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Allergen-free extracts from birch, ragweed, and hazel pollen activate human and guinea-pig submucous and spinal sensory neurons

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Abstract

Background: Non-allergenic, low molecular weight components of pollen grains are suspected to trigger changes in gut functions, sometimes leading to inflammatory conditions. Based on extensive neuroimmune communication in the gut wall, we investigated the effects of aqueous pollen extracts (APE) on enteric and spinal sensory neurons.

Methods: Using Ca^{2+} and fast potentiometric imaging, we recorded the responses of guinea-pig and human submucous and guinea-pig dorsal root ganglion (DRG) neurons to microejection of low (<3 kDa) and high (≥3 kDa) molecular weight APEs of birch, ragweed, and hazel. Histamine was determined pharmacologically and by mass spectrometry (LC–MS/MS).

Key Results: Birch $APE_{<3kDa}$ evoked strong $[Ca^{+2}]_i$ signals in the vast majority of guinea-pig DRG neurons, and in guinea-pig and human enteric neurons. The effect of birch $APE_{\geq 3kDa}$ was much weaker. Fast neuroimaging in human enteric neurons revealed an instantaneous spike discharge after microejection of birch, ragweed, and hazel $APE_{<3kDa}$ [median (interquartile range) at 7.0 Hz (6.2/9.8), 5.7 Hz (4.4/7.1), and 8.4 Hz (4.3/12.5), respectively]. The percentage of responding neurons per ganglion were similar [birch 40.0% (33.3/100.0), ragweed 50.8% (34.4/85.6), and hazel 83.3% (57.1/100.0)]. A mixture of histamine receptor (H1–H3) blockers significantly reduced nerve activation evoked by birch and ragweed $APEs_{<3kDa}$, but was ineffective on hazel. Histamine concentrations in ragweed, birch and hazel APE's < 3 kDa were 0.764, 0.047, and 0.013 μM, respectively.

Conclusions: Allergen-free APEs from birch, ragweed, and hazel evoked strong nerve activation. Altered nerve-immune signaling as a result of severe pollen exposure could be a pathophysiological feature of allergic and non-allergic gut inflammation.

KEYWORDS
allergy, enteric nervous system, gastrointestinal tract, inflammation, neurogastroenterology, pollen sensitization

1 | INTRODUCTION

The last years we have seen increasing evidence for the relevance of allergic sensitization to pollen in chronic inflammatory conditions in the gastrointestinal tract, for example, eosinophilic esophagitis (EoE)[1–6] for review.[7,8] Similarly in regard to Crohn's disease[9] and the irritable bowel syndrome (IBS), pollen sensitization is frequently discussed as a potential factor in the pathogenesis,[10–12] for review.[13] The underlying mechanisms are complex and only partly understood. They may include hypersensitivity responses to pollen cross-reacting foods, the so-called pollen-food-allergy or oral-allergy-syndrome,[14,15] as well other pro-inflammatory effects of pollen exposure.[2,4,5,16,17] Pollen grains, per se, trigger local inflammatory actions in esophageal and/or gastrointestinal mucosa, very similar to effects in the nasal mucosa.[1] This is supported by studies using radiolabeled pollen grains.[18] It was shown that the vast majority (nearly 100%) of pollen grains and high molecular weight pollen extracts in humans do not reach the thoracic respiratory tract but were swallowed and had direct contact with the mucosa of the digestive tract.[18] This is in line with findings from a recent study demonstrating in situ pollen germination in 65.6% of esophageal mucosal biopsies obtained from patients suffering from eosinophilic esophagitis.[17] The authors hypothesized that alteration of the mucosal barrier may facilitate the penetration of pollen grains into deeper layers of the gut wall, thus emphasizing the role of pollen in local inflammation.[17]

The intestinal and respiratory lining are packed with immune cells, such as mast cells, which functionally interact with enteric and spinal sensory nerves. Recently, we have demonstrated in detail signaling between nerves and mast cells at the cellular level in intact human intestinal mucosa.[19]

The complexity of pollen effects is not only a simple function of its allergen content triggering allergic hypersensitivity reactions.[20,21] Along with allergens, pollen grains release multiple other substances that are not allergenic themselves, but act as essential adjuvants, facilitating and enhancing allergic immune responses.[22] These are mainly water-soluble components of low molecular weight (MW) of which only a limited number have been chemically and functionally identified to date. One of these substances is histamine. It was suspected early on that in the case of non-immunological effects of pollen extracts, histamine in particular could be responsible for false-positive reactions in skin tests.[23,24] In an extensive 1992 study, the authors detected histamine at concentrations up to 7.4 μg/mL in 48 of 49 aqueous or phenolic pollen extracts.[25] From a neurophysiological point of view, histamine is interesting because, as we have shown, it has strong nerve excitatory properties in enteric neurons in both humans and rodents.[26] This is one reason why we initially focused on this substance in the present study. Other components are, for example, adenosine,[27] which is known to modulate sensory signaling[28] or phytoprostanes which are structurally related to prostaglandins.[29–32] In these studies, 3 kDa was used as the cutoff value for the separation of the allergenic protein fractions. This was due to the fact that proteins, in order to act as allergens, that is, to form an antibody bond, must have a certain minimum size of about 30 amino acid residues (molecular weight about 3 kDa).[33] For comparison, the molecular weights of the common main pollen allergens of birch and hazel are about 17 kDa,[34,35] those of ragweed allergens are 21–31 kDa.[36]

Key points

- Non-allergenic components of pollen grains are suspected to trigger changes in gut functions, sometimes leading to inflammatory conditions. We investigated the effects of low molecular aqueous pollen extracts on enteric and spinal sensory neurons from humans and guinea-pigs.
- Ca^{2+} and fast potentiometric imaging techniques were used.
- Low molecular extracts of birch, ragweed, and hazel pollen strongly excite neurons in humans and guinea-pigs; nerve excitation was mediated by histamine in birch and ragweed, but not in hazel pollen extracts.
- Altered nerve signaling as a result of severe pollen exposure may be a pathophysiological feature to allergic and non-allergic challenges.

In human airway epithelial cells, low MW fractions of birch, ragweed, and mugwort pollen were found to modulate immune responses and barrier functions.[32,37–39] Additionally, activation of dendritic cell function had been shown.[30,31,40] In a recent study, low MW extract from a.o. birch pollen was shown to compromise the innate antiviral defense of respiratory epithelia during infection with human rhinovirus.[39] Taken together, these results lead to the suggestion that low MW non-allergenic pollen components are responsible for a bundle of symptoms and modulation of innate immune defenses by pollen exposure in both allergic and non-allergic humans.[39]

Whether and, if so, which components of the pollen extracts might play a role in the activation of neurons, particularly in the gastrointestinal wall, is not yet known. Therefore, we performed experiments using different neuronal settings, that is, cell cultures of guinea-pig spinal sensory neurons as well as whole mount preparations of guinea-pig and human intestinal submucous plexus. We recorded on single-cell level slow and fast neuronal responses to

aqueous extracts of birch, ragweed, and hazel pollen. We separated the extracts according to their molecular weight and found that the low MW (protein-free) fractions of all pollen species strongly activated neurons. Using pharmacological methods and mass spectrometry, we identified histamine as a neuroactive component in low MW pollen extracts.

