Apical splenic nerve electrical stimulation discloses an anti-inflammatory pathway relying on adrenergic and nicotinic receptors in myeloid cells

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ABSTRACT

The autonomic nervous system innervates all lymphoid tissues including the spleen therefore providing a link between the central nervous system and the immune system. The only known mechanism of neural inhibition of inflammation in the spleen relies on the production of norepinephrine by splenic catecholaminergic fibers which binds to β2-adrenergic receptors (β2-ARs) of CD4⁺ T cells. These CD4⁺ T cells trigger the release of acetylcholine that inhibits the secretion of inflammatory cytokines by macrophages through α7 nicotinic acetylcholine receptor (α7nAchRs) signaling. While the vagal anti-inflammatory pathway has been extensively studied in rodents, it remains to be determined whether it coexists with other neural pathways. Here, we have found that three nerve branches project to the spleen in mice. While two of these nerves are associated with an artery and contain catecholaminergic fibers, the third is located at the apex of the spleen and contains both catecholaminergic and cholinergic fibers. We found that electrical stimulation of the apical nerve, but not the arterial nerves, inhibited inflammation independently of lymphocytes. In striking contrast to the anti-inflammatory pathway mechanism described so far, we also found that the inhibition of inflammation by apical nerve electrical stimulation relied on signaling by both β2-ARs and α7nAchRs in myeloid cells, with these two signaling pathways acting in parallel. Most importantly, apical splenic nerve electrical stimulation mitigated clinical symptoms in a mouse model of rheumatoid arthritis further providing the proof-of-concept that such an approach could be beneficial in patients with Immune-mediated inflammatory diseases.

1. Introduction

The autonomic nervous system innervates lymphoid tissues, therefore providing an important link between the central nervous system and the immune system. While previous studies have revealed direct autonomic innervation of parenchymal tissue in the thymus, bone marrow, spleen, lymph nodes, and gut-associated lymphoid tissues (Nance and Sanders, 2007), the anatomical and functional characterization of the nerves that project to these lymphoid organs remain incomplete from an anatomical and functional point of view. This is the case for the spleen which is the largest secondary lymphoid organ in the body and the main source of pro-inflammatory cytokines in systemic inflammatory diseases (Murray and Reardon, 2018).

Studies performed in the seventies have shown that the splenic nerve in humans carries approximately 98% sympathetic nerve fibers (Heurlemann and Stutte, 1977; Kudoh et al., 1979). Catecholaminergic postganglionic nerves originating mainly in the superior mesenteric/celiac ganglion enter the spleen accompanying the splenic artery and run along the trabeculae in plexuses. Nerve fibers from the vascular and trabecular plexuses enter the white pulp along the central artery, where they reach their greatest density and end up in the periarterial lymphatic sheath. Sympathetic nerve fibres are co-localized with T-cells, macrophages, as well as B-cells residing in the marginal zone where lymphocytes enter the spleen (Anagnostou, 2007; Hoover et al., 2017). Catecholaminergic innervation is particularly rich in T-cell zones and in areas of mast cells and macrophages, whereas follicular and nodular zones where B cells mature, are poorly innervated. In agreement with what has been observed in humans, Felten et al. have identified nora- drenergic fibers in rat that enter the spleen around the splenic arteries, travel with the vasculature in plexuses, and continue into the spleen.
white pulp (Felten et al., 1987). Experimental studies in rodents have shown that sympathetic nerve terminals in the spleen are able to store and release norepinephrine in response to stimulation (Elenkov and Vizi, 1991; Kees et al., 2003), and that the splenic norepinephrine content dramatically decreased following chemical (Sudo, 1985) or surgical (Vida et al., 2011) sympathectomy.

Further experiment from the Tracey’s group demonstrated that the splenic nerve was a critical component of the vagal anti-inflammatory pathway, a physiological regulatory mechanism whereby afferent vagus nerve stimulation by pathogen-derived products leads to efferent vagus nerve-mediated suppression of proinflammatory cytokine production by spleen macrophages in the red pulp and the marginal zone (Rosas-Ballina, 2008). This was elegantly demonstrated by the surgical ablation of the splenic nerve which abolished the inhibition of LPS-induced TNF production by vagus nerve stimulation in mice. The vagal anti-inflammatory pathway relies on the production of norepinephrine by

