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In-Depth Review

Chemosensory tobacco product toxicology part 2: toxicological testing, assays, and state of the science

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Abstract

The toxicologic impacts on the normative function of the chemosensory system and the loss of its contribution to organism protection and homeostasis remain an underrepresented area of interest in the published literature. The impact of chemical constituents in electronic nicotine delivery system e-liquids or aerosols on the chemosensory system is even less known, as are the effects on product selection and use behavior—and this may be an overlooked impact on the public health. This review is a snapshot of the current state of the science and opportunities for improving and increasing the volume of publications in chemosensory toxicology on the potential impacts of tobacco products. The proposed solutions rely on the determination of the scientific community to take advantage of an unexplored field of opportunity. Active research engagement and use of an integrative, risk-driven planning framework to address harmonization and data gaps in neurosensory research programs would support harmonization, improve scientific visibility in the published literature, and recruit additional investigators to this research community.

Keywords: chemosensory; toxicology; ENDS aerosol constituents; taste; smell; chemesthesis

This two-part series is intended to provide an overview of the sensory anatomy, mechanistic physiology of the chemosensory systems, and sensory perceptions, as well as the state of empirical investigations relative to the toxicology of tobacco products. As discussed in part I of this series, the interconnected olfactory, gustatory, and chemesthetic sensory systems inform the body about changes in internal and external environments, alert the person to potential threats, and produce any aversive, protective, or corrective responses. The processing of chemosensory afferent stimuli in the central cortex also creates the interpretive perception of the exposure and archives memories to guide future responses (Caceres et al. 2009; Tränkner et al. 2014; Avery 2021; Boughter and Fletcher 2021; Di Lorenzo 2021; Green 2021; Harmon et al. 2021; Liman and Kinnamon 2021; Mattes 2021; Mori and Sakano 2021). Given the interaction of these systems and their essential role in the protection of the organism, the potential toxicologic effects of chemicals on the chemosensory system, via environmental, occupational, or intentional routes of exposure, cannot be overlooked (see Fig. 1). Electronic nicotine delivery systems (ENDS) aerosolize e-liquids for inhalation using heat. However, the chemical substances in ENDS aerosol are not limited to intentional e-liquid ingredients but also include thermal degradation products and products formed by chemical reactions in stored e-liquids, such as carbonyls or volatile organic compounds. The amount of carbonyls and other toxicants in ENDS aerosol generated from thermal degradation of propylene glycol (PG) and vegetable glycerin (VG), as well as flavorants, such as cinnamaldehyde, eugenol, menthol, and vanillin, are known to increase with higher device heating temperatures (Beauval et al. 2017; Bitzer et al. 2018; Beauval et al. 2019; Kuehl et al. 2022). Despite this, toxicological testing of chemicals used in e-liquids and aerosols from ENDS products remains incomplete.

This article, the second in the series, reviews the in vitro, in vivo, and in silico methodologies currently available for

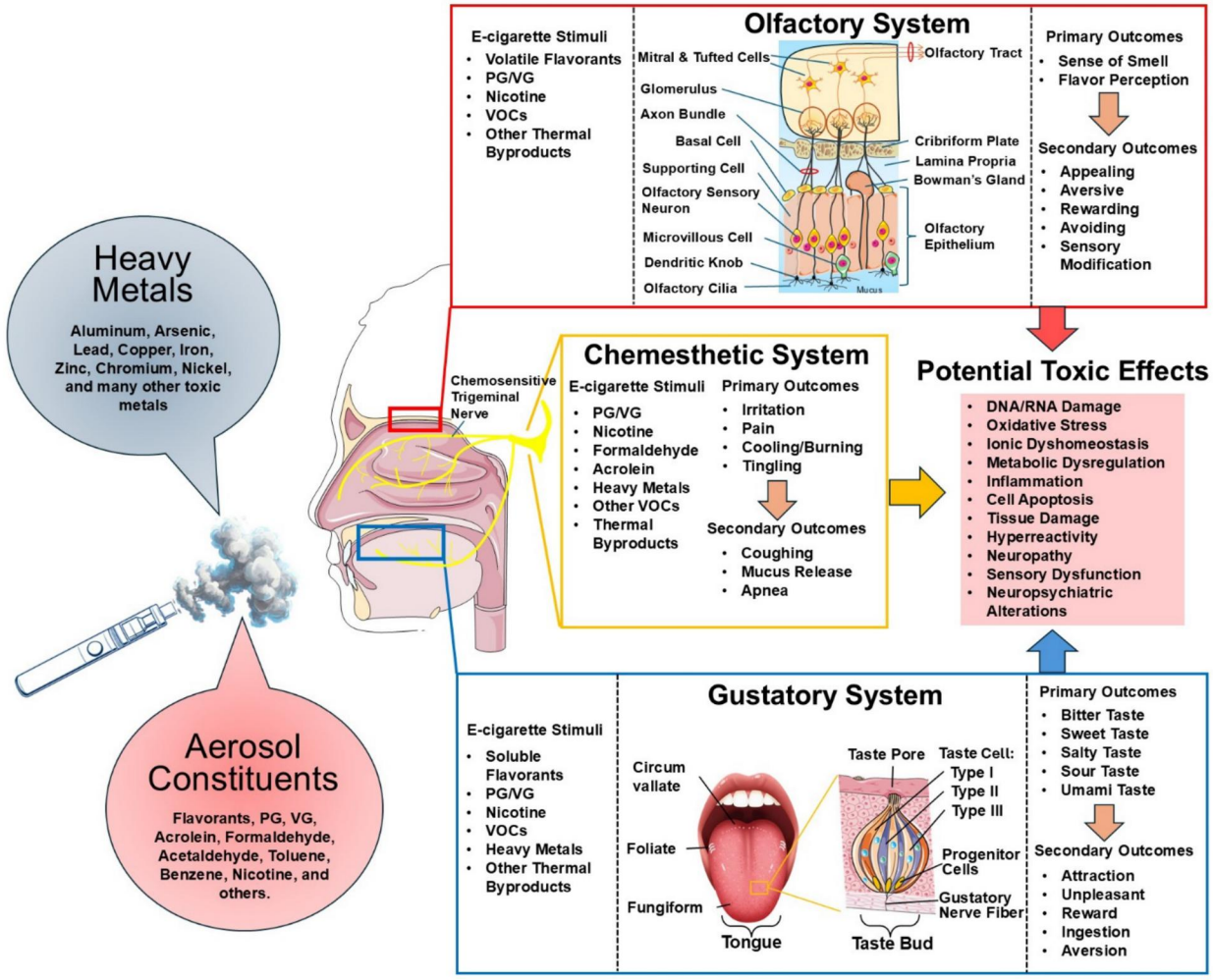

Fig. 1. Chemosensory interaction of ENDS aerosol constituents, physiological and potential toxicological outcomes (adapted drawings from Servier Medical Art [https://smart.servier.com/], licensed under CC BY 4.0 [https://creativecommons.org/licenses/by/4.0/]). The schematic drawing illustrates the olfactory, gustatory, and chemesthetic systems in the oral and nasal cavities and their peripheral sensory structures. Chemesthetic sensation in these regions is mediated by the trigeminal nerve fibers, which connect to the millions of sensory neurons in the oral and nasal mucosa. The chemesthetic sensations of irritation, tingling, pain, burning, and cooling are mediated primarily by free trigeminal nerve endings. The peripheral sensory structure of the gustatory system (i.e. taste buds) can be found in fungiform, foliate, and circumvallate papillae on the dorsal surface of the tongue. Note that chemicals may interact with multiple systems and may create an adverse effect directly on the receptor or indirectly by affecting the connecting neural networks. This figure highlights the potential for acute and long-term health consequences associated with exposure to known constituents of ENDS e-liquids and aerosols.

assessment of toxicant impact on the chemosensory pathways, the methodological challenges that remain, and potential innovations being explored to resolve them. As part of the review, existing toxicity data for biological and chemical mediators will be presented, along with known impacts on chemosensory biology and categories of human perception.

