Published in final edited form as:

Physiol Behav. 2020 November 01; 226: 113124. doi:10.1016/j.physbeh.2020.113124.

Non-neuronal crosstalk promotes an inflammatory response in nodose ganglia cultures after exposure to byproducts from gram positive, high-fat-diet-associated gut bacteria

Carolina R. Cawthon^a, Rebecca A. Kirkland^a, Shreya Pandya^a, Nigel A. Brinson^a, Claire B. de La Serre^{a,*}

^aDepartment of Foods and Nutrition, The University of Georgia, Athens, Georgia 30602, United States

Abstract

Vagal afferent neurons (VAN) projecting to the lamina propria of the digestive tract are the primary source of gut-originating signals to the central nervous system (CNS). VAN cell bodies are found in the nodose ganglia (NG). Responsiveness of VAN to gut-originating signals is altered by feeding status with sensitivity to satiety signals such as cholecystokinin (CCK) increasing in the fed state. Chronic high-fat (HF) feeding results in inflammation at the level of the NG associated with a loss of VAN ability to switch phenotype from the fasted to the fed state. HF feeding also leads to compositional changes in the gut microbiota. HF diet consumption notably drives increased *Firmicutes* to *Bacteroidetes* phyla ratio and increased members of the *Actinobacteria* phylum. *Firmicutes* and *Actinobacteria* are largely gram positive (GP). In this study, we aimed to determine if byproducts from GP bacteria can induce an inflammatory response in cultured NG and to characterize the mechanism and cell types involved in the response. NG were collected from male Wistar rats and cultured for a total of 72 hours. At 48–68 hours after plating, cultures were treated with neuronal culture media in which *Serinicoccus chungangensis* had been grown and removed (SUP), lipoteichoic acid (LTA), or meso-diaminopimelic acid meso-DAP). Some treatments included the glial inhibitors minocycline (MINO) and/or fluorocitrate (FC). The responses were evaluated using immunocytochemistry, qPCR, and electrochemiluminescence. We found that SUP induced an inflammatory response characterized by increased interleukin (IL)-6 staining and increased expression of genes for IL-6, interferon (IFN) γ , and tumor necrosis factor (TNF) α along with genes associated with cell-to-cell communication such as C-C motif chemokine ligand-2 (CCL2). Inclusion of inhibitors attenuated some responses but failed to completely normalize all indications of response, highlighting the role of immunocompetent cellular crosstalk in regulating the inflammatory response. LTA and meso-DAP produced responses that shared characteristics with SUP but were not identical. Our

*Corresponding author.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

⁷Conflict of Interest

The authors declare no conflicts of interest.

results support a role for HF associated GP bacterial byproducts' ability to contribute to vagal inflammation and to engage signaling from nonneuronal cells.

Keywords

Nodose ganglion; cell-to-cell communication; bacterial byproducts; inflammation

1. Introduction

Vagal afferent neurons (VAN) are the primary sensory neurons relaying gut-originating signals to the central nervous system (CNS). Vagal afferent terminals are positioned in the gastrointestinal lamina propria [1] and respond to stretch and distension along with chemically mediated signals generated in response to feeding [2–4]. VAN cell bodies are located in the nodose ganglion (NG) and project to secondary neurons of the nucleus of the solitary tract (NTS), one of the brain's main feeding and energy balance centers [5–7].

VAN have receptors for anorexigenic signals such as cholecystokinin (CCK) [8, 9] and leptin [10, 11], and orexigenic signals such as ghrelin [12] and endocannabinoids [13]. VAN also have receptors for ATP [14], bacterial byproducts (e.g. lipopolysaccharide, LPS) [15], and cytokines including interferon- γ (IFN γ) and tumor necrosis factor (TNF) α [16]. VAN integrate these signals leading to a fine-tuned response which can be modulated by energy-balance status. Activation of VAN ghrelin receptors leads to reduced vagal sensitivity to stretch [17] and to CCK- induced satiety [18] while leptin receptor activation increases VAN sensitivity to CCK [19]. The ability of energy-balance status to induce phenotypic changes in VAN resulting in altered sensitivity to satiety signals has been referred to as vagal plasticity [20, 21]. Interestingly, chronic HF diet consumption leads to a loss of leptin-CCK synergy [22] and reduced sensitivity to anorectic peptides [19, 20]. In rats, these changes in gut-brain signaling coincide with the development of hyperphagia and are associated with inflammation at the level of the NG [15, 19]. Chronic administration of HF-diet associated bacterial byproducts leads to insensitivity to exogenous CCK- induced satiety [15] while prebiotics supplementation [23] prevents HF-diet-driven loss in CCK sensitivity supporting a potential role for the gut microbiota in driving vagal alterations.

Dysbiosis refers to alterations in gut microbiota composition associated with the development of poor health [24]. In human and rodent models, diet-induced obesity leads to dysbiosis and is characterized by an increased ratio of *Firmicutes* to *Bacteroidetes*, the two main bacterial phyla found in the gastrointestinal tract [25, 26]. The obesity-associated increase in *Firmicutes* is frequently driven by an increase in the class *Clostridia* [27, 28]. Increases in the phylum *Actinobacteria* have also been associated with HF feeding [27, 28]. Members of the *Firmicutes* and *Actinobacteria* phyla are largely GP bacteria [29]. LPS, a component of the gram-negative bacterial cell wall, has previously been shown to induce an inflammatory response and alter leptin signaling in cultured VAN [20] and *in vivo* [15] but GP bacterial byproducts have not been investigated.

GP bacteria cell wall structures are not uniform and can include a variety of molecules including peptidoglycans, lipoproteins, diaminopimelic (DAP) acids, and teichoic acids such

as lipoteichoic acid (LTA) which can activate nucleotide-binding oligomerization domain (NOD)-1 and -2 and/or toll-like receptor (TLR)-2 heterodimers [30, 31]. NOD1, NOD2, and TLR2 may be found on neurons and non-neuronal cell types and their activation induces an inflammatory response [30, 32–34]. Insult or injury to the central or peripheral nervous system produces responses from a variety of cell types. Several of these cells are present in peripheral ganglia including neurons, satellite glia (SG), Schwann cells, endothelial cells (endoneurial, perineurial, and epineurial) cells, and microglia/macrophages [35, 36]. Each of these cell types has an ability to participate in immune responding [37–39] and may contribute any response induced by GP bacterial byproducts. Based upon observations that 2–3 SG surround each neuron and the initial portion of its axon [40], it is likely that SG are a numerous non-neuronal cell type in primary cultures of dissociated NG. SG are sometimes viewed as the peripheral equivalent to astrocytes [41] and share features with their CNS counterparts including glial fibrillary acidic protein (GFAP) immunostaining and the ability to participate in the tricarboxylic acid-glutamine cycle [42]; however, macrophage- and/or dendritic cell-associated cellular markers including cluster of differentiation (CD)11b, CD11c, and CD86 have been identified in trigeminal ganglia SG [43]. Crosstalk, a term used here to describe the ability of the response from one cell type to influence the responses of a different cell type, between non-neuronal cell types modulates their respective responses. In the CNS, microglia-astrocyte communication can enhance or inhibit the other's signaling [44]. Microglia-originating vascular endothelial growth factor-B (VEGFB) and transforming growth factor (TGF)- α promote or inhibit astrocytes' activity, respectively [45]. C-C motif chemokine ligand-2 (CCL2) and lipocalin-2 (LCN2) released by astrocytes increase microglia activity while astrocytic TGF β reduces microglia activation [46]. We have previously found that HF diet consumption leads to increased ionized calcium-binding adapter molecule-1 (Iba1, traditionally a microglia/macrophage marker) staining in the NG accompanied by changes in vagal innervation [28]. Additionally, LPS induces phenotypic and functional changes in SG of mouse NG [47] demonstrating a role for non-neuronal cell types in mediating gut-driven NG inflammation and potential changes in vagal signaling.

We hypothesize that byproducts of HF diet associated GP bacteria can induce a response that may be mediated by multiple cell types in the NG resulting in inflammation. To begin testing this hypothesis, the present study aimed to determine if byproducts from GP bacteria can induce an inflammatory response in VAN and to characterize the mechanisms and cell types involved in the response.

2. Materials and Methods

2.1 Animals and Cultures

All animal experiments were carried out in compliance with the University of Georgia's Institutional Animal Care and Use Committee guidelines. Male Wistar rats (6–10 weeks old, Envigo, Indianapolis, IN USA) were euthanized in CO₂ and exsanguinated by severing one femoral artery. The left and right NG were then collected and immediately placed into ice cold Hibernate A without Ca²⁺ (Brainbits, Springfield, IL USA) for transport to the lab.

Upon return to the lab, NG were de-sheathed and digested in 2 mg/mL collagenase (Sigma, St. Louis, MO USA) and dispase (Roche, Basel Switzerland) in Hank's Balanced Salt

Solution (Sigma, St. Louis, MO USA) with 1% Penicillin Streptomycin Glutamine solution (Hyclone, Pittsburgh, PA USA) for 90m at 37° C. After digestion, cells were transferred to a 15 mL conical tube and washed twice in MEDIA composed of Dulbecco's Modified Eagle's Media (Sigma, St. Louis, MO USA) with HEPES, 1% Penicillin Streptomycin Glutamine solution (Hyclone, Pittsburgh, PA USA) and 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA USA). After centrifugation (4m, 850g) and removal of excess supernatant, the remaining volume (1–2 mL) was plated evenly (approximately 125 µL each) onto poly-L-lysine (PLL, Sigma, St. Louis, MO USA) coated slides in sterile 4-well culture plates (Greiner Bio-one, Monroe, NC USA) or PLL coated 20 mm coverslips in 35 mm round culture dishes. Plates were incubated at 37° C in 5% CO₂ for 150–180 minutes and 3–5 mL of fresh, warm MEDIA was added to each well. Cultures were returned to the incubator. The next morning, all MEDIA was aspirated from wells and replaced with 3–5 mL fresh, warm MEDIA. Treatment durations of 2–24 hours were timed so all cultures were collected 71–73 hours after plating. The timing of treatments was selected from a review of the literature where timings ranging from 73 hours to one week were used for work in related fields [19, 20, 48]. Cultures were treated with MEDIA in which *Serinicoccus chungangensis* (DSMZ, Braunschweig, Germany) was cultured and removed (SUP, dose was 50% for both immunochemistry and PCR experiments), meso-diaminopimelic acid (meso-DAP, 250 µg/mL, Sigma, St. Louis, MO USA), known to be found in *S. chungangensis* [49] or LTA from *Bacillus subtilis* (0.015–20 µg/mL, Sigma, St. Louis, MO USA) as a representative GP cell envelope component. The microglial inhibitor minocycline (MINO, 10 µM, Tocris, Minneapolis, MN USA) and/or the astrocyte/SG inhibitor fluorocitrate (FC, 3 nM, Sigma, St. Louis, MO USA) were included in some treatment groups. Inhibitor doses were chosen from the literature [50, 51] and FC was prepared according to Paulsen and colleagues [52]. All treatment groups are *n* = 3 and numbers for each group are included with figures. After treatment, 1–2 mL of MEDIA from each well was harvested and stored at –80° C for lactate measurement and quantification of inflammation-related proteins. Remaining MEDIA was aspirated, and cultures were either fixed for immunocytochemistry in 1% paraformaldehyde (PFA) for 15m at room temperature or scraped from slides into sterile filtered phosphate buffered saline (PBS, pH 7.4) and transferred to a sterile 12 × 75 mm tube (Corning, Corning NY USA). After centrifugation at 400 rcf for 5m, supernatant was removed, and cells were lightly fixed by resuspending in 0.2% PFA and incubating at room temperature for 10 mins. Cells were washed twice in sterile filtered PBS then resuspended in a 1.7 mL centrifuge tube in RNA extraction kit melting buffer (Invitrogen, Carlsbad CA USA).

