  to its mature form, are present in the CB [1]. Interestingly, it has been demonstrated that Chronic intermittent hypoxia [87, 38] and hypoxia [53] will increase the expression of IL-1 $\beta$  and IL-1R in the rat CB. In isolated rat glomus cells, ex vivo, it was determined that IL-1 $\beta$  would

enhance the intracellular calcium spike in response to hypoxia [53] and chronic intermittent hypoxia [87]. Additionally, it was confirmed that the CB can functionally respond to IL-1 $\beta$  [148]. The glomus cells of rats were isolated and patch clamp experiments were performed to measure intracellular potassium, it was determined that IL-1 $\beta$  could mediate a decrease in intracellular potassium and a rise in intracellular calcium in the glomus cells [148]. These two fluctuations in ions by IL-1 $\beta$  are indicative of CB glomus cell activation as similar events occur during hypoxia. Furthermore, the functional capacity of IL-1 $\beta$  activation of the CB was assessed *in vivo*. Topical application of IL-1 $\beta$  to the exposed CB induced increased CSN activity, which could be blocked by non-membrane bound applied IL-1R and an ATP receptor blocker, but not a dopamine receptor blocker [148]. Overall these results are indicative, in rats, that the CB responds to IL-1 $\beta$  functionally and that the activation of the CSN will presumably continue to the brainstem to evoke an efferent response.

Similar to IL-1 $\beta$ , transcriptome analysis via microarray determined that the human CB express IL-6 and IL-6R, whereas two strains of mice did not [109]. It was established that IL-6R $\alpha$ , a subunit of IL-6R, was present in rat *ex vivo* glomus cells via western blot. Additionally, it was demonstrated via confocal microscopy that the IL-6R $\alpha$  colocalised with tyrosine hydroxylase expression indicating that the IL-6R is found on glomus cells [164]. Hypoxia [53] and chronic intermittent hypoxia [87] in rats induce increased expression of IL-6 and IL-6R in the CB. A rise in intracellular calcium induced by hypoxia or chronic intermittent hypoxia was enhanced by prior application of IL-6 to rat glomus cells *ex vivo* [87, 53]. The functional role of IL-6 on rat glomus cell activity was assessed *ex vivo*. Fluorometric measurement of intracellular calcium was analysed in the presence of IL-6: IL-6 induces an increase in intracellular calcium in *ex vivo* rat glomus cells. Additionally, IL-6 can engender the release of catecholamines from glomus cells and this release was dependent on the IL-6 mediated increase in intracellular calcium [45]. Unfortunately, there is no *in vivo* data for the activity of IL-6 on the CB, however, it can be inferred that there is a role for IL-6 in CB activity – again, particularly in the rat.

The presence of TNF, TNFR1 and TNFR2 were assessed by microarray analysis in humans and two strains of mice. In neither mice nor humans was TNF evident, however TNFR1/2 were for both mice and humans [109]. One study examining the cat CB found TNF and TNFR1 in the cat CB localised to glomus cells via immunocytochemistry, mRNA for TNFR1 and TNFR2 was found in the cat CB. It was evaluated if TNF augmented chemosensory discharge in a perfused *ex vivo* cat CB, it did not, even in hypoxic conditions [46]. In a follow up study, in rats, the role

of LPS-induced TNF in the CB was investigated. TNF and TNFR2 were constitutively expressed in rat glomus cells and enhanced by LPS, this result was demonstrated by confocal microscopy, however, similar results were obtained for mRNA and protein expression by western immunoblotting [48]. Hypoxia [53] and chronic intermittent hypoxia [87, 38] will increase the expression of TNF and TNFR1 in the *ex vivo* rat glomus cells. Furthermore, application of TNF to glomus cells before a hypoxic event or chronic intermittent hypoxia leads to an increased intracellular calcium spike [87, 53]. TNF's role in the CB is a little harder to characterise than the role of IL-6 and IL-1 $\beta$ , as it may not induce functional chemosensory signalling in the CB/CSN. It should be noted; however, the authors do point to non-significant data indicating that TNF has a negative role in signalling frequency [46, 172]. Additionally, consider the transcriptome analysis demonstrating that basal TNF was not found in the mice or human CB [109], but it is in both the cat [46] and the rat [48] suggests a species dependent difference in the relationship to inflammation, however more studies will have to ascertain the veracity of this statement. Overall cytokines – TNF, IL-6 and IL-1 $\beta$  – perform a functional role in CB activity as modulators of hypoxic signalling and perhaps even aid in CB activity during chronic hypoxia [124]. Additionally, however, the CB definitively responds to IL-1 $\beta$ , and potentially IL-6, in the rat *in vivo* engendering enhanced CSN activity. Perhaps the role of TNF is not to induce CSN activity but to enhance the local production of IL-1 $\beta$ , as the inflammasome for cleavage to the mature form of IL-1 $\beta$  is found in the rat CB [1] this is speculation and more studies need to be conducted to determine the true role of functional CB sensing of cytokines. Alternatively, there is evidence of the CB detecting bacterial products such as LPS and zymosan.

LPS activates TLR4 in the body. Transcriptome studies of mice and humans via microarray analysis reveal that both species have mRNA for TLR4 [109]. In the rat, it has been demonstrated via confocal microscopy that glomus cells colocalise with TLR4 [1]. Similar results have been shown for TLR4 expression in rats [48]. Furthermore, in rats, it has been demonstrated by western immunoblotting that the signalling units for a functional TLR4 response are present – CD14 and MYD88. Additionally, in the CB glomus cells, LPS is inducing degradation of I $\kappa$ B $\alpha$ , an inhibitor of NF- $\kappa$ B. Whereas, NF- $\kappa$ B concentration in the cytosol of glomus cells reduced following LPS, presumably the NF- $\kappa$ B went into the nucleus to perform its transcriptional role [48]. LPS applied topically to the CB induces functional changes in its histology: The glomerulus appeared to be more oedematous, which is indicative of an

immune response, and there was increased infiltration of immune cells, though oddly not macrophages. Functionally, *in vivo*, LPS applied topically or injected intravenously produced a long-lasting increase in the frequency of breathing. This result suggests that the LPS is activating the CB, chemosensory discharges were also measured in the CSN and it was discovered that *in vivo* LPS enhances CSN activity directly in the cat [46,1]. It is evident that LPS induces activity in the CB, though further studies should be considered. Interestingly, TLR4 was expressed in multiple species – human, rat, mouse and potentially cats. Perhaps CB recognition of PAMPs is more universal, than cytokines.

In addition to LPS, one more PAMP and its influence on the CB have been studied – zymosan. Zymosan is detected by TLR2, which is found in the rat glomus cells. It was demonstrated, *ex vivo*, that mouse CB/CSN respond to zymosan by enhancing chemoafferent discharge in the CSN when coupled with hypoxia. Unfortunately, this result was not replicable *in vivo*, but the authors noted that while overall discharge was unaffected by zymosan there appeared to be more recruited active fibres in the CSN. It was previously mentioned that zymosan can induce the expression of IL-1 $\beta$  and NLRP3 in rat glomus cells, however it was not explored if NLRP3 was active in mice [172]. Perhaps IL-1 $\beta$  is required for *in vivo* activity of the CSN in response to zymosan and as mice do not have IL-1R, this could be the reason zymosan did not alter chemoafferent discharge *in vivo*. Further research is necessary to ascertain the exact signalling roles, however, overall it is indicative that zymosan has a role in modulating CB activity, expanding the immunosensing capabilities of the CB.

The focus of this investigation is the immunosensing capabilities of the CB and what has been discussed here is the core of the data determining that the CB is an immunosensor, however it has been demonstrated that the CB also has multiple other proteins associated with inflammation. The transcriptome analysis of humans and mice CB further displayed that humans have expression of TLR1, NF- $\kappa$ B, IL-10R and HMGB1, but not IL-10 and iNOS. Whereas, mice have mRNA for IL-10R, HMGB1 and NF- $\kappa$ B, but not TLR1, IL-10 and iNOS [109]. Interestingly, in rats iNOS, an enzyme for producing free radicals, has been discovered basally and it increases in response to chronic intermittent hypoxia [38]. There is some additional further evidence of certain immune components being increased in the CB, typically rat, in response to hypoxia, but no functional evidence of their effect on the CB has been demonstrated [124].

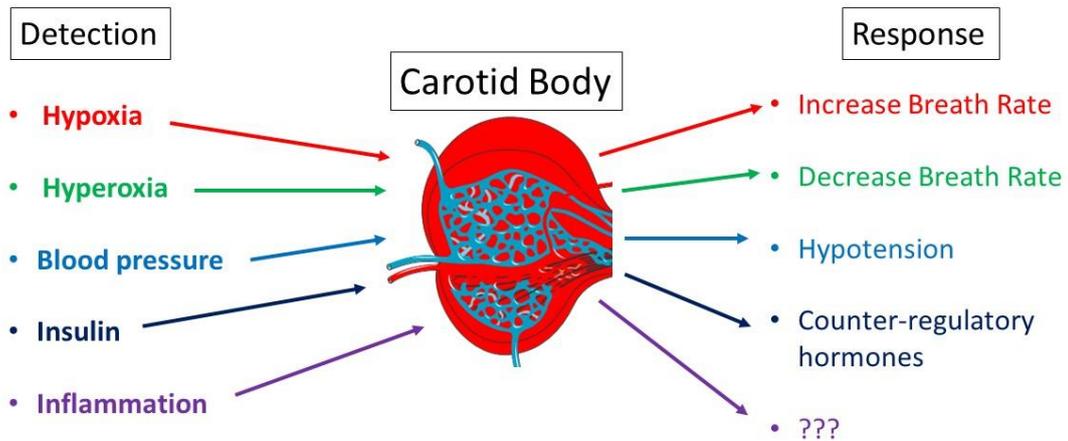
Admittedly more research must be performed on the immunosensing capabilities of the CB

to determine the true role of inflammation on the CB, however it is indicative of an overall role, which has been further examined physiologically by BCN in one study. This study found that after BCN LPS-induced production of TNF was enhanced compared to sham LPS-induced production of TNF and BCN mice receiving saline. Furthermore, it was demonstrated that BCN rats had significantly diminished survival to LPS intraperitoneal injection compared to BCN rats receiving saline or sham rats receiving LPS [111]. Interestingly, these results demonstrate that, in rats, there is a regulation of the immune response upon immune activation but not necessarily in basal conditions. It is worth noting that there were some odd methodological choices in this study, adolescent 5-week-old rats were used and an incredibly high dose of LPS (15 mg/Kg; Rat weight average – 110g) was administered for measuring immune activity. Overall, this study indicates that the rat CB is activated by an immune response, presumably by LPS directly, and loss of this detection leads to a rampant pro-inflammatory response and diminished survivability to infection.

There is one final paper examining the CB and the immune system, however, to understand their results, it is necessary to explain some associated papers. As discussed in section 2.C.ii, vagal afferent sensors have been proposed to be the instigating stimuli for the inflammatory reflex. There is evidence for and against this hypothesis. One group has proposed that the CB is actually the afferent source of the inflammatory reflex [47]. There is some evidence to suggest that this may be the case, aside from unsatisfactory evidence on afferent paraganglia, which will be listed briefly: The CB detects inflammation and responds to it [148, 46, 1]; BCN enhances TNF and reduces survival to LPS [111]; CB afferent signals and vagal afferent signals are integrated in similar regions of the NTS in the brainstem [47]; LPS induces c-fos activation in the NTS, even if the vagus is cervically bilaterally sectioned [176] and BCN prevents c-fos expression in the NTS [132]. The evidence while not overwhelming, certainly provides a place to begin an investigation. The study used electrostimulation of the rat CSN consciously *in vivo* to assess the LPS-mediated increase in pro-inflammatory cytokines. CSN stimulation reduced the production of TNF, IL-6 and IL-1 $\beta$ , however it also increased plasma IL-10 concentration. These results were abrogated by the selective removal of the chemoreceptors from the rat CB keeping the CSN intact. Furthermore, the efferent activity of this role was investigated utilising methylatropine, a mAChR blocker, and propranolol, a  $\beta$ AR blocker, both drugs were capable of blocking the CSN stimulation mediated decrease in pro-inflammatory cytokines by LPS [141]. The authors compared this result to both the idea of the cholinergic anti-

inflammatory reflex and the splanchnic anti-inflammatory reflex, suggesting the involvement of the CB/CSN as the afferent instigator in the two reflexes. This was an incredibly interesting paper suggesting at the possibilities of electrical stimulation in the CSN, however, there were some alarming methodical choices, or perhaps oversights, performed in this study – this topic and paper will be explored further in the discussion.

To conclude it is readily apparent that the CB is involved with the immune system in some capacity, whether it is the afferent sensor for the inflammatory reflex or not, or whether it is in a species dependent manner remains to be seen. Overall, researchers are at the beginning of exploring the CB and inflammation.



#### Illustration 10: Functional carotid body

The CB is a polymodal sensor capable of detecting multiple stimuli and example of which is presented here. The CB will detect hypoxia and induce a reflexive increase in breathing, whereas hyperoxia will induce a reflexive decrease in breathing. The carotid sinus baroreceptors will detect stretch in the internal carotid artery wall and induce reflexive parasympathetic activity to mediate a hypotensive response. Insulin is detected by the CB additionally and causes a believed increase in counter regulatory hormones. Interestingly, it is known the CB can sense inflammation but the exact relationship between the CB and the immune system is currently unknown and the focus of this investigation. Images adapted from Servier Medical Art.

### **3.D.0 – Summary**

The CB is an incredibly interesting micro-organ which has been researched for over a century and new information is still being produced today. The diverse array of functional roles the CB performs is almost unparalleled for a peripheral organ in the body. Interest and research focus are gathering in recent years on the CB and the CSN as new therapeutics around the organ may soon be developed. These therapeutics, pre-clinical and clinical, will be discussed in the following section on bioelectronic medicine.

#### **4.A.0 – Bioelectronic Medicine Overview**

Bioelectronic medicine is the concept of using electricity to treat disorders in the body. Bioelectronic medicine has a fascinating history in medical science; it is probably one of the oldest-newest disciplines in science. There is historical evidence of bioelectronic medicine being used back in ancient Rome as a headache cure and then it received quite a bad reputation with the idea of electroshock therapy for mentally ill patients. In recent years bioelectronic medicine has seen a resurgence, partially because of the capabilities of the inflammatory reflex. It is worth noting, however, that for severe depression and epilepsy stimulation of the vagus nerve has been approved for decades [170]. Furthermore, pacemakers in the heart have been in clinical use since 1958 [108]. Current ongoing research in this field is searching for target nerves that upon stimulation may treat for chronically high blood pressure, both forms of diabetes, bladder contention, IMIDs and additional disorders [15]. There are currently pharmacologic solutions for the listed disorders and new ones are being developed continuously – what make bioelectronic medicine a more desirable therapeutic? There are multiple reasons to investigate bioelectronic medicine over conventional medicine. Virtually all areas of the body are innervated by functional nerves in some capacity. Nerves use action potentials to carry signals and induce their effects on the target area. Action potentials are current flows of ions in and out of a cell altering its electrical potential. Target nerves can be activated or deactivated purely through regulating the induction of action potentials via electricity from an external source. This activation, or deactivation, will augment the activity of the nerve in its target area with unique precision [44]. Using the inflammatory reflex as an example: Stimulate the splenic nerve and there is decreased inflammation in response to LPS [159]; block splenic nerve activity and there will be increased inflammation [71]. Conventional drugs generally cannot compare to that level of specificity, as they often have off-target effects [108]. This form of bioelectronic medicine, electrically activating specific target nerves, is called an electroceutical.

Electroceuticals are currently dependent upon electrodes. Electrodes are the devices implanted onto nerves to interface with the nerve and ultimately facilitate the change from electron-based electricity in the mechanical device to ion-based electricity in the nerve [157]. Currently, electrodes have lead wires that trail away from the device to a source of power or to an external head-cap that can be connected to a source of power. Electrical activity going

through the electrode's wires, into the electrode and then ultimate conversion into nervous activity is provided by a waveform. Waveforms can take multiple shapes; however, square is most common form for modern electrostimulation. Typically, the three main considerations are current, frequency and duration – these make up the waveform. As of yet, there is no consensus on the best waveform to use when stimulating nerves. Partially, because there are multiple factors that can influence the response to stimulation including the type of nerve or brain region stimulated [157].

Ultimately, bioelectronic medicine is a developing field and requires dedicated researchers to discover new target nerves and potentially treatable disorders. There have been some early successes in modern bioelectronic medicine in the vagus nerve and the CSN, which will be addressed in sections 4.B.0 and 4.C.0 respectively.

#### **4.B.0 – Bioelectronic medicine to inhibit inflammation: The vagus nerve**

Electrostimulation of the vagus nerve is currently used for patients with chronic treatment-resistant depression and treatment-resistant epilepsy [170]. Electrostimulation of the vagus has been investigated in multiple disorders – obesity, Alzheimer’s disease, stroke and metabolic imbalance [170]. Primarily, however, this investigation is interested in the inflammatory reflex component of vagus nerve stimulation and its potency against IMIDs in pre-clinical and clinical cases [105, 92].

#### **4.B.i – Bioelectronic medicine to inhibit inflammation: The vagus nerve – Pre-clinical**

The inflammatory reflex is the theory that the vagus afferently detects inflammation, signals to the brainstem, which processes the information and induces reflex efferent activity. The efferent activity carries through the vagus and synapses with the celiac ganglion, where it activates the splenic nerve to inhibit the activity of immune cells within the spleen and ultimately attenuate inflammation [25]. It is unsurprising that such a powerful physiological response would be exploited for its potential clinical benefit. Two pre-clinical studies highlight the possibility of using the inflammatory reflex in humans via vagal nerve stimulation. The first focuses on colitis [105] and the second on arthritis [92].

