Mrgrps on vagal sensory neurons contribute to bronchoconstriction and airway hyper-responsiveness

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Asthma, accompanied by lung inflammation, bronchoconstriction and airway hyper-responsiveness, is a significant public health burden. Here we report that Mas-related G protein-coupled receptors (Mrgrps) are expressed in a subset of vagal sensory neurons innervating the airway and mediates cholinergic bronchoconstriction and airway hyper-responsiveness. These findings provide insights into the neural mechanisms underlying the pathogenesis of asthma.

The lung is densely innervated by sensory nerves, most of which are derived from vagal sensory neurons. Emerging evidence suggests that sensory nerves in the airway play an important role in the pathogenesis of asthma. MrgrpC11, a previously identified itch receptor, is expressed in dorsal root ganglion (DRG) sensory neurons innervating the skin and mediates nonhistaminergic itch. We found that MrgrpC11 is specifically expressed in vagal ganglia, outside the DRG (Supplementary Fig. 1a). Vagal ganglia consist of two different ganglia, termed nodose and jugular ganglia, with distinct embryonic origins: nodose neurons are derived from the epibranchial placode, while jugular neurons are derived from the neural crest. Using Wnt1-Cre;R26-lod-LacZ mice, in which all neural crest–derived neurons express LacZ, we found that MrgrpC11 is selectively expressed in jugular sensory neurons, but not in nodose sensory neurons (Fig. 1a–c and Supplementary Figs. 1c–e and 2). Notably, hMrgrpX1, the functional ortholog of MrgrpC11, is also selectively expressed in jugular ganglion (Supplementary Fig. 1b).

MrgrpC11 immunoreactivity was detected in 6.15% (84 of 1,365) of jugular sensory neurons and in 2.00% (84 of 4,190) of total vagal sensory neurons. MrgrpC11+ jugular sensory neurons were of small diameter (average: 200.27 ± 9.24 μm2), positive for nociceptor marker TRPV1 (Fig. 1d–f) and negative for myelinated neuron marker neurofilament 200kD (Supplementary Fig. 3). We performed retrograde tracing to determine whether MrgrpC11+ jugular sensory neurons innervate the airway. We observed a total of 750 airway-innervating vagal sensory neurons labeled by retrograde tracer CTB-488 (cholera toxin B subunit conjugated with Alexa Fluor-488) and found that 64 of them were MrgrpC11+ (Fig. 1g–i), demonstrating that MrgrpC11+ jugular sensory neurons innervate the airway. However, none of the 201 airway-innervating DRG neurons were MrgrpC11+ (Fig. 1j–l). Therefore, we concluded that MrgrpC11+ primary sensory neurons in different sensory ganglia exhibit different peripheral innervation patterns: MrgrpC11+ jugular sensory neurons innervate the airway, whereas MrgrpC11+ DRG sensory neurons innervate the skin (Fig. 1m). These results allow us to attribute the physiological responses in the airway induced by the activation of MrgrpC11 to jugular sensory neurons in the following experiments.

Bam8-22, a specific peptide agonist for MrgrpC11, can effectively excite MrgrpC11+ jugular sensory neurons in both calcium imaging assay and whole-cell patch-clamp recordings (Supplementary Fig. 4a–d). The vagal sensory neurons isolated from Mrgrp-clusterΔ−/− mice, in which 12 Mrgrp-encoding sequences including MrgrpC11 were deleted, failed to respond to Bam8-22 (Supplementary Fig. 4a). Among the 12 mouse Mrgrp genes, MrgrpC11 is the only receptor that can respond to Bam8-22 in a heterologous system (Supplementary Fig. 5a,b) and mediate Bam8-22-induced responses in the vagal sensory neurons (Supplementary Fig. 4e,f). To determine whether Bam8-22 can activate airway-innervating vagal sensory neurons, we performed ex vivo GCaMP imaging of vagal ganglia (Supplementary Fig. 6) using Pirt-Cre;R26-GCaMP6 mice, in which the pan-sensory-neuron Pirt promoter drives the expression of GCaMP6 in > 95% of sensory neurons. Administration of Bam8-22 onto the airway induced robust Ca2+ increase in 2.67% (33 of 1,237 cells from 10 mice) of vagal sensory neurons (Supplementary Fig. 6b,c), suggesting that Bam8-22 activated a subset of jugular sensory neurons innervating the airway.