2 | METHODS

2.1 | Ethics statement

Procedures for the work with human gut samples were approved by the local ethics committee of the Technische Universität München (project approval 5242/11). Informed written consent was obtained from all subjects, and the studies conformed to the standards set by the Declaration of Helsinki except for registration in a database.

All animal work with guinea-pigs was approved by the Animal Welfare Commissioner of TUM School of Life Sciences (reference number 32-568-2) and are in line with methods stated in Annex IV of Directive 2010/63/EU and German animal welfare law and conform to the principles and regulations as described in the "Principles and standards for reporting animal experiments".[41]

2.2 | Human tissue samples

Studies were performed using macroscopically normal surgical intestinal specimens of human colon ($n=19$), ileum ($n=11$), jejunum ($n=3$), or duodenum ($n=4$), obtained from 37 patients (19 male, 18 females, mean age 67 ± 14) undergoing abdominal surgery at the Medical Clinic in Freising and the Medical Clinic of the Technische Universität München. Diagnoses that led to the surgery were as follows: colon carcinoma/adenoma (16/1), diverticular disease (4), pancreas carcinoma/pancreatitis (3/2), stenosis (3), Crohn's Disease (2), and one each for stoma reversal, small intestinal carcinoma, leukemia, gangrene, ischemia, and unknown diagnosis. Samples were taken from macroscopically normal, unaffected areas as determined by visual inspection by the pathologists. After removal, the surgical specimens were placed in cold aerated sterile HEPES-Krebs solution containing in mM: 135 NaCl, 5.4 KCl; 1.0 Mg $Cl_2 \cdot 6H_2O$, 1.2 NaH_2PO_4, 1.25 $CaCl_2 \cdot 2H_2O$, 12.2 Glucose, and 3 HEPES (all from Sigma-Aldrich Chemie GmbH) and 10 mL/L antibiotic-antimycotic mix (mg/L: 25 amphotericin B, 107 U/L penicillin G, 10,000 streptomycin in physiological saline; CCPro). They were immediately transported to the laboratory for experiments. Under continuous superfusion with ice-cold carbogen-aerated Krebs solution (pH 7.4) containing in mM: 117 NaCl, 4.7 KCl, 1.2 $MgCl_2 \cdot 6H_2O$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 2.5 $CaCl_2 \cdot 2H_2O$, and 11 glucose (all from Sigma), the surgical specimens were microscopically dissected by removing the mucosa and the muscular layers to obtain a whole mount preparation of the inner submucosal plexus. The final preparations (10×20 mm) were pinned on silicone rings.[42]

2.3 | Guinea-pig specimens

Eleven male "Dunkin-Hartley" guinea-pigs (strain HsdDhl:DH, Envigo GmbH; strain 051, Charles River) of an average weight of 390 ± 49 g were used. Animals were killed by a percussive blow to the head followed by exsanguination. From two animals, DRGs were isolated under a stereomicroscope and collected in a small glass bottle filled with HEPES buffer containing in mM: 1 $MgCl_2$, 2 $CaCl_2$, 150 NaCl, 10 HEPES, 10 Glucose, and 5 KCl. They were taken from thoracic segments T2–L2 because in rodents, these ganglia provide the major splanchnic afferent innervation to bronchia, lung, and intestine.[43] The DRGs were digested in protease type I (8 U/mL, Gibco), collagenase type II (516.2 U/mL, Gibco) and bovine serum albumin (BSA fraction V 0.37%, Serva). The incubation step lasted 40–50 min. After several washing steps, the pellet was suspended in 600–1200 µL culture medium (M199 GlutaMAX, Gibco/Invitrogen) containing 1% Penicillin/Streptomycin (Gibco/Invitrogen), 30 mM glucose, and 10% fetal bovine serum (Gibco/Invitrogen). Each culture dish was inoculated with 300 µL ganglia suspension. For the present experiments, we used three culture dishes (two from one guinea-pig and one from the other; ibidi dish 35 mm with ibiTreat-coating, Ibidi). Freshly dissociated and short-term cultured DRG neurons often became dislocated after local application of substances. DRGs were therefore maintained in culture for at least 7 days during which the neurons adhered to the optical bottom of the dish. Medium was changed every 2–3 days and contained 2 µM cytosine β-D-arabinofuranoside (Ara-C; Sigma) to suppress proliferation of other cell types.

Submucous plexus activity was assessed on segments of the proximal part of the distal colon (length 2 cm), which were quickly removed after killing the animals. The segments were dissected in ice-cold carbogen-aerated Krebs solution similarly to the procedure described above for human tissue. The mucosa and the muscle layers were carefully removed for preparation of the submucous plexus. The final preparations (10×20 mm) were pinned on silicone rings.

2.4 | Ca^{2+} sensitive dye imaging

To initially screen for neuronal effects of the aqueous pollen extracts, we used Ca^{2+} imaging technique as a common method to assess long-lasting changes in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). The methods and techniques have been previously described in the enteric nervous system,[19,44] as well as for DRG neurons.[45] Briefly, the primary culture dishes of DRG neurons were incubated for 20 min at room temperature in the dark with the fluorescent calcium indicator Fluo 4-acetoxymethyl (AM) (Invitrogen) at a final concentration of 10 µM diluted in HEPES buffer containing 1.25 mM probenecid (Sigma) to prevent dye leakage. The staining solution was washed out for 10 min, and the preparation was mounted in a self-made recording chamber and continuously perfused with 37°C HEPES buffer. For human and guinea-pig submucous plexus preparations, the staining procedure was similar to that of the DRG neurons,

except a longer incubation period (45 min) and the use of Krebs buffer containing 500 µM probenecid instead of HEPES buffer for incubation, washing, and perfusion during the experiments.

The recording chambers were mounted on an inverted epifluorescence microscope (Zeiss Axio Observer A1, Carl Zeiss) equipped with a high-speed monochrome camera (Zeiss AxioCam HSm) and software (Zeiss Axio Vision 4.8) for acquisition and analysis. Fluo-4 AM was excited using a blue light emitting diode (LED) Luxeon III (3 W, 470 nm dominant wavelength, Phillips Lumiled, Phillips), and the signals were detected with a filter cube F26-514 Bright Line FITC BP (excitation: HC475/35, dichroic: 499, emission: HC530/43, AHF Analysentechnik) using 20× objective (A-Plan, NA = 0.25, Zeiss).[46] The system measured relative changes in fluorescence (ΔF/F) of Fluo-4 AM by monitoring changes in $[Ca^{2+}]_i$. Depending on the stimulus, $[Ca^{2+}]_i$ transients were recorded for 35 s up to 60 s using a frame rate of 0.5 or 1 Hz, in selected cases 6.2 Hz. Under basal condition, the variation in background fluorescent was ±1%ΔF/F. A threefold standard deviation (3%) was defined as threshold for genuine cell activation.[19] Basal, non-stimulated $[Ca^{2+}]_i$ signals were recorded for 35 s.