In the first study an electrode was implanted around the vagus nerve and the carotid artery of rats connected to a head-cap for stimulation. Nearly two weeks later the TNBS method of inducing colitis was performed. The rats were consciously stimulated for the next five days, three hours each day in the morning (1 mA, 5 Hz, 0.5 ms). On the fifth day, rats were euthanised and the colon was removed and assessed for damage. Examining the colon histologically with a H and E stain it was determined that sham-stimulated TNBS rats had a higher degree of inflammation than the Stimulated TNBS rats. Furthermore, a multivariate index of colitis was performed: compiling information on body weight, temperature, histological inflammation and colon cytokine expression. It was revealed that the Stim TNBS rats had a significantly lower score on the multivariate index of colitis than sham-stim TNBS rats [105]. These results indicate the possibility of using vagal nerve stimulation in the treatment of colitis and potentially easing the suffering of patients afflicted with colitis.

In the second study, vagal nerve stimulation was assessed in a collagen-induced model of

arthritis (CIA) in rats. An electrode was placed on the vagus nerve, with externalised leads protected by a Velcro jacket around the neck of the rat. When stimulation or sham stimulation was required the rats were consciously stimulated via alligator clips connecting to the externalised leads of the electrode. Stimulation (3 mA, 10 Hz, 0.2ms – 60 seconds) occurred once daily for a week, nine days after surgery. Prior to stimulation, collagen was injected daily for one week after initial surgery. Ankle diameter in the sham-stim CIA rats increased steadily up until day 16 after surgery, whereas stim CIA rats had significantly smaller ankle diameter, indicative of reduced joint inflammation. Ankles were further examined histologically, and it was found that all clinical measures of inflammation were reduced in the vagal nerve stimulated CIA rats in comparison to the sham-stim CIA rats [92]. These results clearly demonstrate the possibility of vagal nerve stimulation being used to diminish the symptoms of arthritis in patients. Subsequent clinical trials have been performed on vagal nerve stimulation in arthritis and colitis, which will be addressed in the following section.

#### **4.B.ii – Bioelectronic medicine to inhibit inflammation: The vagus nerve – Clinical**

Continuing the pre-clinical work on IBD and arthritis, there have been clinical studies investigating the two IMIDs – by direct or transcutaneous electrostimulation. This investigation will focus on direct vagal electrostimulation.

The first clinical trial to be discussed (NCT01569503), investigates vagus nerve stimulation in Chron's disease. It is a clinical trial directly continued by the researchers who demonstrated the pre-clinical capacity of vagal nerve stimulation in Chron's disease [105]. The trial recruited seven individuals with Chron's disease who were mostly naïve of prior treatment, but certainly had no experience of a prior anti-TNF agent. A small pulse generator was implanted in the chest, with leads going to the cervical vagus. The primary aim of this investigation was to observe tolerability of the generator and continuous stimulation. The vagus nerve was stimulated (1.25 mA, 10 Hz, 0.5 ms) every five minutes for 30 seconds – similar to how epilepsy and depression are treated with vagal nerve stimulation [69]. Two patients had to be removed from the trial due to worsening symptoms, however the other five participants saw reduced levels of Chron's disease activity index and Chron's disease index of severity [18]. There appears to be benefit to the chronic stimulation of the vagus nerve in Chron's disease

- one patient even entered remission. Unfortunately, however, this clinical trial was terminated in phase 3 without explanation (NCT01569503). To speculate, maybe it was due to peripheral side-effects as the cervical vagus is connected to many regions of the body. There is however another clinical trial currently ongoing for vagal nerve stimulation and Chron's disease (NCT02311660).

Another clinical trial assessed the safety and tolerability of vagal stimulation, however this time in the context of rheumatoid arthritis (NCT01552941). The trial timeline consisted of 105 days, day 0 being the first day of stimulation. Day -21 was used for bloodwork, day -14 was for implantation of the electrode, which was activated by an external magnet. The participants received once daily stimulation for 60 seconds, beginning at day 7 and continuing to day 42 (0.25 mA – 2mA, 10 Hz, 0.25 ms). Participants received no stimulation between day 42-56, at day 56 stimulation was restarted until the study's final endpoint at day 84. Previously, it had been shown that vagal nerve stimulation reduced whole blood expression of pro-inflammatory cytokines to LPS (TNF, IL-1 $\beta$ , IL-6) in epileptic patients receiving their device. In this clinical trial, whole blood was taken from the patients at day 0, 42, 56 and 84 and exposed to LPS; TNF was subsequently measured. It was discovered at day 42, patient's whole blood did not produce as much TNF in response to LPS as day 0, on day 56 (after no stimulation for two weeks) TNF went back up, but this could again be decreased with daily stimulation as seen in day 84. Similar results were obtained for C reactive protein – a typical clinical indicator of inflammation. There was indication that the majority of patients responded to the treatment, whereas a sizeable minority, 33%, did not. There were some documented adverse effects, in particular dysphonia (hoarseness) was seen after surgery and during the course of stimulation, there were no serious side effects [83]. This clinical trial was opted to continue initially (NCT01552538), however there has been no new information since late 2016. Perhaps, the adverse effects tilted the risk-benefit ratio. There was, however, a new clinical trial investigating vagus nerve stimulation against arthritis from the same company (NCT03437473) in 2018 – no results have yet been posted about this trial. In conclusion, while not overwhelmingly positive, these clinical trials act as a good starting point to explore bioelectronic medicine. It encourages researchers to discover more precise target nerves or inventing mechanisms for stimulating individual nerve fibres. Perhaps, for the inflammatory reflex to be effectively utilised in medicine, the splenic nerve is the better target than the vagus nerve.

#### **4.C.0 – Bioelectronic medicine: The carotid sinus nerve**

Before the concept of bioelectronic medicine had been revamped in recent years due to the inflammatory reflex, researchers had been working on CSN stimulation for the treatment of drug-resistant hypertension. Recent pre-clinical data on CSN stimulation for the treatment of metabolic disorders will be addressed first and then the current and past trials for CSN stimulation for hypertension will be outlined.

#### **4.C.i – Bioelectronic medicine to treat metabolic disorders: The carotid sinus nerve – pre-clinical**

The CB is involved with the regulation of multiple physiological properties and due to this it is a natural target for bioelectronic medicine. In recent years, potentially due to the resurgence of bioelectronic medicine, the CB has been assessed in this regard. The first study to mention in this regard is the electrical activation of the rat CSN to prevent inflammation [141], previously outlined in section 3.C.iv. While the authors performed this experiment primarily as a physiological exploration of CB activity, it is interesting to consider the therapeutic implications of CSN stimulation. Regardless of whether or not the CB acts as an afferent sensor for the inflammatory reflex, there is a known connection between the hypothalamus and the CB [47]. Perhaps CSN stimulation could induce a more varied and potent anti-inflammatory effect than was reported in the original study [141].

The second study focuses on the treatment of type 2 diabetes and insulin resistance by bioelectronic medicine. There is enhanced CB activity in obese and insulin resistant rats [133], which engenders a surge in sympathetic outflow to the periphery, further influencing metabolic disorders [29]. Insulin resistance is one disorder, heavily influenced by overt CB activity. As insulin resistance develops, more insulin is required to activate partially unresponsive insulin receptors to increase the uptake of glucose into cells. This build-up of insulin presumably acts on the CB and induces increased CB activity, which causes sympathetic outflow and provokes the worsening insulin resistance [29]. Functional removal of the CB, via BCN, helps restore insulin sensitivity and tolerance to glucose [133, 140], ultimately allowing rats to be metabolically healthy while fat. It has been proposed that BCN in humans may be a good therapeutic for treating patients with drug resistant metabolic

disorders. This would engender, however, a host of additional problems considering the varied impact the CB is known to induce in the body.

Fortunately, there is a more elegant solution in the form of bioelectronic medicine, which was explored in the following study. Rats were obtained, electrodes were placed bilaterally on their CSNs and glued into place with physiological glue with the connector wires wrapping dorsally under the neck to a head cap connector. Rats were fed a high fat, high sucrose diet for 14 weeks to induce a type 2 diabetes-like disorder. Stimulation (1-2 mA, 50 KHz) of the rats was performed to block nervous activity. It was found that nine weeks of this stimulation pattern improved insulin sensitivity and glucose tolerance. Additionally, once stimulation stopped, insulin sensitivity and glucose tolerance returned to baseline levels after five weeks. Furthermore, it was assessed if this stimulation pattern induced long lasting impact on the CSN. The CSN of stimulated rats were unharmed and returned to full normal function one week after electrostimulation [139]. This study is interesting because it highlights the capabilities of bioelectronic medicine with the CSN - particularly, the fact that stimulation could be stopped allowing the CB to respond naturally again. This likely prevents alternative compensation mechanisms from developing. While it is particularly interesting that an electrode can be safely tolerated on the CSN, there is a concern that the functional blocking of the CB may impact on the additional roles the CB performs.

#### **4.C.ii – Bioelectronic medicine to treat hypertension: The carotid sinus nerve – clinical**

There was a considerable amount of human experimentation performed in the early 1970's with regards to CSN electrostimulation and hypertension. Initial results showed promise, but no consensus was achieved for the optimal methods for unilateral or bilateral placement, duration of stimulation or location of electrode. With the advent of powerful molecular drugs for treating hypertension; electrical stimulation of the CSN was mostly forgotten about for a few decades [142]. In the last decade, however, there been several promising clinical trials which attenuate hypertension via CSN electrostimulation [168]. Treatment of hypertension via CSN electrostimulation was first assessed acutely and it was determined that devices were safe to implant and their efficacy at lowering blood pressure was confirmed. One such clinical trial (NCT00442286), while successful, proved untenable as batteries had to be replaced for the devices every year [4]. The trial, however, did prove quite successful, with 75% of the 322

active participants reporting a decrease in their treatment resistant hypertension - with 55% of patients reaching their goal of systolic blood pressure  $\leq 140$  mmHg, which was maintained for more than 40 months, confirming long-term viability. The procedure was quite invasive as the generator was left in the participant's chest with leads to both carotid areas covering the CB, CSN and the carotid sinus [4]. A more minimalistic device was proposed which is currently being investigated [66].

This current device is called the Barostim *neo* system, it is a second-generation device for inducing baroreceptor activation therapy (BAT) to lower blood pressure – with an expected battery life of  $2.8 \pm 1.4$  years. The design of the Barostim *neo* system is minimalistic with a small pulse generator in the chest, externally activated via radiofrequency telemetry from a laptop program, connected to a wire with a small disk at the end sutured to the internal carotid artery above the baroreceptors in the carotid sinus. An initial trial using this device involved 30 participants with treatment-resistant hypertension. The participants had an average systolic blood pressure of  $172 \pm 20$  mm Hg and diastolic blood pressure of  $100 \pm 14$  mm Hg. After six months of stimulation, average systolic blood pressure was down  $26 \pm 4.4$  mm Hg, bringing 43% of patients below the desirable 140 mm Hg Systolic blood pressure. Furthermore, the device displayed remarkably few adverse side effects with 90% of participants stating no adverse side effects [66]. The Barostim *neo* system may prove to be an incredibly effective therapeutic for patients with treatment-resistant hypertension. Phase 2 and 3 clinical trials will have to be performed but initial results appear positive. Moving forward with these trials, a recent paper demonstrated that electrical activation of the CSN/carotid sinus not only induces baroreceptor signalling but also chemoreceptor signalling from the CB [76]. This could potentially be a serious concern and the device manufacturers will have to ensure there is no electrical spread from their device to the CB. Overall, BAT is an interesting approach to bioelectronic medicine and may yet prove to be a viable electroceutical.

#### **4.D.0 – The Future of Bioelectronic Medicine**

Bioelectronic medicine is a fascinating discipline. There is something particular about its potential simplicity that appeals to researchers. It is an elegant solution to a variety of physiological disorders. While, bioelectronic medicine is in the middle of a resurgence currently, there is a lot to discover before bioelectronic medicine can reach its full potential. The creation of an “atlas” of nerves has been proposed to ascertain the function of all nerves in the body. Once this has been developed there will be few remaining physiological disorders that cannot be treated by electronic medicine. New miniaturised devices will need to be designed and manufactured to stimulate individual nerve fibres within a nerve bundle to further reduce adverse side effects, potentially, even creating electrodes that function as chemoreceptors, as well as pulse generators. Perhaps, even the future of bioelectronic medicine may be non-invasive stimulation rather than direct placement on a nerve. There is a lot of new information to discover and my thesis plays its part in the required research to get electroceuticals into modern medicine.

# Hypothesis

It is our hypothesis that electrostimulation of the CSN will attenuate inflammation in mice.

## Aims of the investigation:

1. Discover location of CSN in mice and develop a successful isolation surgery.
2. Confirm physiologically the CSN was found.
3. Determine physiological relationship between the CB and inflammation via BCN.
4. Develop a method for CSN stimulation with best parameters.
5. Ascertain if electrostimulation of the CSN has an impact on pro-inflammatory cytokines.
6. Certify that CSN stimulation is independent of electrical spread to the vagus nerve.
7. Investigate if attenuated inflammation via CSN stimulation is an afferent source for the inflammatory reflex.
8. If CSN stimulation is independent of the inflammatory reflex, discover its true pathway.
9. Design a method for conscious stimulation of the CSN in mice.
10. Establish if previous results in semi-conscious mice can be replicated in conscious mice.
11. Observe if CSN stimulation enhances survivability to endotoxemic shock by lethal LPS injection.

# **Electrostimulation of the Carotid Sinus Nerve in Mice attenuates Inflammation via Glucocorticoid Receptor on Myeloid cells.**

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## **FOOTNOTES**

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## **Abstract**

The carotid bodies are polymodal sensors capable of detecting various physiological parameters that signal to brain via the afferent carotid sinus nerve (CSN) for physiological adjustment by efferent pathways. Recent evidence has suggested that there is a relationship between the immune system and the carotid body. We hypothesized that activation of the carotid body would attenuate inflammation. We found that electrical stimulation of CSN attenuated the expression of lipopolysaccharide (LPS)-induced pro-inflammatory cytokines, including Tumor Necrosis Factor (TNF). The inhibition of inflammation mediated by CSN was not mediated by efferent parasympathetic nor sympathetic nerves but rather acted through glucocorticoid receptor (GR). In particular, the stimulation of the CSN led to the increase of the spontaneous discharge activity of the hypothalamic paraventricular nucleus likening the CSN activity to the hypothalamic-pituitary adrenal axis. Finally, using a high dose of LPS, it was found that CSN electrostimulated mice were more likely to survive than control mice. These results provide a rationale for the use of CSN electrostimulation as a therapeutic approach for immune-mediated inflammatory diseases.

## Introduction

The immune system performs the critical role of defending the body against threats such as pathogens and cancerous cells. Occasionally, however the immune system can be too active and engender damage within the body. Fortunately, the immune system continuously regulates its pro-inflammatory response to prevent overt inflammation, in part, via regulatory T-cells and anti-inflammatory cytokines - which can be potent inhibitors of the immune response [8]. Dysregulation of the immune response can lead to disorders such as autoimmune disease and immune-mediated inflammatory diseases (IMIDs). IMIDs form a class of disparate disorders - osteoarthritis and inflammatory bowel disease, being two such examples [6]. In addition, to the immune system, to prevent immune disorders there are two main mechanisms – the hypothalamic-pituitary-adrenal (HPA) axis and the inflammatory reflex [19].

The HPA axis is the mechanism by which the body regulates glucocorticoid production – a hormone supremely capable at inducing immune system quiescence [24]. In brief, the HPA axis involves the paraventricular nucleus (PVN) of the hypothalamus releasing cortisol releasing hormone (CRH) into the anterior pituitary inducing a release of adrenocorticotrophic hormone (ACTH). ACTH engenders the release of glucocorticoid from the adrenal glands to mediate an attenuation of the immune response [24]. Whereas, the inflammatory reflex involves the vagus nerve sensing inflammation via an afferent source, signalling to the brain this detection of immune activity and inducing a reflex efferent response. This reflex efferent activity is mediated again by the vagus nerve, synapsing at the coeliac ganglion, which in turn activates sympathetic nerve fibres to the spleen. These sympathetic nerves release norepinephrine into the spleen activating a subset of T-cells, via the  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) that can produce acetylcholine (ACh) [2] – a known anti-inflammatory agent [25]. This locally produced ACh inhibits macrophages, via nicotinic ACh receptors (nAChR), ultimately reducing inflammation [2].

In recent years, efforts to convert this inflammatory reflex into a therapeutic have been conducted via electrical activation of the vagus nerve. This form of therapeutic can be described as bioelectronic medicine and clinical trials have been launched to investigate its anti-inflammatory properties against various IMIDs [5, 15]. Partially due to the studies on the inflammatory reflex, bioelectronic medicine has seen a resurgence in recent years [4]. There

is a lot of interest in discovering the anti-inflammatory potential of additional nerves. One interesting target is potentially the carotid body (CB) and its innervating nerve the carotid sinus nerve (CSN).

The CB is a paraganglia located bilaterally in the neck at the bifurcation of the carotid artery into the internal and external artery. The CSN runs afferently to the glossopharyngeal nerve [16]. The CB is a polymodal sensor and is capable of detecting oxygen concentration in the blood, blood pressure and insulin [7, 16]. Once these stimuli are detected, the CB signals via the CSN to augment these physiological variables as required. In recent years, it is becoming increasingly evident that the CB can detect inflammation. The CB can detect cytokines – TNF, IL-1B, IL-6 [9, 10, 22] – and bacterial components – lipopolysaccharide (LPS), zymosan [1, 10, 12]. This detection causes molecular activation of the CB [9, 22] as well as inducing signalling in the CSN [10, 14]. Additionally, in rats, it has been shown that the removal of the CSN bilaterally causes decreased survival in animals exposed to high bacterial load [20]. Furthermore, it has been suggested that the CB may be the afferent source for the vagal anti-inflammatory reflex [11] – there is even preliminary evidence to suggest this in rats [21]. Considering the evidence, it suggests that selective activation of the CSN may attenuate inflammation. It is our hypothesis that electrical activation of the CSN in mice will attenuate inflammation and protect against IMIDs.