Chemical constituents in ENDS aerosol can stimulate 1 or multiple chemosensory systems, initiating distinctive chemical sensations. Exemplars will be presented for both odorant and irritant effects, which can be dependent on concentration as well as chemical properties. Examples will also be presented regarding flavorants with appealing sensations that mask their inherent irritant effect and toxicity, in addition to those of other constituents in the ENDS aerosol. While there is the potential for adverse effects of toxicants on other sensory systems, such as the auditory or vestibular system, those are considered outside the scope of this article and will not be discussed.

Review of the literature

Chemesthetic system—in vitro toxicology assessment

Current state

As reviewed in part 1 (Lin et al. 2024) of this series, various ion channels and receptors in nociceptive nerve fibers are responsible for chemical detection and initiating chemesthesis. In particular, the TRP channels, such as TRPA1, TRPM8, and TRPV1, are well-characterized for their chemosensitivity. For example, TRPA1 is broadly sensitive to many exogenous irritants, toxicants, endogenous inflammatory agents, and oxidative compounds (Jordt and Ehrlich 2007; Bessac and Jordt 2008, 2010). Among them are acrolein, acetaldehyde, and formaldehyde, which are generated through thermal degradation of PG/VG by the ENDS heat coil and volatilized into ENDS aerosol (Beauval et al. 2017, 2019; Li et al. 2021; Lorkiewicz et al. 2022). Nicotine and various e-liquid flavorants, including cinnamaldehyde

(cinnamon), vanillin (vanilla), ethyl vanillin (vanilla), benzaldehyde (berry), and their flavorant-solvent adducts (VG and PG acetals), as well as eugenol and toxicant formaldehyde, acrolein, and acetaldehyde, activate TRPA1 (Bandell et al. 2004; Bautista et al. 2006; Bang et al. 2007; McNamara et al. 2007; Talavera et al. 2009; Chung et al. 2014; Wu et al. 2017; Erythropel et al. 2019). Furthermore, a high dose of menthol evokes a sense of irritation via activating TRPA1 (Macpherson et al. 2006; Karashima et al. 2007), although a low dose of menthol may inhibit TRPA1 and activate TRPV3 (Macpherson et al. 2006). The primary chemesthetic sensation of cooling/soothing elicited by menthol, methyl lactate (peppermint oil), and l-carvone (spearmint oil) is through the activation of the cold temperature-sensitive TRPM8 and is thought to be the molecular basis for this masking effect for sensory irritation (McKemy et al. 2002; Peier et al. 2002; Bautista et al. 2007). Another popular channel sensitive to flavorants is TRPV1, which can be activated by eugenol (clove flavor) (Saunders et al. 2013; Chung et al. 2014; Takahashi et al. 2021). TRPV1 is well known for its sensitivity to capsaicin, noxious heat, low pH, and its role in local inflammation and allodynia after physical injury (Tominaga et al. 1998). Although some of the referenced in vitro studies were not performed in the context of ENDS aerosol exposure per se, they provide molecular insight into the probable chemesthetic activation by ENDS aerosolized nicotine, organic volatiles, and irritants, including irritation due to high flavorant concentrations in e-liquids.

In addition to activating TRPA1, nicotine is known to stimulate nicotinic acetylcholine receptors expressed in the nociceptive nerve fibers (Alimohammadi and Silver 2000). Nicotine-elicited chemesthetic sensation is described as an irritation in the back of the throat, a so-called "throat hit" described by people who smoke and use ENDS. A stronger throat hit is associated with increasing levels of nicotine (Ni et al. 2020). Complicatedly, nicotine also evokes an unfavorable bitter taste in a dose-dependent manner, which has influenced the industry to include combinatorial chemical additives, such as artificial sweeteners, in their tobacco and e-liquid formulations to mask the bitterness of the nicotine without impairing the "desirable" chemesthetic throat hit. Interestingly, freebased or protonated nicotine shows different potency levels in airway chemesthetic activation (Ni et al. 2020). Some newer ENDS products on the market, such as "Spree Bar" have been switching from nicotine to synthetic nicotine analogs, such as 6-methyl nicotine (Erythropel et al. 2024), possibly to push back against regulatory oversight (Jordt et al. 2025). However, little is known about the pharmacological and toxicological effects of these nicotine analogues, and neither the pharmacology nor the chemosensory effects of 6-methyl nicotine in humans have been reported or characterized.

In addition to the chemicals mentioned above, 2 additional groups of ENDS aerosol constituents are important to discuss. One is synthetic cooling agents, or ice flavors, denoted as "koolada," "kool/cool," "ice," or WS-3/WS-23 in e-liquids or cigarette products, which are used to produce the same soothing effect found in withdrawn tobacco products with a menthol characterizing flavor designation (Brown et al. 2022; Jabba et al. 2022; Leventhal et al. 2023). These synthetic cooling agents, when included as tobacco product ingredients, robustly stimulate the heterologously expressed cold/menthol receptor TRPM8 (Talavera et al. 2009; Jabba et al. 2023). The other chemical group of concern is the flavor aldehyde PG/VG acetals. These chemicals are not added to the e-liquids but are adducts formed when flavor aldehydes, such as vanillin and benzaldehyde, react with PG/VG in pods or refill bottles and are readily transferred into the aerosol during use of the ENDS device (Erythropel et al. 2019; Jabba et al. 2020). In vitro calcium imaging studies have demonstrated that such acetals robustly activate sensory irritant receptors TRPA1 and TRPV1, some with unexpectedly high receptor activation efficacy (Erythropel et al. 2019). An in vitro study using cultured respiratory cells found that, as a group, flavor aldehyde PG/VG acetals potently suppress cell proliferation, and many are more cytotoxic than their parent flavor aldehydes (Jabba et al. 2020).

Knowledge and technological gaps

In the context of in vitro assays conducted in respiratory cell lines, ENDS aerosol exposure is reported to cause oxidative stress, apoptosis, and necrosis (Rebuli et al. 2023). The released ATP, oxidative substances, and inflammatory mediators are known to act in vivo as endogenous stimuli for the airway nociceptive nerve fibers, inducing those for irritation and pain (Rang et al. 1991). However, the degree to which the symptoms of these effects are noted during the development and clinical presentation of lung injury, such as EVALI, has not been well documented. Popular 2D- and 3D-platforms for the culture of respiratory cells do not yet incorporate the neural cell types or sensory microstructures necessary to investigate or quantify chemesthetic receptor activation due to individual chemicals or the mixture effects of chemicals in ENDS aerosols. The in vitro approach using artificial expression of heterologous sensors in single cell lines (i.e. TRPM8, TRPA1, and TRPV1 ion channels) provides a basic molecular understanding of irritant and toxicant stimulation. However, this system has limitations when applied to the study of the mixture effects associated with ENDS aerosol that have been proposed to create the peripheral masking effect in vivo. In addition, the heterologous expression of sensors in cell culture as an assay platform does not integrate well into studies intended to characterize tissue-level or higher biological impact of chemical substances (i.e. nicotine and menthol) that simultaneously interact with multiple cell types, ion channels, and receptors in the whole organism.

Emerging approaches, platforms, and technology

These limitations may be partially addressed by a new culture system approach with sensory ganglion organoids. A recent study has reported successful generation of dorsal root ganglia (DRG) organoids using human-induced pluripotent stem cells (Mazzara et al. 2020). In situ, the DRG lies in the intervertebral foramen alongside the spinal column and has a molecular signature and physiological functions like those in the trigeminal and vagal ganglia. The authors were able to co-culture DRG organoids with human intrafusal muscle fibers to reconstitute the muscle spindle proprioceptive receptors, which is a multiple cell-type construct with specific 3D-anatomy (Mazzara et al. 2020). Due to the similarity of the tissues in situ, the same technique could be used to develop organoids of the trigeminal and vagal ganglia and co-culture them with respiratory epithelium in a 3D structure to investigate specific, tissue-level effects of ENDS aerosol-induced chemesthesis and chemical masking in airway tissues.