Aliquots of low and high MW fractions of aqueous birch pollen extract (diluted 1:1 with buffer) were locally perfused on DRG neuronal clusters or submucous neurons, respectively, via a micro perfusion pump (Micro4, WPI Sarasota FL) lasting for 30 s at a speed of 0.1 µL/s. According to our previous studies using spritz pulse application, a 1:10 dilution of the applied substance has to be considered for the local perfusion as well.[26]

To check the vitality of submucous neurons, we stimulated the entire preparation via transmural electrical field stimulation (EFS_{TM}).[19] The EFS_{TM} of the nerves was achieved by two platinum electrodes and a constant-voltage stimulator (Typ 215/I, Hugo Sachs Elektronik, Hayard-Apparatus GmbH). The stimulus parameters were 50 Hz pulse train for 10 s with pulse duration of 0.5 ms at 25 V. Cultured cell vitality was tested at the end of each experiment using the calcium ionophore ionomycin (15 µM, Sigma) which was applied for 2 s followed by 60 s recording period. Those cells showing no Ca^{2+} signals in response to ionomycin were exclude from further analysis.

2.5 | Fast neuroimaging technique

Fast neuroimaging allowed detection of action potential discharge at a single-cell level and was used in preparations of human submucous plexus only. This multisite optical recording technique (MSORT) was previously described in detail.[47–49] Briefly, individual ganglia were stained with the fluorescent voltage-sensitive dye 1-(3-sulfonatopropyl)-4-[β[2-(di-*n*-octylamino)-6-naphthyl]vinyl]pyridinium betaine (di-8-ANEPPS, 20 µM dissolved in DMSO (0.135%) and Pluronic F-127 (0.0135%) containing Krebs buffer; Molecular Probes Mobitec). The dye was excited by a green LED (LET S2W, Osram), and the recordings from the stained neurons were performed using an epifluorescence Olympus IX 50 microscope (Olympus Corporation) equipped with a 40× oil immersion objective (UApo 340 nm, Olympus) and an appropriate filter cube (AHF Analysetechnik AG). The technique allows for measurement of the relative changes in fluorescence intensity (ΔF/F), which is linearly related to changes in the membrane potential.[47] Signals were acquired with a frequency of 1 kHz, detected using a cooled charge coupled device (CCD) camera (80 × 80 pixels; Neuro CCD-SMQ imaging system, Red Shirt Imaging LLC Decatur) and processed by the Neuroplex 10.1.2 software (RedShirt Imaging). With the 40× oil objective, the spatial resolution of the CCD camera was 24 μm^2/pixel.

The viability and the staining of the neurons were tested either by recording spontaneous neuronal activity or by evoking fast excitatory postsynaptic potentials with a single electrical stimulus applied to an interganglionic fiber tract via[42] a teflon-coated platinum electrode (diameter 25 µm, Science products GmbH) connected to a constant-voltage stimulator (A360, WPI; 400 µs pulse duration, amplitudes ranging from 10 to 100 µA). Aqueous pollen extracts (diluted 1:1 with Krebs buffer) were applied onto a single submucous ganglion by pressure pulse ejection (20 PSI, ejection speed 55 ± 27 nL/s 400–800 ms duration) from glass microejection pipettes that were placed close to the ganglion (approximate distance: 100–200 µm). Signals were recorded for a 2000 ms period. For statistical analysis and comparison, only experiments with an application duration of 800 ms were used.

2.6 | Aqueous pollen extracts and pharmacology

Pollen from birch (*Betula pendula*) and hazel (*Corylus avellana*) was isolated from flowering catkins in the early pollen seasons of 2018 (birch) and 2019 (hazel). Pollen from Ragweed (*Ambrosia artemisiifolia*) was isolated from inflorescences of flowering *Ambrosia* plants grown in the greenhouse at the Helmholtz Center Munich (kindly provided by Dr. Ulrike Frank, Institute of Biochemical Plant Pathology). Aqueous pollen extracts (APEs) were prepared as described before.[39,50] Briefly, pollen grains were suspended in HEPES-Krebs buffer without supplements at 30 mg pollen per ml. After incubation in a shaking water-bath (250 rpm) at 37°C with agitation for 30 min and vigorous vortexing every 10 min, the suspensions were centrifuged (10 min, 3000×*g*), sterile filtered (0.2 µm pore-size) and the pollen-free supernatant (=aqueous pollen extract) stored in aliquots at −80°C until use. As previously described in detail,[22,37] aliquots of the pollen extracts were separated by ultrafiltration using centrifugal filter units (Amicon Ultra) into two fractions: a low MW, protein-free fraction ($APE_{<3kDa}$), which is rich in small molecules, such as lipids, sugars, nucleosides, and secondary plant metabolites,[40] and a high MW fraction ($APE_{\geq 3kDa}$), which contains the pollen allergens and other proteins, but also residual small molecules that remain bound to proteins. Before use, the samples were diluted 1:1 with Krebs buffer and adjusted to pH 7.4, if necessary. The osmolarity was 291 mosmol/kg for birch $APE_{<3kDa,}$ 286 mosmol/kg for birch $APE_{\geq 3}$ kDa, 298 mosmol/kg for ragweed $APE_{<3kDa}$, and 296 mosmol/kg for hazel $APE_{<3kDa}$; the osmolarity of Krebs buffer

was 296 mosmol/kg. In some experiments, we tested the effects of $APE_{<3kDa}$ in the presence of a mixture of specific histamine receptor antagonists, that is, the H1 receptor antagonist pyrilamine (1 μmol/L; Sigma), the H2 antagonist ranitidine (10 μmol/L; Sigma), and the H3 antagonist clobenpropit (10 μmol/L; Biotrend)[26] or in the presence of the neurotoxin tetrodotoxin (TTX 0.5 μM). Antagonists were added to the perfusing Krebs solution 20 mins before reapplication of the respective pollen extract. The washout periods were at least 60 mins.

2.7 | Histamine analysis in the aqueous pollen extracts

Histamine concentrations in the different fractions of the APEs of birch, ragweed, and hazel pollen ($APE_{<3kDa}$, $APE_{\geq 3kDa}$ and total APE) were quantified using targeted liquid chromatography tandem-mass spectrometry (LC–MS/MS). Therefore, 50 mL of the extracts was mixed with 10 μL of internal standard mixture, consisting of 0.5 μM Histamine-d4 (internal standards, Santa Cruz Biotechnology). Samples were subsequently dried in a SpeedVac vacuum concentrator. Our method is described in detail in.[19] Briefly, pre-column derivatization of all samples was done using phenylisothiocyanate (PITC). A 200 μL mixture consisting of 5% PITC dissolved in pyridine, ethanol, and 0.1% NH3 in a 1/1/1 ratio was added to the dried samples. Samples were shaken at 25°C for 20 min and were subsequently dried and then resuspended in methanol containing 5 mM ammonium acetate and finally diluted with water to reach a methanol/water ratio of 70:30. The analysis was performed on a triple quadrupole QTRAP 5500 LC–MS/MS system operating in positive ESI mode (AB Sciex) equipped with a 1200 series binary pump (Agilent) and coupled to an HTC pal autosampler (CTC Analytics). Chromatographic separation was achieved using a Zorbax Eclipse XDB-C18 column (length 150 mm, internal diameter 3.0 mm, particle size 3.5 μm; Agilent). Eluent A consisted of 0.2% formic acid in water. Eluent B consisted of 0.2% formic acid in acetonitrile. The histamine concentrations were determined in scheduled multiple reaction monitoring (sMRM). For quantification, a 7-point calibration was performed (0–2 μM) using pure histamine. Data analysis was done using Analyst 1.5.1® software (AB Sciex).

