Nicotine & Tobacco Research, 2020, S35–S44
doi:10.1093/ntr/ntaa174
Supplement Article
Received April 24, 2020; Editorial Decision September 4, 2020; Accepted October 1, 2020

Supplement Article

Electronic Cigarette Liquid Constituents Induce Nasal and Tracheal Sensory Irritation in Mice in Regionally Dependent Fashion

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Abstract

Introduction: Electronic cigarettes (e-cigs) are currently used by millions of adults and adolescents worldwide. Major respiratory symptoms, such as coughing reported by e-cig users, including patients with e-cig, or vaping, product use-associated lung injury (EVALI), indicate e-cig constituent-induced sensory irritation. However, e-cig constituent-induced nociceptive activity in nasal and tracheal respiratory epithelia (RE) and neuronal activation in the trigeminal ganglia and brainstem nuclei, which receive airway chemosensory inputs have not been examined and compared. Comparisons of physiological responses between freebase nicotine and nicotine salts are also missing.

Aims and Methods: Event-related potential (ERP) was recorded electrophysiologically to assess mouse nasal and tracheal RE chemosensory responses to various flavorings, nicotine, including freebase and nicotine salts, e-liquid mixtures, and tussigenic stimuli. Also, mice were subjected to inhalation exposure to aerosol of a vanilla-flavored e-liquid or air (control), and the activated-trigeminal nociceptive neurons and brainstem neurons were examined using immunohistochemistry.

Results: Individual constituents and mixtures of e-liquids, capsaicin, and citric and acetic acids evoked significantly larger ERP in the nose than in the trachea with the exception of menthol. ERP responses to freebase nicotine were significantly larger than protonated nicotine. Four nicotine salts (benzoate, lactate, levulinate, and salicylate) induced similar responses. Compared with air-exposed mice, e-liquid aerosol-exposed mice showed a significant increase in numbers of activated trigeminal nociceptive neurons and brainstem neurons in the spinal trigeminal nucleus, paratrigeminal nucleus, and nucleus tractus solitarius.

Conclusions: E-liquid constituents region-dependently stimulate airway nociceptive chemosensory systems, and freebase nicotine is more potent than protonated nicotine.

Implications: Neural abnormalities have been implicated in the development of nasal and respiratory illnesses. The higher sensitivity of the nasal nociceptive chemosensory system to nicotine and flavorings may indicate a health risk for e-liquid aerosol-induced upper airway illnesses via neurogenic alteration and warrants further investigation.

Introduction

Commercial e-liquids typically contain nicotine, propylene glycol, and vegetable glycerin (PG/VG), and flavorings that make electronic cigarettes (e-cigs) appealing for use and sale. Nicotine and high levels of flavorings can irritate the airway, and some flavorings are also cytotoxic.[1–3] Heating aerosolizes e-liquids, which also generates additional irritants and toxicants such as PG/VG metabolites, formaldehyde and acrolein, and heavy metals.[4] These harmful chemicals stimulate

the nose, trachea, and lungs by either orthonasal or retronasal routes, causing sensory irritation and adverse respiratory health effects.[5,6] E-cig users, including patients of e-cig, or vaping, product use-associated lung injury (EVALI), frequently report symptoms of cough, headache or migraine, shortness of breath, fatigue, nasal discharge, and congestion in epidemiological surveys and online forums.[7–9] Chronic vaping further increases the risk for respiratory diseases, inflammation, and susceptibility to bacterial and viral infection.[3,10,11]

Cough and dyspnea can be reflexively triggered by sensory irritation mediated by nociceptive nerve fibers innervating the airway respiratory epithelium (RE).[12–14] Three cranial nerves, ie, the trigeminal, glossopharyngeal, and vagal nerves, supply nociceptive nerve fibers along the upper and lower airway[15] and play an important role in monitoring air quality and airway defense reactions.[14,16] These nerve fibers express a variety of ion channels and receptors, interacting with exogenous odor irritants and toxicants and also endogenous inflammatory mediators.[14,17] Airway trigeminal and vagal nociceptive sensory signals are sent to the caudal part of the spinal trigeminal nucleus (Sp5C), paratrigeminal nucleus (Pa5), and nucleus tractus solitaries (NTS) in the brainstem and higher-ordered brain regions for information processing.[14,18,19] The initiated sense of irritation or pain triggers protective reflexes and innate epithelial immune defenses to minimize chemical-induced damage.[20,21] Many nociceptive nerve fibers release substance P upon activation to stimulate the secretion of mucus and pro-inflammatory cytokines from mast cells, which augment inflammation, vasodilation, edema, cough, and dyspnea.[22,23] This neurogenic mechanism plays an important role in chronic chemical exposure-induced airway inflammation and hypersensitivity known to be caused by tobacco smoke.[24,25]

Little or no direct measurements of sensory activity from intact RE of different airway regions have been taken to evaluate irritation caused by e-cig constituents, especially flavorings, which are used abusively both in the dosages and varieties.[1,2,26] Previous e-cig toxicological studies were mostly done using epithelial cell cultures,[5,6,27] which does not have intact nerve innervation to assess sensory irritation. Furthermore, some e-liquid products contain a high concentration of nicotine salt instead of freebase nicotine.[28] Physiological comparisons of chemosensory responses to these products are missing.

Sensory irritation has been used as a biomarker for setting occupational exposure limits.[29,30] Chemically stimulated trigeminal activity in the nose has been monitored electrophysiologically using event-related potential (ERP) recordings.[13] Here, we assessed the airway sensory irritation caused by e-cig constituents using ERP recordings from nasal and tracheal RE. We also confirmed sensory activation using an antibody against phospho-S6 ribosomal protein (pS6) to immunolabel activated neurons in trigeminal ganglion (TG) and brainstem nuclei receiving airway nociceptive sensory input. Together, our results demonstrate airway nociceptive activation caused by e-liquid constituents, providing evidence for their adverse respiratory health effects.