2.2 Immunocytochemistry

Coverslips with PFA-fixed cultures were washed in PBS three times for 15m each. Next, cultures were permeabilized with 0.1% Triton X for 10m at room temperature and washed in PBS three times for 15m each. After blocking for 1h at 37° C in 10% donkey serum in PBS, primary antibodies (Table 1) were applied and incubated at 4° C overnight. The next morning, cultures were washed three times in PBS for 15m each and secondary antibodies were applied. After 30m incubation at 37° C, slides were washed three times in PBS for 15m each. After removing excess PBS, coverslips were mounted to slides with Fluorogold II with DAPI (Electron Microscopy Sciences, Hatfield, PA). Slides were imaged on a Zeiss Axioscope A1 Microscope (Zeiss, Jena, Germany) with a Zeiss Axiocam 305 color camera

(Zeiss) in the University of Georgia's microscopy core at 10 or 20x magnification. ImageJ (US National Institutes of Health, Bethesda, MD) cell counter plug-in was used to mark cells as they were counted and the proportions of cells positive for each stain or combination were determined from total DAPI counts. Antibodies selected are well-referenced in the literature (except for SOX10; validation images are provided on the manufacturer's website) and antibody specificity was confirmed by 1) double staining with other cell specific markers, and 2) omitting the primary antibody resulting in lack of staining.

2.3 RNA Extraction

Immediately after cultures were collected, fixed, and washed, RNA was extracted from cells using the PureLink™ FFPE RNA Extraction Kit (Invitrogen, Carlsbad, CA USA) according to the manufacturer's protocol with a 30 min incubation in proteinase K. Extracted RNA was stored at -80°C .

2.4 cDNA

After quantification by Nanodrop, the extracted RNA was used as the template for cDNA production with the SSIV or SSIV VILO cDNA kit (Thermo Fisher, Waltham, MA USA) following the manufacturer's protocol. cDNA was stored at -80°C until use.

2.5 PCR (qPCR)

We used qPCR to examine gene expression of IFN γ , interleukin (IL)-1 β , IL-6, TNF α , TGF α , TGF β , CCL2, LCN2, VEGFB, microtubule-associated protein-2 (MAP2), Iba1, GFAP, and 18S (reference) genes. Primers were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA USA) and then checked with PrimerBLAST (National Center for Biotechnology Information) to ensure target specificity (Table 2). 10 μL reactions were set up using KAPA SYBR (Roche, Basel Switzerland) and 200 nm primers. Plates were run on a Roche LightCycler 480 in the Georgia Genomics and Bioinformatics Core (GGBC) using the following protocol: Preincubation 95°C for 10m followed by 45 cycles: Denaturation 95°C 10s, Annealing $57-59^{\circ}\text{C}$ 20s, Extension 72°C 30s. Fold change expression compared to controls was calculated using the $\Delta\Delta\text{Ct}$ method.

2.6 Inflammation-associated Proteins Assay

Media collected from completed culture experiments was concentrated to approximately 15% original volume by centrifugation in a Pierce 3,000 MWCO Concentrator (Thermo Fisher, Waltham, Ma USA) and resuspended in sterile filtered PBS to a final volume of approximately 145 μL . The concentration/resuspension process also reduced phenol red concentration to avoid interference with total protein concentration analysis by Coomassie Plus assay (Thermo Fisher), which was performed according to manufacturer's instructions. After total protein quantification, Mesoscale Discovery (MSD) Rat V-Plex Proinflammatory Panel 2 and Rat Inflammation Panel 1 (Mesoscale Diagnostics LLC, Rockville, MD) were performed according to manufacturer's protocol with samples diluted 2-fold for the V-Plex Proinflammatory Panel 2 assay and 50-fold for Inflammation Panel 1 assay. Samples were run in duplicate to assess inflammatory protein concentrations in the media relative to total

protein in each sample for, chemokine C-X-C motif ligand 1 (CXCL1), CCL2, and thrombospondin-1 (TSP-1).

2.7 Lactate Assay

Lactate released into cell culture media was assessed using an enzymatic colorimetric assay kit (BioVision Incorporated, Milpitas, CA USA) per the manufacturer's instructions.

2.8 Statistical analysis

Statistical analyses were conducted with GraphPad Prism 8 (GraphPad Software, San Diego CA USA) using one-way ANOVA with Fisher's LSD post-hoc or non-parametric test when appropriate. Differences are considered significant when $P < .05$. All data are shown as mean $\pm$ SEM.

3. Results and Discussion

3.1 SUP induces an inflammatory response in primary NG cultures

We investigated SUP driven changes in gene and protein expression in our cultures using immunohistochemistry and PCR. Iba1⁺ staining has been used extensively as a marker of microglia activation as staining increases when microglia change from a ramified to an activated amoeboid morphology. Macrophages also express Iba1 and could be positively stained in our cultures. There is now evidence that microglia can display a variety of complex morphofunctional stages, beyond the traditional "activated" state [60]. Recent advances in imaging technologies have shown that microglia can extend and retract their processes in the order of minutes and can perform several macrophage-like functions such as phagocytosis, inflammatory and anti-inflammatory cytokine production, and antigen presentation [61]. Like macrophages, microglia exist in both pro- and anti-inflammatory states which may be characterized by expression of specific molecules [62–64]. The M1/M2 polarization categories are widely used for both microglia and macrophages [65, 66]. The M1, or classically activated, proinflammatory phenotype can be induced by a variety of factors including bacterial products like LPS or cytokines such as IL-1 β and TNF α [64, 67] and is characterized by production of inflammatory cytokines including CXCL1, IFN γ , IL-1 β , IL-2, IL-6, TNF α [64], and CD86 [63, 64]. The M2 phenotypes are associated with resolution of inflammation, repair, and immunomodulation [64]. The M2a, a repair and regeneration phenotype reliably induced by IL-4 [64], is recognized by anti-inflammatory markers including arginase 1 (Arg1), CD206, fractalkine receptor (CX3CR1), IL-4, CCL2, and TGF β [64]. The M2b phenotype is characterized by immunomodulatory factors including CD32, CD86 [63], IL-10, and suppressor of cytokine signaling (SOCS)-3 [64]. The M2b immunomodulatory phenotype may be induced simultaneously with the M1 response, with the overall response determined by the ratio between the two types [64]. It is also possible for microglia to switch phenotypes in response to changes in stimulus [64]. Immunohistochemistry for IL-6, IL-10, CCL2, and CD206 and inflammation-associated gene expression were used to better characterize the nature of the microglia/macrophage response to SUP.

Since IL-6 is a recognized hallmark of M1 macrophage/microglia polarization, we counted Iba1⁺/IL-6⁺ cells as a marker of a pro-inflammatory M1 state. SUP 4h significantly increased the proportion of Iba1⁺/IL-6⁺ cells (Figure 2AC). Mino 4h produced a trend toward increased proportions of Iba1⁺/IL-6⁺ cells but neither MINO 4h nor FC 4h significantly altered this proportion while MINO + FC 4h significantly increased Iba1⁺/IL-6⁺ cells similarly to SUP4 (Figure 2AC). It appears that more microglia/macrophages were M1 polarized by SUP 4h and MINO + FC 4h compared to other treatments (Figure 2AC). It is not clear why MINO + FC 4h would increase M1 microglia/macrophage polarization but combined with the lack of effect from FC 4h alone, it is possible that lack of modulatory signals from FC-inhibited cells exacerbated the trend toward M1 polarization produced by MINO 4h. Importantly, we observed very little IL-6⁺ staining in other cell groups suggesting the reported changes in IL-6 staining are likely driven by Iba1⁺ cells.

Gene expression of the M1 markers IFN γ , IL-6, and TNF α was increased by SUP 4h (Figure 2EFG). IL-1 β expression levels were significantly increased by SUP 24h, while the other measured cytokines had returned to control levels at SUP 24h (see Supplementary Figure 1). These changes in gene expression match our immunohistochemistry data showing that SUP 4h increased Iba1⁺/IL-6⁺ cells (Figure 2AC). If protein expression follows gene expression with SUP 24h as it did with SUP 4h, our inflammatory cytokines gene expression findings support the hypothesis that SUP 4h induces M1 microglia/macrophage polarization followed by a decrease in proportion of M1 microglia/macrophages between 4h and 24h after treatment with SUP. A SUP driven M1 microglia/macrophage polarization at 4h is further evidenced by the increased CD86 and IFN γ gene expression [63, 64, 68] (Figure 2EI). In this model, we investigate the results of an acute dose of bacterial byproducts while HF feeding and obesity are associated with chronic low-grade inflammation. There is no literature specifically investigating chronic exposure to GP bacterial products on inflammation in the NG, but investigations into the effects of chronic low-dose LPS administration [15] found increased expression in the counter regulatory anti-inflammatory protein SOCS3, which can inhibit leptin signaling. In the CNS, under acute inflammatory conditions, microglia are thought to moderate potential neuronal damage and support remodeling. However, chronic microglia activation and microglia-induced inflammation are observed in numerous brain pathologies and there is evidence that chronically activated microglia exert direct deleterious effects on neurons, contributing to disease progression [69–71]. Chronic HF feeding leads to increased expression of pathogen pattern recognition receptors TLR2 and TLR4 as well as increased M1 microglia/macrophage staining in the NG [72]. These *in vivo* experiments support a role for HF feeding associated bacterial byproducts in promoting inflammation in the NG and, like our findings, suggest that M1 polarized microglia have a role in the inflammatory process.

Our observations of increased gene and protein expression of M1 microglia/macrophage markers coincides with changes in gene expression of the neuronal marker MAP2, which was also increased by SUP 4h (Figure 6D). Since there was no difference in the number of NeuN⁺ cells identified with immunohistochemistry after SUP 4h, this increase is unlikely to be a SUP driven change in the number of neurons. The inclusion of MINO or FC attenuated the MaP2 increase suggesting a potential influence of non-neuronally-originating signals such as IFN γ . IFN γ expression was also increased by SUP and attenuated by inclusion of MINO

or FC (Figure 2E). Interestingly, the Janus kinase / signal transducer and activator of transcription proteins (JAK/STAT) pathway, which can be activated by IFN γ or IL-6, can increase expression of MAP2 [73]. At 24h, MAP2 expression was returned to control levels, as was expression of IFN γ (See Supplementary Figure 1), supporting this potential pathway. Furthermore, SUP 4h was the only condition in which both IL-6 and IFN γ were significantly increased suggesting a possible additive or synergistic effect. MAP2 is thought to have a key role in cargo sorting and axonal transport [74]. Conceivably, the upregulation in MAP2 gene expression in response to SUP 4h may be a direct response to SUP or a response to signaling from M1 polarized microglia/macrophages, also evidenced in this condition, to increase transport of the chemical responses to pro-inflammatory stimuli.

We used 10 μ M minocycline (MINO) as a microglia/macrophage inhibitor and 3 nM fluorocitrate (FC), known to inhibit astrocytes in the CNS, as an inhibitor of SG. Neither MINO 4h nor FC 4h alone induced significant changes in cytokine gene expression (Figure 2EFGH) with the exception of a trend of TNF α toward increase with FC 4h (Figure 2G). Because both microglia/macrophages and SG can produce a cytokine response, inhibition of only one cell type may not be sufficient to decrease overall expression. Or, it may be that although some of the reactive functions of these cell types were inhibited in the presence of MINO or Fc, in the absence of a stimulus, baseline expression of reactive genes is not inhibited by MINO or FC.

