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Sensibilité des neurones nocicepteurs aux signaux immunitaires dans l'inflammation de type 2

Sensitivity of Airway Nociceptor Neurons to Immune Signals in Type 2 Inflammation

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Thèse intitulée

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Présenté par

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Résumé: Les neurones nocicepteurs jouent un rôle clé dans la défense de l'organisme. Dans le cas des réactions inflammatoires, ils initient des réflexes protecteurs tels que la toux, les vomissements, où les démangeaisons, et participent à la régulation de plusieurs mécanismes physiologiques, notamment la réponse immunitaire. Ils jouent ainsi un rôle prépondérant dans l'inflammation de type 2, souvent associée aux allergies. Mais les mécanismes qui permettent l'activation de ces neurones dans ce contexte sont encore mal connus. Au cours de ce projet de recherche, nous avons exploré la capacité des neurones nocicepteurs à détecter les signaux immunitaires spécifiquement associés à l'asthme. Nous avons ainsi identifié les caractéristiques des nocicepteurs des voies aériennes. Nous avons également démontré leur sensibilité aux allergènes grâce à l'expression du récepteur aux immunoglobulines de type E, FcεR1, ainsi que leur capacité à modifier leur transcriptome en réponse aux cytokines IL-4 et IL-13. Ces travaux soutiennent l'importance de la communication entre systèmes nerveux et immunitaires, et mettent en évidence de nouvelles cibles pour limiter la contribution neuronale aux réactions allergiques.

Mots-clés: Nocicepteur, Neurone Sensoriel, Asthme, Inflammation, Cytokines, IL-13, FcεR1, Complexe Nodose-Jugulaire, Ganglions spinaux, Voies Respiratoires

Abstract: Nociceptor neurons play a major role in organism defense. In the context of inflammation, they initiate protective reflexes such as cough, vomiting, or itch, and participate in the regulation of various physiological mechanisms, including the immune response. They notably participate in type 2 inflammation, often associated with allergies. But the mechanisms driving the activation of nociceptor neurons in this context are still elusive. During this research project, we investigated the ability of nociceptor neurons to sense immune signals specifically associated with asthma. We identified the characteristics of airway innervating nociceptors. We also demonstrated their sensitivity to allergens through the expression of the Immunoglobulin E receptor FcεR1, as well as their ability to change their transcriptome in response to IL-4 and IL-13. This work supports the importance of bidirectional communication between the nervous and immune systems and unravels new targets to regulate neuronal contribution to inflammation.

Keywords: Nociceptor, Sensory Neurons, Asthma, Inflammation, Cytokines, IL-13, FcεR1, Jugular-Nodose Complex, Dorsal Root Ganglia, Airways

CV

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Project focused on the ability of airway sensory neurons to detect immune signals and change their phenotype in the context of asthma and allergies. The objectives were to:

- Characterize the molecular profile of airway sensory neurons in naive and pathological states
- Identify new immune signals detected by these neurons
- Demonstrate the impact of neuromediators on immune cells

First author publications:

1. Crosson T, Bhat S, Wang JC, Salaun C, Roversi K, Herzog H, Rafei M, Blunck R, and Talbot S. IL-13 reprograms airway nociceptors transcriptome in allergic airway inflammation. (under review, Journal of Clinical Investigation, BiorXiv)

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Co-author publications:

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Abbreviations

AAI: Allergic airway inflammation
Ach: Acetylcholine
ASIC: Acid-sensing ion channel
ATP: Adenosine triphosphate
BALF: Bonchoalveolar lavage fluid
BDNF: Brain-derived neurotrophic factor
Ca^{++}: Calcium
cAMP: Cyclic adenosine monophosphate
CD: Cluster of differentiation
CGRP: Calcitonin gene-related peptide
CNS: Central nervous system
CREB: C-AMP Response Element-binding protein
CRL: Calcitonin receptor like
DNA: Deoxyribonucleic acid
DRG: Dorsal root ganglion
ERK: Extracellular signal-regulated kinase
GPCR: G protein-coupled receptors
IFN-γ: Interferon gamma
IgE : Immunoglobulin E
IL: interleukin
ILC: Innate lymphoid cell
JNC: Jugular Nodose Complex
K^{+}: Potassium
LTMR: Low threshold mechanoreceptor
MAPK: Mitogen-activated protein kinases
MRGPR: Mas-related G-protein coupled receptor member
mTOR: mechanistic target of rapamycin
Na^{+}: Sodium
NGF: Nerve growth factor
NK1R: Neurokinin receptor 1
NMU: Neuromedin U
NE: Norepinephrine
NPPB: Natriuretic peptide B
NPY: Neuropeptide Y
NTRK1: Neurotrophic receptor tyrosine kinase 1
NTRK2: Neurotrophic receptor tyrosine kinase 2
NTS: Neurotensin S
PACAP: Pituitary adenylate cyclase-activating peptide
PKA: Protein Kinase A
PKC: Protein Kinase C
preBötC: preBötzinger complex
RAMP1: Receptor activity modifying protein 1
RNA: Ribonucleic acid
SP: Substance P
SST: Somatostatin
STAT: Signal transducer and activator of transcription
STING1: Stimulator of interferon response CGAMP interactor 1
TG: Trigeminal Ganglion
TH: Tyrosine Hydroxylase
Th1, 2 and 17: T helper 1, 2 and 17
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor alpha
TRKA: Tropomyosin receptor kinase A

TRPA1: Transient receptor potential ankyrin 1
TRPM: Transient receptor potential melastatin
TRPV1: Transient receptor potential vanilloid 1
VIP: Vasoactive intestinal peptide
VPAC: Vasoactive intestinal peptide receptor type 1

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I - General Introduction

Sensory neurons form an essential source of information for the organism to perceive and interact with its environment. These specialized cells are part of the peripheral nervous system, and most of the sensory neurons have their cell bodies located in peripheral ganglions. Sensory neurons are defined by their ability to detect physical or chemical stimuli, translate it into electric signal, and relay this information to the central nervous system. Amongst the sensory neurons, nociceptors are specialized in detecting threatening stimuli, that can eventually be processed into the sensation of pain. They form a key line of defense of the organism, and their activation triggers protective reflexes such as withdrawal, cough, vomiting or scratching. In addition, the feeling of pain eventually shapes the brain's memory and helps anticipating and preventing harmful situations. Nociceptors are also involved in autonomic reflexes, with activation of smooth muscle, regulation of energy homeostasis, body temperature or blood pressure. Finally, nociceptors can have an efferent action, through the release of neuromediators in the periphery, thus affecting various physiological functions, including the regulation of inflammation and immune responses. Overall, these cells play an important and diverse role in protecting the organism which includes but is not limited to sensation.

In the context of allergies and asthma, nociceptor neurons play an essential role to mount the inflammation. Yet, the mechanisms driving their activation in this type of immune responses have not been elucidated. The main objective of this thesis is to address what type of signals activate the nociceptors during asthma and allergies. We review the roles and functions of nociceptors, from molecular to physiological scale, and define their different subtypes. We also review the current literature regarding the mechanisms driving asthma and allergies, and their connection with the peripheral nervous system with a focus on the lung mucosa. Both neurons and immune cells communicate and affect each other's phenotype, and we detail the state of literature regarding the mechanisms of this crosstalk.

We reveal new evidence demonstrating the characteristics of airway nociceptors, their role in lung inflammation, and their sensitivity to immunoglobulins and cytokines. Finally, we discuss how this new work integrates into the current knowledge, and its possible clinical implications. This work provides new evidence supporting the crosstalk between sensory neurons and immune cells, and unravels potential targets to inhibit maladaptive allergic reactions.

II - Sensory neurons and pain

Cellular biology of sensation and nociception

A principal characteristic of sensory neurons is their ability to detect physical or chemical information and convert it into electrical signal as trains of action potentials (**Figure 1**). To do so, sensory neurons express ionotropic receptors sensitive to these stimuli. Once activated, these ion channels open, allowing influxes of Na^+ and Ca^{++} ions into the neurons, which depolarize the membrane, activate voltage-gated ion channels ($Na_V1.1$, $Na_V1.6$, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$)[3], and trigger action potentials. Transient receptor potential channels TRPV1, TRPM3, TRPM8 and TRPA1 are key receptors in temperature sensing[4-9]. Acid-sensing ion channels (ASIC1, ASIC2, and ASIC3)[10,11] are activated by pH changes. PIEZO2 is the main mechanoreceptor[12], which opens in response to changes in pressure or membrane tension. In addition, TRPA1 was also shown to mediate responses to electrophilic pungent chemicals[13]. Finally, P2X receptors are ATP-sensitive ionotropic receptors that allow the activation of sensory neurons by neighboring sensory cells. This is the case for instance in complex neuroepithelial structures like taste buds[14].

Anatomy of sensory and pain peripheral processing

Sensory neurons are pseudo-unipolar neurons. They have a single axon, that split in two branches, one branch innervating the periphery, and the other reaching the central nervous system (CNS) to communicate with other neurons. Virtually all primary afferent sensory neurons somas are located in peripheral ganglia, namely dorsal root ganglia (DRG), Nodose-Jugular complex (JNC) and Trigeminal Ganglia (TG) (**Figure 2**). DRGs innervate mostly the skin, muscles, and joints, plus some internal organs[15]. TG neurons innervate the head and face[16]. Finally, JNC neurons innervate most internal organs[15]. Interestingly, JNC neurons innervate the mucosa in the lung and intestines, but also organs devoid of mucosa such as liver, heart, or spleen[15,17]. Then, some of these neurons are

not meant to detect exogenous threats, but rather to monitor physical and chemical parameters within the organism. For this reason, the concepts of interoception versus exteroception have emerged[18], underlying the complex and diverse role of sensory neurons. Consequently, it also involves that some sensory neurons signal to the CNS to trigger mechanisms regulating the body's homeostasis, without necessarily triggering pain or sensation[15,19].

DRG sensory neurons are the most abundant in the body. They project to the spinal cord, where they signal to secondary neurons through the release of glutamate. Activated secondary neurons either trigger reflexes by directly activating motor neurons[20,21], or transmit the information to the brain. Most sensory information enters the brain at the level of the brainstem. Spinal cord interneurons project to the thalamus, the periaqueductal grey (PAG), and the parabrachial area (PB)[22]. JNC neurons project mostly to the nucleus of the solitary tract[15], and TG neurons all project to rostral principal nucleus and the caudal spinal nucleus[23]. The sensory information is subsequently processed in various parts of the brain, in order to trigger the sensation of pain.

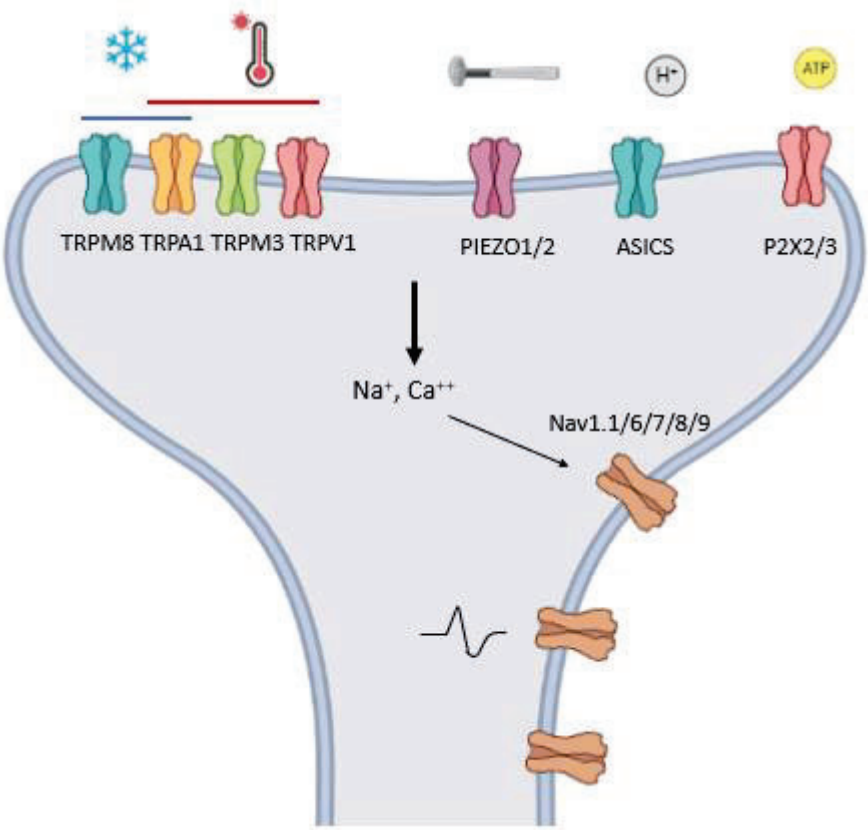

Figure 1 Conversion of physical and chemical stimuli to action potentials In peripheral nerve endings of sensory neurons, TRP, PIEZO, ASICS and P2X channels open in response to temperature, pressure, pH or ATP stimulation. This allows the entry of cations in the neurons, depolarizing the membrane. If the membrane voltage reaches a given threshold, voltage gated channels (Na_V) are activated, and propagate an action potential through the axon.

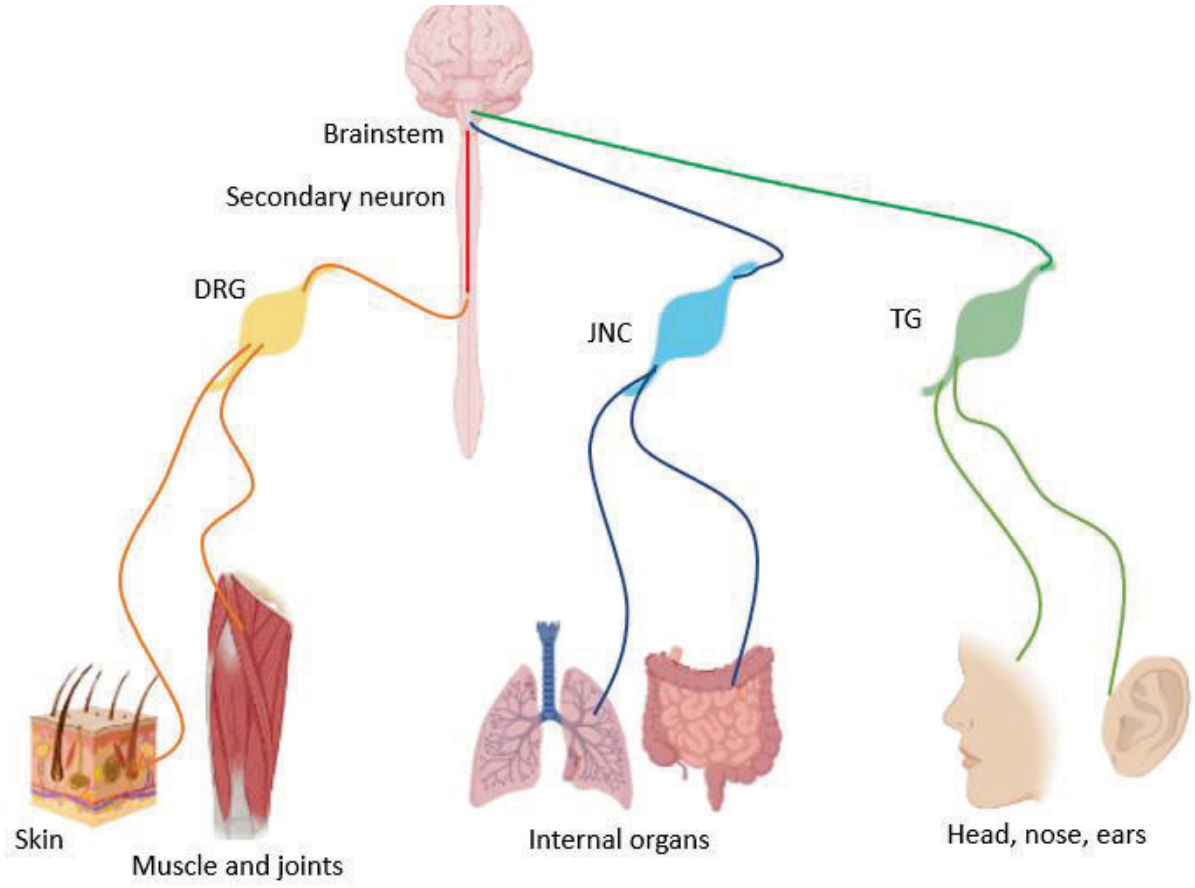

Figure 2 Anatomy of sensory peripheral innervation Primary afferent sensory neurons are located in DRG, JNC, and TG, which respectively innervate the skin and muscle, the internal organs, and the face. They project to the central nervous system, in the spinal cord and brainstem.

Nociceptors classification

Sensory neurons, and in particular nociceptors, form a very diverse and heterogenous group of neurons. They differ in their molecular profile, anatomic position, site of innervation, level of myelination, the cues they are sensitive to, and their electrophysiological properties. This heterogeneity contributes, in part, to the diversity of sensations they can trigger. Various studies have aimed at defining subpopulations, and consensus has recently emerged, supported by the generalization of single-cell sequencing technologies. DRG, Jugular and Trigeminal neurons originate from the neural crest and display similar molecular profiles, while Nodose neurons, originating from the placode, stand apart[24]. While several markers and functions are shared in neurons of these ganglia, those different developmental origins induce specificities, in part because of a differential contribution of neurotrophic factors. Nerve growth factor (NGF) is notably essential in the development of peripheral sensory neurons in the neural crest, but not in placode derived sensory neurons[25,26].

The classification of the neural crest derived neurons has then been more thoroughly established, with the definition of 5 large groups (Low threshold mechanoreceptors (LTMR), Proprioceptors, Non-peptidergic nociceptors, peptidergic nociceptors, and C-LTMRs), further divided for a total of 10-12 subgroups of neural crest derived sensory neurons[1] (**Figure 3**). This classification is rather well conserved across mammalian species[1]. The sodium channel $Na_V1.8$ is the most specific marker to identify nociceptors, while other sensory neurons rather express $Na_V1.1$[1,24]. These-non nociceptor sensory neurons are more thickly myelinated, have a larger diameter, express NEFH and parvalbumin, and are divided into LTMRs, which relay gentle touch sensation, and proprioceptors.

Nociceptor neurons are divided into 3 subgroups, peptidergic, non-peptidergic, and C-LTMR. This last group stands apart as Tyrosine hydroxylase (Th)+ Piezo2+ fibers that can transmit pleasant touch signals[27,28]. Peptidergic and non-peptidergic nociceptors used to be defined based on the presence or absence of the neuropeptides Calcitonin gene-related peptide (CGRP) and Substance P (SP). This classification may however be considered outdated since new omics studies do not identify these markers as the most specific to segregate neuronal subgroups. In addition to that, other neuropeptides can be released by non-peptidergic nociceptors such as Somatostatin (SST), TAFA4, NPPB, and the CGRP isoform CGRPβ. Yet, this terminology is still widely used and will be applied in this thesis. Peptidergic neurons have a small diameter and express high CGRP levels[1]. They are subdivided into 2 groups, one non-myelinated thermosensitive subtype expressing SP, and another one lightly myelinated responsive to mechanical stimuli. Non-peptidergic nociceptors are divided in three subtypes, all likely to be involved in itch and pruritus[29-31], and express various neuropeptides, G protein-coupled receptors (GPCRs), and ionotropic receptors.

Placode-derived nodose neurons have been less thoroughly described. They share the expression of most markers identified in neural crest derived neurons ($Na_V1.8$, $Na_V1.1$, $Na_V1.7$, TRPV1, TRPA1…), but are divided into different subtypes. Despite the low total number of JNC neurons (about 6000 in mice), they display an even greater diversity than neural crest sensory neurons, with 20 to 30 different populations depending on the study[24,32]. The classical distinction between small peptidergic nociceptors and large myelinated low threshold mechanoreceptors does not apply to nodose sensory neurons. As depicted in **Article 2 PMID: 35382899**, they are larger in size than neural crest neurons, display higher sensitivity to thermal and chemical cues, are responsive to different neurotrophins, and express different sets and amounts of neuropeptides. We can hypothesize that this broad diversity of neurons is due to the diversity of tissues innervated by the nodose ganglia. The innervation of airways, gut, liver, and other internal organs may thus require different types of sensors to regulate their physiology. An elegant study recently unraveled that 3 major dimensions, i.e., organ innervated, layer of mucosa innervated, and modality sensed, explain this heterogeneity in the JNC[17]. In this thesis, we will focus on the neuronal subtypes that have been identified in the airways.

Subtype	LTMR1	LTMR2	LTMR3	Proprioceptor	NP nociceptor 1	NP nociceptor 2	NP nociceptor 3	PEP nociceptor 1	PEP nociceptor 2	C-LTMR
Function	Gentle touch			Proprioception	Pain/ itch	Pain/ itch/ neurogenic inflammatio	Pain/itch	Pain/ neurogenic inflammation	Pain/ neurogenic inflammation	Pleasant touch / Pain
Stimuli	Mechanical			Mechanical	Irritant/ mechanical/ acid/ ATP	temperature / ATP/ acid	Temperature/ mechanical/ ATP/ acid/ irritant	Temperature/ irritant	Mechanical/ ATP/ acid	Mechanical/ irritant
Nav	Nav1.1/7			Nav1.1	Nav1.7/8/9	Nav1.7/8/9	Nav1.7/8/9	Nav1.7/8/9	Nav1.7/8/9	Nav1.7/8/9
Myelin	Yes	High	High	High	No	No	No	No	Yes	No
Neuropeptide					CGRPβ	CGRPα/ CGRPβ/ NTS	SST/ NTS/ NPPB	SP/ CGRPα/ CGRPβ/ NTS/ PACAP	CGRPα/CGRPβ	CGRPβ
specific marker	Necab2	Calb1	?	Runx3	Mrgprd	Mrgpra3	NPPB/ SST	SP/PACAP	TAFA1	TAFA4
Abundance	5%	8%	2%	8%	20%	5%	2%	10%	3%	37%

Figure 3 Classification of neural crest derived sensory neurons. The table depicts the 10 sensory neurons subtypes often described in the literature, along with their markers and characteristics. The table is adapted from the work of Usoskin et al.[1].

Sensory innervation of the airways

Innervation of the respiratory system plays an essential role to regulate breathing and protect the airways. Most of the sensory innervation of the airways, which include the lung and trachea, originates from the Jugular-Nodose complex (JNC) through the vagus nerve[33-35]. Despite a limited number of neurons (500-800 in mice), vagal sensory subtypes innervating the airways are still heterogenous. This heterogeneity aims to provide a fully functional innervation in a complex organ, with a variety of sensory fibers characterized by the type of airway innervated (trachea, bronchi, alveoli), the physiological function of the neuron (breath regulation, expiratory reflexes, pain, neurogenic inflammation), the layer of mucosa innervated (smooth muscle, epithelium), or the sensory modality (mechanical, chemical, thermal)[17,33,35]. Consensus has emerged to define three large groups of airway sensory neurons, which we will name here as follows: stretch mechanoreceptors (essential to the respiratory cycle), cough mechanoreceptors, and airway vagal nociceptors.

Electrophysiological studies have defined stretch mechanoreceptors as slowly adapting receptors (SARs) and rapidly adapting receptors (RARs), based on their pattern of response to lung inflation or deflation[36,37]. SARs and RARs are myelinated Aβ mechanosensitive fibers that are active throughout the respiratory cycle[33,38], and show increased activity in response to deep inspiration or lung deflation. These afferent signals are transmitted to the nucleus of the solitary tract in the brainstem, which in turn relays the information to the phrenic motor neurons that control the diaphragm, thus playing a key role in the normal physiological control of breathing[38-40]. As a result, complete vagotomy causes severe respiratory depression which eventually leads to death[41]. However, it is still debated whether SAR and RAR are two distinct classes of neurons, or similar neurons innervating distinct tissue layers[37]. These mechanosensitive fibers sense the degree of airway stretch with the mechanosensitive ion channel PIEZO2. This channel is then essential to the respiratory cycle, as patients with dysfunctional PIEZO2 display breathing difficulties, while PIEZO2 knockout mice suffer respiratory distress and die within a day after birth[39,42].

Another class of airway innervating mechanosensory neurons, referred to as cough mechanoreceptors, are lightly myelinated Aδ fibers that sense punctate mechanical stimulation rather than airway stretch[43]. As a result, these sensory neurons are meant to trigger cough reflexes to expel a physical intrusion in the airways. They rather innervate the larger airways and trachea[33]. In addition to mechanical stimulation, they were also shown to be activated by acidic pH[33].

Airway sensory neurons responsive to thermal and chemical cues are largely unmyelinated C-fibers neurons and lightly myelinated Aδ-fibers neurons[33]. They represent the majority of airway vagal afferent fibers[44], and their sensitivity to cues relies mostly on the expression of TRPV1, TRPA1, or acid-sensing ion channels (ASICs)[45]. They are recruited in response to tissue injury, inflammation, noxious chemicals or temperatures, and their activation can trigger different types of reflexes including apnea, glottic closure, bronchoconstriction, cough, sneeze, or protective swallowing. This group is likely to be divided in different nociceptor subtypes.

Airway sensory neurons have been deeply investigated in laboratory animals, but similar innervation patterns have been observed in human airways, suggesting it would be conserved across mammals[46,47].

This consensus classification of airway vagal neurons relies mostly on observed phenotypic responses to various stimulation[33], in addition to immunostaining and electrophysiological studies. But a complete classification of the airway neurons at the molecular level is still lacking. This area of research is still ongoing[17,48].

Other innervation of the airways

Motor neurons and respiratory regulation

The peripheral nervous system, and by extension the airway innervation, is not limited to sensory neurons (**Figure 4**). Motor neurons control the diaphragm and intercostal muscle to trigger inspirations and expirations. These neurons are located in the ventral and dorsal respiratory groups in the brainstem and innervate these muscles through the phrenic nerve[49]. These neurons are controlled by the preBötzinger complex (preBötC), in the brainstem, which processes information such as blood CO_2 concentration, pH, temperature, airway and muscle stretch (some of this information originating from sensory neurons) to regulate the breath rate[49,50].

Autonomic innervation of the airways

Airways are also largely innervated by autonomic sympathetic and parasympathetic fibers. Parasympathetic fibers share the vagus nerve with JNC sensory neurons, but their cell body are located in the brainstem[40,51]. They project to the intramural ganglia, from which postganglionic cholinergic neurons, innervate the airway smooth muscle[51]. They represent the predominant bronchoconstrictor pathway[52] by releasing of acetylcholine (ACh), which through muscarinic receptors triggers the contraction of airway smooth muscle cells. Sympathetic innervation of the airways originates mostly from the superior cervical ganglia and stellate ganglia in the sympathetic trunk[51]. They innervate the blood vessels and airway smooth muscle cells. They release primarily noradrenaline and are positive for neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP). Sympathetic nerve activation has an opposite effect to parasympathetic fibers, eliciting airway smooth muscle relaxation and bronchodilation[53,54].

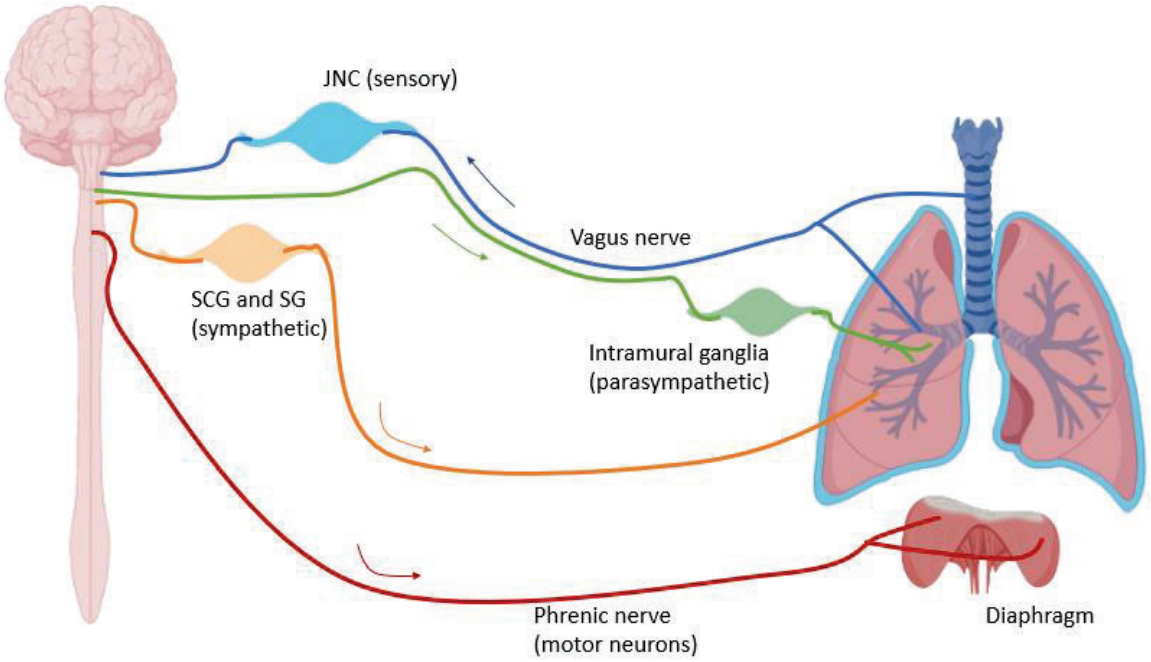

Figure 4 Innervation of the airways Motor, sympathetic, parasympathetic, and sensory neurons innervate the airways. Sensory neurons are the only neuronal type with afferent function, transmitting signal to the brain in both steady state and injury conditions. The sensory signals influence the efferent peripheral neurons to regulate lung physiology.

III - Lung immunology and asthma.

Mucosal and immune defenses in the airways.

The respiratory tract carries out the crucial function of gas exchange. Airways are thus characterized by a vulnerable mucosa, which can get in contact with noxious pathogens or particles. Several layers of defense, including the immune system, are meant to sense and eliminate these threats without altering the lung respiratory function. The intrusion of these exogenous particles can induce mucosal damage, inflammatory response, and possibly severe pneumonia. Several specialized cells participate in maintaining lung homeostasis[55]. The epithelium and secreted mucus provide a physical and chemical barrier. Goblet cells produce mucus that covers the mucosa and can entrap foreign particles. Ciliated cells are abundant epithelial cells that activate their cilia and continuously propel the mucus toward the larger airways where it can be expelled via cough or swallowing. Finally, club cells secrete antimicrobial compounds. In the airway space and within the mucus, alveolar macrophages are a specialized type of immune cells that clear particulates, apoptotic cells, and cellular debris from the airways[56].

Mucus is composed of water, ions, lipids, proteins, and complex macromolecular glycoproteins called mucins[57]. Secreted mucins are synthesized in goblet cells and released by exocytosis upon stimulation. MUC5AC and

MUC5B are the most prominent secreted mucins in the respiratory tract. When secreted, these gel-forming mucins form a dense macromolecular matrix providing the adhesive properties of the mucous layer. Mucus provides a physical barrier, trapping pathogens and particles before they reach the epithelium.

In the context of an infection, or intrusion of pathogens, allergens, viruses, venoms or parasites, the threat has first to be detected, following which signaling molecules are secreted to mount a protective response. The variety of types of threats has led to the development of tuned immune responses with distinct features. Three major types of immune responses are usually distinguished, namely type 1, type 2, and type 17 inflammation[55,58]. Type 1 inflammation is commonly activated in response to intracellular pathogens including bacteria, fungus, and viruses, and is also active in the context of cancer. Type 2 inflammation is triggered in response to venom, pluricellular pathogens and parasites, or allergens. More recently, type 17 inflammation has been associated with infection by extracellular bacteria and fungus. Each type of immune response involves different types of immune cells, cytokines, antibodies, and may induce different physiological pathway in non-immune cells (mucus secretion, pain and itch, vasodilation…). The diversity of immune responses allows fighting the threats more efficiently, while minimizing the damage of the tissue[55].

A tuned immune response involves that the lung mucosa can both detect and segregate the different types of pathogens, to trigger the proper set of immune cells. Type 1 and type 17 immune responses can be triggered by dendritic cells, macrophages, or even epithelial cells. The intrusion of a foreign body is sensed by pattern recognition receptors (PRR); a large family of receptors that recognize a variety of molecules found in pathogens[59]. This detection drives the release of first order cytokines (IL-12, IL-23, IL-1β)[55], which activate a range of other immune cells, amongst which CD4 T cells and innate lymphoid cells 1 and 3 (ILC1 and ILC3). These orchestrate the immune response through the release of second order cytokines. In type 1 inflammation, CD4 T cells polarize into T helper 1 (Th1) cells, and, along with ILC1, release IFN-γ. In case of type 3 inflammation, γδT cells, ILC3, and Th17 polarized CD4 T cells secrete IL-17 and IL-22. Various effector immune cells are activated, or recruited in the inflamed tissue, amongst which CD8 T cells and neutrophils are major effectors in these types of immune responses. Neutrophils are rapidly recruited by chemoattractants released by sensor immune cells and act through phagocytosis, and through the release of neutrophils extracellular traps (NET), which contain DNA, histones, proteases and antimicrobial proteins[60]. CD8 T cells recognize and eliminate infected cells[61].

Th2 immune responses are directed against extracellular pathogens or parasites and rely less on detection by PRR. Instead, it is triggered by tissue damage and by the recognition of proteases[55,62] often released by these extracellular pathogens. Damaged cells in the epithelium release first order cytokines, also called alarmins: IL-33, IL-25 and Thymic stromal lymphopoietin (TSLP)[63]. Mast cells are also key sensor cells that detect proteases, and in turn release effector molecules like proteases, histamine, and prostaglandins, along with cytokines. These signals, in particular type 2 specific alarmins, activate ILC2, and trigger the polarization of CD4 T cells in Th2. These lymphocytes then release type 2 specific second order cytokines, amongst which IL-4, IL-5 and IL-13. IL-4 drives the type 2 immune response and activates a variety of immune cells. IL-13 is notably associated with increased mucus secretion by epithelial cells[64]. IL-5 activates and recruits eosinophils, a type 2 specific effector cell type[65]. Eosinophils degranulate to release compounds that are toxic to helminths: major basic protein (MBP) and eosinophil cationic protein (ECP). Finally, IL-5 and IL-13 also promote epithelial regrowth.

Of note, type 1, type 2, and type 17 immune responses are not always entirely segregated based on the type of pathogens detected. These types of inflammation can have complementary functions. For instance, type 2 immunity triggers more processes associated with tissue repair and regeneration, and it is postulated that type 2 mechanisms are also involved to in the resolution phase following type 1 and type 17 immune responses[66].

Upon reinfection, or repeated exposure to the foreign organism, adaptive immunity will develop. Memory B cells recognize specific antigens with the B cell receptor (BCR), while memory CD4 T cells recognize antigens presented by dendritic cells with the T cell receptor (TCR). In all type 1, type 2 and type 17 immune responses, B cells then produce and secrete antigen-specific antibodies, although type 2 inflammation is commonly associated with higher antibody titers[67]. Conversely, Type 1 immunity relies more on cell-mediated immune response. Indeed, the neutralization and expulsion of large extracellular parasites are more efficiently achieved by antibody mediated immunity, while intracellular pathogens are cleared by CD8 cytotoxicity to eliminate infected cells[58].

Antibody classes and function

Antibodies are composed of an antibody binding fragment (Fab), and a crystallizable fragment (Fc) that deploys effector functions[68]. The Fab contains the variable domains that makes the specificity of the antibody for a given

antigen. The Fc defines the class of antibody. B cells differentiate into producing a specific class of antibody by DNA recombination[69]. There are 5 different isotypes IgA, IgM, IgG, IgE and IgD, further divided into subclasses. IgM takes a pentameric form and can bind to the complement, a cascade of small proteins that has the ability to break into the membrane of a pathogen[68,70]. IgG is an abundant monomer that plays an important neutralization role, and can also activates the complement, opsonization (a mechanism promoting phagocytosis), and antibody dependant cytotoxicity by NK cells (ADCC)[71]. IgA is a secreted form of antibodies, that is actively transported in mucus, saliva, and other secretions, where it plays mostly a role of neutralization. The IgD function has been a puzzle in the immunology area but is thought to interact with basophils and mast cells and to play a role in mucosal immunity[72].

Finally, IgE is the least abundant isotype, and has a short half-life[68]. This isotype is specific to type 2 immune responses, and its production by B cells is driven by the Th2 cytokine IL-4[73]. While IgE is maintained at a low level in the serum, it binds to FcεRI on mast cells where it can remain fixed for months[74]. Upon antigen sensing by IgE bound to FcεRI, mast cells degranulate to release histamine, proteases, and cytokines[73]. This mechanism allows a quick and antigen-specific detection and effector response mediated by these cells. FcεRI is also expressed on basophils, where its activation triggers the production and release of leukotrienes and cytokines[73]. FcεRI is a multimeric receptor with three different subunits. FcεR1α binds to IgE, whicle FcεR1β and FcεR1γ are signaling subunits that activate Src kinases[75]. It should be noted that another less characterized IgE receptor exists, FcεR2. It has lower affinity for IgE and is thought to regulate IgE production by B cells[76].

Allergies

Immune responses are meant to protect organisms against harmful pathogens. But it may in some circumstances become maladaptive and trigger misdirected inflammatory responses. This is the case for auto-immune conditions, and allergies. Allergic disorders are rising in developed countries, with 47.1% of the American population now reporting to have allergies[77]. These are defined as “an abnormal adaptive immune response directed against non-infectious environmental substances (allergens), including non-infectious components of certain infectious organisms”[78].

The reason for the increased prevalence of allergies is not clearly established. One of the main theories is the “hygiene hypothesis”[78], according to which the limited exposure to infections during childhood would result in the development of biased immune responses against non-harmful substances. The mechanism explaining this phenomenon is still elusive, but one possibility is that persistent immune challenges would promote robust anti-inflammatory mechanisms, for instance the release of IL-10, that prevent maladaptive immune responses. The limited exposure to pathogens would dampen these regulatory functions. This proposed mechanism is backed by the observation that elevated anti-inflammatory cytokines, known to be induced by helminth infections, inversely correlates with allergies[79].

Allergies often develop when the immune system in epithelial barriers encounter an allergen that triggers a type 2 immune response. Whether or not long-term allergic responses develop depends on several factors. The quantity, route, and frequency of exposure to the antigen will impact the development of allergies. In addition, the propension of this antigen, and surrounding molecules, to trigger a type 2 immune response explains why some substances are more allergenic than others. For instance, proteases and certain air pollutants promote this type of inflammation[62,80]. Finally, the host genetics, as well as environmental factors, affect the barrier integrity of the epithelium and mucosa, which greatly impact the development of immune responses. Genetic mutations altering skin integrity are often associated with increased prevalence of allergic disorders[81,82]. In addition, genomic studies showed that mutations in genes associated with type 2 immune responses, such as genes involved in alarmin signaling, are often observed in patients with allergic disorders[83].

Allergic disorders manifest differently depending on the tissue exposed. Atopic dermatitis, asthma, food allergies, or allergic chronic rhinitis are all consequences of allergies. Interestingly, it was shown that these manifestations are often connected, for instance children with dermatitis often develop asthma later in life. This phenomenon is described as “the atopic march”[84]. It is thought that sensitization to an allergen in one site may result later in the onset of systemic Th2 inflammation[84], increasing the risk of developing other types of allergies.

Overall, the mechanisms explaining the development of allergies are still elusive. Great advances have been made lately on the exogenous signals promoting the mounting of a type 2 immune responses, in which sensory neurons may play a role. In the next section, we focus on the pathophysiology of asthma.

Asthma pathophysiology and lung immunology.

Asthma is an umbrella diagnosis that encompasses a collection of different pathologies and phenotypes[85]. Clinically, asthma is defined by the presence of symptoms such as wheezing, shortness of breath, chest tightness, and cough[86]. Several mechanisms, called endotypes, can trigger those symptoms[86]. This heterogeneity often results in differences of appreciation by the scientific community in the literature as to what exactly defines asthma and what are the mechanisms of its pathophysiology. Most asthma cases are associated with airway inflammation[85,86], but cases of exercise-induced bronchoconstriction independent of immune responses are also reported, which may or may not be considered as asthma depending on the definition that is applied.

Various causes have been investigated to explain the development of asthma. Genetic variations have been found in patients for genes such as IL33, IL1RL1/IL18R1, HLA-DQ, and IL2RB9[83] suggesting a genetic predisposition to dysregulated mucosal Th2 immune responses. In addition, risk factors such as smoking and obesity can increase the chance to develop late-onset asthma[85].

Asthma can be divided in two different major subtypes, namely eosinophilic and non-eosinophilic (or neutrophilic) asthma. Eosinophilic asthma is characterized by type 2 inflammation in the airways. It is the most prevalent form, with an even higher prevalence amongst children and early onset forms of asthma. It is in most cases triggered by exposure to an allergen[85]. Eosinophilic asthma in young patients is commonly associated with other atopic pathologies such as eczema, rhinitis, or food allergy.

Non-eosinophilic asthma is characterized by type 1/type 17 immune responses, and neutrophil infiltrations in the lung. It usually develops later in life, and although it is less prevalent than eosinophilic asthma, it can lead to more severe exacerbations. The pathogenesis of this endotype is less characterized, but the identified risk factors include smoking, bacterial infections, or obesity.

In this thesis, we focus on the most common type of asthma, induced by type 2 allergic airway inflammation.

Pathophysiology

As described in previous sections, tissue damage, pathogen intrusion, or allergens, trigger immune responses in the airways. The inflammation associated with the immune response results in several physiological changes that obstruct airflow and ultimately lead to an asthma phenotype. Different alterations modify the physical properties of the airways: the quantity and type of mucus secreted, the bronchoconstriction by smooth muscles, and the thickening of the epithelial layer.

Mucus

The two major gel-forming mucins produced in mammals' airways are MUC5B and MUC5AC. At baseline, only MUC5B is expressed and secreted[87], while both MUC5B and MUC5AC are secreted at high levels in the context of airway inflammation. Such hypersecretion is meant to trap foreign organisms, before their killing, and their removal by ascension to the pharynx before swallowing or coughing. In type 2 immune responses, IL-13 is the most recognized inducer of mucus hypersecretion[64]. This cytokine is detected by goblet cells through IL4RII, driving the drastic overexpression of MUC5AC and a more moderate overexpression of MUC5B[88]. Mucus hyperproduction is also present in neutrophilic asthma[89], where potential activators suggested in the literature are IL-6[90], IL-17[90], TNF-a[91] or neutrophil elastase[92]. While mucus hypersecretion is beneficial for the neutralization, killing and removal of pathogens, an excessive or misdirected activation, like in the case of allergies, will contribute to airflow obstruction.

Bronchoconstriction

Airway smooth muscles (ASM) surround most of the airways from the trachea to the bronchioles and are located in a deeper layer around the epithelium. While they have the ability to narrow or enlarge the airways, their actual function remains unclear[93]. The proposed ASM roles include peristalsis to assist mucus expulsion, enhancing the effectiveness of cough, protection in response to irritants[94], or lung embryonic development[95]. It has also been postulated that airway smooth muscle is a vestigial organ that no longer provide an evolutionary advantage[96],

since no known disease or physiological deficit associated with loss of ASM function have been reported[96,97]. ASM in asthmatic patients are excessively contractile in response to little provocation, a phenomenon called airway hyperreactivity (AHR), which is a key defining clinical feature of asthma[98]. ASM can contract in response to chemical or physical stimuli, such as heat or cold[99,100], an effect that is exacerbated in patients with asthma. In addition, ASM contractility is largely driven by the autonomic nervous system, adrenergic sympathetic fibers inducing bronchodilation, while cholinergic parasympathetic fibers can trigger bronchoconstriction. Inflammatory derived mediators are thought to play a key role in modulating ASM function and responsiveness. Mast cell-released histamine, leukotrienes (LTC4 and LTD4), and prostaglandin D2 (PGD2), have long been recognized as potent bronchoconstrictors[101-103]. In addition, various cytokines can modulate the contraction of ASM such as TNF-α, IL-1β[104], IL-5 and IL-13[93,105-108].

Airway remodeling

Reduced airflow can also result from a thickening of the epithelial layer, a phenomenon associated with the airway remodeling[109]. Airway remodeling refers to the structural alterations in the airways of asthma patients, and involves a wide array of pathophysiologic features, including epithelial changes, increased smooth muscle mass, increased numbers of activated fibroblasts/myofibroblasts, subepithelial fibrosis, and vascular changes[110]. Inflammation is believed to be an important driver of these changes. Mediators such as TGF-β[110], IL-5 and IL-13 can promote epithelial regrowth[55]. TGF-β is also known to be pro-fibrotic. Overall, repeated or impaired repair process in the epithelium over time, with inflammatory cytokines and growth factors, can explain the development of airway remodeling[111]. It should be noted that remodeling was also sometimes shown to be present in childhood before the signs of inflammation, suggesting possible other causes, notably genetic[112].

Treatment of asthma

Several classes of drugs are used as treatments for asthma. Short- and long-acting β2-adrenoceptor agonists target the airway smooth muscles, mimicking the action of adrenaline, which is a strong bronchodilator, to restore the airflow[113]. In addition, corticosteroids are used as control therapies. They interact with glucocorticoid receptors in immune cells, where it notably inhibits NF-κB, thus blocking the transcription of several inflammatory genes coding for cytokines, chemokines, or adhesion molecules[113]. β2-agonists provide rapid relief of acute symptoms, while maintenance with daily inhaled corticosteroids is the standard of care for persistent asthma[114]. More recently, monoclonal antibody therapies have reached the approval by regulatory agencies. These are now used when classic treatments are not sufficient to manage the disease[115]. Most of these new biologics inhibits the type 2 immune responses. Benralizumab targets the IL-5 receptor IL5RA, while Mepolizumab and Reslizumab targets IL-5[116]. Given the action of IL-5 on eosinophiles, these are especially efficient for patients with eosinophilia. Omalizumab targets IgE to prevents its binding to FcεRI, and Tezepelumab targets the first order cytokine TSLP[117]. Finally, Dupilumab is a drug targeting IL4RII, hence preventing the action of both IL-13 and IL-4, that was originally developed against atopic dermatitis. It was recently shown to be also efficient to treat asthma[118]. Despite this variety of treatments, progress can still be made. Several patients still suffer uncontrolled exacerbations[115]. In addition, asthma is still an important burden for the society given its large prevalence (300 million people worldwide)[115,119], it complicates the daily life of the patients, and no treatment is truly curative. It is then still important to investigate the mechanisms of this pathology to understand both how it develops and how to treat it more effectively.

Animal models of asthma

Rodent models of asthma have provided useful insights in the pathophysiology of this condition. Most models require a first step of sensitization, during which the immune system detects the allergens, mounts an adaptive immune response with selection of antigen specific T cells and B cells, and develops a memory of the allergen. Another step of those protocols is the challenge, where the animal is exposed, usually intranasally, to the allergen, which triggers an allergic airway inflammation, mimicking an allergic reaction observed in humans asthma patients[120]. Allergens typically used are ovalbumin (OVA), house dust mite (HDM) extracts, fungi (Aspergillus fumigatus, Alternaria alternata) or cockroach extracts[120]. The OVA and HDM mouse models are the most common in the literature. The OVA model requires intraperitoneal sensitization with aluminum hydroxide as

an adjuvant. Airway OVA challenges subsequently induce robust pulmonary inflammation and IgE secretion[120]. This model is convenient for researchers as several tools are commercially available such as OVA-specific IgE, but its relevance is sometimes questioned as OVA allergies in humans do not lead to airway inflammation. The HDM model is thus considered more clinically relevant as it does not require adjuvants and is also a common allergen in human asthma.

IV - Nociceptor neurons and airway inflammation

Nociceptors and inflammation

Inflammation is an ancient medical term that refers to a set of clinical signs and symptoms. Inflammation is not synonymous with immune response, but is a broader concept that englobes all physiological changes that take place in response to an injury or a threat, and which involves several cell types and mechanisms. Two thousand years ago, Celsus defined inflammation as involving four cardinal signs: *dolor* (pain), *calor* (heat), *rubor* (redness) and *tumor* (swelling)[121]. All those 4 signs are deeply connected to the nervous system, and particularly to nociceptor neurons.

In the context of inflammation, sensory neurons can trigger unpleasant sensations such as pain, itch, or sore throat. They are also essential to trigger motor reflexes like scratching and coughing. In addition, their peripheral nerve endings locally release mediators in the inflamed tissue, mainly neuropeptides, that have various effects on the tissue physiology.