To elucidate the function of MrgrpC11+ jugular sensory nerves in the airway, we performed head-out, whole-body plethysmography. Aspiration of Bam8-22 into the airway evoked a change in the respiratory pattern, characterized by an increase in the amplitude of the respiratory waveform and a slight but significant increase in the respiratory rate (Fig. 1n–p and Supplementary Fig. 7a–c). In contrast, Bam8-22 did not induce any change in respiration in Mrgrp-clusterΔ−/− mice (Fig. 1o,p and Supplementary Fig. 7a–c). The Bam8-22-induced respiratory effect was similar to that observed upon the administration of methacholine, a bronchoconstrictor.
Fig. 1 | Activation of MrgprC11+ sensory nerves in the airway changes the respiratory pattern in mice. a–c, Sections of vagal ganglia from Wnt1-ΔC;ROSA26lacZ mice were stained with antibodies against MrgprC11 (red) and LacZ (green). MrgprC11 is only expressed in jugular sensory neurons labeled by LacZ. d–f, MrgprC11+ jugular sensory neurons are positive for nociceptor marker TRPV1. g–i, Sections of vagal ganglia (g–i) and DRG (j–l) from animals intratracheally injected with retrograde neuronal tracer CTB-488 were stained with antibodies against MrgprC11 (red). Airway-innervating neurons are indicated by CTB-488 fluorescence (green). Lower magnification images of DRG sections are presented to show the sparse airway-innervating neurons. Arrowheads mark representative MrgprC11+ neurons in DRG and vagal ganglia. MrgprC11+ jugular sensory neurons express TRPV1, the respiratory reflexes evoked by Bam8-22 and capsaicin are quite different. These results suggest that activation of a few afferents by Bam8-22 versus activation of a large population by capsaicin can produce nonlinear effects in lung responses.

acting on muscarinic acetylcholine receptor (mAChR) in airway smooth muscle (Fig. 1n,q,r and Supplementary Fig. 7d,e). Both Bam8-22- and methacholine-induced respiratory effects were blocked by ipratropium bromide, a mAChR blocker (Fig. 1q,r and Supplementary Fig. 7d,e). Administration of capsaicin to activate TRPV1+ vagal sensory nerves induced a different respiratory response, characterized by a cessation in breathing that persisted for an average of 5.66±0.81 s, followed by a secondary phase with diminished frequency and prolonged braking after each breath (Fig. 1n). This is a prototypical response induced by the administration of respiratory sensory irritants such as cigarette smoke and cinnamonaldehyde into the respiratory tract11,14. We note that although MrgprC11+ jugular sensory neurons express TRPV1, the respiratory reflexes evoked by Bam8-22 and capsaicin are quite different. These results suggest that activation of a few afferents by Bam8-22 versus activation of a large population by capsaicin can produce nonlinear effects in lung responses.

The similarity between Bam8-22-induced and methacholine-induced respiratory responses led us to hypothesize that Bam8-22 may induce bronchoconstriction, which we tested by measuring airway mechanics. Retro-orbital intravenous injection of Bam8-22 significantly increased lung resistance in wild-type (WT) mice but not in Mrgpr-clusterΔ−/− mice (Fig. 2b and Supplementary Fig. 8a,b), demonstrating that Bam8-22 induces bronchoconstriction. Stimulation

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Electrical field stimulation (12 V, 8 Hz, 0.5 ms, 10 s) induced robust contraction of mouse trachea, but Bam8-22 (10 μM) did not. Arrow indicates the addition of Bam8-22; black dots indicate the electrical field stimulation. The results were repeated in three animals. Cholinergic contractile response of mouse trachea to electrical field stimulation (12 V, 8 Hz, 0.5 ms, 10 s) was not changed by the addition of Bam8-22 (28.3 ± 3.0 vs. 30.2 ± 1.80 μL, 20 mg/mL) induced bronchoconstriction in guinea pigs. (vehicle (phosphate-buffered saline or PBS) vs. Bam8-22, n = 8; WT i.v., n = 8; KO i.v., n = 7; WT reversed, n = 6; WT subQ, n = 6; WT i.t., n = 6; WT i.v. baseline vs. Bam8-22, P = 5.36 × 10⁻²). c, Bam8-22-induced bronchoconstriction was blocked by cholinergic blocker ipratropium bromide (naive, n = 6; ipra, n = 5; P = 0.00085). d, Bam8-22-induced bronchoconstriction was blocked by vagotomy (sham, n = 7; vagotomy, n = 7; P = 0.004). e, Representative trace of airway smooth muscle contraction in response to electrical field stimulation and Bam8-22. Electrical field stimulation (12 V, 8 Hz, 0.5 ms, 10 s) induced robust contraction of mouse trachea, but Bam8-22 (10 μM) did not. Arrow indicates the addition of Bam8-22; black dots indicate the electrical field stimulation. The results were repeated in three animals. Cholinergic contractile response of mouse trachea to electrical field stimulation (12 V, 8 Hz, 0.5 ms, 10 s) was not changed by the addition of Bam8-22 (28.3 ± 1.80 vs. 30.2 ± 2.75% of maximum contraction). f, Retro-orbital i.v. injection of Bam8-22 (400 μL, 20 mg/mL) induced bronchoconstriction in guinea pigs. (vehicle (phosphate-buffered saline or PBS), n = 5; Bam8-22, n = 6; PBS vs. Bam8-22, P = 0.018). *P < 0.05, ***P < 0.005; two-tailed unpaired Student’s t test. Data are presented as mean ± s.e.m.