Inclusion of MINO or FC with SUP 4h prevented increased expression of the M1 microglia/macrophage markers IFN γ and CD86 (Figure 2EI) and attenuated IL-6 expression compared to SUP alone but did not prevent an increase compared to control levels (Figure 2F). On the contrary, expression of TNF α and IL-1 β , also M1 markers, was not reduced by the addition of MINO or FC alone to SUP (Figure 2GH) but was moderated at 24h when both inhibitors were used suggesting that either uninhibited cell types were driving the increases when only one inhibitor was present or that M1 polarization was resolving by 24h. The increased CD86 gene expression induced by SUP and its regulation by inclusion of MINO or FC is expected, however it is not entirely clear why MINO alone increased CD86 gene expression. One explanation is that CD86 is associated with both the M1 and M2b phenotype [75] and MINO is believed to specifically inhibit the M1 phenotype [76]. The M1 and M2b phenotypes may coexist [64]; thus, the observed increase in CD86 is not contradictory to inhibition of M1 polarization by MINO. This possibility is in agreement with the lack of MINO-induced gene expression of IFN γ , IL-6, and TNF α which are also M1 markers [64]. We also found that CXCL1 levels, an M1 marker, released into the culture media was increased by inclusion of MINO with SUP 4h or MINO + FC with SUP 24h (Figure 3C). CXCL1 is known as a neutrophil attractant [77] but in a bone cancer pain model, it was found to be involved in crosstalk from astrocytes to neurons and was associated with increased pain hypersensitivity [78]. While it is plausible that the increased CXCL1 in our model is indeed to attract peripheral immune cells, it could also be part of an inflammation-induced cell-to-cell communication. The increases seen with inclusion of glial inhibitors supports the role of communication between cell types in mediating the inflammatory response.

Iba1 gene expression was significantly decreased after SUP exposure (Figure 2D). While this result may seem surprising, increased expression of cytokine genes precedes increased

expression of Iba1 in systemic LPS- induced inflammation [60, 79] and in some brain regions in LPS-injected mice, Iba1 gene expression is significantly reduced at 4h concurrently with increased Iba1 immunostaining, activated morphology of the microglia and increased serum levels of proinflammatory cytokines [60]. In our study, we found an increase in the proportion of Iba1⁺ cells also positively stained for IL-6, an M1 phenotype marker. Our findings may result from an imbalance between mRNA and protein levels (Figure 2ABD) and suggest that inflammatory stimuli can induce a reduction in the Iba1 transcription rate and/or an increase in translation rate. In LPS-activated microglia, the most abundant mRNAs are not the most translated mRNAs [80], supporting the possibility of inflammation-induced changes in mRNA turnover and translation. This decrease in Iba1 mRNA expression may act as a stop signal preventing further microglia activation.

Gene expression of Iba1 was also significantly decreased by MINO 4h which was not unexpected (Figure 2D). We also found a trend toward reduced Iba1⁺ cells via immunocytochemistry in response to MINO 4h (Figure 2), supporting some degree of microglial/macrophage inhibition by MINO.

Importantly, our results do not suggest that these differences in gene expression are driven by differences in the numbers of cells in our cultures. We did not find differences in total cells at 4 or 24h associated with our treatments (Figure 4AB). Treatment with SUP resulted in a non-significant reduction in cells stained with the neuronal marker NeuN (Figure 6AB). This effect was not cell specific as SUP also led to non-significant decreases in Iba1⁺ (Figure 2AB) and GFAP⁺ cells (Figure 9AB). Similarly, MINO and FC did not significantly affect total NeuN⁺ (Figure 6AB), GFAP⁺ (Figure 9AB and Figure 7EF), or Iba1⁺ cells (Figure 2AB and Figure 7AB) compared to control. The lack of differences in numbers of cells and proportions of cell types compared to controls suggests our results are not driven by differences in cell viability.

Interestingly, although SUP 4h and MINO 4h individually led to decreased Iba1 gene expression (Figure 2D), the combination SUP + MINO 4h led to a significant increase in Iba1 gene expression, as did FC 4h. In our immunocytochemistry results, MINO 4h and FC 4h both led to a trend toward a decreased proportion of Iba1⁺ cells but with MINO there was an increase in the proportion of Iba1⁺ cells that were also IL-6⁺ (Figure 2). These differences in gene expression and positively stained cells provide additional evidence of an inflammation induced alteration of the balance between transcription and translation. These results also support the idea that altered responses from other cell types can influence the microglial response. To be more precise, treatment of primary NG cultures with SUP 4h will likely produce some reaction from all cell types in the culture. If all cells respond appropriately to stimulation with SUP 4h, cell-to-cell signals will promote a defined set of responses that, in concert, reduce Iba1 gene expression. When glial cells are inhibited—either completely or partially—the lack of confirmatory modulatory signaling between cell types alters responding (i.e. the ability of SUP 4h to reduce Iba1 gene expression requires signaling from another cell type and the signaling from the other cell types depends on the microglial response; thus, if either cell type is inhibited the concerted response is altered). Since we did not observe extensive neuronal networks in our cultures and there is evidence that NG neurons are not likely to form synapses in culture if SG from the NG are also present [81,

82], as they were in our primary cultures, we believe release of signaling molecules to be the primary means of interaction between cells in our model. However, we also cannot fully exclude direct cell-to-cell communication in our experiments.

While alterations in non-neuronal crosstalk may be driving some of the apparent lack of inhibition by our inhibitors, it is also possible that our results reflect lack of and/or off-target effects from the inhibitors we chose. The limitations of drug-induced cellular inhibition are an important consideration and both MINO and FC have reported crossreactivity, and variability in their inhibitory effects [50–52, 75, 83–101]. MINO is a commonly used microglial inhibitor [50, 75, 83] and FC has been extensively used as an astrocyte [51, 52, 96] and SG inhibitor [97–99, 101]. The doses chosen for our experiments are in line with effective doses found in the literature [50, 51]. However, much higher doses have also been used [75, 96]. Therefore, it is possible that the doses we used did not lead to complete microglia/macrophage or SG inhibition.

The mechanism of MINO's inhibition of microglia has been attributed specifically to inhibition of the p38-MAPK pathway, and may not include other inflammatory pathways (e.g. NF- κ B) [85]; thus, even if our dose was adequate, incomplete inhibition is possible. The characteristics of MINO inhibition of microglia/macrophages also indicate partial inhibition. For example, in rat models of facial crush injury [89] and cardiac arrest [90] MINO did not inhibit microglial proliferation and in studies of bacterially-induced injury inclusion of MINO reduced Iba1 staining but, depending on dose and timing, led to increased TNF- α and CCL2 expression [93]. Some of this variability may be explained by evidence that MINO selectively inhibits microglia polarization towards an M1 phenotype while up-regulating expression of M2 phenotype markers [76, 102]. Iba1 is expressed by both M1 and M2 microglia and Iba1 expression increases in parallel with increased activity of both microglia phenotypes [103]. We found, using IL-6 staining, an increased proportion of M1 polarized Iba1⁺ cells after treatment with SUP 4h (Figure 2ABC). Importantly, our IL-6 staining was largely concurrent with Iba1⁺ staining suggesting changes in IL-6 gene expression are likely driven by microglia and/or macrophages. In Figure 2, we found that SUP induced a strong increase in M1 marker gene expression, notably IL-6, IFN- γ and CD86. Expression of these M1 genes was significantly reduced (but not normalized) by the addition of MINO. This provides evidence of some inhibition of the pro-inflammatory M1 state in the SUP + MINO group. Similarly to our study, Iba1⁺ staining in the cortex decreased with MINO treatment in mice with cerebral hypoperfusion injury but increased when an inflammatory stimulus (LPS) and MINO were administered concurrently [104]. Not unlike our discussion here, the authors concluded that MINO may have indirect and off-target effects on microglia. Not only are the effects of MINO on microglia/macrophages sometimes unexpected, MINO may also affect the responses of other cell types, including astrocytes and SG [84, 87–93]. Overall, our findings support 1) that MINO is an incomplete microglia inhibitor, 2) that Iba1 gene expression and protein expression may not be temporally matched, 3) Iba1 expression may not be an immediately essential feature of microglia/macrophage activation, and 4) MINO inhibits to some degree M1 phenotype polarization in macrophages/microglia.

The inhibitory effects of FC occur via inhibition of aconitase, a key enzyme of the citric acid cycle (TCA). FC has reduced GFAP staining in the dorsal root ganglia in rat models of spinal nerve ligation [97] and in the trigeminal ganglia in a rat model of temporomandibular joint pain [100]. However, FC doses thousands of times ours were unable to fully inhibit the TCA cycle in brain aggregates used to study anoxia [96] evidenced by lactate production and tracing metabolic pathways with ^{13}C labeled glucose and acetate [96].

Astrocytes release more than 6 times more lactate than neurons [105]. Astrocytes and SG are not completely identical, but our cell culture media lactate measurements (Figure 5) found lactate at control levels with FC 4h and reduced with SUP and SUP + FC. These results suggest that we did not measurably inhibit glucose metabolism with our 3 nM FC dose. However, it appears the treatment with SUP decreased SG metabolism which is supported by the trend toward reduced GFAP⁺ cells with SUP 4h and either inhibitor alone (Figure 9). Lactate production by SG has not been investigated so it is possible this result is driven by another cell type. Another important difference between our study and the referenced anoxia study is that FC was given 6h before the induction of anoxia, which those authors identified as the greatest point of FC inhibition. In our experiments, FC was included with the immune stimulant; thus, our results may be better described as the response to activation followed by inhibition, which is likely a different outcome compared to inhibition prior to activation.

We quantified IL-10⁺ cells to assess anti-inflammatory status in our cultures (Figure 6AC). IL-10⁺ cells were significantly reduced by all 4h treatments suggesting a decrease in anti-inflammatory signaling. We only performed IL-10⁺ colocalization with NeuN and the relative proportion of NeuN⁺ cells that are also IL-10⁺ was not changed by any treatments (Figure 6AC), suggesting that changes in IL-10 are driven by non-neuronal cell types. While we cannot say with certainty that our IL-10 staining is solely associated with microglia/macrophage populations, our data support the idea that microglia/macrophages are in less of an immunomodulatory M2b state after all 4h treatments [64]. Although our immunocytochemistry did not find increased IL-10⁺ cells after MINO 4h (Figure 6), there is a trend toward increased IL-10 gene expression induced by MINO 4h (Figure 6E) which is in agreement with MINO promotion of the M2 phenotype.

SOCS3 expression was significantly increased by SUP + MINO or SUP + FC at 4h and 24h, and by SUP + MINO + FC 24h (Figure 6F). Although SOCS3 expression was not increased by either MINO or FC at 4h, FC or MINO + FC at 24h increased SOCS3 expression. Quantification of inflammatory protein levels in the culture media revealed that TSP-1 release was significantly decreased by SUP 4h, SUP + MINO 4h, SUP + FC 4h, and SUP + MINO + FC 24h while FC 4h significantly increased TSP-1 (Figure 3B). In the CNS, STAT3 activation promotes increased TSP-1 expression in astrocytes leading to synaptic remodeling after axonal injury [106] and activation of the STAT3 pathway in this condition is supported by the trend toward increasing SOCS3 (Figure 6F) and significantly increased GFAP (Figure 7I) gene expression. However, the patterns of SOCS3 and GFAP expression across our experimental conditions are not consistent with TSP-1 release. This could be a discordance in gene expression and protein expression, noted with Iba1 [60], or it could indicate that the combined influence of additional factors collectively promote alternate responses. These results could also be related to the previously mentioned variability in the response to FC

dose and timing (Section 3.1). IL-10 expression, overall, was not changed by any of the 4h treatments except MINO + FC combined (Figure 6E). This pattern of SOCS3 and IL-10 expression may indicate that, in our model, there is a temporal component at play (e.g. we induce an M1 phenotype at 4h transitioning to an M2 phenotype later) and/or these anti-inflammatory genes are derived from different cell types (e.g. SOCS3 is largely a neuronal response while IL-10 is largely produced by non-neuronal cell types) resulting in differences in expression patterns when inhibitors are included with SUP 4h. At 24h, SUP, with or without MINO and/or FC led to increased IL-10 gene expression. A 24h exposure to SUP did not affect total Iba1⁺ cell numbers (Figure 7AB) but CCL2⁺ and CD206⁺ cells were increased compared to controls (Figure 7CG). CCL2 and CD206 are recognized markers of the M2a microglia/macrophage phenotype, associated with repair and regeneration [64]. This increase in IL-10 gene expression at 24h and our immunocytochemistry findings (Figure 6) suggest an anti-inflammatory M2 microglia/macrophage polarization at 24h. It should be noted however that counts of Iba1⁺/CD206⁺ cells only trend toward increase at SUP 24h, so we cannot rule out that an additional cell type is expressing CD206. CCL2 was double stained with GFAP in SUP 24h, and the proportion of CCL2⁺/GFAP⁺ cells was not different from controls at SUP 24h, suggesting that the increase in CCL2⁺ cells is associated with non-GFAP⁺ cells, which includes microglia/macrophages and supports our conclusion of the M2a phenotype increasing over 24h. In some agreement with these findings, our measurements of inflammation-associated proteins released into cell culture media found CCL2 levels, associated with astrocytic promotion of reactive microglia [46] as well as of M2a microglia/macrophage activation [64], were significantly increased by inclusion of MINO with SUP 24h and trended toward increase with SUP + FC 24h, MINO + FC 24h, and SUP + MINO + FC 24h (Figure 3A); CCL2 gene expression also trended toward increase with SUP + MINO + FC 24h (Figure 7J).