Fig. 1. Anatomical, histological and functional characterization of murine splenic nerves. (a) Location of splenic arteries in an anaesthetized mouse using laser speckle contrast imaging. White arrows indicate the location of nerve-like structures. (b) Representative microscopy imaging of the indicated nerves stained for Tyrosine Hydroxylase (TH, red), Choline Acetyl Transferase (ChAT, green) and Neuro Filament (NF, blue). Scale bar = 30 µm. (c, d) Light sheet imaging of whole spleen (c) and apical region (d) after staining for TH (black (c); red (d)) or ChAT (black (c); green (d)). Red and green arrows indicate points of entry of arterial and apical splenic nerves, respectively (e). Scale bar = 1 mm (d). (e, f) Arterial or apical splenic nerve from WT (e) and Rag1−/− (f) mice were electrically stimulated (STIM) or not (SHAM-operated), and norepinephrine (e) or acetylcholine (f) spleen contents were measured. (e, f) Data show mean ± S.E.M. of 2 independent experiments. (e, f) One-way ANOVA followed by Tukey’s post hoc test were performed. *, p < 0.05; **, p < 0.01.
splanic catecholaminergic fibers (Rosas-Ballina, 2008), which triggers the release of acetylcholine by CD4+ T cells (Rosas-Ballina, 2011) via β2 adrenergic receptors (β2-AR) (Vida, 2011). Acetylcholine then binds to the α7 nicotinic acetylcholine receptors (α7nAChR) on myeloid cells resulting in the inhibition of LPS-mediated production of pro-inflammatory cytokines (Olofsson, 2012). In apparent contrast to the mechanism described above, an anatomical and functional connection between the vagus and the splenic nerve could not be demonstrated by injecting anterograde tracers in the dorsal motor nucleus (DMV) of the vagus, and retrograde tracers in the spleen (Bratton, 2012; Cailotto, 2012). Furthermore, action potentials in the splenic nerve could not be detected in rats following vagal electrical stimulation (Bratton, 2012).

Whereas the presence of catecholaminergic fibers in the spleen is well established, the presence of cholinergic fibers has been debated for many years (Nance and Burns, 1989; Schäfer et al., 1998; Bellinger et al., 1993; Cano et al., 2001; Kooijman, 2015; Anderson et al., 2015), in part due to the various technical limitations that make the visualization of cholinergic structures by histochemical means challenging. While cholinergic markers have been widely used as an attempt to label cells, axons, and terminals in peripheral tissues, their lack of sensitivity has repeatedly been noted. Furthermore, immunostaining of cholinergic markers in spleen has been described as highly non-specific and variable by many investigators (Nance and Sanders, 2007; Stevens-Felten and Bellinger, 1997; Thayer and Sternberg, 2010). To overcome this problem, Gautron et al. used transgenic reporter mice in which the tdTomato fluorescent protein was selectively expressed in choline acetyltransferase (ChAT)-expressing cells, therefore allowing for the labeling of cholinergic neurons and their projections to the spleen (Gautron, 2013). Data showed the presence of few tdTomato-positive neuronal fibers around arterioles and not in association with the vasculature, diverging into lymphocyte-containing areas of the white pulp. However, this study may be questioned because the presence of cholinergic fibers revealed by this approach could have been due to the transient expression of the Cre

Fig. 2. Functional characterization of spleen nerves. (a, d) Inhibition of TNF production by nerve electrical stimulation in freely moving mice. WT (a), Foxn1−/− (d) were implanted with electrodes onto the indicated nerves on day 0, and injected with LPS on day 7. Electrical stimulation was applied (STIM) or not (SHAM) and serum TNF levels were assessed in samples collected 90 min after LPS injection. Data show percent serum TNF levels in individual mice normalized to mean levels determined in SHAM control mice. (b, c) Differential effect of targeted nerve electrical stimulation on carotid blood pressure and heart rate in anesthetized mice before and after initiation of electrical stimulation. Representative heart rate pattern of an individual animal (b) and mean percent post-stimulation blood pressure (c, upper panel) and heart rate (c, lower panel) values of 6 mice. Baseline mean values were 432 ± 42.7 beats/min and 85 ± 8 mm Hg for heart rate and blood pressure respectively. Data show mean ± S.E.M. of 2-3 independent experiments (n ≥ 6 mice/group) in non-electrically stimulated (SHAM) and electrically stimulated (STIM) mice. (a, c, d) One-way ANOVA followed by Tukey’s post hoc test were performed. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
recombinase at an earlier stage of development of the neural fibers. While some of the data described above may appear contradictory, it should be noted that several nerve-like structures project to the spleen in rodents. Unfortunately, most authors have referred to “the splenic nerve” in their publications without specifying the branch (or branches) that they were studying. Such an issue may be particularly problematic in experiments in which the function of the nerve fibers that project to the spleen was investigated by electrical stimulation or surgical ablation. This prompted us to perform an exhaustive anatomical and functional analysis of the nerves that project to the spleen in mice.