of a subset of vagal sensory neurons causes release of acetylcholine from parasympathetic nerve fibers, which in turn acts on the mAChR in airway smooth muscle to induce bronchoconstriction (Fig. 2a)13. This parasympathetic cholinergic pathway is the major neural mechanism controlling bronchoconstriction in the lung15. The Bam8-22-induced increase in lung resistance was completely blocked by ipratropium bromide (Fig. 2c) and vagotomy surgery (Fig. 2d), which severs the vagus nerve, demonstrating that Bam8-22 induced cholinergic bronchoconstriction in WT animals. However, Bam8-22-induced increase in lung resistance was not affected by the deletion of TRPA1 or TRPV1 (Supplementary Fig. 8c). Bam8-22 did not cause tracheal smooth muscle contraction (Fig. 2e), ruling out the possibility that Bam8-22 directly stimulates airway smooth muscle. Taken together, these data suggest that Bam8-22 activates Mrgrp Cluster Δ−/− vagal sensory neurons, which in turn stimulate the parasympathetic system and induce cholinergic bronchoconstriction.

We also observed similar Bam8-22-induced effects in guinea pigs, another species frequently used to study respiratory reflex and to model respiratory diseases. gpMrgrp1 (clone XM_003462608), the predicted Mrgrp11 ortholog, is expressed in airway-innervating jugular and nodose neurons (Supplementary Fig. 9a,b). Bam8-22 activated guinea pig vagal sensory neurons in a gpMrgrp1-dependent way (Supplementary Fig. 9c–e). Notably, intravenous injection of Bam8-22 in guinea pigs induced robust bronchoconstriction (Fig. 2f and Supplementary Fig. 9f). However, whether Bam8-22 also activates a vagal–cholinergic reflex to induce bronchoconstriction in guinea pigs, as it does in mice, requires further investigation.

Anaphylaxis is an acute, potentially life-threatening allergic reaction mediated by mast cell activation. Anaphylactic symptoms in the respiratory system include coughing, airway swelling and bronchoconstriction16. Retro-orbital injection of ovalbumin (OVA) resulted in a pronounced increase in lung resistance in OVA-sensitized WT mice (Fig. 3a). This effect was significantly inhibited in sensitized Mrgrp Cluster Δ−/− mice (Fig. 3a and Supplementary Fig. 8d), suggesting that Mrgrps mediate anaphylactic bronchoconstriction. Anaphylactic bronchoconstriction is evoked mainly by mast cell mediators acting on both airway smooth muscle and sensory nerves17. Notably, neuropeptide FF, an Mrgrp11 agonist that can be released by activated mouse bone marrow-derived mast cells18, also induced bronchoconstriction (Fig. 3b and Supplementary Fig. 8e). Additional studies are required to determine whether Mrgrps play a role in the sensitization of mast cells, their activation or both.
Influenza virus infection causes characteristic effects within the respiratory tract, including airway inflammation and airway hyper-responsiveness. It is a major risk factor for exacerbation of asthma and causes significant morbidity and mortality annually. We infected WT and Mrgpr-clusterΔ−/− mice with influenza A virus (IAV) to examine whether sensory nerves contribute to IAV-induced respiratory symptoms. Both WT and Mrgpr-clusterΔ−/− mice displayed robust immune responses after inoculation (Fig. 3d and Supplementary Fig. 10a,b). However, we observed significantly lower IAV-induced airway hyper-responsiveness in Mrgpr-clusterΔ−/− mice compared to WT mice (Fig. 3c), demonstrating that Mrgprs are required for IAV-induced airway hyper-responsiveness. The deletion of Mrgpr genes did not affect the airway hyper-responsiveness in a dust mite–induced allergic asthma model (Supplementary Fig. 11). These data suggest that distinct mechanisms exist for the development of airway hyper-responsiveness under different pathological conditions.

We next asked whether direct stimulation of MrgprC11+ jugular sensory neurons could induce airway hyper-responsiveness in the absence of lung inflammation. We pretreated WT and Mrgpr-clusterΔ−/− mice with Bam8-22 to activate MrgprC11+ jugular sensory neurons. Five minutes after Bam8-22 treatment, when the airway resistance had returned to its baseline level, airway responsiveness to methacholine were measured in WT and Mrgpr-clusterΔ−/− mice. We found that pretreatment with Bam8-22 caused a remarkable increase in airway responsiveness in WT mice, but not in Mrgpr-clusterΔ−/− mice (Fig. 3e). As expected, the enhanced airway sensitivity was abolished by vagotomy in WT mice (Fig. 3f).