SUP 4h significantly increased CCL2 gene expression (Figure 7J) which was at control levels by 24h (see Supplementary Figure 1). CCL2 in this setting may also be a SG-derived signal promoting microglia/macrophage activation. Interestingly, the reciprocating microglia-derived, astrocyte and presumably SG-activating signal VEGFB remained at control levels after treatment with SUP and was only increased when inhibitors were included with the treatment (Figure 8A) supporting the possibility that microglia/macrophages promote reactivity from other cell types when crosstalk is altered. A concurrent increase in TGF α , notably associated with microglial downregulation of reactive astrocytes, in the presence of SUP + MINO 4h or SUP + FC 4h (Figure 8B) suggests that signals for activation of astrocytes/SG are counteracted by their opposing signals, similar to the way SOCS3 expression may increase in response to an inflammatory insult [107]. In fact, like STAT3 being an inducer of SOCS3, which then inhibits the JAK/STAT pathway, activation of the NF- κ B pathway promotes expression of TGF α along with inflammatory cytokines [108].

FC 4h significantly increased gene expression of CCL2 (Figure 7J); this is not unexpected as FC also increased expression of GFAP (Figure 7I). CCL2 gene expression was also increased by SUP 4h and trended toward control levels with SUP 24h (Figure 7J and Supplementary Figure 1). Inclusion of MINO but not FC attenuated SUP-induced increases in CCL2 expression at 4h, suggesting a role for microglia/macrophages in this result. MINO

or FC alone or with SUP significantly increased TGF β gene expression at 4h (Figure 7K). TGF β is associated with the M2a microglia/macrophage phenotype [64] and MINO is thought to inhibit M1 while promoting M2 activation [76]. Besides being a microglia-derived signal, TGF β released from astrocytes reduces proinflammatory microglia activation [46] but gene expression of signals associated with increased activation of microglia and astrocytes are also increased in this condition.

VEGFB (Figure 8A) and LCN2 (Figure 8C), factors that promote astrocyte or microglial activation respectively [45, 46], showed increased expression compared to controls and SUP 4h when MINO or FC was included with the treatment. The apparent failure of glial inhibitors to fully regulate these activation-promoting responses in our model suggests that uninhibited cell types, for example the neurons themselves, can upregulate inflammatory gene expression. LCN2, although associated with communication between non-neuronal cell types, may also be released as an alarm signal by injured neurons [109]. We found increased, or trends toward increased, LCN2 gene expression in several treatments that included SUP with an inhibitor (Figure 8C). The potential role of LCN2 as a neuronal alarm signal is intriguing because LCN2 gene expression was not increased by SUP alone (Figure 8C). This supports the idea that in normal functioning, a molecular response from nonneuronal cells may downregulate this neuronal alarm and that cell-to-cell communication is important for the characteristics of the inflammatory response produced by treatment with GP bacterial byproducts. This escalation of activation-promoting factors may be the result of altered cross signaling between microglia/macrophages and other cell types when inhibitors are present.

There are limitations in using cell markers, as they may not be population specific. Here, we have used three cell identity markers to delineate a population of more than three cell types. SG and nonmyelinating Schwann cells (NSC) are believed to come from the same precursor cell type and express many of the same markers [110] making differentiation a significant challenge. However, there are an estimated 2–3 SG per neuron in peripheral ganglia [41] and they could drive GFAP⁺ staining within the NG. There is evidence that SOX10 may be specific for NSC vs SG, therefore we included SOX10 staining (Figure 9). If SOX10 is indeed NSC specific, our results suggest that in control conditions, SG:NSC is roughly 1:1. Treatment with either MINO 4h or FC 4h more than doubles the ratio of SG:NSC and is combined with a trend toward a decreased proportion of GFAP⁺ cells. This data points to a few scenarios. It could be that our inhibitors, MINO and FC, inhibit NSC in addition to or instead of the cell types we hoped to target. It may also be that both SG and NSC express SOX10 and our colocalization gives us more insight about the induced response than it does about the specific cell types. In fact, SG have been found to be phenotypically adaptable [111–113]. While it is not clear if this adaptability extends into adulthood and across species, increased SOX10 has been indicated as driving SG adoption of an oligodendrocyte phenotype [111] and adaptability has been observed in SG from P90 female rats [113] which is an age similar to the rats used in our cultures. Thus, while it is inappropriate to assume all GFAP⁺ cells are the same type, the fact that SG and NSC come from a common precursor combined with SG adaptability raise doubts 1) that it is possible to confidently differentiate these cell types *in vitro* and 2) that it may not be necessary given their overlapping features.

3.2 Treatment with LTA alters expression of some inflammation- and crosstalk-associated genes.

LTA is a component of the gram-positive bacterial cell wall that can interact with TLR2 [31]. While there is no body of research investigating its interaction with gut-brain signaling, LTA can induce an apparent M1 microglia phenotype in vitro [114], has been implicated in the development of non-alcoholic steatohepatitis via its interaction with TLR2 [115], and may have a role in the development HF diet-associated enteric neuropathy [116]. LTA is therefore a good candidate to serve as our representative GP bacterial byproduct with potential deleterious effects on host neurological and metabolic health.

LTA did not change gene expression of GFAP or mAP2 at either dose tested; however, there was a non-significant downward trend in Iba1 gene expression after exposure to LTA 0.015 µg/mL (Figure 10ABC), similar to our findings with SUP. IFN γ and IL-6 gene expression do not differ significantly from control levels after exposure to LTA at 0.015 µg/mL or 20 µg/mL but TNF α and IL-1 β gene expression were significantly increased by LTA 20 µg/mL (Figure 10DEFG). These results have similarities to the response to SUP and support a role for specific GP bacterial byproducts in the induction of NG inflammation. The anti-inflammation associated genes did not change in response to either LTA dose. SUP did not produce a significant change in anti-inflammation associated genes until 24h, which we did not measure with LTA. Genes associated with microglia-to-astrocyte crosstalk, VEGFB and TGF α , were not significantly changed by either dose of LTA (Figure 10JK) and this is similar to the effect of SUP on these genes (Figure 2). CCL2, a gene associated with astrocyte-to-microglia crosstalk, was significantly increased by LTA 20 µg/mL (Figure 10L). Interestingly, LTA 0.015 µg/mL + FC increased expression of TGF β but LTA 0.015 µg/mL + MINO resulted in significantly decreased TGF β gene expression (Figure 10N). It may be that there is a difference in the way our dose of LTA promotes an inflammatory response compared to SUP because MINO and FC, alone or with SUP, result in significantly increased TGF β (Figure 7K).

3.3 Treatment with meso-DAP alters expression of inflammation- and crosstalk-associated genes

Meso-DAP, an identified *S. chungangensis* [49] cell wall component, is a NOD1 [117] agonist making it a good choice for a potentially *S. chungangensis*-derived inflammation inducing molecule for our experiments. IFN γ , IL-1 β , and TNF α but not IL-6 gene expression were significantly increased by meso-DAP (Figure 10DEFG), showing similarity to the response seen with SUP (Figure 2EGH). However, measurement of released inflammation-associated proteins found CXCL1, an M1 marker, trended toward decrease by treatment with meso-DAP 4h (Figure 3C). Unlike the response to SUP, inclusion of miNO did not attenuate IFN γ or TNF α gene expression in response to meso-DAP but did cause IL-1 β expression to trend downward (Figure 10DFG). This could be due to differences in the amount of meso-DAP in SUP compared to our chosen dose. The differences in response may also mean that *S. chungangensis* byproducts include more than one inflammation-inducing moiety. Not unlike SUP and LTA, genes associated with microglia-to-astrocyte crosstalk were maintained at control levels in response to meso-DAP (Figure 10J). CCL2 and TGF β , genes associated with astrocyte-to-microglia crosstalk, were both significantly

increased by meso-DAP (Figure 10LN) and this response was reduced when MINO was included with the treatment. The CCL2 response to meso-DAP was similar to the response induced by SUP. LCN2 gene expression did not change in response to meso-DAP (Figure 10M) and this is consistent with the response we found with SUP. Although there are some differences in the inflammatory response to meso-DAP compared to SUP, our results show that byproducts from a HFD-associated *Actinobacteria* could play a role in gut-originating vagal inflammation.

4. Conclusion

We have shown that byproducts from GP bacteria are able to induce inflammatory and counteracting responses in cultured NG. This was seen in an increased proportion of cells that stained positive for Iba1⁺ + IL-6⁺ and increased expression of IFN γ , IL-6, TNF α , and IL-1 β suggesting treatment with SUP induces classic, M1 microglia/macrophage polarization. Including glial inhibitors with SUP altered the response and our results support the role of non-neuronal crosstalk in mediating and moderating the inflammatory responses. LTA, a representative GP byproduct and meso-DAP, a *S. chungangensis* specific byproduct, were both able to produce some responses similar to those induced by SUP, supporting a potential role for GP bacteria, particularly *Actinobacteria*, in gut-originating vagal inflammation.