The rationale proposed to explain the evolutionary advantage conferred by the recruitment of sensory neurons in inflammation relies on their ability to quickly signal through action potentials, while immune and other cells secrete molecules that require more time to diffuse through the tissue. This was highlighted by the discovery of the “axon reflex”: following the activation of a nociceptor nerve ending, the action potential propagates toward the soma and the brain (orthodromically), but also antidromically when it reaches a nerve branching, resulting in local neuropeptide release in all nerve endings from this same neuron. This allows a quick and localized signaling in the injured tissue following the detection of the threat[122-124] (**Figure 5)**. The role of nociceptors in inflammation can also be indirect, with the triggering of reflexes mediated by the autonomic nervous system[125].

All the mechanisms associated with the neuronal contribution to inflammation are qualified as part of the neurogenic inflammation. Here, we detail the mechanisms by which nociceptor neurons mediate physiological inflammatory responses, including motor reflexes, immune modulation, and other physiological reactions.

Nociceptors and cough

Nociceptors are essential to initiate protective reflexes, such as withdrawal reflexes in response to physical or chemical stimuli. In the context of inflammation, skin-innervating nociceptors are responsible for itch and scratching, while in the airways, they initiate the reflex of coughing. Two different types of airway fibers described in previous sections can initiate cough: thinly myelinated Aδ-fibers “cough mechanoreceptors”, and C-fibers nociceptors. They are believed to innervate mostly the larger airways (larynx, trachea, bronchi), but studies also suggest a modest innervation of deeper lung airways[126]. The cough mechanoreceptors are activated by punctate mechanical stimulation, and by acid stimulation, and help to protect against foreign body intrusion, or to expel excessive mucus secretions[33,126]. The C-fibers nociceptors involved in cough are more responsive to irritant chemicals, inflammation, and tissue damage[33,126].

Nociceptors, neuropeptides, and inflammation

Increasing evidence demonstrate that the role of nociceptors in modulating inflammation is not limited to motor reflexes. Peripheral nerve endings also communicate locally with other cells in the tissue through the release of neurotransmitters and neuropeptides. The most abundant neuropeptides identified in primary afferent sensory neurons and released in the periphery are Substance P and Calcitonin gene-related peptide (CGRP), to which we can add Vasoactive Intestinal Peptide (VIP) in vagal neurons[121]. Other neuropeptides include neuropeptide Y, Somatostatin, pituitary adenylate cyclase-activating peptide (PACAP), neurotensin S (NTS), gastrin releasing peptide, neuromedin U (NMU) and natriuretic peptide B (NPPB) but their expression and peripheral release by

sensory neurons is less established. Notably, some of these neuropeptides also play a role in pain and inflammation at the level of the CNS nerve ending of the neurons, and regulate signal transmission to the brain or central sensitization[127-129].

Vasodilation

Neuropeptides released in the periphery by nociceptors were originally identified for their vasoactive properties[130-133]. CGRP is an extremely potent microvascular vasodilator[132], found to be very active in the skin of all species[134]. Through the RAMP1-CLR receptor, it activates endothelial and vascular smooth muscle cells, thus triggering vasorelaxation[132]. Substance P, through its receptor NK1R, is also detected by endothelial and smooth muscle cells, but is more prone to increase vascular permeability and plasma extravasation[135,136]. Through these vasoactive functions, these neuropeptides promote the infiltration of immune cells and protein mediators in the inflamed tissue[137,138] and participate to the formation of oedema[135]. VIP and PACAP are also potent vasodilators[139,140]

Mucus secretion

Nociceptor-released neuropeptides are also extensively reported to influence mucus secretion in various inflammation models. Substance P was found to increase bronchial mucus secretion in different species[141-143], although the mechanisms at play are not yet elucidated. Substance P was also associated with mucus secretion in guts in the context of inflammatory bowel disease. Albeit less established, CGRP was also found to promote gut mucus secretion[144,145], with a strikingly specific expression of CGRP receptor (CRL-RAMP1) in the goblet cells of the guts[145].

Bronchoconstriction

Nociceptor ablation was shown to reduce airway hyperresponsiveness[146]. However, little evidence supports a role for neuropeptides released by sensory neurons to directly promote bronchoconstriction. VIP, and its receptor VPAC2, were found to promote bronchodilation[147], while Substance P and Neurokinin A were also described as a potential bronchoconstrictor[148-150]. Overall, it appears more likely that the bronchoconstrictive action of sensory neurons is indirect and mediated by autonomic parasympathetic reflexes[33].

Immunomodulation

Over the past three decades, increasing evidence have also demonstrated the ability of neuropeptides to directly promote the activation and infiltration of immune cells in the inflamed tissue. Several studies using genetic ablation or pharmacological silencing of nociceptors, or of the neuropeptides they release, have observed altered immune responses in mice models of disease[151-157]. Optogenetic activation of nociceptor fibers in the skin, in the absence of injury or infection, was also shown to be sufficient to induce the activation of immune cells[122], while neuropeptide injection in the skin induced inflammatory cells infiltration[158]. The mechanisms at play *in vivo* are still elusive given the diversity of types of immune responses, neuropeptides, immune cells at play, and the possibility of indirect effects (i.e., vasodilation promoting immune cell infiltration). The action of nociceptors on inflammation differs depending on the inflammation model and the immune cells involved[159], which supports the hypothesis of a direct communication with immune cells. Overall, *in vitro* data have shown a more established direct effect of neuropeptides on innate or tissue resident immune cells, consistent with the function of nociceptor to signal rapidly and locally in case of threat detection. In this section, we focus on the demonstrated effects of direct immunomodulatory action of neuropeptides on immune cells.

Mast cells

Mast cells are tissue resident cells that detect antigens and proteases and release proinflammatory mediators. They are probably the most thoroughly investigated cell type for neuro-immune crosstalk[160,161]. Accumulated evidence demonstrated proximity and contact between mast cells and nociceptor fibers *in* vivo[154,160-163]. SP has been thoroughly reported as an activator of mast cell degranulation and chemokine production[164,165]. It triggers intracellular pathways in mast cells, although not through the classical SP receptor NK1R, but instead through the

GPCR MRGPRX2, or its mouse homolog MRGPRB2, which is strongly and specifically expressed in mast cells[166-172]. VIP was also found to activate mast cell degranulation through the activation of both VPAC1 and VPAC2 receptors[164,173]. Other reports have mentioned a possible role for somatostatin and neurotensin in neuron-mast cells communication[164].

Macrophages and monocytes

Most nociceptor-released neuropeptides appear to limit the inflammatory phenotype of macrophage and enhance their pro-regenerative functions. A newly described neuropeptide, TAFA4, released by C-LTMR promotes macrophage repair functions[174]. Macrophages also express the receptor NK1R[175-177], through which substance P was shown to induce their polarization toward the anti-inflammatory M2 type[178]. The activation of toll like receptors (TLR) expressed by macrophage induces the expression of the VIP receptor VPAC2, allowing a VIP mediated deactivation of these cells[179]. Peritoneal macrophage incubated with gastrin releasing peptide (GRP) released higher levels of TNF-α[180], while CGRP had the opposite effect[151].

Dendritic cells

Dendritic cells (DC) are often found in proximity to nociceptor nerve endings[153,156,181], but the role of this interaction appears complex and remains to be elucidated. Substance P was shown to activate dendritic cells in type 2 immune response through MRGPRA1 to promote their migration to lymph nodes[156]. In contrast, another study showed that the activation of the substance P receptor NK1R biased dendritic cells to induce type 1 immunity[182]. CGRP was shown to promote DC function for activation of Th2 response[183], but also to prevent antigen presentation[184].

Neutrophils

In vivo studies extensively showed that neutrophil infiltration was suppressed by nociceptors, a phenomenon often attributed to CGRP signaling[155,185]. *In vitro*, CGRP was suggested to dampen bacterial clearance by neutrophils[185]. VIP was shown to block the activation of neutrophils induced by IFN-γ[186]. Gastrin releasing peptide (GRP) was described as a chemoattractant for neutrophils[180].

Innate lymphoid cells

Neuromedin U (NMU) was recently found to activate ILC2 and stimulate their cytokine production and proliferation[187-189]. A result that has been abundantly confirmed since[190,191]. However, the expression and release of NMU in sensory neurons have not been evidenced. It appears likely that the NMU regulating ILC2 function originates from other cell types, including enteric and parasympathetic neurons[187,188]. VIP enhances IL-5 production in ILC2 through VPAC2[192]. CGRP appears to have a complex modulatory role on ILC2s: it enhances IL-5 expression[193-195], but inhibits their proliferation and IL-13 expression[194,195]. To add another layer of complexity, CGRP is also found to be strongly expressed by ILC2s themselves[194,195].

Adaptive immune cells

The effect of neuropeptides on adaptive lymphocytes is less established as a limited number of studies have demonstrated a direct action on these cells. CD4 T cell secretion of cytokines was suggested to be induced by SP, CGRP, and SST[196], but was also shown to be inhibited by CGRP and VIP in a contradictory study[197]. SP was shown to increase immunoglobulin release by B cells[198] while CGRP seems to dampen CD8 T cell activity[199].

Discussion regarding the role of Nociceptors in inflammation

The evolutionary advantage conferred by the recruitment of nociceptors in inflammation and immune responses can be questioned since it is immune cells' role to detect and fight infections. Several hypotheses have been proposed. Increasing pain sensitivity in infected wound promotes protective reflexes and behavior to prevent further damage and infections by other pathogens. Itch and scratching reflexes are also thought to be protective in the case of insects' bites[200] and were even proposed as a "mechanical adjuvant" allowing disruption of the skin to promote the mounting of an immune response[201]. Cough allows to expel mucus and the pathogens it may contain.

Regarding the inflammatory modulation by neuropeptides, the main hypothesis is that sensory neurons can signal rapidly by the mean of action potentials, promoting early responses, while immune cells communication depends on paracrine secretion and molecule diffusion which is a lot slower. For instance, in case of a wound or injury,

sensory neurons can rapidly trigger the formation of an oedema. But this hypothesis is not sufficient since the pro-inflammatory action of sensory neurons is not limited to early immune responses[202,203] and is in part secondary to signals released by immune cells[204,205]. Another interesting feature of nociceptors is that their peripheral nerve projections form a dense grid in all skin and mucosa, with each neuron covering individually a given area (10-400mm^2 for each sensory neuron in human skin)[206,207]. We can then hypothesize that the activation of sensory neurons allows to drive a localized inflammatory response, limited to the area innervated by the activated neurons.

Discussion regarding the opposite effects of nociceptors in inflammation

Sensory neurons function in inflammation appears to depend greatly on the context, the time point observed, the pathology or the type of immunity and immune cells at play. To explain this discrepancy, the consensus emerging from the literature is that nociceptor neurons enhance immune responses in type 2 inflammation, and in innate and humoral immunity. On the other hand, they have inhibitory action in type 1 immune responses and in cell-mediated immunity[159]. Their effect in type 17 immune responses is still controversial[122,153,155]. Such discrepancy is also observed in the world of immune cells, for instance IL-4 is considered pro-inflammatory in type 2 immune responses, but anti-inflammatory in type 1 immunity[208], while IFN-γ works the other way around[209].

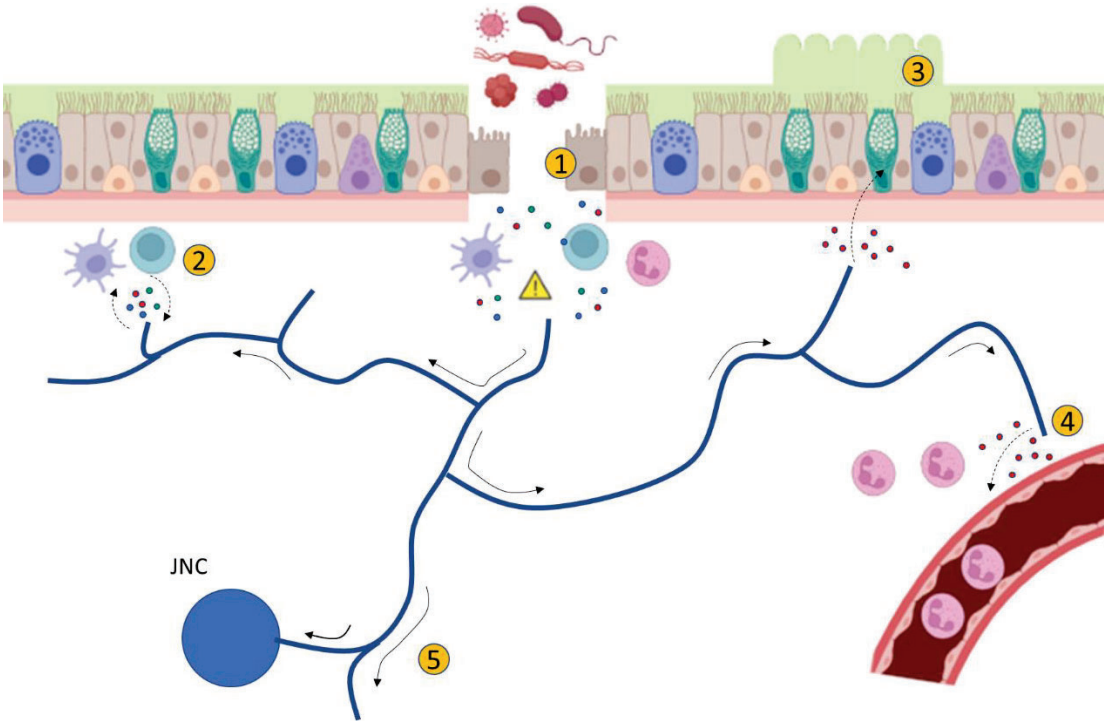

Figure 5 The neurogenic inflammation Upon epithelium damage or infection, various signals can activate nociceptor neurons (1). An action potential is triggered, which propagates toward all the branches of the neurons. It triggers the release of neuropeptides in the nerve endings, promoting neuro-immune crosstalk (2), mucus secretion (3), vasodilation and immune cell infiltration (4). The action potential also propagates to the soma and to the brain, to trigger protective reflexes (5).

V - Nociceptors sensing of immune signals.

Pain sensitization and inflammation

Sensory neurons and nociceptors are meant to detect physical or chemical stimuli, allowing the organism to sense its environment. But these functions can be adapted or modified depending on the context. For instance, sensory neurons adapt or become desensitized if the external temperature changes. In a situation of injury, wound, or inflammation, their sensitivity and excitability can also be miodified, to promote protective reflexes and behaviors.

This phenomenon is regulated by various signals and influenced by different levels of the sensory processing pathway. These changes in pain sensitivity may last for a few hours or days, and their dysregulation can cause long term neuropathic pain[210]. Two types of pain sensitization are distinguished: allodynia, when innocuous stimuli trigger the feeling of pain, and hyperalgesia, when noxious stimuli elicit exaggerated pain responses[210].

Mechanisms of peripheral sensitization

Peripheral sensitization typically involves either an increased sensitivity of ionotropic receptors to environmental cues, a decreased threshold of action potential activation, or a reduced action of inhibitory channels. A wide range of signaling molecules can mediate peripheral sensitization, including protons, bradykinin, histamine, prostaglandins, leukotrienes, endocannabinoids, neurotrophins (NGF and BDNF), cytokines (IL-6, IL-1β, TNFα, G-CSF, GM-CSF, SCF), chemokines, lipids, and diverse proteases[211]. Most of these signaling molecules are associated with inflammation and immune responses, making neuro-immune crosstalk a major driver of nociceptors sensitization. We have reviewed the literature extensively for these inflammatory signals sensed by sensory neurons in the **Article 1** included this thesis.

GPCRs can have a rapid action on nociceptors sensitization. Gq signaling activates protein kinase C (PKC) which can phosphorylate ion channels to promote nociceptors sensitivity. cAMP, which synthesis is enhanced by Gs-GPCR and blocked by Gi-GPCR, also sensitize nociceptors[212-217], an effect mediated by protein kinase A (PKA)[218].

Tyrosine Kinase receptors, and cytokine receptors, trigger the phosphorylation and activation of synapse-to-nucleus intracellular messengers, such as ERK1/2, STAT3, CREB, mTOR or MAPKs, which can change neuronal activity at the transcription level. While regulation of neuronal activity in the soma induced by signals sensed in the periphery is established, the studies focusing on the mechanisms of this retrograde signaling are still ongoing. Indeed, such signals must travel through axons for a large distance (centimeters to meters in humans), before reaching the nucleus[219,220]. The case of NGF, a very potent nociceptor sensitizer, has been thoroughly described. NGF binds to its receptor TRKA, and the ligand/receptor complex is endocytosed, before being transported to the soma[220]. Retrograde transport was also evidenced for STAT3 and CREB[221-224].

Nociceptors sensitivity can then be regulated at the transcriptional level, or in the peripheral nerve endings (**Figure 6**). PKC and PKA can phosphorylate TRPV1[225,226] lowering its threshold of activation, with a drop of its activating temperature from 42° to 33-38°[227-230]. Sensitization can also result from an increased transcription of TRPV1[231] or an increased trafficking and translocation of this ionotropic receptor to the membrane[226,232]. Similar patterns of sensitization occur with TRPA1[233-236]. $Na_V1.7$ and $Na_V1.8$ can also be both upregulated and phosphorylated, lowering the membrane potential threshold of activation to generate action potentials[218,237-241].

Finally, a variety of modulatory ion channels, including voltage-gated K^+ channels, large-conductance K^+ channels, voltage-gated Ca^{2+} channels, Cl^- channels, are expressed on nociceptors[242,243]. Their distribution and relative density also influence the sensitivity of the neurons[242-244].

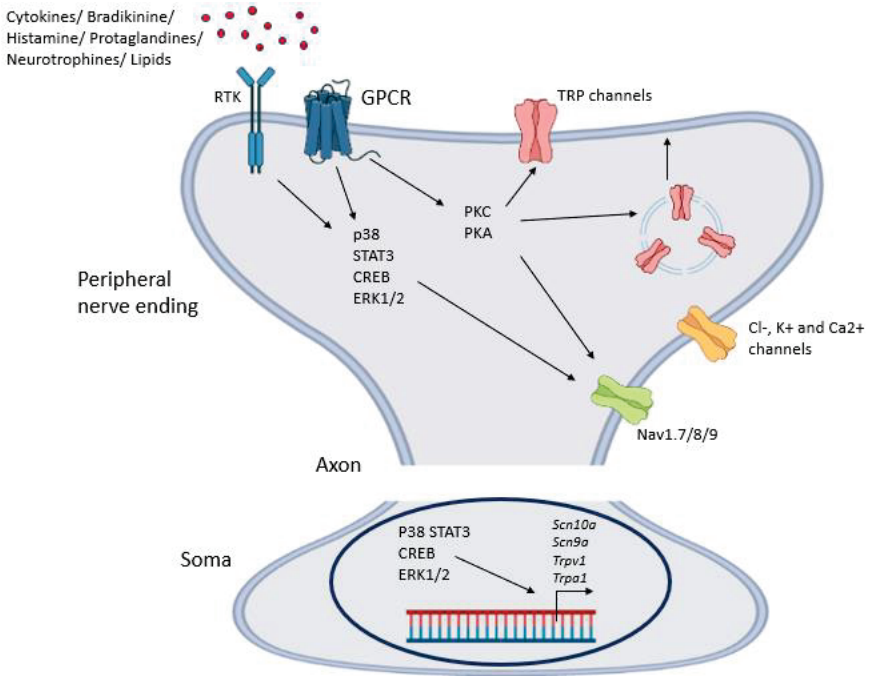

Figure 6 Peripheral sensitization Various signals sensed in the periphery regulates sensory neurons sensitivity. GPCR can rapidly modulate ion channel properties. Tyrosine kinase receptors activate intracellular kinase, that regulate nociceptors' function both in the nerve ending and in the soma.

These complex interplay affecting nociceptors sensitivity may have different physiological outcomes, depending notably on the type of neurons targeted[245]. Sensitization of peptidergic nociceptors can result in thermal and mechanical hyperalgesia, release of neuropeptides, and thus promote neurogenic inflammation[246,247]. On the other hand, non-peptidergic nociceptors sensitization is often associated with itching sensation and scratching[245].

Finally, peripheral sensitization can be induced by anatomical changes and hyperinnervation of a wounded or inflamed area. This hypothesis relies extensively on the expression and release of neurotrophins in various injury models[248-254]. These neurotrophins also play a role in regeneration and sensitization of the peripheral neurons. Increasing evidence of hyperinnervation in inflammation models *in vivo* are now available[204,255-259], although further investigations are required to decipher in what context it occurs (chronic or acute inflammation, type of injury and immune response). Hyperinnervation is thought to increase sensitivity of sensory neurons by increasing their area of contact.

Central sensitization

Sensory inputs are also in some situations sensitized in the central nervous system, in particular in the spinal cord. This phenomenon differs from peripheral sensitization, as it may result in sensation of pain from an area

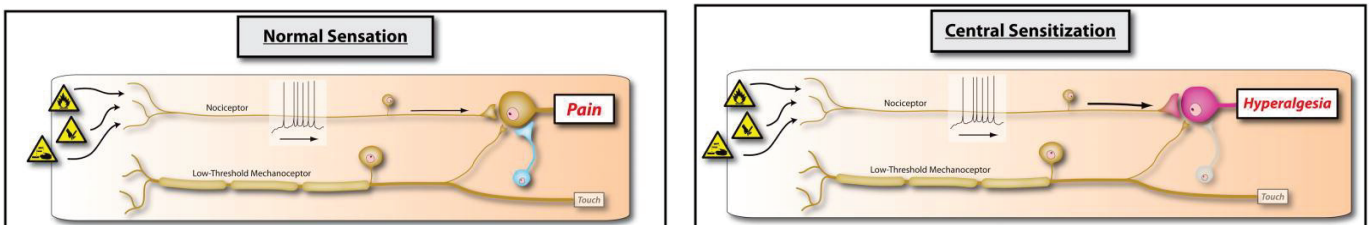

Figure 7 Mechanisms of central sensitization. Copied from the review of CJ Woolf[2]. Central sensitization in somatosensory pathways results from increased synaptic efficacy, and reduced inhibitory mechanisms, which enhance the nociceptor responses to noxious stimuli, and activates normally ineffective synapses between low threshold mechanosensory neurons and pain associated secondary neurons.

larger than the one effectively injured. Several mechanisms have been evidenced, which we describe briefly here. An increased synaptic efficiency between primary afferent and secondary neuron in the spinal cord can result in long lasting allodynia or hyperalgesia (**Figure 7**). This can be induced by increased presynaptic release of neurotransmitters, by an increased post synaptic sensitivity to these neurotransmitters[260-263], by a reduced activity of a type of inhibitory interneurons also located in the spinal cord[264-266], or by glial cells activation toward an inflammatory phenotype[267-270]. As a result, afferent nociceptors activation will trigger increased electrophysiological activity in secondary neurons (allodynia), and non-nociceptive afferent fibers activation can trigger pain processing pathways (hyperalgesia). These mechanisms apply for areas innervated by dorsal root ganglion sensory neurons, which project to the spinal cord. For vagal nociceptor neurons, which project to the brainstem, a similar synaptic regulation is thought to occur in the nucleus of the solitary tract[271,272].

VI- Neuro-immunology in the context of asthma

Several mechanisms of neuro-immune communication described in the previous sections are important in the context of asthma and allergies. In the past decade, studies investigating different aspects of the asthma pathophysiology have demonstrated the proinflammatory role of neurons in asthma. Ablation of nociceptors in mouse models of asthma[152,157] was shown to reduce the number of immune cells (eosinophils, CD4 T cells) infiltrating in the airways, and to dampen airway hyperreactivity[146] and mucus secretion[143]. Various mechanisms and pathways have been suggested and investigated. The expression of ionotropic receptors TRPV1 and TRPA1 were shown to be essential in the neuronal contribution to allergic airway inflammation[157,273-275]. Neuropeptides are also involved, as Substance P promotes mucus secretion[142,276] and VIP enhances lymphocytes activation[152]. Although it was not always demonstrated specifically in the context of asthma, it is very likely that the other immunomodulatory effects of neuropeptides on immune cells described in chapter IV participate in the neuronal proinflammatory action. Several type 2 specific immune cells activated in the context of asthma and allergies are known targets of neuropeptides. This is notably the case of mast cells, ILC2s, and some dendritic cell subtypes.

Several hypothesis and pathways are suggested to explain how sensory neurons are activated in the context of asthma. Most data available was generated in skin models of allergies, but similar mechanisms are hypothesized to occur in allergic airway inflammation. Direct sensing of allergens was suggested in some contexts. Notably, proteases released by multicellular pathogens such as house dust mites are strong inducers of type 2 immunity and appear to directly activate nociceptor neurons[154,156,277]. Limited evidence also suggests that alarmins released by epithelial cells upon damage could also activate nociceptors neurons[278,279], although this observation is not supported by the expression of alarmin receptors in sensory neurons. Antibodies, including the type 2 specific IgE, were shown to be detected by Fc receptors expressed on sensory neurons[280-283]. This was not tested in the context of asthma or airway inflammation, but given the key role of immunoglobulin in type 2 immune responses, we can hypothesize this mechanism to be another way for neurons to be activated in the context of allergen intrusion. Finally, signals released by immune cells such as prostaglandins, histamine, IL-4, IL-5, and IL-13 were shown to activate sensory neurons[284].

Overall, the current consensus is that upon allergen exposure, sensory neurons are activated by various signals, likely occurring at different stages of the type 2 immune response, and having different effects. In response, sensory neurons promote various inflammatory pathways, through mechanisms that require neuropeptide release and the activation of TRP channels. How these various pathways are connected each others remains to be clarified. In addition, most of the studies investigating the mechanisms of neuronal activation in allergies were carried out with different in different tissues and models, and the characterization of airway nociceptors and their ability to sense such type 2 immune signals is still lacking.

VII - Rationale, hypothesis, and research objectives

Lung immune cells and sensory neurons form complex systems that reciprocally interact and influence each other. Sensory neurons are sensitive to various immune and inflammatory signals, and in turn display physiological responses which include but are not limited to hyperalgesia and pain. They also play an active role to fight the threat, through protective reflexes and the initiation of neurogenic inflammation. However, several complex mechanisms drive these neuro-immune interactions, resulting in a very context-dependent influence of nociceptors. In type 2 immune responses, such as in the case of asthma, nociceptors promote the inflammatory

response, worsening the symptoms of asthma. In other contexts, such as type 1 inflammation, they rather dampen the immune response. Such discrepancy may involve that nociceptors are informed of the type of threat and ongoing immune response and adapt their phenotype accordingly. In the context of asthma, we then hypothesized that airway-innervating vagal nociceptors can detect type 2 specific inflammatory signals, and consequently adapt their phenotype to enhance the inflammatory response.

The main objectives of this thesis were to: i) define the characteristics and molecular profile of airway innervating nociceptors; ii) determine which immune signals are sensed by these neurons during asthma and iii) define how these signals affect nociceptor phenotype.

In a first article, we reviewed the state of literature to describe what signals are sensed by nociceptor neurons in the context of immune responses. In a second one, we investigated the specificities of JNC vagal nociceptors neurons, compared to DRG nociceptors that are more largely characterized. In the third and fourth articles, we investigated the sensitivity of JNC nociceptors to type 2 immune signals. First, using a functional approach, we demonstrate their ability to detect allergens through the expression of FcεRI. Then, using transcriptomics and a screening approach, we show how IL-13/IL-4 reprograms nociceptors in the context of allergic airway inflammation.

VIII- Article 1. Profiling of how nociceptor neurons detect danger, new and old foes

Crosson T, Roversi K, Balood M, Othman R, Ahmadi M, Wang JC, Pereira PJS, Couture R, Latini AS, Prediger RDS, Rangachari M, Seehus CR, Foster SL, Talbot S. Journal of Internal Medicine, 2019.

In this publication, we reviewed the literature for all types of immune signals reported to be detected by nociceptors in various inflammatory models and conditions.

Contribution: The student has contributed to the design and setting the focus of the review. He has written several parts, notably those related to cytokines and immune cells signals. He was involved in the making of all figures displayed. He has also played a role in proof reading and assembling all the different sections.

Abstract:
The host evolves redundant mechanisms to preserve physiological processing and homeostasis. These functions range from sensing internal and external threats, creating a memory of the insult and generating reflexes, which aim to resolve inflammation. Impairment in such functioning leads to chronic inflammatory diseases. By interacting through a common language of ligands and receptors, the immune and sensory nervous systems work in concert to accomplish such protective functions. Whilst this bidirectional communication helps to protect from danger, it can contribute to disease pathophysiology. Thus, the somatosensory nervous system is anatomically positioned within primary and secondary lymphoid tissues and mucosa to modulate immunity directly. Upstream of this interplay, neurons detect danger, which prompts the release of neuropeptides initiating (i) defensive reflexes (ranging from withdrawal response to coughing) and (ii) chemotaxis, adhesion and local infiltration of immune cells. The resulting outcome of such neuro-immune interplay is still ill-defined, but consensual findings start to emerge and support neuropeptides not only as blockers of TH1-mediated immunity but also as drivers of TH2 immune responses. However, the modalities detected by nociceptors revealed broader than mechanical pressure and temperature sensing and include signals as various as cytokines and pathogens to immunoglobulins and even microRNAs. Along these lines, we aggregated various dorsal root ganglion sensory neuron expression profiling datasets supporting such wide-ranging sensing capabilities to help identifying new danger detection modalities of these cells. Thus, revealing unexpected aspects of nociceptor neuron biology might prompt the identification of novel drivers of immunity, means to resolve inflammation and strategies to safeguard homeostasis.

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Profiling of how nociceptor neurons detect danger – new and old foes

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Abstract. Crosson T, Roversi K, Balood M, Othman R, Ahmadi M, Wang J-C, Seadi Pereira PJ, Tabatabaei M, Couture R, Eichwald T, Latini A, Prediger RD, Rangachari M, Seehus CR, Foster SL, Talbot S (Université de Montréal, Montréal, QC, Canada; Universidade Federal de Santa Catarina, Florianópolis, Brazil; Université Laval, Québec, QC, Canada; National Taiwan University, Taipei, Taiwan; Citoxlab, Laval, QC, Canada; Universidade Federal de Santa Catarina, Florianópolis, Brazil; Children's Hospital, Boston, MA, USA; Massachusetts General Hospital, Boston, MA, USA). Profiling of how nociceptor neurons detect danger – new and old foes (Review). *J Intern Med* 2019; **286**: 268–289.

The host evolves redundant mechanisms to preserve physiological processing and homeostasis. These functions range from sensing internal and external threats, creating a memory of the insult and generating reflexes, which aim to resolve inflammation. Impairment in such functioning leads to chronic inflammatory diseases. By interacting through a common language of ligands and receptors, the immune and sensory nervous systems work in concert to accomplish such protective functions. Whilst this bidirectional communication helps to protect from danger, it can contribute to disease pathophysiology. Thus, the somatosensory nervous system is anatomically positioned within primary and secondary lymphoid tissues and mucosa to modulate immunity directly. Upstream of this interplay, neurons detect danger, which prompts the release of neuropeptides initiating (i) defensive reflexes (ranging from withdrawal response to coughing) and (ii) chemotaxis, adhesion and local infiltration of immune cells. The resulting outcome of such neuro-immune interplay is still ill-defined, but consensual findings start to emerge and support neuropeptides not only as blockers of T_H1-mediated immunity but also as drivers of T_H2 immune responses. However, the modalities detected by nociceptors revealed broader than mechanical pressure and temperature sensing and include signals as various as cytokines and pathogens to immunoglobulins and even microRNAs. Along these lines, we aggregated various dorsal root ganglion sensory neuron expression profiling datasets supporting such wide-ranging sensing capabilities to help identifying new danger detection modalities of these cells. Thus, revealing unexpected aspects of nociceptor neuron biology might prompt the identification of novel drivers of immunity, means to resolve inflammation and strategies to safeguard homeostasis.

Keywords: cytokine, immunoglobulin, neuro-immunity, sensory neuron.

Background

The peripheral sensory nervous system can be divided into (i) afferent neurons, which are responsible for sensing peripheral information to be transmitted to the brain for processing, and (ii) efferent neurons, which carry electrical signals back to the periphery to generate reflex responses such as cough, scratching and vomiting [1–4].

[†]Authors contributed equally to this manuscript.

These afferent neurons are made up of several classes of fibres, of various calibre, myelination and protein expression, each specialized to detect and transmit specific types of signals. Amongst these, C and Aδ fibres are un- or lightly myelinated small to medium size pseudounipolar fibres. C fibres can be further divided into peptidergic (NGF-sensitive) and nonpeptidergic (GDNF-sensitive) neurons [5]. These sensory neurons or high-threshold noxious stimulus detectors represent the first line of defence against environmental and internal danger [6]. They accomplish these functions using defined types of ion channel transducers such as TRP, ATP, acid-sensing and Piezo channels (as reviewed by Dubin and Patapoutian [6]). The activation of such pore channels leads to the influx of cations [sodium (Na^+), calcium (Ca^{2+})] and the engagement of voltage-gated sodium channels (Na_Vs) which convert detected threats into electrical signals (Fig. 1). A key feature of these ion channel transducers is their high plasticity and context-dependent activity. Thus, the sensitivity and expression levels of these transducers are often modified following tissue injury and inflammation. Whilst this process is meant to adapt to changes in the environment, these changes have limited reversibility, persisting in time and contributing to inflammation. Thus, in response to chornic or repeated stimuli, sensory neurons are sensitized, which results in decreased activation threshold or spontaneous firing [7] (Fig. 1). Phenotypically, these changes can be manifested by chronic itch, pain and cough as well as tissue hypersensitivity.

Cell death secondary to tissue insult leads to the activation of resident monocytes, the influx of various immunocytes and the local release of histamine, serotonin, prostaglandins, bradykinin, ATP, protons, nerve growth factor (NGF), anandamide and cytokines [8]. These mediators then activate nociceptor neurons through their receptors and lead to the overexpression and phosphorylation of ion channel transducers [9,10]. Specifically, TRPV1 and Na_V responses are respectively enhanced by downstream signalling cascades involving PKC or PLA_2/lipoxygenase pathway (Fig. 1). For instance, the action of (i) activin [11], (ii) CCL3 on CCR1 [12], (iii) artemin [13] and (iv) kinins on B_1R [14,15] enhances PKC signalling, whilst histamine activation of H_1R [16], PGE_2 acting on the EP2 receptor and PLb3 [17] enhance PLA_2/lipoxygenase activity. Also, external insults such as pathogens, allergens and air pollution can sensitize nociceptors [18–21] (Fig. 1).

Similar to these processes, the central sensitization of nociceptor neurons also occurs. For example, microglial cells are recruited at the neuron synapse and, by releasing various pro-inflammatory mediators, drive the phosphorylation of glutamatergic receptors (NMDA). This effect enhances NMDA trafficking to the neuron membrane and augments the channel kinetics resulting in the enhancement of synaptic strength (reviewed by Latremoliere and Woolf [22]). Functionally, that sensitization results in long-term potentiation (LTP) of the synaptic transmission between primary and second-order sensory neurons, rendering the CNS hypersensitive to innocuous inputs and, in turn, producing exaggerated responses. Clinically, higher excitability is associated with chronic pain (hyperalgesia) [22–24] and heightened airway defensive reflexes [25] (Fig. 1).

Neuro-immunity

Homeostatic mechanisms and regulators work together to maintain physiological processes. Conversely, chronic inflammatory disease often results from impairment in such functioning [26]. To maintain homeostasis, we evolved various redundant means to sense internal and external threats and generate protective reflexes, which aimed to resolve inflammation [1]. Recent data reveal that the immune and sensory nervous systems accomplished these functions together [27]. Thus, several case reports support a neuronal control of immune responses. For instance, the denervation of a limb following a nerve injury reversed arthritis-induced inflammation in that limb [28]. In addition to noxious stimulus detection and prompting defensive reflexes (scratching), somatosensory neurons also promote host defences, in part, through their direct interaction with immune cells [27]. Both systems share metabolic pathways [29] and dialogue using a common language of receptors, cytokines and neuropeptides. Indeed, the somatosensory nervous system is anatomically positioned in primary and secondary lymphoid tissues, and mucosa to directly modulate immune responses [1,27,30]. The local depolarization produced by noxious stimulus activation of membrane transducers initiates action potential firing [31]. These electrical signals travel from the periphery to the central nervous system to initiate reflexes and sensations [1,27,30]. When these action potentials reach the axon branch points, they are also transmitted back to the peripheral terminals triggering the release of neuropeptides in the neuron

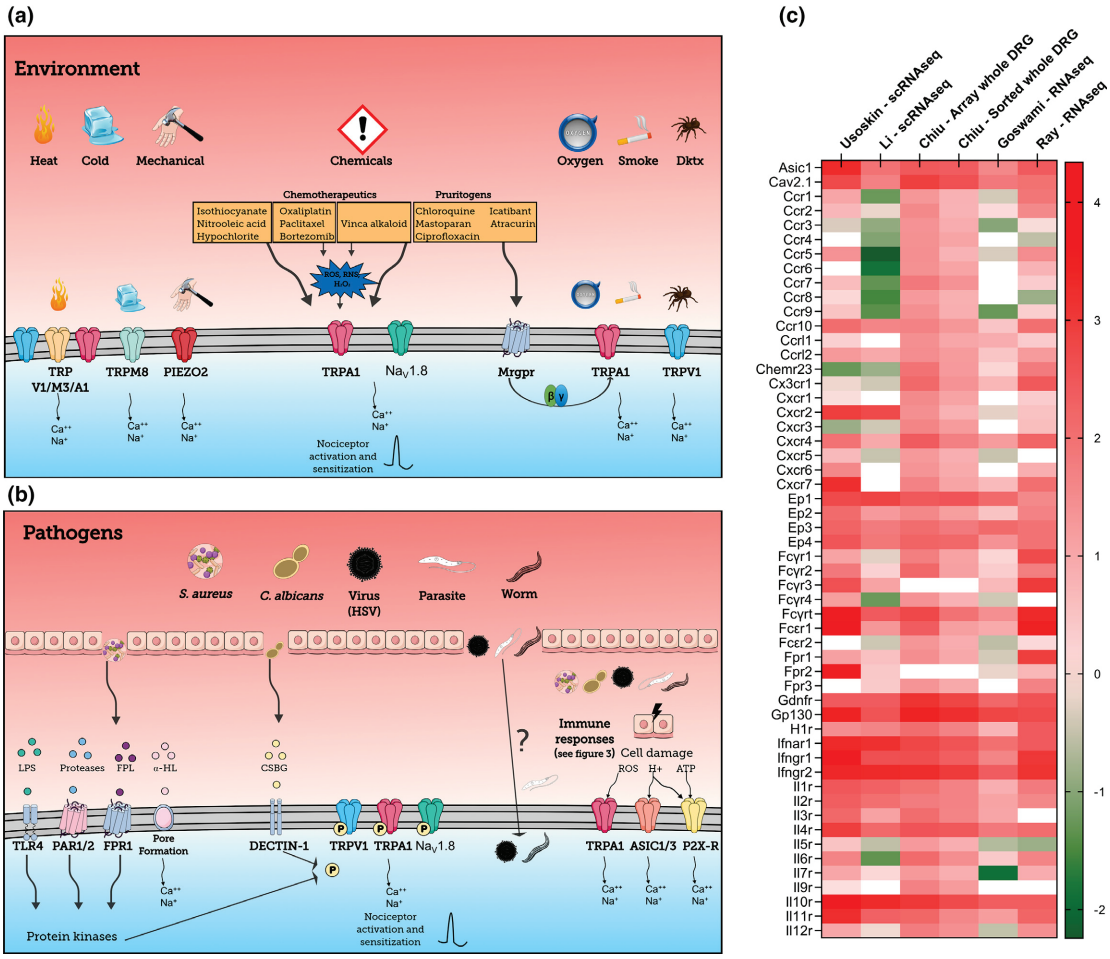

Fig. 1 *Modalities of sensory neuron danger detection. Nociceptor neurons express ion channel transducers, such as TRP, Piezo and ASIC channels, to detect a variety of exogenous dangers ranging from extreme temperature, chemicals and mechanical pressure to toxins and acidic milieu. TRPV1, TRPA1 and MrgpR can also perceive environmental perturbation such as pollutants and hypoxia, as well as various drugs like chemotherapeutics and antimicrobial. Activation of these transducers triggers the influx of sodium and calcium, increasing neuronal resting membrane potential which leads to action potential firing (a). The expression of pathogen-associated molecular pattern receptors helps nociceptor neurons to detect invading microbes (β-glucans (CSBG), lipopolysaccharides (LPS)). Following TRP and Na_V channel phosphorylation, the activation of TLRs and FPRs leads to sensory neuron sensitization. Concomitantly, microbes' secreted toxins, as in the case of the pore-forming toxin α-haemolysin, can also directly drive neuronal activation. Finally, microbes interplay with the cells of the immune system or the mucosal barrier generates various mediators ranging from cytokines and ROS to protons and ATP, all of which can activate neuron (b). Thus, sensory neurons bear cytokine, chemokine, histamine and immunoglobulin receptors, which allow them to respond to cues produced by ILCs and T and B cells, as well as neutrophils, macrophages and mast cells. In general, the activation of these receptors mainly leads to the sensitization of sensory neurons terminals (d). Finally, neurons are found to detect microRNAs present in exosome or PDL1 present on cancer cells. However, not all these mediators result in neuronal sensitization or activation. Thus, the endogenous lipid mediator resolvins help stop the inflammatory process, by silencing TRP channels (e). Heatmaps (c, f; log_{10} colour scale) show the relative transcript expressions of these various danger detectors from six expression profile data sets (see Section for more details). Usoskin [55] and Li [56] used single-cell RNA-sequencing of lumbar neurons. Chiu [52] – Array Whole DRG and Chiu – sorted whole DRG respectively refer to microarrays of whole and FACS-sorted $Na_V1.8^+$ neurons. Goswami [54] performed RNA-sequencing of $TRPV1^+$ neurons, whilst Ray [53] performed RNA-sequencing of human lumbar neurons. The expression levels of the relative gene of interest (GOI) were normalized on* Trpv1 *and, for ease of interpretation, and multiplied by 1000.*

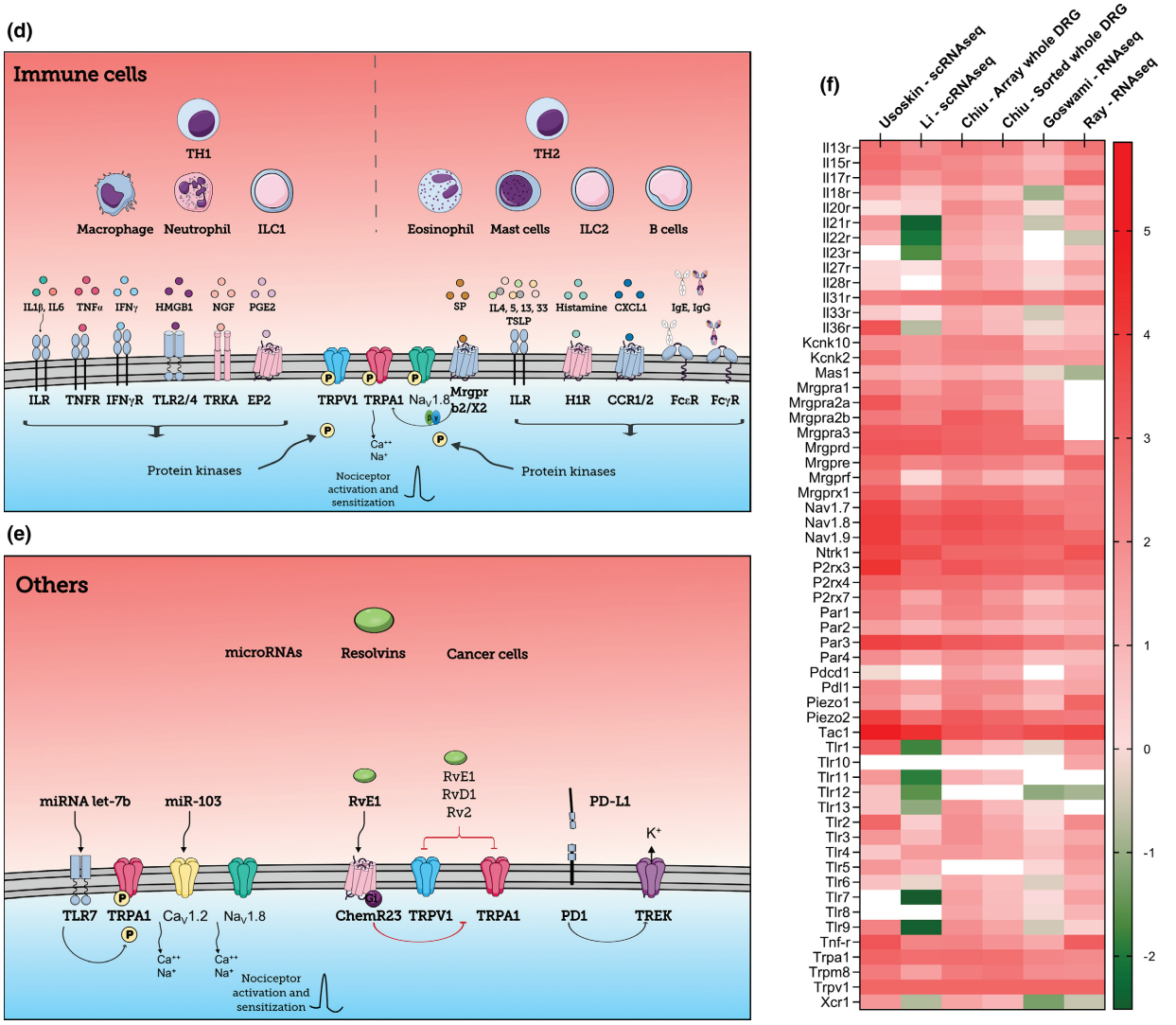

Fig. 1 *Continued.*

microenvironment, which leads to inflammation [1]. Thus, neuropeptides trigger pain by sensitizing neurons, redness and heat secondary to the vasodilatation that increases blood flow, and oedema by increasing vascular permeability. This sets the table for the nervous regulation of immune responses. However, whilst this interplay helps to protect the organism from danger, it also contributes to disease pathophysiology [1,27,32–36].

The overall impact of nociceptor neurons on immunity appears to be context-dependent and varies between T_H1/T_H17 and T_H2 immunity, the sensory ganglia involved, the subset of neurons driving these effects and the making of the neuropeptides released. Such a complex regulation is exemplified by the neuronal control of $CD8^+$ T-cell activity. For example, substance P (SP) directly promotes $CD8^+$ T cells' (i) proliferation and (ii) activation (Granzyme B) and reduces (iii) their adhesion [37–41]. It can also regulate CD8+ T-cell function indirectly by enhancing dendritic cell co-stimulatory function. Thus, it increases DC recruitment, recognition of non-self-antigens, and acts as a co-signal to

enhance IL-2 secretion [41,42]. CGRP (calcitonin gene-related peptide) and VIP (vasoactive intestinal peptide) typically have opposite effects to those of SP: (i) increasing T-cell adhesion, (ii) blocking $CD8^+$ T-cell proliferation when exposed to antigen-pulsed APC, (iii) decreasing OVA-specific T-cell response, (iv) reducing DC migration to lymph node and (v) suppressing FAS-L expression to prolong T-cell responses [41–43]. NPY (neuropeptide Y) inhibits T-cell activation [44], whilst SST (somatostatin) suppresses infiltration and activation of CD8 [45]. In sum, nociceptor peripheral terminals interact with immune cells in locations where they are stationed [46–48] and the peptides they produced to control the chemotaxis and polarization of lymphocytes, regulates the localization, duration and type of inflammation at play [18,38,49–51].

Neuron expression profile

Nociceptors detect not just features typically thought of as causing a painful sensation, such as noxious pressure and temperature, but also atypical signals ranging from cytokines and pathogens to immunoglobulins and even microRNAs. In the light of these data, we aim to paint a revised picture of what constitutes 'danger detection' and 'threat' by nociceptors. In the following section, we summarize the recent attempts using transcriptional/translational profiling to characterize subpopulations of nociceptors, the transcriptional differences between neurons from different anatomical regions and inflammatory contexts, and the threats they may specialize in detecting.

Meta-analysis

Nociceptors have been reported in signal studies to express a variety of 'danger detecting' receptors, but as of yet, there has not been a cumulative summary of these findings. Figure 1 shows the curated meta-analysis we generated from the examination of four naïve mice and one human dorsal root ganglia profiling data sets.