## Methods

### Animals

C57BL/6 wildtype mice were routinely bought from Charles River, France at the age of 6-7 Weeks old. Mice were given a minimum of one week to acclimatise to the animal facility. All experiments were conducted on mice age 8-10 weeks old and exact age is indicated for each experiment. Lysm-Cre:GR<sup>fl/fl</sup> mice were bred in house and confirmed to be double positive by genomic PCR (GoTaq Green Master Mix, Promega). All Mice were given access to food and water *ad libitum* and maintained on a 12-hour light and dark schedule. Ethical approval was obtained and abided by for all procedures by Ethical Committee France.

### Reagents

Liquid isoflurane (Piramal, CSP, France) was used in an isoflurane machine (Anesteo). LPS was obtained and aliquoted to 5 mg/ml. The aliquots were frozen at -20 degrees Celsius and defrosted to be prepared fresh with PBS to a concentration of 100 µg in 200 µl IP. Morphine, Buprecare (Axience, Centravet, France), at 1 ml was mixed with 12 ml PBS and stored at 4 degrees Celsius to be used as required. Prior to surgery buprecare was injected at 100 µl IP, during recovery mice were injected 50 µl subcutaneously the following day. Dolethal (Vétoquinol, Centravet, France) was used to induce lethal unconsciousness prior to intracardial blood extraction. Hexamethonium (Sigma Aldrich) was used at a concentration of 10 mg/Kg in PBS, injected IP 30 minutes prior to LPS at 200 µl. Atropine (Sigma Aldrich) was injected IP (200 µl) at a concentration of 1 mg/Kg in PBS prior to LPS injection. Propranolol (Sigma Aldrich) was used at a concentration of 2.5 mg/Kg in PBS and injected IP 200 µl, 30 minutes prior to LPS. Mifepristone (Sigma Aldrich) was used a concentration of 80 mg/Kg in a PBS/DMSO mix (10%) (Sigma Aldrich).

### Carotid Sinus Nerve Electrostimulation – Semi-Conscious

The C57BL/6 mice were injected intraperitoneally (IP) with buprecare (100 µl – Axience, Centravet, France), as an analgesic, at least 10 minutes prior to surgery. Isoflurane (Piramal, CSP, France) mixed with air at 2.5% was used to induce mice into unconsciousness. Consciousness was tested by mice reflex – pinch on the tail, hind leg and foreleg – if no

response, surgery was commenced. Once unconscious was confirmed, mice were maintained at 2.5% isoflurane (Anesteo) for the course of surgery.

Prior to use surgical instruments were sterilized using a bead sterilizer (Steri 250, SimonKeller). The neck region of the mice was shaved using an electric razor. A sterile scalpel blade (Swann Moston) was used to open the mouse in parallel to the trachea. Skin, and underlying muscle was gently parted and held back by clamps. The left carotid artery was found and followed upwards to the branch of the internal and external carotid artery, additionally the meeting place of the carotid body artery. Continuing towards the brain, the hypoglossal nerve was located, which partially covers the carotid bifurcation. Furthermore, the muscle covering the hypoglossal nerve was located and cut solely. Following this, the hypoglossal was gently disassociated from the surrounding carotid sheath without damage and placed above the glossopharyngeal nerve. Underneath the hypoglossal lies the pharyngeal and glossopharyngeal nerve, typically running in parallel to each other. The CSN was located via its connection from the glossopharyngeal nerve to the CB. In sham and experimental mice, a nichrome electrode of two individual wires (A&M Systems) were placed correctly under and around the CSN. Experimental mice only received electrostimulation, sham mice did not. Electrical stimulation was administered by a Plexon stimulator (PlexStim Electrical Stimulator System) at 200-600  $\mu$ A, 5-10 Hz for 2 minutes, 5 minutes before and after a 100  $\mu$ g LPS (Sigma Aldrich) IP injection of 200  $\mu$ l. Exact stimulation patterns are outlined per experiment. To confirm location of the CSN, breath rate was recorded before and during stimulation – to ensure the electrode was on the CSN. Once stimulation was complete, mice were sutured and allowed to recover in a heated cage. Blood was collected 90 minutes after LPS injection retro-orbitally. Serum was obtained via centrifugation (15 minutes – 10 000 RPM) and stored at -20 degrees Celsius for assay analysis.

In particular experiments, additional measures were performed as outlined (SFig.1 - C). Receptor Blocking drugs were used – Hexamethonium (10 mg/Kg), Atropine (1 mg/Kg), Propranolol (2.5 mg/Kg) and Mifepristone (80 mg/Kg) – at 200  $\mu$ l IP, 30 minutes prior to LPS injection. Additionally, in specific, outlined, instances the spleen, adrenal glands and cervical vagus was excised. The spleen excision was performed by tying a suture knot into the three major blood vessels into the spleen and then gently separating out the spleen. In a separate experiment, the bilateral adrenal glands were specifically cauterised by a biological cauterising tool. In another separate experiment, prior to CSN stimulation the cervical vagus

nerve closest was excised unilaterally. Unilateral excision of the cervical vagus was performed as bilateral cervical vagus excision will kill mice. All mice were adequately sutured as required.

### **Vagus Stimulation**

In one instance vagus nerve stimulation was performed. Similar methods as described for CSN stimulation were used, except the vagus was isolated and dual nichrome wires were placed underneath it. Electrical stimulation was as before - two minutes (600  $\mu$ A, 10 Hz), five minutes before and after an IP LPS (100  $\mu$ g) injection of 200  $\mu$ l. Animals were allowed to recover as before. Additionally, 30 minutes prior to LPS injection hexamethonium (10 mg/Kg) or sham PBS was IP injected (200  $\mu$ l) into the mice.

### **Paraventricular Nucleus Recording**

C57BL/6 Mice of minimum 20 g were obtained. Mice were anaesthetised with ketamine (130 mg/kg, Imalgene 1000) and xylazine (10 mg/kg) injection IP. Once, unconsciousness was confirmed through hind and fore paw pinch surgery was commenced. The CSN was isolated in a similar manner to as described, physiological glue was used to ensure electrodes remained in place during the stereotaxic surgery. The mice were placed in a stereotaxic frame (SR-6, Narishige, Japan) in which the head of the animal was fixed with a pair of ear bars and a perpendicular tooth bar. Heads were shaved with vetedine (Vétoquinol, Centramet, France), prior to a skin incision was made along the sagittal midline of the scalp. Craniotomy was made to target our structures of interest the hypothalamic PVN. Craniotomy windows were repeatedly superfused with physiological saline solution. Stereotaxic coordinates for the PVN were determined from the Paxinos and Watson rat brain atlas (2013) and stainless-steel recording electrodes (platinum/iridium wires) were implanted, under aseptic conditions, into the area of the PVN – which was confirmed by dye placement. A baseline recording of PVN activity for five minutes was recorded and subsequently continuous stimulation (600  $\mu$ A, 10 Hz, 0.1 ms) of the CSN was started and PVN activity was recorded for five minutes using a Multichannel system (Multi Channel Systems MCS GmbH, Germany). Directly after stimulation, the mice were removed from the stereotaxic frame and cervical dislocation was

performed. The brain was removed and immediately sectioned using a vibratome, to view dye placement in the sections.

### **Conscious Stimulation**

Similarly, to semi-conscious stimulation, C57BL/6 mice were injected IP with morphine (100  $\mu$ l), as an analgesic, at least 10 minutes prior to surgery. Isoflurane mixed with air at 2.5% was used to induce mice into unconsciousness. Consciousness was tested by mice reflex – pinch on the tail, hind leg and foreleg – if no response, surgery was commenced. Once unconscious was confirmed, mice were usually maintained at 2.5% isoflurane for the course of surgery, but occasionally if the course of the surgery continued beyond 40 minutes, isoflurane concentration was lowered to ease recovery.

Prior to use surgical instruments were sterilized using a bead sterilizer. Mice were shaved at the neck and skull, subsequently these areas were cleaned with 70% ethanol and vetedine (Vétoquinol, Centravet, France). A small circular cut was made in the skin above the skull, the underlying tissue was cleaned, and the skull exposed. On the skull, many delicate tracings were made with a sterile scalpel to aid later head cap installation. The open skull was covered with sterile gauze prior to a midline cervical incision, parallel to the trachea. The electrode was placed underneath the skin connecting the frontal incision with circular cut on the skull. The CSN was exposed as already described and the electrode of nichrome wires were put underneath the CSN in a similar manner as before. An additional step, to ensure electrode placement was for the wires to be more greatly curved, this enabled further anchoring of the physiological glue (Kwik-cast & Kwik-sil, World Precision Instruments) to ensure the wires were kept safely in place for the required period of time. The animal was cervically sutured and gently flipped to place dental cement (Super Bond C&B) around the electrode end to build the head cap (SFig.2 – C-D), binding was allowed to occur for a few minutes prior to placing the mouse in a heated cage for recovery.

The following day, mice were given 50  $\mu$ l morphine subcutaneously and the subsequent two days were undisturbed. On the fourth day, mice were placed into individual cages and a stimulating wire was connected to their head caps. Mice were stimulated (200  $\mu$ A, 5 Hz) for two minutes, five minutes before and after an IP LPS (100  $\mu$ g) injection of 200  $\mu$ l while consciously roaming around their cage. Sham mice received the same treatment without the

stimulation. All mice once finished were returned to the mother cage and were left undisturbed until 90 minutes after LPS injection. At 90 minutes, mice were given dolethal (Vétoquinol, Centravet, France) and once confirmed to be unconscious blood was extracted intracardially. Blood was centrifuged (15 minutes, 10 000 RPM) for serum and was stored at -20 degrees Celsius for assay analysis.

In one instance, survival to lethal LPS injection (1 mg) was assessed. Conscious stimulation surgery was performed as described, following timeline G (SFig.1 - G). For this experiment, however, on the first day mice were stimulated (200  $\mu$ A, 5 Hz, 0.1 ms) for five minutes at 11:00 and again at 18:00. LPS was injected at 13:00, mice were watched for the next 72 hours and when a mouse died the time was noted.

### **Assays**

As outlined previously, blood was collected for serum analysis. Routinely, a TNF ELISA (DY410 R&D Systems) was conducted as described within the kit. Corticosterone was additionally measured using a kit (ADI-900-097, Enzo LifeSciences). Finally, a meso scale discovery kit (V-PLEX Plus Proinflammatory Panel1 Mouse Kit) was used to assess a wide variety of cytokines.

### **Statistics**

In all instances, except the survival study, experiments were replicated (n = 2) and in some instances twice (n =3). Typically, all experiments contained 6 – 8 animals per group. Data is pooled and individual points represent one animal, results are expressed as means  $\pm$  standard deviation (SD). Student's *t*-test was used if results were shown to have a normal distribution (Shapiro-wilks test), if not a Mann-Whitney test was used. When comparison between more than two columns was necessary, One-way ANOVA was performed. In all instances  $p < 0.05$  is represented by \*,  $p < 0.01$  - \*\*,  $p < 0.001$  - \*\*\* and  $p < 0.0001$  - \*\*\*\*.

## Results

### **Electrical stimulation of the CSN attenuates LPS-induced production of pro-inflammatory cytokines independently of the cholinergic anti-inflammatory reflex.**

Preliminary evidence in rats has shown that the CB is fulfilling the role of afferent sensor for inflammation [21]. The CSN was isolated in mice and electrodes were placed underneath (SFig.2 – A - B). To confirm location and electrode placement breath rate was assessed before and after stimulation. With stimulation there was a significant increase in breath rate (Fig.1 – A:  $p < 0.001$ , \*\*\*, paired students *t*-test,  $n = 7$ ), which returned to baseline levels. We further investigated if electrical activation of the CSN in mice attenuated inflammation and the mechanism by which CSN stimulation is mediating a decrease in inflammation. Timeline A (SFig.1 - A) was used for these experiments. Stimulation of the CSN significantly reduced the LPS-dependent production of TNF (Fig.1 - B:  $p < 0.0001$ , \*\*\*\*, students *t*-test,  $n = 20-21$ ), as well as for IL-1 $\beta$  (Fig.1 - C), IL-6 (Fig.1 - D), IL-12p70 (Fig.1 - E) and IL-2 (Fig.1 - F). Furthermore, this result was replicable with the presence of oil (SFig.3 - A:  $p < 0.01$ , \*\*, Students *t*-test,  $n = 19$ ) and, following timeline B (SFig.1 – B), unilateral vagus excision (SFig.3 – B:  $p < 0.05$ , \*, Students *t*-test,  $n = 10-12$ ). Atropine (1 mg/Kg), a Muscarinic ACh receptor (mAChR) antagonist, hexamethonium (10 mg/Kg), a nicotinic ACh receptor (nAChR) antagonist, and propranolol (2.5 mg/Kg), a  $\beta$  adrenergic receptor blocker, were used to assess the mechanism of action of CSN stimulation – following timeline C (SFig.1 - C). The attenuation of LPS-induced TNF production was not prevented by propranolol (Fig.1 - G:  $p < 0.01$ , \*\*, Students *t*-test,  $n = 14$ ), atropine (Fig.1 - H:  $p < 0.01$ , \*\*, One-way ANOVA,  $n = 13-15$ ) nor hexamethonium (Fig.1 - H:  $p < 0.001$ , \*\*\*, One-way ANOVA,  $n = 12-13$ ). Hexamethonium was further assessed, following Timeline D (SFig.1 - D), the vagus was stimulated, which significantly attenuated LPS-dependent TNF production (SFig.4 -A:  $p < 0.001$ , \*\*\*, One-way ANOVA,  $n = 12-13$ ), however hexamethonium prevented this decrease in inflammation (SFig.4 – A:  $p > 0.05$ , One-Way ANOVA,  $n = 12-13$ ). Following timeline B (SFig.1 - B), the spleen was removed and the CSN was stimulated. Two experiments were performed and normalised to mean control at 100%. TNF concentration was decreased by CSN stimulation in the absence of the spleen (SFig.5 – A:  $p < 0.05$ , \*, Students *t*-test,  $n = 14$ ). Overall these results indicate that the effect of CSN stimulation is independent of the cholinergic anti-inflammatory reflex.

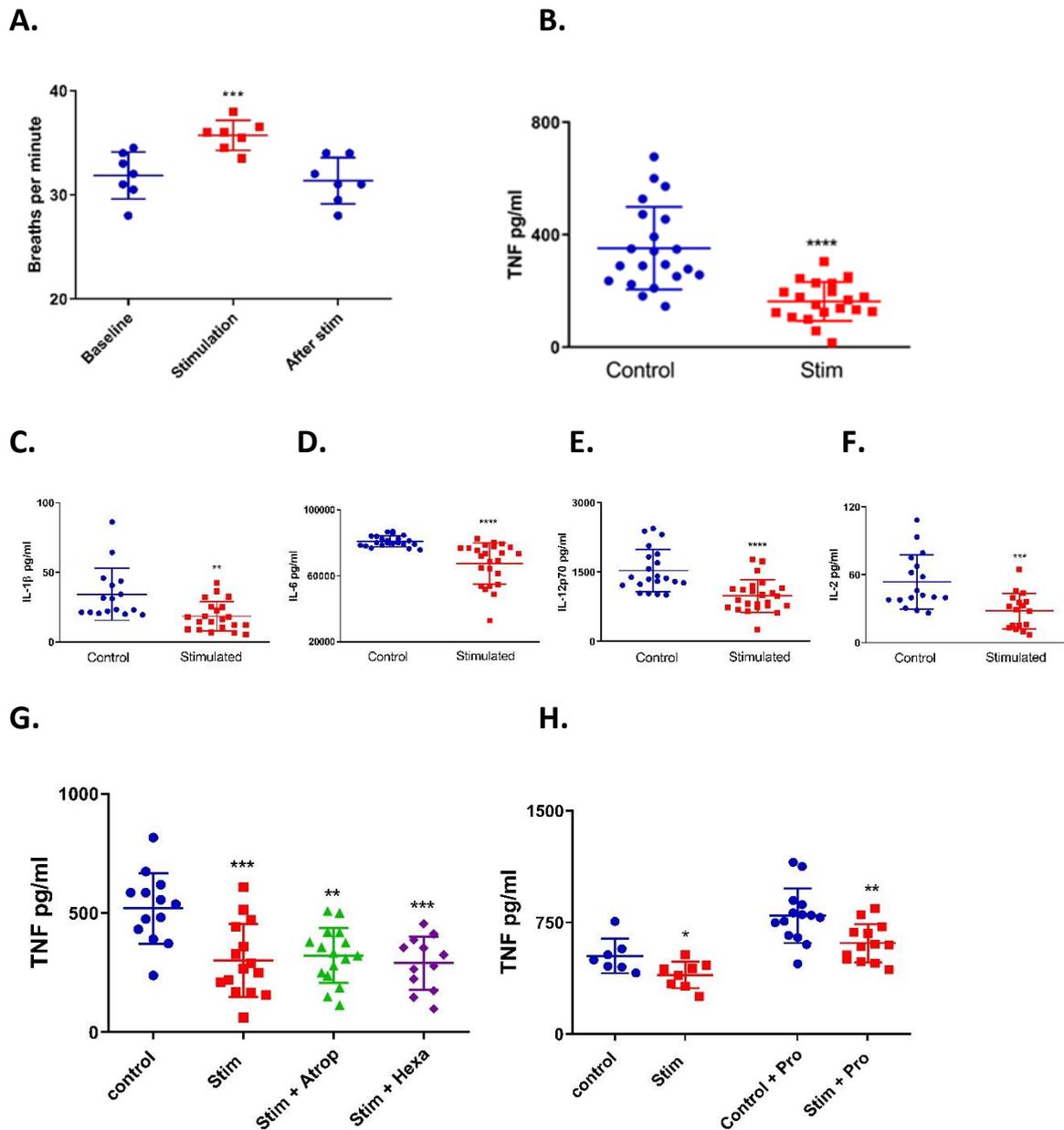
**CSN stimulation increases corticosterone, which acts via the GR on myeloid immune cells to attenuate pro-inflammatory cytokines.**

Since the attenuated production of LPS-induced TNF via CSN stimulation is not associated with current inflammatory reflex theories, we considered how else CSN stimulation may be instigating its effect. The HPA axis seemed a likely alternative so we investigated if CSN stimulation was involved with the axis. It was first assessed, using timeline B (SFig.1 - B), if the adrenal glands were necessary for the effect of CSN stimulation. It was found that the adrenal glands were necessary for the CSN stimulation-mediated decrease in LPS-induced TNF (SFig.6 – A:  $p < 0.05$ , Students *t*-test,  $n = 19-20$ ). Following this result, using timeline A (SFig.1 - A), it was assessed if CSN stimulation altered Corticosterone. CSN stimulation significantly increases the production of Corticosterone (Fig.2 – A:  $p < 0.01$ , \*\*, Students *t*-test,  $n = 21-24$ ). Mifepristone (80 mg/Kg) a glucocorticoid receptor (GR) antagonist, using timeline C (SFig.1 - C), was administered to mice and it was found that the GR was required for CSN stimulation to decrease LPS-dependent TNF production (Fig.2 – B:  $p > 0.05$ , Students *t*-test,  $n = 30-31$ ). To test whether GR signalling in myeloid cells was necessary to mediate the anti-inflammatory effect of CSN electrostimulation, we used LysM-Cre:GR<sup>fl/fl</sup> transgenic mice in which myeloid cells are selectively deficient in GR. Following timeline A (SFig.1 - A); their littermate controls, GR<sup>fl/fl</sup>, responded positively to CSN stimulation (Fig.2 – C:  $p < 0.01$ , \*\*, Mann-Whitney,  $n = 13-14$ ), whereas the LysM-Cre:GR<sup>fl/fl</sup> mice did not (Fig.2 – C:  $p > 0.05$ , Students *t*-test,  $n = 17-18$ ). Following timeline E, (SFig.1 - E), it was assessed if CSN stimulation was activating the PVN of the hypothalamus. Mice that were receiving electrical stimulation of the CSN were determined to have increased activity in their PVN (Fig.2 – D:  $p < 0.01$ , \*\*, Mann-Whitney,  $n = 7$ ). Example traces of baseline and CSN-stimulated PVN activity are shown (SFig.7 - A) and representative images of the marked recording location in the PVN of the hypothalamus (SFig.7 – B - C). Altogether it is evident that CSN stimulation is activating the PVN which initiates the HPA axis response and ultimately causes increased production of corticosterone, which in turn acts on the GR of myeloid cells.