Materials and Methods

Animals

Adult male and female C57BL/6 background wild-type and transgenic mice of 3–6 months were used. To facilitate the identification of brainstem nuclei, we used ChAT$^{(BAC)}$-eGFP mice,[31] which express the enhanced green fluorescent protein (eGFP) driven by endogenous choline acetyltransferase (ChAT) transcriptional regulatory elements within a bacterial artificial chromosome (BAC). ChAT$^{(BAC)}$-eGFP mice provide excellent visualization of cholinergic motor neurons and interneurons.[31,32] All animal care and procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (2011) and approved by the Animal Care and Use Committee of University of Maryland, Baltimore County (approval# WL011051821).

ERP Recordings

Stimulus Chemicals and Solutions

Chemicals including individual flavorings were purchased from Sigma-Aldrich at the highest purity available. For electrophysiological recording, the Ringer's saline contains (in mM) 145 NaCl, 5 KCl, 10 HEPES, 1 $MgCl_2$, 1 $CaCl_2$, 1 Na pyruvate, and 5 D-glucose (pH 7.2). The original stocks of individual stimuli, including capsaicin, menthol, and cinnamaldehyde and lab-made e-liquid mixtures were made by first dissolving the chemicals with small amounts of ethanol (EtOH) with concentrations based on the published flavoring concentrations in commercial e-liquids.[2,33,34] The final EtOH concentrations in working solutions were <0.5%, which did not induce responses itself. Mixtures contained a popular nicotine dose of 18 mg/mL. Stimulus solutions were freshly diluted from stocks. Citric and acetic acids were prepared with Ringer's saline. For measurement of freebase versus protonated nicotine, 5 N NaOH or HCl was used to adjust the pH values.

ERP Recordings from Nasal and Tracheal Mucosae

Mice were euthanized by CO_2 inhalation followed by cervical dislocation and exsanguination through an open heart to minimize blood in the recording tissues. The nasal ERP recording from the anterior RE and instrument setting were described and adapted from our previous publications.[35,36] For tracheal ERP recording, the trachea was exposed ventrally after euthanasia, cut open longitudinally, and the recording electrode was placed on the mucosal surface between the fifth and seventh cartilage rings. Photomicrographs of the nasal and trachea RE and schematic ERP recording locations are shown in Figure 1A and B. Each stimulant was presented three times (1 s duration, 1 min interval), and the largest response amplitude among the three was measured for data analysis.

Immunocytochemistry

E-Liquid Aerosol Inhalation Exposure

Individual ChAT$^{(BAC)}$-eGFP mice were transferred to a clean cage and housed for at least 6 h. The vanilla-flavored e-liquid mix 1 was made in PG/VG (50:50), and aerosol was generated using a refill-tank-type device, a commercial product for human users (Evod pro V2, 30 W with 0.5 Ω coil and refill tank, Kangertech Inc. Shenzhen, China, 60 mL/s flow rate). Aerosol (180 mL each) was collected using a 60-mL syringe and injected three times at 0, 8, and 21 min during a 30-min session through the water inlet into the mouse home cage (in cm: $28L \times 19W \times 18H$) covered by another cage of the same size. The aerosol was diffused through gaps between cages and the inlet and subsequently exhausted in a fume hood. Control mice were injected with clean air. We used mouse home cages and noncontinuously injected aerosol to minimize unrelated neural activity and sensory adaptation (Figure 3A and B: schematic exposure setup and timeline). The presence of e-liquid components in the aerosol was confirmed using a previously established method for analyzing e-liquid aerosol[37] and

PerkinElmer Clarus 680 Gas Chromatograph and SQ 8C Mass Spectrometer in our core facility (mcac.umbc.edu/).

Tissue Preparation

Following the e-liquid aerosol exposure, mice were perfusion-fixed transcardially, and tissues were cryoprotected using our published method.[32,38] The TG and brainstem were dissected out and embedded in optimal cutting temperature compound (Sakura finetek USA Inc., Torrance, CA). Tissue from aerosol-exposed and air-exposed mice were paired and embedded together to ensure the same processing conditions. TGs were serially cut into in 14 µm-thick sections using a cryostat (Microm HM 550), mounted on plus-charged slides (Globe Scientific Inc., Mahwah, NJ), and stored at −80 °C until use. Brainstem tissue was cut into free-floating 30 µm-thick transverse sections, sequentially placed into a six-well culture dish, and one every six sections was immunolabeled.

Immunohistochemistry

Our immunolabeling protocol was previously described.[38] The primary antibody against pS6 (1:5000; cat# 5364, Cell Signaling Technology, Danvers, MA, RRID:AB_10694233) was used to label brainstem sections, and both pS6 and substance P (1:1000; cat#ab10353, Abcam, RRID:AB_ 297089) antibodies were used to label TG sections overnight at 4 °C. The secondary antibodies conjugated with Alexa 488 (1:400; Invitrogen, Eugene, OR, RRID:AB_2535792) or DyLight 549 (1:400; Cat#706-505-148, Jackson ImmunoResearch, West Grove, PA) were used (1 h at room temperature). Sections were coverslipped with Fluoromount-G containing DAPI, which stains nuclei (SouthernBiotech, Birmingham, AL).

Image Acquisition

Fluorescence images were taken using a 4× or 10× lens of Olympus BX-41 epifluorescence microscope equipped with a Retiga 4000DC digital camera (Qimaging, British Columbia, Canada) and QCapture Pro software (Qimaging). Exposure and gain were held consistent across imaging sessions. Confocal images were also taken using an Olympus BX 61 epifluorescence microscope with a spinning disk confocal unit and Slidebook 5.0 software (3I, Denver, CO).