2.8 | Immunohistochemistry

Our method for immunohistochemically staining of cultured DRG neurons was previously described in detail.[51] Briefly after the experiments, the DRG neurons were fixed overnight at 4°C in 4% phosphate-buffered formaldehyde containing 0.2% picric acid, washed (3 × 10 min) with phosphate buffer, and permeabilized for 1 h at room temperature in phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 0.1% NaN3, and 4% horse serum. After further washing (3 × 10 min with phosphate buffer), tissues were incubated with the primary antibody sheep anti-PGP 9.5 (1:20,000; The Binding Site) for selectively labeling enteric neurons. This was followed by incubation with the secondary antibody Cy5 (1:500; Dianova). The fluorescence was examined by an epifluorescence microscope (BX61W1; Olympus).

2.9 | Data analysis and statistics

As described previously, the $[Ca^{2+}]_i$ imaging technique enables the detection of cell outlines during the rise of intracellular $[Ca^{+2}]$ accompanied by the increase in Fluo-4 AM fluorescence.[44] In the DRG cell culture, the neurons were identified by their response to nicotine (Sigma; 100 μM, 600 ms pressure pulse application)[42] and/or by their PGP 9.5 immunoreactivity. As parameter for cell activation the maximum intracellular $[Ca^{2+}]_i$ increase relative to resting light level (%ΔF/F) was determined for each stimulus. Further, the time interval (s) from beginning of a stimulus to the peak $[Ca^{2+}]_i$ responses and the relative proportion of responding neurons in the field of view using a ×20 objective (0.08 mm^2) was determined taking the number of those cells responding to ionomycin as 100%.

For the $[Ca^{2+}]$i imaging experiments in submucous plexus preparations, we used EFS_{TM} to identify the neuronal area since only neurons respond directly to electrical stimulation.[19,44,52] As a parameter for evoked activation, the percentage of responding neuronal area per ganglion (taking the neuronal area responding to EFS_{TM} as 100%) as well as the amplitude of the $[Ca^{2+}]_i$ peak per ganglion and the time to peak were calculated.

The analysis of the fast neuroimaging experiments was performed as described before in detail.[44,53] Briefly, individual neurons can be visualized since the voltage-sensitive dye (Di-8-ANEPPS) incorporates into the membrane revealing the outline of individual cell bodies. The overlay of signals with the ganglion image allowed us to analyze the response of individual neurons. As parameters for the substance-evoked activation of the submucous neurons, we calculated the percentages of responding neurons per ganglion (setting the number of neurons per ganglion which either responded to electrical stimulation or were spontaneously active as 100%) together with their average spike discharge frequency [Hz] per ganglion.

Numbers of tissues (equivalent to number of patients or guinea-pigs), ganglia, and neurons are given in sequence without further specification, for example, a result based on experiments from four tissues, five ganglia, and 20 neurons is presented as (4/5/20). Data are expressed as median values with the 25th and 75th quartiles given in brackets. Statistical analysis and graphs were performed using SigmaPlot 12.5 (Systat Software Inc.). Data were tested for normal distribution using the Shapiro–Wilk test. To determine whether an effect produced by a substance was statistically significantly different from zero, a one-sample signed rank was performed. Data derived from the $[Ca^{2+}]_i$ imaging experiments were compared using the Mann–Whitney rank sum test. Data derived from the voltage-sensitive dye imaging experiments in the human submucous plexus were tested with the Kruskal–Wallis one-way analysis of variance on ranks (comparing neuronal

effects evoked by different pollen species) or by the Friedman repeated measures analysis of variance on ranks combined with the Student–Newman–Keuls Method as pairwise multiple comparison procedure, (comparing results from pharmacology experiments, which were obtained in a paired experimental design). For all tests, a p value of <0.05 was considered significant. Data availability statement: All data $n \leq 30$ are presented and available in the manuscript.

3 | RESULTS

3.1 | Low MW aqueous birch pollen extracts activate cultured guinea-pig dorsal root ganglia

In a first set of experiments, we used cultured guinea-pig DRG's and Ca^{2+} imaging to screen for neuroactive properties of the low MW aqueous pollen fractions ($APE_{<3kDa}$), first focusing on the birch pollen (Figure 1A). Results were based on DRGs from two guinea-pigs, three primary cultures, 10 fields of view containing clusters of in total 35 DGR neurons (guinea-pig no.1/one culture/five fields of view/19 DRG neurons; guinea-pig no. 2/two cultures/five fields of view/16 DRG neurons). The size of the DRG neurons ranged from 14 to 34 µm. According to Lawson et al.[54] DRGs were divided into small (<25 µm) and medium/large cells (25–35/>35 µM). Thirty-one DRG neurons responded to the application of birch $APE_{<3kDa}$ and showed a strong $[Ca^{+2}]_i$ transit of 77.5 %ΔF/F (48.7/128.7) which was significantly different from zero ($p<0.001$); one-sample signed rank test; 81% ($n=25$) belonging to the small type and 19% ($n=6$) to the medium/large type. However, both types responded equally [DRG neurons <25 µm: 79.1%ΔF/F (42.7/133.3) vs. DRG neurons ≥35 µm: 51.0%ΔF/F (49.2/101.6; $p=0.382$, Mann–Whitney rank sum test)]. The signal from DRG neurons peaked shortly after stimulus onset [6.0 s (4.0/10.3), $n=31$]. In six small-sized DRG neurons, we tested the involvement of TTX sensitive sodium channels. Neurons still responded to birch $APE_{<3kDa}$ in TTX (0.5 µM), but the $[Ca^{+2}]_i$ peak was greatly reduced from 59.8%ΔF/F (37.8/117.2) to 2.9 (0.0/6.3), thus indicating additional TTX insensitive mechanisms underlying the Ca^{2+} signaling in response to birch pollen extracts.

3.2 | Aqueous birch pollen extracts activate neurons in freshly dissected submucous plexus preparations

Using Ca^{2+} imaging technique, we checked whether the same sample would also activate neurons in intact tissue, that is, in fresh, whole mount preparations of the enteric submucous plexus of guinea-pig intestine and also in human intestinal preparations. As shown in Figure 1B, birch $APE_{<3kDa}$ evoked a strong $[Ca^{2+}]_i$ transient in all neurons of guinea-pig submucous ganglia. In this approach, we additionally tested for effects of the corresponding high MW fraction ($APE_{\geq 3kDa}$) containing the allergen fraction. Although the $APE_{\geq 3kDa}$ fraction also activated a majority of neurons per ganglion [100.0% (83.3/100.0); $n=4$ tissues and 4 ganglia], the peak response was clearly lower compared to the effects of $APE3_{<3kDa}$ (Figure 1B). The time to peak, however, did not differ [$APE_{<3kDa}$: 28.7 s (25.2/32.3) vs. $APE_{\geq 3kDa}$: 30.0 s (28.5/32.8), $p=0.648$, Mann–Whitney rank sum test]. Also, in human submucous plexus preparations ($n=8$ tissues, 11 ganglia) the low MW birch pollen fraction evoked a clear $[Ca^{2+}]_i$ signal [46.7%ΔF/F (31.5/66.0)] in almost all vital neurons per ganglion [100.0% (40.8/100.0) peaking at 21.4 s (16.0/27.5)]. All $[Ca^{2+}]$ i signals elicited by pollen extracts were significantly different from zero ($p<0.02$; one-sample signed rank test).