2. Results

Gross anatomy of spleen revealed three nerve-like structures among which two were associated with arteries as demonstrated by laser speckle imaging (Supp. Fig. 1, Fig. 1a). The two arterial nerves subdivided into two branches each and entered the spleen at non-apical locations (Fig. 1a). The third nerve referred to hereafter as the “apical splenic nerve” was not associated with an artery and entered the spleen at its apex (Fig. 1a). Both arterial and apical nerves were catecholaminergic as demonstrated by immunostaining. In contrast, the apical nerve but not the arterial nerves contained cholinergic fibers (Fig. 1b). Light sheet imaging of whole clarified spleen showed a dense network of catecholaminergic fibers across the entire spleen, and cholinergic fibers mainly located at the apex (Fig. 1c, d). In agreement with immunostaining data, stimulation of either the apical nerve or one of the two arterial nerves increased norepinephrine levels in spleen (5.82 ± 0.2 ng/mg unstimulated; 9.64 ± 0.76 ng/mg for vagus nerve stimulation; 10.3 ± 1.89 ng/mg for the arterial nerve; 10.9 ± 1.1 ng/mg for the apical nerve) (Fig. 1e). In contrast, only apical nerve stimulation induced the release of acetylcholine spleen (21.8 ± 3.1 µg/mg unstimulated; 38.8 ± 4.9 ng/mg for the arterial nerve; 73.4 ± 11.9 ng/mg for the apical nerve) in lymphocyte-deficient mice (Rag1−/−)(Fig. 1f) further suggesting that cholinergic fibers, and not T cells, were the source of acetylcholine in these experiments.

We next investigated whether electrostimulation of the apical and arterial nerves could inhibit TNF production induced by lipopolysaccharide (LPS) injection in freely moving mice. We implanted mice with electrodes on intact apical, arterial or vagus (as a control) nerves, and applied electrostimulation one week later to stimulate both afferent and efferent fibers. We first checked that electrode implantation had no effect on LPS-induced TNF production (Supp. Fig. 3). All nerves were equally efficient at inhibiting LPS-induced TNF release when stimulated with the same electrical parameters (53.6 ± 8.5% for VN, 42.7 ± 6.7% for the arterial nerve, 49.2 ± 2.9% for the apical nerve) (Fig. 2a). However, and at variance with vagus nerve stimulation, neither arterial nor apical nerve stimulation caused changes in arterial blood pressure or heart rate (Fig. 2b, c, Supp. Fig. 2). Therefore, both arterial and apical splenic nerve stimulation triggered an anti-

![Fig. 3. Role of β 2 adrenergic and α 7 cholinergic receptors in apical splenic nerve-mediated inhibition of LPS-induced TNF production. Wild-type (a), Adb2−/− mice and control littermates (b), Adb2fl/fl (c), Chrma7fl/fl (d), and Adb2fl/fl Chrma7fl/fl mice (e), carrying (+) or not (−) the LysM-Cre transgene were implanted onto the apical splenic nerve with a micro-cuff electrode one week before electrostimulation was applied. Mice were treated (a) or not (b-e) with the indicated pharmacological antagonists (blue or red) or vehicles (open and black). LPS was injected one week later and electrical stimulation was conducted or not as described in Fig. 1e. Data show the percentage of TNF in individual mice normalized to mean levels in SHAM mice. Data show mean ± S.E.M. of 2–3 experiments (n ≥ 5/group) in non-electrically stimulated (SHAM) and electrically stimulated (STIM) mice. (a, b, c, d, e) One-way ANOVA followed by Tukey’s post hoc test were performed. *, p < 0.05; **, p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

![Graphs a, b, c, d, e showing the percentage of TNF normalized to mean SHAM-implanted for different conditions.](image-url)
inflammatory pathway without appreciable cardiovascular off-target effects.