These data demonstrate that activation of MrgprC11+ jugular sensory neurons can potentiate airway responsiveness in the absence of inflammation. Airway responsiveness to methacholine is the result of both direct action on airway smooth muscle and muscle contraction-induced vagal reflexes. Our data, which is consistent with previous studies, supports the hypothesis that the activation of vagal C fibers by Bam8-22 may lead to the sensitization of the vagal afferent–brainstem–parasympathetic reflex pathway and enhance airway responsiveness.

In summary, we demonstrated that MrgprC11+ jugular sensory neurons, constituting only 2% of vagal sensory neurons, mediate cholinergic bronchoconstriction and play a role in the development of airway hyper-responsiveness in mice. Therapeutic approaches targeting this small population will avoid the possible side effects of antagonizing all vagal nociceptors, which play important roles in maintaining the physiological functions of multiple internal organs. Future studies investigating the endogenous agonists of MrgprC11, the peripheral and central axonal projections of MrgprC11+ neurons, and the neural circuit in which these neurons reside will further reveal the neural mechanisms of respiratory diseases. The Mrgpr-clusterΔ−/− mice exhibited decreased anaphylactic bronchoconstriction and reduced airway hyper-responsiveness after influenza virus infection. As 12 Mrgpr genes were deleted in the Mrgpr-clusterΔ−/− mice, the phenotypes observed may not be mediated solely by MrgprC11. Whether the other Mrgpr genes are involved in the tested models requires further investigation.

Fig. 3 | Mrgprs mediate anaphylactic bronchoconstriction and airway hyper-responsiveness. a, Mrgpr-clusterΔ−/− mice (n = 8) exhibited attenuated anaphylactic bronchoconstriction compared to WT mice (n = 6; P = 0.024). b, Retro-orbital i.v. injection of neuropeptide FF (NPFF; 50 µL, 10 mg/mL) induced bronchoconstriction in WT (n = 6) but not Mrgpr-clusterΔ−/− mice (n = 6; P = 0.014). c, Mrgpr-clusterΔ−/− mice exhibited reduced airway hyper-responsiveness after influenza A virus inoculation. (WT + control (ctrl), n = 9; WT + IAV, n = 15; KO + ctrl, n = 10; KO + IAV, n = 12; WT + IAV vs. KO + IAV, P = 0.005). d, Representative hemotoxylin and eosin (H&E)-stained lung sections showing that WT and Mrgpr-clusterΔ−/− mice presented similar lung inflammation responses after IAV inoculation. IFV inoculation experiment was repeated independently two times with similar results. Scale bar, 100 µm. e, Bam8-22 (50 µL, 10 mg/mL) enhanced the airway responsiveness to methacholine in WT but not in Mrgpr-clusterΔ−/− mice. (WT + PBS, n = 6; WT + Bam8-22, n = 5; KO + PBS, n = 7; KO + Bam8-22, n = 5; WT + PBS vs. WT + Bam8-22, P = 0.043; WT + Bam8-22 vs. KO + Bam8-22, P = 0.036). f, Vagotomy blocked Bam8-22-induced enhancement of airway responsiveness. (WT + PBS, n = 6; WT + Bam8-22, n = 5; P = 0.79). *P < 0.05, **P < 0.01, ***P < 0.005; two-tailed unpaired Student’s t test. Data are presented as mean ± s.e.m.
Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-018-0074-8.

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Author contributions

L.H. designed and performed all experiments, except where noted. N.L. and L.H. designed and performed all the airway mechanics and IAV infection experiments. F.R. performed the ex vivo vagal ganglia GCaMP imaging and RT-PCR of guinea pig airway-innervating vagal sensory neurons. Z.L. performed electrophysiological recording of vagal sensory neurons. O.J.L. assisted with the IAV infection experiments. H.S. and J.W. performed immunostaining and calcium imaging with HEK293 cells. Y.Z. performed calcium imaging on vagal sensory neurons. W.M. supervised the airway mechanics experiments. M.K supervised the ex vivo vagal ganglia GCaMP imaging and RT-PCR of guinea pig airway-innervating vagal sensory neurons. B.J.U. supervised the plethysmography experiments. B.J.C. conceived and supervised the plethysmography and airway mechanics experiments. X.D. conceived and supervised the project. The manuscript was written by L.H. and X.D. and edited by N.L., W.M., B.J.U. and B.J.C.