**CSN stimulation in conscious animals attenuates LPS-induced production of TNF and increases survival to endotoxemic shock.**

It is well documented that anaesthesia causes an anti-inflammatory effect [13], therefore it

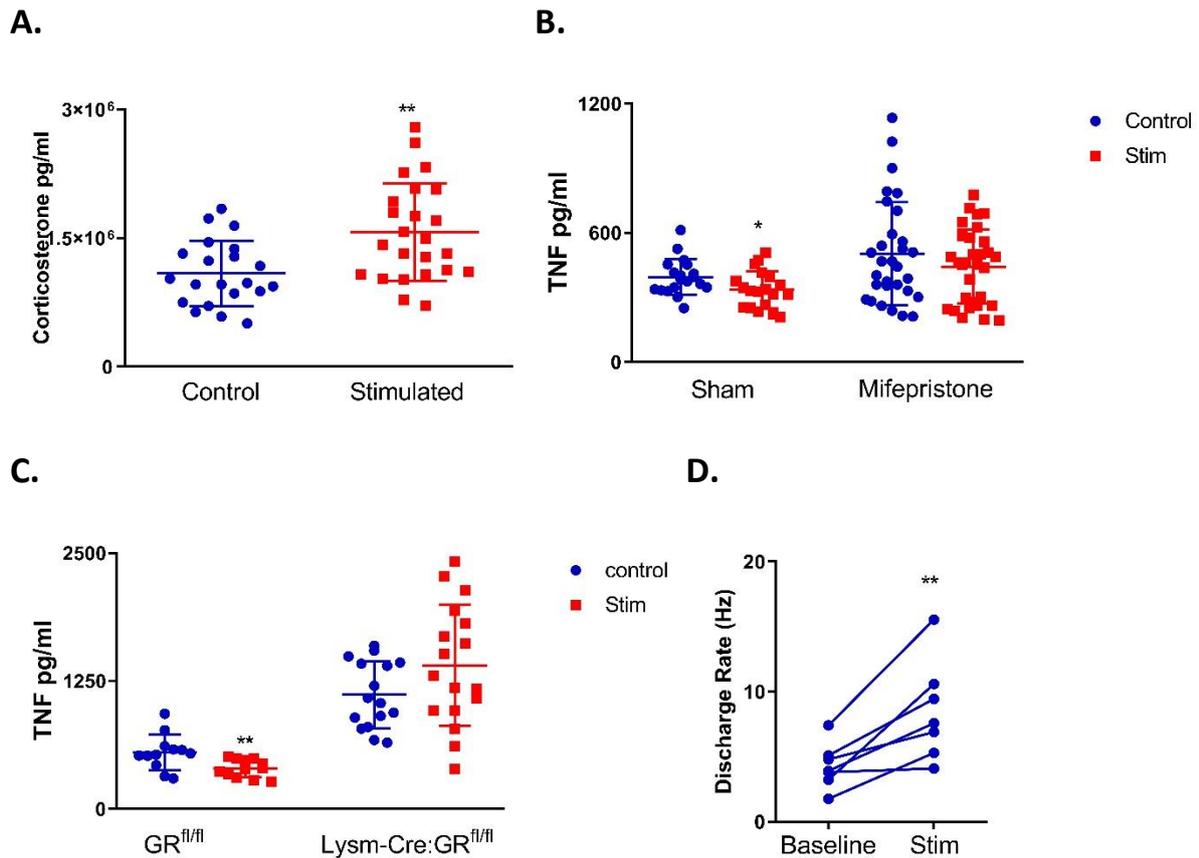
was necessary to confirm the previous results in fully conscious, non-anaesthetised animals. Following timeline F (SFig.1 - F) for conscious stimulation surgery, wildtype mice were used to determine if the decrease in pro-inflammatory cytokines could be replicated during conscious stimulation, similar to unconscious animals (Fig.1 - B). CSN stimulation in conscious animals significantly decreased TNF concentration in the blood (Fig.3 -A:  $p < 0.001$ , \*\*\*, Mann-Whitney,  $n = 23-29$ ). Furthermore, to replicate our results obtained in unconscious Lysm-Cre:GR<sup>fl/fl</sup> mice (Fig.2 - C): Lysm-Cre:GR<sup>fl/fl</sup> mice and their littermate controls, GR<sup>fl/fl</sup>, were obtained and stimulated or not. While the littermate controls demonstrated a tendency to a decrease in TNF concentration following CSN electrostimulation although not significant (Fig.3 – B:  $p = 0.057$ , Mann-Whitney,  $n = 3-4$ ), whereas CSN-stimulated Lysm-Cre:GR<sup>fl/fl</sup> did not show a decrease, significant or otherwise, in TNF concentration compared to sham stimulated mice (Fig.3 – B:  $p > 0.05$ , Mann-Whitney,  $n = 6$ ). Knowing that conscious stimulation was effective at decreasing TNF concentration in the blood, it was assessed if this decreased presence of TNF translates into physiological benefit. Following timeline G (SFig.1 - G), mice had electrodes implanted and were stimulated twice a day for three days. Additionally, on the first day, mice were injected with a lethal dose of LPS (1 mg). Conscious CSN stimulation of mice protected against endotoxemic shock induced death (Fig.3 – C:  $p < 0.01$ , \*, Gehan-Breslow-Wilcoxon test,  $n = 7-9$ ). Overall, CSN stimulation may prove to be a successful therapeutic for inflammatory disorders.



**Figure 1: CSN electrostimulation attenuates production of pro-inflammatory cytokines.**

C57BL/6 mice were obtained, 8-10 weeks of age, and CSN isolation surgery was performed, followed by electrical stimulation 200  $\mu$ A + 5 Hz (A) and 400-600  $\mu$ A + 10 Hz (B-H). Prior to surgery sham PBS or Atropine (1 mg/Kg), Hexamethonium (10 mg/Kg) or Propranolol (2.5 mg/Kg) were administered IP (200  $\mu$ l – G-H). Blood was collected 90 minutes after an IP LPS injection (100  $\mu$ g – 200  $\mu$ l) for serum analysis by ELISA and Meso scale discovery. **A.** Stimulation induced an increase in breath rate ( $p < 0.001$ , \*\*\*, paired students  $t$ -test,  $n = 7$ ), which returned to baseline levels after. **B.** Electrical activation of the CSN significantly ( $p < 0.0001$ , \*\*\*\*, Students  $t$ -test,  $n = 20-21$ ) decreases LPS induced production of TNF and (**C**) IL-6 ( $p < 0.0001$ , \*\*\*\*, Mann-Whitney,  $n = 22-24$ ), (**D**) IL-1 $\beta$  ( $p < 0.01$ , \*\*, Mann-Whitney,  $n = 16-$

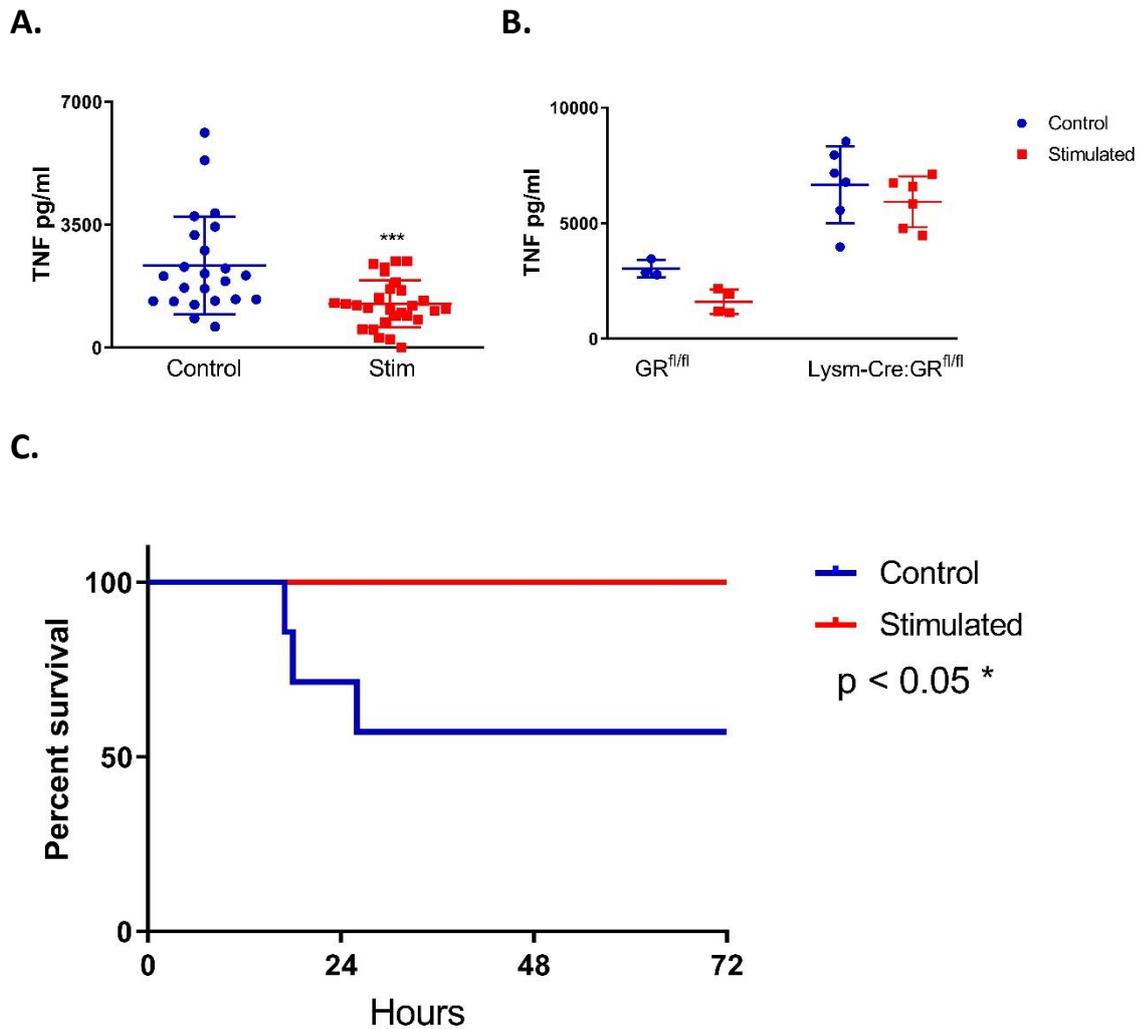
21), (E) IL-12p70 ( $p < 0.001$ , \*\*\*, Mann-Whitney,  $n = 21-24$ ) and (F) IL-2 ( $p < 0.001$ , \*\*\*, Students  $t$ -test,  $n = 17-18$ ). G. Electrical activation of the CSN attenuated the LPS induced production of TNF ( $p < 0.001$ , \*\*\*, One-way ANOVA,  $n = 13-15$ ) and this effect was not blocked by atropine ( $p < 0.01$ , \*\*, One-way ANOVA,  $n = 13-15$ ) nor hexamethonium ( $p < 0.001$ , \*\*\*, One-way ANOVA,  $n = 12-13$ ). H. CSN stimulation attenuated LPS induced TNF production regardless of propranolol ( $p < 0.01$ , \*\*, Students  $t$ -test,  $n = 14$ ). Each individual point represents an animal and data is expressed as means  $\pm$  SD.



**Figure 2: CSN electrostimulation increases corticosterone, which attenuates inflammation via the glucocorticoid receptor of myeloid cells.**

C57BL/6 mice (A, B, D) or  $Lysm-Cre:GR^{fl/fl}$  mice (C) were obtained, aged 8-10 weeks, and CSN isolation and stimulation (600  $\mu A$  + 10 Hz) were conducted. An LPS IP injection (100  $\mu g$  – 200  $\mu l$ ) was administered in all animals, 90 minutes later blood was collected for serum analysis by assay (A-C). **A.** The CSN was electrically stimulated, this stimulation significantly increased the production of corticosterone ( $p < 0.01$ , \*\*, Students  $t$ -test,  $n = 21-24$ ). **B.** Mifepristone or sham 10% DMSO was administered prior to CSN stimulation (200  $\mu l$ , IP). The sham group, receiving electrostimulation still elicited a significant decrease ( $p < 0.05$ , \*, Students  $t$ -test,  $n = 19-21$ ), however in the group receiving mifepristone, this attenuation by electrostimulation was not apparent ( $p > 0.05$ , Students  $t$ -test,  $n = 30-31$ ). **C.**  $Lysm-Cre:GR^{fl/fl}$  mice and their littermate controls,  $GR^{fl/fl}$ , were obtained and both received electrostimulation of the CSN. The littermate controls receiving electrostimulation had significantly attenuated production of LPS-dependent TNF ( $p < 0.01$ , \*\*, Mann-Whitney,  $n = 13-14$ ), however this decrease was not present in  $Lysm-Cre:GR^{fl/fl}$  mice ( $p > 0.05$ , Students  $t$ -test,  $n = 17-18$ ). **D.** Mice were fixed

into a stereotaxic frame and PVN recordings were taken during stimulation of the CSN. The average discharge was acquired per minute for baseline recordings and during stimulation. CSN stimulation significantly increased the activity of the PVN ( $p < 0.01$ , \*\*, Mann-Whitney,  $n = 7$ ). All individual traces, or points, represent one animal. Means are represented as  $\pm$  SD.



**Figure 3: Conscious CSN stimulation is protective against LPS-induced shock.**

C57BL/6 mice were acquired, 8-10 weeks of age, and underwent surgery 4 days prior to stimulation. Stimulation (200  $\mu$ A + 5 Hz) was of free-moving mice and LPS (100  $\mu$ g - 200  $\mu$ l) was injected IP. **A.** CSN stimulation in conscious animals significantly reduced TNF concentration ( $p < 0.001$ , \*\*\*, Mann-Whitney,  $n = 23-29$ ). **B.** Lysm-Cre:GR<sup>fl/fl</sup> mice and littermate controls, aged 12 weeks, underwent the same procedure. Lysm-Cre:GR<sup>fl/fl</sup> mice receiving conscious CSN stimulation were determined to not have decreased concentrations of LPS-induced TNF ( $p > 0.05$ , Mann-Whitney,  $n = 6$ ) compared to sham stimulated mice. **C.** C57BL/6 mice were acquired, 10-12 weeks of age, were obtained. CSN Conscious stimulation surgery was performed and mice were allowed to recover. Subsequently, mice were transferred to individual cages and stimulated (200  $\mu$ A, 5 Hz, 0.01 mS, 5 Minutes) twice a day for the next three days. A lethal dose of LPS was administered to the mice (1 mg, 200  $\mu$ l) IP. Animal survival was routinely monitored and recorded. Stimulated mice demonstrated an

improved ability to survive a lethal injection of LPS compared to control mice ( $p < 0.01$ , \*, Gehan-Breslow-Wilcoxon test,  $n = 7-9$ ). All individual points represent one animal and means are expressed as  $\pm$  SD.

## Discussion

CSN stimulation attenuates the production of pro-inflammatory cytokines via the increased production of corticosterone in mice, acting on the GR of myeloid cells. Interestingly, this evoked response translates to physiological benefit and helps protect mice from endotoxemic shock.