Quantification of Activated Neurons

Trigeminal neurons positive for substance P only or both substance P and pS6 were manually counted from two to three sections per mouse (at least 70 µm apart). For counting pS6 expressing neurons in specific brainstem nuclei, we referenced the mouse brain atlas[39] and locations of GFP (ChAT)-positive motor nuclei. Counts were performed on either the right or the left side (Sp5C and Pa5) or average of both sides of the nuclei (NTS, X, and XII nuclei) in multiple regions per animal. To facilitate cell count in Sp5C, we used the particle analysis function of ImageJ and verified the count manually.

Data Analysis

A student's *t*-test was performed to compare two experimental groups if the *F*-test was not significant and homogeneity of variance was assumed. If the criteria were not assumed, Welch's *t*-test was used instead. If normality could not be assumed, Mann–Whitney *U*-test was used. For analyzing data from three or more groups, we performed ANOVA test and post hoc Fisher's LSD multiple comparison test. If the data were collected from the same animals, we used repeated measures of ANOVA test. If normality could not be assumed, the Friedman test and *post hoc* Dunn's test were used instead. $p < .05$ was considered statistically significant.

Results

Nasal and tracheal ERP responses to individual and mixtures of e-liquid major constituents and noxious chemicals.

Sensory irritation evoked by flavorings and flavored e-liquids has not been objectively measured in native RE with intact nerve innervation. We thus recorded nasal and tracheal ERP in responses to nicotine, commonly used flavorings and lab-made e-liquid mixture (Figure 1A and B, nasal and tracheal preparations and recording positions. Figure 1C, representative ERP traces). Nicotine alone (1 mM) evoked larger responses in the anterior nose than in the trachea (Figure 1E, *t*-test $t(6.04) = 2.754$, $p = .0329$, $n = 7$ for nasal and 8 for trachea, respectively). The average nasal ERP response amplitude induced by cinnamaldehyde but not menthol (1 mM each) was also significantly larger than that of trachea (*U*-test, $U = 219$, $p = .0009$, $n = 14$ for nasal and 11 for trachea, respectively). For controls, we tested known noxious and tussigenic stimuli, capsaicin (10–100 µM), acetic acid and citric acid (pH 4.3 in Ringer's saline), and an odorant pentyl acetate (0.5 mM). These chemicals elicited ERP responses in both nasal and tracheal RE with nasal responses being significantly larger (Figure 1E, *t*- or *U*-test $p = .0027$–$.0317$, $n = 6$–22). Furthermore, the averaged nasal ERP responses to various lab-made e-liquid mixtures (1:1000 dilution; representative ERP traces in Figure 1D) were also significantly larger than tracheal responses, excepting Vanilla Mix1 (Figure 1F, *t*- or *U*-test $p = .0006$–0.0063, $n = 5$–9; Table 1 lists the mixture contents). These results indicate that the selected flavorings, nicotine, and mixtures activate sensory nerves innervating the RE and their responses were airway regional dependent.

To evaluate whether the ERP responses differed between sexes, we analyzed nasal ERP responses to 0.5 mM penthylacetate, 10 µM capsaicin, 0.5 mM cinnamaldehyde, 0.5 mM menthol, and 1:1000 menthol-flavored nicotine e-liquid mixture and tracheal ERP responses to 0.5 mM penthylacetate, and 10 µM capsaicin. We found no significant sex differences in responses to each of these stimuli ($p = .237$–$.902$ and $.175$–$.793$ for mean amplitude and mean deviation of nasal ERPs, respectively, $p = .408$–$.873$ and $.585$–$.651$ for mean amplitude and mean deviation of tracheal ERPs, respectively). Based on these analyses, we pooled the data from males and females for all our experiments.

ERP Responses to Protonated Versus Freebase Nicotine at Different pH Levels

Some commercial e-liquids contain a high concentration of protonated nicotine (nicotine salt) by adding organic acids to freebase nicotine.[28] Nicotine salts reportedly reduce respiratory harshness (irritation) from nicotine, allowing a high level of its usage. The percentage of protonated nicotine in a nicotine solution is pH-dependent. Therefore, we examined nasal ERP responses to nicotine (0.5 mM) in Ringer's solutions at 5 pH values (6.0, 6.5, 7.2, 8.0, and 8.46) from the same animals. Based on the Henderson–Hasselbalch equation,[40] the estimated percentages of protonated nicotine were 1.3, 3.9, 17, 56.3, and 60.8, respectively. We selected this pH range to avoid epithelial damage caused by low or high pH. The ERP response amplitude elicited by 500 µM nicotine increased significantly at higher pHs tested (Figure 2A and B, representative traces and plot of average response amplitude). Friedman's test, $p < .0001$, $n = 8$,