Chemesthetic system—in vivo/ex-vivo toxicology assessment

Current state

Numerous survey data and online forums reported chemesthetic experiences of people who use ENDS (Sapru et al. 2020; Rest et al. 2022). For example, 63.5% of 438 participants reported that ENDS aerosol caused mouth and throat irritation (Hajek et al. 2019).

It is worth noting that flavorants can also behave as irritants at higher concentrations (Doty et al. 1978; Silver et al. 1986) through activation of their molecular targets, such TRP ion channels expressed in the vagal and trigeminal sensory nerve endings distributed throughout the surface lining of the oral cavity and upper airways. However, such effects are often either underestimated or neglected by subjects in self-report surveys, and such surveys often utilize a "yes or no" dichotomous score or a quantitative Likert scale of a specific effect for participants to describe their feelings or sensations (Arts et al. 2006), which does not capture all potential descriptors that may be of interest to others using the data. Furthermore, cognitive biases can greatly influence the perceived volatile levels and individual responses to them (Dalton et al. 1997). Thus, it is necessary to evaluate ENDS ingredients' chemesthetic effects independently from olfaction and gustation using a more controlled subject setting where sensory activity can be assessed in real time.

Nasal chemesthetic activity can be monitored electrically using event-related potential (ERP) or negative mucosal potential (NMP) recording techniques (Hummel et al. 1996; Rombaux et al. 2006). In this approach, single chemical substances or mixtures are applied at various concentrations to the nasal mucosa, and the afferent response signals from nociceptive nerve fibers or specialized sensory cells are recorded using an electrophysiologic sensor probe (or electrode) placed on the mucosal surface (Hummel et al. 1996). This non-invasive ERP/NMP recording method has been used extensively in human subjects to investigate human chemical sensitivity and its alteration in the nose (Hummel et al. 1996; Dalton et al. 2006; Doerfler et al. 2006; Burghardt et al. 2023). In 1 study, NMP from human subjects was monitored to document the effects of long-term exposure to acetic acid aerosol (a known irritant) and found chemesthetic responses to the irritant developed after a series of repetitive exposures (Dalton et al. 2006). This type of human intranasal recording is often combined with psychophysical measurements, such as asking the subject to scale their perception of increasing dose exposures to determine chemical sensitivity thresholds (Doty et al. 1978; Silver et al. 1986; Dalton et al. 2006). In animal studies, assessing nasal chemesthetic trigeminal responses, ERP is recorded from the respiratory mucosa in ex vivo with a semi-nose preparation (ERP) (Ni et al. 2020) or in vivo directly from a branch of the ethmoid trigeminal nerve (Silver and Moulton 1982). These early studies demonstrated that almost all odorants evoke chemesthetic activity at specific dose thresholds that are independent of or above the level eliciting stimulation of the olfactory afferents (Hummel 2000).

Chemesthetic responses from mouse nasal and tracheal respiratory epithelia in ex vivo models have been recorded for select e-liquid constituents using the ERP technique (Ni et al. 2020). The findings suggested that popular flavorants, freebase nicotine, and nicotine salts induce ERP responses in a dose-dependent manner. Interestingly, when stimulated with the same substance and concentrations, the anterior nasal epithelium produced significantly larger ERPs than the tracheal epithelium. The authors also noted that at the same nicotine concentrations, the freebase form of nicotine is more potent than the salt form in inducing ERP responses (Ni et al. 2020). This finding is consistent with data from self-report surveys that e-liquid formulations containing freebase nicotine are considered more irritating or harsh (Kechter et al. 2021).

Monitoring activation of nociceptor neurons in airway afferent pathways is currently being used to assess sensory airway irritation and chemesthesis. One such approach utilizes immunolabeled cellular activation markers c-Fos and phospho S6 to identify and quantify activated neurons. In a recent report, mice exposed to aerosol from a vanilla-flavored e-liquid containing 18 mg/ml nicotine for 30 min showed a significant increase in the number of activated substance P-expressing trigeminal chemosensory neurons compared to the air-exposed controls (Ni et al. 2020). The authors also reported significant activation in 3 brainstem locations (nuclei Sp5c, Pa5, and rNST) that received trigeminal, glossopharyngeal, and vagal nociceptive inputs.

Knowledge and technological gaps

No known in vivo animal studies quantitatively assess oral trigeminal activation and modulation following long-term ENDS usage. Therefore, the mechanistic contribution of toxicant oral trigeminal chemosensory response to the pathogenesis of respiratory illnesses remains poorly defined (Mukerjee et al. 2024). Activated chemesthetic nerve fibers release pro-inflammatory mediators, including neuropeptide substance P and calcitonin gene-related peptide (CGRP), which can lead to peripheral sensitization and airway hyperactivity (Willis et al. 2011; Ha et al. 2015; Matsuda et al. 2019). However, non-chemical factors, such as the relatively high temperatures of the inhaled aerosol generated from ENDS, can also stimulate the same thermosensitive TRP channels responsive to menthol or irritant detection, consequently altering the accuracy of the chemesthetic sensation (Leijon et al. 2019). This temperature effect, which is beyond the scope of this review, is complicated to study for ENDS, as marketed devices do not all have the same fixed or variable power settings, and the airflow is a factor of device design and individual use behavior.

Only a limited number of animal studies assessing chemesthetic effects of PG and VG, the most common carrier solvents in e-liquid formulations, have been published (Niedermirtl et al. 2018). In 2001, naïve human subjects exposed to PG mist (e.g. average 309 mg/m^3) for 1 min in an aviation crash training session developed eye and throat irritation and began coughing (Wieslander et al. 2001). Burning or stinging sensations in the nose have been linked to PG exposure during pharmaceutical studies of inhaler devices (Meltzer et al. 1990). Aerosol analysis of JUUL pods showed PG levels of 0.68 mg per 30 ml puff volume (Omaiye et al. 2019). Another ENDS, the shisha-pen, is reported to generate an aerosol containing 0.71 mg PG in a 50 to 70 ml puff volume from an e-liquid solvent containing 54% PG and 46% VG, producing an estimated maximum inhaled alveolar concentration of 430 to 603 mg PG per cubic meter (Kienhuis et al. 2015). This estimated maximum alveolar concentration of 403 to 603 mg per cubic meter is equivalent to or higher than the irritation threshold in humans (Wieslander et al. 2001). Given that some people puff ENDS >300 per day on average (Dautzenberg and Bricard 2015; Yingst et al. 2020), knowledge of sensory irritation induced by e-liquid solvents is necessary to fully understand the acute and chronic effects of ENDS exposure and to relay that information to the public.

Emerging approaches, platforms, and technology

In addition to the less invasive ERP/NMP recording methods that can be used in human subjects, the degree of airway sensory irritation resulting from tobacco product use can be indirectly analyzed by biologic monitoring of cough or shortness of breath (dyspnea), as these are known reflexive responses to sensory irritation (Fryman et al. 2020). In human subjects, this minimally invasive monitoring approach can be done by temporarily placing sensors within the nostril that record CO_2 level and

temperature changes during inhalation and exhalation. Piezoelectric plethysmograph sensor and pulse oximetry, used routinely in monitoring clinical inpatients, can provide additional information regarding breathing rates, patterns, and efficiency (Pereira et al. 2015; Scott and Kaur 2020). For animal studies of sensory toxicology, there is an array of methodologies of varying invasiveness and technological complexity to measure respiratory mechanics and function, including in vivo imaging of neuronal activity at the cellular level. Using these imaging techniques, researchers have monitored in vivo intracellular Ca^{2+} responses to chemical substance exposures within the sensory neurons of trigeminal and nodose/jugular ganglia of transgenic mice expressing a calcium indicator GCaMP (Hu 2019; Kim et al. 2020). In these invasive experiments, mice are anesthetized, the ganglions of sensory neurons are surgically exposed, and any resulting Ca^{2+} signals are recorded and digitally quantified using fluorescence microscopy. In such studies, trigeminal ganglion neurons were robustly activated by menthol, allyl isothiocyanate (responsible for mustard and wasabi spice), or capsaicin exposure to the oral mucosa (Leijon et al. 2019). This demonstrated in vivo method is adaptable for the assessment of chemical irritation, sensitization, or neurotoxicity arising from exposure to e-liquid ingredients as well as ENDS aerosol. Recently developed culture platforms of somatosensory ganglion organoids may also provide a viable non-animal alternative to assess ENDS products' sensory irritation and chemical toxicity (Mazzara et al. 2020; Xiao et al. 2020). However, due to their lack of supporting epithelial interactions that occur in the intact animal, the data generated from these newly developed alternative methods still need cross-validation with animal studies to determine the applicability of the findings.