We first verified if the CSN was discovered in mice via electrical activation of the nerve. It was determined that CSN location was found via an increase in breathing during electrostimulation of the CSN, which returned to baseline breath rate immediately after stimulation (Fig.1 -A). Having discovered the CSN, using timeline A (SFig.1 - A) it was assessed if CSN electrostimulation would mitigate inflammation. It was found that LPS-induced TNF production was significantly decreased because of CSN stimulation (Fig.1 - B). Furthermore, CSN stimulation decreased the expression of LPS-induced IL-1 $\beta$  (Fig.1 - C), IL-6 (Fig.1 - D), IL-12p70 (Fig.1 - E) and IL-2 (Fig.1 - F). Importantly, the effect of CSN stimulation was demonstrated to be independent of electrical spread (SFig.3 - A) to the vagus nerve (SFig.3 - B).

It has been suggested that the afferent source for the vagal anti-inflammatory reflex is the CB [11]. The CB as the instigator of the inflammatory reflex is an enticing possibility, which was investigated by one group. The authors demonstrated that electrical activation of the rat CSN attenuates inflammation dependently on nAChR and  $\beta_2$ AR, they went on to conclude that the CB is the afferent source of the vagal anti-inflammatory reflex [21]. Regarding this study, we investigated the mechanism by which CSN stimulation mitigates inflammation. In mice, we demonstrated that the effect of CSN stimulation is independent of both nAChR and  $\beta_2$ AR, as well as mAChR (Fig.1 - G - H). Furthermore, we confirmed that our nAChR antagonist, hexamethonium, was functional because it could prevent the anti-inflammatory effect of vagus nerve stimulation (SFig.4 - A). Additionally, the effect of CSN stimulation in mice is mostly independent of the spleen (SFig.5 -A). It is evident that in mice the evoked decrease of anti-inflammatory cytokines is distinct of the known vagal anti-inflammatory reflex.

The reason for this difference between two close species is unknown and little research has investigated the mouse CB, however what is known suggests fundamental difference in cytokine receptor profiles between mice and rats [17, 26, 27]. Perhaps the discrepancy in results is just species differences. Alternatively, the methods chosen by the authors who

demonstrated that rat CSN stimulation attenuates inflammation via the inflammatory reflex are questionable [21]. The authors have chosen a stronger than necessary signal and a protracted duration for activating a nerve. Regarding this information, and their placement for electrode implantation it increases the potential for electrical spread to the vagus nerve. Ideally, the authors should have performed a unilateral removal of the vagus nerve to confirm their attenuation of inflammation by rat CSN electrostimulation was independent of the vagus nerve.

Considering that CSN stimulation in mice is independent of the vagal anti-inflammatory reflex, we investigated other potential mechanisms for the effect of CSN stimulation. One possible mechanism for the attenuation of pro-inflammatory cytokines in mice by CSN electrostimulation is the HPA axis. There is evidence of a connection between the CB and the PVN of the hypothalamus [3, 18, 23] – perhaps electrical activation of the CSN activates the PVN and instigates the HPA axis. It was first demonstrated that bilateral cauterisation of the adrenal glands prevented the effect of CSN stimulation (SFig.6 - A). Furthermore, it was shown that CSN electrostimulation is increasing serum corticosterone concentration (Fig.2 - A). This result suggests that corticosterone is the ultimate mediator of the effect of CSN stimulation, which was confirmed when mifepristone prevented the anti-inflammatory effect of CSN stimulation (Fig.2 - B). As mifepristone is a general antagonist of GR, it provides no information on the location of action for the CSN stimulation-mediated increase in corticosterone. We used a transgenic mouse model, in which all Lysm-containing cells, primarily myeloid cells – such as macrophages, had no functioning GR. *Lysm-Cre:GR<sup>fl/fl</sup>* mice no longer responded to the effect of CSN electrostimulation, however their littermate controls, *GR<sup>fl/fl</sup>*, did (Fig.2 - C). Overall, these results confirm that CSN stimulation acts via corticosterone activating GR on myeloid cells.

As referred to previously, it is known that there is a connection between the CB and the PVN [3, 18, 23]. The evidence, however, is not incredibly convincing and we wanted to confirm that CSN electrostimulation instigates the HPA axis by increasing activity in the PVN. Subsequently, we developed an experiment following timeline E (SFig.1 - E) that put mice in a stereotaxic frame with a recording electrode in their PVN. Additionally, implantation of an electrode on the CSN was performed. A baseline recording of PVN activity was taken. Directly following this, CSN stimulation was initiated while PVN activity was being recorded. It was discovered that CSN electrostimulation was increasing the electrical activity of the PVN (Fig.2

-D). This result confirms the link between the CB and the PVN, but also verifies the mechanism by which CSN electrostimulation in mice inhibits inflammation. CSN stimulation induces activity in the PVN which instigates the HPA axis and ultimate increase in corticosterone, which in turn mitigates inflammation by activating the GR of myeloid cells.

It is well known that anaesthesia decreases immune activity [13]. Therefore, it was a concern with all results described so far that anaesthesia may have been influencing the outcome of CSN electrostimulation. A method for conscious stimulation of the mouse CSN was developed and following timeline F (SFig.1 - F) it was demonstrated that conscious CSN stimulation can attenuate LPS-induced TNF production (Fig.3 - A). Additionally, it was investigated if CSN stimulation in *Lysm-Cre:GR<sup>fl/fl</sup>* produced an effect. It was found that there was no difference between sham-stimulated and CSN stimulated *Lysm-Cre:GR<sup>fl/fl</sup>* mice (Fig.3 - B). Indicating that the pathway for the effect of conscious CSN stimulation is concordant with that for semi-conscious stimulation.

We additionally investigated if this mitigation of inflammation by CSN electrostimulation could translate to physiological benefit - using a model of endotoxemic shock by lethal LPS injection. Following timeline G, mice were stimulated twice daily for five minutes over three days, on the first day having received a lethal LPS injection. It was discovered that CSN stimulation significantly increased the chance of survival in mice (Fig.3 - C). Endotoxemic shock is representative of immune system dysregulation and as CSN stimulation can endow protection onto mice against it, it suggests that CSN stimulation may prove effective against additional IMIDs.

The attenuation of inflammation by CSN stimulation is particularly exciting because it is the first instance of a potential anti-inflammatory bioelectronic medicine pathway using the HPA axis. Due to this, it is likely that an electroceutical, bioelectronic medicine therapeutic, would be particularly interesting for patients requiring long-term administration of glucocorticoids. If evoked use of endogenous glucocorticoids by CSN stimulation proved as effective as exogenous glucocorticoids, perhaps this electroceutical would be adapted clinically. Further studies will have to be performed, but the initial results prove positive for CSN stimulation.

In conclusion, CSN stimulation in mice activates the HPA axis enhancing the production of corticosterone, which in turn activates GR on myeloid immune cells ultimately mediating a

decrease in inflammation, which aids survival in mice against endotoxemic shock by lethal LPS injection. Further studies will be required but CSN stimulation may prove to be a viable therapeutic and aid long-term users of glucocorticoid.

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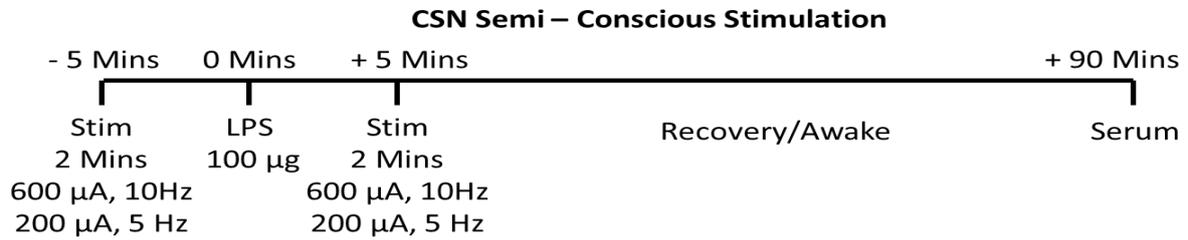
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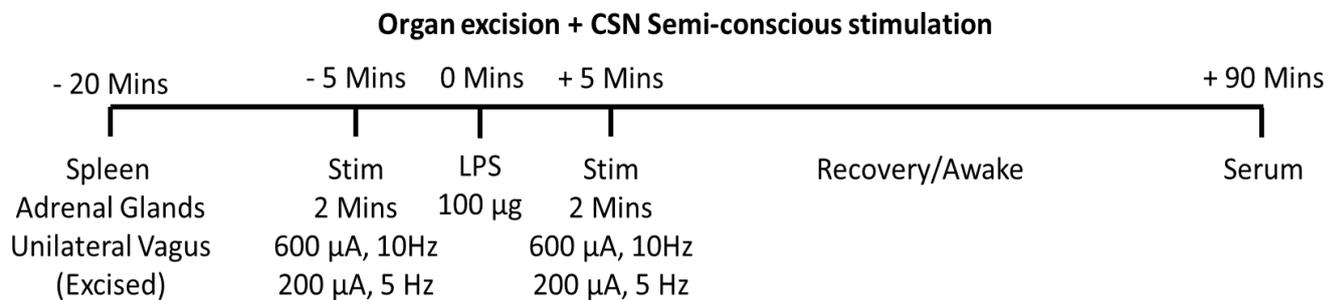
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# Supplementary Figures

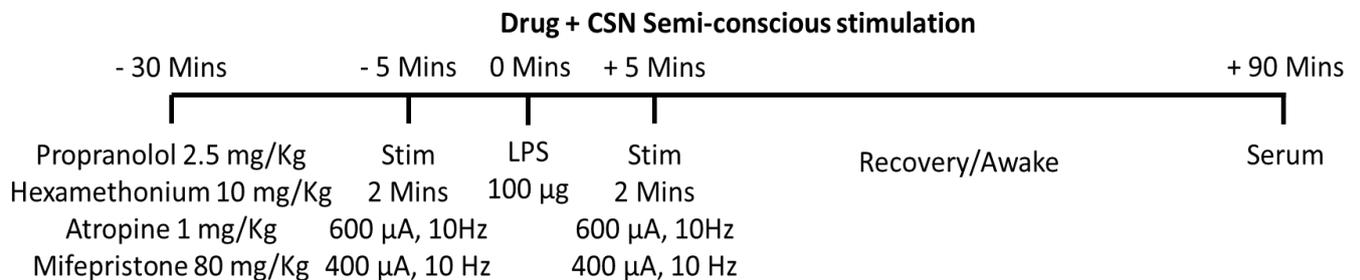
**A.**



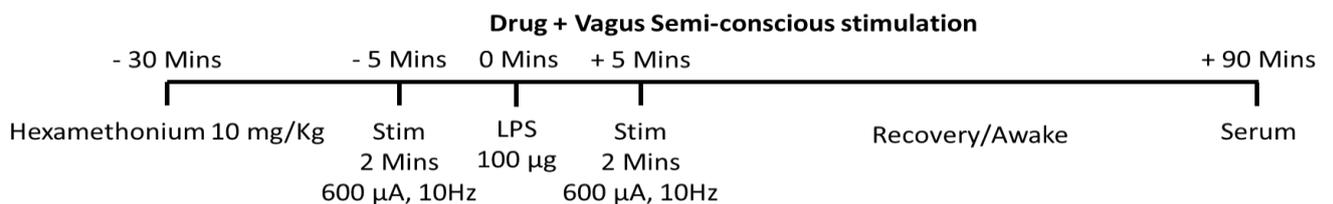
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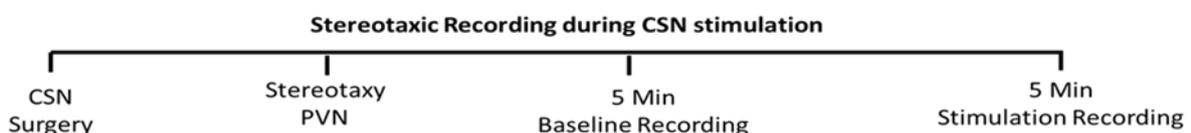
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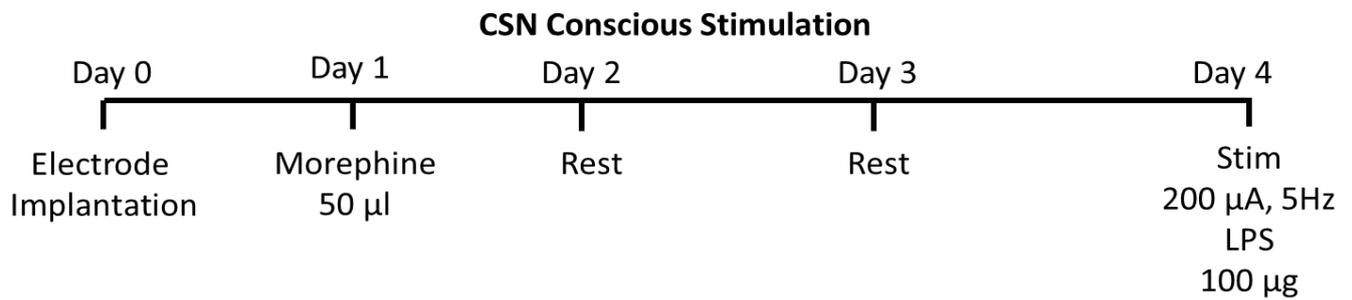
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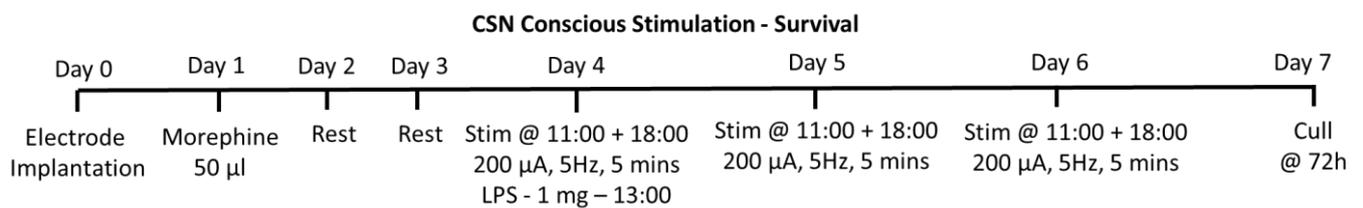
**E.**



**F.**

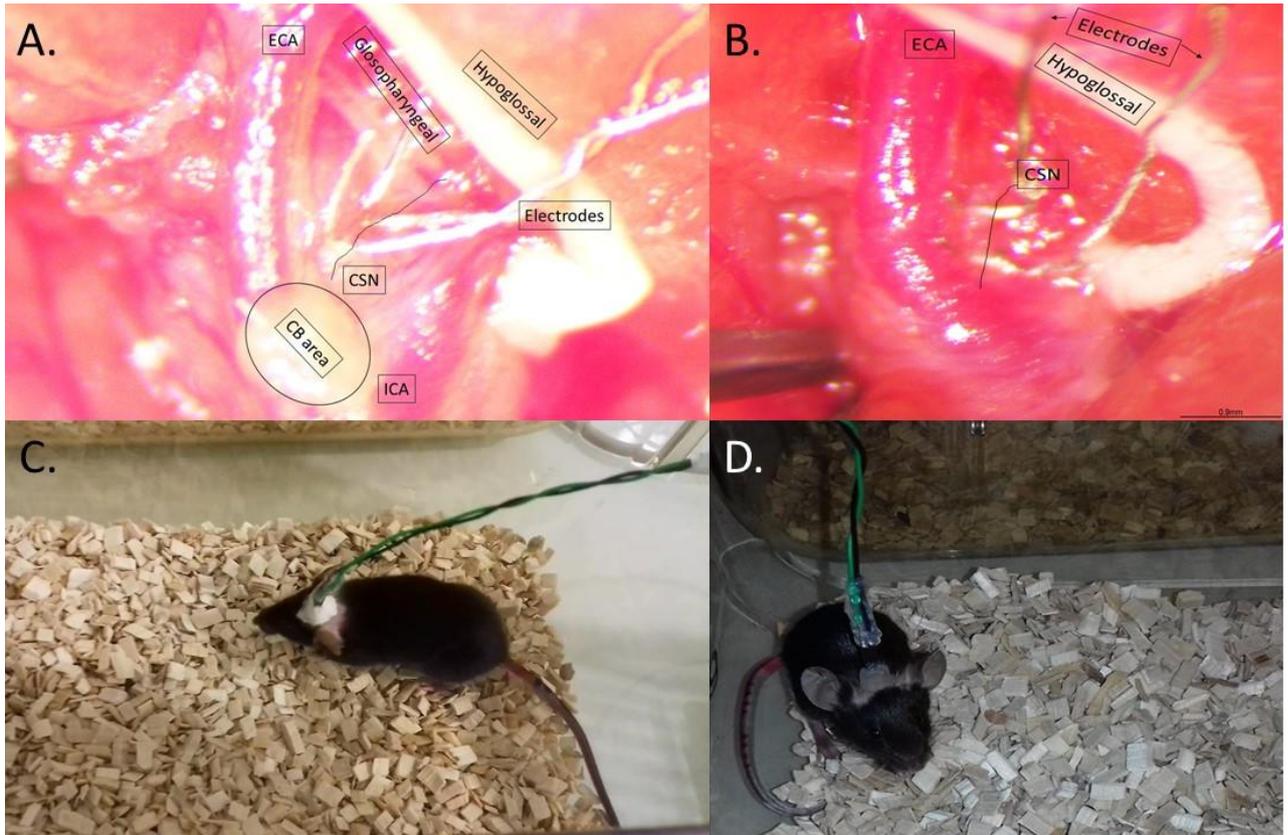


**G.**



**Supplementary Figure 1: Timelines for Experimental Protocols**

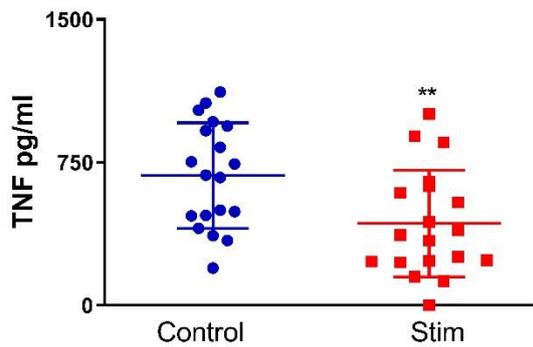
C57BL/6 mice, 8-10 weeks of age were used in various experimental protocols. Routinely, morphine (100 µl) was given IP before surgery – A-D, F-G. Morphine (50 µl) was additionally given subcutaneously for long-term recovery experiments – F-G. Prior to CSN surgery, some experiments had organ/nerve excisions – B – and others blocking drugs were administered – C & D. In all instances with recovery, mice recovered in a heated cage – A-D, F-G. LPS was administered IP (200 µl) in all instances at 100 µg – A-D, F, except, G, when a lethal dose of LPS was used (1 mg – 200 µl). When stimulation was required – A-G – it was administered at 600 µA + 10 Hz or 400 µA + 10 Hz or 200 µA + 5 Hz & 0.01 ms in all instances. Stimulation occurred during unconsciousness – A-E and consciousness – F-G. Experiments were typically conducted at least twice with an n number of 6-8.