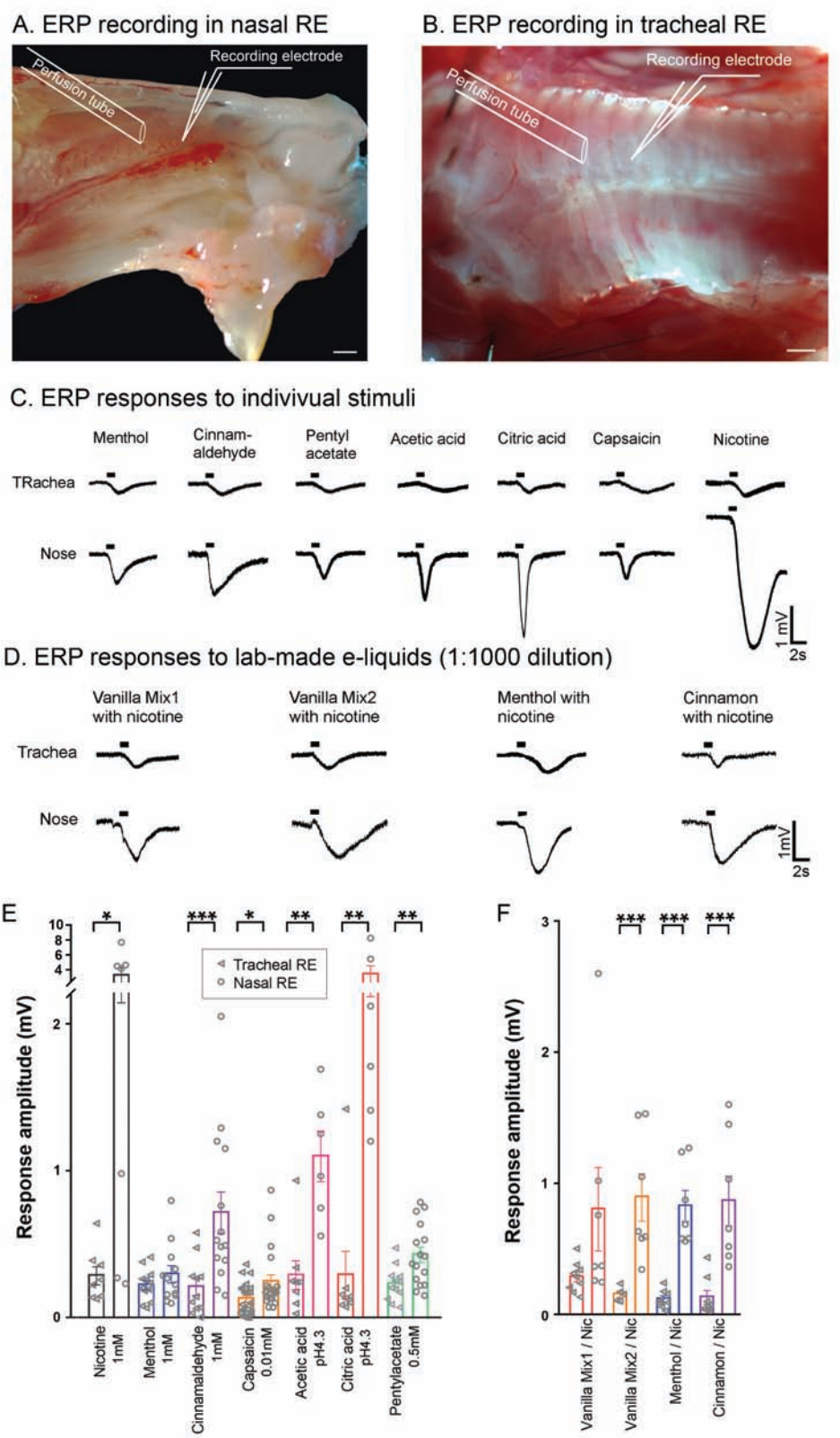

Figure. 1. Responses to nicotine, flavorings, e-liquids, and noxious stimuli in tracheal and nasal respiratory epithelia (RE). (A,B) Microphotographs of the anterior hemi-nose and trachea and schematic positions of ERP recordings. Scale: 0.5 mm. (C,D) Representative ERP records measured from tracheal and nasal RE. (E) Average peak amplitudes of ERP responses to individual stimuli. The nasal ERP responses to nicotine, cinnamaldehyde, capsaicin, acetic acid, citric acid, and pentylacetate are significantly larger than tracheal responses (*$p < .05$, **$p < .001$, ***$p < .001$, *t*- or *U*-test, $n = 6–22$ mice/data point). (F) Average peak ERP response amplitudes evoked by four lab-made e-liquid mixtures. Nasal responses to three of the four e-liquid mixtures are larger than tracheal responses (*** $p < .001$, *t*- or *U*-test, $n = 5–9$). Triangles and circles in (E,F) indicate individual mouse responses from tracheal and nasal REs, respectively. Bar graphs: mean ± SEM.

Table 1. Constituents and concentrations of the lab-made mixtures

Lab e-liquid name	Original brand/ company	Nicotine, mg/mL, mM	Cinnam aldehyde, mg/mL, mM	Menthol, mg/mL, mM	Vanillin, mg/mL, mM	Ethyl vanillin, mg/mL, mM	Ethyl maltol, mg/mL, mM
Vanilla Mix1/ nicotine	Double-dark chocolate[a] Zeus e-Juice	18, 111			33, 217	1.3, 7.8	5.6, 40
Vanilla Mix2/ nicotine	Caramel[b]Freedom Smoke USA	18, 111			9.9, 65		1.1, 7.8
Cinnamon/ nicotine	Cinnamon Ceylon[b] FlavourArt /Freedom smoke USA	18, 111	26, 197				
Menthol/ nicotine	Menthol arctic[b]Freedom Smoke USA	18, 111		84, 538			

[a]Chemical concentrations except for ethyl maltol are according to Tierney *et al.*[2]
[b]Chemical concentrations are according to Bahl *et al.*[33] and Behar *et al.*[34]

post hoc multiple comparison with Dunn's test, p = .0001, pH 6 versus 8; p < .0001, pH 6 versus 8.46; p = .0009, pH 6.5 versus 8; p = .0003, pH 6.5 versus 8.46; p = .026 pH 7.2 versus 8.46). pH 8.46 saline without nicotine yielded small ERP responses in the same animals, which on average, were about 0.74% of response amplitude to nicotine at the same pH (n = 17; Supplemental Figure 1). Therefore, the significant differences in ERP responses were due to the ratio of free-base to protonated nicotine.