Because the vagal anti-inflammatory pathway was shown to be dependent on CD4 T cells (Rosas-Ballina, 2011), we investigated the role of these cells in the inhibition of inflammation by apical and arterial spleen nerve stimulation. As expected (Rosas-Ballina, 2011), the inhibition of LPS-induced TNF production by vagus nerve stimulation was abolished in T cell-deficient mice (Foxn1−/−) and the same was also the case following arterial nerve stimulation (Foxn1−/−) demonstrating that T cells were not required. Thus, the anti-inflammatory effects of arterial and apical splenic nerve stimulation are mediated by distinct mechanisms. This prompted us to further investigate how apical nerve stimulation inhibited LPS-induced TNF secretion.

The inhibition of LPS-induced TNF production by apical nerve stimulation was abolished by both pranolol and methyllycaconitine (MLA), but not by atropine further suggesting that β2/β2-ARs and α7nAchRs, but not muscarinic AchRs were involved (Fig. 3a). Of note, a complete restoration of TNF production was achieved only when pranolol and MLA were administered together suggesting the existence of two signaling pathways acting in parallel (Fig. 3a). TNF production was partially restored in Adrb2−/− further confirming a role for β2-ARs in the anti-inflammatory effects of apical nerve stimulation (Fig. 3b).

Since the inhibition of LPS-induced TNF production by apical nerve stimulation did not require lymphocytes, we made the hypothesis that norepinephrine and acetylcholine acted directly on myeloid cells. To test this, we used LysM-Cre:Adrb2fl/fl and LysM-Cre:Chrna7fl/fl mice in which myeloid cells (Clausen et al., 1999) are selectively deficient in β-2 ARs and α7nAchRs respectively. While apical nerve stimulation did reduce LPS-induced TNF level in both LysM-Cre:Adrb2fl/fl and Adrb2fl/fl mice, LysM-Cre:Chrna7fl/fl mice exhibited less TNF than Adrb2fl/fl mice compared to Adrb2fl/fl mice further demonstrating a role for β2-ARs signaling in myeloid cells (81.6 ± 5.8% in LysM-Cre:Adrb2fl/fl mice versus 60.9 ± 5.7% in Adrb2fl/fl mice) (Fig. 3c). Furthermore, TNF production was partially restored in LysM-Cre:Chrna7fl/fl further demonstrating a role for α7nAchRs signaling in myeloid cells (67.1 ± 5.5% in LysM-Cre:Chrna7fl/fl mice versus 48.6 ± 8.1% in Chrna7fl/fl controls) (Fig. 3d). Lastly, TNF secretion was fully restored in LysM-Cre:Adrb2fl/fl:Chrna7fl/fl mice in which myeloid cells lack both β2-ARs and α7-nAchRs (121.0 ± 17.8% in LysM-Cre:Adrb2fl/fl:Chrna7fl/fl mice versus 60.5 ± 11.7% in Adrb2fl/fl:Chrna7fl/fl controls) (Fig. 3e). Altogether, these results demonstrated that the inhibition of LPS-induced TNF secretion by apical nerve stimulation did not require the presence of lymphocytes and was dependent on β-2 ARs and α7nAchRs signaling in myeloid cells, with these two signaling pathways acting in parallel.

While the vagus anti-inflammatory pathway was first identified in rodents injected with LPS, vagus nerve stimulation (VNS) was eventually shown to ameliorate collagen-induced arthritis (CIA) development in rats (Levine, 2014). Promising effects have also been reported in patients with Rheumatoid arthritis (RA) (Koopman, 2016). Because apical nerve stimulation was as efficient as VNS at inhibiting LPS-induced TNF production, we investigated whether apical splenic nerve could also inhibit CIA in arthritis-prone DBA mice. Mice were immunized with collagen II on day 0, and implanted with electrodes applied onto the apical nerve on day 11. At day 21, animals were boosted with collagen II and followed for clinical symptoms until 35 days (Fig. 4a). Electrical stimulation was conducted every 4 h starting on day 16. Surgical implantation of electrodes onto the apical splenic nerve neither modified disease incidence nor progression compared to non-operated animals (Supp. Fig. 4). While all SHAM mice developed arthritis within 28 days, disease onset was significantly delayed in stimulated animals, 16% of which showed complete protection throughout the study period (Fig. 4b). Apical nerve stimulation reduced disease severity (Fig. 4c, d) and joint swelling (Fig. 4e). This was accompanied by a reduction in synovial inflammation in the hind paw and reduction in tibiotalar and tarsus bone erosions in stimulated compared to SHAM mice. (Fig. 4f, g). Further, both the frequency and numbers of inflammatory monocytes were markedly reduced in the spleen of stimulated animals (Fig. 4h, i). Altogether, our results demonstrate that apical nerve stimulation inhibited CIA in mice.