First, Chiu [52] and colleagues' microarray (28 000 coding and 7000 noncoding transcripts) profiled whole and FACS-sorted $Na_V1.8^+$ DRGs neurons. The cells used were pooled from 7- to 20-week-old age-matched male and female cervical (C1–C8), thoracic (T1–T13) and lumbar (L1–L6) ganglia. Relative gene expression was reported as robust multiarray average (RMA). Ray *et al.*, [53] RNA-sequenced L2 DRG neurons from three Caucasian women aged 30–60 years and reported expression levels as transcripts per kilobase million (TPM). Goswami *et al.*, [54] RNA-sequenced FACS-sorted $TRPV1^+$ L3-L5 DRG neurons dissected from three groups of ten 8-week-old male and female C57Bl6 mice and reported expression levels as reads per kilobase per million mapped reads (RPKM). Usoskin and colleague's [55] RNA-sequenced (1.14 million reads were mapped to 3574 ± 2010 genes/cell) single neurons from L4–L6 lumbar DRGs harvested from 6- to 8-week-old male and female adult mice. The 799 cells sequenced were randomly picked, of which 622 cells were classified as neurons, 68 cells had an ambiguous assignment, and 109 cells were non-neuronal. Data were reported as numbers of read per million (RPM), which, for comparison, were transformed as RPKM (Fig. 1). Li and colleagues [56] performed high-coverage single-cell RNA-sequencing ($10\,950 \pm 1218$ genes/neuron) of 64 $IB4^+$ neurons, 69 $IB4^-$ small neurons and 64 large neurons collected from nineteen 8- to 10-week-old male C57BL/6 mice, and data are expressed as RPKM. Refer to https://www.talbotlab.com/data set to download the complete and up-to-date dataset.

Anatomical difference

Sensory neurons can be categorized based on size, myelination and anatomical origins, namely lumbar, vagal and trigeminal ganglion. In brief, trigeminal neurons innervate the face, the neurons of nodose and jugular ganglia (ND) innervate the visceral organs, and somatosensory neurons are located in the dorsal root ganglions (DRGs) and innervate the skin, muscles and joints [24,57]. Whilst the neurons from these various origins are similar in shape, they accomplished very different functions [58,59] and are therefore highly heterogeneous. In support of this, Peeters and colleagues' microarray [60] compared the expression profile of whole ND and DRG neurons and recorded 5049 genes, of which 4386 genes were not differentially expressed between the two sites (0.5-fold–2.0-fold). However, 272 genes (5.8% of the total genes) were found to be expressed twice or more in ND neurons and 391 (8.9%) in DRG. To name a few, transcript levels of *Grik1* (33.0-fold), *Scn1a* (21.9-fold), *Tac1* (12.4-fold), *Ptgfr* (8.7-fold), *Npy2r* (4.9-fold), *Scn9a* (3.0-fold) and *Nppb* (2.1-fold) were increased in DRG neurons, whilst *Gpcr25* (73.7-fold), *Phox2b* (19.7-fold), *Avpr1a* (10.4-fold), *Edn1*

(8.0-fold), *Il4ra* (3.8-fold), *Ednrb* (2.5-fold) and *Tlr6* (2.1-fold) were elevated in ND neurons. These transcriptional differences reflect heterogeneity that has implications for the different types of environmental danger that the nociceptors from the ND and the DRG encounter.

Context dependence

Genome-wide changes in sensory neuron expression profiles in pathologic conditions have been described in models of nerve injury, another situation which may indicate threat and requires a coordinated immune/tissue injury response. Microarray analysis of rat DRG after axotomy has revealed expression changes to members of distinct families, including neuropeptides, receptors, ion channels, signal transduction molecules and synaptic vesicle proteins [61]. More recently, sequencing of single DRG neurons after nerve injury has shown a large heterogeneity in expression changes, leading either to cell death, regeneration or physiological pain, the last being associated with lower expression of potassium channels [62]. As expected, sensory neuron sensitivity is not only regulated at the transcriptional level. Using a chemotherapy-induced neuropathic pain model, Megat *et al.* [63] have compared translating ribosome affinity purification sequencing (TRAP-seq) and RNA-seq data to assess gene regulation changes in mice DRG neurons. They demonstrated that whilst only five genes are differentially transcribed in their model, 452 genes had differential translation levels. Because of their axonal length, sensory neuron plasticity also relies on local (dendritic) protein expression regulation. Overall, protein expression is determined at many levels, including (i) RNA expression, (ii) translation efficiency and (iii) protein stability [64], suggesting that a more global approach to profiling might also be essential.

Cluster

Principal component analysis of the various expression profile data sets revealed the existence of multiple neuronal clusters. For example, scRNA-seq Usoskin [55] found eleven DRG sensory neuron subtypes: three distinct low-threshold mechanoreceptor neurons, two proprioceptive and six principal types of thermosensitive, itch-sensitive, type C low-threshold mechanosensitive and nociceptive neurons. On the other hand, Hockley [65] performed unbiased single-cell RNA-sequencing on retrogradely traced 314 colonic sensory neurons clustered into five subtypes of thoracolumbar neurons defined as Mrgprd (neurofilament-b), Cbln2 (neurofilament-a), Smr2 (peptidergic A), Fam19a1 (nonpeptidergic) and Trpa1 (peptidergic B), and two lumbosacral subtypes defined as Hpse (neurofilament) and neurotrimin (peptidergic). Whilst most of these clusters vary between datasets, they support the overarching idea of dedicated sensory lines to carry certain types of signals. Whilst this remains to be formally proven, such circuits could include pruritic triggers, temperature detectors and sensors for immune cells. Studies focused on identifying and delineating markers of these lines could help inform the design of targeted therapies to block the transmission of specific danger signals.

Variety of danger detectors

The heatmaps presented in Fig. 1 depict the relative expression of classic noxious stimulus detectors such as (i) ion channel transducers (TRP, P2X, ASIC, Piezo) and (ii) GPCR for locally sensitizing mediators (MrgpR, PG, H, BK, etc.), as well as atypical threat detectors including (iii) receptors for immunocyte-produced mediators (interleukins, chemokines, immunoglobulins) and (iv) DAMP detector (PAR, TLR, PRR). Overall, these transcriptional data highlight the immense diversity of danger detector capabilities of nociceptor neurons ranging from pressure and temperature to cytokines and microbes. To supplement this, we reviewed evidence supporting the detection of such threats, the molecular cascade engaged upon their detection and the resulting immunomodulatory outcome. Below we segregated dangers as endogenous (cytokines, cancers) or exogenous (temperature, microbes, etc.) triggers and discuss findings from these individual studies.

Exogenous triggers

Mechanical

Sensory neurons are capable of detecting mechanical force, from innocuous touch to noxious pain [66,67]. A large number of channels are involved in mechanical sensing [68,69], including the Piezo channels [70,71]. Piezo2 is expressed in many classes of peripheral sensory neurons [72] and is the primary mechanotransducer channel for touch detection [73] and proprioception in mice [74] and humans [75,76]. Also, changes in Piezo2 activity have been linked to mechanical hyperalgesia [77,78]. In brief, Piezo2 confers mechanically evoked ionic currents [70], and it is intrinsically

gated by force [79]. In the lung, Piezo2-expressing vagal sensory neurons act as airway stretch receptor potentially involved in the onset of respiratory diseases [80]. Finally, TRP channel subtypes such as TRPA1 and TRPV4 can also contribute to mechanical hypersensitivity [81,82].

Temperature

TRP channels constitute the central heat transducers expressed by nociceptors. TRPV1 and TRPV4 are activated by temperatures ranging from 42 to 52°C [83,84] (more details reviewed by Schepers and Ringkamp [85]). Although the TRPV1 channel is the prototypical heat sensory [83], a recent study indicated that TRPV1, TRPM3 and TRPA1 are necessary to detect acute noxious heat [86]. In addition to TRP channels, calcium-activated chloride channels of the anoctamin/TMEM16 family [87] and the two-pore potassium channels TREK and TRAAK [88,89] may also contribute to heat sensing. In the respiratory tract, where the TRPV1 heat threshold (43°C) is unlikely to be reached, hyperthermic temperatures (~40°C) might increase sensitivity to other stimuli such as capsaicin.[90] On the other hand of the spectrum, noxious cold is sensed by the TRPM8 channel expressed by Aδ and C fibres [91–93]. TRPM8 detects temperature below 26°C and down to 8°C as well as agents of the menthol family (reviewed by McKemy [94]). In the respiratory tract (for more details: Grace *et al.* [95]), inhaling cold air leads to TRPM8 activation, which triggers coughing and airway constriction [96,97]. TRPM8 is also found to overexpress in vagal sensory neurons of asthmatic patients [97]. Besides TRPM8, potassium [98] and sodium channels [99,100] help transmit cold noxious information to the brain. Although some studies point out for a role of TRPA1 in such processes [101,102], it appears that cold sensitizes TRPA1 receptors to various other stimuli [103].

Chemicals

TRPA1 is sensitive to various chemicals such as isothiocyanate, nitro-oleic acid and hypochlorite [104,105]. TRPA1 also mediates inflammatory responses to environmental insults [104,106,107]. Vagal TRPA1 activation leads to systemic protective reflexes such as coughing [108], respiratory depression [109], neuronal hyperexcitability [110,111] and initiating allergic airway inflammation and hyperresponsiveness [112,113] (reviewed by Geppetti *et al.* [114]).

Microbes

Bacteria

Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs), and Toll-like receptors (TLRs) are expressed by innate immune cell subsets. However, such receptors were also identified on neurons subsets. Typically, TLRs are responsible for sensing extracellular pathogens [115]. For example, neuronal TLR4 senses lipopolysaccharides (LPS) of the gram-negative *porphyromonas gingivalis* bacterial membrane. TLR4 activation can lead to action potential firing [116], overexpression and sensitization of TRPV1, and local release of neuropeptides, all of which contribute to LPS-induced tactile allodynia [10,117,118]. The bacterial membrane flagellin activates TLR5, whilst the lipoteichoic acid present on gram-positive bacteria drives neuronal activation through TLR2 [10]. Finally, evidence suggests that components of gram-negative bacteria might directly stimulate TRPA1 [119].

The Chiu and Woolf Labs have shed light on various TLR-independent pathways through which bacteria activate sensory neurons. For example, *methicillin-resistant Staphylococcus aureus (MRSA)* membrane-bound N-formulated peptide activates neuronal PRR-1 and PRR-2. Concomitantly, MRSA-released α-haemolysin toxin activates nociceptors by perforating their lipid bilayer membrane. In turn, the activated neurons respond using calcium flux, action potential firing and CGRP release [120]. Of note, these effects were prevented by sensory neuron silencing with QX-314 [121]. *Escherichia coli*, a gram-negative bacterium, lead to painful urinary tract infection through the action of pore-forming toxins. *Staphylococcus Aureus pneumonia* mediated CGRP release by $TRPV1^+$ vagal sensory neurons, which, in turn, blocks the recruitment of neutrophils and pulmonary γδ T cells [122]. *S. Pyogenes*, the pathogen responsible for necrotizing fasciitis, secrete streptolysin S (SLS), which activates nociceptor neurons. In turn, *S. Pyogenes* leads to pain and CGRP release, which inhibit neutrophil recruitment and facilitate bacterial survival during infection. Therefore, augmenting neuro-immune interactions could be used to treat infection [123]. Using a novel culture system, Yissachar and colleagues [124] found that, despite belonging to the same Clostridium bacterial subset, up to 60% of nociceptors showed calcium flux when exposed to the Treg-inducing bacteria *C. Ramosum*, whilst

only a few nociceptors were sensitive to *P. Magnus*, which do not affect Tregs *in vivo*. The neuropeptides expressed by and released from nociceptors also depend on the type of bacteria activating them. As such, *C. Ramosum* downregulates genes encoding for SP, secretogranin III and galanin, whilst the T_H17 inducer-segmented filamentous bacteria (SFB) had the opposite effect. Finally, sensory neuron activation by *C. Ramosum* correlates with colonic $ROR\gamma^+$ Treg induction, increasing in mice lacking SP and decreasing in capsaicin diet-fed mice. These data indicate that gut commensal microbes augment nociceptor activity, contributing to immunity [124].

Virus

Neurotropic viruses such as herpes simplex virus type 1 (HSV-1) target the peripheral nervous system, triggering painful neuropathy. The glycoproteins present on the viral envelope bind to heparan sulphate [125] and nectin-1 [126,127] expressed on the neuron cell surface, initiating the fusion of HSV-1 viral envelope with the neuron plasma membrane. The virus capsid is then retrogradely transported to the cell body, which serves as a viral reservoir [128,129]. Overall, more work is warranted to define the exact mechanism through which virus target and enter neurons as well as the way they modulate their functions.

Parasites

Depending on the strain, leishmanial infections can lead to either hypoalgesia or hyperalgesia [130,131]. Thus, the inflammatory mediators found in the nerve [130,132,133], spinal cord [134] and brain [135] of infected mice can trigger peripheral and central sensitization. Intestinal schistosomiasis infection increases mucosal mast cell number and intestinal lamina propria CGRP-immunoreactive nerve fibres [136], whilst desensitization or ablation of these neurons reduced intestinal mast cell content [136].

Fungus

Fungi are the largest group of organisms after insects. Of whom, *Candida albicans* is a commensal fungus that colonizes the reproductive tract, skin and mucosa, but can also lead to skin and oral pain in immunodeficient and diabetic patients. As such, *Candida* colonization can have harmful consequences as in the case of oropharyngeal candidiasis, which affects 90% of HIV-infected patients [137], osteo-inflammation [138], disseminated candidiasis, chronic mucocutaneous candidiasis [139] and candidiasis-induced itch [140]. Mucocutaneous candidiasis drives protective skin immunity through nociceptor-mediated dermal dendritic cell activation and IL-17A induction. *C. albicans* activates skin $TRPV1^+$ neurons, leading to CGRP release, $CD301b^+$ dermal dendritic cell activation and IL-23 production. The latter activates tissue-resident IL-17-secreting γδ T cells, resulting in neutrophil activation and liberation of antimicrobial peptides, promoting the resistance to cutaneous candidiasis [34,139,141,142]. In parallel, *C. albicans* can stimulate $Na_v1.8^+$ nociceptors via the Dectin-1-TRPV1/TRPA1 axis, leading to CGRP release [138]. The latter inhibits β-glucan-induced NF-κB activation via the transcriptional repressor Jdp2, suppressing cytokine production in myeloid cells [138]. Dectin-1 signalling pathway also plays a role in allodynia, where *C. albicans*-derived β-glucan stimulates nociceptors through two Dectin-1-dependent inflammatory pathways [143]. The first acts through extracellular ATP and the ATP transporter VNUT, and second, through the phospholipase C (PLC)-TRPV1/TRPA1 axis.

Proteases

Through its enzymatic action, the proteases released by damaged cell and bacteria activate nociceptor-expressing protease-activated receptors (PARs) [144,145]. Evidence has shown that PAR activation can modulate nociception (reviewed by Cenac and Vergnolle [146]). PAR1 and PAR2 activation is related to calcium mobilization and neuron sensitization, which lead to inflammatory and visceral pain in humans and rodents [147,148], whilst PAR4 signalling pathways can be pro- [149] or anti-nociceptive [150] (reviewed by Bao *et al.* [151]).

Drugs

Chloroquine (CQ), a medicine used to treat malaria, often induces histamine-independent itch [152,153]. CQ activates Mas-related G protein-coupled receptor (Mrgpr), causing TRPA1 activation and calcium flux, and triggers the reflex to scratch [17,154,155]. CQ can also trigger itch by acting directly on TRPV1, TRPC3 and TRPM8, and therefore mediate this effect via a diverse set of DRG neuron subsets [156]. Besides, other small molecules such as icatibant, mastoparan, atracurium and ciprofloxacin were found to activate MrgprB2 (mice)/MrgprX2 (humans) and drive systemic pseudo-allergic reactions [157]. Clinical data revealed that chemotherapy agents, such as

oxaliplatin [158], vincristine [159], paclitaxel [160] and bortezomib [161], are a significant cause of peripheral neuropathy (as reviewed by Boyette-Davis *et al.* [162]). Although the mechanisms underlying this effect are poorly understood, chemotherapeutics were found to alter Na_Vs activity by mobilizing intraneuronal calcium [163]. Chemotherapeutics-induced ROS production might also lead to TRPA1 and TRPV1 sensitization [161,164–166]. Finally, vinca alkaloids were found to activate TRPA1 [167], whilst paclitaxel-mediated TLR4 activation might sensitize TRPV1 [166]. Overall, the direct action of chemotherapeutics on sensory neurons is still debatable [82,168], but it is plausible that exogenous drugs may directly bind to TRP channels, or that, through intracellular signalling, may substantially modify their activity.

Endogenous triggers

Cytokines

Through their interplay with leucocytes, sensory neurons can indirectly detect perturbation to the host homeostasis. As such, most cytokines, chemokines and growth factors of leucocytic origin can directly activate and sensitize nociceptors. The consequences of these interactions have been associated with chronic pain and hypersensitivity in conditions ranging from asthma [18], atopic dermatitis and psoriasis [169] to rheumatoid arthritis [170]. For example, lumbar and vagal sensory neurons express IL-1 and TNF receptors. IL-1β and TNF-α can thus cause hyperalgesia and neuropeptide release [171–174]. IL-1R/IL-1β induces $Na_V1.8$ phosphorylation, decreasing neuronal action potential firing threshold [175,176]. The cell surface receptor to IL-6 is a heterodimer consisting of IL-6Ra and gp130. Whilst IL-6Ra is not expressed on sensory neurons, soluble IL-6-IL6-Ra complexes can bind to GP130 on nociceptors, thereby sensitizing them [177]. Furthermore, the IL-6 produced by innate immune cells can drive the expression of TRPV1 on tumour-innervating sensory neurons [178]. C-fibre sensitization can also result from neuronal IL-17R activation by IL-17, which triggers PKB and ERK phosphorylation in neurons [179,180].

Several T_H2-driving cytokines were also found to sensitize nociceptors. For example, IL-5 activation of vagal nociceptor IL-5R induces the release of VIP and drives airway hyperresponsiveness [18]. Also, by binding to its cognate receptors, IL-31 induces neuronal ERK phosphorylation and itching [33,181,182]. When subjected to an environmental stressor, the epithelial cells of the skin mucosa release the thymic stromal lymphopoietin (TSLP), which activate its cognate receptor on neurons, triggering TRPA1-dependent itching [183]. Finally, poison ivy-mediated skin inflammation promotes IL-33 release which, by binding to neuron-expressing ST2, triggers scratching reflex [184]. Both found in DRGs, IL-4R and IL-13R activation also mediate itch [33,185].

A combination of whole nerve electrophysiology recording of vagal neurons and advanced electrical signal decoding resulted in the discovery of cytokine-specific neural signals, also known as neurogram [172]. Given that the electrochemical transmission of cytokine signals likely utilizes a relatively homogenous set of sensory nerve fibres, the encoding of cytokine-specific electrical signal supports the existence of a supraspinal capacity to interpret such signals. Deciphering each of these signals opens the door for the targeting of cytokine-specific electrical signatures rather than silencing the whole population of nociceptor neurons.

Protons

Monitoring acidosis is an essential aspect of sensory neuron control of homeostasis [186,187]. Acid-sensing ion channels (ASICs) ASIC1 and ASIC3 are the main proton sensors, and their activation leads to neuronal depolarization [188], inducing pain [189–192] and airway hyperreactivity [25,193,194]. Protons can also bind to the ATP-binding site of P2X channels [195]. Finally, TRPV1 also acts as an acid sensor [196].

Chemicals

Along with its role as an exogenous chemical sensor, TRPA1 can also sense circulating levels of O_2, revealing an unexpected aspect of homeostatic control of hyperoxia and hypoxia [197,198]. TRPA1 also partakes in the detection of microenvironmental oxidative stress [199] (for review, see Kozai *et al.* [200]), including H_2O_2 [201,202]. ROS sensing by TRPA1 also results in hypersensitivity to cold stimuli [203].

Resolvins

During the resolution phase of inflammation, lipidic mediators such as polyunsaturated fatty

acids and docosahexaenoic acid [204,205] are metabolized to produce resolvins, including RvE1, RvD1, RvD2. These endogenous mediators directly inhibit TRPV1 and TRPA1 channel [205–208]. They also modulate the function of these channels indirectly, through their action on specific GPCRs, such as ChemR23 receptor [207,209]. Of note, RvD2 is notably more potent than the TRPV1 antagonist AMG9810 to reverse inflammatory pain [206].

Exosome

Another modality sensed by sensory neurons are exosomes containing noncoding microRNAs (miRs). Spinal cord and DRG miRNA repertoires were drastically changed in the context of peripheral nerve injury [210–212] and inflammatory pain [210,213,214], whereas miR-124, miR-103 and miR-23b are known to attenuate inflammatory and neuropathic pain by altering intracellular neuronal, astrocytic and microglial functions [215–217], miR-103 exerts a pro-nociceptive effect by directly activating TRPA1 and Cav1.2 [215,218]. The endogenous miRNA let7b, which is highly expressed in DRG tissues, induces action potential firing in neurons co-expressing TLR7 and TRPA1 [218]. Therefore, miRNA emerged as a dynamic and endogenous axis of modulating neuronal excitability, independent of their effect on modulating gene expression. Anti-miRNAs and miRNA mimics might be considered to reverse neuropathic pain (reviewed by Dai *et al.* [219]).

DAMPs

The high-mobility group box one protein (HMGB1), a DNA-binding protein, is released actively by immune cells or passively by injured cells [220], and acts as an endogenous ligand of TLR2 and TLR4. It has been shown to lead to calcium mobilization and increases the excitability of primary DRG neurons [221], driving pain hypersensitivity [222–224]. HMGB1-TLR4 interplay drives clinical conditions such as sepsis, arthritis [225,226], cancer [227,228] and potentially asthma [229–231] (reviewed by Imbalzano *et al.* [232]). S100 proteins are another type of endogenous proteins with various intracellular functions, but which also act as DAMPs when excreted in the extracellular milieu. Of which, S100A8 is released in the spinal cord of neuropathic pain models [233–235], whilst S100A9 has an anti-nociceptive effect [236].

Cancer

Within the microenvironment, tumour growth is accompanied by the apoptosis of fast-dividing cells as well as compression of the surrounding tissue, all of which drive cancer-induced pain and itch [237]. Clinically, it has long been appreciated that nerves are often found close to tumour cells, as in the case of breast cancer [238] and choroidal melanoma [239]. In a process known as neuroneoplastic synapses, it was postulated that the mediators released by the nerve endings regulate the proliferation, progression and metastasis of cancerous cells [240–242]. In support of such a function, oesophageal, colorectal cancer [45], breast cancer [243] and cardiac carcinoma [244] were also found to promote neuritogenesis *in vitro*. More precisely, it was determined that VEGFα released by breast cancer carcinoma [245] drives neuritogenesis, whilst neuropeptides released by sensory neurons enhance chemokinesis [243]. Besides VEGFα, cancer cells released NGF and neurturin can also trigger neurite outgrowth. Overall, the similarities between cancer generation and neuro-regeneration support a nerve dependence of cancer cell growth [246].

Interestingly, the levels of tumour-infiltrating neutrophils and eosinophils, as well as sensory neuron innervation, correlate with the severity of pain and itch experienced by patients with melanoma [247]. Finally, the pharmacological inhibition [248] and genetic depletion [249] of $TRPV1^{+}$ neurons support an essential role in these fibres in transmitting bone cancer-induced pain. Thus, in a mouse model of this disease, blocking CB2R dampens morphine tolerance [250]. Given the increasing prevalence of cancer and the relative inefficacy of pain killers for cancer-associated pain, a deeper understanding of cancer-induced pain mechanism is warranted to develop better interventions [251]. For instance, agonist-mediated cannabinoid receptor CB2 stimulation inhibits capsaicin responses in human sensory neurons (238).

In addition to pain and itch, neurons may play other roles in tumours. Through the secretion of humoral modulators such as neurotransmitters, neuropeptides and neurotrophic factors, neurons regulate tumour metastasis and angiogenesis [252–254]. For example, locally released CGRP in the tumour environment acts on epithelium enhancing VEGF release and subsequent tumour angiogenesis [255]. Secondly, tumour cells can

evolve the capacity to invade peripheral nerve and using their projection as a mean to spread to other parts of the body [256]. In models of nasopharyngeal [257] or prostate [258] cancer, neuron-produced noradrenaline enhances tumour invasiveness by driving matrix metalloproteinase activity. Tumour-innervating vagal neurons can also release neural-associated factors, like netrin-1, which increase cancer metastasis [259–261]. Also, noradrenaline suppressed the migration of ES-2 ovarian carcinoma [262] and prostate cancer cells (reviewed by Isaacs [263]). Finally, acetylcholine and noradrenaline released from autonomic nerve were also found to stimulate tumour growth directly (reviewed by Boilly [246]).

Supporting a crucial role of neuro-neoplastic communication in the development of cancer, the ablation of pancreatic sensory neurons inhibited neurogenic inflammation and slowed pancreas tumour progression [264]. Similarly, surgical gastric denervation or pharmacological silencing using the botulinum toxin suppresses gastric tumorigenesis and reduced inflammation [265]. It is, however, to be noted that the role of nerve in cancer is not always consistent and appeared to be tumour- and context-dependent. Thus, colorectal and breast cancer models were not found hyperinnervated, whilst tumour neo-angiogenesis was thought of to be independent of the nervous system [266]. Moreover, the amount of neurogenesis inversely correlates with the survival of colorectal cancer patient [45]. Consistently in $APC^{Min/+}$ mice, it has been demonstrated that vagal nerve innervation promotes tumour growth in the small intestine [267]. Vagal neurons drive the production of protein trefoil factor 2 by splenic memory T cells helping to suppress myeloid-derived suppressor cell (MDSC) activity. As such, splenic denervation promotes the expansion of MDSC, helping the progression of colorectal cancer [268]. In sum, this double-edged-sword role of neuro-neoplastic synapses in cancer progression muddied the potential of neurons as a target in the management of malignancy and, as such, warrants further research.

Finally, due to their clinical trial successes (reviewed by Szostak [269]), immune checkpoint receptors become an area of intense research. As such, it is now well recognized that cancer cells evolve the capacity to express specific ligands for these immune checkpoint receptors and that their interplay with T cells inhibits their activation [270]. However, recent findings revealed that the programmed cell death ligand-1 (PD-L1) was also expressed by DRG sensory neurons [271], tumour-associated nerves [272] and Crohn's patient innervation of subepithelial mucosa [273]. Elegant work by Chen and colleagues revealed that PD-L1/PD-1 signalling in neurons drives SHP-1 phosphorylation, which activates the potassium channel TREK2, resulting in the suppression of nociceptive neuron excitability [271]. Overall, the simultaneous interplay between i) nociceptors and tumour cells and ii) nociceptors and immune cells supports the idea that cancer cells might co-opt neuro-immune interplay to modulate host anti-tumour immunity.

Immunoglobulins

Immunoglobulins (Ig) are plasma cells-secreted proteins with a variable antigen-binding domain and a constant region (Fc domain). Five main isotypes of Ig have been identified in mammals: IgA, IgD, IgE, IgM and IgG, which are recognized by the Fc receptors (FcRs). When activated on immune cells, FcRs send an intracellular signal which results in the production of cytokines, phagocytosis and degranulation. In addition to their homeostatic roles in the context of infection, injury and immune-related diseases, FcRs are gradually being recognized for their involvement in neurological disorders. Thus, pieces of evidence support the expression of FcRs on microglia, astrocytes, oligodendrocytes and even neurons [274]. The IgG receptors, FcyRs, were found to be present on human peripheral neurons as early as in foetus aged from 20 to 38 weeks [275–277], supporting a role for IgG-FcyR in CNS development. Recent studies also reported an implication of neuronal-Ig system in Alzheimer's disease [274,278], chronic pain [274,278,279], allergy [280–283] and antigen-specific autoimmune diseases [284–288]. Overall, Ig sensory neuron crosstalk may serve a role in pathophysiology [274,277] and, therefore, deserves closer scrutiny.

Autoantibodies have been associated with chronic pain states present in autoimmune diseases. Thus, in the context of Guillain–Barré syndrome (GBS), autoantibodies were found to recognize antigens, such as gangliosides, which are expressed on DRG nociceptor neuronal cell bodies (review in McMahon *et al.* [289]). Such detection alters the function of the somatosensory nervous system and leads to neuropathic pain [284]. In a mouse model of GBS,

the Fc portion of anti-ganglioside immune complex (IC) interacts with FcγRIII expressed on injured nerves mediating peripheral neuropathy [285] and inhibiting axonal regeneration [290]. The contactin-associated protein 2 (CASPR2) is another example of a protein which generates autoantibodies known to drive neuropathic pain in humans [291] and mice [292]. Interestingly, when human-purified CASPR2 antibodies are injected to mice, they hypersensitize sensory neurons by decreasing Kv1 channel function [292]. In rheumatoid arthritis (RA), a condition characterized by cartilage and bone destruction [286,293], it was found that the denervation of a limb, following a nerve injury, reverses the RA development in that limb, providing a clear clinical indication that nociceptors regulate such inflammatory processes [28,294]. Thus, RA treatment shuts down inflammation; however, severe RA pain often persists in its absence [286]. Accordingly, recent studies highlighted the implication of autoantibodies in arthralgia [295], a clinical manifestation that usually precedes the onset of RA [296]. Anti-citrullinated protein antibodies (ACPAs), a family of autoantibodies that is common in patients with RA, directly bind to FcγR on nociceptors [296]. Once injected to healthy mice, human-purified ACPAs bound to osteoclasts and led to CXCL1/IL-8 release. In turn, such complex activates sensory neurons expressed CXCR2 and induces mechanical and thermal hypersensitivity [295,296].

More recently, dorsal root ganglia were found not to synthesize the antigen-specific antibodies found in peripheral sensory neurons, but rather sequester primarily IgG_1 subtype antibodies. Thus, dorsal root ganglion sensory neurons harvested from either naïve or immunized mice lack the enzymes RAG1, RAG2, AID and UNG, which are necessary to produce these various antibodies and failed to express *Ighg1* transcripts in DRG sensory neurons [297]. Whilst the role and consequences of neuronal sequestration of IgG1 remain unclear, it may serve as a local pool of releasable antigen-specific antibody [297].

Mechanical and thermal hypersensitivity can also be regulated and amplified by circulating immune complex formed between IgG and IC. Thus, an IgG stimulation of cultured DRG neurons increased calcium signalling and neuronal excitability, whilst the intradermal injections of an IgG-IC increased C-fibre firing [281,286]. FcγRI am expressed in mice [281], rats [298] and human peripheral nerves [299–301]. Once activated, FcγRI leads to an increase in intracellular calcium, which enhances neuron excitability trigger action potential firing [298], and leads to substance P and CGRP release [281,298]. Similarly, by directly activating primary sensory neurons and reducing action potential firing threshold, Ig-free light chains (IgLC) induce severe pain and neurogenic inflammation [280,302].

Circulating IgE mediates host defence responses such as itching, coughing and mucus secretion [280], and unexpectedly binds to FcεRI-expressing nociceptors [280]. In line with this, functionally active FcγRI and FcεRI were expressed on rodents' superior cervical ganglion neurons and myenteric plexus neurons [287]. Moreover, in a mouse model of cutaneous anaphylaxis; IgE-IC drives thermal sensitivity and severe pain [282]. IgE was also implicated in the pathogenesis of food allergy, where allergic mice showed an upregulation of FcεRI on the abdominal vagus [303]. In this context, IgE-IC activates the vagal neurons, leading to glutamate release and T_H2 immunity [294]. The topical application of blocking antibody to FcεRIα was more efficient than mast cell stabilizer or histamine I receptor antagonist to abolish antigen-induced scratching behaviour [283]. Thus, the authors found that allergen-sensitized mice upregulate FcεRI expression on trigeminal ganglion neurons and that its activation by circulating IgE drives allergic ocular pruritus [283]. Moreover, immunization, such as in the case of exposure to ragweed pollens, drives the nociceptor overexpression of both FcγRI [281] and FcεRI [281].

Clinical relevance

The question remains open as to whether a physician should attempt to amplify or silence neuroimmune interplay. Whilst the overall impact of such dialogue on the course of disease remains murky, consensus are emerging. Nociceptor neurons appear to amplify T_H2 immunity as observed in the context of allergic airway inflammation [18,112,304–306], allergic dermatitis [183,307] and psoriasis [308]. They do so by driving ILC2 cells [18,112,304], CD4 [18], or dermal dendritic cells [308] activity. However, somatosensory neurons appear to block T_H1 or T_H17 immunity as observed in the context of bacterial [120,122,124] and fungal [34] infection as well as anti-tumour immunity [45,267,268]. Neurons initiate such effects by

secreting CGRP, which blocks the activity of neutrophils [120] and CD301b$^+$ dermal dendritic cells [34].

One can wonder if, and how, impaired sensory neuron functioning impacts the progression of inflammatory diseases. Thus, such dysfunction is common in chronic pathologies ranging from diabetes, and spinal and peripheral nerve injury to mycobacterium infection. For example, the *Mycobacterium Leprae* (*M. Leprae*), an intracellular-infecting bacterium, preferentially infect Schwann cells using the interaction between the phenolic glycolipid-1 (PGL-1), a component of the cell wall of *M. Leprae*, and laminin-2, found in the basal lamina of Schwann cells. Upon such infection, *M. Leprae* induces the demyelination and axonal degeneration of peripheral sensory neurons [309]. Besides, PGL-1 activates macrophages and NO release damaging neurons' axon and leading to demyelination [310]. Beyond those, macrophages activated by *M. Leprae* trigger T-cell response, in a STAT-4/6-, CREB-, Jagged1- and Notch-dependent manner [311,312]. *M. Leprae*-infected Schwann cells produce IL-12 and NO in a TLR6-dependent manner, which implies that *M. Leprae* could evoke neural damage-mediated immunity [313]. Thus, leprosy patient frequently suffers from neuropathic pain [314], which can be a consequence of the interplay between tissue-infiltrating macrophages and nociceptors [315]. Altogether, *M. Leprae* causes neural damage both directly, by infecting Schwann cells, and indirectly, through innate and adaptive immunity. Therefore, the progression and establishment of *M. Leprae*-mediated inflammation might be secondary to the interplay between sensory neurons and the cells of the immune system.

Conclusions

Highlighting novel aspects of danger sensing will likely reveal unexpected aspects of host defence and, in doing so, ways by which neurons and immune cells dialogue to preserve homeostasis. Whilst this interplay appears to be self-driven in the context of T_H2 immunity, preventing the onset of allergy by targeting sensory neurons might constitute an attractive therapeutic strategy. In parallel, the adoption of OMICs approaches should define subsets of vagal, lumbar and trigeminal nociceptor neurons, and help design targeted (low side effects) approach to modulate the activity of these sensory neuron subpopulations. Finally, to inform and efficiently target this interplay, systematic context-dependent (naïve and inflammatory phenotype) co-culture of neurons and specific types of immune cells could help map the underlying biology controlling these phenotypes.

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Contributions

TC, KR and ST designed the study; TC, KR, ST, MB, RO, MA, JCW, TE and PJSP drafted the manuscript; and TC, KR, RC, AL, RDP, MR, CRS, SLF and ST wrote the manuscript.

Conflict of interest

The authors have declared that no conflicts of interest exist.

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IX - Article 2. Anatomical differences in nociceptors sensitivity

Crosson T and Talbot S, Journal of Bioelectronic medicine, 2022.

In, this publication, we defined the specificities of vagal nociceptors, compared to DRG nociceptors that are more largely characterized.

The student has conducted all the experiments and analysis in the study. Both the student and supervisor designed the study, drafted, and proofread the manuscript.

Abstract:
Background: Dorsal Root Ganglia (DRG) neurons are derived from the neural crest and mainly innervate the skin, while Jugular Nodose Complex (JNC) neurons originate from the placode and innervate internal organs. These ganglia are composed of highly heterogeneous groups of neurons aimed at assessing and preserving homeostasis. Among other subtypes, nociceptor neurons are specialized in sensing and responding to environmental dangers. As form typically follows function, we hypothesized that JNC and DRG neurons would be phenotypically and transcriptomically different.

Methods: Mouse JNC and DRG neurons were cultured ex vivo. Using calcium imaging, qPCR and neurite outgrowth assay, we compared the sensitivity of JNC and DRG neurons. Using in-silico analysis of existing RNA sequencing datasets, we confronted our results to transcriptomic differences found between both ganglia.

Results: We found drastically different expression levels of Transient Receptor Potential (TRP) channels, growth factor receptors and neuropeptides in JNC and DRG neurons. Functionally, naïve JNC neurons' TRP channels are more sensitive to thermal cues than the ones from DRG neurons. However, DRG neurons showed increased TRP channel responsiveness, neuropeptide release and neurite outgrowth when exposed to Nerve Growth Factor (NGF). In contrast, JNC neurons preferentially responded to Brain-derived neurotrophic factor (BDNF).

Conclusion: Our data show that JNC and DRG neurons are transcriptomically and functionally unique and that pain sensitivity is different across anatomical sites. Drugs targeting NGF signaling may have limited efficacy to treat visceral pain. Bioelectronics nerve stimulation should also be adjusted to the ganglia being targeted and their different expression profile.

Bioelectronic Medicine

SHORT REPORT Open Access

Anatomical differences in nociceptor neurons sensitivity

Theo Crosson and Sebastien Talbot*

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Keywords: Nociceptor neurons, Neuro-immunity, Dorsal root ganglia, Jugular Nodose ganglia, Brain-Derived Neurotrophic Factor, Nerve growth factor, Ntkr1, TRPV1, TRPM8, TRPA1

Background

Nociceptor neurons, a subtype of sensory neurons, form a key line of defense against environmental dangers. They detect a broad range of thermal, mechanical, and chemical threats and respond by means of protective reflexes and by modulating the cells of the microenvironment. Nociceptor neurons can be differentiated based on their expression profiles (Prescott et al., 2020; Usoskin et al., 2015; Kupari et al., 2019; Kaelberer et al., 2020; Sapio et al., 2020), degree of myelination, type of cues to which they are sensitive (Crosson et al., 2019), the reflexes they initiate, the anatomical location of their soma, or the anatomical site they innervate (Mazzone & Undem, 2016). For instance, dorsal root ganglia (DRG) neurons are derived from the neural crest and innervate the skin, muscles, and joints, while the Jugular Nodose Complex (JNC) neurons originate from the neural crest (Jugular) and placode (Nodose) and innervate internal organs (Chavan et al., 2017; Baker & Schlosser, 2005).

Nerve growth factor (NGF) was discovered for its role in the development of the peripheral nervous system (Levi-Montalcini & Hamburger, 1951; Crowley et al., 1994), including nociceptors. In adulthood, NGF is

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released by glial, epithelial, or immune cells (Lindholm et al., 1987; Heumann et al., 1987; Leon et al., 1994; Donnerer et al., 1992; Woolf et al., 1994; Longo et al., 2013; Braun et al., 1999; Stanzel et al., 2008) in response to injury or inflammation. In turn, by acting on its specific receptors, TrkA, which is highly expressed on nociceptors, NGF promotes nerve regeneration (Onger et al., 2017; Sofroniew et al., 2001), tissue innervation (Kinkelin et al., 2000; Ghilardi et al., 2012; Reinert et al., 1998), and increases nociceptors sensitivity (Ji et al., 2002; Chuang et al., 2001; Shu & Mendell, 1999; Nicholas et al., 1999; Koltzenburg et al., 1999; Zhang et al., 2005). As a result, several drugs, notably monoclonal antibodies, targeting NGF signaling are developed to treat chronic pain (Wise et al., 2021). Despite TrkA expression in JNC neurons (Verge et al., 1992; Zhuo & Helke, 1996), the impact of NGF on vagal nociceptors remains controversial. On the one hand, NGF does not affect the survival of JNC neurons (Lindsay & Rohrer, 1985; MacLean et al., 1988), but it was found to increase their expression of Substance P (SP) (MacLean et al., 1988; Hunter et al., 2000).

As the site they innervate (skin, joints) can be readily studied using standard behavioral tests (i.e., Von Frey, Hot plate), DRG neurons' function, sensitivity and biophysical properties is well characterized. In contrast, airway or gut pain is difficult to assess; leaving JNC neuron's function poorly defined. To address these shortcomings, specific ganglia can be isolated, cultured *ex vivo* and neurons' sensitivity to various stimuli can be studied using calcium microscopy as a mean to infer pain sensitivity. Using such an approach, we discovered that JNC neurons have an increased sensitivity to thermal stimuli. We also found that while NGF enhanced DRG neurons sensitivity and neurite outgrowth of nociceptors, it had limited effect on JNC neurons.

Our data shows that JNC and DRG neurons are transcriptomically and phenotypically different, revealing that targeted pain blocking molecules, such as NGF blockers, will likely be ineffective to blunt visceral pain. In addition, JNC neurons express different neuropeptides than the ones from DRG. These data suggest that JNC neuron immunomodulatory actions are also likely to be different from the one produced by DRG neurons. Altogether, these findings support an anatomical-dependent neuronal control of immunity and pain.

Results and discussion

Upon sensing danger, nociceptors initiate defensive reflexes (ranging from withdrawal response to coughing); and release neuropeptides, which regulate vasodilation, mucus secretion, and local infiltration of immune cells (Chiu et al., 2012). The resulting outcome of such neuro-immune interplay is still ill-defined, but data support neuropeptides as blockers of T_H1-mediated immunity but also as drivers of T_H2 immune responses (Talbot et al., 2015; Crosson et al., 2021; Foster et al., 2017; Trankner et al., 2014; Chu et al., 2020; Baral et al., 2018; Chiu et al., 2013; Serhan et al., 2019).

As lumbar and vagal neurons innervate different mucosa, we hypothesized that these neurons are tuned to detect different modalities. As a result, we expect these neurons to express different ion channel receptors and neuropeptides. To uncover whether it is the case, we proceed to the *in-silico* analysis of the RNA sequencing dataset published by Kaelberer and colleagues (Kaelberer et al., 2020). As posited, naïve mouse JNC neurons express different neuropeptides (Fig. 1A), ion channel receptors (Fig. 1B), and growth factor receptors (Fig. 1C) than the ones found in DRG. For instance, JNC neurons are enriched for *Vip,* and *Ntrk2,* while DRG neurons show higher levels of *Calca,* and *Ntrk1* (Fig. 1A and C). Furthermore, naïve JNC neurons overexpress the heat receptors *Trpv1* and cold/heat receptor *Trpa1*, while the DRG neurons are enriched for the cold receptor *Trpm8* and heat receptor *Trpm3* (Fig. 1B). Of note, these genes were selected as they are prototypical markers of sensory neurons' functionality.

Kaelberer and colleagues sequencing used freshly harvested ganglia. Since JNC or DRG neuron phenotyping is often carried out with primary cultures, we've investigated if cultured neurons show the same expression pattern as freshly dissociated cells. After 24h in culture, JNC neurons show higher *Trpa1* expression than DRG neurons and lower one for *Trpm8* and *Calca* (Fig. 1D). *Trpv1* expression was comparable in both cultures. Overall, these results largely match the one from the sequencing of fresh ganglia - but differs slightly for *Trpv1*.

To assess whether these transcriptomic differences translate to physiology, we set up a side-by-side culture system to compare the sensitivity of JNC and DRG neurons (2,500 neurons/dish) to menthol (TRPM8 agonist, 100 μM), capsaicin (TRPV1 agonist, 300 nM) and JT010 (TRPA1 agonist (Takaya et al., 2015), 1μM). Using calcium microscopy as a proxy for neuron's sensitivity, we found that in comparison to DRG, JNC neurons cultures show higher numbers of calcium responsive neurons when exposed to capsaicin (Fig. 2A-B), menthol (Fig. 2C-D) and JT010 (Fig. 2E-F).

While the respective responses to capsaicin and JT010 matches with the higher expression of *Trpv1* and *Trpa1* in JNC neurons, the menthol data are opposite to one predicted by the transcriptomic profile of *Trpm8* (Fig. 1B). Such discrepancy between *Trpm8* gene expression and calcium response to menthol demonstrates that bulk gene expression cannot be used as a sole indicator of neuronal sensitivity. Indeed, the activation of sensory neurons also depends on the number of neurons

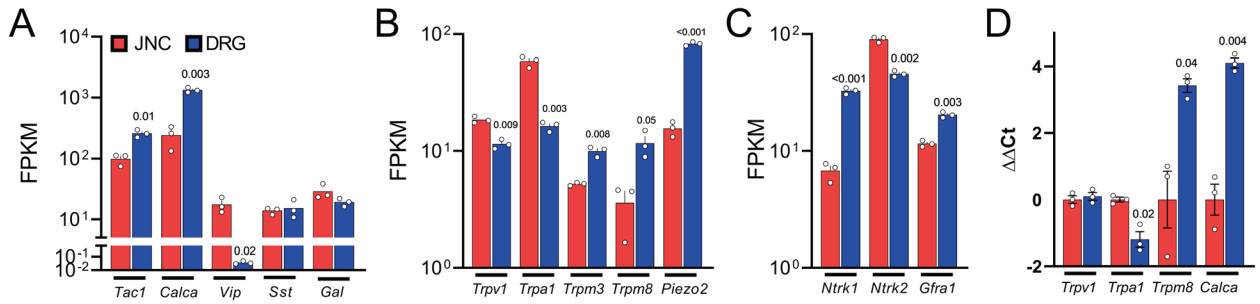

Fig. 1 Lumbar and vagal neurons transcriptomes are different. (**A**-**C**) The whole jugular nodose ganglia complex (JNC; n=3; red) and dorsal root ganglia (DRG; n=3; blue) mouse transcriptomes were profiled using RNA sequencing (RNA-seq; Illumina HiSeq 2500) by *Kaelberer et al., 2020*. *In-silico* analysis of the neuron's transcriptome revealed that naïve mouse JNC nociceptor neurons express different neuropeptides (**A**), ion channel receptors (**B**), and growth factor receptors (**C**) than the one found in DRG. JNC neurons are enriched for *Vip*, *Trpv1*, *Trpa1*, and *Ntrk2*, while DRG neurons show higher higher levels of *Calca*, *Tac1*, *Trpm8*, *Trpm3*, *Piezo2*, *Gfra1* and *Ntrk1*. (**D**) Gene expression was also assessed by RT-qPCR after 24 hours of culture (**D**). ΔΔCt was calculated in comparison to JNC. *Trpm8* and *Calca* were enriched in DRG neurons, while *Trpa1* was enriched in JNC neurons (**D**). *Expression across datasets is shown as Fragments Per Kilobase of transcript per Million reads mapped (FPKM) (**A**-**C**) or ΔΔCt with JNC as the control condition (**D**). Data are shown as means ± S.E.M. N=3/groups. P-values are shown in the figure and determined by unpaired multiple Student t-test with Hidom-Sidak corrections (**A**-**D**). Experimental details were defined in Kaelberer et al., 2020 (**A**-**C**)*

expressing the thermoreceptor, the relative membrane expression of each ion channel on a given neuron, their phosphorylation status, their activation threshold and the one of the voltage-gated sodium and calcium channels bore by these neurons. In addition, reports suggest the possible action of menthol on TRPA1 (Karashima et al., 2007; Lemon et al., 2019; Macpherson et al., 2006), which could explain the inconsistency we observed between gene *Trpm8* expression and menthol responsiveness.

We next sought to investigate the sensitivity of nociceptors to neurotrophins, including Nerve Growth Factor (NGF) that is widely used in nociceptor cultures. By acting on its receptor TrkA (*Ntkr1)*, NGF is known to promote kinase activation (Ji et al., 2002; Zhang et al., 2005), ion channel phosphorylation, and increased trafficking to the plasma membrane (Ji et al., 2002; Stein et al., 2006), all of which resulting in heightened TRP channels sensitivity. First, we noticed that *Ntkr1* is preferentially expressed in DRG neurons (Fig. 1C) and, as expected, adding NGF to the DRG cultures enhanced their responsiveness to capsaicin (Fig. 3A). In contrast, NGF surprisingly had no impact on JNC neurons' responses to capsaicin.

Another feature of neuron activation is their release of neuropeptides. This allows sensory neurons to modulate the synaptic transmission of electrical signals and play a crucial part in promoting pain chronicity. It is also an essential feature by which nociceptor neurons modulate immune cells activity. Similar to calcium flux, we discovered that NGF supplementation increased capsaicin-induced CGRP release from DRG neurons (Fig. 3B). Interestingly, the increased capsaicin sensitivity and CGRP release in response to NGF was not associated with increase in *Trpv1* and *Calca* transcripts (Supplementary Figure 1A-B). This result is in line with previous findings suggesting that nociceptor sensitization by NGF exclusively relies on post-transcriptional mechanisms (Ji et al., 2002).