Comparison Between Nicotine and Organic Nicotine Salts

Benzoic, lactic, levulinic, and salicylic acids are commonly used to make nicotine salts in e-liquids.[28] We compared nasal ERP responses to nicotine without benzoate, nicotine benzoate, Na benzoate, and benzoic acid at concentrations of 50, 100, and 500 µM, respectively. The nicotine benzoate solution was made by adding equimolar benzoic acid and nicotine to the solution (pH 7.2). Nicotine alone and nicotine benzoate elicited concentration-dependent nasal ERP responses while Na benzoate and benzoic acid at 100 to 500 µM evoked only small responses (Figure 2C, representative traces). Responses to the nicotine solution at 100 and 500 µM were significantly larger than the responses to Na benzoate or benzoic acid solutions of the same concentrations in the same animals (Figure 2D, Friedman p = .0276, Dunn's p = .0253–.0442 for 100 µM; Friedman p = .0012, Dunn's p = .0073–.0253 for 500 µM, n = 6). Because we adjusted the Ringer's solution to pH 7.2 with NaOH, the responses between sodium benzoate and benzoic acid solutions were not significantly different (Dunn's p = .655, .823, and .823 at 50, 100, and 500 µM, respectively, n = 6). Also, ERP responses to nicotine without benzoate and nicotine benzoate solutions were not significantly different at the three concentrations tested (Dunn's p = .502, .823, and .823 at 50, 100, and 500 µM, respectively, n = 6). These data suggest that organic acid used to make protonated nicotine in e-liquids contributes minimally to nasal ERP responses compared with nicotine at the same concentrations.

We further measured ERP responses to other nicotine salts and found that at pH 7.2, lactate, levulinate, and salicylate nicotine salts (500 µM each) elicited similar average ERP responses to those of nicotine benzoate (Figure 2C and E, representative traces and plot; one-way ANOVA among four nicotine salts $F(3, 29)$ = 0.6777 and p = .5728, Fisher's LSD multiple comparison p = .19–.95, n = 6–14).

Quantitative Analysis of e-Liquid Aerosol Exposure-Activated Neurons in TG and Brainstem Nuclei

Trigeminal nerve fibers detect mechanical, thermal, and noxious chemical stimuli from the head and neck regions including the nasal mucosa. A subset of nociceptive fibers that express neuropeptide substance P plays an important role in initiating sensory irritation and protective reflexes, and airway health and diseases.[14,41,42] We immunolabeled TG sections of both air control and aerosol-exposed mice (Figure 3A and B, exposure setup and timeline), using antibodies against substance P and a cell activity marker pS6. We found numerous pS6 immunolabeled (pS6+) TG neurons, including presumed mechanosensitive neurons with large-diameter cell bodies (Figure 3C and F). Substance P immunoreactivity was found in small-diameter nociceptive neurons and their nerves (Figure 3D and G). Apparently, more pS6+ neurons were found in TG sections of e-liquid aerosol-exposed mice compared with the control. We did not count the total number of pS6+ cells because most of them lacked substance P expression and their activation could be due to mechanical and thermal stimulation. The numbers of substance P-labeled TG neurons in control and aerosol-exposed mice were comparable. E-liquid aerosol exposure significantly increased the number of TG neurons expressing both substance P and pS6 (Figure 3I, t-test p = .0001, n = 4 per group), indicating that e-liquid constituents activated some trigeminal nociceptive neurons.

Nociceptive TG neurons innervating the nasal mucosa send their information to Sp5C and additionally to Pa5, which also receive sensory inputs from the glossopharyngeal and vagal nerves innervating pharynx and lower airway and regulate the respiratory reflexes.[43,44] Vagal nociceptive input from the lower airway and lungs is primarily sent to NTS.[14,22] Therefore, we immunolabeled brainstem sections from air- and aerosol-exposed mice with the anti-pS6 antibody (Figure 4A–C, brainstem images in low magnification. Figure 4 D–F, representative images of Sp5C, Pa5, and NTS, respectively). We counted pS6-expressing neurons in Sp5C (Bregma-7.92, -7.76, and -7.56 mm), Pa5 (Bregma –7.48 to –7.32, –7.20 to 7.08 mm), and NTS (Bregma –7.76, –7.56, –7.48, and –7.08 mm). These approximate regions were defined by overlaying the mouse brain atlas[39] onto the sections and by referencing ChAT (eGFP)-expressing motor nuclei (Figure 4C). Aerosol exposure significantly increased the numbers of pS6-labeled neurons in many regions of these nuclei compared with air-exposed mice (Figure 4G–I, t-test, $^{***}p$ < .001, $^{*}p$ < .05, n = 3–4 animals). These results provide evidence for the e-liquid aerosol-exposure induced sensory irritation or nociception in both the upper and the lower airways.