Our model investigates the response to administration of an acute, high dose of limited bacterial byproducts which does not accurately represent the milieu that would be found *in vivo* or the response that would be found in the chronic low-dose setting of consistent HF feeding. *In vivo*, multiple studies find that HF-feeding leads to increased gut permeability [28] and an increase in circulating levels of the bacterial byproduct LPS [23, 28]. Vagal afferents' terminals in the lamina propria may respond directly to bacterial byproducts or react to signals originating from enteric glia or peripheral immune cells. It is also possible that gut-originating bacterial byproducts in circulation induce an inflammatory response in the NG (i.e. vaga, inflammatory reflex [59]). *In vivo* studies have investigated diet-induced inflammation in the NG of rats [20, 28, 72, 118] and male mice [72, 119, 120]. In the latter, mice were fed a 60% HF diet and effects were evaluated at 24h [119], 2 weeks [120], 4 weeks [120], and 12 weeks [72]. After 24h HF feeding, Iba1 and CD86 immunostaining and Iba1, IL-6, and TNF α gene expression increased in the NG [119]. Vagotomy or ghrelin treatment prevented the inflammatory response to 24h HF feeding, directly implicating vagal activation rather than systemic circulating factors in the early observed inflammatory response [119]. This immediate inflammatory response in the NG subsides by 2 weeks [120] but re-emerges by 4 weeks and remains at 12 weeks [72, 120]. This re-emergence of vagal inflammation by 4 weeks corresponds to the time frame we have observed hyperphagia in rats on HF diet [23] and supports the hypothesis that vagal inflammation promotes impaired feeding regulation. We can wonder if bacterially-driven inflammation may be part of the early response, the later response or both. HF diet consumption leads to microbiota dysbiosis in as little as one to seven days [28, 121–123]. However, short-term HF feeding has also been shown to promote gut-barrier integrity in the very short term in rat models of hemorrhagic shock and sepsis [124–127]. Our current results may therefore represent interactions that occur after chronic HF feeding. Our data broadly support the idea that the

gut microbiome communicates with VAN by way of inflammation. Additionally, while GP bacterial byproducts have been implicated in NASH [115] and enteric enteropathy [116], both of which are HF-feeding associated diseases, to our knowledge we are the first to demonstrate that GP bacterial byproducts can induce inflammation in the NG; whether GP bacterially induced NG inflammation induces feeding dysregulation remains to be investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors acknowledge the equipment and technical expertise provided by Dr. Robert Gogal, College of Veterinary Medicine, University of Georgia and the assistance with immunochemistry image acquisition provided by Dr. Elizabeth Klingbeil, Department of Foods and Nutrition, University of Georgia.

Funding

This work was supported by NIDDK grant #1021RR581526 from the National Institutes of Health to CDLS.

List of abbreviations:

Arg1	arginase 1
CCL2	C-C motif chemokine ligand-2
CNS	central nervous system
CXCL1	chemokine C-X-C motif ligand 1
CCK	cholecystokinin
CD	cluster of differentiation
DAP	diaminopimelic
FC	fluorocitrate
CX3CR1	fractalkine receptor
GGBC	Georgia Genomics and Bioinformatics Core
GFAP	glial fibrillary acidic protein
IFNγ	interferon- γ
IL	interleukin
Iba1	ionized calcium-binding adapter molecule-1
JAK/SAT	Janus kinase / signal transducer and activator of transcription proteins pathway
LCN2	lipocalin-2

LPS	lipopolysaccharide
LTA	lipoteichoic acid
meso-DAP	meso-diaminopimelic acid
MSD	Mesoscale Discovery
MAP2	microtubule-associated protein-2
MiNO	minocycline
NG	nodose ganglion
NSC	nonmyelinating Schwann cells
NOD	nucleotide-binding oligomerization domain
NTS	nucleus of the solitary tract
PFA	paraformaldehyde
PBS	phosphate buffered saline
PLL	poly-L-lysine
SG	satellite glia
SOCS-3	suppressor of cytokine signaling-3
TCA	the citric acid cycle
TSP1	thrombospondin-1
TLR	toll-like receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
VAN	vagal afferent neurons
VEGFB	vascular endothelial growth factor-B

8. References

- [1]. Berthoud H-R, Powley TL Characterization of vagal innervation to the rat celiac, suprarenal, and mesenteric ganglia. *J. Auton. Nerv. Syst* 1993;42:153–70. [PubMed: 8450174]
- [2]. Berthoud HR The vagus nerve, food intake and obesity. *Regul. Pept* 2008;149:15–25. [PubMed: 18482776]
- [3]. Berthoud HR, Lynn PA, Blackshaw LA Vagal and spinal mechanosensors in the rat stomach and colon have multiple receptive fields. *Am J Physiol Regulatory Integrative Comp Physiol*. 2001;280:R1371–R81.
- [4]. Cawthon CR, de La Serre CB Gut bacteria interaction with vagal afferents. *Brain Res*. 2018;1693:134–9. [PubMed: 29360469]

- [5]. Rinaman L Ascending projections from the caudal visceral nucleus of the solitary tract to brain regions involved in food intake and energy expenditure. *Brain Res.* 2010,1350:18–34. [PubMed: 20353764]
- [6]. Mayer EA Gut feelings: the emerging biology of gut-brain communication. *Nat. Rev. Neurosci* 2011,12:453–66. [PubMed: 21750565]
- [7]. Grill HJ, Hayes MR Hindbrain neurons as an essential hub in the neuroanatomically distributed control of energy balance. *Cell Metab.* 2012,16:296–309. [PubMed: 22902836]
- [8]. Zarbin MA, Wamsley JK, Innis RB, Kuhar MJ Cholecystokinin Receptors: Prescense and Axonal Flow in the Rat Vagus Nerve. *Life Sci.* 1981,29:697–705. [PubMed: 6168883]
- [9]. Moran TH, Smith GP, Hostetler AM, McHugh PR Transport of cholecystokinin (CCK) binding sites in subdiaphragmatic vagal branches *Brain Res.* 1987,415:149–52. [PubMed: 2441809]
- [10]. Buyse M, Ovesjo M-L, Goiot H, Guilmeau S, Peranzi G, Moizo L, et al. Expression and regulation of leptin receptor proteins in afferent and efferent neurons of the vagus nerve. *Eur. J. Neurosci* 2001,14:64–72. [PubMed: 11488950]
- [11]. Burdyga G, Spiller D, Morris R, Lal S, Thompson DG, Saeed S, et al. Expression of the Leptin Receptor in Rat and Human Nodose Ganglion Neurons. *Neuroscience.* 2002,109:339–47. [PubMed: 11801369]
- [12]. Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. *Am J Physiol Gastrointest Liver Physiol.* 2006,290:G1289–97. [PubMed: 16423919]
- [13]. Burdyga G, Lal S, Varro A, Dimaline R, Thompson DG, Dockray GJ Expression of cannabinoid CB1 receptors by vagal afferent neurons is inhibited by cholecystokinin. *J Neurosci.* 2004,24:2708–15. [PubMed: 15028763]
- [14]. Blanke EN, Stella SL Jr., Ruiz-Velasco V, Holmes GM Purinergic receptor expression and function in rat vagal sensory neurons innervating the stomach. *Neurosci. Lett* 2019,706:182–8. [PubMed: 31085293]
- [15]. de La Serre CB, de Lartigue G, Raybould HE Chronic exposure to Low dose bacterial lipopolysaccharide inhibits leptin signaling in vagal afferent neurons. *Physiol. Behav* 2015,139:188–94. [PubMed: 25446227]
- [16]. Wang J, Kollarik M, Ru F, Sun H, McNeil B, Dong X, et al. Distinct and common expression of receptors for inflammatory mediators in vagal nodose versus jugular capsaicin-sensitive/TRPV1-positive neurons detected by low input RNA sequencing. *PLoS One.* 2017,12:e0185985. [PubMed: 28982197]
- [17]. Kentish S, Li H, Philp LK, O'Donnell TA, Isaacs NJ, Young RL, et al. Diet-induced adaptation of vagal afferent function. *J. Physiol* 2012,590:209–21. [PubMed: 22063628]
- [18]. de Lartigue G, Dimaline R, Raybould H, Dockray GJ, Varro A, Lur G EGR1 Is a Target for Cooperative Interactions between Cholecystokinin and Leptin, and Inhibition by Ghrelin, in Vagal Afferent Neurons *Endocrinology.* 2010,151:3589. [PubMed: 20534729]
- [19]. de Lartigue G, de La Serre CB, Espero E, Lee J, Raybould HE Leptin Resistance in Vagal Afferent Neurons Inhibits Cholecystokinin Signaling and Satiety in Diet Induced Obese Rats. *PLoS One.* 2012,7.
- [20]. de Lartigue G, Raybould HE, Lee J, de la Serre CB, Espero E Diet-induced obesity leads to the development of leptin resistance in vagal afferent neurons. *AJP: Endocrinology and Metabolism.* 2011,301:E187. [PubMed: 21521717]
- [21]. Ronveaux CC, de Lartigue G, Raybould HE Ability of GLP-1 to decrease food intake is dependent on nutritional status. *Physiol. Behav* 2014,135:222. [PubMed: 24955496]
- [22]. de Lartigue G, Ronveaux CC, Raybould HE Deletion of leptin signaling in vagal afferent neurons results in hyperphagia and obesity. *Mol Metab.* 2014,3:595. [PubMed: 25161883]
- [23]. Klingbeil EA, Cawthon C, Kirkland R, de La Serre CB Potato-Resistant Starch Supplementation Improves Microbiota Dysbiosis, Inflammation, and Gut-Brain Signaling in High Fat-Fed Rats. *Nutrients.* 2019,11.
- [24]. Petersen C, Round JL Defining dysbiosis and its influence on host immunity and disease. *Cell. Microbiol* 2014,16:1024–33. [PubMed: 24798552]

- [25]. Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. *PNAS*. 2004;101:15718. [PubMed: 15505215]
- [26]. Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI Obesity alters gut microbial ecology. *PNAS*. 2005;102:11070–5. [PubMed: 16033867]
- [27]. Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen Y-Y, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. *Gastroenterology*. 2009;137:1716–24. [PubMed: 19706296]
- [28]. Sen T, Cawthon CR, Ihde BT, Hajnal A, DiLorenzo PM, de La Serre CB, et al. Diet-driven microbiota dysbiosis is associated with vagal remodeling and obesity. *Physiol. Behav* 2017;173:305–17. [PubMed: 28249783]
- [29]. Hansen AK, Nielsen DS Handbook of Laboratory Animal Bacteriology, 2nd Edition Boca Raton, FL: CRC Press; 2015.
- [30]. Lampron A, Elali A, Rivest S Innate immunity in the CNS: redefining the relationship between the CNS and Its environment. *Neuron*. 2013;78:214–32. [PubMed: 23622060]
- [31]. Oliveira-Nascimento L, Massari P, Wetzler LM The Role of TLR2 in Infection and Immunity. *Front. Immunol* 2012;3:79. [PubMed: 22566960]
- [32]. Lehnardt S Innate immunity and neuroinflammation in the CNS: the role of microglia in Toll-like receptor-mediated neuronal injury. *Glia*. 2010;58:253–63. [PubMed: 19705460]
- [33]. Trudler D, Farfara D, Frenkel D Toll-like receptors expression and signaling in glia cells in neuro-amyloidogenic diseases: towards future therapeutic application. *Mediators Inflamm*. 2010;2010.
- [34]. Mitterreiter JG, Ouwendijk WJD, van Velzen M, van Nierop GP, Osterhaus A, Verjans G Satellite glial cells in human trigeminal ganglia have a broad expression of functional Toll-like receptors. *Eur. J. Immunol* 2017;47:1181–7. [PubMed: 28508449]
- [35]. Waise TMZ, Dranse HJ, Lam TKT The metabolic role of vagal afferent innervation. *Nat. Rev. Gastroenterol. Hepatol* 2018;15:625–36. [PubMed: 30185916]
- [36]. Thomas PK The connective tissue of peripheral nerve: an electron microscope study. *Journal of Anatomy, London*. 1963;97:35–44.
- [37]. Hayward JH, Lee SJ A Decade of Research on TLR2 Discovering Its Pivotal Role in Glial Activation and Neuroinflammation in Neurodegenerative Diseases. *Exp. Neurobiol* 2014;23:138–47. [PubMed: 24963278]
- [38]. Kim D, You B, Lim H, Lee SJ Toll-like receptor 2 contributes to chemokine gene expression and macrophage infiltration in the dorsal root ganglia after peripheral nerve injury. *Mol. Pain* 2011;7:74. [PubMed: 21951975]
- [39]. Ubogu EE The molecular and biophysical characterization of the human blood-nerve barrier: current concepts. *J. Vasc. Res* 2013;50:289–303. [PubMed: 23839247]
- [40]. Pannese E Biology and Pathology of Perineuronal Satellite Cells in Sensory Ganglia. Cham, Switzerland: Springer International Publishing AG part of Springer Nature; 2018.
- [41]. Hanani M Satellite glial cells in sensory ganglia: from form to function. *Brain Res. Brain Res. Rev* 2005;48:457–76. [PubMed: 15914252]
- [42]. Miller KE, Richards BA, Kriebel RM Glutamine-, glutamine synthetase-, glutamate dehydrogenase- and pyruvate carboxylase-immunoreactivities in the rat dorsal root ganglion and peripheral nerve. *Brain Res*. 2002;945:202–11. [PubMed: 12126882]
- [43]. van Velzen M, Laman JD, Kleinjan A, Poot A, Osterhaus AD, Verjans GM Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype. *J Immunol*. 2009;183:2456–61. [PubMed: 19635905]
- [44]. Jha MK, Jo M, Kim JH, Suk K Microglia-Astrocyte Crosstalk: An Intimate Molecular Conversation. *Neuroscientist*. 2018;1073858418783959.
- [45]. Rothhammer V, Borucki DM, Tjon EC, Takenaka MC, Chao CC, Arduro-Fabregat A, et al. Microglial control of astrocytes in response to microbial metabolites. *Nature*. 2018;557:724–8. [PubMed: 29769726]
- [46]. Norden DM, Fenn AM, Dugan A, Godbout JP TGFbeta produced by IL-10 redirected astrocytes attenuates microglial activation. *Glia*. 2014;62:881–95. [PubMed: 24616125]