3.3 | Low MW ragweed, birch, and hazel pollen extracts induce fast neuronal responses

To check for fast onset responses, we continued our experiments using an ultra-fast neuroimaging approach with a voltage-sensitive dye as a reporter. Here, we focused exclusively on human submucous plexus preparations and included the low MW fractions of two further pollen taxa (ragweed and hazel). The spritz application of $APE_{<3kDa}$ of all three pollen types evoked a fast onset action potential discharge in human submucous neurons (Figure 2). The spike discharge was comparable between the three pollen extracts [median for birch: 7.0 Hz (6.2/9.8), ragweed: 5.7 Hz (4.4/7.1), and hazel $APE_{<3kDa}$ 8.4 Hz (4.3/12.5), respectively]; all effects were significantly different from zero, $p<0.016$; one-sample signed rank test. Likewise, the percentage of neurons responding per ganglion to the extracts were similar [median birch: 40.0% (33.3/100.0), ragweed: 50.8% (34.4/85.6), and hazel: $APE_{<3kDa}$ 83.3% (57.1/100.0), respectively; Figure 2]. Next, we investigated which substance might be involved in the nerve activation. We focused on histamine as the most likely candidate. As seen in Figure 3A,B, the spike discharge of those neurons that responded to birch and ragweed $APE_{<3kDa}$ was significantly reduced by a mixture of H1, H2, and H3 receptor antagonists and recovered after wash out. The response to corresponding hazel $APE_{<3kDa}$ was only marginally affected by histamine receptor antagonists.

3.4 | Aqueous ragweed, birch, and hazel pollen extracts contain histamine

Using targeted liquid chromatography-mass spectrometry, we identified histamine in the extracts of the different pollen species (Table 1). The analysis revealed a higher histamine concentration for ragweed APE in all fractions compared to birch and hazel APE, respectively. In APE<3 kDa, the histamine concentration was highest for ragweed (0.764 µM), lowest for hazel pollen (0.013 µM). Regarding the histamine concentration in the non-fractionated total extracts, it was found that the deviations between the measured values and the calculated data (concentrations in the $APE_{<3kDa}$ plus $APE_{\geq 3kDa}$) were small and ranged from 5.6 to 13.4%. This underlines the reliability of the quantitative analysis.

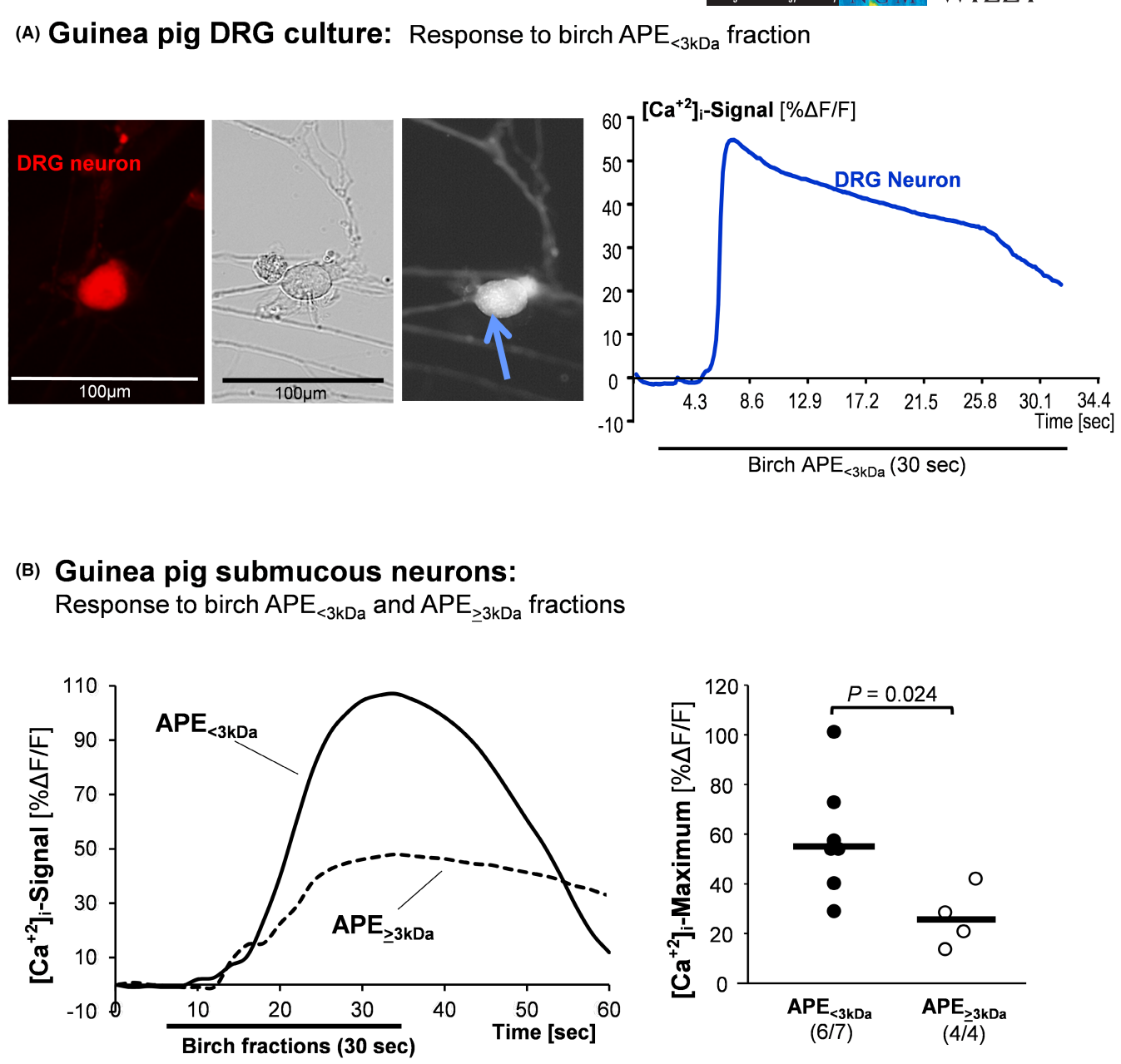


FIGURE 1 (A) Effects of birch low MW aqueous pollen fractions ($APE_{<3kDa}$) on cultured guinea-pig DRG neurons. Identification of anti-PGP 9.5 positive neurons (red) in the cell culture (left image; epifluorescence microscope Olympus BX61W1; objective ×40). Calcium image of the cell culture stained with Ca^{2+} sensitive dye Fluo-4 AM brightfield image in the middle, fluorescence image on the right panel [epifluorescence microscope Zeiss Axio Observer A1; objective ×20; DRG neuron (blue arrow)]. Cell activation was reported by imaging changes in intracellular calcium $[Ca^{2+}]_i$. The graph (right panel) illustrates the traces of the $[Ca^{2+}]_i$ signals in response to the $APE_{<3kDa}$ fraction of birch pollen. The signal of the DRG neuron appeared instantaneously after the onset of the stimulus. The sample frequency in this example was 6.2 Hz. (B) Effects of low ($APE_{<3kDa}$) and high ($APE_{\geq 3kDa}$) MW birch pollen fractions on guinea-pig submucous neurons. The traces on the left panel illustrate that the birch $APE_{<3kDa}$ fraction, containing no protein allergens, evoked a stronger neuronal activation compared to the corresponding $APE_{\geq 3kDa}$ fraction. The amplitudes of the $[Ca2^{+}]_i$ signals (right panel) differed significantly. The bare below the traces indicate the duration of the stimulus. Statistics were based on the number of ganglia. *p*-values are based on Mann–Whitney rank sum test; numbers in parentheses indicate number of tissues/ganglia studied.