Olfactory system—in vitro toxicology assessment

Current state

Despite the limited amount of toxicity studies, the chemicals in e-liquids and ENDS aerosols that have been identified suggest there is a pressing need to evaluate their effects on human sensory neurons. This is particularly important for ENDS aerosol constituents on the FDA's Harmful and Potentially Harmful Chemicals list, such as acrolein, toluene, acetaldehyde, benzene, formaldehyde, and the heavy metals (U.S. Food and Drug Administration 2019). Previous studies have shown that inhaled heavy metal toxicants directly access the olfactory bulb and the rest of the brain via axonal transport by olfactory afferent nerves (Moberly et al. 2012; Ibanez et al. 2014; Huynh et al. 2022). Furthermore, a previous toxicological study established that intranasal inhalation exposure of rats to acrolein, acetaldehyde, or formaldehyde causes epithelial inflammation and loss of olfactory neurons (Cassee et al. 1996). These in vivo data suggest metal and carbonyls cytotoxicity to olfactory sensory neurons (OSNs) and other cell types of the main olfactory epithelium (MOE), holding tremendous value for unraveling the impact of these chemicals on sensory function and the pathophysiology of neurologic diseases. Dosing of cells to acrolein in vitro, a simple aldehyde emitted in tobacco product smoke and aerosols, is known to deplete stores of glutathione and glyceraldehyde 3-phosphate dehydrogenase, induce protein–protein adducts, lipid peroxidation, and damage the mitochondria, ultimately leading to chronic oxidative stress and apoptosis (Achanta and Jordt 2017). However, direct in vitro studies showing metals or carbonyls from ENDS aerosol exposure entering OSNs or other MOE cell types, such as supporting cells (also called sustentacular cells), are missing. Specific to ENDS tobacco products, nicotine can act on OSNs as an odorant (Bryant et al. 2010) and is also known to induce oxidative stress and accelerate cellular senescence in bronchial epithelial cells (Bodas et al. 2016; Merecz-Sadowska et al. 2020). However, the incidence and degree of oxidative stress or cellular senescence after exposure to nicotine or its metabolites *N*-nitrosonornicotine and nitrosamine ketone have never been explored in the olfactory epithelial cells or tissues. The additional findings that neuronal stem cells exposed to e-liquid or ENDS aerosols develop mitochondrial dysmorphology and dysregulation (Zahedi et al. 2019) also strongly suggest the impact of these constituents be examined relative to their effects on neurogenesis of OSNs due to their prominent role in both olfactory function, structural integrity, and overall brain health (Bhatia-Dey and Heinbockel 2021; Dan et al. 2021).

As discussed in detail in the first manuscript of this two-part series, ENDS aerosol contains a wide range of volatile flavorants that can stimulate the OSNs. While it is anticipated that frequent or prolonged exposure to ENDS flavorants would reduce olfactory sensitivity and signaling based on the known rapid olfactory desensitization, in vitro studies of how and to what degree flavorant exposure from ENDS use may alter OSNs' normative signaling and output remain to be done, and no studies have characterized the effect(s) of ENDS use frequency, duration, flavorant types, and dosage. Bridging these knowledge gaps, however, may be critical in understanding ENDS use patterns and product choices.

Knowledge and technological gaps

Although 3D and air–liquid cultures of human nasal respiratory epithelial cells have been examined for changes in cell growth, inflammation, and cytotoxicity induced by exposure to ENDS aerosol (Wiest et al. 2021), similar research has not been done with cultured olfactory sensory cells. In addition, human and rodent olfactory epithelium (OE) are known to express various xenobiotic-metabolizing enzymes (XME) (Dhamankar et al. 2015; Heydel et al. 2019; Takaoka et al. 2022). Data derived from recombinant olfactory XME suggests that aldehyde-based flavorings and odorants alter the metabolic capacity of the supporting cells and subsequently increase oxidative stress in the peripheral olfactory system (Takaoka et al. 2022). Therefore, metabolic capacity should be considered in the hazard assessments for ENDS and tobacco product use (Heydel et al. 2019). The importance of metabolic capacity to the olfactory system is evident when considering their essential role in modulating and terminating olfactory signals by metabolizing odorants and, in some cases, enhancing the peri-receptor activities to facilitate odor-receptor binding. For example, nicotine oxidation product nicotyrine has been detected in ENDS aerosol (Son et al. 2018) and is also known to inhibit the cytochrome P450 CYP2A13, leading to a reduction in xenobiotic clearance from OE. In vitro platforms can be leveraged to demonstrate the inhibitory or synergistic effects of ENDS aerosol chemical substances and metabolites on the metabolic capacity of the OE or the potential of producing more toxic compounds (Ji et al. 2018). These data gaps can best be bridged by leveraging the newer 3D tissue platforms, engineered tissue systems, and co-culture microfluidics to replicate organ-specific functionality or approximate absorption, distribution, metabolism, and excretion of ENDS aerosol constituents.

Emerging approaches, platforms, and technology

Multiplex bioassays have been used for quickly quantifying inflammation, cell signaling, growth and apoptosis, toxicity markers, and xenobiotic transportation, but these approaches

have not been applied to evaluate the effects of ENDS aerosols on the olfactory system. Leveraging a multiplex assay platform for olfactory studies might support rapid hazard identification and help characterize adverse outcomes resulting from acute or chronic toxicant exposures to the peripheral and central olfactory systems. Use of multiplex bioassay platforms is also known to shorten time to discovery, support biologically relevant and harmonized exposure regimens between studies and collaborating laboratories, and provide considerable savings compared to traditional liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS)-based proteomics and RNA-seq analysis.

In vitro culture of OSNs, explants, and olfactory epithelial organoids can be adapted for toxicological testing. These approaches take advantage of olfactory progenitor cells that can proliferate and generate all olfactory epithelial cells during development and throughout life. For mouse primary OSN culture, embryonic olfactory tissue containing olfactory progenitor cells was harvested, dissociated, and co-cultured with a feeder layer of astrocytes to promote neuron survival (Gong 2012). Also, olfactory explants harvested embryonically or neonatally can develop and differentiate into mature OSNs and other cells in the OE (Mathé et al. 1975; Josephson et al. 2004). These in vitro cultures generally are short-term except for the OE-bulb co-cultures, which last 68 days in vitro (Josephson et al. 2004). The primary olfactory cell culture may be suited for acute toxicological testing. Still, using them to investigate the long-term effect of ENDS exposure will likely be challenging, especially trying to mimic inhalation exposure. Another avenue is to use "ODORA," a conditionally immortalized cell line derived from the OSN lineage (Murrell and Hunter 1999). The ODORA culture, however, lacks other major non-neuronal cell types in the OE. The recently published olfactory organoids or colony culture may overcome this limit. Olfactory organoid culture can be established from murine and human adult olfactory progenitor cells using a specific culture medium (Ren et al. 2021). These organoids include OSNs and other major OE cell types, such as supporting cells. Thus, they can potentially recapitulate native olfactory cell gene expression and functions. Advantageously, human olfactory organoid culture can be generated from olfactory progenitor cells obtained through biopsies from the superior nasal septum (Garcia et al. 2022), representing an emerging approach for in vitro study of human olfactory-specific toxicological testing (Murrell and Hunter 1999; Goetze et al. 2002; Josephson et al. 2004; Gong 2012; Ren et al. 2021; Garcia et al. 2022).