So far, we probed NGF-mediated sensitization by measuring whether the neurons show heightened responsiveness to capsaicin (calcium influx, neuropeptide release). Along with such an important feature of pain-sensing and transmission, we next sought to test whether NGF modulates JNC and DRG neurite outgrowth. The latter is a process characterized by the growing or branching of neurons in response to guidance cues, which occurs in the developing nervous system and during nerve regeneration. As previously reported, NGF increased the neurite length of cultured DRG $Na_V1.8$ positive nociceptors (Fig. 4B, D, G). In line with the other features we had previously tested, we found that neurite outgrowth of JNC nociceptors were very mildly affected by NGF (Fig. 4A, C, G), but was strongly enhanced by Brain-Derived Neurotrophic Factor (BDNF; Fig. 4E, G). DRG nociceptors' neurite growth was not changed by BDNF supplementation (Fig. 4F, G). While NGF is an important driver of neurogenic inflammation (Donnerer et al., 1992; Woolf et al., 1994; Longo et al., 2013; Braun et al., 1999), our data suggest that it would have limited impact on vagal sensory neuron activity and growth, but would rather target DRG and sympathetic neurons (Kinkelin et al., 2000; Ghilardi et al., 2012; Aloe et al., 1992).

Since NGF showed a limited, but not null, effect on JNC nociceptor neurite growth, we hypothesized that only a subtype of JNC neurons would be sensitive to this neurotrophin (Nassenstein et al., 2010). To address whether this is the case, we analyzed Prescott *and colleagues* (Prescott et al., 2020) single JNC neurons RNA

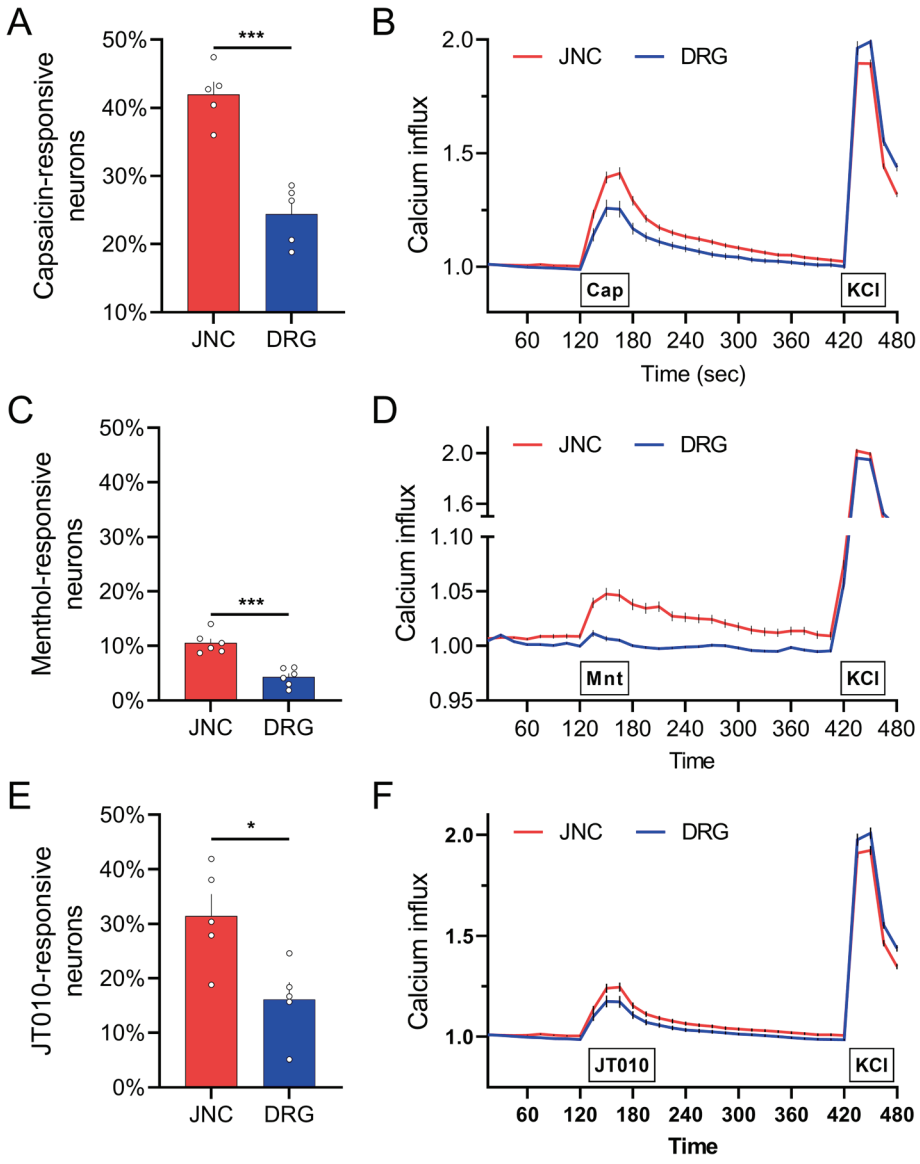

Fig. 2 JNC neurons are more sensitive to TRPV1, TRPA1 and TRPM8 stimulation. Naïve C57BL/6 mouse JNC and DRG neurons were harvested, cultured (2,500 neurons/plate) for 24h, loaded with a calcium responsive dye (Fura-2AM), and sequentially exposed to the TRPV1 agonist capsaicin (300nM; **A-B**), the TRPA1 agonist menthol (100uM; **C-D**), or the TRPA1 agonist JT010 (1uM, **E-F**) followed by KCl (40mM; positive control; **A-F**). The number of responsive (**A, C, E**) cells to capsaicin, menthol, and JT010 is increased in JNC neurons. The average amplitude of responses are shown in (**B, D, F**). *Data are shown as mean ± S.E.M. Number of dishes tested is shown. N are as follows:* ***A-C-E****: n=5-6 dishes/group,* ***B-D-F****: n=290-1200 neurons/group. P-values are shown in the figure and determined by unpaired Student's t-test (****A, C, E****)*

sequencing dataset. We found that *Calca*, *Tac1*, *Ntrk1* are enriched in jugular (neural crest derived) neurons, considered transcriptomically close to DRG neurons (Prescott et al., 2020; Kupari et al., 2019), whereas *Vip* and *Ntrk2* are preferentially expressed by nodose (placode derived) neurons (Fig. 5 A-B). Within the JNC, *Trpv1* (*n*=6424) is expressed in jugular and nodose neurons (Fig. 5C). However, *Ntkr2*$^{+}$*Trpv1*$^{+}$ neurons (*n*=5495) are 2.4-fold more abundant than *Ntkr1*$^{+}$*Trpv1*$^{+}$ (*n*=2262) neurons (Fig. 5C). Along with our calcium imaging recordings, these data suggest that the JNC contains a large population of Vip^{+} capsaicin-responsive nodose neurons that are insensitive to NGF. In line with the functional and gene expression data we described here, this is an important finding as JNC neuron's function is typically studied using *ex vivo* cultures supplemented with NGF. Thus, JNC cultures would likely be best model using other neurotrophins. JNC neurons (Fig. 1C) and specifically nodose neurons (Fig. 5C) overexpress the BDNF receptor *Ntkr2,* and BDNF enhance

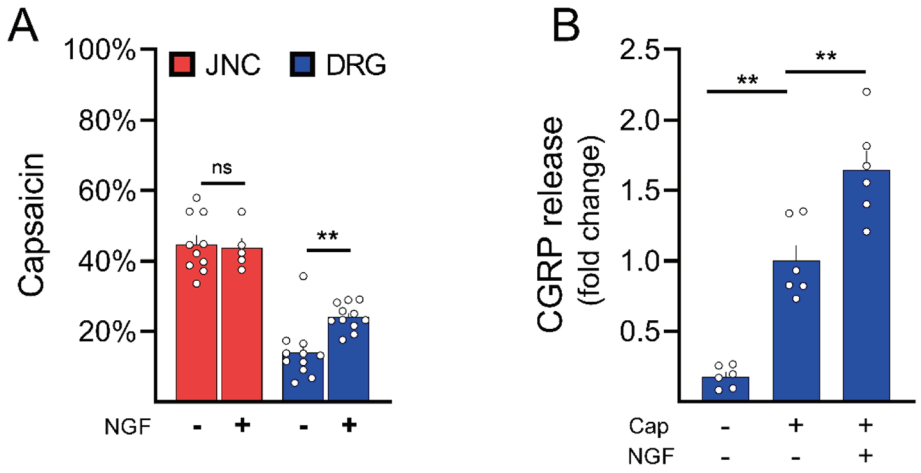

Fig. 3 JNC neurons are insensitive to NGF. Naïve C57BL/6 mouse JNC and DRG neurons were harvested, cultured for 24h in the presence of Nerve Growth Factor (NGF, 50 ng/mL). The cells were then loaded with a calcium responsive dye (Fura-2AM) and sequentially exposed to capsaicin (100 nM) followed by KCl (40mM; positive control; **A)**. The addition of NGF increased the number of capsaicin-responsive neurons in DRG cultures but not in the one from the JNC (**A**). Similarly, NGF increased capsaicin-induced CGRP release from cultured DRG neurons **(B)**. *Data are shown as mean ± S.E.M. Number of dishes tested is shown. N are as follows:* ***A****: n=5-10 dishes/group from two independent experiments,* ***B****: n=6 wells from different animals/group from two independent experiments. P-values are shown in the figure and determined by unpaired Student's t-test (**A**) or one-way ANOVA post hoc Dunnett (**B**)*

their neurite growth (Fig. 4E). Given that BDNF is also released in inflammatory contexts (Braun et al., 1999), it is likely to impact vagal nociceptor phenotype to a larger extent than NGF.

Finally, we found that cultured $Na_V1.8$ positive JNC nociceptors are larger than DRG nociceptors (Fig. 4H-I). These data further support a different composition of the neuronal subtypes between these two ganglia, and this is likely a function of the mucosal danger each is exposed to. Thus, along with the anatomical differences in nociceptor neurons' ability to sense danger and the molecule driving their sensitization, we also found a substantial discrepancy in the neuropeptide produced by JNC and DRG neurons. Thus, DRG neurons preferentially express SP and CGRP while JNC neurons, specifically the one from the nodose, express VIP (Figs. 1A and 5). Interestingly, these three neuropeptides are reported to regulate immune responses, but their role differs depending on the type of inflammation (T_h1, T_h2) and the type of immune cells involved (Foster et al., 2017).

Conclusions

By innervating locations ranging from skin to visceral mucosa, sensory neurons are exposed to different environments and types of danger. Here, we found significant transcriptomic and phenotypic differences between JNC and DRG neurons. In addition to these, our data revealed that JNC neurons have an intrinsic and heightened capacity to sense heat and cold dangers.

System modelling using *ex vivo* culture systems of JNC or DRG should take into account that JNC and DRG neurons differentially respond to neurotrophins, DRG being more sensitive to NGF, while JNC is more sensitive to BDNF. The management of visceral and skin inflammation and pain should also be tailored differently. Among other differentiator, the selective targeting of these growth factor receptors could help preferentially act on one of these systems while sparing the other. Finally, bioelectronics nerve stimulation should also be adjusted to ganglia being targeted and, depending on the pathological situation, could be accompanied by pharmacological blockers of the NGF or the BNDF receptors.

Methods

In silico analysis of whole DRG and JNC transcriptome

RNA sequencing data of mouse nociceptors expressed as Fragments per Kilobase Per Million (FPKM) were downloaded from NCBI Gene Expression Omnibus (GSE141395). Experimental procedures are detailed in the original study published by Kaelberer et al (Kaelberer et al., 2020). The authors extracted whole JNC and DRG from C57BL6 mice, which were then flash-frozen in liquid nitrogen before RNA isolation and sequenced on an Illumina HiSeq 2500. TopHat was used to map reads to mouse reference genome mm9. Genes coding for neuropeptides, ion channels and neurotrophins receptors were processed using Microsoft Excel.

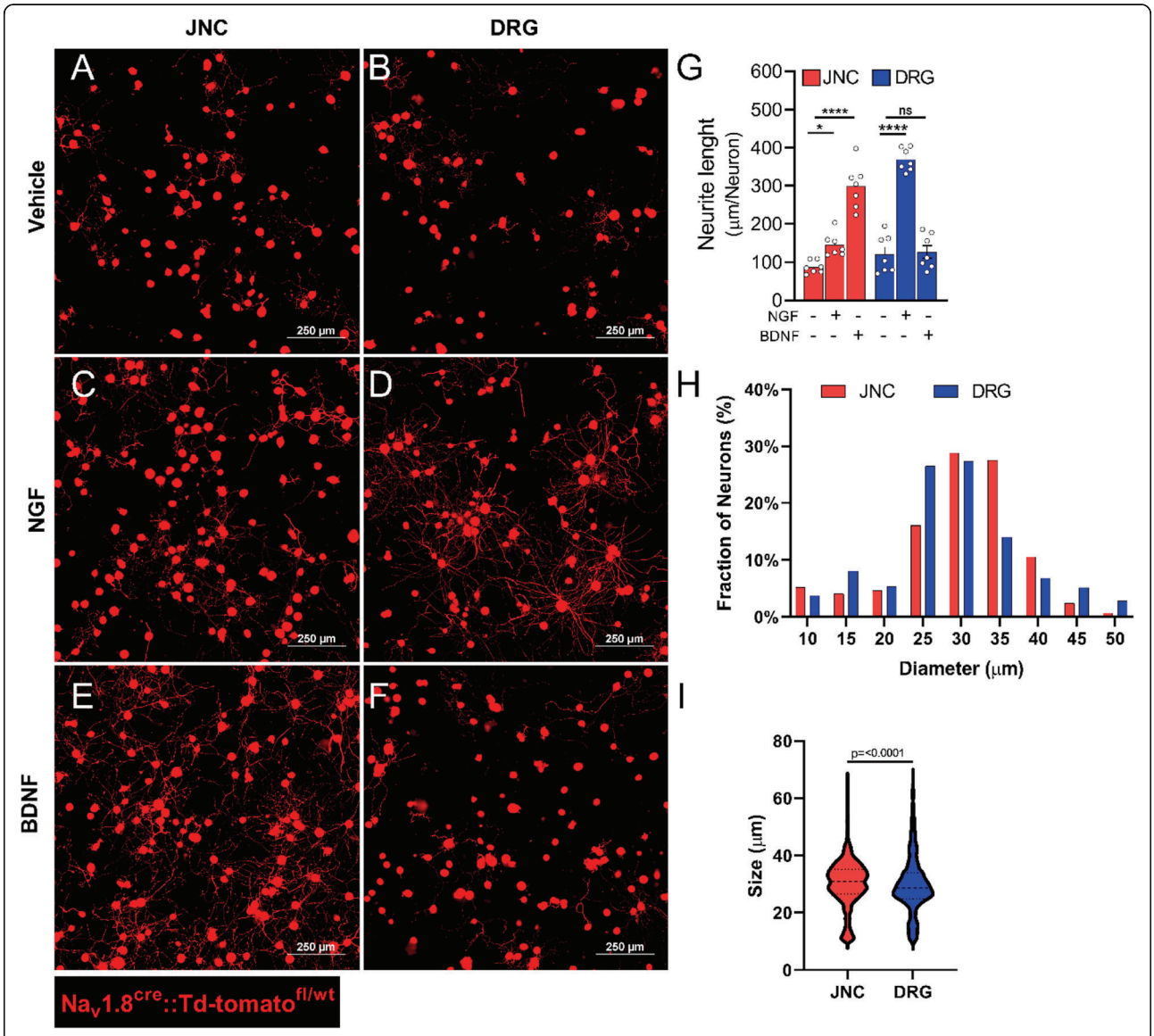

Fig. 4 Lumbar and vagal neurons are phenotypically different and respond differently to NGF and BDNF. $Na_V1.8^{cre}$::Td-tomato$^{fl/wt}$ (red) mice JNC (**A, C, E**) and DRG (**B, D, F**) nociceptor neurons were harvested, and cultured (1,500 neurons/plate) for 24h in the presence of vehicle (**A**-**B**), Nerve Growth Factor (NGF, 50 ng/mL; **C**-**D**), or Brain-Derived Neurotrophic Factor (BDNF, 50 ng/mL; **E**-**F**). In comparison to vehicle, NGF increased neurite outgrowth in cultured DRG neurons, and had limited impact in JNC neurons (**G**). BDNF increased neurite outgrowth in cultured JNC neurons but had no impact in DRG neurons (**G**). In vehicle-exposed culture, JNC neurons have a larger diameter than DRG neurons (**H**-**I**; n>1000 neurons). *Data are shown as mean ± S.E.M. N=7 dishes/group from two independent experiments* (***A***-***I***). *P-values are shown in the figure and determined by one-way ANOVA post hoc Dunnett* (***G***) *or Mann-Whitney Test* (***I***). *Scale = 250 µm* (***A***-***F***). NaV1.8$^+$ *nociceptors are labeled in red* (***A***-***F***)

In-silico analysis of JNC neurons single-cell transcriptome

Prescott et al. (2020) generated single cell sequencing data of nodose ganglia cells from 40 mice using 10X Genomics platform. The data was downloaded from NCBI Gene Expression Omnibus (GSE145216) and analyzed using Seurat. Neuronal cells were selected based on *Slc17a6* (Vglut2) expression (raw count ≥ 2). Standard workflow was used for quality control, preprocessing, normalization and clustering (resolution = 0.5, PCs = 1:30). Neurons were considered positive for other markers if their raw count was ≥ 1.

Animals

Mice were housed in standard environmental conditions (12h light/dark cycle; 23oC; food and water ad libitum) at facilities accredited by CCPA. Parental strain C57BL6 (Jax, # 000664) and Td-Tomato$^{fl/fl}$ (Jax, # 007908) were purchased from Jackson Laboratory. Parental strain Nav1.8$^{cre/cre}$ mice were generously supplied by Professor John Wood (UCL). Male and female mice C57Bl6 and Nav1.8$^{cre/wt}$::Td-Tomato$^{fl/wt}$ were bred in-house and used between 6 and 12 weeks of age.

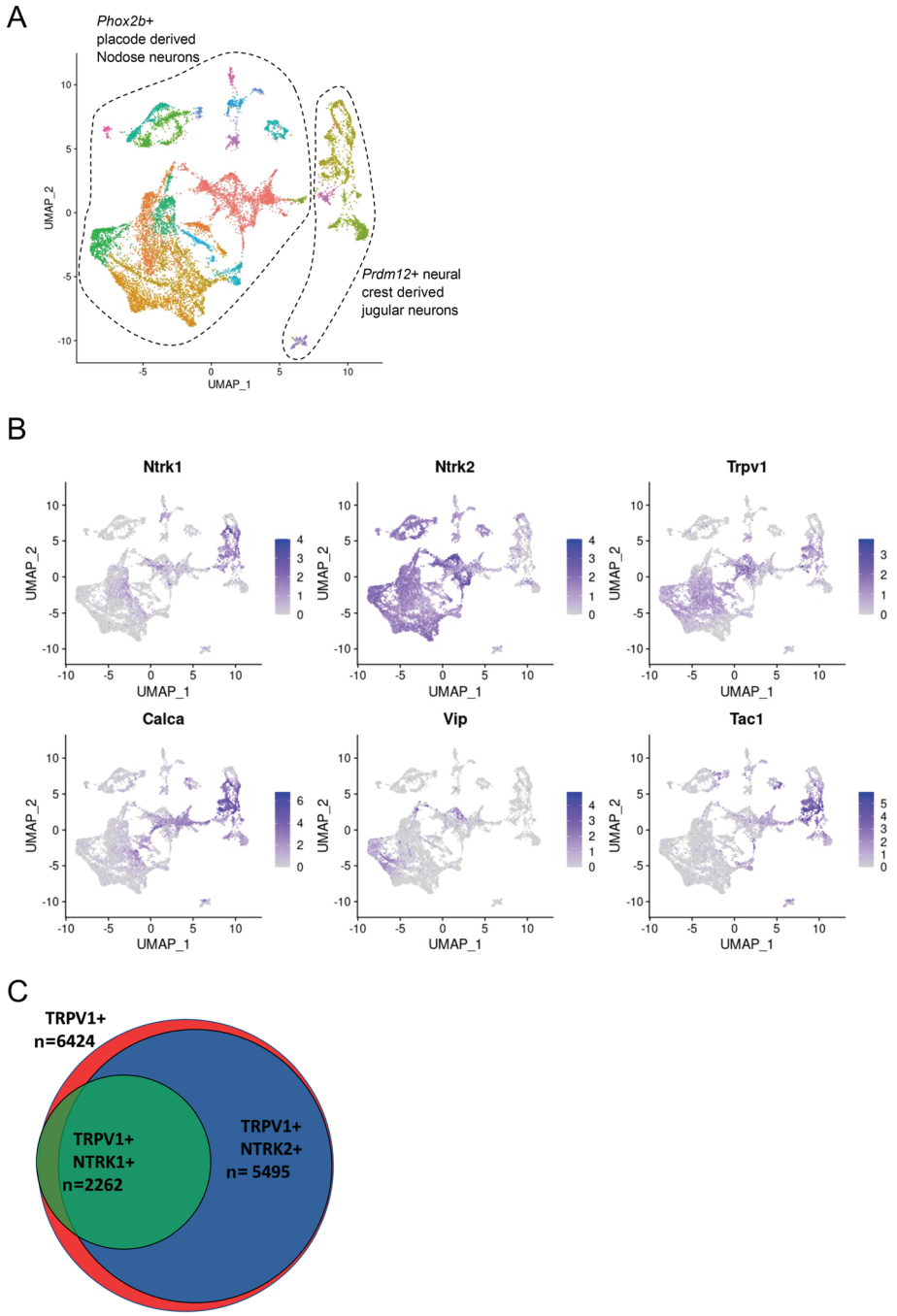

Fig. 5 Nodose neurons have low expression of *Ntrk1* but are enriched for *Ntrk2*. *In-silico* analysis of single-cell RNA sequencing of 15,142 *Slc17a6*$^{+}$ *(Vglut2)* JNC sensory neurons (**A**). *Phox2b*$^{+}$ neurons are derived from the placode which are designated as nodose neurons, while *Prdm12*$^{+}$ neurons are derived from the neural crest and indicate jugular neurons (**A**). Uniform Manifold Approximation and Projection (UMAP) were generated, featuring normalized expression of neurotrophins receptors (*Ntrk1, Ntrk2*), ion channel receptor (*Trpv1*) and neuropeptides (*Calca, Vip, Tac1*; **B**). *Calca, Tac1, Ntrk1* are enriched in jugular (neural crest) neurons whereas *Vip* and *Ntrk2* are preferentially expressed by nodose (placode) neurons **(B)**. *Trpv1* (*n*=6424) is expressed in jugular and nodose neurons, *Ntkr2*$^{+}$*Trpv1*$^{+}$ neurons (*n*=5495) are 2.4-fold more abundant than *Ntkr1*$^{+}$*Trpv1*$^{+}$ (*n*=2262) neurons (**C**). Experimental details were defined in (Prescott et al., 2020)

Neuron culture

Mice were sacrificed and JNC ganglia and DRG were dissected out into ice-cold DMEM medium (Corning, # 10-013-CV), completed with 100 U/mL penicillin and 100 μg/mL streptomycin (Fisher, # MT-3001-Cl), and 10% FBS (VWR, #10799-390). JNC ganglia were pooled

from several mice. Cells were then transferred in PBS completed with 1 mg/mL collagenase IV (Sigma, #C5138) + 2.4 U/mL dispase II (Sigma, # 04942078001) and incubated for 80 minutes at 37°C. Ganglia were triturated with glass Pasteur pipettes of decreasing size in supplemented DMEM medium, then centrifuged (200g) over a 15% BSA gradient in PBS to eliminate debris. Neurons were then plated on Laminin (Sigma, # L2020) coated cell culture dishes. The cells were cultured at 37° with Neurobasal-A medium (Gibco, # 21103-049) 0.01 mM AraC (Sigma, # C6645) and 200nM L-Glutamine (VWR, # 02-0131), without neurotrophins unless otherwise indicated. Culture densities and durations are further described below for each application.

Calcium microscopy

2,500 neurons from C57BL6 DRG or JNC in a 10ul drop were plated on laminin-coated glass bottom 35mm dishes (ibidi #81218). 1ml of Neurobasal media supplemented or not with 50ng/mL Nerve Growth Factor (NGF, ThermoFisher #13257019) was added after 1-hour incubation at 37°. Neurons were then cultured overnight before being used for calcium imaging. Cells were loaded with 5μM Fura-2-AM (Biovision #125280) at 37°C for 45-60min in the culture medium then washed into Standard Extracellular Solution (SES, 145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH 7.5), and imaged at room temperature. Capsaicin 100nM or 300nM (Tocris #0462), Menthol 100uM (medchemexpress #HY-N1369), JT010 1uM (Sigma #SML1672) were prepared in SES and were flowed (30sec) directly onto neurons using perfusion barrels followed by buffer washout (5 minutes). 40mM KCl solution was then flowed on the cells for 20 seconds. Cells were illuminated by a UV light source (Cool LED, pE-340) alternating 340 nm and 380 nm excitation, and a camera (Photometrics Prime 95B 25mm) captured fluorescence emission (515/45nm) with a 20X objective. For analysis, circular Regions of interest (ROI) were manually drawn on neurons based on their 380nm fluorescence. 340/380 fluorescence ratios were then calculated and exported. Microsoft Excel was used for further analyses (Microsoft, USA). Basically, 380/340 ratio values for each ROI were normalized by their baseline. Neurons were considered responsive if the fluorescence ratio increased by at least 10% within 1 minute after injection. Live neurons were defined based on their response to KCl.

CGRP release

Ten thousand neurons from C57BL6 mice DRG were plated on laminin-coated 96 well plates in 200ul media supplemented or not with 50ng/mL NGF. Biological replicates were made using a different mouse's preparation for each replicate. After 48 hours of culture, neurons were washed one time with a fresh Neurobasal medium. The medium was then replaced by Neurobasal media, either with or without 300nM capsaicin, before incubation at 37° for 20 minutes. Culture media were then harvested, centrifuged at 700g to remove cellular debris, and used freshly for CGRP Elisa (Cayman #589001) following manufacturer instructions.

Neurite growth

One thousand five hundred neurons from Nav1.8$^{cre/wt}$:: Td-Tomato$^{fl/wt}$ mice DRG or JNC in a 15 ul drop were plated on laminin-coated glass bottom 35mm dishes. 1ml of Neurobasal media supplemented or not with 50ng/mL Nerve Growth Factor (NGF) or Brain Derived Neurotrophic factor (BDNF, Peprotech #450-02) was added after 1-hour incubation at 37°. Pictures of the whole plating area were taken after 24 hours of culture using a 20X objective to collect Td-Tomato fluorescence (Excitation 554/23nm; Emission:609/54nm). For the analysis of neurite outgrowth, an in-house developed method was used. Using Nikon Elements software, a fluorescence threshold was used to define the Td-tomato positive neurites and soma. The somas were then defined and excluded based on fluorescence intensity, size, and circularity. The total size of neurites was then divided by the number of somas for each culture dish. The soma diameters of the culture without neurotrohpin were used to compare JNC and DRG nociceptors size distribution.

RT-qPCR

Five thousand neurons from C57BL6 mice DRG or JNC were plated on laminin-coated 96 well plates in 200ul media supplemented or not with 50ng/mL NGF. Biological replicates were made using a different mouse's preparation for each replicate. After 24 hours of culture, the culture medium was removed, and the cells harvested in 500ul Trizol (Thermofisher #15596018). RNA was then extracted using the kit PureLink™ RNA Micro Scale (Thermofisher # 12183016) following manufacturer's instructions. RNA was reversed transcribed using the SuperScript™ VILO™ Master Mix (Thermofisher #11755250). The cDNA was then subjected to 2-step thermocycling using Power up qPCR SYBR Green mix (Thermofisher # A25742) and data collection was performed on a Mic qPCR machine (Bio Molecular Systems). Expression levels were normalized using the ΔΔCt method with *Actb* as the reference gene. The primers used were *Trpv1* Forward: GGCCGAGTTT CAGGGAGAAA; *Trpv1* Reverse: TATCTCGAGTGC TTGCGTCC; *Calca* Forward: AGGCACCGCTCA CCAG; *Calca* Reverse: CCTGGGCTGCTTTCCAAG

AT; *Actb* Forward: TGTCGAGTCGCGTCCACC; *Actb* Reverse: TATCGTCATCCATGGCGAACTGG; *Trpa1* Forward: TCCAAATTTTCCAACAGAAAAGGA; *Trpa1* Reverse: CGCTATTGCTCCACATTGCC; Trpm8 Forward: AGCAGTGGAGTTGTTCACCG, Trpm8 Reverse: GCTTCGCAGGAGTAGACCAG.

Statistical analysis

Results are expressed as mean ± standard error of the mean (S.E.M.) in all experiments. The statistical significance was tested by one-way or two-way ANOVA, two-tail unpaired Student T-test, or Mann-Whitney test as indicated in figure legends. Values were considered significantly different when $p < 0.05$. Statistical computations and graphs were made with GraphPad 8.1 software (GraphPad Software).

Abbreviations

BDNF: Brain-derived neurotrophic factor; CGRP: Calcitonin gene-related peptide; DRG: Dorsal root ganglia; JNC: Jugular nodose ganglia; NGF: Nerve growth factor; SP: Substance P; Tac1: Tachykinin peptide 1; T_h1: Type 1 helper cells; T_h2: Type 2 helper cells; TRPA1: Transient receptor potential ankyrin subtype 1 protein; TRPM8: Transient receptor potential cation channel subfamily melastatin member 8; TRPV1: Transient receptor potential vanilloid type 1; VIP: Vasoactive Intestinal Peptide

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42234-022-00088-w.

Additional file 1: Supplementary Figure 1. NGF does not impact gene transcription in cultured sensory neurons. Nociceptor neurons were harvested and cultured (5000 neurons/well) for 24h in the presence of Nerve Growth Factor (NGF, 50 ng/mL; denoted *as* +) or its vehicle (*denoted as* -). ΔΔCt were calculated for *Trpv1* (**A**) and *Calca* (**B**) and fold change were calculated in comparison to untreated JNC neurons. NGF did not impact *Trpv1* (**A**) and *Calca* (**B**) gene expression in JNC or DRG neurons (**A, B**). *Data are shown as mean ± S.E.M. Number of dishes tested is shown. N=3 biological replicates/group. P-values were determined by unpaired Student's t-test.*

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Authors' contributions

TC performed experiments. TC and ST designed the study, analyzed the data, and wrote the manuscript. All authors participated in revising the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

All data generated during this study are included in this published article and its supplementary information files. Some of the data analyzed from previous publications were obtained from public database NCBI Gene Expression Omnibus (GSE141395 and GSE145216).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

X - Article 3. FcεR1 expressing nociceptors trigger allergic airway inflammation.

Crosson T, Wang JC, Doyle B, Merrison H, Balood M, Parrin A, Pascal M, Mindt BC, Seehus CR, Ozcan A, Huang X, Semenara E, Lai N, Majdoubi A, Abdulnour RE, Rajchgot T, Rafei M, Foster SL, Thibodeau J, Fritz JH, Levy BD, Woolf CJ, and Talbot S. Journal of Allergy and Clinical Immunology, 2021.

In this publication, we unravel new type 2 signals that are sensed by nociceptors. Using a functional approach, we show that nociceptors can directly respond to antigens through the expression of FcεRI.

This publication is the result of a collaborative study involving several labs and scientists. The student finished the study and addressed reviewers' comments. His main contribution was the generation and characterization of the mice with conditional knock-out of FcεR1γ in nociceptors (TRPV1^{Cre}::FcεR1γ$^{fl/fl}$) and the experimentations performed with this mouse strain.

Abstract:
Background: Lung nociceptor neurons amplify immune cell activity and mucus metaplasia in response to an inhaled allergen challenge in sensitized mice.

Objective: We sought to identify the cellular mechanisms by which these sensory neurons are activated subsequent to allergen exposure.

Methods: We used calcium microscopy and electrophysiologic recording to assess whether vagal neurons directly respond to the model allergen ovalbumin (OVA). Next, we generated the first nociceptor-specific FcεR1γ knockdown (TRPV1^{Cre}::FcεR1γ$^{fl/fl}$) mice to assess whether this targeted invalidation would affect the severity of allergic inflammation in response to allergen challenges.

Results: Lung-innervating jugular nodose complex ganglion neurons express the high-affinity IgE receptor FcεR1, the levels of which increase in OVA-sensitized mice. FcεR1γ-expressing vagal nociceptor neurons respond directly to OVA complexed with IgE with depolarization, action potential firing, calcium influx, and neuropeptide release. Activation of vagal neurons by IgE-allergen immune complexes, through the release of substance P from their peripheral terminals, directly amplifies TH2 cell influx and polarization in the airways. Allergic airway inflammation is decreased in TRPV1^{Cre}::FcεR1γ$^{fl/fl}$ mice and in FcεR1a–/– mice into which bone marrow has been transplanted. Finally, increased in vivo circulating levels of IgE following allergen sensitization enhances the responsiveness of FcεR1 to immune complexes in both mouse jugular nodose complex ganglion neurons and human induced pluripotent stem cell–derived nociceptors.

Conclusions: Allergen sensitization triggers a feedforward inflammatory loop between IgE-producing plasma cells, FcεR1- expressing vagal sensory neurons, and TH2 cells, which helps to both initiate and amplify allergic airway inflammation. These data highlight a novel target for reducing allergy, namely, FcεR1γ expressed by nociceptors.

FcεR1-expressing nociceptors trigger allergic airway inflammation

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Conclusions: Allergen sensitization triggers a feedforward inflammatory loop between IgE-producing plasma cells, FcεR1-expressing vagal sensory neurons, and T_H2 cells, which helps to both initiate and amplify allergic airway inflammation. These data highlight a novel target for reducing allergy, namely, FcεR1γ expressed by nociceptors. (J Allergy Clin Immunol 2021;147:2330-42.)

Key words: *Neuroimmunity, nociceptor neurons, allergy, asthma, T_H2, FcεR1, TRPV1, allergen detection, vagal sensing, substance P*

Trigeminal and lumbar nociceptor somatosensory neurons express antibody-sensing Fc receptors,[1] namely, Fcγ receptor for IgG and Fcε receptor for IgE.[2,3] This phenomenon may explain why sensory neurons in a sensitized animal fire action potentials when exposed to an allergen.[4,5] After binding to the antigen for which they were raised, antibodies form immune complexes that activate Fc receptors expressed by nociceptors. Such stimuli generate calcium flux, action potential firing, and neuropeptide release, triggering pain or itching.[6-8] However, whether vagal sensory neurons innervating visceral organs also sense immune complexes, and what the consequences are if they do, are unknown. We set out to examine these questions.

Nociceptor neurons respond to immune cell cues such as cytokines, and they drive immune responses.[9-13] For example, in the ovalbumin (OVA) and house dust mite (HDM) mouse models of allergic airway inflammation (AAI), sensory neurons innervating the lung drive $CD4^+$ T and group 2 innate lymphoid cell (ILC2) activation through a vasoactive intestinal peptide (VIP)-VPAC2 neuropeptide axis.[14-16] In turn, these immune cells release the cytokines IL-5 and IL-13, which activate lung sensory neurons as part of a feedforward proinflammatory loop that amplifies the adaptive immune response to allergen exposure[14] and also drives mucus metaplasia.[17] However, the specific

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Disclosure of potential conflict of interest: S. Talbot, B. D. Levy, and C. J. Woolf have an equity stake in Nocion Therapeutics. The rest of the authors declare that they have no relevant conflicts of interest.

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Abbreviations used
AAI: Allergic airway inflammation
BALF: Bronchoalveolar lavage fluid
BL6: C57BL6
CGRP: Calcitonin gene–related peptide
DRG: Dorsal root ganglion
DTA: Diptheria toxin
HDM: House dust mite
IC: IgE-Ovalbumin complex
ILC2: Group 2 innate lymphoid cell
JNC: Jugular nodose ganglion complex
KCl: Potassium chloride
NMU: Neuromedin U
OVA: Ovalbumin
qPCR: Quantitative PCR
QX-314: Membrane-impermeable form of lidocaine
SP: Substance P
TRPA1: Transient receptor potential ankyrin 1
TRPC3: Transient receptor potential cation channel subfamily C member 3
TRPV1: Transient receptor potential vanilloid subtype 1
VIP: Vasoactive intestinal peptide

mechanisms by which an antigen initiates type 2 airway inflammation and the point at which nociceptors are engaged remain to be determined. To address this issue, we have now used genetic approaches and AAI models to examine (1) whether and how jugular nodose ganglion complex (JNC) nociceptor neurons sense allergens; (2) what the consequences for immune cells may be if they do; and (3) whether allergen sensitization sets off an interaction between adaptive immune responses and nociceptor activity that triggers AAI.

METHODS

Detailed information on materials and methods are available in the Methods section in this article's Online Repository (available at www.jacionline.org). In brief, all procedures were approved by the Institutional Animal Care and Use Committees of Boston Children's Hospital and Université de Montréal. AAI was studied in an OVA-based model.[18] On days 0 and 7, mice were sensitized by 200-μL intraperitoneal injections of a solution containing 1 mg/mL of OVA (Sigma, St Louis, Mo; catalog no. A5503-25G) and 5 mg/mL of aluminum hydroxide (Sigma; catalog no. 239186-500G). On days 14 to 17 (at 10:00 AM), mice were exposed to a 6% OVA aerosol for 20-25 minutes or intranasal OVA instillation (50 μg/50 μL). Mice were humanely killed on days 0, 3, 6, 9, 13, 14, 15, 18, 20, 21, or 26; JNC neurons were harvested, cultured, and analyzed by using calcium microscopy, electrophysiology,[14] and single-cell quantitative PCR (qPCR).[19] Culture media were analyzed by using ELISA.[14] Bronchoalveolar lavage fluid (BALF) was harvested, and cells were isolated, counted, and immunophenotyped by using fluorescence-activated cell sorting.[14] Alternatively, the membrane-impermeable form of lidocaine (QX-314, Tocris, Bristol, UK [100 μM]) and capsaicin (Tocris, 1 μM) were nebulized on day 14, and the mice were typically humanely killed on day 15. Another type 2 inflammation model was induced via HDMs. Lightly anesthetized (by using isoflurane) mice were sensitized (on days 1-5), challenged (on days 8-10) with HDMs (CiteQ, Groningen, The Netherlands; catalog no. 15J01 [20 μg/50 μL, administered intranasally), and humanely killed on day 11, after which their BALF was harvested and the cells were isolated, counted, and immunophenotyped by flow cytometry.

RESULTS

Vagal nociceptors initiate airway inflammation

Type 2 allergic inflammation was induced in mice by an initial sensitization to OVA with aluminum hydroxide as an adjuvant (administered intraperitoneally on days 0 and 7), followed 2 weeks later by inhaled OVA challenges.[14] A single inhaled OVA exposure on day 14 after sensitization resulted in increased numbers of $CD45^+$ cells in the BALF when tested 24 hours later (Fig 1, *A*). To establish whether nociceptor activation at the time of the allergen exposure contributes to the inflammatory response, we tested whether capsaicin (1 μM, administered intranasally), an exogenous transient receptor potential vanilloid subtype 1 (TRPV1) ligand that activates lung afferents nociceptors, would modify the extent of the immune infiltrate. Capsaicin given 3 hours before the initial allergen challenge doubled the OVA-mediated influx of $CD45^+$ cells in the BALF (Fig 1, *A*).

Next, to specifically explore whether nociceptors have a role in initiating the inflammation resulting from allergen exposure in sensitized mice, we used a strategy to selectively silence lung nociceptors before the allergen exposure. This approach used nonselective large-pore ion channels (TRPA1 and TRPV1) as cell-specific drug entry ports to deliver a charged, membrane-impermeable form of lidocaine (QX-314) to pulmonary sensory fibers to block sodium currents and, thereby, action potentials.[14] A 3-hour pretreatment with QX-314, in the presence of capsaicin as a TRPV1 channel opener reduced the OVA-mediated airway inflammatory response to the subsequent allergen inhalation (Fig 1, *A*). QX-314 pretreatment without capsaicin, which does not silence nociceptors, did not affect the numbers of $CD45^+$ cells in BALF (Fig 1, *A*).

To test for nociceptor activation by an allergen challenge, we probed phosphorylated p38 MAPK activation levels as an activity marker in JNC neurons.[20-24] An acute inhaled OVA challenge in sensitized mice produced a time-dependent increase in phosphorylated p38–positive JNC neurons 1 hour after OVA challenge that continued for 24 hours (Fig 1, *B-D*). To determine whether this effect occurred in the airway-innervating neurons, $TRPV1^{Cre}$ mice were injected intranasally with AAV9-FLEX-ChR2-Tdtomato (10^{12} titers, 50μL) virus 2 weeks before allergen sensitization. Using single-cell qPCR, we found that *p38* and *Tac1* transcript expression increased in airway-innervating neurons (Td-tomato–positive) obtained from OVA-challenged mice (1 hour [Fig 1, *E*]). The finding that silencing sensory neurons before the first allergen exposure reduced the inflammatory response while activating the nociceptors had the opposite effect raises the possibility that vagal nociceptors might be directly engaged by the allergen challenge and the possibility that such activation may contribute to immune cell recruitment/activation.

Vagal nociceptors express FcεR1

In silico analysis of 7 previously published expression profiling data sets[25] of $TRPV1^+$ neurons shows that in addition to sensory neuron markers (*Trpv1* and *Trpa1*) and nociceptor neuropeptides (*Tac1* which encode for substance P [SP]), *Vip*, neuromedin U [*Nmu*]), and calcitonin gene–related peptide [*Calca*], these afferents express the immunoglobulin receptors (*Fcεr1γ*, *Fcεr2β*, *Fr1*, *Fcγr2α*, and *Fcγr3α*). In the case of *Fcεr1γ*, we found higher relative expression levels than of the pattern recognition receptor *Fpr1* and similar levels to the one of the P2y purinoceptor 1 *(P2yr1)* [Fig 2, *A*]. Of note, FPR1 and P2YR1were found to be

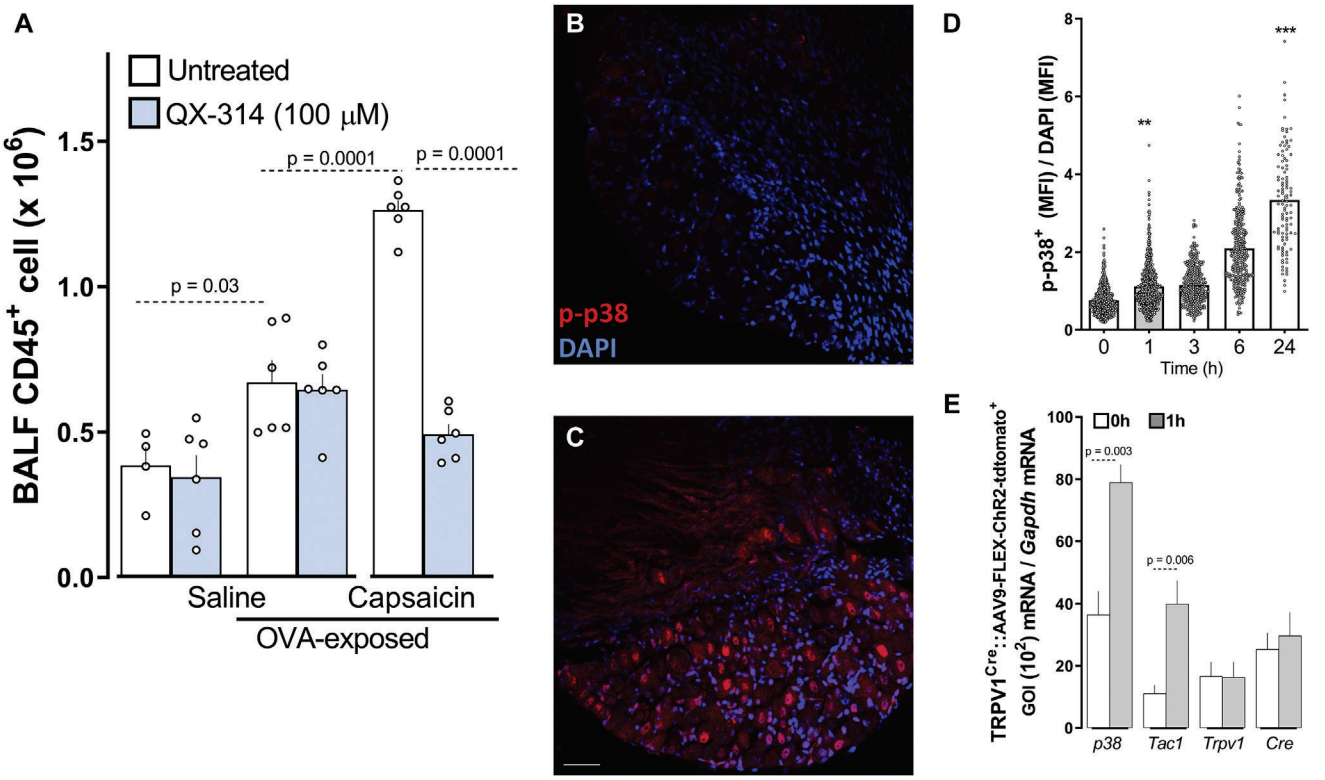

FIG 1. Vagal nociceptors initiate airway inflammation. **A,** An acute inhaled OVA challenge (day 14) increased the numbers of $CD45^+$ cells in the BALF of previously sensitized mice. A 3-hour pretreatment with intranasal 1 μM capsaicin led to a further increase in number of $CD45^+$ cells over the number after OVA treatment alone. QX-314 treatment 3 hours before the first OVA challenge did not alter the allergen-induced inflammation, but cotreatment with capsaicin to open TRP channels and allow entry of QX-314 in airway nociceptors to silence their electric activity prevented the onset of allergic airway inflammation. **B-D,** Allergen challenge (day 14) with OVA increased (in a time-dependent manner) the number of neurons that were positive for phosphorylated p38 (p-p38), which is an activity marker in JNC neurons. **E,** To track airway-innervating neurons, a cre-dependent AAV9-FLEX-Tdtomato virus (10^{12} titers [50 μL]) was injected intranasally 2 weeks before sensitization. Through use of single-cell qPCR, *p38* and *Tac1* transcript expression were increased in airway-innervating neurons (Td-tomato–positive) from OVA-challenged mice (1 hour). *Trpv1* and C*re* expression were not affected. Data are shown as means ± SEMs. **A,** One-way ANOVA and *post hoc* Bonferroni correction. **D** and **E,** Two-tailed unpaired Student *t* test. n = 4 to 10 animals/group; 1 to 3 cohorts. **B** and **C,** p-p38 is labeled in red, and nuclei are labeled in blue, Scale bar = 100 μm. *DAPI,* 4′,6-Diamino-2-phenylindole; *GOI,* gene of interest; *MFI,* mean fluorescence intensity.

functionally active in these neurons.[11,19,25-31] Next, *ex vivo* JNC neurons from naive and allergen-sensitized animals ($Tac1^{cre}$::G-$CaMP6^{fl/wt}$ reporter mice) were cocultured (1:1 mix). In this context, we found that 87% of all FcεR1γ transcript–expressing neurons originated from allergen-sensitized mice ($GCaMP6^+$ [see Fig E1, *A* in this article's Online Repository at www.jacionline.org]). We then measured the level of *Fcεr1* expression on vagal sensory neurons by using fluorescence *in situ* hybridization (Fig 2, *B-D*), immunofluorescence (Fig 2, *E* and see Fig E1, *B-H*), and qPCR performed on fluorescence-activated cell sorting–purified $TRPV1^+$ JNC neurons ($TRPV1^{cre}$::Td-$Tomato^{fl/wt}$ mice [see Fig E1, *I*]). As in the coculture setting (see Fig E1, *A*), we found that FcεR1α/γ transcript and protein levels were expressed in naive mouse JNC neurons but this level is further increased in neurons from allergen-sensitized mice (Fig 2, *B-E* and see Fig E1, *B-I*).