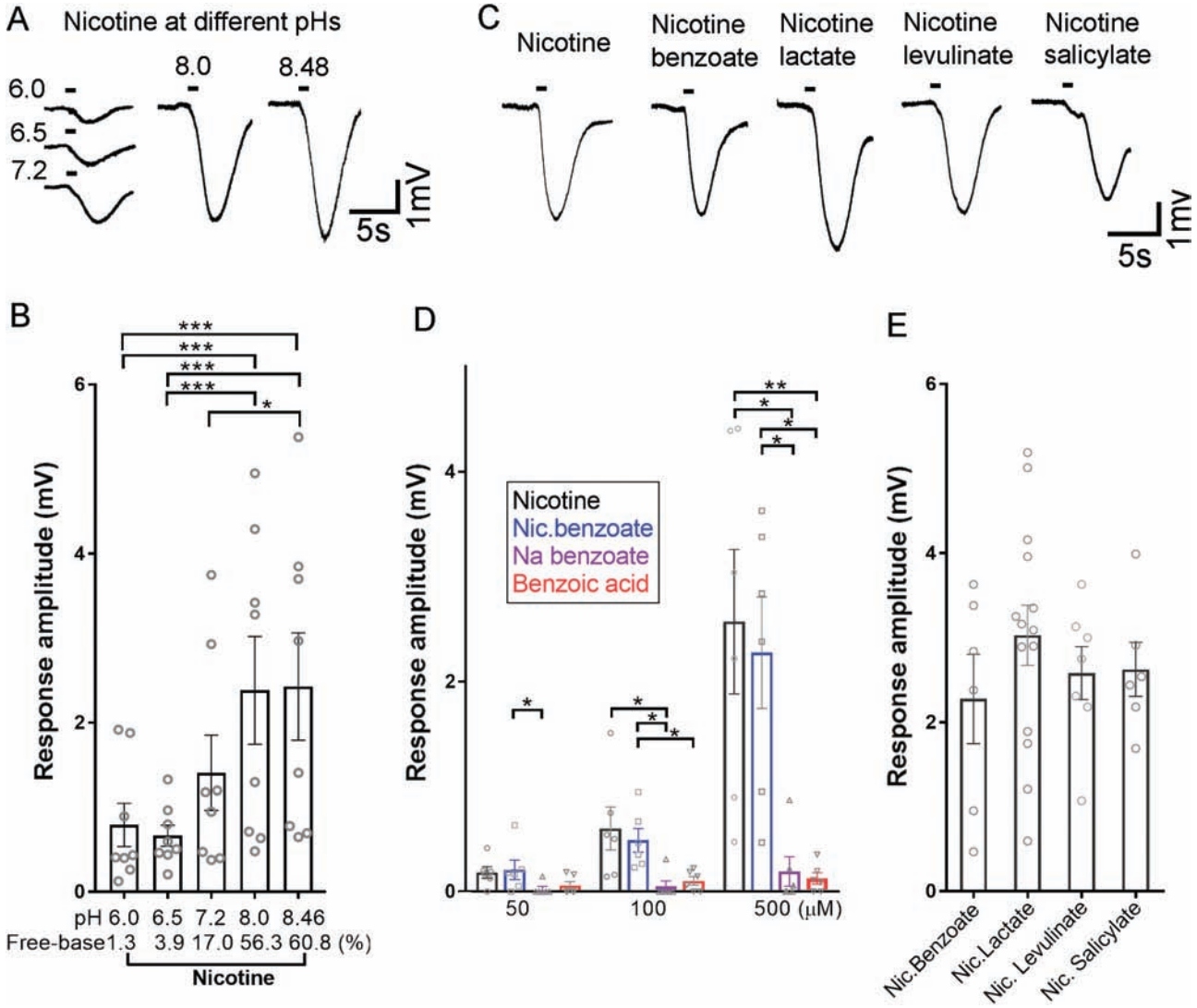

Figure 2. Nasal ERP responses to protonated versus freebase nicotine at different pH and to nicotine salts. (A,B) Representative response traces and average response amplitudes to nicotine (0.5 mM) at different pH values from the same animals. Estimated percentages of freebase nicotine at the tested pH were indicated at *x*-axis. ERP response amplitude increases at higher pH values due to the increased freebase form of nicotine (*$p < .05$, *** $p < .001$, Friedman's test for repeat measurement followed by Dunn's multiple comparison tests. $n = 8$ animals). (C) Representative ERP traces to nicotine and nicotine salts. (D) Responses to different concentrations (50, 100, and 500 μM) of nicotine, nicotine benzoate, Na benzoate, and benzoic acid at pH 7.2 from the same animals. Note the strong concentration-dependent responses to nicotine alone and nicotine benzoate, which are significantly larger than those to Na benzoate and benzoic acid at 100 and 500 μM (*$p < .05$, **$p < .01$, Friedman's test followed by Dunn's multiple comparison, n=6 animals). Responses to nicotine alone and nicotine benzoate were not significantly different at the three concentrations tested ($p = .502 – 0.823$, Friedman's test followed by Dunn's multiple comparison, $n = 6$ animals). (E) Responses to four common nicotine salts (500 μM each, pH 7.2) made of benzoic, lactic, levulinic, and salicylic acids, respectively. Their response amplitudes are not significantly different ($p = .528$, one-way ANOVA, $n = 6–14$). Bar graphs: mean ± SEM.

Discussion

We have provided evidence for the stimulatory effects of various e-liquid constituents on airway chemosensory systems using electrophysiological recordings and immunohistochemistry. Our comparative analysis demonstrated significant regional differences with nasal ERP responses being larger than those of trachea across almost all examined stimuli. We also demonstrated that freebase nicotine is a more potent trigeminal stimulus than protonated nicotine and there were no significant differences in ERP responses to the four organic nicotine salts at the same concentration. Furthermore, we showed e-liquid aerosol exposure significantly increased numbers of activated neurons in TG and brainstem nuclei receiving nociceptive input from the airway. To the best of our knowledge, these data represent the first comparative assessment of the sensory irritation caused by e-cig constituents from intact nasal and tracheal RE with nerve innervation.

The significantly larger ERP responses in the nose than the trachea may reflect differences in nociceptive nerve density in these two regions and/or expression of ion channels and receptors that are sensitive to irritants and toxicants.[13,14,41,45,46] The anterior nasal RE are densely innervated by trigeminal nociceptive nerve fibers,[13,46] many of which also receive sensory signals from