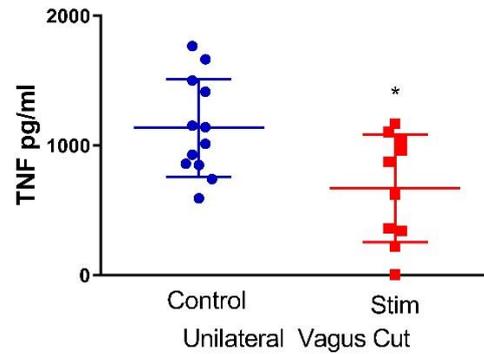
**Supplementary Figure 2: Representative photos of electrode placement on the CSN and conscious stimulation.**

The CSN in mice is represented here with a bipolar electrode placed underneath – this is typical for all experiments **A - B**. Small modifications were made for conscious stimulation of the CSN following timeline **F & G** (SFig.1 – **F - G**). Mice during conscious stimulation are represented in **C – D**.

**A.**



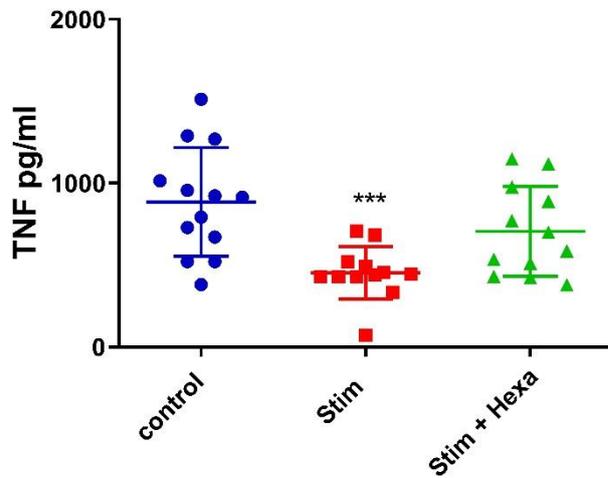
**B.**



**Supplementary Figure 3: Attenuated TNF production via CSN electrostimulation is independent of electrical spread.**

C57BL/6 wildtype mice were obtained, 8-10 weeks of age, CSN isolation surgery was performed and mice were stimulated (200 – 600  $\mu$ A, 5-10 Hz). LPS was injected IP (200  $\mu$ l – 100  $\mu$ g) during surgery. **A.** Isolating the CSN stimulation with oil did not prevent the electrostimulated-mediated decrease in TNF concentration (\*\*,  $p < 0.01$ , Students  $t$ -test,  $n = 19$ ). **B.** Unilateral vagal removal did not prevent the decrease in TNF concentration by CSN electrostimulation (\*,  $p < 0.05$ , Students  $t$ -test,  $n = 10-12$ ). All individual points represent one animal and data is expressed as means  $\pm$  SD.

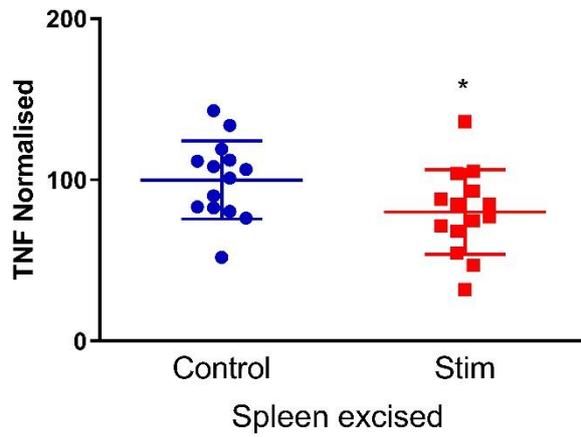
A.



**Supplementary Figure 4: Hexamethonium prevents vagus electrostimulation decreasing TNF concentration.**

C57BL/6 wildtype were obtained, 8-10 weeks of age, vagus isolation surgery was performed and stimulated (600  $\mu$ A, 10 Hz) with or without hexamethonium (10 mg/Kg). The Vagus nerve was stimulated and LPS induced production of TNF was decreased ( $p < 0.001$ , \*\*\*, One-way ANOVA,  $n = 12-13$ ), however, this effect was prevented by hexamethonium ( $p > 0.05$ , One-way ANOVA,  $n = 12-13$ ). All individual points represent one animal and data are expressed as means  $\pm$  SD.

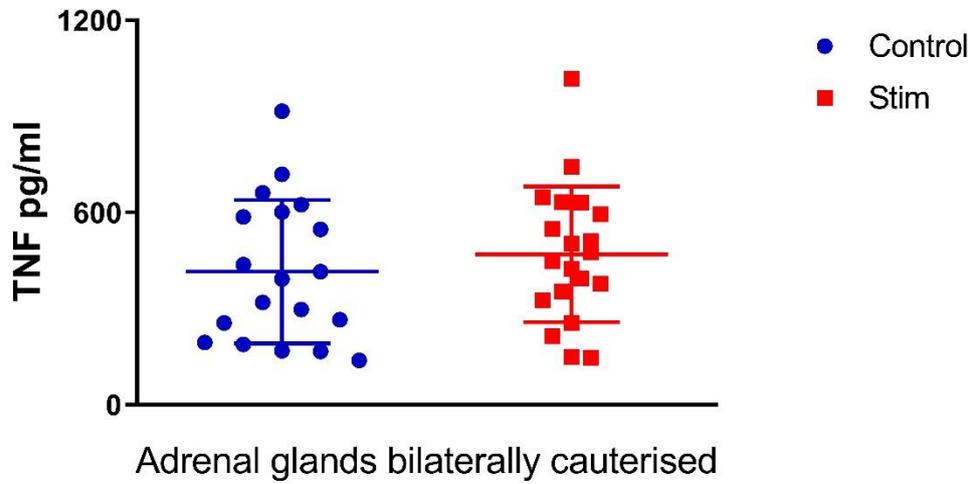
A.



**Supplementary Figure 5: TNF decrease by CSN electrostimulation is independent of the spleen.**

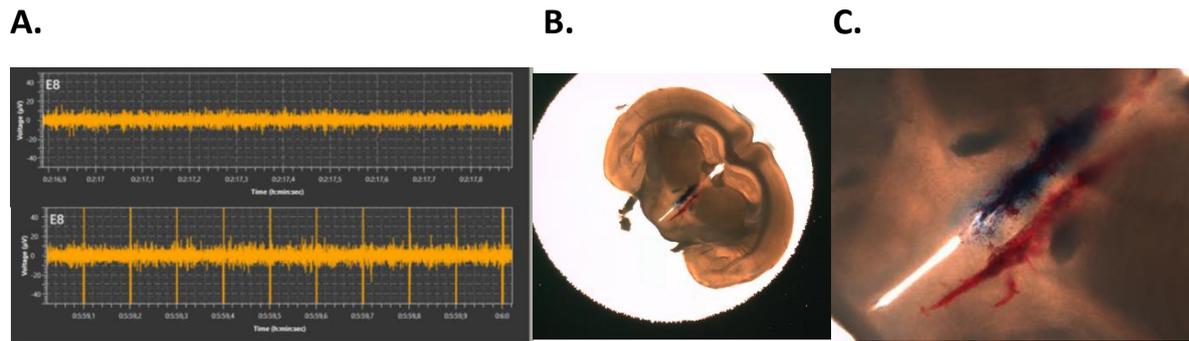
C57BL/6 wildtype mice were obtained, 8-10 weeks of age, the spleen was surgically removed and the CSN was isolated. Electrostimulation (200-600  $\mu$ A, 5-10 Hz) was performed and it significantly decreased TNF concentration ( $p < 0.05$ , \*, Students t-test,  $n = 14$ ). All points represent normalised data of individual mice. Normalised data is expressed as means  $\pm$  SD.

A.



**Supplementary Figure 6: Bilateral cauterisation of the adrenal glands prevent a decrease in TNF by CSN electrostimulation.**

C57BL/6 wildtype mice were obtained, 8-10 weeks of age, bilaterally the adrenal glands were cauterised, prior to CSN isolation and stimulation (600  $\mu$ A, 10 Hz). CSN stimulation did not produce a difference in TNF concentration after bilateral adrenal gland cauterisation ( $p > 0.05$ , Students  $t$ -test,  $n = 19-20$ ). All points represent individual animals and data is expressed as means  $\pm$  SD.



**Supplementary Figure 7: CSN electrostimulation activates the paraventricular nucleus of the hypothalamus.**

C57BL/6 wildtype mice were obtained, 8-10 weeks of age, the CSN was isolated and stimulated (600  $\mu$ A, 10 Hz). Electrical activity recordings of the PVN of the hypothalamus were taken using a stereotaxic frame. **A.** A representative trace of activity in the PVN during CSN stimulation. **B.** The frontal plane of a representative brain with dye to mark PVN location(**B**) and a zoomed in image of the dye in the PVN (**C**). The dye was used to show location of the recording electrode. All images shown are representative of the whole.

## **Additional Results**

### **Methods**

#### **Bilateral carotid neurotomy (BCN)**

The C57BL/6 mice were injected IP with buprecare (100 µl – Axience, Centravet, France), as an analgesic, at least 10 minutes prior to surgery. Following this, mice were anaesthetised using isoflurane (Piramal, CSP, France) mixed with air at 4% for induction and maintained at 2.5% isoflurane throughout the course of surgery. Consciousness was tested by mice reflex – pinch on the tail, hind leg and foreleg – if no response, surgery was commenced. Mice were shaved, using 70% ethanol, with a razor. Further 70% ethanol was used to clean the area, as well as vetedine (Vétoquinol, Centravet, France).

Prior to use surgical instruments were sterilized using a bead sterilizer (Steri 250, SimonKeller). A sterile scalpel blade was used to open the mouse in parallel to the trachea. Skin, and underlying muscle was gently parted and held back by clamps. The carotid artery was found and followed upwards to the bifurcation of the internal and external carotid artery. Continuing towards the brain, the hypoglossal nerve was located, which covers the internal and external carotid artery – as well as the glossopharyngeal nerve. Furthermore, the muscle covering the hypoglossal nerve was located and cut solely. Following this, the hypoglossal was gently disassociated from the surrounding carotid sheath without damage and placed above the glossopharyngeal nerve. Underneath the hypoglossal, typically the pharyngeal and glossopharyngeal nerve run parallel to one another. The CSN was located via its connection from the glossopharyngeal nerve to the CB. In the sham mice the nerve was just located, however in the experimental mice the nerve was sectioned. This exact procedure was repeated bilaterally. Mice were sutured and allowed to recover in a fresh cage.

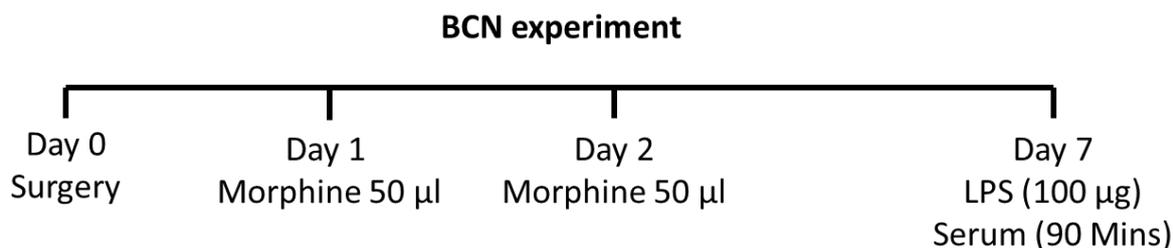
The subsequent two days mice were injected with buprecare (50 µl) subcutaneously. Additionally, it was ensured that the mice were recovering well and that the sutures were intact. One week later, LPS (Sigma Aldrich) was administered to all mice at 100 µg in 200 µl IP. Blood was collected from the mice 90 minutes later and was centrifuged (15 minutes – 10 000 RPM) - serum was obtained.

## Results

### BCN does not augment the immune response in mice

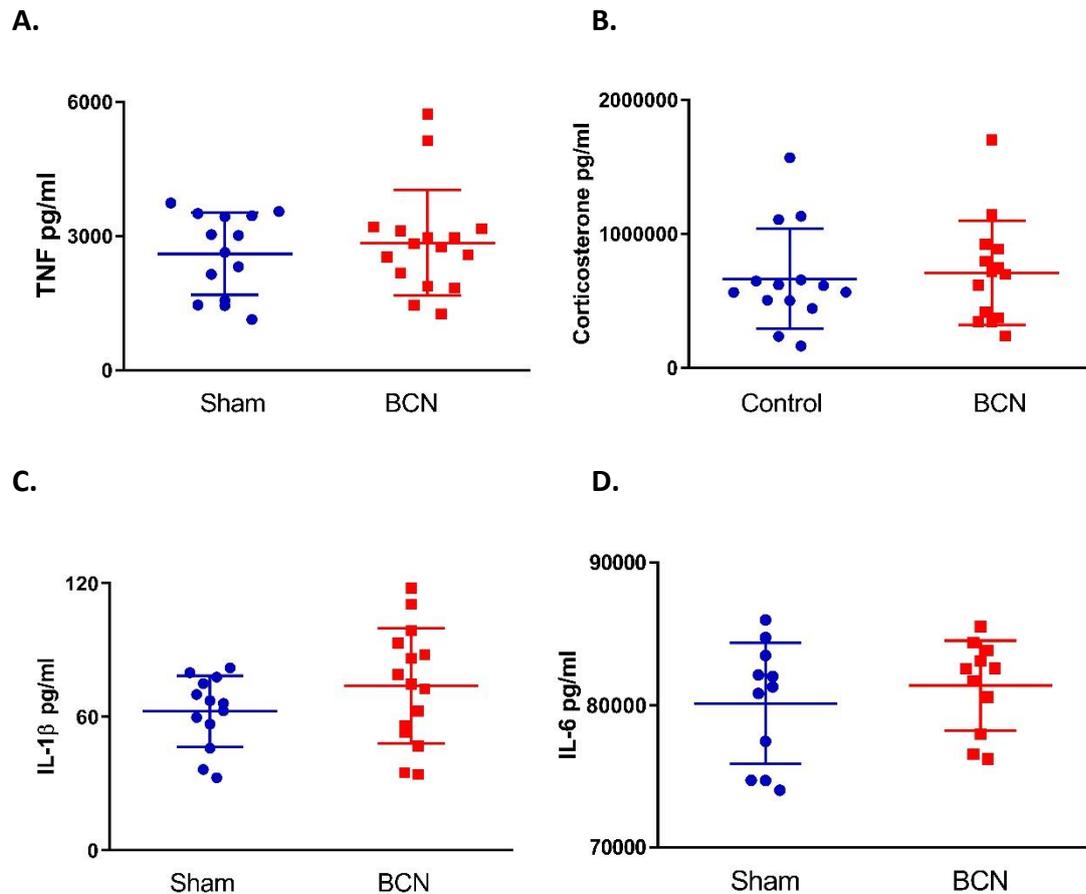
Previously it has been shown that a bilateral resection of the CSN in rats leads to an enhanced pro-inflammatory cytokine profile [20]. We set out to determine if this result was replicable in mice. Following timeline A (SFig.8 - A), a BCN surgery was conducted, BCN had no effect on LPS-induced TNF production (SFig.9 – A:  $p > 0.05$ , Mann-Whitney,  $n = 14-16$ ), IL-1 $\beta$  (SFig.9 – C:  $p > 0.05$ , Mann-Whitney,  $n = 13-15$ ) nor IL-6 (SFig.9 – D:  $p > 0.05$ , Mann-Whitney,  $n = 11$ ). Additionally, it was assessed if BCN had an impact on LPS-induced corticosterone production, it was found that it did not (SFig.9 – B:  $p > 0.05$ , Mann-Whitney,  $n = 14$ ). Together, our results indicate that there are some differences in the rat and mice CB - with its physiological relationship to inflammation (without electrostimulation).

#### A.



#### Supplementary Figure 8: Timeline for BCN

Surgery was commenced on day 0, using wildtype C57BL/6 mice, aged 8-10 weeks old. Surgery was performed as described and the mice were allowed to recover in a heated cage. The subsequent two days morphine, buprecare, was administered to the mice subcutaneously (50  $\mu$ l). On day 7, LPS (100  $\mu$ g, 200  $\mu$ l) was injected IP into the mice, 90 minutes later blood was collected.



**Supplementary Figure 9: BCN has no effect on immune components**

C57BL/6 mice, 8-10 weeks of age, underwent BCN surgery. A week after BCN surgery, LPS (100  $\mu$ g – 200  $\mu$ l) was administered IP, 90 minutes later, blood was collected for serum analysis by ELISA & Meso scale discovery. **A.** BCN did not alter the LPS induced production of TNF ( $p > 0.05$ , Mann-Whitney,  $n = 14-16$ ) **B.** corticosterone ( $p > 0.05$ , Mann-Whitney,  $n = 14$ ) **C.** nor IL-1 $\beta$ , ( $p > 0.05$ , Mann-Whitney,  $n = 13-15$ ) **D.** nor IL-6 ( $p > 0.05$ , Mann-Whitney,  $n = 11$ ). Each individual point represents an animal and data is expressed as means  $\pm$  SD.