FcεR1-expressing vagal nociceptors respond to immune complexes

To assess whether vagal sensory neurons respond directly to IgE-OVA complexes, we exposed primary JNC neuron cultures to a range of concentrations of IgE, OVA, and IgE-OVA complex (IC) and tested calcium flux as a proxy of neuronal activation. Approximately 12% to 15% of isolated neurons from a sensitized mouse responded to the allergen-antibody complex (a >10% increase in Fura-2AM fluorescence), with a significantly lower response in nonsensitized JNC neurons (Fig 3, *A*). The increased immune complex sensitivity of JNC neurons from isolated allergen-sensitized mice was confirmed in $Na_V1.8^{Cre}$::G-$CaMP6^{fl/wt}$ mice and Fura-2AM–loaded neurons (see Fig E2, *A* in the Online Repository at www.jacionline.org). Immune complex–responsive neurons were mostly small-diameter mustard oil– and capsaicin-sensitive neurons (TRPA1- and

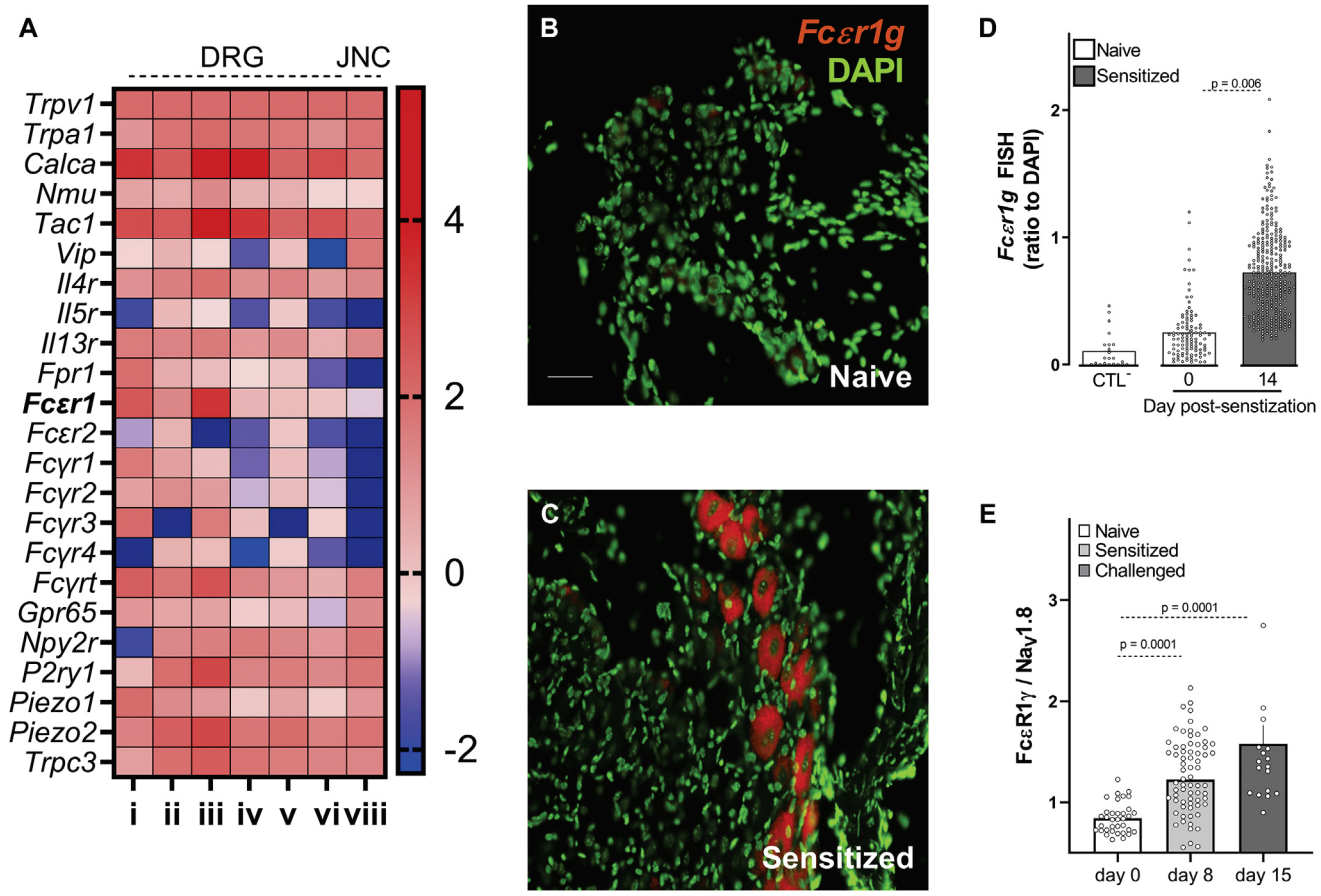

FIG 2. Vagal nociceptors express FcεR1. **A,** A meta-analysis of 7 published nociceptor expression profiling data sets[25] showed basal expression of sensory neuron markers (*Trpv1* and *Trpa1*), neuropeptides (*Tac1, Vip, Nmu, Calca*), asthma-driving cytokine receptors (*Il4rα, Il5rα, Il13rα1*), and the immunoglobulin receptor *Fcer1γ*. Fluorescence *in situ* hybridization (FISH) (**B-D**), and immunohistochemistry (**E**) was used to analyze the levels of FcεR1 transcript and protein expression in JNC neurons (days 0 and 14 [**B-D**]) and days 0, 8, and 15 [**E**]). The data revealed that these levels were increased in allergen-sensitized mice neurons relative to those in naive mice (**B-E**). Data are shown as means ± SEMs. **D-E,** Two-tailed unpaired Student *t* test. n = 4-8 animals/group; 1-2 cohorts. **A**, RNA sequencing of human lumbar neurons (i)[27]; microarrays of mouse fluorescence-activated cell sorting–sorted $Na_V1.8^+$ neurons (ii)[19]; single-cell RNA sequencing of mouse lumbar neurons (iii and iv)[28,29]; microarray profiling of mouse whole DRG (v)[31]; RNA sequencing of mouse $TRPV1^+$ neurons (vi)[30]; and single-cell RNA sequencing of mouse vagal ganglia (vii).[31] Expression across data sets was ratioed over Trpv1 and multiplied by 100; $\log_{10}$ of these values presented as a heatmap. **B** and **C,** *Fcεr1γ* is labeled in red, and nuclei are labeled in green. Scale bar = 30 μm. *CTL*-, Negative control; *DAPI*, 4′,6-diamino-2-phenylindole.

TRPV1-responsive, respectively [Fig 3, *B-C* and see Fig E2, *B*]). The identity of the responsive neurons as nociceptors was confirmed by using cultured JNC neurons from $TRPV1^{Cre}$::diphtheria toxin $(DTA)^{fl/wt}$ mice or $Na_V1.8^{Cre}$::$DTA^{fl/wt}$ nociceptor-depleted mice, with the response to IgE-OVA absent in the latter and massively reduced in the former (Fig 3, *D*). The IgE-OVA–induced calcium flux was dose-dependent and higher than that produced by exposure to IgE or OVA alone (~1%-4% [Fig 3, *E*]). Immune complex–stimulated allergen-sensitized JNC neurons release SP and VIPs but not CGRP (Fig 3, *F*). IgE or OVA exposure alone had no impact on SP secretion from allergen-sensitized mouse neurons (Fig 3, *F* and see Fig E2, *C*).

Next, neurons isolated from naive and allergen-sensitized mice were cocultured (1:1 mix) to directly compare their responsiveness to immune complex exposure in the same experimental conditions. In this setting, we found approximately 3-fold more IgE-OVA–responsive neurons and a higher amplitude of response in allergen-sensitized neurons from a reporter mouse ($TRPV1^{Cre}$::Td-$tomato^{fl/wt}$) than in nonsensitized (no reporter) naive neurons (see Fig E2, *D* and *E* and Movie E1 [in the Online Repository at www.jacionline.org] showing decay in unbound cytoplasmic Ca^{2+} in response to IgE-OVA).

IgE controls FcεR1 expression

Given the increased immune complex–mediated calcium flux in JNC neurons from sensitized mice, we set out to test which component of allergen sensitization might be responsible for this effect. To do this, we used unbiased single-cell qPCR to probe changes in transcript expression in $TRPV1^+$ JNC neurons (capsaicin-responsive) at various times during the asthma protocol. We found elevated *Fcεr1γ* transcript expression as early as 3 days after sensitization, as well as elevated *Il-5r* and *Tslp-r* levels, which peaked on day 26 (Fig 3, *G*). Given that in addition to

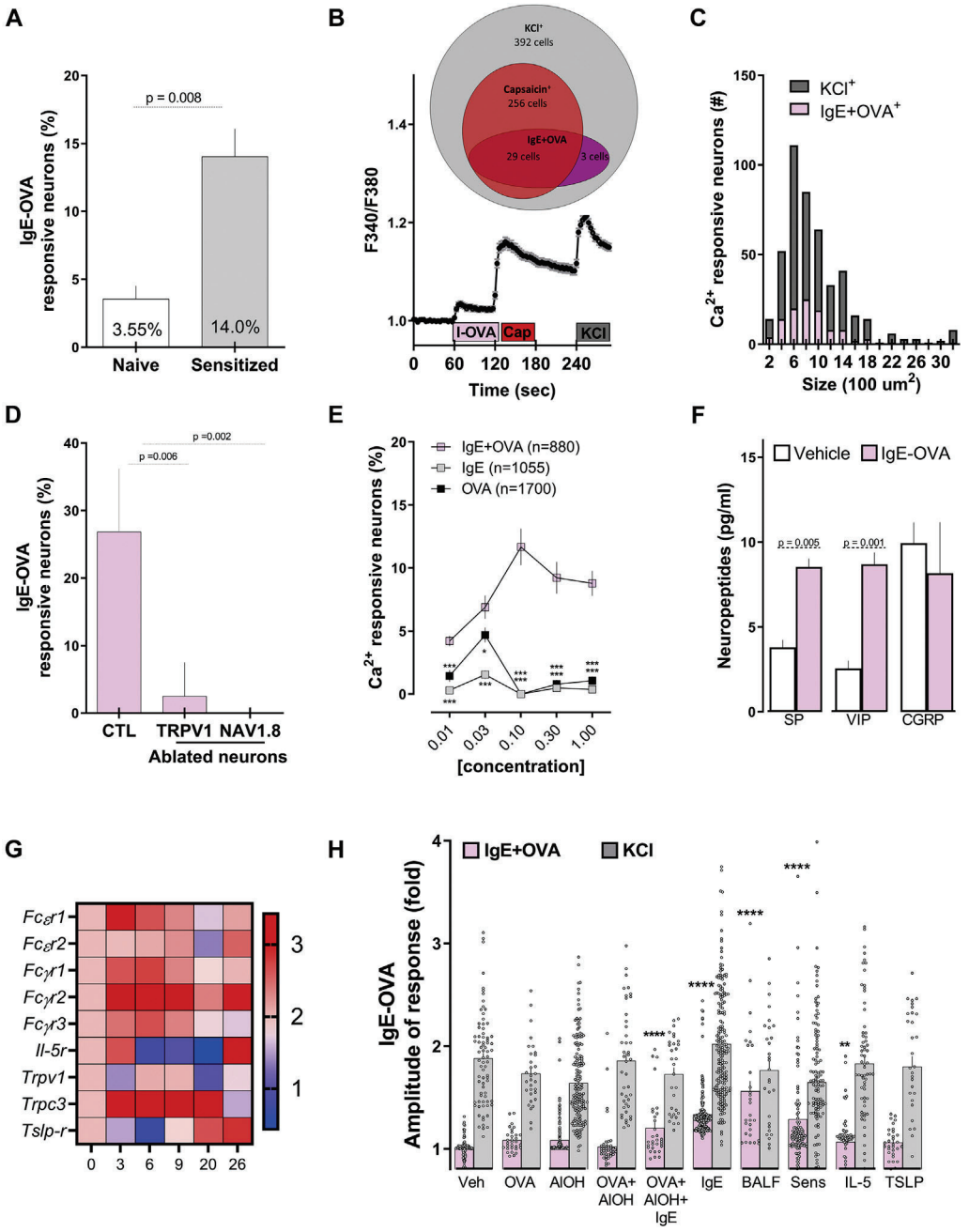

FIG 3. IgE-OVA complex evokes calcium flux in vagal nociceptors. **A,** Ultrapure OVA (1%), when complexed with mouse recombinant IgE (0.001%), triggered larger calcium transients in cultured JNC neurons obtained from allergen-sensitized mice (day 14) than in those obtained from naive mice. **B,** An example of the response evoked in capsaicin-sensitive neurons. **C and D,** IgE-OVA-induced calcium influx was mostly observed in small-sized neurons and capsaicin-sensitive neurons and was absent in cultured neurons obtained from allergen-sensitized (day 14) TRPV1 ($TRPV1^{cre}$::$DTA^{fl/wt}$) or $Na_V1.8$ ($Na_V1.8^{cre}$::$DTA^{fl/wt}$) nociceptor ablated mice. **E and F**, The IgE-OVA complex evoked (in a dose-dependent manner) a calcium response greater than that for IgE or OVA alone (**E**) and triggered the release of SP and VIP (**F**). G, Capsaicin-responsive neurons were picked at various time points of the allergy induction protocol and analyzed by single-cell qPCR. Allergen sensitization transiently drove the expression of *Fcεr1*, which peaked on day 3. **H,** Compared with naive vagal neurons, we found an increased number of immune complex–responsive neurons in cells pretreated (24 hours) with IgE, OVA plus AlOH plus IgE, IL-5, or asthmatic BALF or harvested from allergen-sensitized (day 14) mice. Data are shown as means ± SEMs. **A**, **D**, and **F**, Two-tailed unpaired Student *t* test. **H,** Two-tailed Mann-Whitney *U* test. **E,** One-way ANOVA and *post hoc* Bonferroni correction. n = 3 to 8 animals/group; 2 or 3 cohorts. **B,** IgE-OVA (1% + 0.001% [60-75 seconds]) is represented by the pink box, capsaicin (1 μM [120-135 seconds]) is represented by the red box, and the gray box represents KCl (40 mM [240-255 seconds]). Relative gene expression was ratioed over day 0 and multiplied by 1000; log10 of these values presented as a heatmap (**G**). *CTL*, Littermate control.

immunologically "arming" mast cells to undergo antigen-mediated activation, IgE stabilizes and enhances FcεR1 expression on immune cells[32-34]; we posited that circulating IgE might be responsible for the neuronal expression of FcεR1. We tested whether immunoglobulins, interleukins, the adjuvant, the model allergen, or a combination of these induced the expression of functional FcεR1 in primary cultured JNC neurons. Although potassium chloride (KCl) responses were similar across the various treatment groups, we found an increased response to the immune complex when JNC neurons were preexposed to IgE, IgE plus OVA plus AlOH, or BALF extracted from a mouse with asthma (Fig 3, *H*). As expected, allergen sensitization before harvesting of the neurons had a similar effect (Fig 3, *H*). Interestingly, human induced pluripotent stem cell–derived nociceptor neurons that were preexposed to IgE for 24 hours also showed a calcium flux (11%) in response to the immune complex, an effect that was absent in non–IgE-exposed human induced pluripotent stem cell–derived nociceptor neurons (see Fig E2, *F* and *G*). These findings reveal the context-dependent expression of FcεR1 on nociceptor neurons, which is modulated by IgE after allergen sensitization, highlighting a linkage between plasma B cells and vagal nociceptors.

Immune complex modulation of neuron excitability

To probe whether the immune complex modulates nociceptor neurons' excitability, we first identified those allergen-sensitized cultured JNC neurons that responded to the immune complex with a calcium flux (see Fig E3, *A* in the Online Repository at www.jacionline.org). We then tested their electrophysiological response to the complex by whole cell patch recording. Despite both groups having similar baseline resting membrane potentials (~60 mV), the responder group became depolarized when reexposed to the allergen-antibody complex (8 of 8) (Fig 4, *A*), an effect that was virtually absent in the nonresponder group (present in only 1 of 24) (Fig 4, *B*). Furthermore, the depolarization was sufficient to evoke action potential firing in some allergen-antibody–sensitive neurons (3 of 8 [Fig 4, *C*]), an effect that was never observed when the cells were treated with OVA or IgE alone (Fig 4, *D*).

To test whether allergen sensing by vagal nociceptors increases lung allergic responses, we first used whole nerve electrophysiologic recording of vagal neurons to check whether immune complexes were sensed *in vivo*. Similar to the findings in an *in vitro* setting, allergen-sensitized wild-type mice showed vagal compound action potential responses to the immune complex when injected intraperitoneally. Importantly, a similar effect was also found in allergen-sensitized mast cell–depleted mice (c-Kit$^{W\text{-}sh/W\text{-}sh}$),[35] supporting a mast cell–independent effect. The immune complex had no effect in naive wild-type mice or allergen-sensitized FcεR1γ$^{-/-}$ mice (Fig 4, *E* and see Fig E3, *B-E*).

The FcεR1–transient receptor potential cation channel subfamily C member 3 axis controls IC-induced calcium flux

To specifically explore whether activation of vagal nociceptors by the IgE-OVA complex occurred though FcεR1, we identified small-diameter (≤25 μm) immune complex–responsive and immune complex–nonresponsive neurons in culture from sensitized and challenged mice (see Fig E3, *A*) and then profiled them by using single-cell qPCR. The responsive neurons showed higher *Fcεr1* transcript expression than that in nonresponsive neurons, even though both expressed similar transcript levels of *Trpv1* and *Trpa1* (Fig 4, *F*). Next, we used FcεR1α null mice to confirm the role of FcεR1 in sensing the immune complex. We found that although cultured JNC neurons from FcεR1α$^{-/-}$ mice respond normally to KCl or capsaicin, they were virtually insensitive to the immune complex (see Fig E4, *A-D* in the Online Repository at www.jacionline.org). Similarly, 2 different FcεR1α-blocking antibodies abolished the neuronal response to the immune complex, but not to KCl or capsaicin (MAR1 clone [see Fig E4, *E*] and CRA1 clone [see Fig E4, *F*]).

Immune complex–induced calcium flux was absent when allergen-sensitized neurons were cultured in the absence of extracellular calcium (see Fig E4, *G* and *H*) and when treated with the nonselective TRP channel blocker ruthenium red (see Fig E4, *I*) or with a transient receptor potential cation channel subfamily C member 3 (TRPC3) antagonist (see Fig 4, *J* and *K*). Similar to the IgG-antigen complex activation of lumbar dorsal root ganglion (DRG) neurons[36] and in accordance with their co-expression in a subset of peptidergic neurons (see Fig E5 in the Online Repository at www.jacionline.org),[37-39] our data indicate that the allergen-IgE complex produces a calcium influx through an FcεR1-TRPC3 axis.

Neuron-sensing allergen helps prime T_H2 cell activity

Although detection of allergen immune complexes by vagal neurons constitutes a novel feature of danger detection and may elicit sensations and reflexes, we tested whether such early sensing also affects local immune responses. To do this, we focused on ILC2s and T_H2 cells, which are early drivers of AAI that are modulated by sensory neurons.[14,40,41] T_H2 cells (Fig 5, *A* and see Fig E6, *A* and *B* in the Online Repository at www.jacionline.org) or BALF CD4 T (cells Fig 5, *B*) harvested from OVA-challenged mice showed increased *Tacr1* transcript expression compared with the corresponding cells from naive mice. Therefore, these cells are poised to respond to nociceptor cues.

Splenocyte-harvested naive CD4$^+$ T cells were polarized into T_H2 cells. In the presence of peptidase inhibitor, the T_H2 cells were then either exposed to IgE-OVA, cocultured with JNC neurons from allergen-sensitized mice before stimulation with IgE-OVA, exposed to conditioned media harvested from IgE-OVA stimulated allergen-sensitized JNC neurons (1:2 dilution), or exposed to SP (1μM). T_H2 cell activation was then profiled by flow cytometry. Compared with IgE-OVA–stimulated T_H2 cells, T_H2 cells cocultured with IgE-OVA–stimulated neurons, T_H2 cells exposed to IgE-OVA–induced neuron-conditioned media, and T_H2 cells exposed to SP showed increased levels of IL-5$^+$ (Fig 5, *C*), IL-13$^+$ (Fig 5, *D*), and IL-5$^+$IL-13$^+$ (Fig 5, *E*). Also, SP or JNC neuron coculture increased T_H2 expression of IL-4$^+$IL-13$^+$ (see Figs E6, *C* and E7 in the Online Repository at www.jacionline.org). These treatments had no impact on the proportion of GATA3$^+$ and IL-4$^+$ in CD4$^+$ T cells (see Figs E6, *E* and *F* and E7). T_H2 cell polarization was also unaffected when cocultured with naive JNC neurons (see Fig E8 in the Online Repository at www.jacionline.org). Whereas ILC2s also express various neuropeptide receptors (see Fig E9, *A* in the Online Repository at www.jacionline.org), IL-7–stimulated

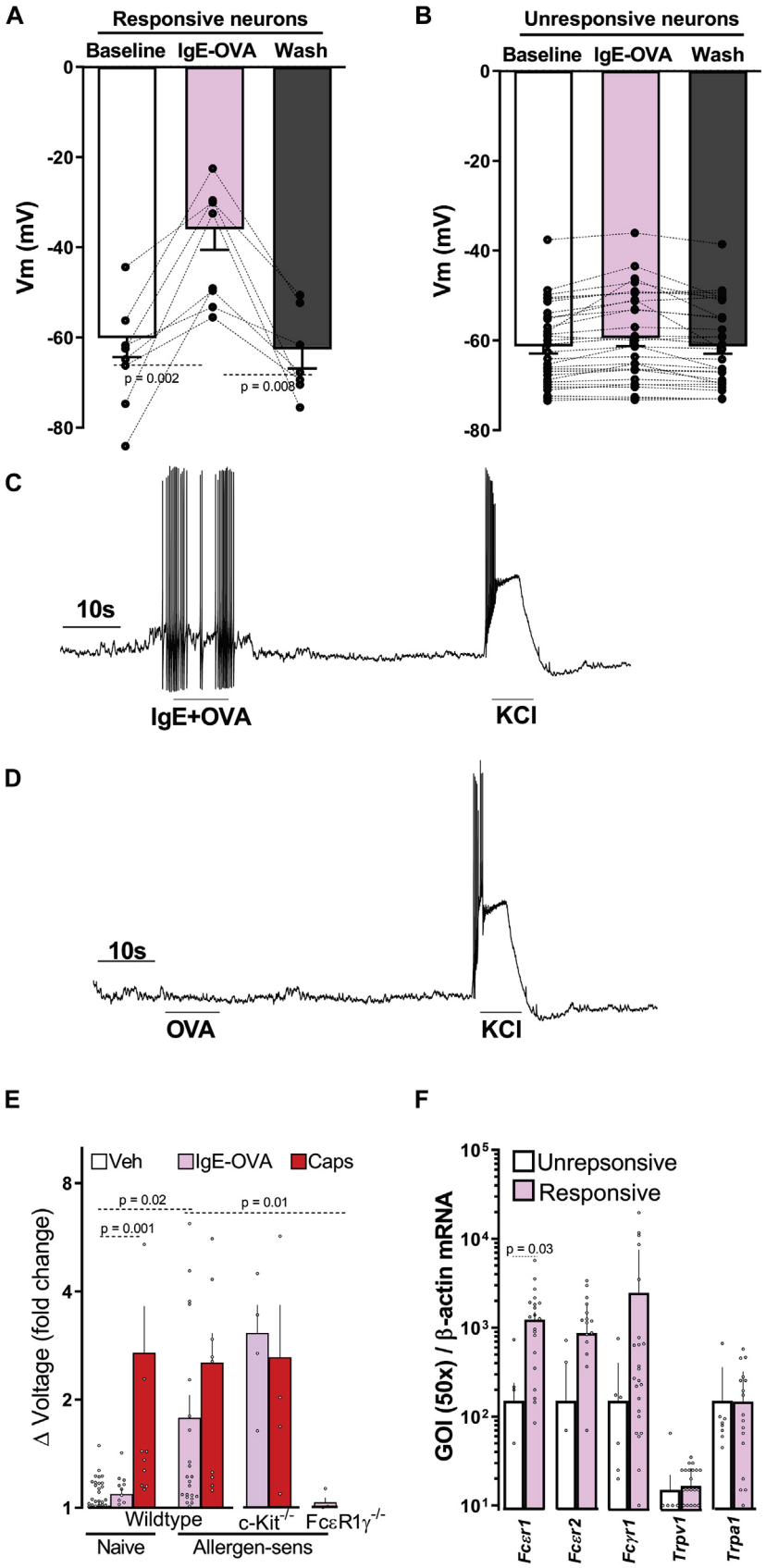

FIG 4. Direct detection of allergens by nociceptors. **A-C,** Compared with IC-unresponsive neurons, IgE-OVA–sensitive neurons (day 14) showed increased depolarization of the membrane potential (**A** and **B**) and action potential firing following IgE-OVA reexposure (**C**). **D,** OVA alone did not induce action potential firing. **E**, Whole nerve electrophysiology was used to assess *in vivo* allergen sensing by vagal neurons and

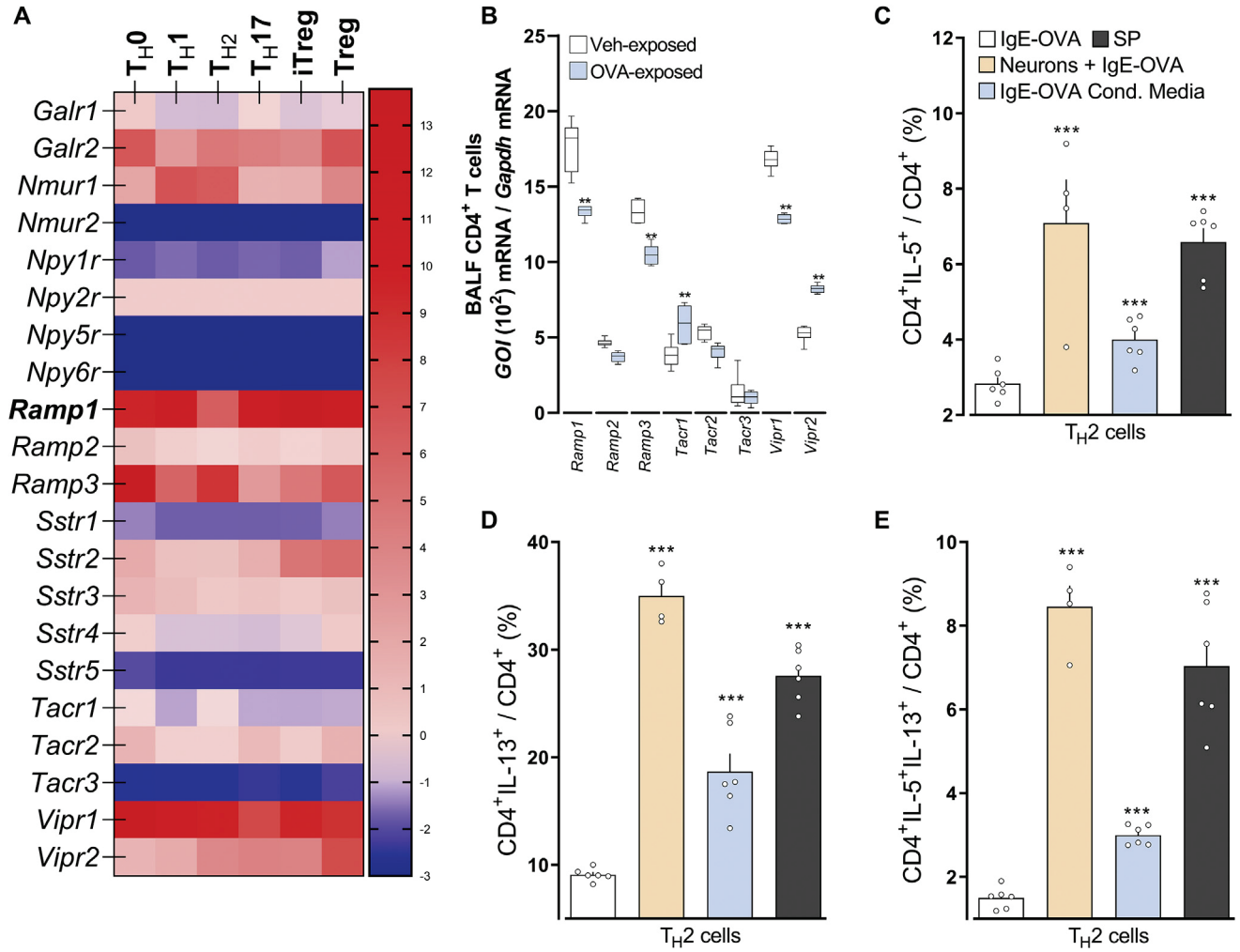

FIG 5. Following allergen sensing, FcεR1$^+$ nociceptor neurons drive T_H2 cell polarization. **A** and **B,** Compared with naive, T_H1, T_H17, induced regulatory T, and regulatory T cells (**A**) or naive CD4 T cells (**B**), T_H2 cells (*in silico* analysis of Immgen's RNA sequencing data [**A**]) or BALF CD4 T cells harvested from OVA-challenged mice (day 15-16 [**B**]) showed increased *Tacr1* transcript expression (**A** and **B**). **C-E,** Purified naive CD4 spleen cells were polarized into T_H2 cells, after which they were cocultured with allergen-sensitized mice JNC neurons (*beige bar*) or stimulated with conditioned media (Cond.) harvested from IgE-OVA-stimulated allergen-sensitized JNC neurons (*blue bar*) or with SP (*gray bar* C-E). Compared with IgE-OVA–stimulated T_H2 cells, T_H2 cells cocultured with neurons and then stimulated with neuron-conditioned media or SP showed increased levels of IL-5$^+$ (**C**), IL-13$^+$ (**D**), and IL-5$^+$IL-13$^+$ cells (**E**). Data are shown as means ± SEMs. **B-E,** Two-tailed unpaired Student *t* test. **A,** Transcript expression is shown as DESeq2 normalized counts. **C-E,** Stimulation and coculture were done in the presence of a cocktail of protease inhibitors (1/1000). n = 4-6 animals/group; 1-2 cohorts.

lung ILC2s were insensitive to both IgE-OVA–triggered neuron conditioned media and SP (see Fig E9, *B-D*).

Allergen sensing by vagal nociceptors amplifies allergic lung inflammation

To assess the impact of vagal neuronal sensing of allergens, we transplanted C57BL6 (BL6) bone marrow into whole body–irradiated BL6 (BL6→BL6) and FcεR1α$^{-/-}$ (BL6→FcεR1α$^{-/-}$) mice. We found that spleen CD45$^+$ immunocytes had a similar expression of FcεR1α (see Fig E10, *A* in the Online Repository at www.jacionline.org) in allergen-sensitized BL6→FcεR1α$^{-/-}$ chimeric mice and that the JNC neurons were less sensitive to immune complex (see Fig E10, *B*). We also found that 1 day after an acute OVA challenge, allergen-sensitized BL6→FcεR1α$^{-/-}$ chimeric mice had reduced numbers of BALF-infiltrating total

revealed that allergen-sensitized wild-type and mast cell–depleted mice (c-Kit$^{-/-}$) demonstrated compound action potential when exposed to IgE-OVA (0.001%-1%, intraperitoneally), which are effects that were absent in naive or allergen-sensitized FcεR1γ$^{-/-}$ mice. Capsaicin (1 μM) showed compound action potential in all tested mice lines. **F,** IgE-OVA responsive neurons overexpressed *Fcεr1* transcript, as evidenced by single-cell qPCR. Data are shown as means ± SEMs. **A, B,** and **F,** Two-tailed paired (**A** and **B**) or unpaired (**F**) Student *t* test. **E,** One-way ANOVA and *post hoc* Bonferroni correction. n = 3 to 24 animals/group; 1 to 8 cohorts. *GOI*, Gene of interest; *Veh*, vehicle; *Vm,* membrane potential.

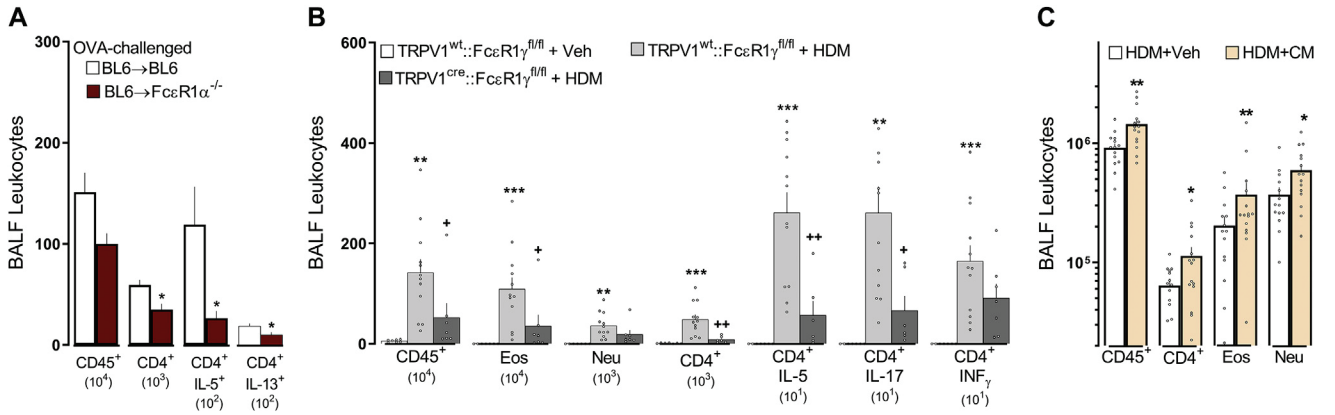

FIG 6. FcεR1$^+$ nociceptor neurons initiate type 2 airway inflammation. **A,** One day after an acute OVA challenge, allergen-sensitized FcεR1α$^{-/-}$ chimeric mice (BL6→FcεR1α$^{-/-}$) displayed a reduced level of AAI (lowered numbers of BALF-infiltrating CD4$^+$ cells) and polarization (IL-5$^+$ and IL-13$^+$) in comparison with the allergen-sensitized control (BL6→BL6) mice. **B,** Next, we used the cre/lox toolbox to generate the first cell-specific FcεR1γ knockdown (TRPV1^{Cre}::FcεR1γ$^{fl/fl}$) and littermate control (TRPV1^{wt}::FcεR1γ$^{fl/fl}$) mice. The TRPV1^{Cre}::FcεR1γ$^{fl/fl}$ mice were protected from HDM challenge–induced BALF infiltration of CD45$^+$ cells, eosinophils, and CD4$^+$ cells, as well as from CD4 polarization (IL-5 and IL-17). **C,** HDM-challenged mice exposed to neuron-conditioned media harvested from IgE-OVA–stimulated allergen-sensitized JNC neurons demonstrated increased AAI (BALF influx of CD45$^+$ cells, CD4$^+$ cells, eosinophils, and neutrophils). Data are shown as means ± SEMs. **A** and **C,** Two-tailed unpaired Student *t* test. **B,** One-way ANOVA and *post hoc* Bonferroni correction. n = 5 to 14 animals/group; 1-2 cohorts. *CM,* Conditioned media; *Veh,* vehicle.

CD4$^+$, and CD4$^+$IL-5$^+$ cells compared with control chimeric mice (Fig 6, *A*). These findings support a key upstream role for FcεR1$^+$ nonhematopoietic cells (eg, nociceptors) in inducing AAI.

To directly pinpoint a role for FcεR1γ$^+$ nociceptors from radio-resistant hematopoietic cells, we used cre/lox technology to generate nociceptor-specific FcεR1γ knockdown (TRPV1^{Cre}::FcεR1γ$^{fl/fl}$) mice. The TRPV1^{Cre}::FcεR1γ$^{fl/fl}$ mice showed a 3.2-fold decrease in HDM-induced AAI (BALF numbers of CD45$^+$ cells, eosinophils, and total and IL-5$^+$ CD4$^+$ T cells [Fig 6, *B* and see Figs E12 and E13 in the Online Repository at www.jacionline.org), as well as BALF cytokines levels (IL-13 [see Fig E11, *A* in the Online Repository at www.jacionline.org]) when compared with littermate control mice (TRPV1^{wt}::FcεR1γ$^{fl/fl}$). Heterozygote knockout mice (TRPV1^{Cre}::FcεR1γ$^{fl/wt}$) also showed a, albeit lower, reduction in HDM-induced AAI (~1.58-fold) and OVA-induced AAI (~1.65-fold [see Fig E11, *B* and *C*]). We then tested whether the nocifensive behavior of the TRPV1^{cre} mice remained intact. We confirmed that cre recombinase expression in TRPV1$^+$ neurons had no impact on the thermal nociceptive threshold of the mice or on the neurons' sensitivity to noxious stimuli (see Fig E14 in the Online Repository at www.jacionline.org).

An *in silico* analysis of 5 mouse expression profiling data sets (single-cell RNA sequencing, bulk RNA sequencing, and microarray [see Figs E15-E18 in the Online Repository at www.jacionline.org]) revealed that TRPV1 is not expressed by airway stromal or immune cells (including T_H2 and mast cells). In addition, TRPV1^{cre}FCεR1γ$^{fl/fl}$ peritoneal mast cells degranulate normally in response to IgE-OVA (see Fig E18, *C*). TRPV1^{cre}DTA$^{fl/wt}$ mice have the same proportion of peritoneal mast cells as littermate control mice (see Figs E18, *D* and E19 in the Online Repository at www.jacionline.org), whereas flow cytometry analysis of peritoneal mast cells from TRPV1^{cre}Td-tomato$^{fl/wt}$ mice showed no cre expression (see Figs E18, *E* and E20 in the Online Repository at www.jacionline.org). Although the TRPV1^{cre}FCεR1γ$^{fl/fl}$ mouse T_H2 and mast cells were intact, their JNC neurons did not express *FCεr1γ* following *in vivo* allergen sensitization (see Fig E21, *A-C* in the Online Repository at www.jacionline.org), nor did they respond to IgE-OVA (see Fig E21, *D*). However, capsaicin-induced calcium flux responses were still intact (see Fig E21, *E*).

The reduction in the influx of lung immunocytes in TRPV1^{Cre}::FcεR1γ$^{fl/fl}$ mice was accompanied by similar levels of IgE (not shown), suggesting that although the capacity to mount an allergic antibody response was present in these mice, AAI was reduced. The diminished inflammatory phenotype in the TRPV1^{Cre}::FcεR1γ$^{fl/fl}$ mice was phenocopied by mice whose TRPV1$^+$ sensory neurons were genetically ablated (TRPV1^{Cre}::DTA$^{fl/wt}$ [see Fig E22, *A-C* in the Online Repository at www.jacionline.org]). Although the specific knockdown of FcεR1γ on TRPV1$^+$ sensory neurons did not prevent the polarization of ILC2s (not shown), the genetic ablation of TRPV1$^+$ neurons did (see Fig E22, *D-F*). Finally, coexposing HDM-challenged mice on days 8 and 9 to neuron-conditioned media (supernatant from immune complex–stimulated allergen-sensitized JNC neurons) further augmented the HDM-evoked increases in BALF CD45$^+$ cell, CD4$^+$ cell, eosinophil, and neutrophil cell numbers (Fig 6, *C*).

DISCUSSION

Sensory neurons' involvement in AAI was first suggested by the finding that TRPA1 genetic ablation reversed OVA-induced AAI.[42] How are these neurons activated? IL-5, a type II effector cytokine produced by multiple immune cells during allergy, directly activates airway sensory neurons, leading to the secretion of the neuropeptide VIP. In turn, VIP acts on ILC2 and CD4$^+$ cells to induce cytokine production, including IL-5 and IL-13, to initiate a positive feedforward proinflammatory cycle.[14,15,43] In

parallel, NMU (the ligand of NMUR1) activates ILC2s. Acting with IL-25, it also amplifies allergic inflammation.[40,41,44] In the airway, NMU is found exclusively in lung afferent DRG neurons. A loss of NMU-NMUR1 signaling reduces ILC2 function by altering transcriptional programs following an allergen challenge in vivo, and this loss of signaling prevents the development of AAI.[40]

What sets off the neuroimmune cycle in the setting of AAI? Does it begin with the activation of immune cells or nociceptors by allergen exposure in sensitized animals? The data presented here point to an upstream detection role of allergen-IgE complexes by nociceptors in driving T_H2 cell activation and downstream allergic inflammation. Following allergen sensitization, immune complex sensing by FcεR1γ$^+$ nociceptors sets off the airway neuroimmune interactions that trigger and maintain AAI.

JNC neurons express FcεR1

We identified a subset of mouse JNC neurons that express functional FcεR1. Studies have reported neuronal-Ig interactions in Alzheimer disease, chronic pain, arthritis-induced hypersensitivity, allergy, and antigen-specific autoimmune diseases.[2,3,6,7,45-51] IgG and IgE activate motor neuron release of neurotransmitters and help control antigen trafficking in lymph nodes.[50,52-55] These findings are in line with the expression by neurons of receptors typically expressed by immune cells, such as MHC class I on central nervous system neurons, as well as nociceptor expression of the immune checkpoint receptor PD-1.[56,57]

IgE drives neuronal expression of FcεR1

Sensory neuron responsiveness is highly tunable, changing in response to diverse insults.[15,58] We have now revealed that similar to FcεR1 expression on hematopoietic cells,[32-34,59] IgE drives FcεR1 overexpression in cultured murine JNC nociceptors and in human induced pluripotent stem cell–derived nociceptors. FcεR1 is also upregulated in the abdominal vagus nerve in mice with food allergy,[54] in the trigeminal ganglion neurons of allergen-sensitized mice,[8] and in lumbar DRG neurons following exposure to ragweed pollens[2] or during cutaneous anaphylaxis.[48] Overall, these data support a mechanistically conserved mechanism for the neuronal sensing of immune complexes during allergy.

TRPC3 mediates IC-FcεR1–induced calcium influx

Immune complex sensing leads to TRPC3-dependant calcium influx and the subsequent release of neuropeptides. TRPV1, TRPC3, and FcεR1 are coexpressed in nociceptors, as revealed by the single-cell RNA sequencing of sciatic nerve crushed mouse peptidergic neurons (cluster 2).[39] The FcγR-syk-TRPC3 axis is also responsible for IgG-IC–induced hyperalgesia.[36]

Nociceptor-ILC2 crosstalk

ST2$^+$ ILC2s express transcripts for *Ramp1, Nmur1,* and *Tacr1*. Previous reports have shown that vagal neurons produce VIP[14,60] and lumbar neurons release NMU, both of which control ILC2 activation.[40,41,44] In addition, although CGRP supports ILC2 production of IL-5,[61] it also inhibits alarmin-driven type 2 cytokine production, constrains IL-13 expression, and blocks ILC2 proliferation.[61-63] We found here that the complete ablation of peptidergic neurons (in TRPV1^{Cre}::DTA$^{fl/wt}$ mice) prevents type 2 cytokine production and infiltration of ILC2s. Such findings phenocopy our earlier data obtained with ablation of all nociceptors in $Na_V1.8^{Cre}$::DTA$^{fl/wt}$ mice or nociceptor silencing pharmacologically with QX-314.[14] Although immune complex sensing promotes SP and VIP (but not CGRP) production, IgE-allergen complex–stimulated JNC neurons fail to modulate ILC2 function. The level of immune complex-FcεR1–induced VIP release (0.01 ng/mL) is, however, lower than that produced by IL-5 (0.8 ng/mL) or capsaicin (40 ng/mL). In addition, the ILC2s used here were only costimulated with IL-7, while other studies found that a broader costimulation, including with IL-25 and IL-33, is necessary for these cells to respond to CGRP and NMU. This result suggests that neuron-ILC2 crosstalk requires the maximal engagement of multiple peptidergic neurons to trigger a broad release of neuropeptides, including NMU and CGRP, for their activation, and that such a pattern of neuropeptide release may not occur subsequent to allergen sensing.

Neuropeptides drive T_H2 polarization

Conditioned media harvested from IgE-OVA–stimulated JNC neurons obtained from allergen-sensitized mice drives T_H2 cell polarization *in vitro* and enhances HDM-mediated airway inflammation with increased BALF influx of CD45$^+$ cells, CD4$^+$ cells, and eosinophils *in vivo.* The neuronal detection of immune complexes leads to the release of SP and VIP in the mucosa,[64,65] which can facilitate the local influx and the polarization of these immune cells. Indeed, T_H2 cells and OVA-exposed BALF CD4$^+$ T cells express the receptors for these peptides (ie, *Vipr2* and Tacr1). Although we previously found that recombinant VIP increased T_H2 cell activation and polarization, we have now shown that the SP-Tacr1 axis increases production of IL-5$^+$, IL-13$^+$, and IL-5$^+$IL-13$^+$ T_H2 cells *in vitro*. In addition, we also have found that SP contributes to mucus metaplasia.[1] Overall, all of our current and past data indicate that antigen detection by lung nociceptors sets off systemic defensive reflex responses such as cough or mucus secretion[17] and, by mobilizing T_H2 cells, prompt engagement of the adaptive immune system.

Neurons control AAI

Fcεr1γ acts as an ITAM adapter protein, transducing signals of immunoreceptors such as Fc receptors and IL-3R.[66,67] Consistent with the immune complex sensing by FcεR1 nociceptors as a major upstream driver of T_H2-mediated AAI, we found that nociceptors lacking FcεR1γ (TRPV1^{cre}::FcεR1γ$^{fl/fl}$) are protected from IgE-OVA–induced calcium flux. In addition, mice whose nociceptors lack FcεR1γ are protected from airway inflammation induced by OVA (a classic mouse model of allergic asthma) and HDMs (a more clinically relevant model of patient allergy). These effects phenocopy the findings that we observed in irradiated FcεR1α knockout mice into which bone marrow had been transplanted (a model resistant to IgE-induced systemic anaphylaxis). In addition, the detection of allergens by nociceptors preceded an immune response because silencing nociceptors before the allergen challenge reduced the inflammation. *In vivo* immune complex challenges in sensitized mice triggered action potentials in vagal neurons within 5 minutes of exposure, which is an effect independent of mast cells. These findings are similar to that regarding the role of IL-4R$^+$ skin nociceptors in atopic

dermatitis–induced skin inflammation via infiltration of T_H2 cells and basophils.[9]

TRPV1 does not affect mast cell function

Along with the irradiated mice into which bone marrow had been transplanted, we generated nociceptors lacking FcεR1γ by using the well-characterized TRPV1^{cre} mouse line.[15,17,42,68-74] Whereas immortalized human mast cells (from mast cell leukemia) might bear TRPV1, detailed genetic findings reveal that mouse mast cells do not express this ion channel in functional assays[74] and that mouse mast cells do not express either the TRPV1 transcript or protein,[69,75-77] do not degranulate in response to heat[76] or capsaicin,[76,78] and are insensitive to resiniferatoxin chemoablation, which occurs through TRPV1 activation.[75]

From 5 unbiased expression profiling data sets (microarray, bulk RNA sequencing, and single-cell RNA sequencing), we have now confirmed that mouse mast cell populations do not express TRPV1. In addition, peritoneal mast cells from TRPV1^{cre}FCεR1γ$^{fl/fl}$ mice degranulate normally in response to IgE-OVA and TRPV1^{cre}DTA$^{fl/wt}$ mice have the same proportion of peritoneal mast cells as controls, whereas flow cytometry analysis of peritoneal mast cell from TRPV1^{cre}Td-tomato$^{fl/wt}$ mice shows no Cre expression. Overall, although an indirect activation of nociceptors by mast cell–produced histamine is likely,[79] our data suggest that the earliest activation of nociceptors by allergens is actually direct, occurring via the immune complex. Both IgE-OVA and capsaicin (TRPV1 agonist) induce vagal nerve compound action potentials *in vivo,* and this activity is present in mast cell–depleted mice (c-Kit$^{W\text{-}sh/W\text{-}sh}$)[30] but absent in the FCεR1γ$^{-/-}$ mouse.

Immunologic sensitization drives B cells to initiate antibody class switching, the generation of memory B cells, and the release of specific immunoglobulins.[80] After antigen recall, the specific antibodies bind to Fc receptors on innate immune cells, allowing for a swift and precise response.[81] We have now uncovered a novel aspect of allergen detection, namely, that vagal nociceptors expressing FcεR1 detect allergens complexed to IgE, and leads to the activation of lung T_H2 cells. Preventing immune complex sensing by FcεR1 nociceptors reduces antigen-induced airway inflammation. These findings reveal a novel potential therapeutic avenue for the early treatment or prevention of allergic airway disease that targets direct sensory neuron activation by allergens in immune complexes.

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Key messages

- **Vagal nociceptor neurons overexpress FcεR1γ during allergy.**
- **FcεR1γ-expressing neurons sense invading allergens, which initiate allergic inflammation.**
- **Nociceptor neuron–released SP drives T_H2 cell polarization.**

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XI - Article 4. IL-13 promotes sensory-sympathetic neurons crosstalk in asthma

Crosson T, Bhat S, Wang JC, Salaun C, Roversi K, Herzog H, Rafei M, Blunck R and Talbot S. Under review, Journal of Clinical investigation, uploaded on BiorXiv.

In this publication, using transcriptomics and a screening approach, we observed that two major type-2 cytokines, IL-13 and IL-4, induce gene expression changes in airway specific vagal nociceptors during asthma.

The student and supervisor designed the study. The student performed most of the experiments and analysis with the support of other lab members. The electrophysiology experiments on dissociated neurons were performed by S. Bhat. The western blots and some immunofluorescence stainings were performed by C. Salaun. Read mapping and DESeq2 analysis of RNA-seq data were performed by the bioinformatic core of the IRIC genomic platform. The student and supervisor drafted and proofread the manuscript.

