THE ROLE OF DIET-INDUCED TASTE CHANGES AND TASTE PREFERENCE IN ETHANOL INTAKE OF MICE

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THE ROLE OF DIET-INDUCED TASTE CHANGES AND TASTE PREFERENCE IN ETHANOL INTAKE OF MICE

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Ethanol is perceived as having sweet and bitter components, and its acceptance and consumption levels have been linked to taste. While extensive research has been conducted to identify the genetic basis of alcoholism, studies have focused on mechanisms involved in the reward systems due to their outsized involvement in the broader field of substance use-related behaviors. However, given that taste acts as the primary behavioral gateway to alcohol consumption, it is imperative to understand its role in potentially shaping alcohol consumption to manage clinical tactics based on factors such as dietary intake and taste genetics.

This Ph.D. project was designed to expand the current understanding of how dietary intake and taste preference influence the perception of ethanol. Using C57BL/6 mice as an animal model, we combined methods in 2-bottle preference testing, immunohistochemistry, and qRT-PCR to identify intersections between taste and ethanol consumption. In the first arm of the project, we investigated the effects of bitter or sweet diet-induced shifts in ethanol preference, and whether molecular and anatomical changes in taste-related functions were implicated in those behavioral shifts. While the molecular mechanism by which chronic bitter intake raises ethanol preference remains unclear, we demonstrated that a 4-week bitter diet increased the preference for ethanol, and that it had unexpected secondary effects on sweet perception and sweet- or umami-sensing T1R3-positive cells in taste buds. In the second arm of the project,

inherent variation in the mRNA expression levels of taste genes were evaluated against ethanol preference patterns. Ethanol preference was found to be associated with bitter aversion in males, with no genetic association with sweet taste functions but correlations with *T2R26* and *T2R37* bitter receptor and *GNAT3* taste transducing genes.

Collectively, our findings add to the existing body of research on the role of taste in alcohol consumption by ruling out key bitter taste-related genes as molecular drivers of a bitter diet-driven shift in ethanol consumption, and also by demonstrating a genetic link between ethanol preference and bitter sensing or taste transducing capacities of male C57BL/6 mice. The implications of our findings highlight the need for continued research into the association between alcohol consumption and long-term effects of chronic bitter intake, as well as between alcohol consumption and variation in bitter gene expression.

BIOGRAPHICAL SKETCH

Anna Koh received her B.A. in Chemistry at New York University in 2012, where she minored in Molecular and Cell Biology. Following graduation, she joined the Division of Liver Diseases at the Icahn School of Medicine at Mount Sinai, where she honed her technical and analytical skills by working on multidisciplinary projects aimed at genomic characterization and therapeutic target discovery in liver cancer. In 2016, she enrolled in Cornell University's Food Science Ph.D. program. Having developed an interest in behavioral risk factors for liver cancer at her previous position, Anna focused her research on the role of taste in shaping alcohol consumption patterns. In 2021, she joined Impossible Foods' Sensory and Perceptual Research team, where she applies her understanding of taste biology to improve how we measure the sensory effects that guide consumption.

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CHAPTER 1

BITTER AND SWEET DIETS ALTER TASTE RESPONSE AND ALCOHOL CONSUMPTION BEHAVIOR

INTRODUCTION

Taste guides the consumption of alcohol

Alcohol consumption is a confounding risk factor for many medical conditions, including three of the CDC's top five leading causes of death in the United States: cancer, stroke, and unintentional injuries (Centers for Disease Control and Prevention (CDC), 2020). Over 14 million adults in the USA are afflicted with alcohol use disorder, which cost the U.S. $249 billion in 2010 alone (National Institute on Alcohol Abuse and Alcoholism (NIAAA), 2019). To mitigate the disease burden of excessive drinking, extensive research has been conducted in search of the genetic basis for alcoholism. Variations in genes that encode $GABA_A$ receptor α-2 subunit (GABRA2), cholinergic muscarinic 2 receptor (CHRM2), and α-synuclein (SNCA) in the brain are a few loci that have been implicated as proffering a predisposition toward alcohol abuse (Covault et al., 2004; Luo et al., 2005; Foroud et al., 2007). Additionally, chronic drinking reinforces alcohol consumption behavior through modulation of neuroreceptors such as the dopamine D_1 receptor and serotonin receptor 1A ($5\text{-}HT_{1A}$) (El-Ghundi et al., 1998; Belmer et al., 2018). Given that clinical treatment options often target specific receptors, understanding the physiological basis for alcoholism is instrumental in efforts to manage alcohol use disorder.

While alcohol consumption is widely influenced by neurobiological factors, its modulators are not confined to the brain. Research in the last two decades has

expanded this narrative to also include findings concerning the taste system. Perceived as tasting both sweet and bitter in humans and rodents, alcohol is voluntarily consumed with varying degrees of acceptance (Belknap et al., 1993; Scinska et al., 2000; Blizard, 2006), in a manner some have linked to taste (Lanier et al., 2005). In particular, functional polymorphism in human bitter receptor gene *TAS2R38* – and to a lesser extent, *TAS2R13* and *TAS2R16* – are associated with alcohol intake (Duffy et al., 2004; Wang et al., 2007; Dotson et al., 2012; Allen et al., 2014). In a study that established bitter taste sensitivity as a reliable predictor for alcohol intake, Duffy et al. (2004) demonstrated a positive correlation between perceived bitterness intensity of 6-n-propylthiouracil (PROP) – a bitter tastant and emblem for genetic variance in bitter perception, particularly that arising from common mutations in the bitter taste receptor TAS2R38– and bitterness from ethanol in human subjects. This result suggests that greater sensitivity to bitterness, which presumably leads to a higher degree of aversion to bitter taste, may curb alcohol intake.