Competing interests

The authors declare no competing financial interests.

Additional information

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Methods

Animals. All experiments were performed with approval from the Johns Hopkins University Animal Use and Care Committee and the Georgia Institute of Technology Animal Use and Care Committee. The generation of Mrgpr-clusterΔ−/− mice was described previously1, and they had been backcrossed to C57Bl/6 for at least ten generations. Wnt1-Cre and ROSA26-lal-LacZ mouse lines were purchased from Jackson Laboratory. The Pirt-Cre line was generated in X. Dong's laboratory. The ROSA26-lal-GCaMP6f line was kindly provided by D. Bergles at Johns Hopkins University. Three-week-old male animals were used for in vivo imaging experiments. 2- to 3-month-old males were used for all other experiments. We purchased 200- to 250-g male Hartley guinea pigs from Charles River. All the animals were housed in the vivarium with a 12-h light/dark cycle, and all the behavioral tests were performed from 9 a.m. to 1 p.m., during the light cycle. Mice and guinea pigs were housed in groups, with a maximum of five per group for mice and two per group for guinea pigs.

Retrograde tracing of sensory neurons innervating the airway. Mice were anesthetized with pentobarbital (70 mg/kg), and the ventral upper tracheal region was exposed and pierced with a microinjecting needle attached to a Hamilton syringe. Retrograde tracer CTB-488 (cholera toxin B subunit conjugated with Alexa Fluor-488; 20 μL) was directly injected into the tracheal lumen. Vagal ganglia and DRG were collected 7 d after the injection for immunohistochemical analysis. Male Hartley guinea pigs (250 to 300 g) were anesthetized with ketamine/xylazine (50 mg/kg ketamine and 2.5 mg/kg xylazine). Retrograde tracing of sensory neurons innervating the airway. Mice were purchased from Tissue for Research (http://tissue4research.com/). The results were repeated with 10 animals. All results from Tissue for Research (http://tissue4research.com/) were reproduced.

RT-PCR. Total RNA was extracted from various tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Mouse tissues were collected from 2- to 3-month-old C57Bl/6 male mice. Reverse transcription was carried out using the Superscript III first-strand synthesis system (Invitrogen). PCR conditions for mouse Mrgpr genes, hMrgprX1, hTRPV1 and hGAPDH: 95 °C for 3 min, 40 cycles of 95 °C, 30 s at 55 °C and 60 s at 72 °C. All PCR results were confirmed by DNA sequencing. Full-length images of DNA gels used in Supplementary Figs. 1, 5 and 9 are presented in Supplementary Fig. 12.

RT-PCR with human tissues. Human jugular and nodose ganglia were purchased from Tissue for Research (http://tissue4research.com/). The results were repeated five times with vagal ganglia collected from five different donors. The procedure was approved by Georgia Tech Biological Materials Safeguards Committee. Human lung total RNA was purchased from Orgene. Characteristics and medical history of patients from whom the tissues were collected are as follows:

1. Age: 75; sex: female; race: white; height: 5′7″ (165 cm); weight: 170 lbs. (77.1 kg); cause of death: ovarian cancer
2. Age: 60; sex: male; race: white; height: 5′6″ (167 cm); weight: 205 lbs. (92.6 kg); cause of death: melanoma
3. Age: 44; sex: female; race: white; height: 5′3″ (160 cm); weight: 120 lbs. (54.4 kg); cause of death: colon cancer
4. Age: 44; sex: female; race: white; height: 5′3″ (160 cm); weight: 112 lbs. (50.8 kg); cause of death: end-stage COPD
5. Age: 72; sex: male; race: white; height: 5′7″ (170 cm); weight: 140 lbs. (63.5 kg); cause of death: pancreatic cancer.

RT-PCR with guinea pig airway-innervating neurons. Dissociated jugular and nodose ganglia neurons were cultured on coverslips 14 d after the DiI injection. Individual Dil-labeled cells were identified by fluorescent microscope and harvested by glass-pipette into PCR tubes containing 1 μL each of RNAse inhibitor (Invitrogen). The cells were immediately placed on dry ice after collection. About 25 cells were pooled into each PCR tube. Cells were processed using the Superscript III CellsDirect cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions to generate first-strand cDNA. All primers for gpMrgprX1, gpTRPV1 and gpACTB are intron-spanning. PCR conditions: 95 °C for 5 min, 50 cycles of 30 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C. All PCR results were confirmed by DNA sequencing.