Olfactory system—in vivo toxicology assessment

Current state

Olfactory dysfunction is strongly associated with various brain illnesses. For example, olfactory dysfunction is relatively common in patients during early and late stages of both Alzheimer's and Parkinson's diseases (Attems et al. 2014), often decades before motor or cognitive functions visibly decline (Doty 2008). As discussed in part 1 of this series, olfaction is directly connected to brain affective functions in the limbic system that affect psychological behavior. People who use ENDS have a higher risk for developing substance use disorders, depression, anxiety, and low self-esteem (Grant et al. 2019), and the extent to which ENDS usage impacts memory formation, emotional regulation, and mood disorders is a key aspect of future research. However, in vivo study of long-term ENDS exposure leading to impairment or alteration of olfaction or its contributions to development and progression to neurodegenerative and mental diseases remains a limited topic of published research.

The effects of in vivo exposure to ENDS aerosol have been explored over the past decade in mouse and rat models for the lower airways and lung parenchyma, with a focus on cardiovascular and respiratory effects (Tsai et al. 2020). The effects of acute or chronic ENDS use on the OE of the nose, alteration of the olfactory sensitivity, and the resultant effects on ENDS use are not well understood, even though flavor preference, as previously described, drives both ENDS product selection and use behavior (Krüsemann et al. 2020). One study has reported that people who chronically use ENDS do not differ significantly from never smokers in terms of odor threshold detection or discrimination but that both of these groups performed better than the cohort who smoked cigarettes (Majchrzak et al. 2020). While human psychophysical examinations in sensory clinics can provide valuable data, olfactory disorders are generally underestimated in self-reported public surveys (Nordin et al. 1995; Hoffman et al. 2016; Nørgaard and Fjaeldstad 2021). Often, the public also misunderstands olfactory dysfunction as an oral taste disorder (Nørgaard and Fjaeldstad 2021). Self-evaluation of olfactory performance for survey studies is also quite subjective and influenced by many factors, such as prior experience and familiarity with the odor used for testing and training (Kollndorfer et al. 2015). Despite these limitations, study findings demonstrate that chronic cigarette smoking is significantly associated with olfactory impairment or alteration in self-report surveys (Glennon et al. 2019). It is worth noting that the vast majority of flavorants and nicotine used in e-liquids, as well as PG/VG thermal byproducts in ENDS aerosol such as acrolein, acetaldehyde, and formaldehyde (AlMatrouk et al. 2021), are all volatiles with perceptible smells to most humans. In addition, the smell of certain flavorants can mask the bitterness and throat hit of ENDS aerosols and is intentionally added to e-liquid formulations to encourage continued use, which also prolongs aerosol exposure (Johnson et al. 2022). How acute and chronic exposure to these compounds may adaptively modulate olfactory sensitivity and influence ENDS use, in addition to the inherent toxic effects of the aerosol, is another area of consideration that will require chronic studies in animal models as well as human subject data.

The same in vivo study designs and exposure models used for investigating the respiratory and cardiovascular impact of ENDS use can be adapted to study olfactory effects. Most animal exposure protocols investigating ENDS aerosol effects consist of housing animals in chambers (mice) or nasal exposure tubes (rats) connected to apparatus that dispense ENDS aerosols at prescribed intervals and durations into their ambient air, following the CORESTA Standardized puffing method (CORESTA and CCfSRRtT 2015). Animals either undergo exposures to air puff controls or ENDS aerosols from various e-liquid brands, which may vary in PG/VG ratios, nicotine dosages, flavor chemical composition and concentration, and exposure durations, depending on the focus of the experiment (Garcia-Arcos et al. 2016; Chen et al. 2018). In general, most e-cigarette exposures have been whole-body exposures by way of the chamber's ambient air; however, individual "nose only" exposure chambers are increasingly used (Tsai et al. 2020).

Non-chemosensory biologic and toxicologic outcomes from exposure to combustible tobacco products have been documented by examination of changes in tissue morphology or histochemical staining, inflammatory profiles, gene expression profiles, cell metabolism, and behavioral challenge responses (Merecz-Sadowska et al. 2020). In a recent report, mice exposed

to either cigarette smoke or ENDS aerosol for 14 days (150 puffs daily) showed impairment in spatial memory learning and locating of food rewards, which is suggestive of olfactory impairment as one of the toxicant target organs (Prasedya et al. 2020). Histological and molecular analysis were not done on the olfactory tissue from these exposure studies; however, there is no reason why similar study designs and models could not be used in a similar manner to evaluate the chemosensory effects of exposure to ENDS aerosols.

Gustatory system—in vitro toxicology assessment

Current state

The relevance of the gustatory system to toxicology and exposure-related adverse outcomes is grounded in 2 pragmatic functions attributed to gustation: (i) the gustatory detection of toxicants to avoid ingestion and (ii) collateral impacts on biologic integrity due to toxicant-induced loss of normative function. In the detection and avoidance of toxicants, the basic taste modality of bitterness evolved as a warning mechanism for potential toxicants, as many naturally occurring toxicants are present in bitter-tasting vegetation. Consequently, the presence of a bitter taste generally discourages animals from ingesting the substrate, thus avoiding the toxic hazard (Garcia and Hankins 1975). A group of G-protein coupled receptors (T2Rs) is responsible for bitter taste detection of naturally occurring or man-made toxicants, such as nicotine alkaloids (Chandrashekar et al. 2000). Currently, there are several techniques employed to assess and quantify a toxicant-induced bitter response. Classically, freshly isolated taste buds from various animal models or human biopsies are used in electrophysiological recordings and functional imaging to characterize responses to bitter chemical stimuli, including any dose dependence, and the involvement of T2R signaling pathways (Ogura et al. 2002). More recently, artificially created tissue- or cell-based sensors (e.g. "electronic tongues") have been bioengineered into micro-instrumentation devices, using overexpression of T2Rs in the cultured component. The biologic electronic tongues (BioETs) have rapidly become a state-of-the-art platform for in vitro gustatory research into food and beverages, pharmaceutical development and classification (Qin et al. 2019), taste masking (Wang et al. 2021), and investigation of associated signaling pathways (Xiao et al. 2021).

The second functional area of toxicologic import, collateral impacts on biologic integrity, is the result of direct local effect(s) of toxicants on the tissues of the tongue, nasopharynx, and oropharynx. In humans, taste buds are located at the dorsal surface of the tongue, the soft palate, pharynx, larynx, and upper esophagus (Roper and Chaudhari 2017; Gutierrez and Simon 2021). Vaping directly exposes taste buds to nicotine, toxicants, and irritants in the inhaled aerosol. Thus, it is likely that taste bud cells are adversely impacted by ENDS use. Although a recent publication reported no significant taste perception differences between college students who use ENDS and those who do not, there were limitations of the study (e.g. the inclusion of participants with relatively low exposure to ENDS aerosol in the ENDS user cohort) that may impair the ability to detect differences between student vape users and non-users (McCormack et al. 2024). In fact, people who frequently use ENDS report a phenomenon known as "vaper's tongue," a decreased perception of taste that affects enjoyment of food, drinks, and their preferred e-liquid flavors. The symptoms of "vaper's tongue" are self-reported to be temporary or last for a few weeks during routine use of ENDS. Reducing ENDS usage and improving oral hygiene have been reported to mitigate or reduce the severity of sensory impairment. The root cause and pathophysiology of vaper's tongue, taste receptor cell damage, or dry mouth, or a combination of these, remain poorly understood. Other than nicotine, no published studies on specific ENDS constituents or aerosols have reported in vitro findings with taste receptor cells and organoids or T2R-based cell sensors.