- [47]. Feldman-Goriachnik R, Belzer V, Hanani M Systemic inflammation activates satellite glial cells in the mouse nodose ganglion and alters their functions. *Glia*. 2015;63:2121–32. [PubMed: 26109245]
- [48]. Johnston JR, Freeman KG, Edwards GL Activity in nodose ganglia neurons after treatment with CP 55,940 and cholecystokinin. *Physiol Rep*. 2018;6:e13927. [PubMed: 30512249]
- [49]. Traiwan J, Park MH, Kim W *Serinicoccus chungangensis* sp. nov., isolated from tidal flat sediment, and emended description of the genus *Serinicoccus*. *Int. J. Syst. Evol. Microbiol* 2011;61:1299–303. [PubMed: 20639223]
- [50]. Suk K Minocycline suppresses hypoxic activation of rodent microglia in culture. *Neurosci. Lett* 2004;366:167–71. [PubMed: 15276240]
- [51]. Hermann GE, Viard E, Rogers RC Hindbrain glucoprivation effects on gastric vagal reflex circuits and gastric motility in the rat are suppressed by the astrocyte inhibitor fluorocitrate. *J Neurosci*. 2014;34:10488–96. [PubMed: 25100584]
- [52]. Paulsen RE, Contestable A, Villani L, Fonnum F An In Vivo Model for Studying Function of Brain Tissue Temporarily Devoid of Glial Cell Metabolism: The Use of Fluorocitrate. *J. Neurochem* 1987;48:1377–85. [PubMed: 3559554]
- [53]. Rodrigues RS, Ribeiro FF, Ferreira F, Vaz SH, Sebastiao AM, Xapelli S Interaction between Cannabinoid Type 1 and Type 2 Receptors in the Modulation of Subventricular Zone and Dentate Gyrus Neurogenesis. *Front. Pharmacol* 2017;8:516. [PubMed: 28848435]
- [54]. Li S, Hu X, Zhang M, Zhou F, Lin N, Xia Q, et al. Remote ischemic postconditioning improves neurological function by AQP4 down-regulation in astrocytes. *Behav. Brain Res* 2015;289:1–8. [PubMed: 25907740]
- [55]. Zhou S, Zhou Y, Yu J, Du Y, Tan Y, Ke Y, et al. Ophiocordyceps lanpingensis polysaccharides attenuate pulmonary fibrosis in mice. *Biomed. Pharmacother* 2020;126:110058. [PubMed: 32145591]
- [56]. Liu J, Li K, Zhou J, Sun T, Yang C, Wei J, et al. Bisperoxovanadium induces M2-type macrophages and promotes functional recovery after spinal cord injury. *Mol. Immunol* 2019;116:56–62. [PubMed: 31605961]
- [57]. Wang J, Fu X, Zhang D, Yu L, Lu Z, Gao Y, et al. Effects of crenolanib, a nonselective inhibitor of PDGFR, in a mouse model of transient middle cerebral artery occlusion. *Neuroscience*. 2017;364:202–11. [PubMed: 28943249]
- [58]. Peng KT, Liu JF, Chiang YC, Chen PC, Chiang MH, Shih HN, et al. Particulate matter exposure aggravates osteoarthritis severity. *Clin. Sci. (Lond.)* 2019;133:2171–87. [PubMed: 31696218]
- [59]. Shi XD, Chen LW, Li SW, Sun XD, Cui FZ, Ma HM The observed difference of RAW264.7 macrophage phenotype on mineralized collagen and hydroxyapatite. *Biomed Mater*. 2018;13:41001.
- [60]. Silverman HA, Dancho m., Regnier-Golanov A, Nasim M, Ochani M, Olofsson PS, et al. Brain region-specific alterations in the gene expression of cytokines, immune cell markers and cholinergic system components during peripheral endotoxin-induced inflammation. *Mol. Med* 2015;20:601–11. [PubMed: 25299421]
- [61]. Madore C, Joffre C, Delpech JC, De Smedt-Peyrusse V, Aubert A, Coste L, et al. Early morphofunctional plasticity of microglia in response to acute lipopolysaccharide. *Brain, Behavior, and Immunity*. 2013;34:151–8.
- [62]. Cherry JD, Olschowka JA, O'Banion MK Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J. Neuroinflammation* 2014;11:98. [PubMed: 24889886]
- [63]. Toledano Furman NE, Prabhakara KS, Bedi S, Cox CS, Olson SD OMIP-041: Optimized multicolor immunofluorescence panel rat microglial staining protocol. *Cytometry Part A*. 2018;93:182–5.
- [64]. Chhor V, Le Charpentier T, Lebon S, Ore MV, Celador IL, Josserand J, et al. Characterization of phenotype markers and neuronotoxic potential of polarised primary microglia in vitro. *Brain. Behav. Immun* 2013;32:70–85. [PubMed: 23454862]
- [65]. Marshall SA, McClain JA, Kelso ML, Hopkins DM, Pauly JR, Nixon K Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: The importance of microglia phenotype. *Neurobiol. Dis* 2013;54:239–51. [PubMed: 23313316]

- [66]. Raivich G, Bohatschek M, Kloss CUA, Werner A, Jones LL, Kreutzberg GW Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Res.* 1999;30:77–105.
- [67]. Colton CA Heterogeneity of microglial activation in the innate immune response in the brain. *J. Neuroimmune Pharmacol* 2009;4:399–418. [PubMed: 19655259]
- [68]. Kigerl KA, Gensel JC, Ankeny DP, Alexander JK, Donnelly DJ, Popovich PG Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. *J Neurosci.* 2009;29:13435–44. [PubMed: 19864556]
- [69]. Perry VH, Nicoll JA, Holmes C Microglia in neurodegenerative disease. *Nat Rev Neurol.* 2010;6:193–201. [PubMed: 20234358]
- [70]. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A Physiology of microglia. *Physiological reviews.* 2011;91:461–553. [PubMed: 21527731]
- [71]. Kingwell K Neurodegenerative disease: Microglia in early disease stages. *Nat Rev Neurol.* 2012;8:475. [PubMed: 22890215]
- [72]. Naznin F, Toshinai K, Waise TM, NamKoong C, Md Moin AS, Sakoda H, et al. Diet-induced obesity causes peripheral and central ghrelin resistance by promoting inflammation. *J. Endocrinol* 2015;226:81–92. [PubMed: 26016745]
- [73]. Zhou Y, Wu S, Liang C, Lin Y, Zou Y, Li K, et al. Transcriptional upregulation of microtubule-associated protein 2 is involved in the protein kinase A-induced decrease in the invasiveness of glioma cells. *Neuro Oncol.* 2015;17:1578–88. [PubMed: 26014048]
- [74]. Gumy LF, Katrukha EA, Grigoriev I, Jaarsma D, Kapitein LC, Akhmanova A, et al. MAP2 Defines a Pre-axonal Filtering Zone to Regulate KIF1-versus KIF5-Dependent Cargo Transport in Sensory Neurons. *Neuron.* 2017;94:347–62 e7 [PubMed: 28426968]
- [75]. Liu X, Su H, Chu TH, Guo A, Wu W Minocycline inhibited the pro-apoptotic effect of microglia on neural progenitor cells and protected their neuronal differentiation in vitro. *Neurosci. Lett* 2013;542:30–6. [PubMed: 23518153]
- [76]. Kobayashi K, Imagama S, Ohgomori T, Hirano K, Uchimura K, Sakamoto K, et al. Minocycline selectively inhibits M1 polarization of microglia. *Cell Death Dis.* 2013;4:e525. [PubMed: 23470532]
- [77]. Kohler A, De Filippo K, Hasenberg M, van den Brandt C, Nye E, Hosking MP, et al. G-CSF-mediated thrombopoietin release triggers neutrophil motility and mobilization from bone marrow via induction of Cxcr2 ligands. *Blood.* 2011;117:4349–57. [PubMed: 21224471]
- [78]. Ni H, Wang Y, An K, Liu Q, Xu L, Zhu C, et al. Crosstalk between NFkappaB-dependent astrocytic CXCL1 and neuron CXCR2 plays a role in descending pain facilitation. *J. Neuroinflammation* 2019;16:1. [PubMed: 30606213]
- [79]. Norden DM, Trojanowski PJ, Villanueva E, Navarro E, Godbout JP Sequential activation of microglia and astrocyte cytokine expression precedes increased Iba-1 or GFAP immunoreactivity following systemic immune challenge. *Glia.* 2016;64:300–16. [PubMed: 26470014]
- [80]. Boutej H, Rahimian R, Thammisetty SS, Beland LC, Lalancette-Hebert M, Kriz J Diverging mRNA and Protein Networks in Activated Microglia Reveal SRSF3 Suppresses Translation of Highly Upregulated Innate Immune Transcripts. *Cell Rep.* 2017;21:3220–33. [PubMed: 29241548]
- [81]. Cooper E Synapse formation among developing sensory neurones from rat nodose ganglia grown in tissue culture. *J. Physiol* 1984;351:263–74. [PubMed: 6146714]
- [82]. De Koninck P, Carbonetto S, Cooper E NGF Induces Neonatal Rat Sensory Neurons to Extend Dendrites in Culture after Removal of Satellite Cells. *Journal of Neuroscience.* 1993;13.
- [83]. Garrido-Mesa N, Zarzuelo A, Galvez J Minocycline: far beyond an antibiotic. *Br. J. Pharmacol* 2013;169:337–52. [PubMed: 23441623]
- [84]. Moller T, Bard F, Bhattacharya A, Biber K, Campbell B, Dale E, et al. Critical data-based re-evaluation of minocycline as a putative specific microglia inhibitor. *Glia.* 2016;64:1788–94. [PubMed: 27246804]