Abstract:
Nociceptor neurons play a crucial role in maintaining the body's equilibrium by detecting and responding to potential dangers in the environment. However, this function can be detrimental during allergic reactions, since vagal nociceptors can contribute to immune cell infiltration, bronchial hypersensitivity, and mucus imbalance, in addition to causing pain and coughing. Despite this, the specific mechanisms by which nociceptors acquire pro-inflammatory characteristics during allergic reactions are not yet fully understood. In this study, we aimed to investigate the molecular profile of airway nociceptor neurons during allergic airway inflammation and identify the signals driving such reprogramming. Using retrograde tracing and lineage reporting, we identified a class of inflammatory vagal nociceptor neurons that exclusively innervate the airways. Using an ovalbumin mouse model of airway inflammation, we found that these neurons undergo significant reprogramming characterized by the upregulation of the NPY receptor Npy1r, along with Il6. A screening of asthma-driving cytokines revealed that IL-13 drives part of this reprogramming, including Npy1r overexpression via the JAK/STAT6 pathway, while IL-1β induces IL-6 expression and release. Additionally, we observed that sympathetic neurons release NPY in the bronchoalveolar fluid of asthmatic mice, which limits the excitability of nociceptor neurons. In summary, allergic airway inflammation reprograms airway nociceptor neurons to acquire a pro-inflammatory phenotype, characterized by the release of IL-6, while a compensatory mechanism involving NPY1R limits nociceptor neurons' activity.

IL-13 promotes sensory-sympathetic neurons crosstalk in asthma.

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SHORT TITLE: Cytokines reprogram nociceptor transcriptome.

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ABSTRACT. Nociceptor neurons play a crucial role in maintaining the body's equilibrium by detecting and responding to potential dangers in the environment. However, this function can be detrimental during allergic reactions, since vagal nociceptors can contribute to immune cell infiltration, bronchial hypersensitivity, and mucus imbalance, in addition to causing pain and coughing. Despite this, the specific mechanisms by which nociceptors acquire pro-inflammatory characteristics during allergic reactions are not yet fully understood. In this study, we aimed to investigate the molecular profile of airway nociceptor neurons during allergic airway inflammation and identify the signals driving such reprogramming. Using retrograde tracing and lineage reporting, we identified a class of inflammatory vagal nociceptor neurons that exclusively innervate the airways. Using an ovalbumin mouse model of airway inflammation, we found that these neurons undergo significant reprogramming characterized by the upregulation of the NPY receptor *Npy1r*, along with *Il6*. A screening of asthma-driving cytokines revealed that IL-1β, IL-13 and BDNF drive part of this reprogramming. IL-13 triggered *Npy1r* overexpression in nociceptors via the JAK/STAT6 pathway, while IL-1β induces IL-6 expression and release. Additionally, we observed that sympathetic neurons release NPY in the bronchoalveolar fluid of asthmatic mice, which limits the excitability of nociceptor neurons. In summary, allergic airway inflammation reprograms airway nociceptor neurons to acquire a pro-inflammatory phenotype, characterized by the release of IL-6, while a compensatory mechanism involving NPY1R limits nociceptor neurons' activity.

BACKGROUND. Sensory neurons are heterogenous and can be differentiated based on their expression profiles [1-3], degree of myelination [4], the type of cues to which they are sensitive [4,5], the reflexes that they initiate, their anatomical location [6] or the organ that they innervate [7,8]. For instance, internal organs such as the lungs are innervated by the vagus nerve[9-12], whose sensory neurons originate from the jugular-nodose complex ganglia (JNC).

Pain sensory neurons, also known as nociceptor neurons, form a key line of defense against environmental dangers. They detect a broad range of thermal, mechanical, and chemical threats and respond by means of protective reflexes such as the itch and cough reflexes[13,14]. To fulfill their role in host defense, nociceptors are geared to detect and respond to a variety of danger signals ranging from cytokines to inflammatory lipids, allergens, fungi, cancer cells, bacteria, toxins, and even immunoglobulins[5,15,16]. Via the axon reflex, nociceptors respond by locally releasing neuropeptides. While these peptides can have direct inflammatory and immunomodulatory actions[17-23], nociceptors' impact on inflammation is also indirect, via modulation of the autonomic nervous system[24].

In the context of asthma, vagal nociceptors neurons worsen airway hyperreactivity by promoting bronchoconstriction, coughing, mucus imbalance, and immune cell infiltration[18,25-27]. Yet, the specific mechanisms by which nociceptors are activated and acquire this pro-inflammatory phenotype are not fully

understood. Here we sought to define the molecular profile of airway nociceptor neurons during allergic airway inflammation and to identify the signals driving their reprogramming.

Using retrograde tracing and lineage reporting, we first identified the population of nociceptor neurons that innervates the airways. Using the ovalbumin mouse model of allergic airway inflammation, we found a drastic reprogramming of airway nociceptor neurons characterized by the upregulation of the NPY receptor *Npy1r,* and the cytokine *Il6.* Next, we identified that the asthma-driving cytokine IL-13, via pSTAT6 signaling, drives part of this transcriptional reprogramming. Along with *Npy1r* overexpression, we found that NPY was elevated in the bronchoalveolar fluid of asthmatic mice. In cultured JNC neurons, NPY1R agonist decreased nociceptor neurons cAMP and blunted neuronal excitability.

RESULTS.

A class of inflammatory nociceptor specifically innervates the airways

Via vagal projections, the JNC innervates most visceral organs[28]. Single-cell RNA sequencing datasets revealed that JNC neurons are highly heterogenous[2,3,10] and provide a transcriptome-based neuron subtype classification[2,3,6,29]. While genetically guided optogenetic studies identified JNC neuronal subtypes controlling breathing and tracheal reflexes[1,10,30], additional molecular characterization of airway nociceptors in inflammatory context is lacking.

To address this, we tracked airway nociceptors using the $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ nociceptor neuron reporter mice, in which, we back-labeled airway neurons using the retrograde tracer DiD' (i.n. 200 µM). Two weeks after DiD' injection, the JNC neurons were isolated, and airway nociceptor neurons ($Na_V1.8^+DiD^+$), visceral nociceptor neurons ($Na_V1.8^+DiD^-$) and a mixed population of $Na_V1.8^-$ cells (e.g. satellite glial cells, macrophages, $Nav1.8^{neg}$ neurons, and stromal cells), were purified by flow cytometry (**SF. 1A-B**) and RNA-sequenced (**Fig. 1A-B, SF. 1C**). About 5–10% of JNC nociceptors neurons were labeled with DiD' (**SF. 1B, D**). Principal component analysis (PCA) analysis confirmed that the three cell populations were well segregated (**SF. 1C**).

When compared with visceral nociceptor neurons, *Il6, Kcng1*, *Trpa1, Trpv1,* and *Npy1r* were found to be significantly enriched in airway nociceptor neurons (**Fig. 1A-B**). Calcium imaging of JNC neurons showed that while capsaicin sensitivity was similar across neuronal subpopulations, JT010-mediated TRPA1 activation was drastically increased (2.5-fold) in airway neurons (DiD^+) compared to other visceral neurons (**Fig. 1C, SF. 1E-F**). Using JNC neurons from $NPY1R^{Cre}$::$tdTomato^{fl/wt}$ mice, we also confirmed NPY1R enrichment in the airway population (**Fig. 1D**).

We then mapped out the airway nociceptor neuron subsets within the JNC neuron populations using published scRNAseq data from Prescott and colleagues [1]. We reanalyzed these data using *Vglut2, Scn10a* and *Scn1a* to define clusters of nociceptors and low-threshold sensory neurons[2], and *Phox2b* and *Prdm12* to differentiate between jugular and nodose neurons (**Fig. 1E**, **SF. 2A-D**) We found that the marker genes of one cluster, coined *Nodose Nociceptor 8 (NN8)*, were highly enriched (Normalized Enrichment Score (NES)=2.2) in our airway nociceptor neurons sequencing (**Fig. 1E**).

NN8 appears to exclusively innervate the airways. Several of NN8 markers (*Trpv1, Trpa1*; **Fig. 1E**) are known drivers of neurogenic inflammation in the airways[27,31-34], while this cluster also co-expresses *Npy1r* and *Il6* (**Fig. 1E, SF. 2E-J**). Additionally, we identified three other populations of sensory neurons preferentially innervating the airways (**Fig. 1E)**, including a subset that we assume to be cough-inducing mechanoreceptors (NN7; **Supplementary table 1**). An in-silico analysis of Zhao et al.[7] single cell projection-seq data validated this result (**SF. 3A-H**). Thus, these data confirm that one specific lung-innervating neuron subtype co-express *Scn10a* (gene encoding for $Na_V1.8$), *Npy1r, Kcng1, Trpa1* and *Il13ra1* (**SF. 3C-H**).

Airway nociceptors undergo transcriptional reprogramming in asthma

Next, we sought to test whether the airway nociceptor neuron transcriptome is impacted during allergic airway inflammation (AAI). Retrograde tracer-exposed nociceptor reporter mice ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) underwent the classic ovalbumin (OVA) model of asthma[18]. OVA-exposed mice showed significant airway inflammation characterized by eosinophilic infiltration (**Fig. 2A**), but had a similar number of back-labeled airway nociceptor neurons (**SF. 4A**). As previously defined (**SF. 1A-B**), the JNC neurons were isolated, and airway nociceptor

neurons ($Na_V1.8^+DiD^+$), visceral nociceptor neurons ($Na_V1.8^+DiD^-$) and $Na_V1.8^-$ cells were purified by flow cytometry and RNA-sequenced.

DESeq2 analysis revealed that AAI significantly affected the expression of 92 genes in airway nociceptor neurons (**Fig. 2B-E**), compared with only one and two genes in the visceral nociceptor neurons and $Na_V1.8^-$ cell populations, respectively (**Fig. 2B, SF4. B-C**). Seventeen of these upregulated genes had previously been associated with increased pain or nociceptors activity, while six were observed to dampen pain sensation (**Supplementary table 2**). Within the airway nociceptor neurons, AAI notably increased the expression of *Bdnf, Il6, Kcng1*, *Npy1r*, *Sting1,* and *Trpa1* (**Fig. 2C-D**).

Cytokines trigger gene expression changes in nociceptors

Since the transcriptome changes induced by AAI are restricted to airway nociceptors and are virtually absent in other visceral nociceptors and $Na_V1.8^-$ cells, these variations are likely triggered by a mediator in the airways detected by peripheral nerve endings. To identify this neuromodulator, we exposed (24h) cultured JNC neurons to various allergy driving cytokines, inflammatory lipids, and neurotrophins, and used transcription changes to *Npy1r*, *Sting1*, *Bdnf*, and *Il6* as proxies for an AAI-like signature (**Fig. 2C**). This screening approach revealed that IL-4 and IL-13 triggered the overexpression of *Npy1r* (**Fig. 3A)***,* TNF-α and IL-1β induced *Il6* (**Fig. 3B**)*, Bdnf* was triggered by the neurotrophin itself **(Fig. 3C)**, while *Sting1* was induced by IL-1β, BDNF, TNF-α and IgE mixed with its cognate antigen ovalbumin **(Fig. 3D)**. The other cytokines and neurotrophins tested did not impact the expression of the tested genes.

Cytokines and neurotrophins are known to initiate transcription programs in their target cells[35,36]. We then exposed cultures of JNC from of $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ reporter mice to IL-13, IL-1β, BDNF and TNF-α, before purification of the nociceptor neurons by flow cytometry ($tdTomato^+$) and RNA-sequencing. DEGs were triggered in the four different conditions **(Fig. 3E-I, Supplementary table 3)**, with the most drastic changes induced by IL-1β and BDNF.

A gene set enrichment analysis (GSEA) was performed using the most significantly overexpressed genes (FDR<0.2) in all tested conditions. These data revealed that the genes elevated in cultured JNC neurons exposed to IL-1β, IL-13, and BDNF, as well as the ones previously identified in injured neurons[37], were enriched in JNC nociceptors of AAI mice (**Fig. 3J, Supplementary Table 4**). IL-13 signature showed the strongest enrichment (NES=2.0). Specifically, 11 DEGs identified in AAI neurons were then also induced *in vitro* by BDNF*,* and 9 genes induced by IL-1β, among which 4 were induced by these two proteins (**Fig. 3K)**. IL-13 induced 2 genes identified in AAI, *Npy1r* and *Serpina3i* (**Fig. 3K)**. Morphologically, BDNF affected the neurite outgrowth in cultured JNC nociceptors, while the tested cytokines had no impact (**Fig. 3L)**. These results highlight the similarities and specificities between transcription programs inducible in nociceptor neurons, and suggest that IL-1β, BDNF and IL-13 signaling pathways are complementary to induce the AAI signature in our mouse model of asthma.

Nociceptor neurons produce IL6

IL-1β notably led to a strong *Il6* overexpression in cultured JNC neurons (**Fig. 3B, F**). While neuronal expression of *Il6* has been reported in other types of neurons [38], the expression of this cytokine has not yet been evidenced in JNC nociceptor neurons. Along with the transcription changes that we elucidated here, we confirmed that IL-6 is expressed and released by IL-1β-exposed JNC neurons (**Fig. 4A-C)**, with levels comparable to IL-1β-exposed splenocytes (**Fig. 4C)**.

IL-13 reprograms nociceptors through JAK/STAT6

While IL-1β and BDNF effects on nociceptors has been thoroughly investigated[6,34,39-42], the literature regarding IL-13 is scarcer[14,43], and this interaction was not yet reported in vagal sensory neurons. We then aimed to delineate the mechanisms by which IL-13, a type 2 specific cytokine, activates nociceptor neurons and changes their transcriptome. IL-13 (24h) significantly affected the expression of 47 genes, including *Npy1r* (**Fig. 3A, E, Fig. 5A, Supplementary table 4**). Since both IL-4 and IL-13 and had comparable effects *in vitro* (**Fig. 3A),** we used our transcriptomic profiles to identify which IL-13 and IL-4 receptors are expressed by nociceptor neurons and found that only the IL4RII subunits *Il4ra* and *Il13ra1* are detected in these cells (**SF. 4A, Supplementary table 5**). *Il13ra1* expression was higher in nociceptors than in $Nav1.8^-$ cells and was also

found to be expressed in the inflammatory airway nociceptor cluster NN8 (**Fig. 1E**; **SF. 2I**), along with the signaling mediator *Stat6* (**SF. 2J)**.

Since IL4RII is a shared receptor for IL-13 and IL-4 that signals via JAK1/2 and STAT6 to regulate immune cells' transcription[44], we tested whether a similar mechanism was at play in nociceptor neurons. We found that IL-13 (30min) triggered STAT6 phosphorylation in JNC and DRG neuron cultures (**Fig. 5B-D**). In addition, IL-13-mediated induction of *Npy1r* was prevented by the STAT6 inhibitor AS1517499 as well as by the JAK1/2 inhibitor ruxolitinib (**Fig. 5E**). In the tested conditions, IL-13 did not induce calcium flux in nociceptor neurons (**SF. 5B**), but nevertheless resulted in an increased in *Npy1r* transcript expression in cultured DRG neurons (**SF. 5C**). To see if this pathway is responsible for *Npy1r* overexpression *in vivo,* we treated AAI mice with intranasal instillation of AS1517499 (150µg/50µL). AS1517499 prevented *Npy1r* induction in nociceptor neurons without affecting *Il6* overexpression, confirming that the AAI molecular profile depends in part on pSTAT6 activity **(Fig. 5F)**.

Sympathetic neurons release NPY in the airways during asthma

Npy1r appears as a major marker of AAI inflammation in nociceptor neurons. Since the optogenetic activation of NPY1R^{+} JNC neurons trigger expiratory reflexes in mice [1] and NPY1R activation impacts pain and itching reflexes[45-53], we sought to test whether Neuropeptide Y (NPY)/NPY1R interaction could occur in the lung. Using lung cryosections and immunostainings, we observed a strong and specific expression of NPY in nerve fibers often located around the bronchi (**Fig. 6A-B, SF. 6A-C**). While in proximity, the NPY-expressing neuron fibers were mostly distinct from Na$_V$1.8 positive nociceptor nerve endings (**Fig. 6A-C; SF. 6A-C**).

Using triple-labeling, we found that NPY-expressing neurons colocalized with the sympathetic neuron marker tyrosine hydroxylase (TH; **Fig. 6B-C, SF. 6B-C**). Thus, approximately 40% of sympathetic neurons in the stellate ganglia (SG) express NPY (**Fig. 6D-F**) which is in sharp contrast with virtually no expression in the JNC neurons (**Fig. 6D-F**).

The pattern of sympathetic NPY^{+} lung innervation was comparable between naïve and AAI mice, while bronchoalveolar lavage fluid (BALF) CD45^{+} cell number was drastically increased (**Fig. 7A-E, SF. 6D).** Along with airway inflammation (**Fig. 7F**), allergen challenges (day 18) significantly increased the release of NPY in BALF (**Fig. 7G**) and serum (**Fig. 7H**). Of note, NPY levels were normal upon allergen sensitization (day 1 and 7), and while immune cells were still elevated, the neuropeptide concentration returned to baseline during the resolution phase (day 21; **Fig. 7G-H).** A similar pattern was observed for BALF IL-13 level (**Fig. 7C**).

In the lung, *Npy* RNA increased after allergen challenges and remained elevated during the resolution phase, a pattern comparable to the one of the asthma markers *Muc5ac* and *Il13* (**Fig. 7I**). Finally, *Npy1r* overexpression in JNC nociceptors also transiently peaked upon allergen challenges (**Fig. 7J**). We then tested whether these changes were specific to OVA-induced airway inflammation and found a similar rise in BALF NPY in the house dust mite (HDM) model of asthma (**Fig. 7K**).

NPY1R blunts vagal nociceptor excitability

Given that both *Npy1r* and NPY levels were elevated during airway inflammation, we tested whether blocking NPY1R would impact OVA-induced AAI. As opposed to previous studies[54], the NPY1R inhibitor BIBO3301 did not impact AAI immune cell infiltration in our model (**SF. 7A-B**). Similarly, the conditional knock-out of NPY1R on nociceptor neurons (Na$_V$1.8^{cre}::NPY1R$^{fl/fl}$) did not change the number of immune cells in the BALF (**SF. 7C-D**).

NPY1R is a Gi-coupled receptor[55] and the action of NPY on DRG nociceptor neurons promotes either analgesia or noxious hypersensitivity[45-53]. Given this discrepancy we set out to address how NPY-NPY1R modulates cultured JNC nociceptor neuron sensitivity. To do so, we used whole cell patch clamp recording of NPY1R^{+} JNC nociceptor neurons (NPY1R^{cre}::tdTomato$^{fl/wt}$). We measured electrical changes in response to the NPY1R-specific agonist Leu^{31}Pro^{34}NPY. We observed a significant reduction in the nociceptor neurons' excitability (**Fig. 8A-D**), as measured by a reduced number of action potentials in response to current injection. We also measured a reduced level of intracellular cAMP in cultured JNC neurons exposed to Leu^{31}Pro^{34}NPY (**Fig. 8E**). Taken together, NPY1R decreased nociceptor neuron sensitivity (cAMP, current clamp excitability) without impacting allergy-mediated immune cell infiltration, and future work is necessary to measure whether it modulates neuron-induced coughing, bronchoconstriction, or mucus metaplasia.

DISCUSSION. Nociceptor neurons shape host defense at mucosal barriers. They do so by detecting environmental danger, triggering an avoidance response, and by tuning immune responses. In the context of allergy, nociceptor neurons were found to amplify dermatitis[14,19,20,56], conjunctivitis[57] and airway inflammation[18,26,31,32,58,59]. In the lungs, these responses range from coughing and bronchoconstriction to mucus secretion and, depending on the context, amplifying, or taming immunity. Such broad responses are made possible by the highly heterogenous nature of nociceptor neurons.

Airway nociceptors. The exact neuronal subset involved in these responses remained to be defined. Zhao and colleagues[7] posit that the variety of organs innervated by the vagus nerve explains in part the need for JNC sensory neuron heterogeneity. As such, airway sensory neurons[8,9,11,30] were classified as i) low-threshold stretch-sensitive neurons (essential to the respiratory cycle); ii) mechanoreceptors (sensitive to punctate mechanical stimuli); and iii) high threshold thermosensitive and chemosensitive nociceptors (recruited in response to tissue injury, inflammation, noxious chemicals or temperatures).

Using a combination of lineage reporters, retrograde tracing and transcriptomic analysis, we revealed that JNC airway nociceptor neurons have a unique gene signature segregated from that of other visceral nociceptors. We identified a new class of *Kcng1*-expressing inflammatory nociceptors (*Trpa1*$^+$, *Trpv1*$^+$, *Il6*$^+$, *Npy1r*$^+$, *Il13ra1*$^+$) that exclusively innervate the airways (NN8). This confirms the assumption of Kupari and colleagues[2] that this neuron subtype (which they label as NG14) consists of pulmonary afferent unmyelinated neurons. Additionally, we found that the airways are preferentially innervated by a neuronal subset (*Kcnv1*$^+$, *Piezo1*$^+$, *Piezo2*$^+$) reminiscent of cough mechanoreceptors (NN7, NG3 in Kupari et al.'s study) as well as by a subset of polymodal nociceptors (NN2), and an Nav1.8^{low} population possibly belonging to the low-threshold stretch-sensitive neurons[10,11]. These neuronal subtypes and their markers are listed in **supplementary table 1.**

AAI reprogramming. We identified a drastic reprogramming of airway nociceptor neurons in response to allergic airway inflammation. Interestingly, the gene signature that we identified largely overlap with the one typically observed in injured nociceptor neurons. These changes are reminiscent of those observed in LPS model of airway inflammation[60]. We can hypothesize that inflammation induces neuronal damage in the airways, which would explain such similarities.

While immune and glial cell activation or infiltration is sometimes reported in the spinal cord or DRG following peripheral inflammation or nerve injury[51,61-65], this was not the case in the JNC of AAI mice. This implies that the neuro-immune interactions occur at the peripheral nerve ending level rather than in the ganglia. Additionally, the nociceptor neurons innervating other organs were shielded from the transcriptional changes we observed in airway neurons, which also involves that those changes are triggered by signals originating from the nerve ending in the airways.

Cytokines reprograms nociceptor neurons. As we found that AAI reprogrammed airway nociceptor neurons' transcriptome, we tested how various cytokines impact JNC neuron expression profiles. Interestingly, nociceptor neurons showed different gene signatures when exposed to IL-13/IL-4, IL-1β, TNF-α, or BDNF. Thus, it appears that the combination of signaling pathways induced by nerve ending damage, inflammatory cytokines and neurotrophins add up *in vivo* and result in the nociceptors AAI signature. These findings are also indicative of nociceptor neurons' plasticity to various inflammatory conditions and subsequent context-dependent neuro-immune responses[66].

IL-13 mimics some of the transcriptional changes observed during AAI, an effect that involves STAT6 phosphorylation and subsequent regulation of gene expression. In physiology, our findings suggest that IL-4/IL-13 released by airway T_H2 and ILC2 cells are locally sensed by IL4RII-expressing vagal nerve endings. In turn, the intracellular signals are likely to be retrogradely transported to the soma to generate transcriptional changes. Such retrograde transport has been reported in nociceptors for STAT3[67-70] and CREB[71,72], and thus may also occur for STAT6.

IL-4 and IL-13 were previously found to induce calcium flux in dorsal root ganglia nociceptor neurons and to trigger an itching reflex[14,43]. While we did not observe direct calcium flux in JNC nociceptor neurons exposed to IL-13, our data converge regarding the functional expression of IL4RII and JAK1/2 activation in nociceptor neurons[43,73]. IL-13/IL-4/JAK/STAT6 is a key signaling pathway essential to type 2 inflammation and allergies[74,75], and strategies to target it have proven effective to treat atopic dermatitis, asthma and to prevent

itching[76]. We can reason that the sensory relief observed in these patients may, in part, be due to the silencing of this pathway in nociceptor neurons.

IL-6. IL-6 is a pleiotropic cytokine released by a variety of immune cells [77]. Its release is enhanced in asthmatic patients[78], various mouse models of asthma[79] and other models of lung inflammation. IL-6 has a variety of immunomodulatory actions, such as inducing T_H2 and T_H17 differentiation[80,81], suppressing T_H1[82], inhibiting Tregs[83,84], or increasing lung fibrosis[85] and mucus secretion[79]. IL-6 expression was previously reported in enteric neurons[38], and sometimes appears in sensory neurons' transcriptome following nerve injury[86].

Here, we present the first evidence that IL-6 is expressed and released by JNC nociceptors. We found this release to be enhanced by IL-1β stimulation. This finding likely has important physiological consequences, since the amount released by nociceptor neurons was equivalent to that produced by immune cells from the spleen. Since *Il6* expression is increased in airway nociceptors during AAI, it will be of interest to investigate the contribution of vagal nociceptor-released IL-6 to airway inflammation and define which tissue and cells it targets.

NPY, NPY1R, pain and allergy. Pain warns the organism of environmental dangers. Endogenous neuromodulatory mediators can either increase or decrease the organism's perception. In the context of pain, the impact of NPY and NPY1R remains controversial. Nevertheless, a consensus has emerged as for NPY1R expression and antinociceptive effect in the central nervous system[45-48,50].

The effect on primary afferent nociceptor neurons is less established and NPY exerts a complex influence on pain sensitivity and neuropeptide release. This duality is likely explained by nociceptor neurons' co-expression of NPY1R and NPY2R, with the former dampening pain and inflammation while the latter exacerbates it[49,51-53]. Building on these findings, we presented the first set of data suggesting the impact of NPY-NPY1R on vagal neuron sensitivity. We discovered that NPY-NPY1R decreased JNC nociceptor neuron activity by decreasing the levels of cAMP and reducing action potential firing. cAMP has long been recognized as an intracellular messenger promoting nociceptor sensitization[87-92], an effect mediated in part by PKA-induced phosphorylation of $Na_V1.8$ channels[93].

NPY also fulfills numerous physiological functions, ranging from the control of hunger/feeding to energy homeostasis[94,95], vasoconstriction[96] and immunomodulation[97]. As we found to be the case in our OVA- and HDM-challenged mice, other studies showed elevated NPY levels in various rodent models of lung inflammation[54,98-101] as well as in the plasma of elderly asthma patients[102]. While NPY1R was reported as a driver of lung immune cell infiltration[54], we failed to see any such effect. However, airway NPY was also reported to increase methacholine-induced bronchoconstriction[54,98], and $NPY1R^+$ vagal neurons were shown to trigger expiratory reflexes[1]. It would thus be of interest to assess whether this GPCR expression on vagal neurons is involved in cough and bronchoconstriction.

Conclusion. In summary, our data revealed a new class of vagal airway specific nociceptors that acquire an inflammatory gene signature during allergic inflammation or when stimulated with IL-13 and IL-1β. *Npy1r* overexpression was induced by IL-13 in a JAK/STAT6-dependent manner. During allergic airway inflammation, sympathetic nerve fibers, found in proximity to nociceptor fibers within the bronchi, release NPY, which subsequently decrease nociceptor neurons' activity (**SF. 8**). Future work will reveal whether targeting vagal NPY1R constitutes a relevant therapeutic target to quell asthma-induced bronchoconstriction and cough.

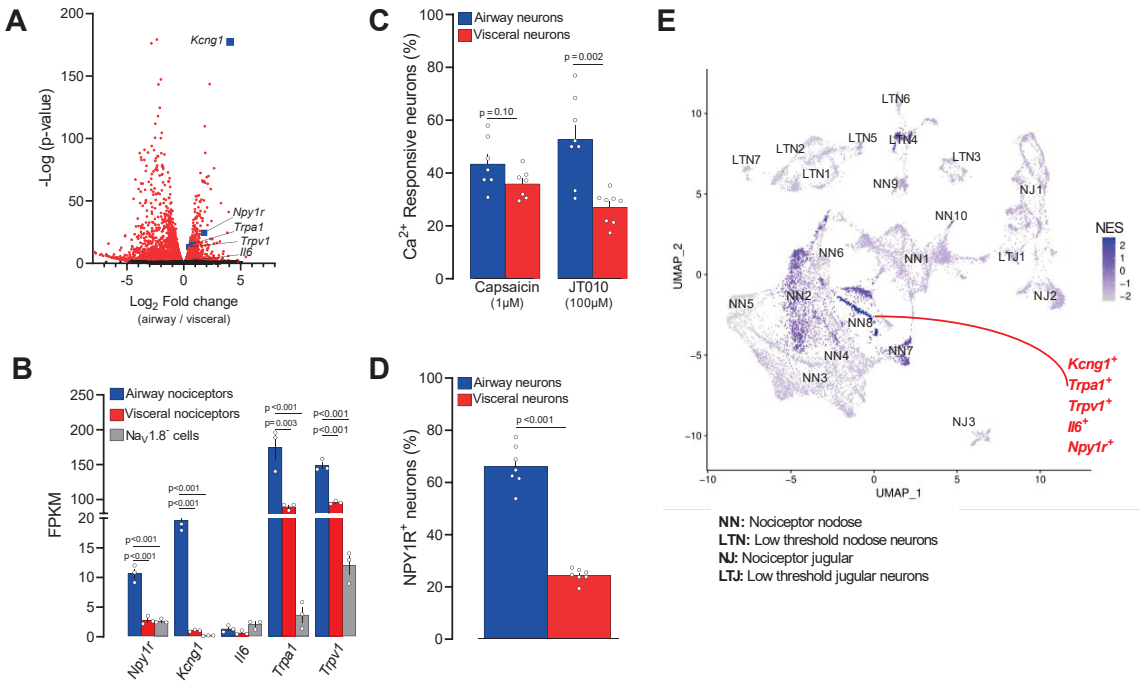


Figure 1. Airway vagal nociceptor neurons have a unique molecular profile.

(A-B) To identify airway-innervating nociceptor neurons, naive 8-week-old male and female nociceptor neurons reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice were injected intranasally with the retrograde tracer DiD' (200 μM). Fourteen days later, the mice were euthanized and their JNC ganglia isolated and dissociated. Airway-innervating nociceptor neurons ($Na_V1.8^+DiD^+$), visceral nociceptors ($Na_V1.8^+DiD^-$) and $Na_V1.8^-$ cells were purified by flow cytometry and their RNA sequenced. A volcano plot of pairwise comparison of airway-innervating nociceptor neurons versus visceral nociceptor neurons shows differentially expressed transcripts in red (adjusted p-value <0.2). Among others, *Npy1r, Kcng1, Trpa1, Trpv1* and *Il6* were enriched in airway-innervating nociceptor neurons (**A**). *Npy1r*, *Kcng1*, *Trpa1* and *Trpv1* were also more expressed in airway nociceptors when compared to $Na_V1.8^-$ cells (**B**).

(C) 8-week-old male and female C57BL6 mice were injected intranasally with the retrograde tracer DiD' (200μM). Fourteen days later, the mice were euthanized and their JNC ganglia isolated and dissociated. JNC neurons were cultured (16h) and responsiveness to noxious stimuli was assessed. While responsiveness to the TRPV1 agonist capsaicin (300 nM) was stable between the two groups, the proportion of neurons responsive to the TRPA1 agonist JT010 (50 μM) was higher in airway-innervating neurons (DiD^+; **C**).

(**D**) Naive 8-week-old male and female NPY1R reporter ($NPY1R^{cre}$::$tdTomato^{fl/wt}$) mice were injected intranasally with the retrograde tracer DID' (200 μM). Fourteen days later, the mice were euthanized and their JNC ganglia isolated and dissociated. JNC neurons were cultured (16h), and neurons defined by KCl calcium responses. Using fluorescent imaging, we found that NPY1R-expressing neurons ($tdTomato^+$) are more frequent in the airway-innervating population (DiD^+; **D**).

(E) UMAP of *Slc17a6*$^+$ (VGLUT2) JNC neurons from single-cell RNA sequencing revealed heterogeneous neuronal subsets. Gene set enrichment analysis was performed to address which neuron subtype preferentially innervated the airways, with normalized enrichment score (NES) indicated in blue. The neuronal cluster NN8 expresses several neuro-inflammatory markers (*Il6, Kcng1, Npy1r, Trpa1, Trpv1*) and exclusively innervates the airways (NES=2.3). Experimental details were defined in *Prescott et al.* and the bioinformatic analysis is described in the method section (**E**).

*Data are shown as a representative volcano plot displaying DESeq2 normalized count fold change and nominal p-values for each gene **(A)**, as mean ± S.E.M (**B-D**), or as normalized enrichment score (**E**). N are as follows:*

***A-B**: n=3 biological replicates (4 mice per sample), **C-D**: n=7–8 dishes per group. P-values were determined by DESeq2 analysis (**A**); one-way ANOVA with post hoc Tukey's (**B**); or two-sided unpaired Student's t-test (**C, D**). P-values are shown in the figure.*

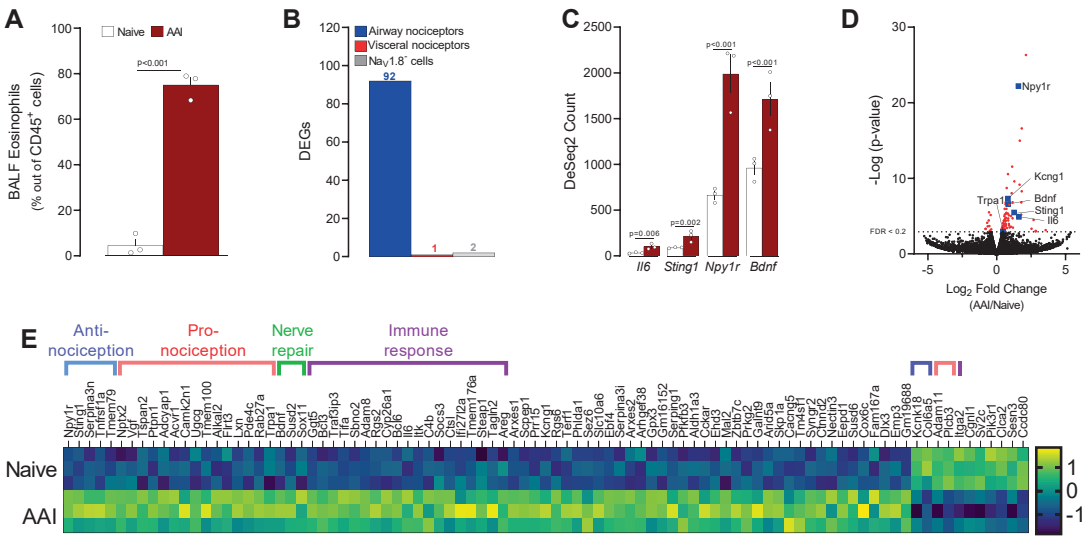


Figure 2. Allergic airway inflammation reprograms airway nociceptors transcriptome.

(**A-E**) 8-week-old male and female nociceptor neuron reporter (Nav1.8^{cre}::tdTomato$^{fl/wt}$) mice underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by inhaled OVA challenges (days 14-17). To identify airway-innervating nociceptor neurons, the mice were injected intranasally with the retrograde tracer DiD' (200 µM) (days 2-4). One day after the last allergen challenge, the bronchoalveolar lavage fluid (BALF) was harvested and immunophenotyped by flow cytometry. OVA-exposed mice showed significant airway inflammation characterized by a significant eosinophilic (CD45^{+}CD11C^{low}SiglecF^{Hi}) infiltration (**A**). The JNC ganglia were isolated and dissociated. Airway-innervating nociceptor neurons (Na$_V$1.8^{+}DiD^{+}), visceral nociceptors (Na$_V$1.8^{+}DiD^{-}) and Na$_V$1.8^{-} cells were purified by flow cytometry and their RNA sequenced. Differentially expressed genes were virtually only observed in airway-innervating nociceptor neurons (**B**). Among others, *Il6, Sting1, Npy1r*, and *Bdnf* were overexpressed in airway-innervating nociceptor neurons (Na$_V$1.8^{+}DiD^{+}; **C**). Pairwise comparison shows differentially expressed transcripts in airway-innervating nociceptor neurons between naive and AAI conditions, shown as a volcano plot (adjusted p-value <0.2 in red; **D**) and heatmap (**E**).

*Data are shown as mean ± S.E.M (**A-C**), a volcano plot displaying DESeq2 normalized count fold change and nominal p-values for each gene **(D),** or a heatmap displaying the z-score of DESeq2 normalized counts **(E).** N are as follows: **A-E**: n=3 biological replicates (4 mice per sample). P-values were determined by a two-sided unpaired Student's t-test (**A**) DESeq2 analysis **(**adjusted p-value in **C** and nominal p-value in **D)**. P-values are shown in the figure.*

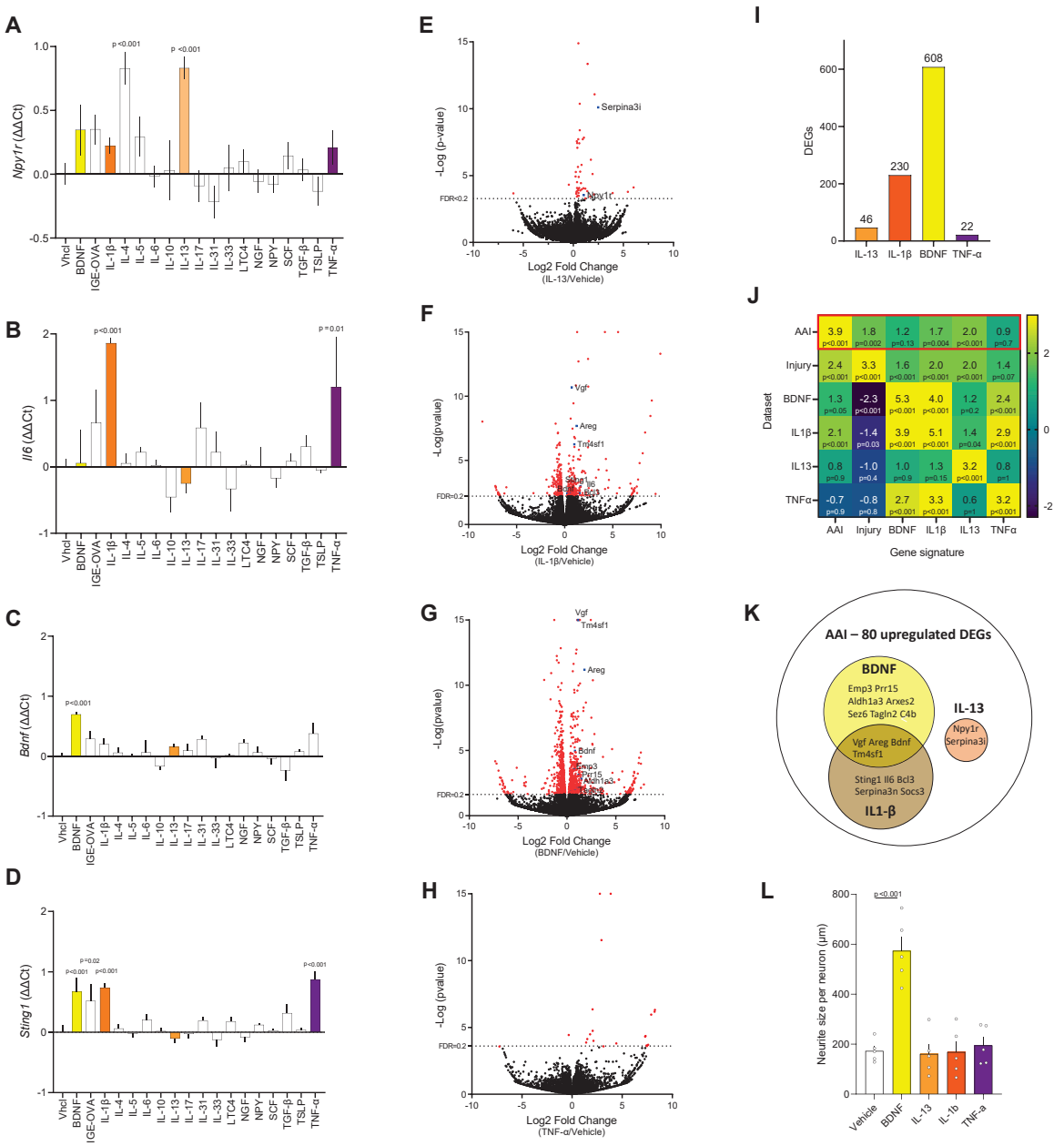


Figure 3. Teasing out the cytokine cocktail driving AAI-induced nociceptor reprogramming.

(**A-D**) 8-week-old male and female naïve C57BL6 mice JNC neurons were cultured and exposed (24h) to various inflammatory mediators. Changes in *Npy1r* (**A**), *Il6* (**B**), *Bdnf* (**C**), and *Sting1* (**D**) transcript expression were assessed by qPCR. In comparison to the vehicle, IL-4 and IL-13 (100 ng/mL) increased JNC neurons' expression of *Npy1r.* IL-1β and TNF-α (100 ng/mL) increased the expression of *Il6*. BDNF induced its own overexpression while IL-1β (100 ng/mL), IgE-OVA (10 ug/mL), BDNF (50 ng/mL) and TNF-α (100 ng/mL) increased the expression of *Sting1*.

(**E-I**) 8-week-old male and female naïve nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice JNC neurons were cultured (24h) in the presence of vehicle, IL-13 (**E**), IL-1β (**F**), BDNF (**G**), or TNF-α (**H**). The nociceptor neurons ($tdTomato^+$) were FACS-purified and their RNA sequenced. Pairwise comparison showed differentially expressed genes induced by these cytokines (adjusted p-value <0.2 in red) and the genes in common with the AAI signature are highlighted in blue. IL-13, IL-1β, BDNF, and TNF-α respectively induced the overexpression of 46, 230, 608 and 22 genes (**I**).

(J) GSEA analysis were performed to compare gene signatures (i.e., overexpressed DEGs) induced by AAI, nerve injury[36], BDNF, IL-1β, IL-13 and TNF-α to their respective whole datasets. The gene signatures induced by nerve injury, IL-13, IL-1β and BDNF were enriched in JNC neurons from mice with AAI (**J**).

(**K**) The common DEG induced by cytokines and AAI are depicted in a Venn diagram. IL-1β and BDNF signatures had similarities, while IL-13 induced two specific AAI genes *Npy1r* and *Serpina3i* (**K**).

(**L**) Nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice JNC neurons were cultured (24h) in the presence of vehicle, IL-13, IL-1β, BDNF, or TNF-α. BDNF, but not cytokines, promoted neurite growth (**L**).

*Data are shown as mean ± S.E.M (**A-D, L**), as a volcano plot displaying DESeq2 normalized count fold change and nominal p-values for each gene (**E-H**) as number of DEGs (**I**), as heatmap displaying normalized enrichment score (NES) (**J**) or as a Venn diagram (**K**). N are as follows: **A-D**: n=3–4 cultures from different mice per group, **E-H:** n=3-4 cultures from different mice per group, **L**: n=6 culture dishes per group. P-values were determined by one-way ANOVA with post hoc Dunnett's (**A-D, L**) or DESeq2 analysis (**E-H**), or GSEA analysis (**J**). P-values are shown in the figure or indicated by * for p ≤ 0.05; ** for p ≤ 0.01; *** for p ≤ 0.001.*

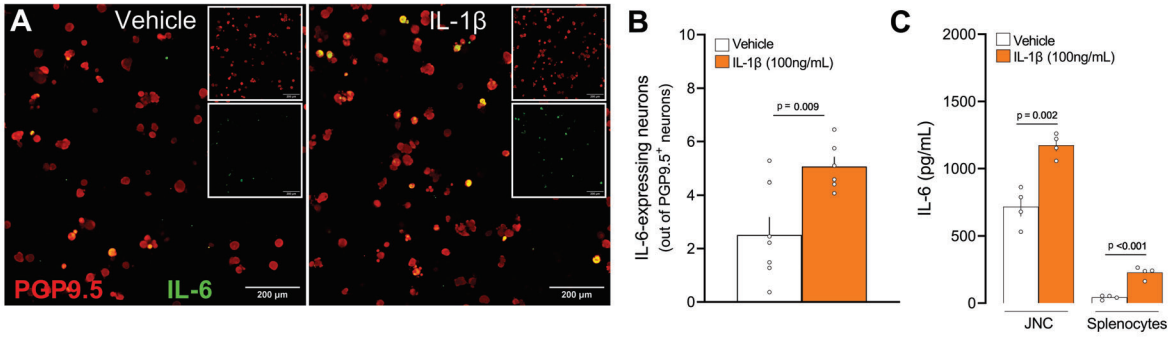


Figure 4. IL-1β induces IL-6 release from JNC nociceptors.

(**A-B**) IL-6 expression was assessed using immunofluorescence in 8-week-old male and female C57BL6 mice JNC neurons cultures exposed to IL-1β (100 ng/mL; 24h). IL-6 (green) was found to be expressed in $PGP9.5^+$ (red) neurons (A), at higher levels in IL-1β-treated than in vehicle-treated neurons (**A**, **B**).

(C) 1.5 x 10^4 JNC neurons or 3 x 10^6 splenocytes from naive 8-weeks-old male and female C57BL6 mice were cultured (36h) in the presence or absence of IL-1β (100 ng/mL), and supernatant was harvested for ELISA. In comparison to the vehicle, IL-1β increased IL-6 release by JNC neurons and splenocytes (**C**).

Data are shown as as cell immunostaining, scale bar 200 µm (**A**), *or mean ± S.E.M (**B-C**). N are as follows: **A-B:** n=6 culture dishes per group, **C**: n=4 culture wells per group. P-values were determined by two-sided unpaired Student's t-test (**B-C**). P-values are shown in the figure.*

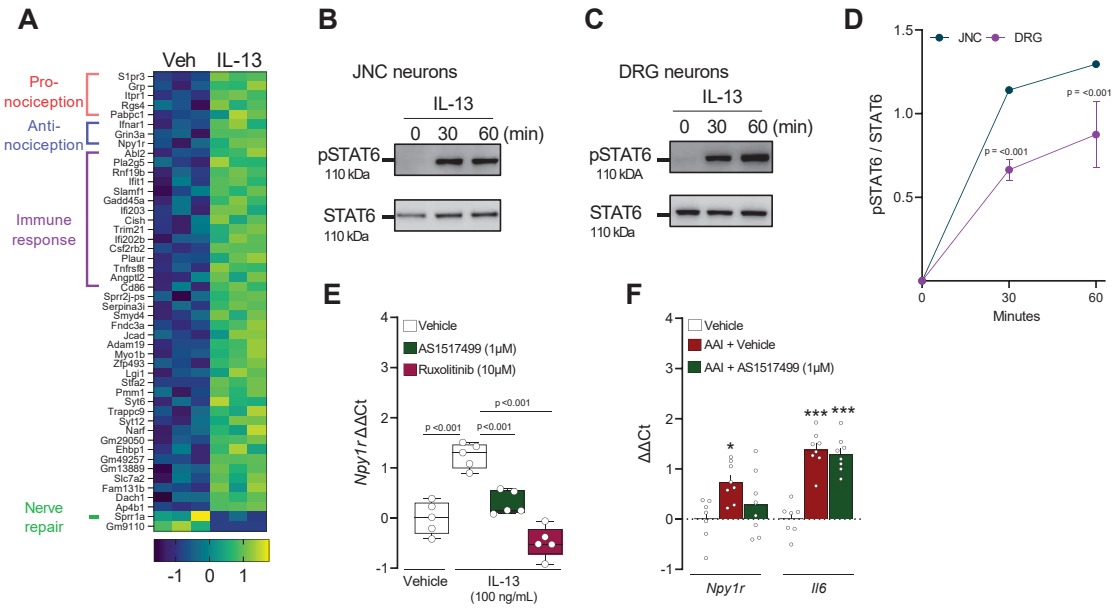


Figure 5. IL-13 reprograms nociceptor neurons.

(**A**) Pairwise comparison shows 48 differentially expressed genes between the vehicle and IL-13 treated neurons, shown as a heatmap with putative gene function (see Supplementary table 4) (**A**).

(**B-D**) Naive 8-week-old male and female C57BL6 mice JNC or DRG neurons were cultured and exposed (30–60 minutes) to IL-13 (100 ng/mL) or vehicle. Changes to pSTAT6 were analyzed by Western blot. IL-13 time-dependently increased the pSTAT6/STAT6 ratio in JNC (**B, D**) and DRG (**C, D**) neurons.

(E) In the presence or absence of the STAT6 inhibitor AS1517499 (1 μM) or the JAK1/2 inhibitor ruxolitinib (10 μM), naive 8-week-old male and female C57BL6 mice JNC neurons were cultured (24h) with IL-13 (100 ng/mL) and changes to *Npy1r* transcript expression was analyzed by qPCR. *Npy1r* overexpression induced by IL-13 was blocked by both AS1517499 and ruxolitinib (**E**).

(**F**) 8-week-old female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice underwent the ovalbumin mouse models of asthma. Allergic airway inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by intranasal instillation with OVA mixed with STAT6 inhibitor AS1517499 (150ug/50ul) or vehicle (days 14-17). One day after the last allergen challenge, mice were sacrificed, and JNC nociceptor neurons ($tdTomato^+$) purified by flow cytometry for RNA extraction and RT-qPCR. OVA-exposed mice nociceptors showed increased expression of *Npy1r* and *Il6*. *Npy1r* overexpression was prevented by AS1517499 (**F**).