4 | DISCUSSION

In the present study, we demonstrated for the first time that low MW aqueous pollen extracts of birch, ragweed, and hazel contain neuroactive components that strongly activate spinal sensory neurons (in guinea-pig DRGs) and enteric neurons of the submucosal plexus of guinea-pig and human. Nerve activation was independent of classic allergic hypersensitivity reactions. Pharmacologically, a significant histaminergic component was shown to mediate the nerve-activating effects of birch and ragweed pollen extracts, but not for

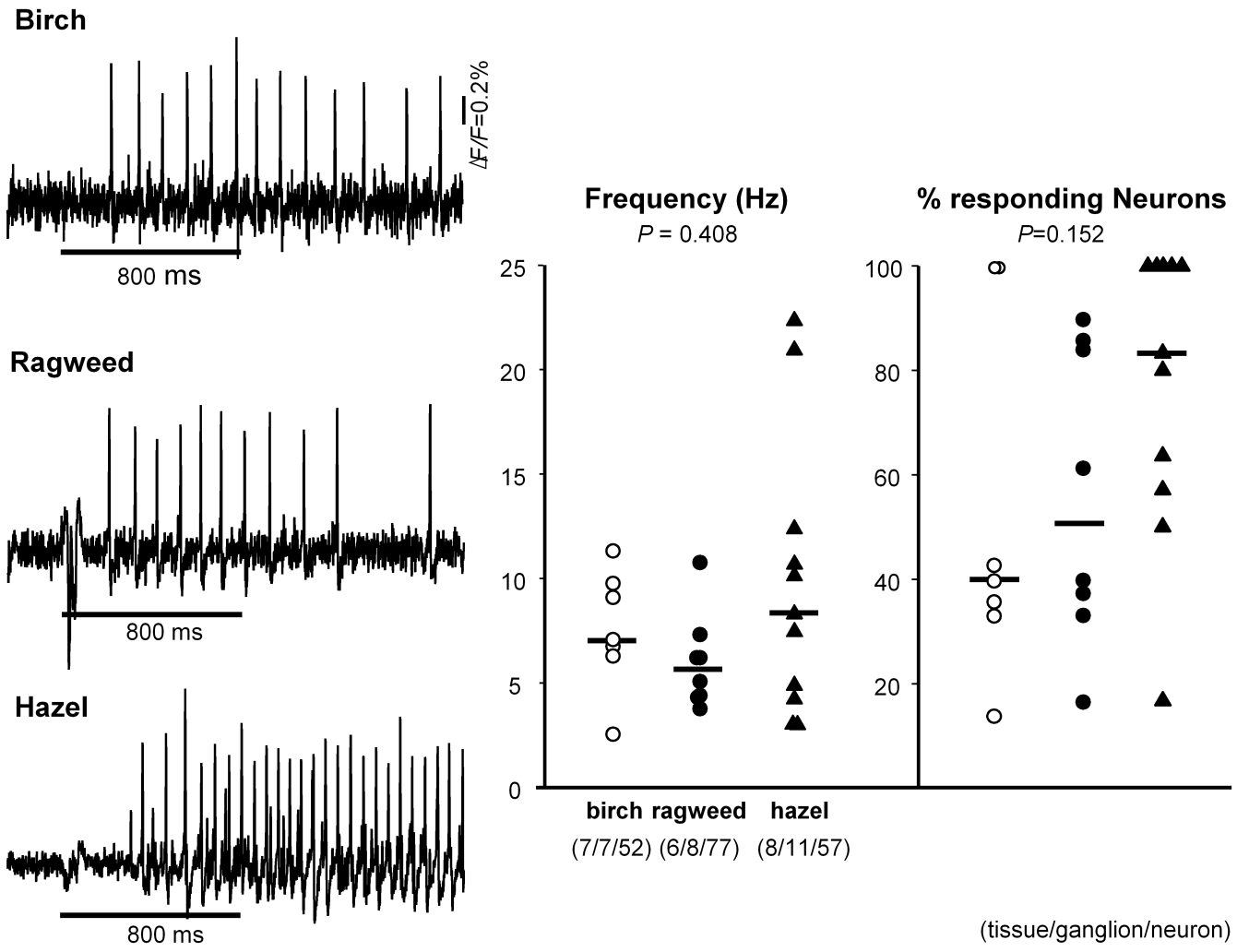
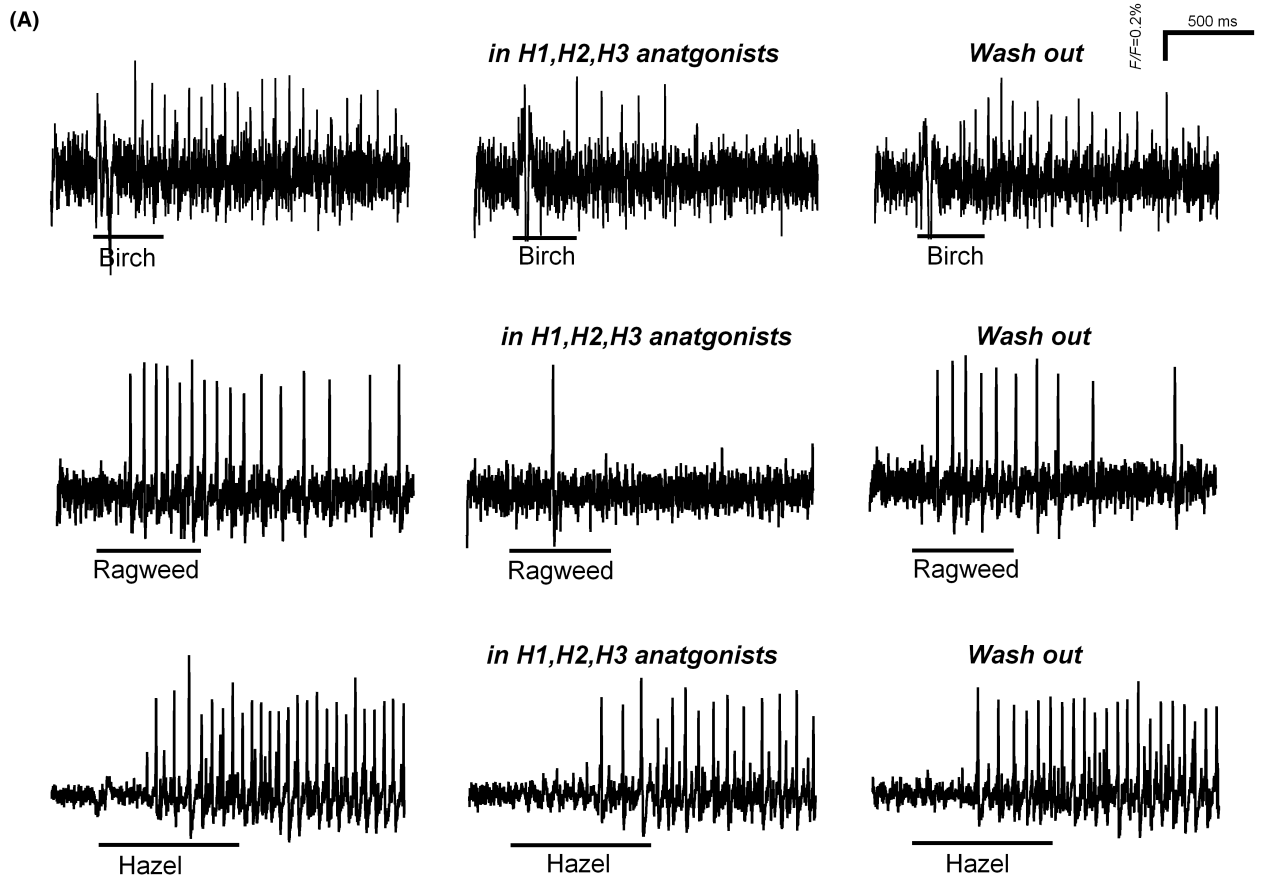