- [85]. Bhatia HS, Roelofs N, Munoz E, Fiebich BL Alleviation of Microglial Activation Induced by p38 MAPK/MK2/PGE2 Axis by Capsaicin: Potential Involvement of other than TRPV1 Mechanism/s. *Sci. Rep* 2017;7:116. [PubMed: 28273917]
- [86]. Song ZP, Xiong BR, Guan XH, Cao F, Manyande A, Zhou YQ, et al. Minocycline attenuates bone cancer pain in rats by inhibiting NF-kappaB in spinal astrocytes. *Acta Pharmacol. Sin* 2016;37:753–62. [PubMed: 27157092]
- [87]. Bernardino AL, Kaushal D, Philipp MT The antibiotics doxycycline and minocycline inhibit the inflammatory responses to the Lyme disease spirochete *Borrelia burgdorferi*. *J. Infect. Dis* 2009;199:1379–88. [PubMed: 19301981]
- [88]. Afroz S, Arakaki R, Iwasa T, Oshima M, Hosoki M, Inoue M, et al. CGRP Induces Differential Regulation of Cytokines from Satellite Glial Cells in Trigeminal Ganglia and Orofacial Nociception. *Int. J. Mol. Sci* 2019;20.
- [89]. Fendrick SE, Miller KR, Streit WJ Minocycline does not inhibit microglia proliferation or neuronal regeneration in the facial nucleus following crush injury. *Neurosci. Lett* 2005;385:220–3. [PubMed: 15964677]
- [90]. Janata A, Magnet IA, Schreiber K, Wilson CD, Stezoski JP, Janesko-Feldman K, et al. Minocycline fails to improve neurologic and histological outcome after ventricular fibrillation cardiac arrest in rats. *World Journal of Critical Care Medicine*. 2019;19:106–19.
- [91]. Hashemi-Monfared A, Firouzi M, Bahrani Z, Zahednasab H, Harirchian MH Minocycline decreases cD36 and increases CD44 in LPS-induced microglia. *J. Neuroimmunol* 2018;317:95–9. [PubMed: 29395319]
- [92]. Silva TM, Chaar LJ, Silva RC, Takakura AC, Camara NO, Antunes VR, et al. Minocycline alters expression of inflammatory markers in autonomic brain areas and ventilatory responses induced by acute hypoxia. *Exp. Physiol* 2018;103:884–95. [PubMed: 29528526]
- [93]. Kielian T, Esen N, Liu S, Phulwani NK, Syed MM, Phillips N, et al. Minocycline modulates neuroinflammation independently of its antimicrobial activity in staphylococcus aureus-induced brain abscess. *Am. J. Pathol* 2007;171:1199–214. [PubMed: 17717149]
- [94]. Gong K, Zou X, Fuchs PN, Lin Q Minocycline inhibits neurogenic inflammation by blocking the effects of tumor necrosis factor-alpha. *Clin. Exp. Pharmacol. Physiol* 2015;42:940–9. [PubMed: 26175075]
- [95]. Kannampalli P, Pochiraju S, Bruckert M, Shaker R, Banerjee B, Sengupta JN Analgesic effect of minocycline in rat model of inflammation-induced visceral pain. *Eur. J. Pharmacol* 2014;727:87–98. [PubMed: 24485889]
- [96]. Sa Santos S, Sonnewald U, Carrondo MJ, Alves PM The role of glia in neuronal recovery following anoxia: In vitro evidence of neuronal adaptation. *Neurochem. Int* 2011;58:665–75. [PubMed: 21316414]
- [97]. Liu FY, Sun YN, Wang FT, Li Q, Su L, Zhao ZF, et al. Activation of satellite glial cells in lumbar dorsal root ganglia contributes to neuropathic pain after spinal nerve ligation. *Brain Res.* 2012;1427:65–77. [PubMed: 22050959]
- [98]. Cao J, Li Z, Zhang Z, Ren X, Zhao Q, Shao J, et al. Intrathecal injection of fluorocitric acid inhibits the activation of glial cells causing reduced mirror pain in rats. *BMC Anesthesiol.* 2014;14:119. [PubMed: 25598703]
- [99]. Lee J, Ohara K, Shinoda M, Hayashi Y, Kubo A, Sugawara S, et al. Involvement of Satellite Cell Activation via Nitric Oxide Signaling in Ectopic Orofacial Hypersensitivity. *Int. J. Mol. Sci* 2020;21.
- [100]. Zhang P, Bi RY, Gan YH Glial interleukin-1beta upregulates neuronal sodium channel 1.7 in trigeminal ganglion contributing to temporomandibular joint inflammatory hypernociception in rats. *J. Neuroinflammation* 2018;15:117. [PubMed: 29678208]
- [101]. Cheng CF, Cheng JK, Chen CY, Lien CC, Chu D, Wang SY, et al. Mirror-image pain is mediated by nerve growth factor produced from tumor necrosis factor alpha-activated satellite glia after peripheral nerve injury. *Pain.* 2014;155:906–20. [PubMed: 24447514]
- [102]. Xu N, Tang X-H, Pan W, Xie Z-M, Zhang G-F, Ji M-H, et al. Spared Nerve Injury Increases the Expression of Microglia M1 Markers in the Prefrontal Cortex of Rats and Provokes Depression-Like Behaviors. *Frontiers in Neuroscience*. 2017;11.

- [103]. Quick ED, Seitz S, Clarke P, Tyler KL Minocycline Has Anti-inflammatory Effects and Reduces Cytotoxicity in an Ex Vivo Spinal Cord Slice Culture Model of West Nile Virus Infection. *J Virol*. 2017;91:e00569–17. [PubMed: 28878079]
- [104]. Ma J, Zhang J, Hou WW, Wu XH, Liao RJ, Chen Y, et al. Early treatment of minocycline alleviates white matter and cognitive impairments after chronic cerebral hypoperfusion. *Scientific reports*. 2015;5:12079-. [PubMed: 26174710]
- [105]. Walz W, Mukerji S Lactate Release From Cultured Astrocytes and Neurons: A Comparison. *Glia*. 1988;1:366–70. [PubMed: 2976396]
- [106]. Tyzack GE, Sitnikov S, Barson D, Adams-Carr KL, Lau NK, Kwok JC, et al. Astrocyte response to motor neuron injury promotes structural synaptic plasticity via STAT3-regulated TSP-1 expression. *Nature Communications*. 2014;5.
- [107]. Duncan SA, Baganizi DR, Sahu R, Singh SR, Dennis VA SOCS Proteins as Regulators of Inflammatory Responses Induced by Bacterial Infections: A Review. *Front. Microbiol* 2017;8:2431. [PubMed: 29312162]
- [108]. Karki P, Johnson J Jr., Son DS, Aschner M, Lee E Transcriptional Regulation of Human Transforming Growth Factor- α in Astrocytes. *Mol. Neurobiol* 2017;54:964–76. [PubMed: 26797516]
- [109]. Xing C, Wang X, Cheng C, Montaner J, Mandeville E, Leung W, et al. Neuronal production of lipocalin-2 as a help-me signal for glial activation. *Stroke*. 2014;45:2085–92. [PubMed: 24916903]
- [110]. Kastriiti ME, Adameyko I Specification, plasticity and evolutionary origin of peripheral glial cells. *Curr. Opin. Neurobiol* 2017;47:196–202. [PubMed: 29161639]
- [111]. Weider M, Wegener A, Schmitt C, Kuspert M, Hillgartner S, Bosl MR, et al. Elevated in vivo levels of a single transcription factor directly convert satellite glia into oligodendrocyte-like cells. *PLoS Genet*. 2015;11:e1005008. [PubMed: 25680202]
- [112]. Fex Svenningsen A, Colman DR, Pedraza L Satellite cells of dorsal root ganglia are multipotential glial precursors. *Neuron Glia Biol*. 2004;1:85–93. [PubMed: 18634609]
- [113]. George D, Ahrens P, Lambert S Satellite glial cells represent a population of developmentally arrested Schwann cells. *Glia*. 2018;66:1496–506. [PubMed: 29520852]
- [114]. Jiang-Shieh YF, Yeh KY, Wei IH, Chang CY, Chien HF, Tsai RY, et al. Responses of microglia in vitro to the gram-positive bacterial component, lipoteichoic acid. *J. Neurosci. Res* 2005;82:515–24. [PubMed: 16237723]
- [115]. Loo TM, Kamachi F, Watanabe Y, Yoshimoto S, Kanda H, Arai Y, et al. Gut Microbiota Promotes Obesity-Associated Liver Cancer through PGE2-Mediated Suppression of Antitumor Immunity. *Cancer Discov*. 2017;7:522–38. [PubMed: 28202625]
- [116]. Nyavor Y, Brands CR, May G, Kuther S, Nicholson J, Tiger K, et al. High-fat diet-induced alterations to gut microbiota and gut-derived lipoteichoic acid contributes to the development of enteric neuropathy. *Neurogastroenterol. Motil* 2020:e13838. [PubMed: 32168415]
- [117]. Correa RG, Milutinovic S, Reed JC Roles of NOD1 (NLRC1) and NOD2 (NLRC2) in innate immunity and inflammatory diseases. *Biosci. Rep* 2012;32:597–608. [PubMed: 22908883]
- [118]. Naznin F, Sakoda H, Okada T, Tsubouchi H, Waise TM, Arakawa K, et al. Canagliflozin, a sodium glucose cotransporter 2 inhibitor, attenuates obesity-induced inflammation in the nodose ganglion, hypothalamus, and skeletal muscle of mice. *Eur. J. Pharmacol* 2017;794:37–44. [PubMed: 27876617]
- [119]. Waise TMZ, Toshinai K, Naznin F, NamKoong C, Moin ASM, Sakoda H, et al. One-day high-fat diet induces inflammation in the nodose ganglion and hypothalamus of mice. *Biochemical and Biophysical Research Communications*. 2015;464:1157. [PubMed: 26208455]
- [120]. Naznin F, Toshinai K, Waise TMZ, Okada T, Sakoda H, Nakazato M Restoration of metabolic inflammation-related ghrelin resistance by weight loss. *J. Mol. Endocrinol* 2018;60:109–18. [PubMed: 29233861]
- [121]. Vaughn AC, Cooper EM, DiLorenzo PM, O'Loughlin LJ, Konkell ME, Peters JH, et al. Energy-dense diet triggers changes in gut microbiota, reorganization of gut-brain vagal communication and increases body fat accumulation. *Acta Neurobiol Exp (Wars)*. 2017;77:18–30. [PubMed: 28379213]

- [122]. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014,505:559–63. [PubMed: 24336217]
- [123]. Turnbaugh P. j., Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med* 2009,1:6ra14.
- [124]. Lubbers T, de Haan JJ, Luyer MD, Verbaeys I, Hadfoune M, Dejong CH, et al. Cholecystokinin/Cholecystokinin-1 receptor-mediated peripheral activation of the afferent vagus by enteral nutrients attenuates inflammation in rats. *Ann. Surg* 2010,252:376–82. [PubMed: 20585240]
- [125]. Luyer MD, Greve JWM, Hadfoune M. h., Jacobs JA, Dejong CH, Buurman WA Nutritional stimulation of cholecystokinin receptors inhibits inflammation via the vagus nerve. *J. Exp. Med* 2005,202:1023–29. [PubMed: 16216887]
- [126]. Luyer MD, Jacobs JA, Vreugdenhil AC, Hadfoune M, Dejong CH, Buurman WA, et al. Enteral administration of high-fat nutrition before and directly after hemorrhagic shock reduces endotoxemia and bacterial translocation. *Ann. Surg* 2004,239:257–64. [PubMed: 14745335]
- [127]. Saia RS, Ribeiro AB, Giusti H Cholecystokinin Modulates the Mucosal Inflammatory Response and Prevents the Lipopolysaccharide-Induced Intestinal Epithelial Barrier Dysfunction. *Shock*. 2020,53:242–51. [PubMed: 30998649]

Highlights

- Gram positive (GP) bacterial byproducts trigger an increase in inflammatory cytokines expression in nodose ganglia cultures.
- The GP bacteria-induced inflammatory response is characterized by early increased expression of proinflammatory genes and later increased expression of genes associated with anti-inflammatory factors (i.e. IL-10).
- The response to GP bacterial byproducts appears to be mediated by signaling between non-neuronal cell types.

Figure 1. Methods Overview

Created with BioRender. Graphical depiction of experimental procedures from tissue collection through treatment and analyses.