The sequences of the primers used were as follows. All primer sequences are

MrgprA1-R: AGACGCGGAGAGTCTAGGTT
MrgprA12-F: TCAAGGGGTCGCTGGAACGC
MrgprA12-R: TCAAGGAGGGAGAGGAAGAG
MrgprA2-F: CTCCTGCACCAAAGCACAA
MrgprA2-R: AACACAATGTTAGAATGTGCT
MrgprA3-F: GCCTACAACGAGCAAGAAGAT
MrgprA3-R: CTCAAACCCATGGAGTGAGGT
MrgprA4-F: CTCTGGTGTGCTTATCCGT
MrgprA4-R: TTCAGGTGTTACTTGGTAC
MrgprA10-F: TCTGGTGCACATCTCCAA

Immunostaining. Adult mice (8–12 weeks old) were anesthetized with pentobarbital and perfused with 4% paraformaldehyde in PBS. Vagal ganglia and dorsal root ganglia (DRG) were dissected from the perfused mice for immunostaining. The tissues were postfixed, cryoprotected in 30% sucrose and sectioned with a cryostat. The primary antibodies used: rabbit polyclonal anti-MrgprC11 (custom-made by ProteinTech Group, Inc.), chicken anti-LacZ (Abcam, ab9361), guinea pig anti-TRPV1 (Chemico, ab5566), chicken anti-NF-200 (Aves, NFH), rat anti-subP (Millipore, MBA356) and anti-tubulin-β3 (Covance, PRB-435D). All secondary antibodies were purchased from Invitrogen: donkey anti-rabbit IgG-Alexa Fluor-555 (A-11035), goat anti-chicken IgG-Alexa Fluor-488 (A-11037), donkey anti-rat IgG-Alexa Fluor-488 (A-21208) and goat anti-mouse IgG-Alexa Fluor-488 (A11001). To detect FITC binding, sections were incubated with 1:200-diluted IB4-Alexa 488 (I-21141, Invitrogen) during secondary antibody incubations. MrgprC11 antibodies were validated with Mrgpr-clusterΔ−/− mice (Supplementary Fig. 2). Validation data for other antibodies are available from the commercial providers.

Calcium imaging. Calcium imaging was performed as previously described1. Briefly, cells (HEK293 cells, vagal sensory neurons from 3- to 4-week-old mice or vagal sensory neurons from 200- to 250-g guinea pigs) were cultured on glass coverslips. Cells were loaded with fura 2-acetoxymethoxy ester ( Molecular Probes) for 30 min in the dark at room temperature (20–21 °C). After washing, cells were imaged at 340- and 380-nm excitation to detect intracellular free calcium, and a 20% increase in F380/F340 was set as threshold. Average fluorescence ratios (F380/F340) were calculated from ≥20 cells, and all population data are presented as mean ± s.e.m.

Whole-cell current-clamp recordings of vagal sensory neurons. Bam8-22-responding vagal sensory neurons were first identified by calcium imaging before they were patched for current clamp recording. Patch pipettes had resistances of 2–4 MΩ. Action potential measurements were performed with an Axon 700B amplifier and the pCLAMP 9.2 software package (Axon Instruments). Neurons were perfused with 2 μM Bam8-22 for 20 s. All experiments were performed at room temperature.

Ex vivo vagal ganglia GCaMP imaging. A modification of a method described previously22 was used for the dissection and tissue preparation. Briefly, mice were killed for analysis by CO2 overdose and exsanguination. The blood from the pulmonary circulation was mostly washed out by in situ perfusion with 20 mL of Krebs-bicarbonate solution (KBS) containing (in mM): 118 NaCl, 5.4 KCL, 1 Na2HPO4, 1.2 MgSO4·7H2O, 25 NaHCO3, 11.1 dextrose. The trachea and lung, with intact vagus nerves and vagal ganglia, were dissected and placed in a two-compartment tissue chamber (Supplemental Fig. S4a). The lung was potted in one compartment (airway chamber) to receive chemical stimuli. The left vagal ganglia were pinned in a second compartment (VG chamber) for multiphoton imaging. Both compartments were perfused with 37°C KBS. There was no fluid exchange between the two compartments. The trachea was cannulated with PE tubing to allow the delivery of chemicals onto the airway. The lungs were punctured with a 27-gauge needle (1–2 mm deep, 2–8 punctures per lobe) to allow perfusing KBS to exit the tissue. The surface of the rostral vagal ganglia was imaged by a multiphoton microscope (Scientifica) with a 20× water-immersion lens.

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Head-out whole-body plethysmography. Head-out whole-body plethysmography was performed with anesthetized and spontaneously breathing mice. Mice were anesthetized with urethane and tracheotomized to allow the aspiration of chemicals (2 μL; 1 mM capsaicin, 1 mM MCh, 5 mM Bam8-22) directly into the trachea through the cannula. Delivering chemicals directly into the trachea allowed us to avoid interference from the upper respiratory tract, which is mainly innervated by trigeminal sensory neurons. The body of the animal was kept in the plethysmography chamber, and respiratory effort was monitored digitally using a Biopac data acquisition system. The respiratory rate and the amplitude of the respiratory waveform were analyzed offline with AcqKnowledge software.