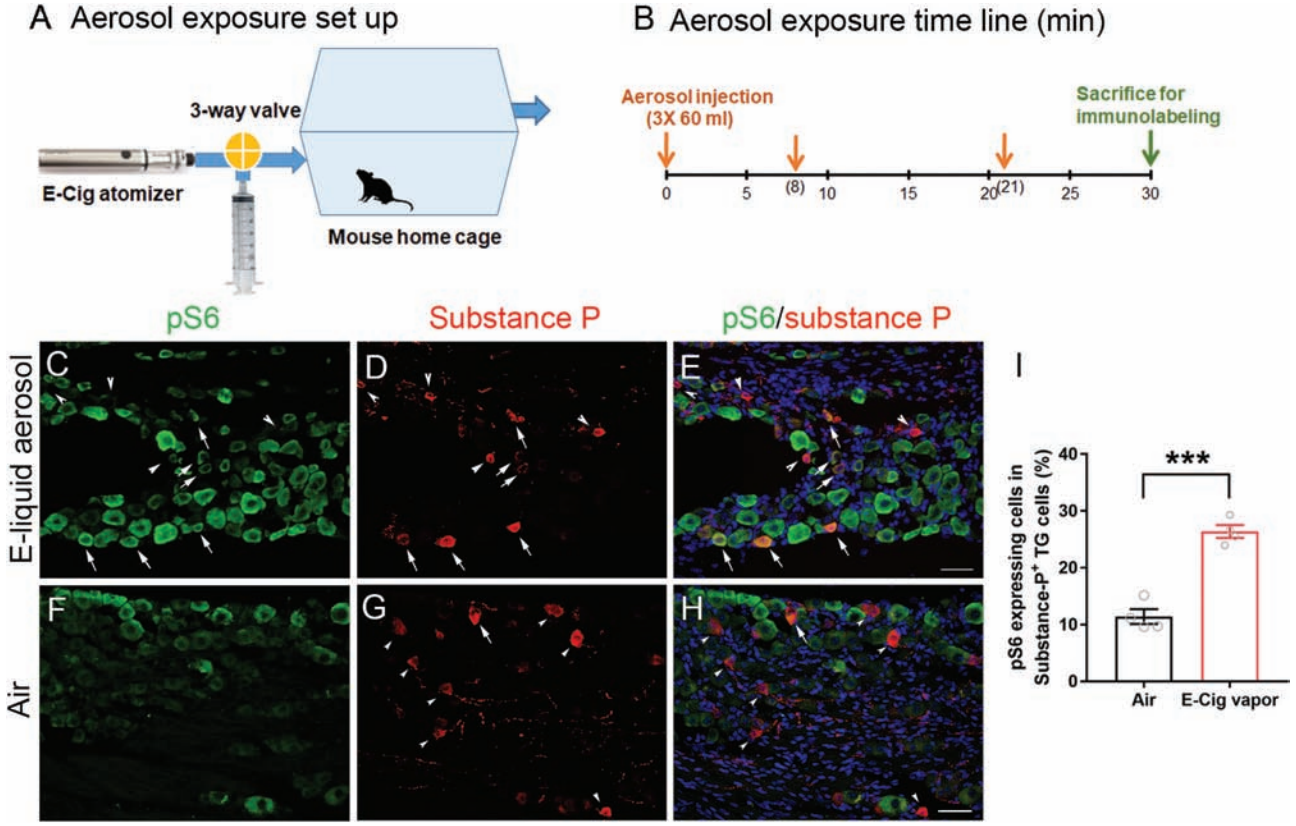

Figure 3. Nociceptive trigeminal neuron activation induced by e-liquid aerosol exposure. (A,B) Schematic aerosol exposure setup and timeline. (C–E) and (F–H) Immunolabeling of pS6 (C,F, green), substance P (D,G, red), and overlay (E,H) from e-liquid aerosol-exposed and control mice, respectively. DAPI stained nuclei (blue). Arrows point to activated cells. Arrowheads point to nonactivated cells. Scale: 50 µm. (I) Percentage plot. Substance P-positive only and both pS6- and substance P-positive neurons were counted from 2 to 3TG sections and summed per mouse. E-liquid aerosol exposure significantly increases the percentage of substance P-positive cells that express the activation marker pS6 compared with that of air-exposed group (***$p = .0001$, *t*-test, $n = 4$).

solitary chemosensory cells (SCCs) that are preferentially located at the region,[35] although they are found throughout the airway.[47] SCCs express bitter receptors and respond to various odor irritants[35] and bitter-taste substances and modulate respiratory rate and patterns.[47,48] The higher density of both nerve fibers and SCCs in the anterior nose (the entrance of the airway) likely allows humans and animals to better detect inhaled harmful chemicals, including those in e-liquid aerosol. The uneven distribution of nociceptive nerves and SCCs may also account for the large individual variations in ERP responses.

We found that nicotine dominantly contributed to the evoked ERP amplitude in our lab-made flavored e-liquid mixtures. This is expected because nociceptive neurons express various nicotinic receptors.[49] The nicotine concentration in the tested lab-made e-liquid mixture was 111 µM at 1:1000 dilution and we stimulated for 1 s duration. JUUL cartridges contain 5% or 308 mM nicotine.[40] The company claims that each cartridge of 0.7 mL generates about 200 puffs and 50 mL puff volume × 200 puffs = 10,000 mL aerosol volume. Based on this, we estimate nicotine concentration of the JUUL aerosol is about 21.6 µM/puff. As chemosensory responses depend on both stimulus concentration and stimulating period, repetitive puffs of around 5 and more likely would accumulate nicotine to an irritating level in the RE. In our ERP recordings, nicotine responses are strongly dose dependent although the average responses to 50 µM nicotine are relatively small (Figure 2D). We further provide physiological evidence that freebase nicotine is more potent in evoking ERP responses than protonated nicotine. One possible explanation is that RE is more permeable to lipophilic freebase nicotine, making it easier to access trigeminal free nerve endings that are close but do not reach the mucosal surface.[46]

ERP recordings from the nasal mucosa of humans and animals have been previously reported to assess sensory irritation by odor irritants.[13] Although we cannot rule out a potential contribution from nonsensory epithelial cells, the same regions also responded to noxious control stimuli. The significant increase in numbers of activated substance P-positive neurons in TG and also brainstem neurons in Sp5C, Pa5, and NTS of e-liquid aerosol-exposed mice compared with controls provides strong supporting evidence for the sensory irritation evoked by e-liquid aerosol constituent nicotine and flavorings and potential other byproducts generated during heating.[4]

In summary, our electrophysiological and immunohistochemical data demonstrate the stimulatory effects of e-liquid major constituents on the airway nociceptive sensory systems. The higher nasal sensitivity to nicotine and flavorings calls for future investigation of neurogenic involvement in upper airway illnesses induced by e-cig exposure.