3. Discussion

In this study, we have performed an exhaustive anatomical and functional analysis of the neural fibers that project to the spleen in mice. As expected, we found that the vast majority of the neural fibers that are present in the spleen are catecholaminergic as demonstrated by TH immunostaining. These fibers originated from three nerve branches among which two run along splenic arteries and one does not. This third nerve branch, which entered the spleen at its apex, has already been described but the nature of the fibers that it contains was not investigated (Buijs et al., 2008; Cailotto, 2012). While the origin of the cholinergic apical fibers is not known, they are likely to originate from either a vagus branch or the splanchic (supra-renal ganglion). In agreement with immunostaining data, we found that electrical stimulation of any of these three nerve branches induced the release of norepinephrine in the spleen.

In addition to catecholaminergic fibers, we found that the apical nerve, but not the two arterial nerves, also contained cholinergic fibers as demonstrated by ChAT immunostaining. While some authors have already reported the presence of ChAT-positive neural fibers in the spleen, these findings have been debated for years in part due to the relatively low sensitivity and selectivity of ChAT immunostaining reagents and procedures. In agreement with our immunostaining data, we found that stimulation of the apical nerve, but not of the arterial nerves, increased acetylcholine spleen content in T lymphocyte-deficient mice, further suggesting that neural fibers, and not other acetylcholine producing cells, were the source of acetylcholine in these experiments.

Pioneer studies by Tracey’s group has demonstrated the existence of a vagus anti-inflammatory reflex in which signals traveling in the vagus nerve modulate the activity of the splenic nerve, which secretes norepinephrine in spleen. In agreement with these data, stimulation of either the vagus nerve or one of the two arterial splenic nerve inhibited LPS-induced inflammation, and this phenomenon was abolished in T cell-deficient nude (Foxn1−/−) mice. In striking contrast, the inhibition of LPS-induced TNF production by apical nerve stimulation was neither abolished in Foxn1−/− mice, nor in Rag2−/− mice that lack both T and B lymphocytes. Therefore, while apical and arterial splenic nerve stimulation were equally efficient at inhibiting LPS-induced secretion, they relied on different underlying mechanisms. We propose to refer to this newly described pathway as “apical splenic anti-inflammatory pathway”, which co-exists with the previously described vagal anti-inflammatory pathway. In contrast to the vagal anti-inflammatory pathway (Chavan et al., 2017), the apical splenic anti-inflammatory pathway does not require the presence of T cells and is dependent on β-2 ARs signaling in myeloid cells. While the apical splenic and the vagal anti-inflammatory pathways are both dependent on α7nAchRs signaling in myeloid cells, the former relies on the β-2 ARs and α7nAchRs pathways acting in parallel while the latter relies on these pathways acting sequentially (Fig. 5). Another important difference between the vagal anti-inflammatory pathway and the new apical splenic anti-inflammatory pathway is that the former impacts a variety of organs (Martelli et al., 2019) including the gastrointestinal tract (Matteoli and Boeckxstaens, 2013) and the kidneys (Inoue, 2016) while the latter targets the spleen only. It remains to be determined whether other vagus-unrelated peripheral nerves could convey an anti-inflammatory signal to specific tissues, and more specifically to organ-draining lymph nodes.
Fig. 4. Apical splenic nerve stimulation inhibits collagen-induced arthritis. (a) Schematic representation of the experimental protocol. DBA mice were immunized with collagen type II on day 0, and electrodes were implanted onto the apical splenic nerve on day 11. Electrical stimulation (650 µA, 10 Hz, 2 min) was conducted (red) or not (black) 6 times a day 4 h apart starting on day 16. Mice were boosted with collagen type II on day 21 and monitored every 2–3 days for clinical symptoms during a period of 35 days. Data show percentage of arthritic mice (b), clinical score (c), number of arthritic paws (d), swelling of the hind paw (e) and histological analysis of hind paw (f, g). Representative histological images are shown (f) and mean values of synovial inflammation (left), tibiotalar erosion (middle) and tarsus erosion (right) scores (g). (h, i) Flow cytometry analysis of spleen cell population after gating on MHC-II−, F4/80−, CD11c− cells. A (h) Representative flow cytometry density plot is presented with numbers indicating the percentage of cells in the gated area. (i) Total number (left) and frequency (right) of inflammatory monocytes. (b, c, d, e, g, i) Data show mean ± S.E.M. of 2 experiments (n = 6–14/group) in non-electrically stimulated (SHAM) and electrically stimulated (STIM) mice. (a) Log-rank test, (c, d) two-way ANOVA followed by Sidak’s post hoc test and (e, g) unpaired t-test were performed. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
RA is a chronic degenerative autoimmune disease characterized by joint synovial inflammation and bone cartilage erosion leading to significant disabilities (Aletaha and Smolen, 2018). While symptomatic relief can be achieved by treatment with anti-TNF antibodies and other biologicals, a sizeable proportion of RA patients do not respond to these treatments (Aletaha and Smolen, 2018). Consistent with the critical role of TNF in the pathogenesis of RA (Vervoordeldonk and Tak, 2002), vagus nerve stimulation ameliorates collagen-induced arthritis (CIA) development in rats (Levine, 2014) and promising effects have been reported in early stage clinical studies in RA patients (Roopman, 2016). While VNS is currently approved for the treatment of drug resistant epilepsy and depression with no major side-effects, it sometimes carries the risks of untoward off-target effects depending on the electrostimulation parameters and the sensitivity of the patient (Ben-Menachem, 2001). VNS produced heart rate reductions in most preclinical species involving rodents as well as large animals (Warner and Russell, 1969; Buschman, 2006). However electrostimulation of apical splenic nerve is not associated with significant impact on cardiovascular parameters in mice in accordance with the absence of detectable afferent fibers in this nerve. Thus, at variance with vagus nerve stimulation, apical nerve stimulation may offer the opportunity to deliver a broader panel of electrical parameters of stimulation that may improve therapeutic efficacy. While it remains to be determined whether apical splenic nerve stimulation is as efficient as VNS in inhibiting CIA, stimulating the splenic nerve instead of the vagus nerve might offer an alternative for RA patients. On another topic, the mechanisms underlying the beneficial impact of apical splenic nerve stimulation on CIA remain to be identified. Based on our data in the LPS model, we could hypothesize that it involves the β 2-ARs and α7nAChRs pathways acting in parallel to inhibit LPS-mediated inflammatory cytokine production.