FIGURE 2 Spritz application of low MW aqueous pollen fractions ($APE_{<3kDa}$) from birch, ragweed, and hazel evoked a strong action potential discharge in human submucous neurons (left panel; duration of the application indicated by the bars below the traces). The quantification of the neuronal activity revealed no significant difference between the responses evoked by the different pollen types, neither in the action potential frequency nor in the percentage of neurons responding per ganglion (right two panels). Statistics were based on the number of ganglia; therefore, the action potential discharges of individual neurons per ganglia were averaged. The total number of neurons activated by birch, ragweed, and hazel pollen extracts was 24, 48, and 41, respectively. *p*-values are based on the Kruskal–Wallis one-way analysis of variance on ranks; numbers in parentheses indicate number of tissues/ganglia/neurons studied.

the corresponding extract from hazel pollen. This novel mechanism must be considered as a relevant feature in chronic inflammatory conditions of the gut.

Our study shows, for the first time, that pollen components which lack allergens activate enteric and spinal sensory neurons. The functional importance of an activation in enteric neurons is obvious as their cell bodies and neurites are located within the gut wall and known to regulate gut functions. The response evoked in spinal sensory neurons is equally relevant as their terminals innervate the submucous layer.[55] Allergic responses have a prominent neurogenic component (for review).[56,57] The focus is usually on allergic hypersensitivity reactions, which essentially involve

FIGURE 3 (A) The spike discharge in human submucous neurons in response to birch (upper traces) and ragweed (middle traces) low MW pollen fractions ($APE_{<3kDa}$) was reduced by a combined application of H1, H2, and H3 receptor antagonists ranitidine, pyrilamine, and clobenpropit, respectively, and recovered after wash out. The response to corresponding hazel pollen fraction was affected only slightly by histamine antagonists (lower traces). (B) The quantitative analysis confirmed a significant (*) reduction of spike frequency by histaminergic blockage in birch and ragweed fractions but not for hazel pollen fractions. The percentage of responding neurons per ganglion was not influenced significantly by histamine receptor antagonists in any of the pollen species samples. Statistics were based on the number of ganglia. Therefore, the action potential discharges of individual neurons per ganglia were averaged. Friedman repeated measures analysis of variance on ranks.



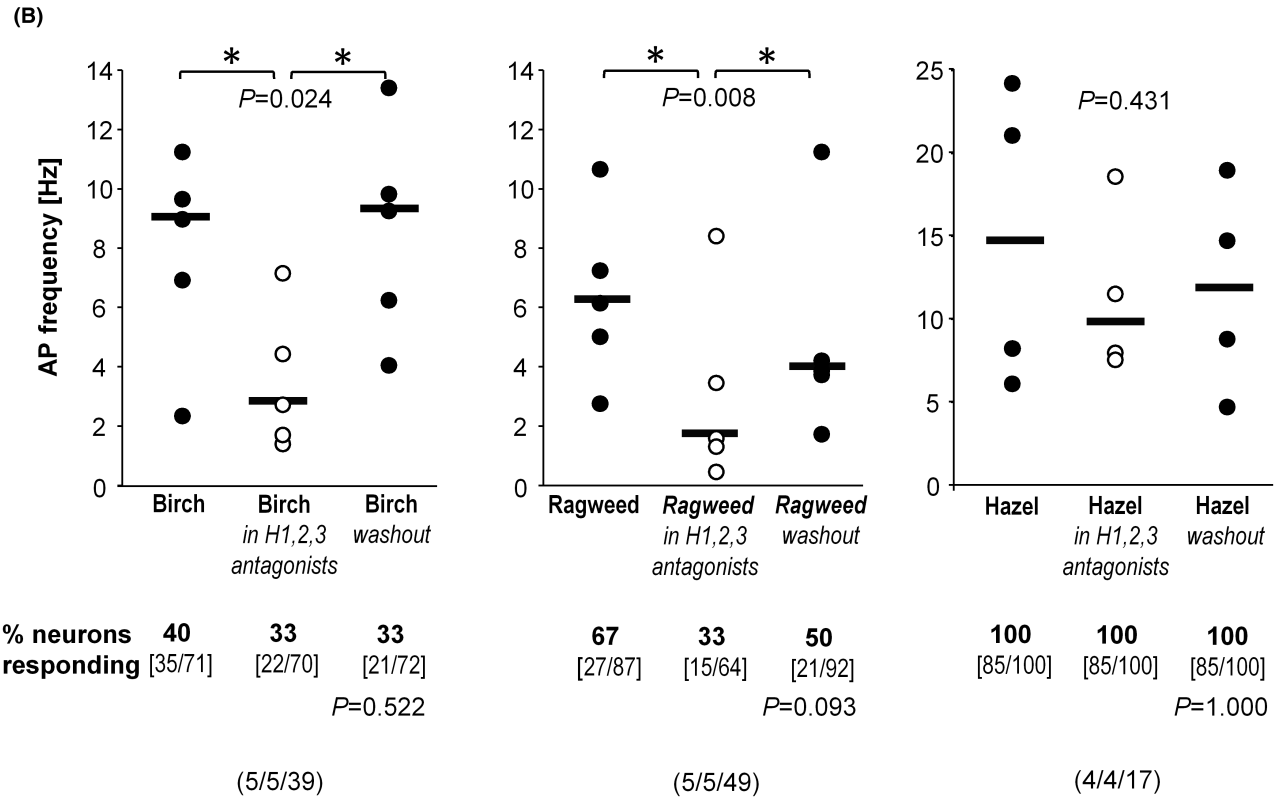


TABLE 1 Histamine concentration in different fractions of the aqueous pollen extracts (APE).

Pollen type	Histamine concentration (μM)		
	Low molecular weight fraction ($APE_{<3kDa}$)	High molecular weight fraction ($APE_{\geq 3kDa}$)	Total APE
Ragweed	0.764	0.168	1.076
Birch	0.047	0.004	0.055
Hazel	0.013	0.004	0.018

signaling between nerves and immune cells, most importantly mast cells.[19,58–60] However, as we have shown in the present study for different pollen types, there appears to be an effective mechanism that ensures the activation of neurons independent of the allergic response of the host. Here, neuronal stimulation is mediated via low MW substances released directly from pollen grains.