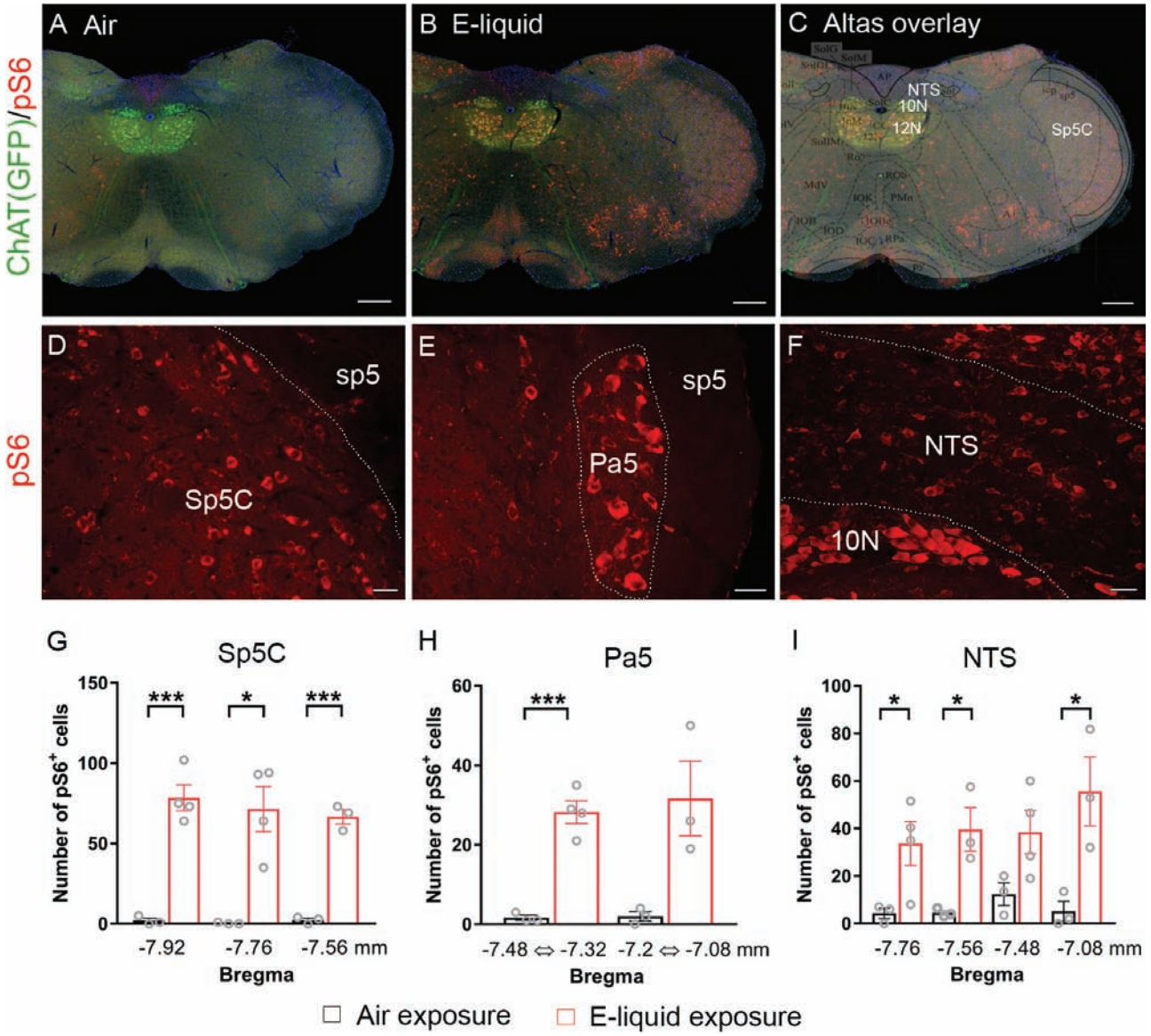

Figure 4. E-liquid aerosol activates brainstem nuclei receiving afferent input from the airway. (A,B) Images of pS6 immunolabeling (red) and ChAT (GFP; green) in brainstem sections from air- and aerosol-exposed mice, respectively. (C) Overlay of the image (B) and a schematic draw of brainstem from the mouse brain atlas (Bregma-7.56 mm).[39] Note the expression of ChAT (eGFP) in motor neurons of vagal (10 N) and hypoglossal nuclei (12 N). (D–F) Higher magnification representative images showing pS6-immunoreactive neurons in Sp5C, Pa5 and NTS, respectively. Scale: A–C, 200 µm; D–F, 30 µm. (G–I) pS6-immunopositive cell counts in Sp5C, Pa5, and NTS at different brainstem regions from caudal to rostral (Bregma –7.92 mm to –7.08 mm). Aerosol exposed group showed a significantly higher numbers of pS6-immunolabeled neurons than the control group (***$p < .001$, *$p < .05$, *t*-test, $n = 3$–4 animals), indicating that these nuclei received nociceptive afferent inputs from the airway during e-liquid aerosol exposure.

Supplementary Material

A Contributorship Form detailing each author's specific involvement with this content, as well as any supplementary data, are available online at https://academic.oup.com/ntr.

Funding

This study was supported by the National Institute of Drug Abuse at the National Institute of Health (NIDA) / Tobacco Regulatory Science Program (TRSP) grant DA046349 to WL.

Declaration of Interest

The authors have no conflicts of interest to declare. The findings and conclusions are those of the authors and do not necessarily represent the official views of the funding agency and the funding program.

Acknowledgment

We thank Ms. Jessica M. Mei and Rakaia Kenney for their critical reading and comments and Mr. Joshua Wilhide and Ms. Maggie LaCourse in the Molecular Characterization and Analysis Complex at UMBC (https://mcac.umbc.edu/) for the gas chromatography and mass spectrometry analysis.

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