Emerging approaches, platforms, and technology

The BioETs use gustatory receptors coupled with bio-impedance sensing technologies to evaluate response to flavor compounds of interest (Qin et al. 2019), which has enabled quantitative real-time investigation of receptor function. Multiple techniques have been reported for constructing BioETs. Some methods report the separation of receptor-containing membranes from cells and using them to engineer synthetic in vitro systems functionalized by specialized electric sensors (Song et al. 2014; Xiao et al. 2021). In 2019, a group developed a novel BioET that measured response to bitter compounds applied to Caco-2 cells that had been treated to artificially develop and overexpress T2R38 receptors (Qin et al. 2019). Cells were seeded onto a novel biosensor platform for cellular impendance recording, and the test compounds were then applied. The presence of the receptor was confirmed with intracellular calcium signaling imagery, and the artificially created ability of this cell line to sense a bitter sensation was analyzed using computational tools. The gustatory response was quantified and reported as the normalized electrical impedance across channels. In 2020, a biosensor created in sperm cells was incorporated with a fluorescent probe for responsive signal readout of bitter substances (Tian et al. 2020), and a T2R-expressing cardiomyocyte sensor has also been used to screen for bitter-flavored cardiotoxicants in tandem with simultaneous changes in myocyte electrophysiological potentials and mechanical beat frequency (AlMatrouk et al. 2021). These sensors have been proposed for screening applications of bitter substances and response kinetics in the food and pharmaceutical industries but have not yet been applied to the study of flavorants or other ingredients in ENDS aerosols. Using heterologous expression of bitter taste receptors and the associated signaling pathway may provide insight into whether taste desensitization at the receptor and signaling pathway levels plays a role in developing vaper's tongue.

Micro-physiological systems have been proposed for gustatory research in the form of a tongue-on-a-chip (Roelse et al. 2024). A recent publication reports use of HEK293 cells engineered to express sweet and bitter taste receptor arrays and a calcium indicator to assay response to taste stimuli in a microfluidics platform—the "tongue-on-a-chip" (Roelse et al. 2024). While these in vitro systems have not been widely used to evaluate inhaled tobacco products, these technologies can be utilized to study gustatory response and toxic effects of compounds present in ENDS aerosol to enhance the science-based regulation of tobacco additives and e-liquids and understand ENDS product designs. For example, nicotine evokes an unfavorable bitter taste in a dose-dependent manner, which has influenced the industry to apply combinatorial chemical additives to their tobacco and e-liquid products to mask the bitterness of the nicotine without impairing its "desirable throat hit" (Pullicin et al. 2020). One limitation of this technique is that the microfluidics-on-a-tongue approach cannot be used to evaluate chemesthetic masking effects of cooling and soothing of bitter stimuli, as this occurs through trigeminal nerve mediation of the masking flavorant, and the microfluidic devices do not have the ability to add trigeminal neuron interaction at this time.

As previously discussed, the gustatory system influences the product preference of people who use ENDS, their perceptions of harm, and vaping behavior. In light of these findings, the lack of published studies on e-liquid ingredients and chemicals found in ENDS aerosol and their effects on gustation is a significant data gap in tobacco regulatory research. The impacts of this data gap, addressing potential gustatory toxicities, extend well beyond tobacco regulatory concerns. As pointed out by Gauvin et al. (2015), effects on the gustatory system are seldom considered during preclinical drug development; the olfactory bulb, nasal epithelium, oropharynx, and tongue are not present in any suggested list of tissues for histology or assay of potential chemosensory impact.

Future work is needed to bridge this gap by correlating receptors, such as from BioETs or cell-based signals, to the toxicological and masking effects of ENDS constituents, from sweet-taste substances on gustatory function to the self-reported changes in human taste perceptions. Continuous improvement of BioETs may help overcome the current limitations of single-receptor monitoring to better address the biological reality of multi-receptor transduction and processing within the human gustatory system. At a molecular level, assays of concern may include those for altered gene expression and epigenetic modification, as well as metabolomic profiling and their potential role(s) in altered taste discrimination and stimuli detection threshold, taste cell turnover rate, and the number and composition of taste buds. Study designs will need to consider that the magnitude of effect may vary by anatomic locations of taste buds on magnitude of effect, as well as vaping topography and variability in the flavorant combinations between marketed e-liquid products.

Gustatory system—in vivo (ex-vivo) toxicology assessment

Current state

The oral cavity is exposed to combusted tobacco smoke and ENDS aerosol immediately upon inhalation. Early psychophysical studies in human subjects indicate chronic and heavy smokers have reduced oral detection thresholds for bitter-tasting substances (Krut et al. 1961; Kaplan et al. 1964). Electrogustometry of taste bud responses in the human tongue has also documented tobacco smoke-induced changes in taste sensitivity (Chéruel et al. 2017) and confirmed morphological changes of fungiform taste buds in the tongue tip region (Pavlos et al. 2009; Khan et al. 2016; Pavlidis et al. 2017). The source of toxicants in ENDS aerosols potentially driving these effects are not limited to PG/VG thermal degradation products and heavy metals. The artificial sweetener sucralose, a common e-liquid ingredient, is known to produce toxic isomers of chloropropanols when heated and aerosolized (El-Hage et al. 2019). However, systematic clinical or epidemiologic surveys documenting the impact of ENDS aerosol on the gustatory system have yet to be published. Similarly, no publications explore the effects of ENDS aerosol on the structure, function, or collaborative physiologic roles of the gustatory system.

Interestingly, polymorphism in both sweet and bitter taste receptors is a critical genetic determinant of sensory discrimination and intensity within receptors. The bitter taste receptor (T2R38) gene variant has been used to classify an individual as a taster or a non-taster based on their intensity rating of bitter substances phenylthiocarbamide and propylthiouracil; the intensity rating for these compounds is much lower in non-tasters (Kim et al. 2003; Risso et al. 2016). Epidemiological surveys found that pregnant female Caucasian smokers and African American male smokers who carry the taster T2R38 haplotype exhibited a significant preference for mentholated cigarettes when a choice is offered (Oncken et al. 2015; Risso et al. 2017). The impact of T2R38 and genetic variants of other taste receptors on preferential selection of flavored ENDS products has yet to be determined.

Rats and mice have taste bud locations very similar to those of humans; however, studies have documented species-specific anatomic differences in regional patterns of taste response and sensitivity (Sollars and Hill 2005). For example, in rodents, the fungiform taste buds are more accessible than foliate and circumvallate taste buds, as they do not lie in deep tissue trenches of the tongue. To date, the correlation of vaping topography with global gustatory or regionally specific taste bud toxicity is an unexplored area. Furthermore, even though the bitter taste sensation evolved in animals and humans to avoid ingestion of poisonous plants or food matter, no studies have examined the impacts on the gustatory system in animal models to assess the adverse effects of ENDS aerosols.

Emerging approaches, platforms, and technology

Commonly used study designs and apparatus for gustatory research in animal models can be leveraged with minimal modifications to assess the effects of either individual chemical constituents or mixtures found in ENDS e-liquids. Because taste-based likes and dislikes drive robust feeding and avoidance behavior in rodents, a commonly used behavioral assay for gustation is the binary or "two-bottle" taste preference test (Bagdas et al. 2022). In this approach, animals are given controlled, equal access to a bottle containing water and another containing water plus the test article to drink from. The amounts consumed from each bottle are measured and compared to calculate a preference index, which is used to quantify taste acceptance or avoidance of the test article (Gaillard and Stratford 2016). A modification of this "two-bottle" assay uses a lickometer to quantify the gustatory likes and dislikes between different solutions or solutions of various concentrations (Jewkes et al. 2017; Melo et al. 2022). In this assay, rodents are trained to lick to obtain water or to be tested in a specified time interval, and the number of licks for any source solution is counted electronically to determine the taste detection threshold, dose-dependent preference, or degree of avoidance (Jewkes et al. 2017; Melo et al. 2022). This approach has been performed to assess taste detection effects attributable to genetic manipulations, environmental exposure, cancer drug side effects, and subject health comorbidities (Delay et al. 2006; Jewkes et al. 2017, 2018; Lemon et al. 2019; Yu et al. 2020).