## Discussion

We have established that electrostimulation of the CSN evokes an anti-inflammatory response that can attenuate inflammation regardless of common parasympathetic or sympathetic nerve activity. Mitigation of inflammation by CSN electrostimulation is, however, dependent upon corticosterone and its action on the GR of myeloid cells. Furthermore, this evoked potential translates into clinical benefit as conscious CSN electrostimulation protected mice from a lethal LPS injection. Our findings prove our initial hypothesis that CSN electrostimulation attenuates inflammation - interestingly, however, in a manner distinct of the results in other species and outlines a new pathway for bioelectronic medicine. These topics will be addressed in the following sections.

### Impact of BCN on LPS-induced response

I first investigated the physiological role of the mouse CB/CSN in regulation of inflammation. Rats who received BCN have shown an increase in inflammation in response to LPS and a diminished survivability [111]. An augmented BCN response to LPS was assessed in unconscious (unshown) and conscious animals a week after initial surgery. LPS-induced TNF production was unchanged between sham and BCN in both experimental conditions (SFig.9 - A). Furthermore, additional cytokines were examined and BCN generated no change in LPS-induced IL-1 $\beta$  (SFig.9 - C) or IL-6, (SFig.9 - D). There was, however, a mild increase in IL-12p70 (unshown) – this can likely be attributed to coincidence as it is not known why there would be any change in IL-12 alone. Additionally, BCN evoked no change in anti-inflammatory cytokines – IL-10 and IL-4 (unshown).

It is worth noting that the inflammatory profile of mice who had undergone a BCN surgery were not assessed in the absence of an immune-stimulant like LPS, nor were non-cytokine based immune elements investigated. It is feasible that BCN alone subtly induces changes in the immune response, however this was not investigated and, as LPS-mediated corticosterone was not augmented by BCN in mice (SFig.9 - B), it remains unlikely. Differing concentrations of LPS were investigated in BCN mice - no concentration of LPS (5  $\mu$ g – 300  $\mu$ g) augmented pro-inflammatory cytokines. Interestingly, however, the one study in rats to show an increase in inflammation after BCN used a very high concentration of LPS – approximately

1500 µg. A considerable number of their rats died before blood collection was possible [111]. Perhaps, BCN induced increases in inflammation are only by very high concentrations of an immune-stimulant, however, we believe that if this were true for mice it would have been evident in our survival study – we determined there was no difference in survival for sham and BCN mice after lethal LPS injection (400 µg - unshown).

Typically, BCN was performed by a gentle excising of the CSN from just below the glossopharyngeal to the CB. In one final experiment, the entire fork of the carotid artery below the glossopharyngeal nerve was cleaned and the internal and external carotid artery were bare – this rough BCN produced no change to LPS-induced inflammation compared to controls. Mice do have expression of TLR4, unlike multiple cytokine receptors [109], and while functional studies have not been performed, with the information currently available there is no reason to think that mice CB is incapable of responding to LPS due to lack of the requisite signalling-machinery. Overall, this indicates that the CB/CSN of mice have a different relationship to inflammation than rats.

It is perhaps unsurprising that there would be some differences between mice and rats in the regulation of inflammation by the CB. Research for the CB is notorious with fundamental species differences [86, 98] – both anatomical [27] and physiological [86, 107], even between mice and rats in response to hypoxia [79, 153]. Furthermore, the one study examining the immune components of the mouse CB suggest crucial fundamental differences between the mouse and the rat. Mice were shown not to have IL-1R nor IL-6R [164, 163], whereas rats express these receptors and they have been shown to be functional [118, 148]. Whether this has an impact on the physiological role of the CB remains unknown. Simply, more research is required to ascertain the true role of afferent sensing in the CB for mice and other species. Our results suggest that there may not be a physiological role of the CB in regulating inflammation. No physiological role, however, is not concomitant with no potential for evoked anti-inflammatory pathways, especially considering the myriad of functions the CB does perform.

### **Impact of CSN electrostimulation on the immune response**

To assess if CSN electrostimulation could attenuate inflammation, regardless of the apparent non-physiological role for CB immunosensing, I developed a method for isolating and

electrically stimulating the CSN in “semi-conscious” mice (SFig.2 – A + B). Semi-conscious or acute stimulation occurs when mice have received analgesia, morphine, and anaesthesia, isoflurane, presumably inducing a partially anti-inflammatory state in the mice [50]. The mice, however, after stimulation were allowed to wake up and recover from the surgery – therefore semi-conscious stimulation, following timeline A (SFig.1 - A).

First it was assessed if I was stimulating the right nerve. Electrostimulation of the CSN is known to cause an increase in breath rate [15] and when we stimulated the nerve (left CSN in all experiments), we could see a clear increase in breath rate, which returned to baseline levels after stimulation (Fig.1 - A). This known physiological response confirms we are working on the CSN and provides further credence to our BCN results. TNF was used as the gold-standard indicator of inflammation in our experiments - as it is frequently used in the same context for the inflammatory reflex [152]. Upon CSN electrostimulation, there was a decrease in LPS-induced TNF serum concentration (Fig.1 - B). This result demonstrates the anti-inflammatory potential of the CSN in mice and replicates a similar result in rats [141]. We further investigated this anti-inflammatory effect on additional cytokines, and it was discovered that CSN electrostimulation decreased LPS-induced IL-1 $\beta$  (Fig.1 - C), IL-6 (Fig.1 - D), IL-12 (Fig.1 - E) and IL-2 (Fig.1 - F), as well as additional pro-inflammatory cytokines (unshown). The decreased expression of these cytokines during an immune response would be profound: Recruitment and extravasation of cells to response areas would be blunted by decreased TNF; there would be a diminished fever response with depressed levels of IL-1 $\beta$  and IL-6; T-cell numbers and effectiveness would be reduced with decreased availability of IL-2 and IL-12 [155]. Overall, the immune response would be sub-optimal, which in cases of mild infection could prove dangerous, however with a severe infection this effect could be crucial for survival.

Electrical spread is a serious concern in bioelectronic medicine, particularly in the region of the CB. For example, within approximately two millimetres of the mouse CSN there is three major cranial nerves – the hypoglossal, sympathetic and vagus nerve. The vagus nerve is a singular concern for electrical spread during CSN electrostimulation because it is well established that vagal electrostimulation attenuates inflammation [152]. To mitigate the risk that our results were due to electrical spread, we performed two experiments. In the first experiment the CSN was isolated and stimulated as before, except surrounded by oil – this decreases the probability of the current spreading to other nerves and activating them – it was found that CSN electrostimulation still attenuated LPS-induced serum TNF concentration

(SFig.3 - A). In the second experiment a unilateral cut of the cervical vagus nearest to the left CSN was performed and CSN stimulation again decreased the LPS-induced production of serum TNF (SFig.3 - B). These control experiments confirm that sole CSN stimulation induces the mitigation of inflammation.

As stated in the introduction (4.A.0), there is no real consensus in the literature on the best waveform to use for stimulation. From my experience with the CSN, I saw that the optimal waveform can vary depending on the equipment used. This is the reason different waveforms are used in different experiments [SFig.1 – A-D]. In the wave of modern bioelectronic medicine stimulation often occurs before and after an event – such as an LPS injection [152]. Considering this information, a twice, two-minute stimulation was chosen for our experiments, five minutes before and after an injection of LPS. This stimulation pattern was shown to be as effective, if not more so, than a continuous ten-minute stimulation using a similar waveform (data unshown). It has been shown that continuous high-frequency stimulation of the CSN is not damaging to the nerve [15], however for translatable bioelectronic medicine a shorter period of stimulation is preferred.

### **CSN electrostimulation attenuates inflammation independently of known inflammatory reflexes**

We have now established that CSN stimulation is effective and distinct of the vagus. The mechanism of action for attenuation of pro-inflammatory cytokines by CSN electrostimulation has yet to be established. Regardless of the later evidence in rats [141], it was natural to first assess if CSN stimulation was an afferent source for the inflammatory reflex as outlined in section 3.C.iv of the introduction. The crucial neurotransmitter receptors in the inflammatory reflex are  $\alpha 7$ nAChR and  $\beta_2$ AR [3]. It was first investigated if parasympathetic fibres were crucial in the mechanism of CSN stimulation: nAChR, hexamethonium, and mAChR, atropine, blockers were used to ascertain the role of these key receptors. Neither hexamethonium nor atropine influenced CSN electrostimulation-mediated attenuation of LPS-induced pro-inflammatory cytokines (Fig.1 - G). As this result was surprising considering the key role nAChR has in the inflammatory reflex [3] – it was discovered that a similar concentration of hexamethonium would block the attenuation of LPS-induced production of serum TNF by vagal electrostimulation (SFig.4 - A). Overall, this

indicates that the effect of CSN stimulation is independent of the inflammatory reflex as described [3], however it was not shown to be independent of the splanchnic anti-inflammatory reflex.

Disregarding the limited evidence for the splanchnic anti-inflammatory reflex [101, 100], a compelling hypothesis could be developed for the CB to be the afferent source of this potential reflex. The CB upon activation has substantial sympathetic outflow [29] and there is evidence of a link between CB chemoreceptor activation and the spleen [123] and splanchnic nerve activity [40]. Propranolol, a  $\beta$ AR blocker, was used to determine if sympathetic fibres and specifically the  $\beta_2$ AR were crucial to the effect of CSN stimulation – it was found that they were not (Fig.1 - H). In addition, to distinguishing the effect of CSN stimulation from the splanchnic anti-inflammatory reflex, this result re-emphasises that the effect of CSN stimulation is independent of the inflammatory reflex.

We further investigated if the spleen was a crucial component to the attenuation of inflammation by CSN stimulation. The spleen is the key organ of both the well-established inflammatory reflex and the alternative splanchnic anti-inflammatory reflex. CSN stimulation, in mice with an excised spleen, decreased the LPS-induced production of TNF (SFig.5 - A). It should be noted, however, that this result was from normalised data as the two cohorts I investigated had significantly different TNF expression. Furthermore, both cohorts had insignificantly attenuated TNF production by CSN electrostimulation, when combined statistical significance was obtained. It is not surprising that the spleen is critical to the attenuation of pro-inflammatory by CSN stimulation, as the spleen is the most important contributor of inflammatory cytokine secretion in response to infection [71], however it is not crucial to the mitigation of inflammation by CSN stimulation. These results confirm that the effect of CSN electrostimulation is independent of the known inflammatory reflexes. This fact is contrary to the initial results discovered in rats [141] and will be addressed in section “CSN electrostimulation in rats: comparison to mice”. This result is quite exciting as new additional inflammatory reflexes are being discovered [25] and potentially, the CB/CSN evoked reduction of inflammation will be added to this growing list.

### **The ultimate mediator of CSN electrostimulation is glucocorticoid**

The effect of CSN stimulation being independent from the inflammatory reflex, raises the

question of how its effect is being mediated. The HPA axis was an enticing alternative as there are no current inflammatory reflexes that function via the action of glucocorticoid. As glucocorticoid is a potent anti-inflammatory agent with a myriad of appreciable functions [166] – any potential inflammatory reflex evoking glucocorticoid would be a powerful therapeutic. Additionally, there is evidence of a connection between the CB and the adrenals and the hypothalamus [98]. Consequently, investigating the attenuation of pro-inflammatory cytokines by CSN electrostimulation regarding the HPA-axis was a logical choice.

We first demonstrated that CSN electrostimulation increased serum concentration of corticosterone in mice (Fig.2 - A). An increase in corticosterone, however, does not mean the mechanism for CSN stimulation is dependent upon it – as there is preliminary evidence for a glucocorticoid increase in the inflammatory reflex [122]. Therefore, it was assessed if the mitigation of inflammation via CSN electrostimulation could be nullified through various physiological and pharmacological means. In one experiment, bilateral cauterisation of the adrenal glands was performed in mice before CSN stimulation. The absence of the adrenal glands prevented the effect of CSN stimulation (SFig.6 - A). Furthermore, mifepristone, a GR blocker, was used in conjunction with CSN stimulation and it was found that mifepristone prevented the decrease in LPS-induced TNF production (Fig.2 - B). These results indicate that the adrenals, the source of glucocorticoid, and GR are crucial to the effect of CSN stimulation – indicating that the HPA axis is activated in some capacity by the stimulation of the CSN and that corticosterone is the ultimate mediator of the effect of CSN stimulation in mice. Blockade of GR by mifepristone, however, is a general block of the receptor and does not offer information on where glucocorticoid is inducing its effect.

Since it is known that the glucocorticoid is inhibiting early stage inflammation, this indicates that it is likely to be acting on the innate immune response and specifically macrophages. Lysm-Cre:GR<sup>fl/fl</sup> mice were obtained, mice using the cre-lox system on Lysm containing cells, myeloid cells, in which GR was knocked out. The Lysm-Cre:GR<sup>fl/fl</sup> and their littermate controls, negative for the Lysm-Cre (GR<sup>fl/fl</sup>) sequence, received CSN stimulation: The littermate controls had decreased expression of LPS-induced TNF expression, whereas the Lysm-Cre:GR<sup>fl/fl</sup> mice did not have a similar response (Fig.2 - C). This inability of Lysm-Cre:GR<sup>fl/fl</sup> to respond positively to the effect of CSN stimulation indicates that glucocorticoid is acting on myeloid immune cells, likely macrophages, to attenuate inflammation. This confirms that the mitigation of inflammation by CSN stimulation is activating some component of the HPA axis to induce the

increased expression of corticosterone – which decreases the expression of pro-inflammatory cytokines.

Interestingly this evoked response is an amalgamation of the nervous and endocrine system, perhaps containing the best elements of the two systems - the speed of the nervous system and the long-lasting impact of hormones. As outlined in the introduction section 2.B.iv, GR can attenuate inflammation via multiple mechanisms. A brief overview, however, the increased production of corticosterone will activate its receptor in myeloid cells, leading to transcription of several anti-inflammatory cytokines. Additionally, the corticosterone-GR complex will block pro-inflammatory transcription factors from functioning and furthermore it will disrupt the mRNA of pro-inflammatory cytokines [166] – which is likely responsible for the rapid attenuation of inflammation following CSN stimulation.

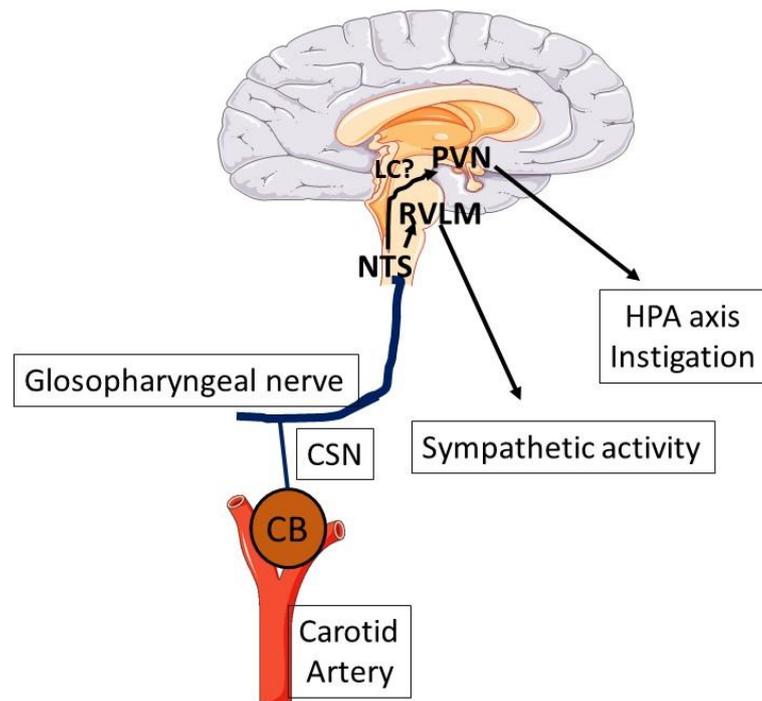
### **CSN electrostimulation directly activates the PVN – instigating the HPA axis**

It is evident that the effect of CSN stimulation is activating the HPA axis in some capacity. There is evidence that this increase in corticosterone could be mediated directly via sympathetic nerves to the adrenal glands or directly to the hypothalamus [98] These options will now be addressed. It is widely accepted that CB activation engenders sympathetic outflow, which is responsible for an increase of serum epinephrine and NE [49]. In dogs it has been shown that hypoxia will induce catecholamine release – epinephrine and NE - from the adrenal medulla, however in denervated dogs this process is delayed [129]. Additionally, in rats, similar results were obtained, but the innervating nerve of the adrenal glands were not assessed - instead it was demonstrated that the hypoxia mediated increase in epinephrine and NE was abolished by BCN [14]. Together, these studies confirm there is a chemoreceptor-mediated increase in epinephrine and NE, likely mediated via the innervating nerve of the adrenal glands. As this connection to the adrenal medulla has been already confirmed, perhaps this is the mechanism in which CSN stimulation increases glucocorticoid.