*Data are shown as a heatmap displaying the z-score of DESeq2 normalized counts **(A),** as Western blots **(B-C),** as mean ± S.E.M (**D, F**), or as box (25th–75th percentile) and whisker (min-to-max) plot (**E**). N are as follows: **A:** n=3 cultures from different mice per group, **B-D**: n=1 JNC culture and n=3 DRG cultures from different mice, **E**: n=5 cultures from different mice per group, **F**: n=7-8 mice per group. P-values were determined by one-way ANOVA with post hoc Dunnett's (**D-F**). P-values are shown in the figure or indicated by * for p ≤ 0.05; ** for p ≤ 0.01; *** for p ≤ 0.001.*

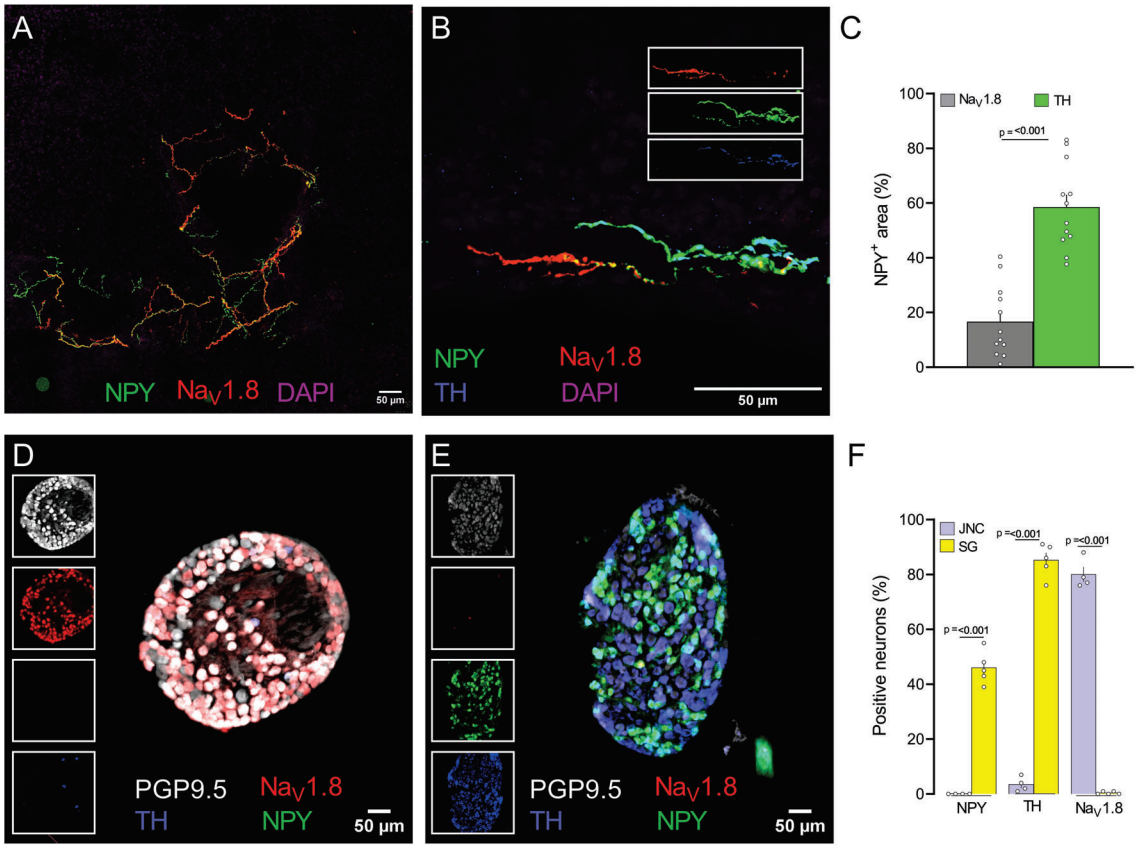


Figure 6. NPY is expressed by sympathetic neurons in the lung

(A-F) Lung (**A–C**), jugular nodose complex ganglia (JNC; **D**) and stellate ganglia (SG; **E**) were harvested from naïve 8-weeks-old male and female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice. The tissues were cryosectioned, and the source of NPY assessed by immunofluorescence. In the lung, NPY (green) and $Na_V1.8$ (tdTomato, red) were expressed in nerve fibers around the bronchi **(A).** While often in proximity, NPY (green) mostly colocalized with the sympathetic neuron marker tyrosine hydroxylase (TH; blue) rather than with $Na_V1.8$ (tdTomato; red) nociceptor fibers **(B-C).** NPY was not expressed in the JNC (**D, F**). In the stellate ganglia (SG), NPY was strongly expressed in TH^+ sympathetic neurons (**E, F**). PGP9.5 (white) was used to define JNC and SG neurons (**D, E, F**)

*Data are shown as immunostained tissue, scale bar 50 µm (**A, B, D, E**), or as mean ± S.E.M (**C, F**). N are as follows: **B, C**: n=12 field of views from 4 different mice, **D-F**: n=4–5 mice per group. P-values were determined by a two-sided unpaired Student's t-test (**C, F**). P-values are shown in the figure.*

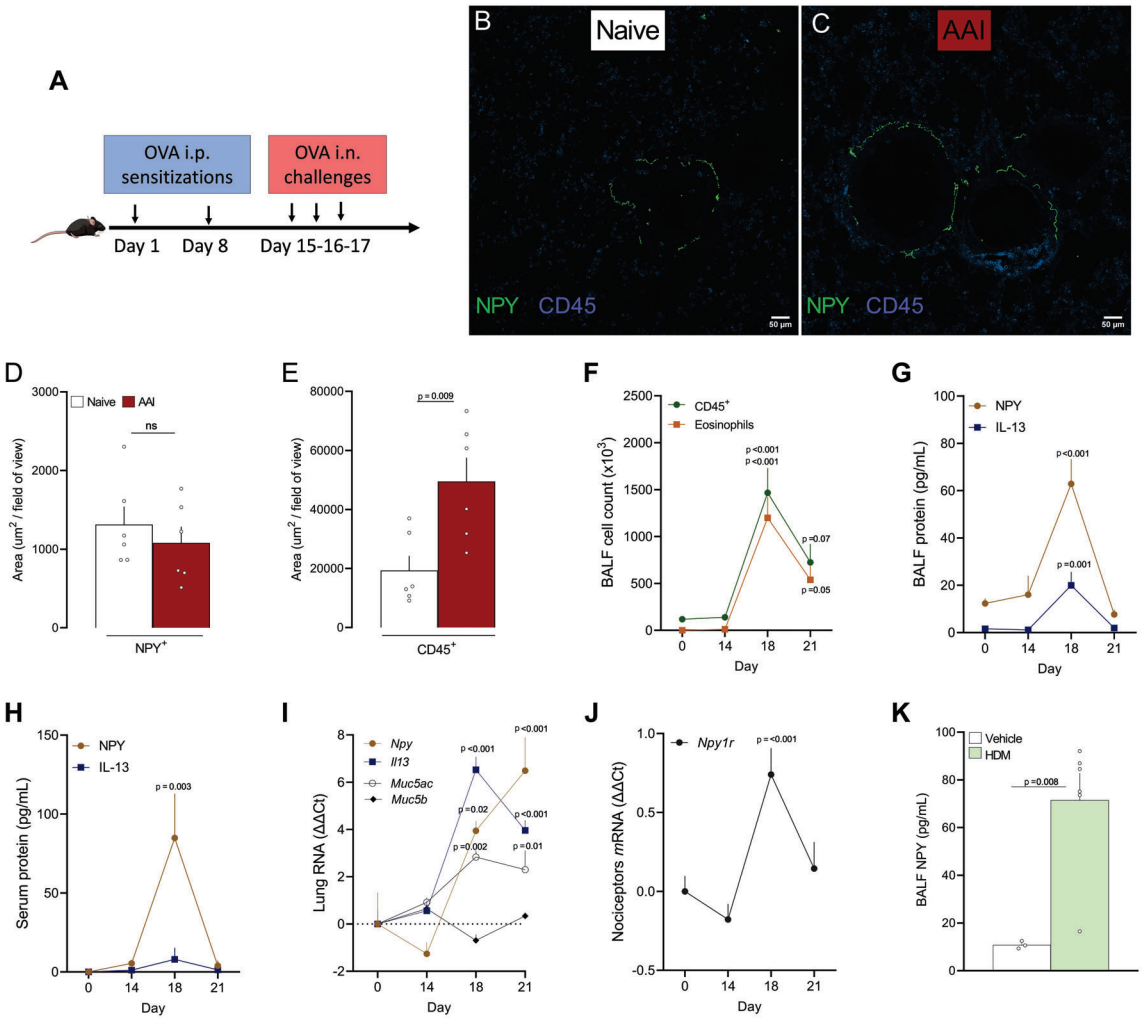


Figure 7. NPY is released in airways during allergic airway inflammation.

(A) Schematic of the AAI protocol. 8-week-old female C57BL6 mice underwent the ovalbumin mouse models of asthma. Allergic airway inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by inhaled OVA challenges (i.n. days 15-17).

(B-E) Naïve and AAI mice (day 18) lung were harvested and cryosections prepared for immunostaining with anti-NPY (green) and anti-CD45 (blue) (**B-C**). NPY was observed in nerve fibers around the bronchi, and the pattern of innervation was comparable between conditions (**D**), while $CD45^+$ cells shown increased infiltration the lung of AAI mice (**E**).

(F-I) 8-week-old female C57BL6 mice underwent the ovalbumin mouse models of asthma. BALF, serum and lung were harvested at different time points (days 0, 14, 18, and 21) and the levels of inflammatory mediators were analyzed by ELISAs and qPCR. OVA-exposed mice showed significant airway inflammation characterized by leukocytes ($CD45^+$) and eosinophil ($CD45^+CD11C^{low}SiglecF^{Hi}$) infiltration on day 18 (**F**). Along with this rise in airway inflammation, we found an increase in BALF (**G**) and serum (**H**) NPY, while lung *Npy* expression was also increased (**I**).

(J) JNC ganglia were harvested from OVA-exposed nociceptor reporter mice ($TRPV1^{cre}$::$tdTomato^{fl/wt}$) at different time points, $TRPV1^+$ neurons were purified ($tdTomato^+$) by flow cytometry, and changes in transcript expression were measured by qPCR. At the peak of inflammation (day 18), we found a transient increase in *Npy1r* expression (**J**).

(K) To induce another model of allergic airway inflammation, 8-weeks-old female C57BL6 mice were challenged (day 1-5 and 8–10) with house dust mite (HDM; 20µg/50µL, i.n.). The mice were sacrificed on day

11, their BALF harvested, and cell free supernatant analyzed by ELISA. HDM-exposed mice showed a significant increase in BALF NPY level (**K**).

*Data are shown as experiment schematics (**A**), as immunostained tissue (**B-C**) or as mean ± S.E.M (**D-K**). N are as follows: **B-E**: n=6 mice per group, **D-I**: n=6-7 mice per group, **J**: n=4-10 mice per group, **K:** n= 3-6 mice per group. P-values were determined by two-sided unpaired Student's t-test (**D, E, K**), one-way ANOVA with post hoc Dunnett's (**B-J,** comparison to day 0). P-values are shown in the figure or indicated by * for p ≤ 0.05; ** for p ≤ 0.01; *** for p ≤ 0.001.*

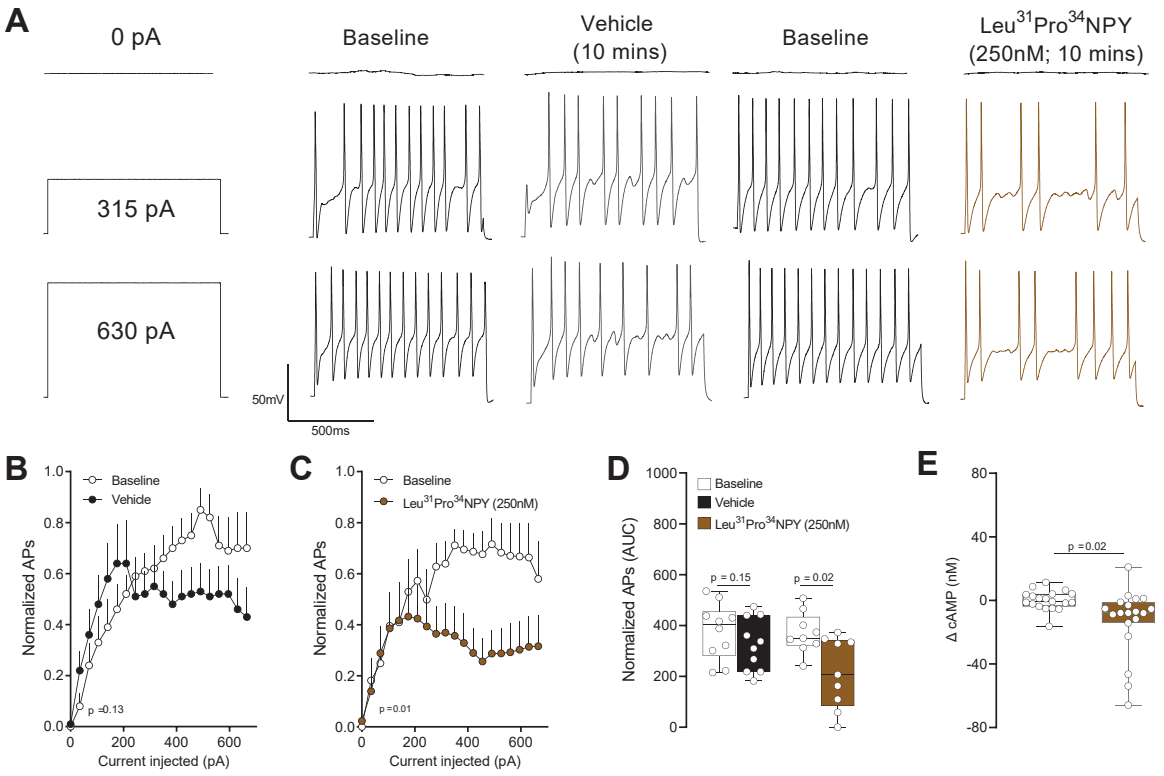
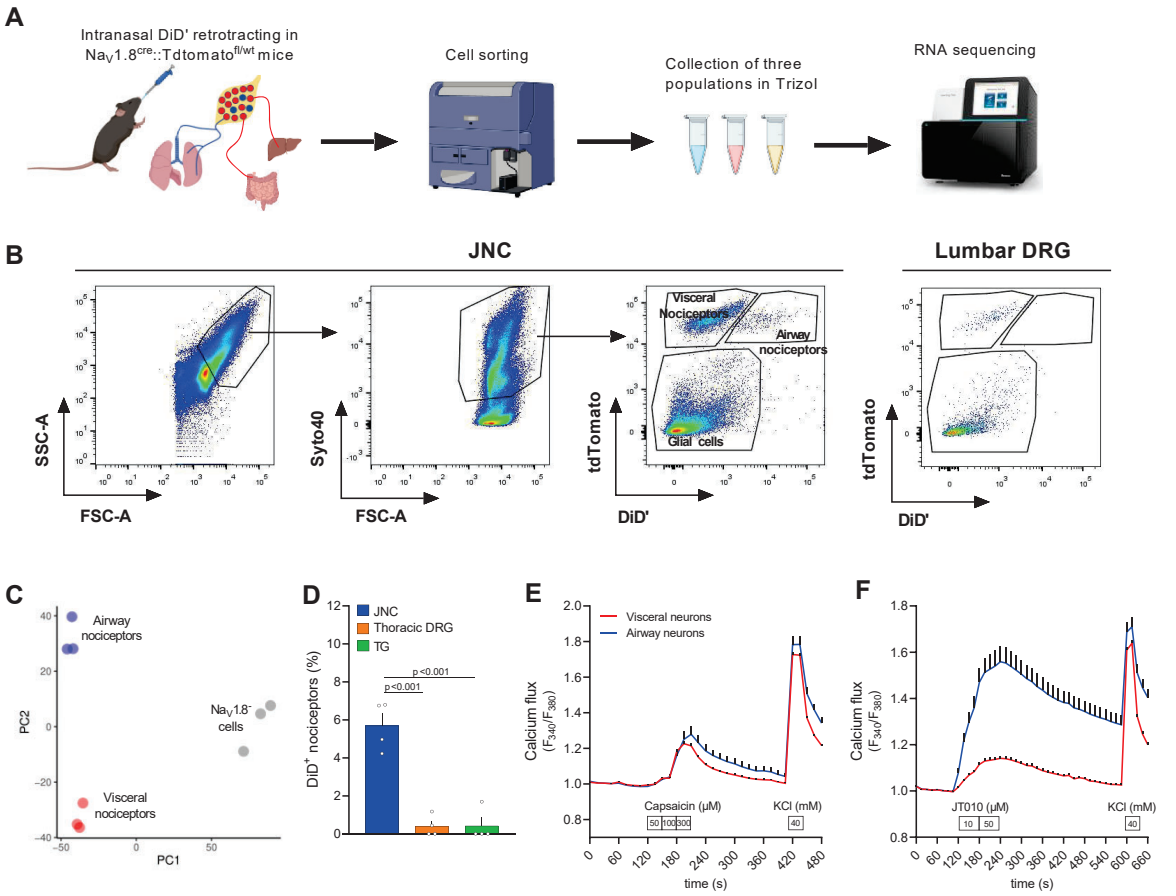


Figure 8. NPY1R blunts JNC nociceptors excitability.

(A-D) 8-week-old male and female NPY1R reporter (NPY1R^{cre}::tdTomato$^{fl/wt}$) mice were sacrificed and their JNC neurons harvested and cultured (16 hours). Whole cell patch clamp electrophysiology was performed on the NPY1R^{+} nociceptor neurons. A current clamp was applied while the neurons' membrane potential was recorded before and after exposing (10 min) the cell to Leu^{31}Pro^{34}NPY (250 nM) or its vehicle. The number of action potentials for each neuron was normalized by the maximum number observed at baseline (**B-D**). The areas under the curve in **(B)** and **(C)** were calculated and plotted **(D)**. While the vehicle had little to no effect on neuronal excitability (**A**, **B, D**), Leu^{31}Pro^{34}NPY reduced the number of action potentials in response to current stimulation in NPY1R^{+} (tdTomato) neurons **(A, C, D).**

(E) 8-week-old C57BL6 male and female mice were sacrificed and their JNC neurons harvested and cultured (16 hours). The neurons were exposed to Leu^{31}Pro^{34}NPY (250 nM) or vehicle for 30 minutes in presence of phosphodiesterase inhibitors. The cells were then lysed and the cAMP concentration assessed by enzymatic assay. Leu^{31}Pro^{34}NPY significantly reduced cAMP concentration **(E)**.

*Data are shown as traces of membrane potential for individual neurons (**A**), mean ± S.E.M **(B, C)**, or box (25th–75th percentile) and whisker (min-to-max) plots **(D-E)**. N are as follows: **B, D**: n=10 vehicle treated neurons, **C, D:** n=9 Leu^{31}Pro^{34}NPY treated neurons, **E**: n=19 culture wells. P-values were determined by two-way ANOVA (**B, C**) or two-sided unpaired Student's t-test **(D-E)**. P-values are shown in the figure.*



Supplementary Figure 1. Airway vagal nociceptor neurons have a unique transcriptome.

(A) Diagram depicting the retrotracing of the airway-innervating nociceptor neurons. To identify airway-innervating nociceptor neurons, naive 8-week-old male and female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice were injected intranasally with the retrograde tracer DID' (200 μM). Fourteen days later, the mice were euthanized and their JNC ganglia isolated, dissociated, and airway nociceptor neurons were purified by flow cytometry. The neurons' transcriptome was then analyzed by RNA sequencing **(A)**.

(B) Jugular nodose complex neurons gating strategy. Small debris were eliminated (FSC/SSC), and the whole cells were identified (nucleus marker SYTO40). Populations of airway-innervating nociceptor neurons ($Na_V1.8^+DiD^+$), visceral nociceptors ($Na_V1.8^+DiD^-$) and $Na_V1.8^-$ cells were then separated. Lumbar DRG were used as gating controls since they do not innervate the airways **(B)**.

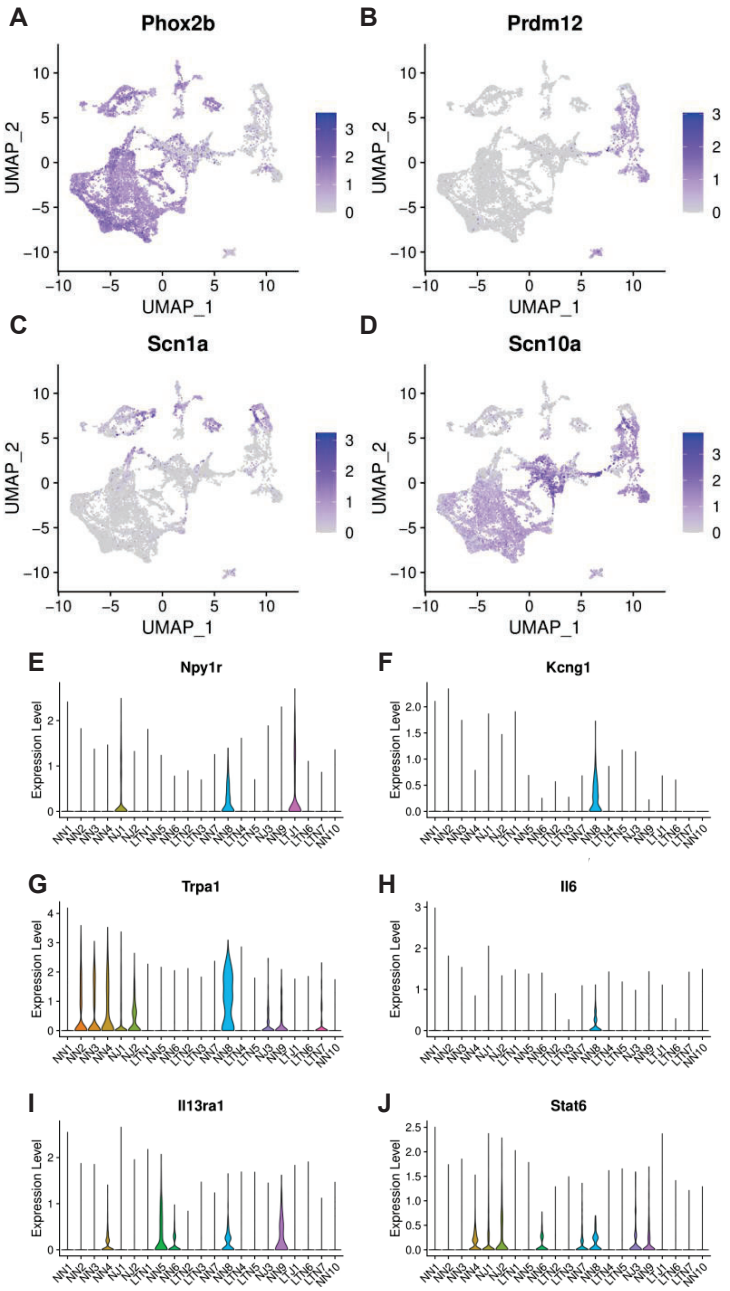
(C) The transcriptome of purified airway-innervating nociceptor neurons ($Na_V1.8^+DiD^+$), visceral nociceptors ($Na_V1.8^+DiD^-$) and glial cells ($Na_V1.8^-DiD^-$) was analyzed by RNA sequencing and population segregation was confirmed using principal component analysis **(C)**.

(**D**) Naive 8-week-old male and female $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ mice were injected intranasally with the retrograde tracer DiD' (200 μM). Fourteen days later, the mice were euthanized and JNC, thoracic DRG, and TG ganglia isolated, dissociated, and imaged with a fluorescence microscope. DiD' retrotracer was detected in JNC nociceptor neurons but virtually absent in other ganglia (**D**).

(**E-F**) Naive 8-week-old male and female C57BL6 mice were injected intranasally with the retrograde tracer DiD' (200 μM). Fourteen days later, the mice were euthanized and their JNC ganglia isolated and dissociated. JNC neurons were cultured (16 h) and calcium responsiveness to noxious stimuli was assessed. While the average neuronal responsiveness to the TRPV1 agonist capsaicin (300 nM) was stable between the two

groups (**E**), the calcium flux induced by the TRPA1 agonist JT010 (50 µM) was higher in airway-innervating nociceptor neurons (**F**).

*Data are shown as a schematic (**A**), flow cytometry dot plot (**B**), principal component analysis (**C**), and mean ± S.E.M (**D–F**). N are as follows: **D**: n=4 culture dishes**, E**: n=116 airway-innervating neurons and 1307 visceral neurons, **F**: n=137 airway-innervating neurons and 1406 visceral neurons. P-values were determined by a two-sided unpaired Student's t-test (**D**) and are indicated in the figure.*

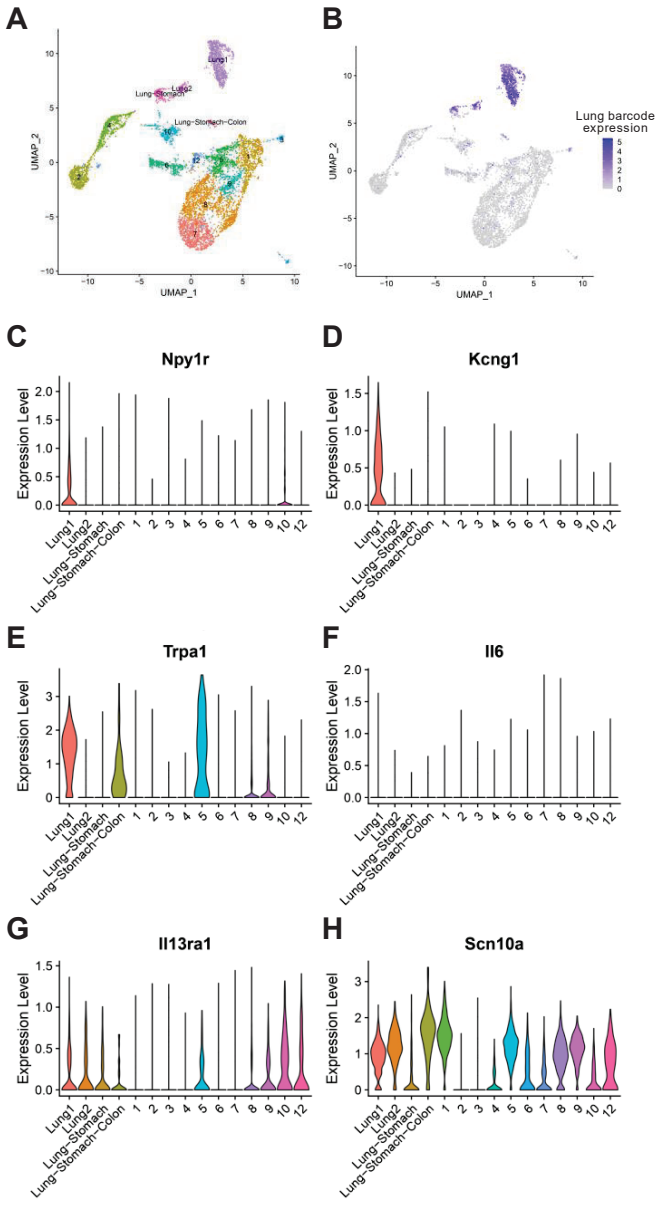


Supplementary Figure 2. JNC airway nociceptor neuron markers

(**A-D**) UMAPs of *Slc17a6*$^{+}$ (VGLUT2) JNC neurons from single-cell RNA sequencing showing expression of *Phox2b* (**A**), *Prdm12* (**B**), *Scn1a* (**C**), and *Scn10a* (**D**). *Phox2b* and *Prdm12* delineate the nodose and jugular neurons, while *Scn1a* and *Scn10a* delineate low-threshold sensory neuron and nociceptor neuron populations. The experimental details were defined in *Prescott et al.* and the bioinformatic analysis is described in the methods section (**E**).

(**E–J**) Violin plot showing expression of *Npy1r* (**E**), *Kcng1* (**F**), *Trpa1* (**G**), *Il6* (**H**), *Il13ra1* (**I**), *Stat6* (**J**) in neuronal cells for each cluster identified by single-cell sequencing. All these genes are co-expressed in the airway-specific nociceptor neuron cluster NN8. The experimental details were defined in *Prescott et al.* and the bioinformatic analysis is described in the methods section.

*Data are shown as UMAPs with log-normalized expression as a feature (**A-D**) or as a violin plot of the log-normalized expression (**E-J**).*

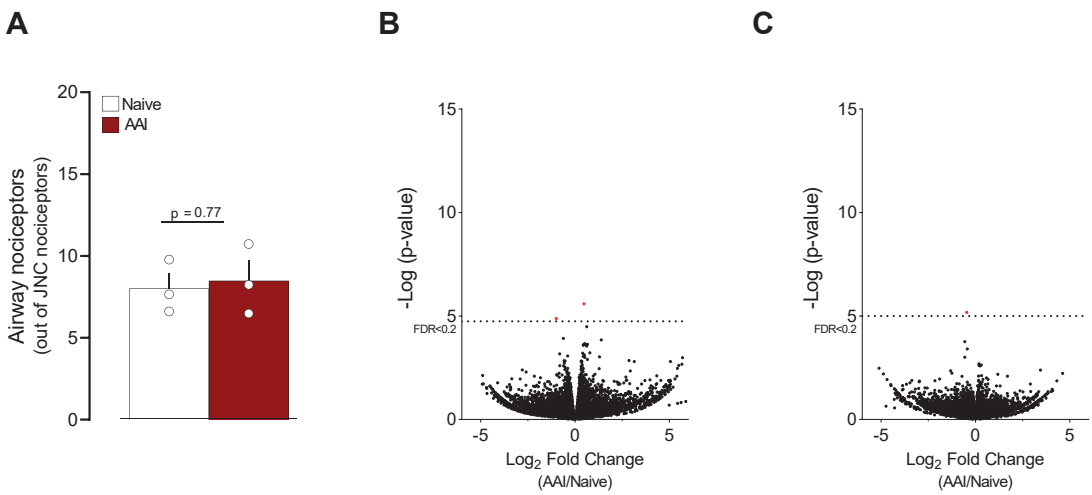


Supplementary Figure 3. Lung projecting neurons single cell RNA sequencing.

(**A-B**) UMAPs of barcode positive JNC neurons cluster from single-cell RNA projection-sequencing[7] (**A**) and lung barcode expression (**B**). 4 clusters innervate the lung, 2 of them being lung specific, the two others also innervating stomach and colon (**B**). The experimental details were defined in Zhao *et al*[7]. and the bioinformatic analysis is described in the methods section.

(**C-H**) Violin plot showing expression of *Npy1r* (**C**), *Kcng1* (**D**), *Trpa1* (**E**), *Il6* (**F**), *Il13ra1* (**G**), *Scn10a* (**H**) in neuronal cells for each cluster identified by single-cell sequencing.

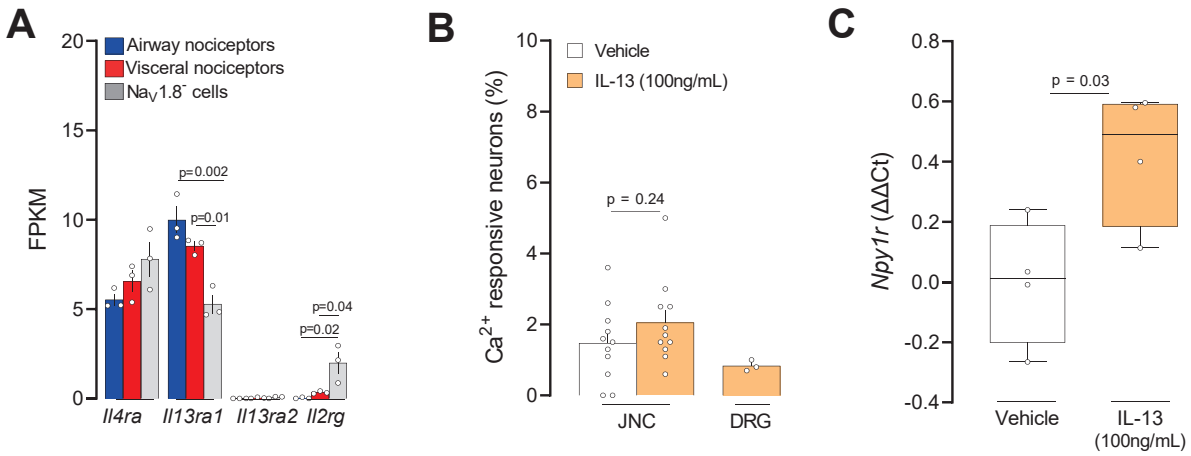
*Data are shown as UMAPs with log-normalized expression as a feature (**A-B**) or as a violin plot of the log-normalized expression (**C-H**).*



Supplementary figure 4. Airway-innervating nociceptor neurons from AAI mice overexpress nerve injury markers

(**A-C**) 8-week-old male and female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by inhaled OVA challenges (days 14–17). On days 2, 3, and 4, mice were injected intranasally with the retrograde tracer DiD' (200 µM). One day after the last allergen challenge, the mice were euthanized and their JNCs isolated and dissociated, and airway-innervating nociceptor neurons ($Na_V1.8^+DiD^+$) were purified by flow cytometry (**A**). Visceral nociceptor ($Na_V1.8^+DiD^-$) and $Na_V1.8^-$ cell transcriptomes were analyzed by RNA sequencing (**B-C**). The number of JNC airway-innervating nociceptor neurons is similar between naïve and OVA-exposed mice ($tdTomato^+DiD^+$) (**A**). A volcano plot (adjusted p-value <0.2 in red) of pairwise comparison of naive versus AAI visceral nociceptors ($Na_V1.8^+DiD^-$) shows one differentially expressed gene (**B**). A volcano plot (adjusted p-value <0.2 in red) of pairwise comparison of naive versus AAI $Na_V1.8^-$ cells shows two differentially expressed genes (**C**).

*Data are shown as mean ± S.E.M (**A**), a volcano plot displaying DESeq2 normalized count fold change and nominal p-values for each gene **(B, C).** N are as follows: **A-C**: n=3 biological replicates (4 mice per sample). P-values were determined by a two-sided unpaired Student's t-test (**A**) or DESeq2 analysis (**B, C**).*



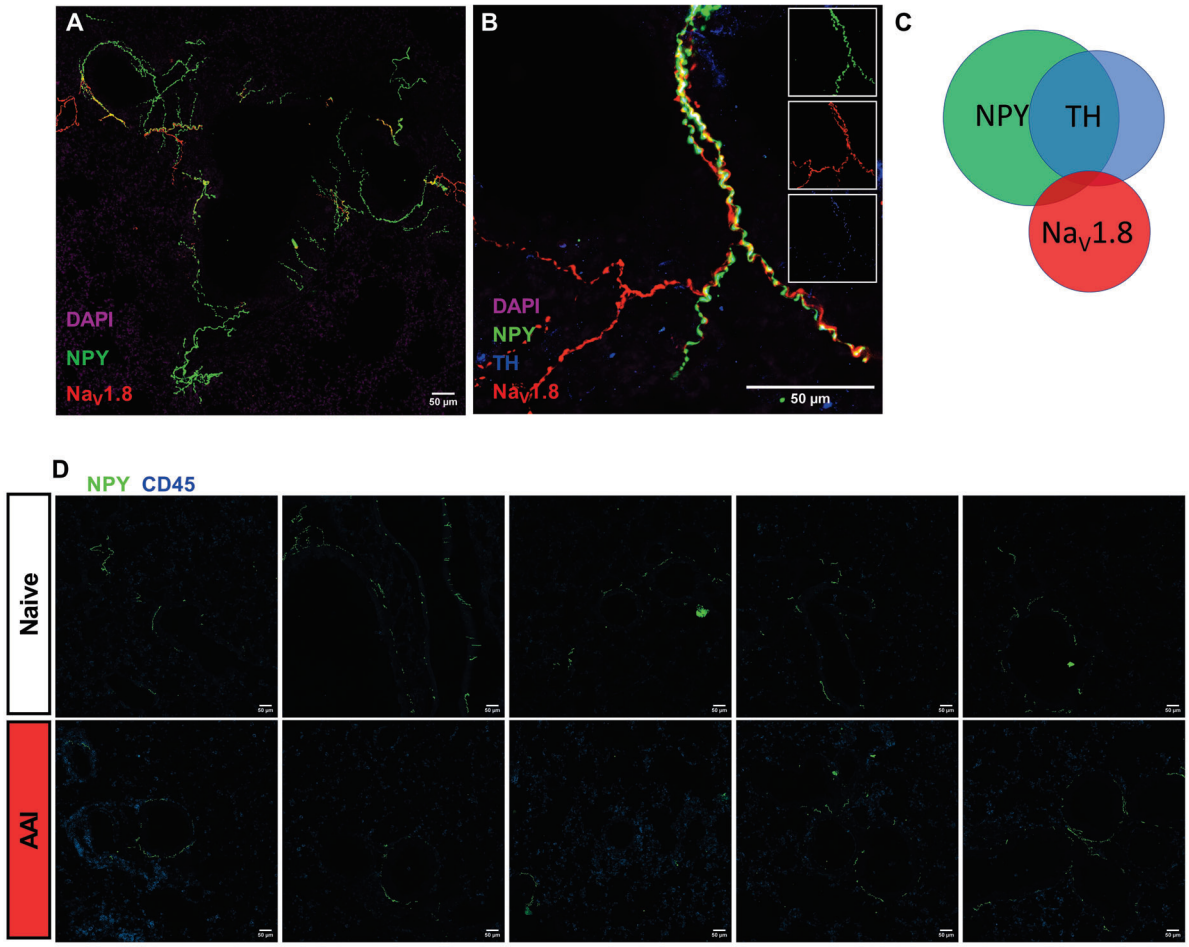
Supplementary figure 5. IL-13 reprogram airway nociceptor neurons through IL4RII.

(**A**) Naive 8-week-old male and female nociceptor neurons reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice were injected intranasally with the retrograde tracer DID' (200 µM). Fourteen days later, the mice were euthanized and their JNC ganglia isolated and dissociated. Airway-innervating nociceptor neurons ($Na_V1.8^+DiD^+$), visceral nociceptors ($Na_V1.8^+DiD^-$) and $Na_V1.8^-$ cells were purified by flow cytometry and RNA sequenced. Both IL4RII subunits, *Il4ra* and *Il13ra1*, were detected in JNC nociceptors. Other IL-13 and IL-4 receptors are not detected. *IL13ra1* transcript expression was higher in nociceptor neurons when compared to levels measured in $Na_V1.8^-$ cells (**A**).

(B) Naive 8-week-old male and female C57Bl6 mice were euthanized and their JNC and DRG ganglia were isolated and cultured (16 h). The neurons were then loaded with the calcium indicator Fura-2AM (5 µM) and their responsiveness to IL-13 (100 ng/mL) was assessed using calcium microscopy. DRG and JNC neurons show limited calcium response when exposed to IL-13 compared to its vehicle **(B)**.

(**C**) Naive 8-week-old male and female C57BL6 mice DRG neurons were isolated and cultured in the presence of IL-13 (100 ng/mL; 24 h) or its vehicle. Transcript levels were assessed by qPCR. In comparison to the vehicle, IL-13 increased DRG neurons' expression of *Npy1r* (**C**).

*Data are shown as mean ± S.E.M (**A-B**), or as box (25th-75th percentile) and whisker (min-to-max) plots (**C**). N are as follows: **A**: n=3 biological replicates (4 mice per sample), **B**: n=11 culture dishes for JNC and 3 culture dishes for DRG, **C**: n=4 cultures from different mice per group. P-values were determined by one-way ANOVA with post hoc Tukey's (**A**); or a two-sided unpaired Student's t-test (**B-C**). P-values are shown in the figure.*

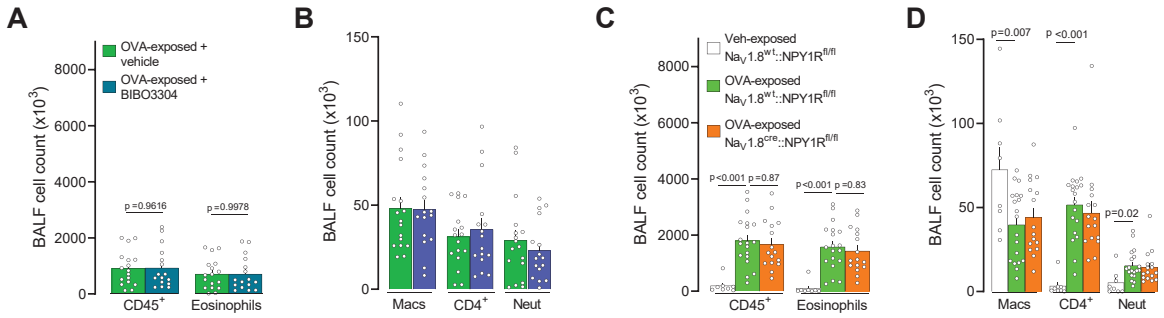


Supplementary figure 6. Airway-innervating sympathetic neurons express NPY.

(A-C) The lungs of 8-week-old male and female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice were harvested, cryosectioned, and NPY expression was assessed by immunofluorescence. Representative lung images (20x) showed NPY (green), nuclei (purple), and $Na_V1.8$ (red) distribution (**A**). Representative lung images (40x) showed NPY (green), nuclei (purple), $Na_V1.8$ (red), and TH (blue) distribution (**B**) A Venn diagram shows that NPY colocalized with lung TH^+ sympathetic fibers, but was largely distinct from $Na_V1.8^+$ nociceptors neurons fibers (**C**).

(D) 8-week-old female C57BL6 mice underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by inhaled OVA challenges (days 14-17). One day after the last allergen challenge, the lungs were harvested, cryosectioned, and NPY and CD45 expression were assessed by immunofluorescence. NPY (green) innervation patterns are comparable between conditions, while CD45 cells (blue) were increased in lung from AAI mice (**D**).

*Data is shown as tissue immunostaining, scale bar = 50µm (**A, B, D**) or a Venn diagram (**C**). N are as follows: **C**: n=12 field of views from 4 different mice; **D**: n=6 field of views from 6 different mice per condition.*

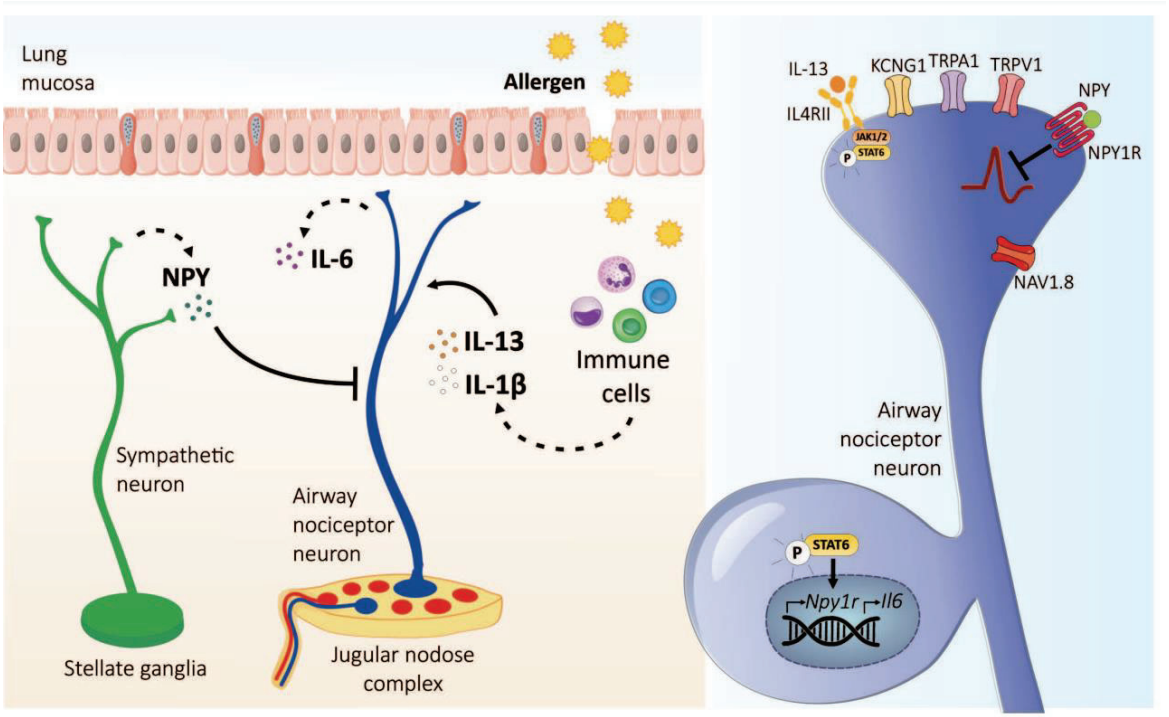


Supplementary figure 7. NPY does not impact immune cell infiltration.

(**A-B**) 8-week-old female C57BL6 mice underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by inhaled OVA challenges (days 14–17). NPY1R blocker BIBO3304 (25 µg/200 µl, i.p.) or its vehicle (DMSO 0.33%) was injected daily (day 13–17). One day after the last allergen challenge, the bronchoalveolar lavage fluid (BALF) was harvested and immunophenotyped by flow cytometry. OVA-exposed mice showed significant airway inflammation characterized by increased BALF infiltration of leukocytes ($CD45^{+}$), eosinophils ($CD45^{+}CD11C^{low}SiglecF^{Hi}$), alveolar macrophages ($CD45^{+}CD11C^{low}SiglecF^{Hi}$), neutrophils ($CD45^{pos}SiglecF^{low}CD11B^{high}LY6G^{high}$), and CD4 T cells ($CD45^{pos}SiglecF^{low}CD11B^{low}CD4^{high}$) (**A-B**). BALF cell numbers were not affected by the NPY1R blocker BIBO3304 (**A-B**).

(**C-D**) 8-week-old female nociceptor neuron NPY1R conditional knockout ($Nav1.8^{wt}::NPY1R^{fl/fl}$) and littermate control mice ($Nav1.8^{wr}::NPY1R^{fl/fl}$) underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. days 0 and 7) followed by inhaled OVA challenges (days 14–17). One day after the last allergen challenge, the bronchoalveolar lavage fluid (BALF) was harvested and immunophenotyped by flow cytometry. OVA-exposed mice showed significant airway inflammation characterized by increased leukocytes infiltration ($CD45^{+}$), among which eosinophils ($CD45^{+}CD11C^{low}SiglecF^{Hi}$), neutrophils ($CD45^{pos}SiglecF^{low}CD11B^{high}LY6G^{high}$), and CD4 T cells ($CD45^{pos}SiglecF^{low}CD11B^{low}CD4^{high}$), while alveolar macrophages ($CD45^{+}CD11C^{high}SiglecF^{Hi}$) were reduced. BALF cell numbers were not affected by the conditional KO of NPY1R (**C-D**).

*Data are shown as mean ± S.E.M (**A-D**). N are as follows: **A-B**: n=17 mice per group; **C-D**: n=6 (naive) and 16–19 (AAI) mice per group. P-values were determined by a two-sided unpaired Student's t-test (**A, B**) or one-way ANOVA with post hoc Tukey's (**C, D**). P-values are shown in the figure.*



Supplementary figure 8. IL-13 promotes sensory-sympathetic neurons crosstalk in asthma.

When allergens are present in the airways, the immune and stromal cells in the area become activated and release inflammatory cytokines such as IL-1β and IL-13. These cytokines are then detected by nociceptor neurons, which leads to specific changes in gene expression. For example, exposure to IL-1β increases the expression and release of IL-6 by airway nociceptor neurons. Additionally, IL-13 signaling through its interaction with IL4RII leads to increased expression of Npy1r via phosphorylation of STAT6. Via the action of neuropeptide Y (NPY) released by sympathetic neurons, this cascade ultimately decreased the sensitivity of NPY1R-expressing nociceptor neurons through reduced intracellular levels of cAMP.

Supplementary Table 1. Airway neuronal subtypes**.** Single cell sequencing data of JNC cells from Prescott et al. were reanalyzed using Seurat. Neuronal cells were selected based on *Slc17a6* (Vglut2) expression. A total of 21 neuronal populations were identified. *Phox2b* and *Prdm12* were used to identify nodose and jugular groups, while nociceptor neurons and low-threshold sensory neurons were defined based on their expression of *Scn10a* and *Scn1a*. The markers identified for each cluster were compared to airway-innervating neurons sequencing by GSEA. A positive normalized enrichment score indicates preferential innervation of the airways for a given neuronal population. Detailed classification of neurons preferentially innervating the airways is displayed in a tab. Other tabs display the average expression of all genes for all neuronal clusters and DESeq2 analysis comparing airway and visceral nociceptor neurons. Another tab shows the average gene expression in clusters identified in reanalysis of projection-seq data from Zhao et al.