Figure 2. SUP increased the proportion of Iba1⁺ cells coexpressing IL-6 and inflammatory gene expression consistent with an M1 microglia/macrophage phenotype

A. Cultures were stained against the inflammatory marker IL-6 (red) and the microglia/macrophage marker Iba1 (green). DAPI was included as nuclear stain. Scale bar is 100 μ M. **B.** The proportion of Iba1⁺ cells was not changed by any treatments. **C.** SUP or MINO + FC significantly increased the number of Iba1⁺ cells coexpressing IL-6. Values are compared to average of controls and shown as mean $\pm$ SEM. One-way ANOVA with Fisher's LSD was performed to identify differences from control. Differences were deemed significant if $P < .05$. **D.** Iba1 gene expression was significantly decreased by SUP 4h and MINO 4h and was significantly increased by SUP + FC 4h. **E.** IFN γ expression was increased by SUP 4h and this was normalized by inclusion of MINO or FC. **F.** IL-6 gene expression was increased by SUP 4h and attenuated by inclusion of MINO or FC but not normalized. **G.**

TNF α gene expression was increased by SUP 4h with no attenuation of this effect by MINO or FC. **H.** Gene expression of IL-1 β was not increased by SUP. **I.** CD86 gene expression was increased by SUP 4h or MINO 4h. Gene expression presented as fold change compared to controls. One-way ANOVA with Fisher's LSD post hoc test or for non-normal data, Kruskal-Wallis tests identified significant differences ($P < .05$) in gene expression. Data are shown as mean $\pm$ SEM. Within each graph, bars with differing letters are significantly different from one another

Figure 3. Inflammation-associated proteins released into MEDIA are altered by treatment SUP, FC, SUP + MINO, SUP + FC.

A. CCL2 release was increased by SUP + MINO and/or FC 24h and MINO + FC 24h. **B.** TSP-1 expression was decreased by SUP 4h, SUP + MINO 4h, SUP + FC 4h, and SUP + MINO + FC 24h and was increased by FC 4h. **C.** CXCL1 release trended toward increase with SUP 24h and was increased when MINO was added. SUP + FC 4h but not SUP + FC 24h also lead to significantly increased CXCL1 while MINO + FC 24h resulted in a trend of CXCL1 toward increase. Treatment with meso-DAP resulted in a trend toward decreased release of CXCL1. Differences in pg/μg total protein/mL in inflammation-associated proteins present in recovered cell culture media analyzed by one-way ANOVA with Fisher's LSD post hoc test. Significant differences ($P < .05$) in protein levels were identified. Data are shown as mean $\pm$ SEM. Within each graph, bars with differing letters are significantly different from one another

Figure 4. Treatments did not alter total cell numbers

Cultures were stained with the nuclear stain, DAPI and counts were made of each well to compare the total number of cells at **A.** 4 hours and **B.** 24 hours. Values are compared to average of controls and shown as mean $\pm$ SEM. One-way ANOVA with Fisher's LSD or one-tailed T-test was performed to identify differences from control. Differences were deemed significant if $P < .05$.

Figure 5. Lactate released into media is reduced by SUP with or without inhibitors

Differences in nm lactate present in recovered cell culture media analyzed by one-way ANOVA with Fisher's LSD post hoc test. Significant differences ($P < .05$) in lactate present were identified. Data are shown as mean $\pm$ SEM. Bars with differing letters are significantly different from one another.

Figure 6. SUP ± inhibitors altered staining and gene expression of M2b phenotype markers but NeuN⁺ cell proportions were not altered by any treatments.

A. Cultures were stained against the anti-inflammatory marker IL-10 (red) and the neuronal marker NeuN (green). DAPI was included as nuclear stain. Scale bar is 100 μ M. **B.** The proportion of NeuN⁺ cells was not changed by any treatments. **C.** All treatments significantly reduced the number of IL-10⁺ cells. Values are compared to average of controls and shown as mean $\pm$ SEM. One-way ANOVA with Fisher's LSD was performed to identify differences from control. Differences were deemed significant if $P < .05$. **D.** MAP2 gene expression was significantly increased only by SUP. **E.** IL-10 expression was increased by SUP 24h and glial inhibitors alone or combined did not attenuate this response. **F.** SOCS3 gene expression was increased in response to SUP with glial inhibitors but not by SUP 4h or SUP 24h. Fold change in gene expression compared to controls. One-way ANOVA with

Fisher's LSD post hoc test or for non-normal data, Kruskal-Wallis tests identified significant differences ($P < .05$) in gene expression. Data are shown as mean $\pm$ SEM. Within each graph, bars with differing letters are significantly different from one another.

Figure 7. Proportions of CD206 and CCL2 cells suggest a less inflammatory profile after 24h treatment with SUP.

Controls and cultures treated with SUP 24h were stained against the M2 markers CD206 (red, **A**) and CCL2 (red **E**) and for the microglia/macrophage marker Iba1 (green, **A**) or GFAP (green, **E**), a marker of both SG and nonmyelinating Schwann cells (NSC). DAPI was included as nuclear stain. Scale bar is 50 μ m. Positive cells were counted, and counts normalized to controls. Graphs (**B**, **C**, **D**, **F**, **G**, **H**) show differences in proportions of positively stained cells. **B**. The proportion of Iba1⁺ cells was not changed by SUP 24h. **C**. The proportion of CD206⁺ cells was increased by SUP 24h. **D**. The proportion of cells coexpressing Iba1 and CD206 trended toward increase at 24h. **F**. The proportion of GFAP⁺ cells was not changed by SUP 24h. **G**. The proportion of CCL2⁺ cells was increased by SUP 24h. **H**. There was no difference in the proportion of GFAP⁺ cells coexpressing CD206 after SUP 24h. Values are compared to average of controls and shown as mean $\pm$ SEM. One-tailed T tests were performed to identify differences from control. Differences were deemed

significant if $P < .05$. **J.** CCL2 gene expression was significantly increased by SUP, FC, and SUP + FC and trended toward increase with SUP + MINO 4h, MINO + FC 24h, and SUP + MINO + FC 24h. **K.** TGF β was increased in the presence of MINO 4h, SUP + MINO 4h, FC 4h, SUP + FC 4h, and SUP + MINO + FC 24h. Fold change in gene expression was compared to controls. one-way anova with Fisher's LSD post hoc test or for non-normal data, Kruskal-Wallis tests identified significant differences ($P < .05$) in gene expression. Data are shown as mean $\pm$ SEM. Within each graph, bars with differing letters are significantly different from one another.

Figure 8. Treatment with SUP $\pm$ glial inhibitors altered expression of genes associated with astrocytic regulation of microglia.

A. VEGFB gene expression was not changed by treatment with SUP. Inclusion of MINO or FC with SUP significantly increased VEGFB gene expression. **B.** TGF α gene expression was increased by SUP + MINO or FC at 4h or FC 4h, but not by SUP. **C.** LCN2 gene expression was not increased by SUP. Inclusion of MINO and/or FC with SUP resulted in increased LCN2 and FC alone increased LCN2 gene expression. Fold change in gene expression compared to controls. One-way ANOVA with Fisher's LSD post hoc test or for non-normal data, Kruskal-Wallis tests identified significant differences ($P < .05$) in gene expression. Data are shown as mean $\pm$ SEM. Within each graph, bars with differing letters are significantly different from one another

Figure 9. The number of GFAP⁺ cells coexpressing SOX10 trended down with SUP treatment at 4h
A. Cultures were stained against the transcription factor SOX10 (red) and the SG and NSC marker GFAP (green). DAPI was included as nuclear stain. Scale bar is 100 μ M. B. The proportion of GFAP⁺ cells was not changed by any treatments although SUP 4h produced a downward trend. C. The proportion of GFAP⁺ cells coexpressing SOX10 trended down with SUP 4h, MINO 4h, or FC 4h. Values are compared to average of controls and shown as mean $\pm$ SEM. One-way ANOVA with Fisher's LSD was performed to identify differences from control. Differences were deemed significant if $P<0.05$.

Figure 10. Treatment with LTA or meso-DAP induced some responses similarly to SUP. ABC. The only cell marker response to LTA was a trend toward Iba1 decrease. **DEFG.** LTA at 20 µg/mL but not at 0.015 µg/mL increased TNF α and IL-1 β gene expression. Meso-DAP increased IFN γ , TNF α , and IL-1 β gene expression at 4h and inclusion of MINO did not hold this response at control levels. **H.** SOCS3 gene expression was reduced by meso-DAP alone and increased by meso-DAP + MINO. **I.** IL-10 gene expression was increased by meso-DAP and this was not attenuated by inclusion of MINO. **JK.** LTA 0.015 µg/mL + MINO and meso-DAP + MINO increased VEGFB gene expression. **L.** LTA 20 µg/mL, LTA 0.015 µg/mL + MINO, meso-DAP, and meso-DAP plus MINO caused CCL2 to trend upward or increase. **M.** LCN2 was not altered by conditions tested. **N.** TGF β was increased by LTA 0.015 µg/mL + FC and by meso-DAP. Fold change in gene expression compared to controls. One-way ANOVA with Fisher's LSD post hoc test or for non-normal data, Kruskal-Wallis tests identified significant differences ($P < .05$) in gene expression. Data are shown as mean $\pm$ SEM. Within each graph, bars with differing letters are significantly different from one another

Table 1**Antibodies**

Anti-	Source and Catalog #	Dilution	Reference
-NeuN (rabbit)	Cell Signaling D3S3I	1:500	[53]
-Iba1 (rabbit)	Waco 019–19741	1:500	[23, 28]
-GFAP (rabbit)	Proteintech 16825–1-AP	1:500	[54]
-IL-6 (mouse)	Proteintech 66146–1-Ig	1:500	[55]
-IL-10 (mouse)	Proteintech 60269–1-Ig	1:500	[56]
-CCL2 (MCP1, mouse)	Proteintech 66272–1-Ig	1:400	[57]
-CD206 (mouse)	Proteintech 60143–1-Ig	1:400	[58, 59]
-SOX10 (mouse)	Proteintech 66786–1-Ig	1:500	
Donkey anti-rabbit Alexa Fluor 488	Life Technologies A-21206	1:1000	
Donkey anti-mouse Alexa Fluor 546	Life Technologies A-10036	1:1000	

Table 2

Primer Sequences

Gene	FWD	REV
18S	GCATGGCCGTCTTAGTT	CATCTAAGGGCATCACAGAC
CCL2	CACCTGCTGCTACTCATTTCA	TACTACAGCTTCTTTGGGACAC
CD86	CCCGTTTCAAAAGGCTCAA	TCTGCGCCCAAATAGTGTTTC
GFAP	AGAGTCCACAACCATCCT	CTAGGCGATACTCCGTACAT
Iba1	GCCTCATCGTCATCTCCCA	AGGAAGTGCTTGTGATCCCA
IFN γ	TGGATGCTATGGAAGGAAAGAG	GACTTCAAAGAGTCTGAGGTAGAA
IL-10	GCTATGTTGCCTGCTCTT	GGAAGTGGGTGCAGTTATT
IL-1 β	CTGACAGGCAACCACTTAC	TCCCATACACACGGACAA
IL-6	AGTGGCTAAGGACCAAGA	TTAGATACCCATCGACAGGA
LCN2	TCTCTGGGCTCAAGGATAA	CTCACCGTCTGTTTCAGTTGT
MAP2	GAGAAGGCCCAAGCTAAAG	AGAGGAGACATTGCTGAGT
SOCS3	CACCTGGACTCCTATGAGAA	TCTTCTGCTGGGCTAACT
TGF α	GCCAGGATGATGTCTTATGT	CCGCTCGTGGATCATTAAACA
TGF β	CCGCAACAACGCAATCTATG	CTTCCCGAATGTCTGACGTATT
TNF α	ACAAGGCTGCTGAAGATG	CCCGGCCTTCCAAATAAA
VEGFB	GCACAAATCAGATGGTGAGAGA	CAGGAGATGGTTGATGGCTTAG