To look for neuronal activation, we first performed calcium imaging experiments. This is the method of choice to visualize slow, long-lasting changes in the intracellular calcium $[Ca2+]_i$ levels and to also detect low-threshold activation.[44] The technique is considered as a very sensitive method minimizing the risk to miss responses or effects. In our study, we primarily focused on extracts from birch pollen, as birch pollen is the most potent aeroallergen in Central and Northern Europe with high seasonal peak concentrations,[61] sensitization rates of 6.4–22.6%, and considerable clinical importance.[62] Birch pollen, in particular, is frequently discussed in the context of the pathophysiology of eosinophilic esophagitis,[1,14,16] (for review).[7,8,63] We have studied two different types of neurons. One is primary cultured guinea-pig DRG neurons in order to study the ability of pollen-derived substances to affect sensory nerves. The DRGs were taken from thoracic segments T2–L2 because these ganglia represent the major splanchnic afferent innervation of the bronchi, lungs, and intestines in rodents.[43] DRGs contain the cell bodies of afferent sensory neurons. The receptors found on peripheral terminals of these neurons in vivo are most often expressed on their cell bodies as well.[64] As we could show in the present study, the $APE_{<3kDa}$ of birch pollen strongly activate DRG sensory neurons. About 80% of the responding neurons belonged to the small size population, which mainly mediate pain-, itch-, and somato-sensation (for review).[56,57] We have previously reported a very similar response of small-sized DRG neurons after application of mucosal biopsy supernatants of patients with irritable bowel syndrome.[45] The evoked calcium peaks were strongly reduced by preincubation of the neurons with TTX, suggesting that opening of voltage-sensitive fast Na-channels are required for initiating intracellular Ca-signals.

Our study showed that similarly to the DRG neurons, guinea-pig and human enteric neurons responded with strong calcium signals to a stimulation with the $APE_{<3kDa}$ of birch pollen. Therefore, nerve activation is a rather general hallmark of the pollen extracts and may not reflect the specificity of a particular neuron type or species. Guinea-pig intestinal plexus preparations were also used to test the effect of the $APE_{\geq 3kDa}$ fraction of birch pollen extracts, which, unlike the $APE_{<3kDa}$ fraction, contain the allergenic proteins. The $APE_{\geq 3kDa}$ fraction also evoked a calcium signal in guinea-pig neurons, but the response was much smaller compared with the effects of the corresponding $APE_{<3kDa}$ birch pollen fraction. A hypersensitivity reaction can be excluded because the guinea-pigs were not sensitized to the pollen, and, moreover, the neuronal response occurred much more rapidly than would be expected from a classical mast cell-triggered event.[19] Thus, either direct effects of proteins or other, as yet unidentified, substances need to be considered. However, some residual low MW substances that may be retained in the high MW fraction, for example, due to binding to filter matrix or protein, could also be responsible for the corresponding neuronal activation. We will come back to this point in detail later in the discussion.

Because of species differences in terms of receptor pharmacology between rodents and humans (for review),[58] we focused exclusively on human ENS preparations as matrix for all further targeted studies on signaling and pharmacology of pollen-induced nerve activation, despite the limited availability of human tissues. We also switched our recording to fast neuroimaging technology. By using a voltage-sensitive dye, this technique allows reliable measurement of action potentials at the level of single cells.[49] Furthermore, we wanted to clarify whether the nerve-activating effect of the low molecular aqueous pollen extracts is specific to birch, or represents a general property of other pollen species as well. To clarify this, we included two more pollen taxa. One of them was hazel, characterized by a marked homology of the major allergen (Cor a 1) to that of birch (Bet v 1),[61,65] and ragweed pollen as a representative of herbaceous plants. Ragweed is in the focus of many studies due to its extreme allergenic potential.[66] The $APE_{<3kDa}$ from all three pollen types elicited an immediate discharge of action potentials with broadly similar frequency and pattern, suggesting that nerve-activating effects may not be a species-specific but a general feature of the water-soluble, non-allergenic fraction of pollen extracts. We identified histamine as an important mediator for two of the pollen extracts based on the finding that the nerve activation was strongly reduced by a combination of H1, H2, and H3 receptor blockers, which primarily mediated histamine effects in human enteric neurons.[26] The histaminergic component was only observed for ragweed and birch pollen extracts. Other, yet to be identified, substances must mediate the effects of hazel pollen extract.

Further, we quantified the histamine concentration in the pollen extracts using LC–MS/MS analysis. A high level was detected in the $APE_{<3kDa}$ fraction of ragweed pollen, which is very consistent with the histamine-mediated nerve activation of this extract. The corresponding concentration in the birch pollen extract was lower,

however, still about 4× higher than in the $APE_{<3kDa}$ extracts from hazel pollen. This could explain the lack of effects of histamine antagonists on nerve activation in hazel $APE_{<3kDa}$. The quantitative analysis confirmed our pharmacological findings and highlights the importance of histamine as a neuroactive low molecular weight component of pollen.

We extended the analyses also to the high molecular weight fractions. It turned out that only for ragweed pollen a significant concentration of histamine could be detected. The level suggests that a pharmacological proof of histamine would also work. This means that in the case of ragweed pollen, a histaminergic neuroactive component may also be involved for the $APE_{\geq3kDa}$ fraction. In contrast, in the $APE_{\geq3kDa}$ fraction of birch (and equally of hazel) pollen, the level of histamine was so low making a pharmacological detection of histamine by antagonists in our setup rather unlikely. Therefore, we believe that mainly non-histaminergic mechanisms are responsible for the observed nerve-activating effect of the birch pollen $APE_{\geq3kDa}$ fraction. In addition, we measured the histamine concentration also in the total extract (not fractionated). The values are comparable in magnitude to the histamine concentrations determined in the study by Williams et al.[25] in aqueous and phenolic pollen extracts of a variety different species. Importantly, the dose of pollen extract used in our experiments is within the dose range applied in nasal allergen challenges (e.g., for birch: 1–40 µg/mL Bet v)[67,68] and can thus be considered clinically relevant.

In the present study, we provided evidence that aqueous pollen extracts of birch, ragweed, and hazel contain neuroactive components which strongly activate spinal sensory neurons and enteric neurons. This nerve activation is independent of the classical allergic hypersensitivity responses. We suggest that the rapid release of bioactive mediators from pollen during contact with the aqueous phase of mucosal membranes may represent a pathophysiological relevant feature contributing to the manifestation or aggravation of acute or chronic inflammatory conditions of the mucosa.

AUTHOR CONTRIBUTIONS

SB designed and drafted the work, conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. SS conducted part of the Ca imaging experiments and analyzed the data. PG performed the targeted liquid chromatography tandem-mass spectrometry, analyzed and interpreted the data, and wrote the corresponding parts of the manuscript. FZ and IED provided human samples and medical council. They were accountable for correct patient characterization and material handling. MS and CT-H critically reviewed the manuscript for important intellectual content. They were involved in the conception, design, and funding of the work. SG designed and drafted the work, interpreted the data, and edited and revised the manuscript. She had the original idea and was involved in the conception and design of the work. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed. This work has been previously presented as a Poster at the 3rd Meeting of the Federation of Neurogastroenterology and Motility 2018 in Amsterdam. Buehner S, Schäuffele S, Gilles-Stein S, Demir IE, Ceyhan G, Zeller F, Traidl-Hoffmann C, Schemann M. Pollen extracts of birch and ragweed activate human and guinea-pig submucous neurons and guinea-pig DRG neurons. P014.

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CONFLICT OF INTEREST STATEMENT

No competing interests declared.

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