These results pave the way for the use of splenic nerve electrical stimulation as a promising alternative to other therapeutic strategies in RA with less side effects.

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Author contributions

P.B. conceived the study. M.G., T.S. and P.B. designed experiments. M.G., T.S., C.P., F.C., D.D., E.Mu., E.Ma. and S.A. performed experiments. M.G., T.S., A.S., M.J.V, N.G. and P.B. interpreted the data. P.B., T.S. and N.G. wrote the manuscript. All the authors read and approved the final manuscript. Correspondence and requests for materials should be addressed to P.B. (blancou@ipmc.cnrs.fr).

Competing financial interest

A patent application is pending.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.03.015.

Methods

Mice: C57BL/6, DBA, Foxn1−/−, Rag1−/−, LysM-Cre:Adrb2fl/fl and LysM-Cre:Chrm2fl/fl mice were purchased from Charles River. ADRB2−/− mice were purchased from The Jackson Laboratory and backcrossed on the C57BL/6 background for at least 10 generations. All experiments were...
performed with 8-12 wk old female mice except for the DBA strain which were 8 wk old male mice. Mice were housed on a 12 h light/dark cycle (lights on/off at 7 am/7 pm) with food ad libitum. Mice were treated in accordance with our local Animal Care and Use Committee guidelines.

**Light sheet imaging:** After anesthesia, the spleen tissue was exposed and placed 30 cm below the Moor-FLPI laser speckle perfusion imager (Moor instruments Ltd.). Blood perfusion images were saved and analyzed by the Image Review software (Moor-FLPI V2.0).

**Immunohistochemistry:** Nerves were excised and immediately submerged in OCT compound (Sakura Finetek, Torrance, CA). Tissues in OCT were quickly frozen using dry ice, then kept at -80 °C for long-term storage. Nerve cryosections were cut in triplicate at 6 μm using a Microm HM 550 cryostat (Thermo Fisher Scientific, Inc.). Sections were fixed with cold acetone/methanol (v/v: 1: 1) for 5 min, then washed in PBS. Primary antibodies (anti-choline acetyltransferase, AB144P, Merck; anti-tyrosine hydroxylase, AB152, Merck; anti-neurofilament, ab4680, Abcam) were diluted 1:50, 1:300 and 1:500 respectively in PBS and incubated for 1 hour in a humidified chamber. Sections were washed twice with PBS, and secondary antibodies were applied at 1:400 dilutions for 45 min (Donkey anti-goat, Donkey Anti-Rabbit, Donkey Anti-Chicken IgY resp. from Jackson Immunoresearch). Slides were washed twice in PBS for 5 min, fixed with 4% paraformaldehyde, treated with 1% glycerol in PBS before covering with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Inc.) and imaged under a fluorescence microscope. Images were analyzed with Volocity image analysis software (PerkinElmer, Waltham, MA).