Airway mechanics. Anesthetized (pentobarbital, 70 mg/kg) animals were tracheotomized, attached to a Flexivent pulmonary mechanics analyzer (SCIREQ) and paralyzed with succinylcholine (2 mg/kg). Anesthetized mice were ventilated at a tidal volume of 9 mL/kg, at a frequency of 150 bpm. Anesthetized guinea pigs were ventilated at a tidal volume of 8 mL/kg, at a frequency of 90 bpm. Positive end-expiratory pressure was set at 3 cm H2O. We assessed theairway responsiveness using a single-compartment model to measure the total lung resistance (Rt), a dynamic variable that is largely dependent on the resistance to airflow through the airways and sensitive to airway smooth muscle contraction. Bam8-22 (50 μL, 10 mg/mL for mice; 400 μL, 20 mg/mL for guinea pigs), NPFF (50 μL, 10 mg/mL), and MCh (50 μL, 30 μg/mL) were administered by retro-orbital i.v. injection. Airway hyper-responsiveness was determined in response to aerosolized methacholine challenges (0, 1, 3, 10 and 30 mg/mL). The nebulizer was on for 10 s to deliver each dose of MCh.

Organ bath studies. Organ bath studies were performed as previously described16. Briefly, whole tracheas were dissected out from mice after they were killed, and placed in oxygenated Krebs-bicarbonate solution containing (in mM): 118 NaCl, 5.4 KCl, 1 NaH2PO4, 1.2 MgSO4, 1.2 CaCl2, 25 NaHCO3, 11.1 dextrose. Tracheas were cleaned of connective tissue and tracheal rings (whole or laterally divided in half), suspended between two tungsten stirrups in 10-mL organ chambers filled with Krebs solution that was warmed to 37 °C and bubbled with 95% O2–5% CO2 to maintain a pH of 7.4. One stirrup was connected to a strain gauge (model FT03; Grass Instruments, Quincy, MA, USA), and tension was recorded on a Grass Model 7 polygraph (Grass Instruments). Preparations were stretched to a resting tension of 0.2 g and washed with fresh Krebs buffer at 15-min intervals during a 60-min equilibration period. After equilibration, tracheas were challenged with either Bam8-22 (10 μM) or electrical field stimulation (12 V, 8 Hz, 0.5 ms, 10 s/100 s). At the end of each experiment, all tracheas were maximally contracted with carbamylcholine (1 mM). The results are expressed as a percentage of maximum contraction.

IAV infection-induced lung inflammation. Mouse-adapted influenza A/California/4/2009 H1N1 was a kind gift from S. Klein at the Department of Molecular Microbiology and Immunology at Johns Hopkins University School of Public Health. Eight- to 12-week old mice were anesthetized with ketamine (100 mg/kg) and xylazine (15 mg/kg) and were infected intranasally with 0.4 50% mouse-lethal dose (MLD50) of IAV in 30 μL of DMEM. Mice in the control group were infected with 30 μL DMEM medium only. At 5 d postinfection, airway mechanics were examined using the Flexivent system, and bronchoalveolar lavage fluid (BALF) was collected to evaluate lung inflammation. BALF was centrifuged, and the cell pellet was resuspended in PBS to perform differential cell counting of leukocytes (macrophages/monocytes, neutrophils, eosinophils and lymphocytes). The supernatant of BALF was used to examine the levels of TNF-α, IFN-γ and IL-6 using enzyme immunoassay kits purchased from R&D Systems (Minneapolis, MN) according to individual kit instructions. Lungs were inflated and fixed with formalin at a pressure of 30 cm H2O, and lung sections were processed and stained with hematoxylin and eosin.

Mouse model of anaphylaxis. Mice were sensitized with 20 μg of OVA emulsified with 2 mg of Imject Alum Adjuvant in 150 μL PBS by i.p. injection. Twenty-one days after OVA sensitization, mice were anesthetized and challenged with 500 μg of OVA in 30 μL of PBS, and airway mechanics were measured using the Flexivent system. The airway resistance increased gradually after the OVA injection, reaching a peak 30–60 s after the injection. To examine the level of OVA-specific IgE, sera of sensitized mice was collected on day 18 (before challenge). The OVA-specific IgE level was determined using the anti-ovalbumin IgE (mouse) ELISA kit (Cayman Chemical, 500840) following the manufacturer’s instructions.