Of particular importance to the use of the binary taste preference tests for evaluation of e-liquids and their components is the fact that this assay has already been published in peer-reviewed journals using fruit flies (*Drosophila melanogaster*) and successfully demonstrated the essential role of taste receptors in detecting toxic compounds or compounds with a flavor preference (Poudel and Lee 2016). Additionally, when using the *Drosophila* model to assess gustation, inhibition of proboscis extension—a negative feeding (avoidance) reaction—can also be leveraged to study the behavioral changes post-exposure to the gustatory detection of a known toxicant administered in a secondary exposure medium (Wang et al. 2004).

Electrophysiological recordings from whole gustatory nerves or single nerve fibers have been done to assess taste sensitivity, coding, and disorders in mice (Lyall et al. 2007) and primates (Hellekant et al. 1997). Chemical compounds are directly applied to gustatory tissues in a controlled manner, and the induced

changes in action potentials, firing rates, and patterns are recorded electronically. Using this method, recordings of the chorda tympani have documented nicotine effects on both salty and sour stimuli in a concentration-dependent manner (Lyall et al. 2007). A newer, indirect method for afferent signal quantification in neural tissue uses imaging technology and fluorescent microscopy to measure intracellular Ca^{2+} flux in taste receptor cells in surgically exposed brain regions of the gustatory circuit while receiving and processing afferent inputs from taste buds in real time. This technique relies on the use of genetically altered animal subjects expressing calcium indicators such as G-CaMP in their neurons to monitor changes in neural activity, which has been successfully bioengineered in mice and *Drosophila* (Marella et al. 2006; Wu et al. 2015). More recent neuroscience publications have employed dual electrophysiology and fiber photometry implants to monitor treatment effects in freely behaving mice (Patel et al. 2020). These advances in brain imaging techniques have made it possible to monitor neural activities in freely behaving animal subjects, rather than unconscious, anesthetized ones.

Controlled chemogustometry in human subjects is essential to detect potential toxic effects of ENDS use on the gustatory system due to flavor-masking effects in marketed ENDS pods and e-liquid mixtures that bias toward an underappreciation of any adverse taste effects during ENDS use. The standard chemogustometry technique involves direct application of chemicals in solution to the regions of oral mucosa containing taste buds (e.g. the tip of the tongue), using a piece of filter paper, and comparing responses to those for prototypical taste standards (e.g. quinine hydrochloride or table salt) (Green et al. 1996; Cavazzana et al. 2019). Subjects are asked to identify a perceived flavor and rate the intensity of the chemicals used. Determination of a gustatory threshold of detection as well as intensity of taste is often included in these study designs. Other than the direct adverse effect on gustatory detection of the exposure compounds, human taste tests can also provide information about the sequential impact of exposures, such as the masking effect of ENDS flavorants, sugars, and artificial sweeteners on gustatory perception. Rodent studies have already demonstrated that artificial sweeteners like sucralose and acesulfame-K, when added to oral nicotine pouch products, can reverse the normative aversion to, and increase the consumption of, nicotine pouch extracts (Jabba et al. 2024). Some food flavorants used in ENDS products can stimulate both the olfactory and taste systems. Qualitative evaluation of the gustatory-mediated masking effect in humans can be done by applying a small nasal clamp or inserting nasal plugs prior to the exposure to block olfactory sensing of flavorants.

Chemesthetic system—in silico toxicology assessment

Current state

Few studies have been published using computational methods or in silico tissue modeling to assess the direct effects of tobacco use on chemesthetic, olfactory, or gustatory sensory systems. Emerging technologies, such as compound structure–activity prediction platforms (Dagan-Wiener et al. 2019) and artificial intelligence-driven machine learning (Pal et al. 2014), are just being developed to attempt in silico models of the chemesthetic system. Computer-driven, quantitative structure–activity relationship (QSAR) models are actively used by the FDA and others to predict potential hazards to human health for selected compounds of interest due to intentional dosing, in the case of pharmaceuticals, or unintentional dosing, in the case of environmental exposures or contaminated consumer products (Zang et al. 2017; Myatt et al. 2018; Schultz et al. 2018; Yoo et al. 2020; Wijeyesakere et al. 2023).

Sensory-based computational predictions of ENDS chemical compound toxicity are limited; however, in silico predictive tools based on bitter-taste receptors have been developed for use in the food industry and to improve drug tolerability (Bahia et al. 2018; Dagan-Wiener et al. 2019). While it is possible to use similar machine learning algorithms to screen databases of ENDS aerosol chemicals to assess gustatory oral effects, adapting the receptor-based strategy to predict the toxicants in the olfactory system is challenging. This is because the olfactory system utilizes a combinatorial scheme for odor reorganization, in which individual receptors can bind to multiple odorants/flavorants that share a specific functional domain (Buck 2004), and toxicants are often mixed with other non-toxic volatiles, such as formaldehyde, in flavored ENDS aerosol during exposure.

Menthol, because of its common use in combustible tobacco and ENDS products, has been investigated in silico using computer-driven molecular docking simulations for TRMP8 receptor binding (Shahoei and Tajkhorshid 2020; Xu et al. 2020). Menthol reportedly enhances the desensitization of human α3β4 nicotinic acetylcholine receptors (nAChR), a subtype found in both the brain and peripheral sensory system (Ton et al. 2015; Bavo et al. 2021), and upregulates their expression in the midbrain and alters the receptor subtype stoichiometry (Ton et al. 2015; Henderson et al. 2016). Using various computational methods to analyze the human α4β2 nicotinic acetylcholine receptor, the most abundant nAChR in the brain, findings suggest menthol binds around the lipid-receptor protein border on the cell membrane and inhibits the nicotine sensitivity of the nAChR. Supporting this hypothesis, the up-regulation of nAChR expression in neural tissue has been demonstrated in people who use menthol cigarettes; the mechanistic receptor interactions by menthol and any analogue compounds have not been documented using experimental data (Brody et al. 2013).

A recent review presented a summary of in silico data streams that have been incorporated into toxicology studies assessing the heart, kidneys, and lungs (Bassan et al. 2021). The limitations of the testing approaches and additional database needs are necessary to improve the predicted accuracy for the dose response of organs to chemical toxicants in specific organ systems. Based on existing limitations and needs to improve predictive accuracy, the authors proposed a framework for data streams needed to support a computational approach to organ toxicity in the heart, kidneys, and lungs. This proposed framework also provides a starting point for developing predictive toxicology models of the sensory system amenable to tobacco smoke and ENDS aerosol exposures. This working framework, adapted from Bassan et al. (2021), is outlined in Fig. 2. As proposed, the risk assessment construct begins with proposed mechanisms of effect or an adverse outcome pathway (AOP). Assessment of toxicant effects may proceed in parallel for chemesthetic, olfactory, and gustatory receptors, using animal or non-animal assays. The key to this chemosensory adaptation is a mechanistic approach to the action of suspected toxicants on the direct or collaborative function of these chemosensory divisions. To do this in a meaningful way, it is essential to consider all anatomic routes of toxicant contact with chemosensory receptors and that effects at different anatomic locations correlate with product use as well as effects on human behavior and adverse outcomes. For example, effective analysis of gustatory (oral) and olfactory (nasal) toxicity requires understanding of how the local anatomic dose of a