**Light-sheet-based fluorescent microscopy:** Animals were perfused using a 4% PFA solution followed by a PBS solution. Excised spleens were digested and clarified using the iDISco+ method (https://idis-co.info/). Briefly, they were dehydrated at room temperature in successive bates of 20% MetOH for 1h, 40% MetOH for 1h, 60% MetOH for 1h, 80% MetOH for 1h, 100% MetOH for 1h and 100% MetOH overnight. Then the organs are incubated in a solution of 33% MetOH and 66% Di-ChloroMethan (DCM, Sigma) overnight and washed twice with 100% methanol for 1h. Organs were then bleached in chilled fresh 5% H2O2 in methanol overnight at 4°C before being rehydrated with methanol/H2O2 series (80%, 60%, 40%, 20% and PBS, 1 h each at RT). Samples were then immunolabelled for 1 hour in a humidity chamber. Sections were washed twice with PBS, and secondary antibodies were applied at 1:400 dilutions for 45 min (Donkey anti-goat, Donkey Anti-Rabbit, Donkey Anti-Chicken IgY resp. from Jackson Immunoresearch). Slides were washed twice in PBS for 5 min, fixed with 4% paraformaldehyde, treated with 1% glycerol in PBS before covering with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Inc.) and imaged under a fluorescent microscope. Images were analyzed with Volocity image analysis software (PerkinElmer, Waltham, MA).

**Electrodes and surgery:** For studies in anaesthetized animals (Fig. 1e, g, h), mice were pre-medicated with buprenorphine (100 μg/kg, i.p.) 30 min before surgery and anaesthetized with isoflurane (2% v/v). A hook electrode was placed under the splenic or the vagus nerve. For studies in freely moving animals, mice were pre-medicated with buprenorphine (100 μg/kg, i.p.) 30 min before surgery and anaesthetized with isoflurane (2% v/v) for the duration of the surgery. For splenic nerve implantation, one mm length 100 μm-sling bipolar micro-cuff electrodes (CorTec) were implanted onto the arterial or apical nerve. For vagus nerve implantation, two mm length 200 μm-tunnel bipolar micro-cuff electrodes (CorTec) were implanted onto the vagus nerve.

**Electrostimulation:** Mice were anaesthetized and either 100 μm sling or 200 μm tunnel Cortec electrodes were implanted onto the splenic nerves (apical or arterial) or the vagus nerve respectively. Seven days following surgery, these animals were injected ip with a sublethal dose of LPS (5 mg/kg) which has been used in previous studies to induce LPS production (Huston, 2006). Electrostimulation was applied using a PlexStim V2.3 (Plexon) starting at −10, 0 and +20 min relative to LPS injection. Sera was collected at 90 min after LPS injection and assessed for TNF levels. Controls consist of fully Cortec implanted mice, which did not receive electrical stimulation (SHAM). Electrostimulation were rectangular charge-balanced biphasic pulses with 650 μA pulse amplitude, 100 μs pulse width (positive and negative) at 10 Hz frequency for 2 min (STIM).

**Carotid blood pressure:** C57/B6J mice were anaesthetized as described above, a catheter was placed into the left carotid, connected to a pressure transducer and recorded using a Acqknowledge 881 (MP100WS) software (Biopac System, Inc.). A tunnel or cuff electrode were placed under the vagus nerve or the splenic respectively and different pattern of stimulation were delivered starting by the lowest intensity. Mean blood pressure and heart rate were analysed using Biopac data acquisition Acqknowledge 881 (MP100WS) software (Biopac System, Inc.).