House dust mite–induced airway allergic inflammation. Mice were sensitized by i.p. injection of 100 μg house dust mite extract in 150 μL PBS on days 1 and 14. Lightly anesthetized mice (isoﬂurane) were challenged by intranasal instillation of 100 μg house dust mite extract in 20 μL PBS on days 18 and 21. Airway mechanics measurements were performed on day 23.

Data analysis and statistics. Statistical analyses were performed using Prism 7 (GraphPad). We did not use statistical methods to predetermine the sample size, but our sample sizes are comparable to those reported in previous publications. Differences between experimental groups were analyzed with two-tailed unpaired Student's t tests. Threshold for significance (α) was set at 0.05. Data are reported as mean ± s.e.m. Normality and equality of variance were analyzed with the Shapiro-Wilk normality test and F-test, respectively. When the sample sizes were too small to calculate normality, the distribution was assumed to be normal. The experimenters were blinded to the genotype of the animals. Animals were assigned randomly to the various experimental groups, and data were collected and processed randomly. N values are indicated for all experiments that required statistical analysis. RT-PCR and immunostaining were performed at least three times each with three different animals. Retrograde tracings were performed three times each using three animals each time.

Life Sciences Reporting Summary. Further information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The authors declare that all data generated or analyzed during this study are included within the article and its supplementary information files.

References
21. Kwong, K. et al. J. Physiol. (Lond.) 586, 1321–1336 (2008).
22. Undem, B. J. et al. J. Physiol. (Lond.) 556, 905–917 (2004).
23. Weigand, L. A., Myers, A. C., Meeker, S. & Undem, B. J. J. Physiol. (Lond.) 587, 3355–3362 (2009).
## Experimental design

1. **Sample size**

   Describe how sample size was determined.

   We did not use statistical methods to predetermine the sample size. Our sample sizes are comparable to those reported in previous publications. All detail were described in the "Data analysis and statistics." in Methods section.

2. **Data exclusions**

   Describe any data exclusions.

   No data were excluded.

3. **Replication**

   Describe whether the experimental findings were reliably reproduced.

   The number of experiments are stated in the Methods section. RT-PCR and immunostaining were performed at least three times with three different animals. Retrograde tracings were performed three times using three animals each time. All attempts at replication were successful.

4. **Randomization**

   Describe how samples/organisms/participants were allocated into experimental groups.

   Animals were randomly chosen for each experimental group. See "Data analysis and statistics." in Methods.

5. **Blinding**

   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   The experimenter was blind to the genotype of the animals. See "Data analysis and statistics." in Methods.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**

   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a

   - **Confirmed**
     - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
     - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
     - A statement indicating how many times each experiment was replicated
     - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
     - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
     - The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
     - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
     - Clearly defined error bars

   See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Prism 7 (GraphPad), pCLAMP 9.2 (Axon Instruments), and AcqKnowledge.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

There is no restriction on availability of unique materials in this study.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

The primary antibodies used: rabbit polyclonal anti-MrgprC11 (made by Proteintech Group, Inc. and validated with knockout animals), chicken anti-LacZ (Abcam, ab9361), guinea-pig anti-TRPV1 (Chemico, ab5566), chicken anti-NF-200 (Aves, NH), rat anti-subP (Millipore, MAB356). All the secondary antibodies were purchased from Invitrogen: Donkey anti-Rabbit IgG-Alexa Fluor 555 (A-31572), Goat anti-Chicken IgY-Alexa Fluor 488 (A-11039), Goat anti-Guinea Pig IgG-Alexa Fluor 488 (A-11073), Donkey anti-Rat IgG Alexa Fluor® 488 (A-21208), Goat anti-mouse IgG-Alexa Fluor 488 (A11001). To detected IB4 binding, sections were incubated with 1:200 diluted IB4-Alexa 488 (I-21411, Invitrogen) during secondary antibody incubations. MrgprC11 antibody was validated with knockout animals in which MrgprC11 was deleted. Validation data for other antibodies are available from the commercial providers.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

HEK293 cells

b. Describe the method of cell line authentication used.

The cell line used was not authenticated.

c. Report whether the cell lines were tested for mycoplasma contamination.

The cell lines used was not tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

ICLAC record indicate that HEK cell line might be contaminated with Hela cells. In our study, HEK293 cells were used as a in vitro system to express 12 different Mrgpr family members in mammalian cells. We used this gene expression system combined with calcium imaging assay to test whether these 12 receptors can be activated by peptide Bam8-22. Hela cells can also be used for the same experiments. The possible contamination of Hela cells in our system does not affect our conclusion.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

All mice used are C57Bl/6J. 3 weeks old male animals were used for Ca2+ imaging assay. 2-3 months old males were used for all other experiments. 200-250 grams male Hartley Guinea pigs were purchased from Charles River.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study does not involve human research participants.