Supplementary Table 2. DESeq2 analysis of JNC cell populations in AAI and Naive conditions. 8-week-old male and female nociceptor neuron reporter ($Nav1.8^{cre}$::$tdTomato^{fl/wt}$) mice underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. days 0 and 7) followed by inhaled OVA challenges (days 14–17). On day 2, 3, and 4, mice were injected intranasally with the retrograde tracer DiD' (200 µM). One day after the last allergen challenge, the JNC neurons were harvested for flow cytometry purification and RNA sequencing. DESeq2 pairwise comparison of airway-innervating ($Na_V1.8^+DiD^+$) naive nociceptor neurons versus AAI nociceptor neurons counterparts shows 92 differentially expressed genes. A literature review for each gene allowed a classification of their expected function [52,77,103-152]. Other tabs display DESeq2 analysis of visceral nociceptors ($Na_V1.8^+DiD^+$) and $Na_V1.8^-$ cells.

Supplementary Table 3. Cytokines reprograms nociceptor transcriptome. 8-week-old male and female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice JNC neurons were cultured (24 h) with IL-13 (100 ng/mL), IL-1β (100 ng/mL), BDNF (50 ng/mL), TNF-α (100 ng/mL) or vehicle, before nociceptor purification by flow cytometry and RNA-sequencing. Data from Cobos and colleagues [37] was also reanalyzed by DESeq2 and shows various differentially expressed genes induced in whole DRG three days after spared nerve injury. The different tabs shows the DESeq2 analysis for each of these conditions.

Supplementary Table 4. DEGs in IL-13 exposed nociceptors. 8-week-old male and female nociceptor neuron reporter ($Na_V1.8^{cre}$::$tdTomato^{fl/wt}$) mice JNC neurons were cultured (24 h) with IL-13 (100 ng/mL) or vehicle. The nociceptor neurons were then purified by flow cytometry and changes to their transcriptome analyzed by RNA sequencing. The tables display the results of the DESeq2 analysis comparing the vehicle and IL-13-exposed conditions. DEGs are showed in a separate tab. A literature review for each gene allowed a classification of their expected function[52,153-175].

Supplementary Table 5. FPKM normalized sequencing data. Fragment per kilobase per million (FPKM) normalized values for all sequencing data produced in this study (airway nociceptors, visceral nociceptors, $Na_V1.8^-$ cells in naive and allergic airway inflammation conditions, cultured nociceptors exposed to vehicle, cytokines, or neurotrophins).

METHODS

Animals. Mice were housed in standard environmental conditions (12h light/dark cycle; 23°C; food and water ad libitum) at facilities accredited by CCPA. Parental strain C57BL6 (Jax, #000664), tdTomato$^{fl/fl}$ (Jax, #007914), Trpv1$^{cre/cre}$ (Jax, #017769), Npy1r$^{cre/cre}$ (Jax, #030544) were purchased from Jackson Laboratory. Parental strain Nav1.8$^{cre/cre}$ mice were generously supplied by Prof. John Wood (UCL). Parental strain NPY1R$^{fl/fl}$ mice were generously supplied by Prof. Herbert Herzog (Gavan Institute of Medical Research). Male and female mice were bred in-house and used between 6 and 12 weeks of age. Cross breeding was used to generate the following genotypes: Na$_V$1.8^{cre}::tdTomato$^{fl/wt}$, NPY1R^{cre}::tdTomato$^{fl/wt}$, TRPV1^{cre}::tdTomato$^{fl/wt}$, Na$_V$1.8^{cre}::NPY1R$^{fl/fl}$, and Na$_V$1.8^{wt}::NPY1R$^{fl/fl}$ (littermate control).

Ovalbumin model of allergic airway inflammation. On days 0 and 7, mice were sensitized by a 200 µL i.p. injections of a solution containing 1 mg/mL ovalbumin (Sigma, #A5503) and 5 mg/ml aluminum hydroxide (Sigma, # 239186). On days 15–17, mice were lightly anesthtetized (isoflurane 2.5 %, CDMV #108737) and instilled daily with 50 µg/50 µL OVA intranasally. Mice were sacrificed on day 18 unless otherwise indicated.

House dust mite model of allergic airway inflammation. Lightly anesthetized (isoflurane 2.5 %, CDMV #108737) mice were challenged (20 µg/50 µL, intranasal) on day 1–5 and 8–10 with house dust mites (CiteQ Biologics, #15J01) and sacrificed on day 11.

Airway neurons' retrograde tracing. Mice were injected (50 µL, 200 µM in PBS, 1% DMSO; intranasally) with DiD' (Thermofisher, #D7757) for 3 consecutive days (D2-D4 of OVA protocol). The mice were sacrificed two weeks after the last injection to allow the tracer to reach the JNC.

BIBO3304: 30-min prior to the OVA/veh challenges, the mice were injected (25 µg / 200 µL; intraperitoneally) with BIBO3304 (Tocris, #2412) or vehicle (0.2% DMSO diluted in PBS) on days 14, 15, 16 and 17.

AS1517499: Ovalbumin was mixed with AS1517499 (150 µg / 50 µL) or vehicle (DMSO), for intranasal co-instillation at each challenge of the OVA protocol (day 15-16-17). Naïve mice received the vehicle (DMSO) without Ovalbumin.

Neuron culture: Mice were sacrificed and JNC or DRG were dissected into an ice-cold HEPES buffered DMEM medium (Thermofisher, #12430062). JNC ganglia from 2-10 mice were pooled in the same tube. The cells were transferred to a HEPES buffered DMEM medium completed with 1 mg/mL collagenase IV (Sigma, #C5138) + 2.4 U/mL dispase II (Sigma, #04942078001) and incubated for 70 minutes at 37 °C. Ganglia were triturated with glass Pasteur pipettes of decreasing size in DMEM medium, then centrifuged (200 g) over a 15% BSA gradient in PBS to eliminate debris. The neurons were plated on laminin (Sigma, #L2020) coated cell culture dishes. The cells were cultured at 37° with Neurobasal-A medium (Gibco, #21103-049), supplemented with 2% B27 (Thermofisher, #17504044) 0.01 mM AraC (Sigma, #C6645), 200 nM L-Glutamine (VWR, # 02-0131), 100 U/mL penicillin and 100 µg/mL streptomycin (Corning, #30002CI) without neurotrophin unless otherwise indicated. Culture densities and durations are described for each application.

Cyclic AMP. JNC neurons were cultured ($1.5x10^3$) in 96 well plates (VWR #10062-900) for 16 hours. The media was removed, and neurons exposed to Leu^{31}Pro^{34}NPY (250nM Tocris #1176) or vehicle (PBS containing cAMP phosphodiesterase inhibitor (100 µM Ro-20-1724; Sigma, #557502 and 500 µM 3-Isobutyl-1-methylxanthine; Sigma, #I5879) for 30 minutes. Cells were lysed for enzymatic cAMP measurement assay using a commercial kit and following manufacturer's instructions (Promega, #V1501).

Calcium microscopy. C57BL6 or NPY1R^{cre}::tdTomato$^{fl/wt}$ DRG or JNC neurons were plated ($2x10^3$) on laminin-coated glass-bottom dishes (35 mm; ibidi, #81218) and cultured overnight. The cells were then loaded with Fura-2-AM (5 µM, 37 °C, Biovision, #125280) for 45min, washed with Standard Extracellular Solution (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5), and imaged at room temperature. Capsaicin 50 nM, 100 nM and 300 nM (Tocris, #0462) or JT010 10 µM and 50 µM (Sigma, #SML1672), or IL-13 (100 ng/mL, Biolegend #575904) were prepared in SES and were flowed (30 seconds, 1 minute and 1 minute, respectively) directly onto neurons using perfusion barrels followed by buffer washout (5 minutes). 40 mM KCl solution was then flowed on the cells for 20 seconds. To test NPY1R effect on neuron

sensitivity, $Leu^{31}Pro^{34}$NPY (250 nM; Tocris, #1176) or vehicle was perfused for 5 minutes before the other compounds. A single field of view was acquired per dish. Cells were illuminated by a UV light source (Cool LED, pE-340) with alternating 340 nm and 380 nm excitation, and a camera (Photometrics Prime 95B 25 mm) captured fluorescence emission (515/45 nm) with a 20X objective on a Ti2 microscope (Nikon). Regions of interest (ROI) were manually drawn and 340/380 fluorescence ratios were exported. Microsoft Excel was used for further analysis (Microsoft, USA). Neurons were considered responsive to a compound if the fluorescence ratio increased by at least 10% within 1 minute after injection.

Electrophysiology. JNC neurons from $NPY1R^{cre}$::$tdTomato^{fl/wt}$ mice were plated ($2x10^3$) onto Poly-D-lysin and laminin-coated glass-bottom 35 mm dishes (Ibidi #81218) and cultured in supplemented neurobasal medium. TdTomato-positive JNC neurons were identified using a Nikon Eclipse Ti microscope. Whole-cell voltage and current-clamp recordings were performed with an EPC 800 patch clamp amplifier (HEKA) and were filtered at 10 kHz with the internal Bessel filter and digitized using an Axon Instruments Digidata 1440A digitizer at 20 kHz. The neurons were placed in standard external solution (163 mM NaCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES, pH = 7.4). Recording pipettes were pulled from soda lime glass pipettes (outer diameter 1.5 mm, Kimble® Chase, #41A2502,) using a P-97 microelectrode puller (Sutter Instrument). Pipettes with resistances of 3–5 MΩ were used for recordings. Pipettes were filled with internal solution (133 mM potassium gluconate, 6 mM NaCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 10 mM HEPES, and 10 mM EGTA, pH = 7.2, final K_{in}^+ concentration –163mM).

For voltage clamp experiments, the neurons were clamped at –60mV. Neuronal currents were recorded before and after drug exposure from a series of depolarization steps ranging from –120 to +70mV in 10 mV intervals. For the current clamp AP protocol, neurons were injected with a series of 35 pA current steps (1 s duration) from 0 to 630 pA. These voltage and current clamp experiments were performed before and 10 mins post-addition with a pipette of external solution (vehicle) or $Leu^{31}Pro^{34}$NPY (250 nM; Tocris, #1176). Current amplitudes and APs were quantified using Clampfit 10.7 (Molecular Devices). Since baseline activity varied among the JNC neurons, the activity of the neurons after application of vehicle or $Leu^{31}Pro^{34}$NPY was normalized to baseline activity of the same cell.

Bronchoalveolar lavage fluid (BALF). Mice were anesthetized by intraperitoneal injection of urethane (250 µL i.p., 20%) and a 20G sterile catheter inserted longitudinally into the trachea. 500 µL followed by 1 mL of ice-cold buffer (PBS, 2% FBS, 1 mM EDTA) containing protease inhibitors (Sigma, #P1860) was injected into the lung, harvested, and stored on ice. BALF underwent a 400g centrifugation (5 min; 4 °C), supernatant of the first flush was harvested and frozen (−80°C) for subsequent ELISA experiments. Cells were resuspended for cell count and flow cytometry analysis.

BALF immunophenotyping. Cells were resuspended in red blood cells lysis buffer (Gibco, #A10492-01) for 1 minute then washed with PBS. Cells were then stained with Zombi Aqua (Biolegend, #423102) for 10 minutes, before staining in a flow cytometry buffer (PBS, 2% FBS, 1 mM EDTA) supplemented with 1% Rat Serum. Staining antibodies included anti-CD45–BV421 (1:400; BioLegend, #103134), anti-Siglec-F–PE (1:400; Thermofisher, #12-1702-82), anti-CD11b-APC–Cy7 (1:400, BioLegend, #101262), anti-CD11c–FITC (1:400, BioLegend, #117306), anti-Ly6G–APC (1:400, BioLegend, #127614), anti-Ly6C–PE-Cy7 (1:400, BioLegend #128018), and anti-CD4-PerCP–Cy5.5 (1:400, BioLegend, #116012) for 30 minutes in the dark at 4 °C. $1x10^4$ counting beads (Biolegend, #424902) were added for absolute quantification before data acquisition on a FACS Canto II (BD Biosciences). Leukocytes were gated as $CD45^{pos}$, eosinophils as $CD45^{pos}SiglecF^{high}CD11C^{low}$, alveolar macrophages as $CD45^{pos}SiglecF^{high}CD11C^{high}$, neutrophils as $CD45^{pos}SiglecF^{low}CD11B^{high}LY6G^{high}$, and CD4 T cells as $CD45^{pos}SiglecF^{low}CD11B^{low}CD4^{high}$.

Flow cytometry sorting of sensory neurons. $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ mice were sacrificed and JNC or DRG were dissected into ice-cold HEPES buffered DMEM medium (Thermofisher, #12430062). For RNA sequencing of airway nociceptor neurons, JNC ganglia were pooled from 4 mice (2 males and 2 females) for each biological replicate. Ganglia were transferred into a HEPES buffered DMEM medium completed with 1 mg/mL collagenase IV (Sigma, #C5138) + 2.4 U/mL dispase II (Sigma, #04942078001) and incubated for 70 minutes at 37°C, then washed in DMEM medium before trituration with glass Pasteur pipettes of decreasing size. Cells were then centrifuged (200g) over a 15% BSA gradient in PBS to eliminate debris. Nucleus were

stained with SYTO40 (10 µM, 5 minutes RT; Thermofisher, #S11351) to differentiate cells from axonal debris, washed with PBS, then cells were resuspended in sterile flow cytometry buffer (PBS, 2% FBS, 1 mM EDTA) and filtered (70 µm; VWR, #10204-924).

For DiD'-injected mice, JNC neurons were sorted directly into Trizol (500 µL; Invitrogen, #15596026) and stored at −80°C for subsequent RNA extraction. Airway nociceptors were gated as Syto40^{hi}tdTomato^{hi}DiD^{pos}, visceral nociceptors as Syto40^{hi}tdTomato^{hi}DiD^{lo}, and glial/stromal Na$_V$1.8$^-$ cells as Syto40^{hi}tdTomato^{lo}. DiD'-injected Na$_V$1.8^{cre}::tdTomato$^{fl/wt}$ mice lumbar DRG neurons and naive Na$_V$1.8^{cre}::tdTomato$^{fl/wt}$ mice JNC neurons were used as gating controls for DiD'. JNC neurons from C57BL6 mice were used as gating controls for tdTomato.

BALF ELISA. Cell free BALF supernatant and serum were used for ELISA quantification after storage at −80°. IL-13 (Thermofisher, #88-7137-88) and NPY (Cusabio, #CSB-E08170M) were measured using commercial kits following manufacturer's instructions.

Nociceptor neuron ELISA. C57BL6 mice JNC neurons were cultured (1.5x10^4) in 96 well plates with vehicle or IL-1β (100 ng/mL). After 36 hours, supernatant was harvested, centrifuged (500g) to remove cellular debris, and IL-6 secretion analyzed using a commercial ELISA kit following the manufacturer's instructions (Biolegend, #431304).

Whole-lung RNA extraction: Whole lungs were minced with a razor blade, and about a quarter of the minced lung was mixed with 500uL Trizol (Thermofisher #15596018) and stored at -80° for subsequent RNA extraction. RNA was separated from protein and DNA by mixing 500 µL of sample in Trizol with 100 µL chloroform before ultracentrifugation (15 min, 16000g, 4°). The upper phase was mixed with a half volume of 100% isopropanol, transferred to a purification column, and the RNA was then purified using the kit E.Z.N.A. Total RNA Kit I (VWR, #CA101319) following manufacturer's instructions.

JNC Neurons RNA extraction: RNA was separated from protein and DNA by mixing 500 µL sample in Trizol with 100 µL chloroform before ultracentrifugation (15 min, 16000g, 4°). The upper phase was mixed with a half-volume of 100° isopropanol, transferred to a purification column, and the RNA purified using the kit PureLink RNA Micro Scale (Thermofisher, #12183016) following manufacturer's instructions.

Screening of cytokine neuromodulatory capacity. JNC neurons were plated (5x10^3; 200 µL) on laminin-coated 96 well plates and exposed to cytokines (100 ng/mL; IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-31, IL-33, SCF, TGF-β, TSLP or TNF-α), lipid mediators (200 ng/mL; leukotriene C4), neurotrophins (50 ng/mL; nerve growth factor or brain-derived neurotrophic factor), IgE-OVA (10 µg/mL) or NPY (100 nM). To investigate IL-13 intracellular signaling, JNC neurons were incubated with IL-13 (100 ng/mL, Biolegend #575904) for 24 hours in presence or not of the STAT6 inhibitor AS1517499 (1 µM MCE #HY-100614) or the JAK1/2 inhibitor Ruxolitinib (10 µM, MCE #HY-50856). Biological replicates were made using different mice preparations for each replicate. After 24 hours of culture, the medium was removed, and the cells harvested in 500 µL Trizol (Thermofisher, #15596018). Samples were then used for RNA extraction and RT-qPCR.

RT-qPCR. RNA was reverse transcribed using the SuperScript VILO Master Mix (Thermofisher, #11755250). The cDNA was then subjected to two-step thermocycling using PowerUp qPCR SYBR Green Mix (Invitrogen, #A25742) and data collection was then performed on a Mic qPCR machine (Bio Molecular Systems). 1–3 ng cDNA was used for qPCR from neuron preparations, and 80–100 ng for qPCR from whole lung RNA. Expression levels were normalized using the ΔΔCt method with *Actb* as the reference gene.

The primers used were : *Actb* Forward: TGTCGAGTCGCGTCCACC; *Actb* Reverse: TATCGTCATCCATGGCGAACTGG; *Trpa1* Forward: TCCAAATTTTCCAACAGAAAAGGA; *Trpa1* Reverse: CGCTATTGCTCCACATTGCC; *Npy1r* Forward: CTCGTCCCGCTTCAACAGAG; *Npy1r* Reverse: TCAAAACGGATCAAATCTTCAGCA; *Il6* Forward: GGATACCACTCCCAACAGACC; *Il6* Reverse: AATTGCCATTGCACAACTCTTTTC; *Bdnf* Forward: CAGGTTCGAGAGGTCTGACG; *Bdnf* Reverse: AAGTGTACAAGTCCGCGTCC; *Sting1* Forward: CTGCCGGACACTTGAGGAAA; *Sting1* Reverse: CCGTCTGTGGGTTCTTGGTAG; *Npy* Forward: CGCTCTGCGACACTACATCA; *Npy* Reverse:

AGGGTCTTCAAGCCTTGTTCT; *Il13* Forward: CCAGACTCCCCTGTGCAAC; *Il13* Reverse: GGCTACACAGAACCCGCC; *Muc5ac* Forward: AAGATCAACTGTCCGCAGGG; *Muc5ac* Reverse: GGTGCACCGTACATTTCTGC; *Muc5b* Forward: AAGAGCACAGAGTGTCAGGC; *Muc5b* Reverse: GTGTGTGGCAGCTTTTGAGG.

Western Blot. C57BL6 mice DRG or JNC neurons ($1.5x10^4$; 200 µL) were plated onto 35mm well plates and cultured overnight. Neurons were exposed to IL-13 or vehicle for 60 minutes (37°), then washed with PBS and lysed in RIPA buffer (Sigma, #20-188) supplemented with phosphatase inhibitor (1/100, Sigma #P0044) and protease inhibitor (1/200, Sigma, #P1860) for 20 minutes at room temperature. Protein concentrations were measured by BCA (Thermofisher, #23227) and equilibrated with a supplemented RIPA buffer. Samples were then mixed 1:1 with loading buffer (0.1M Tris pH 6.8; 4% SDS; 0.25% Bromophenol blue; 20% glycerol; 10% β-mercaptoethanol) and heated to denature proteins (100 °C, 5 minutes). 10 µg of protein were loaded and separated by SDS-PAGE electrophoresis with an acrylamide gel. Proteins were then transferred to a nitrocellulosis membrane, blocked with blocking buffer (TBS-T buffer containing 5% BSA), incubated with primary antibodies in blocking buffer (overnight, 4°), washed, and incubated with secondary antibodies in blocking buffer (1 h, RT). Membranes were then washed and revealed using SuperSignal West Dura Extended Duration Substrate (Thermofisher, #34075). Antibodies used included rabbit anti-pSTAT6 (1/1000, CST, #9361S), rabbit anti-STAT6 (1/1000, CST, #9362S) and HRP anti-rabbit (1/2000, CST, #7074S).

Neurite growth assay $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ JNC neurons were plated ($1.5x10^3$) on laminin-coated glass-bottom dishes (35 mm; ibidi, #81218) and cultured for 24 hours. Neurobasal media was supplemented or not with with IL-13 (100 ng/mL, Biolegend #575904), IL-1β (100 ng/mL, Biolegend #575104), TNF-α (100 ng/mL Biolegend # 575204), BDNF (50 ng/mL Peprotech #450-02) or vehicle. Pictures of the whole plating area were taken after 24 hours of culture using a 20X objective to collect tdTomato fluorescence (Excitation 554/23nm; Emission:609/54nm). For the analysis of neurite outgrowth, an in-house developed method was used. Using Nikon Elements software, a fluorescence threshold was used to define the tdTomato positive neurites and soma. The somas were then defined and excluded based on fluorescence intensity, size, and circularity. The total size of neurites was then divided by the number of somas for each culture dish.

Immunofluorescence. C56BL6 and $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ mice were anesthetized with urethane (20%, 250 µL, i.p.) and perfused with 10 mL PBS, then with 10 mL 4% PFA. Lungs, JNC, DRG and SCG were harvested and incubated in 4% PFA for 24 hours at 4°. Organs were then sequentially transferred in 10%, 20%, then 30% sucrose (24 hours each), before mounting and freezing (−80°) in OCT. Cryosections of 40 µm for lungs and 15 µm for ganglia were prepared. For neuron cultures, the medium was removed, and the cells fixed with 4% PFA for 15 minutes. Sections and cultures were incubated in blocking solution (1X PBS, 0.2% Triton X-100, 50 mg/mL BSA and 5% goat serum) for 3 hours at room temperature, then incubated with primary antibodies in staining solution (PBS, 0.2% Triton X-100 and 3% goat serum) for 48 hours at 4°. Primary antibodies used included rabbit anti-NPY (1/500, CST #11976S), guinea pig anti-PGP9.5 (1/1000, Sigma #AB5898), chicken anti-TH (1/500, Aves Laboratory #TYH-0020), rat anti-CD45 BV421 conjugated (1/300, Biolegend ##103134), rabbit anti-IL6 (1/300, CST #12912T). Cryosections were then washed (3 times, 15 minutes with shaking) with PBS containing 0.2% Triton X-100. Secondary antibodies were incubated in staining solution for 16 hours at 4°. Secondary antibodies included AF488 goat anti-guinea pig (1/1000, Thermofisher #A11073), AF546 goat anti-guinea pig (1/1000, Thermofisher #A-11074) AF647 goat anti-rabbit (1/1000, Thermofisher #A21245), CF488 goat anti-chicken (1/1000, Sigma #SAB4600039), AF405 goat anti-chicken (1/1000, Thermofisher #A48260). Ti2 microscope (Nikon) equipped with a camera (Photometrics Prime 95B 25 mm) was used for imaging. Nerve endings in lung slices were acquired by confocal microscopy (Zeiss, LSM800), with a Z stack of the whole section (40 µm).

Immunofluorescence analysis. Using ImageJ or Nikon Elements, circular ROI were manually defined around each neuron based on PGP9.5 fluorescence for JNC and SG slices. For confocal pictures of lung slices, an orthogonal projection of the z-stack was made, then a threshold-based method was used to define the area of nerve fibers and immune cells for each marker as well as their overlapping areas. Cultured cells were analyzed with a threshold-based method to define neurons based on tdTomato or PGP9.5 expression. Average fluorescence in ROI was exported and all further analysis were performed in Microsoft Excel. When spillover between colors occurred, a compensation was applied using single-stain controls.

Neuron culture for RNA sequencing. Na$_V$1.8^{cre}::tdTomato$^{fl/wt}$ mice were sacrificed and their JNC harvested. For each sample, JNC from an equal number of males and females were pooled and enzymatically dissociated, seeded (1x10^4 / well) in 12 well plate (VWR, #10062-894), and cultured with IL-13 (100 ng/mL), IL-1β (100 ng/mL), TNF-α (100 ng/mL), BDNF (50 ng/mL) or vehicle. After 24 hours, the cells were then mechanically detached with a cell scraper and harvested in flow cytometry buffer (PBS, 2% FBS, 1 mM EDTA), filtered (70 µm), sorted by flow cytometry (FSC^{hi}SSC^{hi}tdTomato^{hi}), and collected into Trizol.

RNA sequencing. RNA libraries preparation and sequencing were carried out at the genomic platform of the Institut de Recherche en Cancérologie et en Immunologie (IRIC). Briefly, RNA quality was assessed using a Bioanalyzer (Agilent), and all preparations had an RIN>7.5. Libraries were prepared using the KAPA mRNA HyperPrep Kit (KapaBiosystems #KR1352). All barcoded samples were then sequenced with a Nextseq500 (Illumina) with 75-cycle single-end read.

Genome alignment and differential expression analysis were carried out (IRIC genomic platform). Sequences were trimmed for sequencing adapters and low quality 3' bases using Trimmomatic version 0.35 and aligned to the reference mouse genome version GRCm38 (gene annotation from Gencode version M25, based on Ensembl 100) using STAR version 2.7.1. Gene expressions were obtained from STAR as readcounts and computed using RSEM to obtain normalized gene and transcript level expression in FPKM. Differential expression analysis for the various comparisons of interest were made using DESeq2 [176] and STAR readcounts. Further analysis and plots were made using RStudio or Microsoft Excel. Genes were considered as differentially expressed if their adjusted p-value (FDR) was less than 0.2.

***In-silico* analysis of JNC neuron single-cell transcriptome:** Prescott et al.[1] generated single-cell sequencing data for jugular-nodose ganglia cells from 40 mice using the 10X Genomics platform. The data was downloaded from the NCBI Gene Expression Omnibus (GSE145216) and analyzed using Seurat. Neuronal cells were selected based on *Slc17a6* (VGLUT2) expression (raw count ≥ 2). A standard workflow was used for quality control, preprocessing, normalization, and clustering (resolution = 0.2, PCs = 1:30). *Phox2b* and *Prdm12* were used to identify nodose and jugular groups, while nociceptor neurons and low-threshold sensory neurons were defined based on their expression of *Scn10a* and *Scn1a* [2]. Xhao et al.[7] generated single-cell projection sequencing data for jugular-nodose ganglia cells from mice in which barcode expressing viruses were used to retrogradely label neurons innervating various internal organs. The data was downloaded from the NCBI Gene Expression Omnibus (GSE192987) and analyzed using Seurat. Neurons expressing at least one barcode (raw count ≥ 2) were selected. A standard workflow was used for quality control, preprocessing, normalization, and clustering (resolution = 0.75, PCs = 1:20).

GSEA analysis: Gene set enrichment analysis [177] was performed to compare similarities between different sequencing results together using the GSEA software. To assess the preferential innervation of airways by scRNAseq neuron clusters, GSEA analysis was performed using the cluster markers identified in Prescott et al.'s dataset as genesets, and the DESeq2 counts of airway and visceral neurons as gene expression dataset. To compare AAI, BDNF, IL-1b, IL-13, TNF-a, and nerve injury sequencing data, gene signatures were constituted by selecting overexpressed genes (FDR<0.2) induced in each condition. For the nerve injury signature, RNAseq data from Cobos et al [37] was downloaded from NCBI Gene Expression Omnibus (GSE102937) and reanalyzed by DESeq2 (**supplementary table 3**). The enrichment of each gene signatures in each DESeq2 count datasets were measured by GSEA.

Data availability. Bulk RNA-seq raw and processed data have been deposited in the NCBI's gene expression omnibus (GSE223355). Processed data can also be accessed in the **supplementary tables 1–5**. Additional information and raw data are available from the lead contact upon reasonable request.

Statistics. No data were excluded. P *values ≤ 0.05* were considered statistically significant**.** One-way ANOVA, two-way ANOVA, and Student t-tests were performed using Graphpad Prism. DESeq2 and Seurat analysis and statistics were performed using RStudio.

Replicates. Replicates (n) are described in the figure legends and represent the number of animals for *in vivo* data. For *in vitro* data, replicates can either be culture wells or dishes, animals, fields-of-view (microscopy), or

neurons (patch-clamp), but always include different preparations from different animals to ensure biological reproducibility.

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XII - Discussion

Our work provided new insights into the regulation of immune responses by nociceptors. We focused specifically on airway-innervating nociceptors and provided new molecular characterization of these neurons. Additionally, we identified two type 2 specific immune signals detected by airway nociceptors: IgE through FcεRI, and the cytokines IL-4/IL-13 through IL4RII. Detection of such signals by nociceptors were already suggested in other studies, but we proved here that these are occurring in vivo in mice models of asthma, and specifically in airway nociceptors. In addition, we evidenced intracellular pathways triggered in sensory neurons by those signals. Interestingly, we also observed that sensory and sympathetic nervous systems are interacting in allergic airway inflammation, which appears to dampen sensory neurons excitability.

Airways and vagal neurons

Our work provides novel insights in the classification of vagal nociceptors. We showed that while vagal nociceptors express a large set of common markers with DRG neurons, they also display important specificities. For instance, they are strikingly more sensitive to thermal and chemical stimulation, and they express higher levels of TRPA1 and TRPV1. We can rationalize that visceral and airway mucosa are more vulnerable than the skin, and thus require more sensitive sensors. In addition, JNC vagal nociceptors appeared largely insensitive to NGF, but they are more responsive to BDNF. Since NGF is an important target of drugs in development to treat pain[285,286], we can hypothesize that these treatments would have a moderate effect on visceral pain and interoception. Finally, the neuron classification classically observed in DRGs does not apply well for JNC neurons, where neuropeptides produced are different and neurons are larger in size.

More specifically, the airway-innervating nociceptors are even more sensitive to TRPA1 mediated chemical stimulations than other visceral nociceptors. We showed that airway vagal nociceptors have a unique profile and defined new airway specific neuronal subpopulations at the molecular level. Such molecular map allows to define pathways or receptors of interest to specifically target vagal airway nociceptors, to treat allergic airway inflammation or potentially other pathologies associated with dysregulated lung functions.

Antigen sensing by nociceptors

Using a functional approach, we observed that vagal nociceptors from mice with allergic airway inflammation were responsive to allergen stimulation. This detection was dependent on the expression of FcεRI in populations of peptidergic nociceptors and participated to the pro-inflammatory function of these neurons by enhancing their release of Substance P and VIP. Knocking out FcεRI in nociceptor neurons reduced the immune cell infiltration in a mouse model of allergic airway inflammation. Our data is in line with other studies demonstrating the functional expression of Fc receptors in nociceptor neurons[282,287,288].

Nociceptor transcriptome during allergic airway inflammation

Using RNA-sequencing, we observed a fairly large number of genes overexpressed in nociceptors in a mouse model of asthma. In the JNC, these changes were restricted to airway-innervating nociceptors, ruling out the possibility of immune cell activation in the ganglia, and suggesting that these changes are induced by signals sensed in the inflamed tissue. Our data suggest that cytokine signaling, along with neuronal damage, are responsible for these gene expression changes.

FcεRI in RNAseq

A notable discrepancy between the results of **Article 3** and **Article 4** must be discussed here. In the RNA sequencing of airway specific nociceptors, we failed to detect the expression of *Fcer1g*. However, we did detect this transcript, as well as its overexpression in asthmatic mice, using a similar approach of neuron purification by flow cytometry and RT-qPCR (Article 3, supplementary figure E1I). These two experiments were performed with

different genetic reporter mice, TRPV1^{cre}::tdTomato$^{fl/fl}$ and Na$_V$1.8^{cre}::tdTomato$^{fl/fl}$. These two populations of sensory neurons largely overlap, but the TPRV1 population is expected to be more restricted and might be enriched in FcεRI. In addition, the RNA sequencing of airway nociceptors was performed with a very limited number of neurons and thus a limited amount of RNA. For this reason, some genes with low expression might not be detected. In our experimental conditions, RT-qPCR is expected to be more sensitive[289]. mRNA sequencing with deeper coverage might be required to measure neuronal *Fcer1g*. Notably, we did detect higher amounts of *Fcer1g* mRNA in cultured nociceptors, which suggest that this gene has higher expression in our *in vitro* experiments. Interestingly, we did also observe that exposing neurons to IgE-OVA did induce some the DEGs observed in AAI (**see Article 4**)

IL-13/JAK/STAT6 in nociceptors

In line with previous findings, we observed the functional expression of the IL-13/IL-4 receptor IL4RII in nociceptors. These cytokines were previously found to enhance itch in mice models of dermatitis[205]. We demonstrate for the first time the functional signaling induced by this receptor in vagal nociceptors. In line with observations made with immune cells and other cell types, IL4RII triggers the phosphorylation of STAT6 in a JAK1/2 dependent fashion, which subsequently regulates transcription. We then identified STAT6 target genes in nociceptors. Our data suggest an ambivalent role of this cytokine signaling pathway in nociceptors, with the induction of the antinociceptive receptor NPY1R. This underlies the complex roles of IL4RII, associated with type 2 inflammation and allergic disorders, as well as with epithelial regeneration following tissue damage[66].

The IL4RII/JAK/STAT6 pathway has been actively investigated by allergy immunologists in the past decade, with development, and commercialization of drugs targeting this pathway to treat allergic disorders[117]. Our data suggest that targeting this pathway also affects the nociceptor function. It would be interesting to address how these drugs affect cough, chest pain, and the respiratory cycle in treated patients.

Cytokine expression by nociceptors

Our sequencing data of airway nociceptors from mice with allergic airway inflammation evidenced the overexpression of a set of genes traditionally associated with immune cells. Among these, IL-6 and amphiregulin (Areg) are two cytokines observed in asthma and allergic disorders[290,291]. The expression and release of these cytokines by nociceptor neurons is still limited. IL-6 functional expression was previously observed in enteric neurons[292], and DRG sensory neurons were also shown to express CSF-1 and CCL2 upon nerve injury[293-295]. Despite a robust IL-6 release observed in vagal neuron cultures, genetic ablation of nociceptors did not impact the amount of IL-6 in the airways of mice with allergic airway inflammation (**Figure 8**), probably because IL-6 is also released by other diverse sets of cells. The physiological relevance of IL-6 release by nociceptors has still to be demonstrated. We can hypothesize that this release is required at specific time points, or in specific tissue layers where target cells are in contact with nociceptors. Interestingly, IL-6 was also extensively described as a driver of neuroinflammation in the central nervous system[296-298]. If the IL-6 produced by sensory neurons has a modest contribution in the peripheral tissue innervated, we can also hypothesize that this cytokine is released by the brainstem terminals of vagal nociceptors, promoting neuroinflammation and central sensitization.

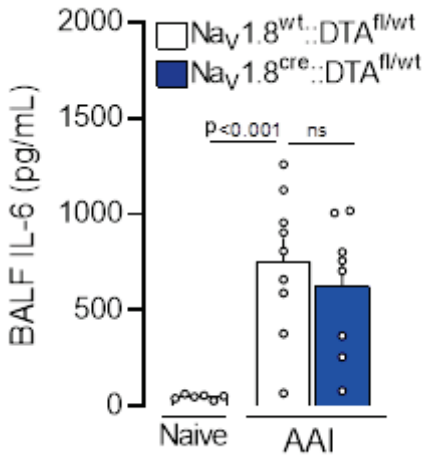

Figure 8 Nociceptors do not contribute to BALF IL-6 increase in AAI 8-week-old female $Na_V1.8^{cre}$::$DTA^{fl/wt}$ and littermate controls $Na_V1.8^{wt}/DTA^{fl/wt}$ mice underwent the ovalbumin mouse models of asthma. Allergic inflammation was induced in mice by an initial sensitization to ovalbumin (OVA) (i.p. day 0 and 7) followed by inhaled OVA challenges (days 14–17). One day after the last allergen challenge, the bronchoalveolar lavage fluid (BALF) was harvested and IL-6 measured in cell free supernatant by ELISA. The genetic ablation of nociceptors did not alter the concentration of IL-6 in the BALF.

Relevance in human biology

As most of the work done in these studies were carried out using mouse vagal and DRG neurons, the question of whether this is applicable to human biology can be raised. Other studies have aimed to access the human sensory neurons biology, with techniques including either post-mortem sequencing or immunostaining of human sensory neurons ganglia, as well as with human stem cells differentiated in sensory neurons[299-302]. Sequencing data is not yet available for human JNC neurons, but the datasets comparing in mice and human DRG unraveled similar neuronal subtypes[301], although the gene expression levels may vary for some specific genes. Interestingly, the dataset released by Jung *et al*[300] reveals that *Npy1r* and *Il13ra1* are co-expressed in a subset of peptidergic nociceptors (PEP1) (**Figure 9**), which profile is similar the one of the airway-specific NN8 we identified in mice JNC. This observation is true in both human and mice, with *Il13ra1* being more expressed in human than mouse DRG, while the opposite occurs for *Npy1r*. Although this should be validated in human JNC nociceptors, we can infer from the RNA data that the pathways described in this thesis are likely to also occur in human sensory neurons.

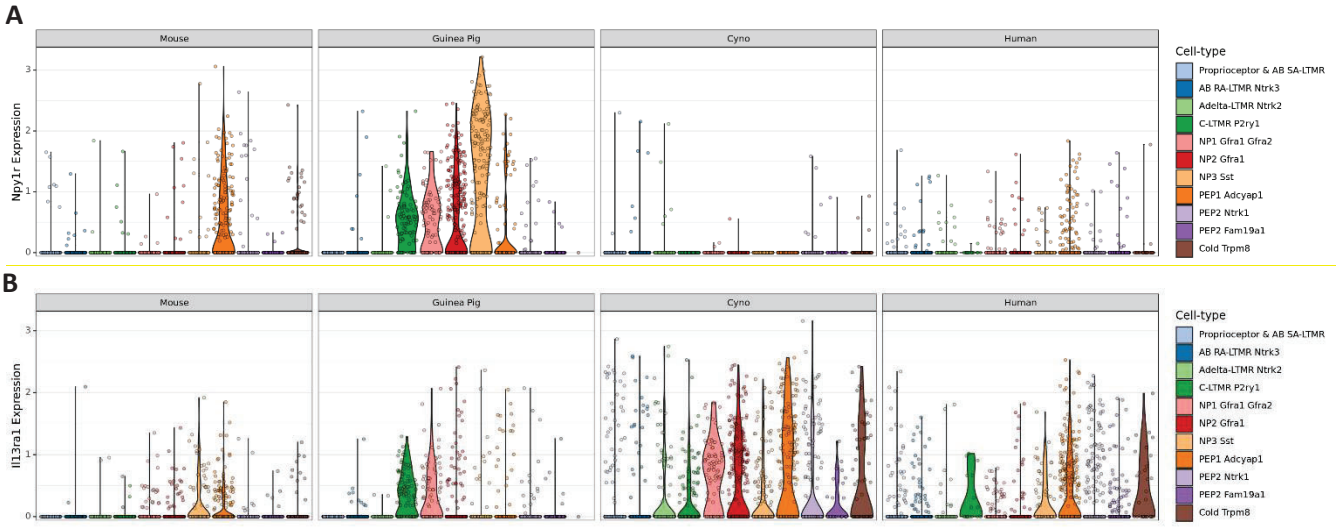

Figure 9 *Npy1r* and *Il13ra1* are co-expressed in PEP1 DRG neurons in mice and human. Single nuclei sequencing was performed with DRG sensory from mice, guinea pig, and cynomolgus monkey[301]. 11 comparable

neuronal subtypes were observed in each species. Expression of *Npy1r* **(A)** and *Il13ra1* **(B)** in clusters from each species is displayed as a violin plots.

Role of nociceptors in the development of allergies

Our work focused on the communication between vagal nociceptor neurons and immune cells in the context of type 2 immunity. In this sense, it does not address the causes of development of human allergies and asthma, but rather focuses on mechanisms at play in the acute phase of inflammation. However, it would be interesting to investigate if such mechanisms can be dysregulated in patients, potentially worsening, or even triggering the development of allergies. NPY, for instance, is found to be elevated in the plasma of elderly asthma patients, even in resting conditions[303]. In genome wide association studies, polymorphisms of genes coding for IL-13, IL-4, and IL-4RA are recurrently found in asthma patients[304]. The contribution of nociceptors themselves is not established in the pathogenesis of asthma, but it would be interesting to explore whether asthma patients' sensory neurons are more sensitive to type 2 signals such as IL-4/IL-13 or IgE.

Future work

Our work has highlighted the functional expression of receptors associated with type 2 inflammation in nociceptors. This supports our initial hypothesis that neurons are informed of the ongoing type of inflammation through neuro-immune crosstalk. Yet, further work is required to obtain a complete understanding of the role of these pathways. The key function of sensory neurons is to signal to the brain to trigger sensations or reflexes. How this function is modified by those immune signals, and what sensations and behaviors are impacted remain to be defined. An essential experiment would be to address the impact of neuronal activation by FcεRI, IL4RII or NPY1R on airway hyperreactivity and bronchoconstriction, using mice with conditional Knock-out of these receptors in nociceptor neurons. Cough behavior cannot be observed in mice, but it would be interesting to measure cough in larger animals such as guinea pigs, using agonists or antagonists of these receptors. Alternatively, since monoclonal antibodies targeting IgE and IL4RII signaling are now used to treat asthma patients, it would be relevant to address the impact of these therapies on symptoms such as cough.

In addition, while we observed that FcεRI promotes neuropeptide release, we still lack data demonstrating how IL-4/IL-13 changes nociceptor physiology. NPY1R, induced by IL4RII activation, appears to dampen the electrophysiological activity of nociceptors, but we do not know how IL-13 globally affects nociceptors' function. In our conditions, we have not observed morphological changes in nociceptor cultures exposed to IL-13 (**Figure 10A**), and this cytokine did not affect TRPV1 and TRPA1 calcium responses (**Figure 10B**). The precise role of IL4RII activation and STAT6 phosphorylation, as well as the axonal retrograde signaling it involves, must be explored. We can hypothesize an action on the sensitivity of other untested TRP channels, on voltage gated channels and neuron electrophysiological properties, or on neuropeptide release. In any case, our sequencing data of IL-13 exposed neurons provides a toolbox for future investigations.

Finally, our work has focused on airway vagal nociceptors. Despite the specificities we observed, these neurons have similarities with gut and skin sensory neurons. It would be of interest to explore whether our observations are also applicable to type 2 inflammation in different tissues. In support of this, we observed that IL-13 also induced *Npy1r* overexpression in DRG neurons. Addressing the molecular profile of nociceptors in mice models of atopic dermatitis or food allergies would be a good way to address common sensory pathways and infer whether the dysregulation of these could be an aggravating factor in allergic disorders. The advantage would also be that pain behavior associated with skin inflammation is more accessible than for airway inflammation, and this could provide an alternative way to explore whether FcεRI, IL4RII, and NPY1R affects pain or itch sensation.

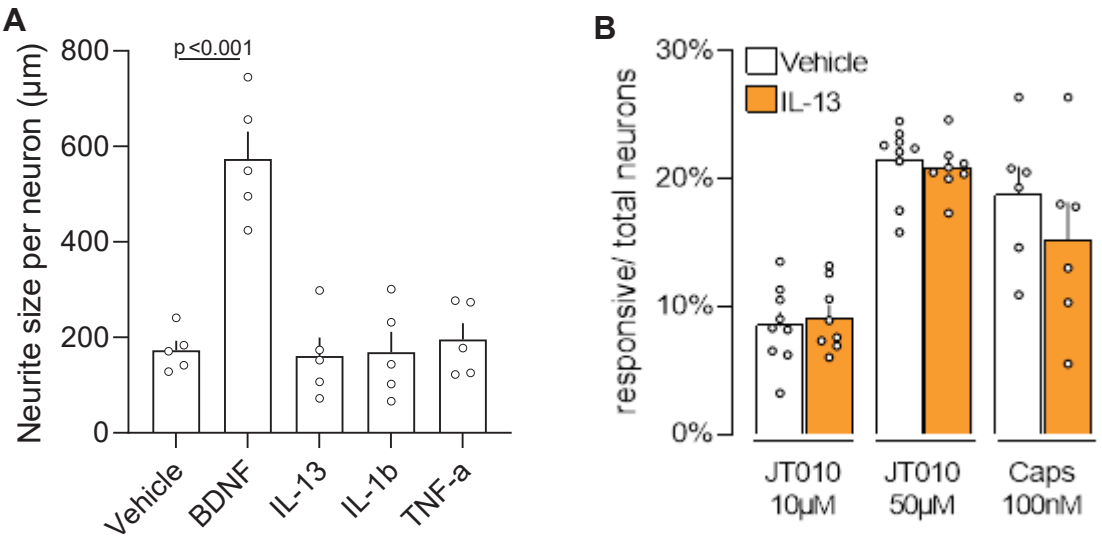

Figure 10 IL-13 does not affect nociceptor neurite growth and TRP channel responses A- JNC sensory neurons from $Na_V1.8^{cre}$::$tdTomato^{fl/wt}$ were cultured for 24 hours in neurobasal media supplemented or not with cytokines IL-13, IL-1β, TNF-α (100 ng/mL), or the neurotrophin BDNF (50 ng/mL). Only BDNF increased the neurite outgrowth in nociceptors (**A**). **B-** JNC neurons were cultured for 24 hours in neurobasal media supplemented or not with IL-13 (100 ng/mL), and responses to JT010 (TRPA1) and capsaicin (TRPV1) were measured by calcium imaging. Calcium responses were not affected by the presence of IL-13 (**B**).

Pain and neuro-immunology, from basic science to pharmaceutical outcome

The research for therapies effectively targeting the chronic pain associated with various pathologies have suffered several drawbacks in the past decades. In particular, the chance of success for new pain drugs is particularly low compared to other pharmaceuticals areas, with only 0.7% of drug candidates in phase I eventually reaching approval and commercialization[305]. Consequently, pharmaceutical companies have reduced their efforts in research and development in this field in the past years. Venture capital in the U.S. invests 40 times less to develop drugs in pain than in oncology[305]. Yet, pain is a major burden for both the patients and the society, with about 116 million people suffering from chronic pain in the United States, for an estimated cost of 721 billion dollars[305]. Chronic itch, cough, and other forms of pain remain largely untreated, with opioids still often being the most effective treatments despite their important side effects and addictive properties[306,307].

However, the fundamental research has made great progress in the past decade, giving hope for the future. The relatively recent discovery of TRP channels[4], and even more recently of the PIEZO channels[12], awarded by the Nobel prize in 2021, have revolutionized the field. This new knowledge has opened new ways to dissect the molecular and cellular mechanisms of pain, with the development of new analytical tools and techniques. In addition, the rapid progress in transcriptomic technologies have provided researchers with complete molecular maps of nociceptor neurons, which identifies several new specific targets to investigate. Finally, the advances in genetic engineering promoted the development of several Cre-Lox mice lines, which are now essential tools to study the function of pain related genes at the physiological level. As a result, considerable progress is being made by fundamental biologists to better understand sensory neurons. More specifically, and concomitantly to our work, others have found the importance of neuro-immune crosstalk in pathologic pain and identified the functional expression of immune related genes in nociceptor neurons[117,308,309]. These studies and ours suggest that repurposing drugs targeting immune cell pathways could be an efficient and cost-effective way to address inflammatory associated pain[117,310,311].

The field of neuro-immunology has notably progressed quickly in the past decade, benefiting from these advances in the sensory neuron field, and from the innovations and techniques in the immunology research area. Evidence of neuron-immune interactions and shared pathways are expanding quickly, with their relevance *in vivo* validated

in several mice models. But intracellular mechanisms at play in primary afferent sensory neurons remain largely to be explored to develop targeted therapies to treat both inflammatory disorders and the associated pain pathways.

Conclusions

Our work has investigated the ability of airway nociceptors to sense immune signals during asthma. In line with our initial hypothesis, it appeared that signals specific to type 2 immunity are detected by these neurons, inferring that they may adapt their phenotype to the different forms of inflammation. In addition, we aimed to provide molecular characterization of the airway neuron subtypes involved in this neuro-immune crosstalk, in both naïve and pathologic conditions. These studies reveal new genes and pathways of interest to target the neuronal component of allergic airway inflammation. Our results fit with the ongoing research in the field of neuro-immunology, but some specificities underline the complex roles of nociceptor neurons in the regulation of physiology. Future investigations will be required to address the relevance of these pathways in preclinical models, their connection with pain sensation and reflexes, as well as their role in patients.

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