**TNF, norepinephrine and acetylcholine levels:** For TNF, retro-orbital blood sampling was performed under isoflurane anesthesia. TNF levels were measured by ELISA (Mouse TNF-alpha DuoSet, R&D Systems) following manufacturer instructions and normalized to those measured in SHAM animals. TNF was below the lower level of detection (LLOD) in control mice that were not injected with LPS. For acetylcholine, mice received 0.1 μg/g of the acetylcholine esterase inhibitor neostigmine intraperitoneally 30 min prior to electrostimulation. Spleen were harvested and snap-frozen in liquid nitrogen immediately after electrostimulation. Protein extracts were prepared using Precelys machine (MP Biomedicals) in Lysing Matrix tube with TNET Buffer (10 mM Tris 150 mM NaCl 5 mM EDTA 1% Triton 10% Glycerol 1%, Protease Phosphatase Inhibitor Cocktail (Pierce, Thermo Fisher Scientific)). Protein concentration were assessed using BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific) following manufacturer instructions. Norepinephrine or acetylcholine levels were measured by ELISA (for norepinephrine: Norepinephrine – Sensitive, DLD Diagnostika GmbH; for acetylcholine: QuickDetect™, Clinisciences) following manufacturer recommendations. LLOD were 15, 75 and 1.2 pg/ml for TNF, nor-epinephrine and acetylcholine respectively.

**Treatment with antagonists:** Antagonists (atropine: 1 mg/kg (Pinardi et al., 2003); Proparanol: 5 mg/kg (Abdin et al., 2014); methyllycaconitine: 5 mg/kg (Lewis et al., 2015)) were given i.p. 30 min prior to LPS injection to allow for the antagonists to inhibit all accessible receptors (Albanus et al., 1968; Hanson et al., 1978; Stegelmeier et al., 2003).

**Induction and assessment of collagen-induced arthritis:** Bovine type II collagen (2 mg/ml in 0.05 M acetic acid; Chondrex, Redmond, WA) was mixed in an equal volume of Freund’s complete adjuvant (2 mg/ml of Mycobacterium tuberculosis; Chondrex). DBA mice were immunized intradermally at the base of the tail with 100 μl of emulsion (100 μg of collagen mixed with complete adjuvant). Mice were injected subcutaneously at the base of tail and monitored daily for clinical signs of arthritis. Mice were euthanized after 21 days of treatment and paws were removed and fixed in formalin. The knee joint was dissected free of surrounding tissue and measured with a caliper to determine the percentage of joint space narrowing (JSN). The joint area was then imaged and used to calculate the JSN.

**Blood pressure and heart rate monitoring:** The splenic nerve was exposed following anaesthesia and a 200 μm-tunnel Cortec electrode was implanted subcutaneously at the base of tail. The implant was connected to a pressure transducer and recorded using a Acqknowledge software (MP100WS). Blood pressure and heart rate were measured and analyzed using Biopac data acquisition Acqknowledge 881 (MP100WS) software (Biopac System, Inc.).
collagen) on day 0 and day 21. At day 11, mice were anaesthetized with isoflurane and the spleen area was exposed. One mm length 100 µm-sling micro-cuff electrode (CorTec) was implanted onto the apical splenic nerve. At day 16, mice were placed in individual cage and connected to a PlexStim V2.3 (Plexon) or MAPS (Axonic) stimulator. The set-up of the electrostimulation were rectangular charged-balanced biphasic pulses with 650 µA pulse amplitude, 2 ms pulse width (positive and negative) at 10 Hz frequency for 2 min 6 times a day (every 4 hours). The severity of arthritis was assessed using an established semiquantitative scoring system of 0–4, where 0 = normal, 1 = swelling in 1 joint, 2 = swelling in > 1 joint, 3 = swelling in the entire paw, and 4 = deformity and/or ankylosis. The cumulative score for all 4 paws of each mouse (maximum possible score 16) was used to represent overall disease severity and progression. For the evaluation of incidence, mice were considered to have arthritis if the clinical arthritis score was at least at 1 point for three consecutive days.

**Flow Cytometry**: Single-cell suspensions were stained with anti-CD11b (clone M1/70, BD Biosciences), anti-CD11c (HL3, BD Biosciences), anti-αMHCII (M5/114,15,2, ebioscience), anti-F4/80 (MB8, ebioscience), anti-Ly6C (AL-21, BD Biosciences). Dead cells were excluded using 7-AAD staining. Data were acquired on a LSRII (BD) flow cytometer and analyzed using the FlowJo software.

**Statistics**: Cria progression was plotted using Kaplan-Meier’s curve and differences between groups were estimated using the log-rank test. Normality of sample distribution was assessed using the Kolmogorov-Smirnov test. For comparison between two groups, statistical significance was assessed using unpaired t-test. For comparison between more than two groups, statistical significance was assessed using one-way ANOVA followed by Tukey’s post hoc test. All statistical analysis were performed using GraphPad Prism v.6.

**References**

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