Alternatively, CSN stimulation could be directly activating the hypothalamus to instigate the HPA axis. It is generally accepted that CB activation will engender a rise in glucocorticoid concentration in the blood [49], however there is no evidence that is singularly convincing, but the overall picture becomes clear when all evidence is compiled. One lab demonstrated in dogs and rats that hypoxia will induce the release of corticosterone/cortisol as well as ACTH

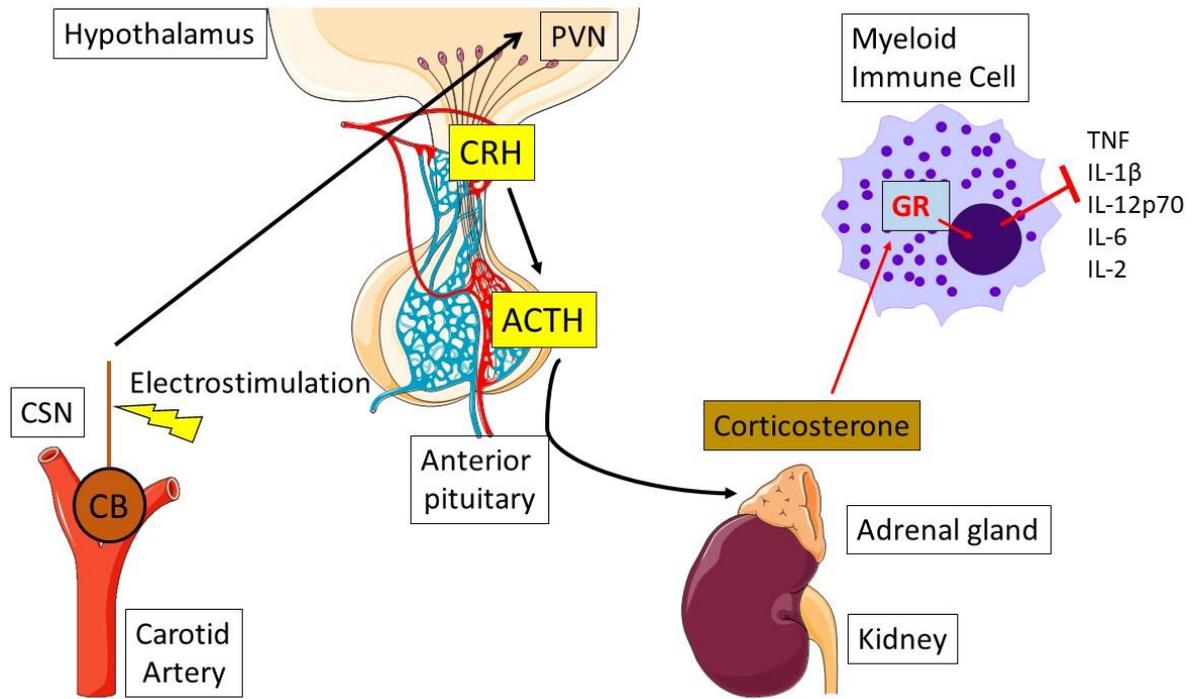
[128, 127, 129], however it is not convincing that the enhanced ACTH is mediating the increase of cortisol - as cortisol appears to be rising more rapidly than ACTH [129]. Moreover, the increase in corticosterone can be interrupted by BCN [128]. Examining histological evidence, it has been shown that CB activation by hypoxia or potassium cyanide will induce c-fos activity in the PVN of the hypothalamus [149, 10 - 34]. Overall, these results establish that there is a direct connection between the CB and the hypothalamus.

Having established previously that bilateral cauterisation of the adrenal glands prevents the effect of CSN stimulation (SFig.6 - A) – our hypothesis was that the effect of CSN stimulation is activating the hypothalamus directly. We were interested, however, in definitively proving that there is a direct connection between the CB and the hypothalamus - specifically the PVN. To assess if CSN stimulation is activating the PVN, the CSN was stimulated while recording electrical activity of the PVN from mice fixed in a stereotaxic frame. It was determined that upon CSN stimulation there was increased activity in the PVN (Fig.2 - D). An example trace of activity, as well as histological controls, confirmed the right stereotaxic coordinates of the PVN have been provided (SFig.7 – A-C). This result indicates a direct connection between the CB and the hypothalamus, indicating that CSN electrostimulation instigates the HPA axis at the source [Illustration.11]. It should be noted that there is a possibility that the activity of the PVN could be feedback from adrenal afferents – while we consider this possibility unlikely a further experiment of denervation of the adrenals could be performed, instead of bilateral cauterisation to assess this hypothesis. The overall mechanism by which CSN electrostimulation attenuates the production of pro-inflammatory cytokines has now been elucidated – CSN stimulation evokes enhanced HPA axis activity to mitigate inflammation [Illustration.12].



**Illustration 11: Carotid Body signalling includes PVN activity**

In a previous illustration it was demonstrated that CB/CSN activity will lead to enhanced sympathetic activity in the body via the NTS and RVLM. It is now known that there is a direct connection between the CSN and PVN. CSN stimulation increase PVN activity instigating the HPA axis. Image partially made via Servier Medical Art.



**Illustration 12: The mechanism by which CSN electrostimulation attenuates inflammation**

Electrical activation of the CSN will enhance PVN activity – releasing CRH, which in turn releases ACTH. ACTH will travel through the vasculature to activate corticosterone producing cells in the mouse adrenal glands. Corticosterone will subsequently activate the GR on myeloid immune cells and attenuate their production of pro-inflammatory cytokines. Images taken from Servier Medical Art.

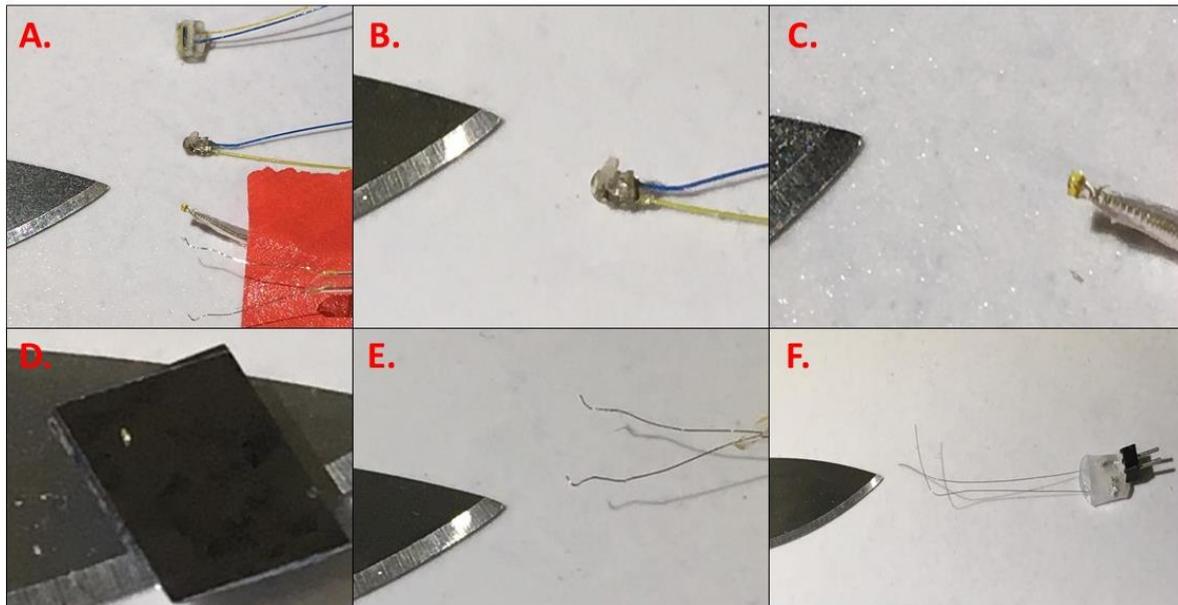
## **CSN electrostimulation attenuates inflammation in conscious mice**

All the experiments described to this point have been with semi-conscious animals. This fact causes a concern, because the efficacy of our results need to be tested on animals that are not under the influence of anaesthesia/analgesia [50]. One of the main critiques of the inflammatory reflex research was the fact that it was performed on anesthetised animals [101, 100]. To avoid the impact anaesthesia has on the animals, conscious stimulation must be performed. Conscious stimulation increases the complexity of the experiments: It requires an electrode to be implanted into an animal for an extended period, repeat stimulations and often a head cap (SFig.2 – C+D). These factors are reasonably well tolerated in rodents and are quite common [15], however chronic implantation of an electrode onto the CSN in mice gives rise to further complexity.

As mentioned, the CSN in mice is a small nerve - by our rough estimation it is 0.8 - 1 mm in length and 30 - 40  $\mu\text{m}$  in diameter. Conventional electrodes for bioelectronic medicine are at their smallest at 1 mm [Illustration.13 – A]. Consequently, we designed and ordered two custom electrodes to fit the mouse CSN. The first electrode from Microprobes for Life Sciences was an elegant design of internal diameter 50  $\mu\text{m}$  and length 0.5 mm [Illustration.13 - C]. The implantation of the electrode was possible, however, due to the depth of location for the CSN and its position between two major arteries – there is not much manoeuvrability. The wires of the Microprobes electrode were designed badly for the CSN, as the wires caused the nerve to flip and twist – perhaps if the wires were placed tightly perpendicular upwards, instead of to the side, this electrode would prove practical. Additionally, from a lab designing micro-electrodes [93], we requested one built to our specifications. We now had the opposite problem, in that it was too small [Illustration.13 - D] and completely impractical for implantation and stimulation of the CSN. Ultimately, we chose to remain with our nichrome wires with a modified design for chronic implantation [Illustration.13 – E-F]. After experimentation and testing I designed a practical method of implantation as described in the methods section.

Conscious CSN stimulation of mice attenuated the production of LPS-induced TNF and it was shown to be as effective as semi-conscious stimulation (Fig.3 - A). This result confirms that the results seen with semi-conscious stimulation can be replicated without anaesthesia. It does not confirm, however, the mechanism discovered for the effect of CSN stimulation. To

test this Lysm-Cre:GR<sup>fl/fl</sup> and their littermate controls were obtained and conscious stimulation was performed. In the littermate mice it appears they responded positively to the CSN stimulation, however, the n number is too small to confirm (Fig.3 - B). The Lysm-Cre:GR<sup>fl/fl</sup> mice did not respond to CSN stimulation - there was no difference in LPS-induced TNF production between sham and stimulated mice (Fig.3 - B). This confirms the mechanism for the effect of CSN stimulation in mice [Illustration.12], nonetheless this result should be replicated to obtain a larger n number of mice. Controversially, our results conflict with the literature for how CSN stimulation is mediated in rats [141]. The potential reason for this will now be addressed in the following section.



### Illustration 13: Electrode selection and design

The above images show representative electrodes of what are typically used and specifically for the mouse CSN, next to the tip of a scalpel blade. **A.** Four electrodes are shown, the topmost is a 2 mm cuff electrode – routinely used on larger nerves like the vagus. Underneath, the cuff electrode is a 1 mm sling electrode, routinely used for the splenic nerves. **B.** Furthermore, it was assessed for practical implantation on the mouse CSN, it was found to be too large and bulky. **C.** A customised cuff electrode was developed to our specifications, it too was found to be too cumbersome for practical use, as it continually twisted the CSN. **D.** Another specialised electrode was sourced from a lab [ref], however while the internal diameter was perfect of the mouse CSN, the electrode was too small for practical use. **E.** Nichrome wires were utilised to acutely stimulate the mouse CSN successfully, their manoeuvrability proved the most practical means to put an electrode on the CSN. **F.** Moreover, it was the only means to chronically stimulate the mouse CSN, when soldered to an external connector.

## **CSN electrostimulation in rats: comparison to mice**

In rats, it was determined that the effect of CSN stimulation is dependent upon parasympathetic and sympathetic pathways and the authors linked it to the inflammatory reflex and the splanchnic anti-inflammatory reflex. Additionally, the authors demonstrated that surgical removal of just the chemoreceptors but maintaining the CSN fibres to the CB and carotid sinus, prevents the effect of CSN stimulation [141]. It is clear there is functional differences between their elucidation of the effect of CSN stimulation in rats and ours in mice [Illustration.14]. Within their paper, questions can be raised about their methodological choices [141]: The stimulation duration was for one hour and while this is comparable to human methods of stimulation [170] and shown to be undamaging separately [15], an hour of stimulation is just unnecessary to activate a nerve directly [117]; stimulation of the conscious rats was only one day after surgery, not allowing full recovery time for the rats and potentially still under the influence of anaesthesia; finally, their method for chronic implantation of wires onto the CSN is non-specific – wires are wrapped in a coil around the CSN and internal carotid artery [75], a positioning that would enhance the possibility of electrical spread.

Further questions can be raised about the authors experimental design [141]. In one experiment, it is shown that rats with bilateral chemoreceptor denervation respond to LPS and that when the CSN is stimulated there is no change in LPS-induced TNF production. Previously, it had been demonstrated, in rats, that there should be an increase in inflammation due to BCN [111]. Considering the functional role of rat chemoreceptors to inflammation [118, 148] a similar increase for bilateral chemoreceptor denervation should have been demonstrated. Moreover, there is no good physiological reason why chemoreceptor denervation would prevent the decrease in LPS-induced TNF production seen by CSN electrostimulation. Activation of an alive, functional, CSN will still signal afferently towards the brain to instigate the anti-inflammatory effect, regardless of the activity of the CB itself – this has been demonstrated with signalling of the efferent vagus nerve [117]. Additionally, the vagus nerve is near the CSN and while the authors took precautions to limit electrical spread, there is known connections between the vagus nerve and the CSN [151, 145]. Furthermore, considering the design on their electrode and the duration of stimulation [75] – it is possible, and our hypothesis, that they may have activated the vagus nerve with

their stimulation. To ascertain if the authors are truly stimulating only the CSN to induce their effect, a unilateral removal of the vagus nerve should have been performed.

It should be noted however, that for the reasons described at the start of the discussion - it may be that there are species differences between the rat and the mouse CB/CSN. There is certainly evidence in the literature to suggest this [109, 163, 164] and our own described results enhance this possibility. Alternatively, there is the enticing possibility that different waveforms will instigate different effects of the CB/CSN, specifically baroreceptor or chemoreceptor activity [98]. A reason for this could be varying degrees of myelination on the nerve fibres, however there is no real strong evidence to suggest this. Perhaps it could be explored in future experiments. Ultimately, the veracity of our experiments and the authors [141] will be validated by external sources and the true nature of CSN stimulation will be confirmed.

	Salgado Lab	Blancou Lab My Thesis
<b>Animal</b>	Rat	Mouse
<b>Decreases Inflammation</b>	Yes	Yes
<b>Stimulation Duration</b>	1 Hour	2 x 2 Minutes
<b>Waveform</b>	1 mA, 30 Hz, 1 ms	200 $\mu$ A, 5 Hz, 0.1 ms
<b>Parasympathetic activity</b>	Yes	No
<b>Sympathetic activity</b>	Yes	No
<b>Spleen dependent</b>	Hypothesised Yes	No
<b>Vagus nerve dependent</b>	Hypothesised Yes – efferently Afferently?	No
<b>HPA &amp; glucocorticoid dependent</b>	No	Yes

#### **Illustration 14: Comparison between rat and mouse CSN stimulation**

It is evident that there are considerable differences between the results obtained with rats when stimulating the CSN and when we stimulate the CSN in mice. The potential reason for this discrepancy will be explored in the main text.

## **CSN conscious electrostimulation prevents endotoxemic shock in mice**

To ensure that the decreased production of pro-inflammatory cytokines mediated by CSN electrostimulation is physiologically relevant to inflammatory disorders, we investigated if chronic CSN stimulation aided survival in mice against endotoxemic shock by a lethal LPS injection. Mice were stimulated five minutes, twice a day for three consecutive days. On the first day mice received a lethal dose of LPS, and it was determined that the stimulated mice were more likely to survive than sham mice (Fig.3 - C). While, this was a statistically significant result, it should be replicated with a larger n number to further confirm its veracity. Our final result confirms the therapeutic potential for CSN electrostimulation, however, there are many more exciting experiments that could be performed with CSN electrostimulation. The effect of CSN stimulation could be assessed in multiple IMIDs or transplant surgeries to find its therapeutic niche in a growing market of anti-inflammatory electroceuticals.

Interestingly, as the effect of CSN stimulation is the only known evoked anti-inflammatory effect to be mediated by glucocorticoids, finding its niche may be readily possible. One such experiment would be to consciously stimulate the CSN in one experimental group and the other administer dexamethasone and then compare survival of immune cells in the spleen. Dexamethasone would induce unneeded apoptosis in the leukocytes [70], whereas CSN stimulation may not. If both therapeutics regulate inflammation comparably, perhaps the risk/benefit ratio would favour the usage of the CSN stimulation in certain disorders – particularly when patients have a long-term requirement of glucocorticoids, such as transplant donors [96]. While this is speculative, it is an interesting possibility that our natural increased production of glucocorticoids may prove as effective as exogenous glucocorticoids. An additional benefit of an electroceutical serving as a glucocorticoid therapeutic is the ability to regulate the stimulation requirements to prevent the onset of the adverse-effects from high-dose glucocorticoids [118]. It is possible that the balance between managing a disorder and high-dose glucocorticoid adverse-effects could be found more readily.

An electroceutical of CSN stimulation as a glucocorticoid therapeutic is an interesting possibility, there is however some hurdles to overcome. For continued long-term investigation in mice, suitable electrodes need to be built for more complicated experiments. Additionally, for translatable therapy there needs to be advances in electrode design. The ideal stimulator for the CB/CSN needs to target individual nerve fibres only. It is feasible, and

being considered, to find the individual fibres of nerve with the required effect, via physiological assessment, and implanting an electrode on that singular fibre [15, 44]. Otherwise, CSN stimulation may induce a myriad of undesired side-effects. Regardless, our results described here highlight the exciting potential future of bioelectronic medicine and the CSN.

## Conclusion

The CB and its innervating nerve the CSN are desirable targets for bioelectronic medicine due to their ability to regulate various physiological functions in the body. We have assessed CSN stimulation in this capacity and have discovered that CSN electrostimulation in mice will attenuate multiple pro-inflammatory cytokines. Furthermore, this effect is independent of common sympathetic and parasympathetic nerves. CSN electrostimulation mobilises the HPA axis to produce glucocorticoids, which act on the GR found in myeloid cells to induce the attenuated inflammation. Moreover, the attenuated inflammation by CSN stimulation has physiological benefit – increasing the survivability of mice from a lethal LPS injection. The mechanism described here is the first known instance of an evoked anti-inflammatory pathway by electrostimulation mediated by glucocorticoids and consequently has a unique role in bioelectronic medicine.

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