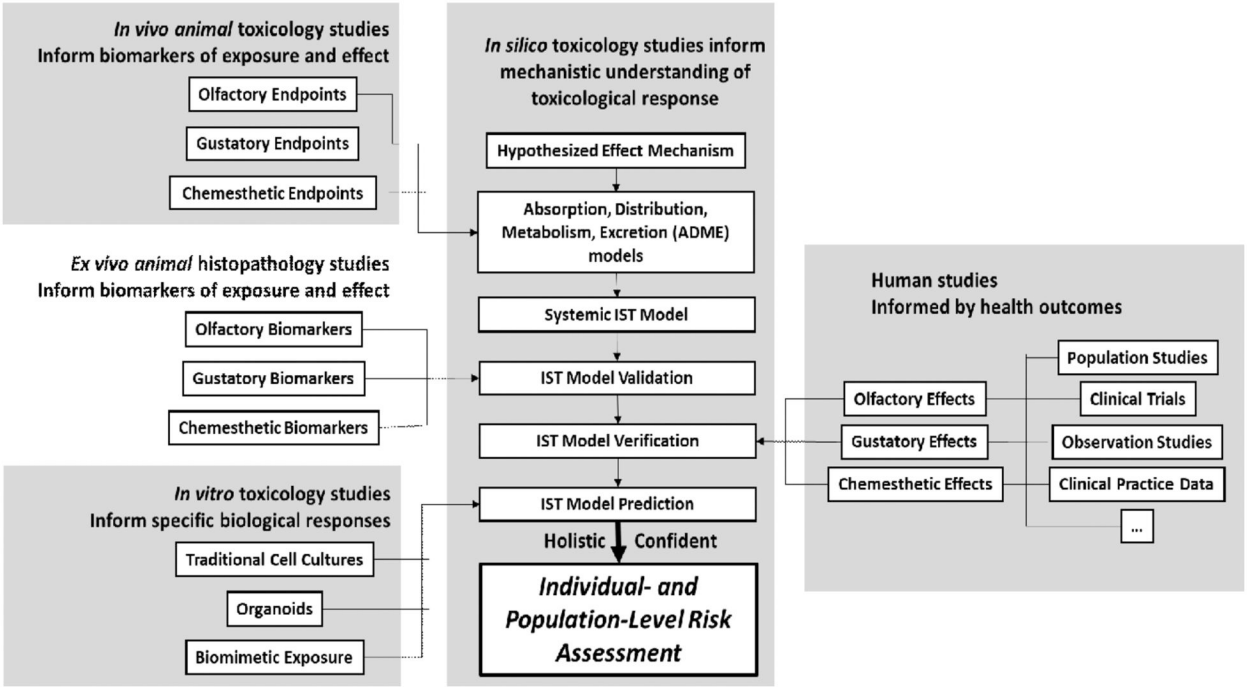

Fig. 2. Proposed risk assessment framework for sensory systems toxicity (adapted from Bassan et al. (2021)).

toxicant delivered to the receptors is dictated by the normal or intended tobacco product use.

The biologically relevant delivery model should also incorporate human product use standards, such as puff number, puff volume, and inhalation pausing, using market devices to quantify biologic concentrations achieved at the olfactory or gustatory receptor sites. These data can be applied to dosing regimens for traditional in vitro studies to ensure adequate surface dose delivery for dose response or hazard detection assays. A better understanding of how exposure translates to concentration at the receptors in the oral cavity can also be used to improve in vivo study of sensory endpoint effects in vivo and ex vivo to characterize mechanisms of distribution, metabolism, and excretion for toxicant studies.

As previously discussed, in silico platforms have already been developed and used by investigators for some organ targets; however, a great deal of work remains to incorporate these techniques into chemosensory toxicology. Effective computerized predictive toxicology models for chemosensory predictions require validation of ADME and QSAR characteristics of interest with probable or confirmed impacts on the chemesthetic, gustatory, and olfactory systems. Chemosensory-relevant ADME and QSAR are not selectable outputs for most computational platforms, and toxicology outcomes are not addressed in most computational platforms. This is due in large part to the low volume of chemosensory publications in general circulation as well as the absence of standardized methods and outcome measures used to assess direct toxicity or masking effects of chemicals on sensory receptor responses within the neurosensory field. Both issues require a greater publication presence of method development and empirical studies using in vitro and in vivo neurosensory techniques in the published literature, which would help harmonization efforts as well as support the creation of a database to train the algorithms for a new predictive output within existing computational software programs currently on the market. Increasing publication of peer-reviewed literature by the members of the neurosensory research community can promote development of these computational platforms and raise awareness of a possible adverse health outcome resulting from tobacco product use (Taboureau and Audouze 2017). In many ways, the situation is similar to the one that promoted the development of the Human Environmental Disease Network, which was recently developed using a computational model to assess effects of environmental contaminants on the public health (Taboureau and Audouze 2017).

Conclusion and future directions

The number and wide variety of ENDS options and their e-liquid chemical components currently on the market are a great burden, demanding scientific assessment of their potential public health effects on all areas of systems biology. This effect is intensified for chemosensory biology, as the normative biology and the impact of impaired function are not widely appreciated in the scientific or medical communities. This is due in large part to the low volume of chemosensory publications in general circulation, as well as the absence of standardized methods and outcome measures used to assess direct toxicity or masking effects of chemicals on sensory receptor response(s). One solution relies on a long-term research commitment to publication that integrates in vivo, in vitro, and in silico data streams to harmonize methodology and enhance the efficiency and effectiveness of studies in chemosensory toxicology. A scientific strategy with a similar decision framework was proposed to address the environmental testing burden of agrichemicals by an international working group in 2013 (Thomas et al. 2013). This early proposal included in vitro-to-in vivo extrapolation, pharmacokinetic modeling, and computational predictive exposure modeling to address variability in environmental exposure situations and to bridge data between species. The parallel development and

evolution of the AOP from a conceptual tool for systems biology into an attractive framework for the development of new approach methodologies (NAMs) and their integration into hazard and risk assessment has expanded the options available to integrative research strategies (Ankley et al. 2010; Burden et al. 2015; Edwards et al. 2016; Conolly et al. 2017; OECD 2017; Wittwehr et al. 2017; Coady et al. 2019). The use of the AOP framework in the regulatory community and the incorporation of NAMs have shortened the study time required for scientific discovery and have been a significant help to the replacement, reduction, and refinement of animal use. To that end, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) has conceptually advanced the 2013 approach by modernizing its focus on toxicity testing and NAMs development from a one-to-one replacement of in vivo tests to NAMs that support integration of findings between multiple in vitro or in chemical assay platforms as well as in silico approaches (ICCVAM 2024).

The toxicologic impacts on normative function of the chemosensory system and the loss of its contribution to organism protection and homeostasis remain an underrepresented in the published literature. The impact of chemical constituents in ENDS e-liquids or aerosols on the chemosensory systems is even less well known, as are the effects on product selection and use behavior. In many ways, the impact of chemicals in ENDS aerosol or e-liquids has much in common with the hidden impacts of environmental chemical exposures on the development of human diseases in terms of public awareness, the nature of individual exposures, and the nature of the chemical actors in play.

This review has offered a snapshot of the current state of the science and opportunities for improving and increasing the volume of publications in the press in the field of chemosensory toxicology, particularly regarding the potential impacts of tobacco products. Funding and experience notwithstanding, the proposed solutions rely on the determination of the scientific community to take advantage of a largely unexplored field of opportunity. Use of AOPs and an integrative approach to all data streams, including NAMs, is a proven means to rapidly find and address gaps in basic science and toxicology of systems biology, and has the potential to enhance knowledge in chemosensory biology as well as attract additional scientists to this area of research. A long-term scientific commitment to increased publication and use of an integrative, risk-driven planning framework to address harmonization and data gaps in neurosensory research programs would improve study visibility in the published literature, help bridge between data streams, and support regulatory trust in new non-animal NAMs. As a side benefit, this increased trust in NAMs within the regulatory community has the potential to improve efficiency across all areas of interest in tobacco regulatory science, while at the same time replacing, reducing, and refining the use of animals in future scientific efforts.

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