On the other end of the spectrum, liking for sweet taste can also drive alcohol intake (Kampov-Polevoy et al., 1999; Avena et al., 2004; Blednov et al., 2008; Brasser et al., 2010). Through a knockout experiment, Blednov et al. showed that mice lacking taste signaling genes α-gustducin (*GNAT3*), transient receptor potential cation channel (*TRPM5*), or taste receptor gene *T1R3* demonstrated lower preference for, and intake of ethanol compared to their wild-type counterparts. A positive correlation between preference for sweet taste and ethanol has also been observed in human subjects (Kampov-Polevoy et al., 1997; Krahn et al., 2006). Additionally, in humans, ethanol is reported as having a burning sensation (Green, 1987). A physiological rationale for this

phenomenon was verified in rodent studies showing that the heat-sensing vanilloid receptor-1 (TRPV1) mediates ethanol intake response in a manner similar to how humans and rodents sense spiciness from capsaicin (Trevisani et al., 2002; Blednov & Harris, 2009), which is sensed independent of the taste buds.

Collectively, these results suggest that taste serves to guide not only our consumption of food but also alcohol. That both bitter and sweet tastes are specifically implicated in mediating alcohol intake was demonstrated in a study by Lanier et al. (2005), which showed that PROP bitterness rating of scotch was inversely correlated with liking of its bitterness and sweetness in college students. Given the mounting evidence, it is fair to deduce that alcohol consumption patterns may be modulated by altering taste perception. Taste perception has been shown to readily change with habitual intake, which can lower sensitivity to a habitually consumed stimulus (Holt et al., 2000; Cornelis et al., 2017; Costanzo et al., 2017; Vennerød et al., 2017). For appetitive tastes such as sweet, umami, and moderate levels of salty, habitual intake reduces perceived intensity and raises the preferred dose of the respective tastant, which may reinforce a loop of unhealthy overconsumption (Pangborn & Pecore, 1982; G. H. Kim & Lee, 2009). Similarly, repeated exposure to aversive tastes such as bitter raises the tolerated intensity of the taste quality, which in turn allows for increased consumption of bitter tasting foods like cruciferous vegetables (Caton et al., 2013; Mohd Nor et al., 2021). Therefore, because taste properties of ethanol – bitter and sweet – can be shaped by dietary habits, we hypothesized that diets rich in bitter or sweet tasting foods may also modulate the perception and consumption of ethanol. Specifically, blunted taste response as a result of chronic bitter or sweet diets may

reduce the perception of the aversive bitter taste or appetitive sweet taste in ethanol, increasing or reducing ethanol consumption, respectively (Figure 1).

Figure 1. Diagram of central hypothesis.

Repeated exposure to taste stimuli causes a blunted taste response

Over the last five decades, a steady rise in food availability has introduced new diet-induced global health challenges (Kearney, 2010). In particular, excessive intake of highly palatable foods, usually containing high levels of salt, sugar and fat, can lead to diets unhealthily high in sodium or sugars. Such diets are implicated in the development of chronic diseases such as diabetes and hypertension, and have overtaken undernutrition as the number one cause of diet-induced death (Tapsell et al., 2016). Burdens of disease tied to such diets are growing at an alarming rate, with Singh et al. (2015) ascribing more than 180,000 mortalities in 2010 to sugar sweetened beverage-induced diabetes, cardiovascular diseases, and cancers, based on a comparative risk assessment model.

The interconnection between food choice and the taste system imposes an additional layer of complexity to the problem. Taste has been rated consistently as the most important factor influencing consumers' purchasing decisions (Kourouniotis et al., 2016; International Food Information Council (IFIC), 2020) and contributes vitally to feelings of satiation that affect meal cessation (Boesveldt & de Graaf, 2017). Further,

overconsumption of highly palatable foods can induce changes in the perception of taste (G. H. Kim & Lee, 2009), thus altering the drives of feeding behavior. Repeated exposure to taste stimuli can suppress taste responses, as in the case of habitual consumption of artificial sweeteners or monosodium glutamate producing a reduction in their perceived intensities in human subjects (Appleton & Blundell, 2007; Noel et al., 2018). A parallel reduction in appetite in both studies points to the possibility that taste adaptation can trigger unforeseen downstream effects that are possibly linked to central feeding circuits or mechanisms of satiation in the gastrointestinal tract.

Diet-driven behavioral changes have been studied in the opposing context as well, in which subjects on a low-sugar diet reported heightened sensitivity to sweet taste (Bertino et al., 1982; Wise et al., 2016). Weighed in conjunction with a potential correlation between reduction in dietary sugar intake and a decrease in body weight (Te Morenga et al., 2013), it is clear that taste adaptation-induced changes in eating behavior should be considered as significant players in the management of diet-related health challenges. A change in eating behavior can also be observed with habitual intake of foods high in fat, widely considered as a potential sixth basic taste modality (Besnard et al., 2016; Costanzo et al., 2017), adding evidence to the fact that taste perception and food consumption patterns are closely linked.

Investigation into adaptation-induced physiological changes that drive consumption patterns lags behind the extensive reporting that focuses on the effects of behavioral changes. Because food consumption is so closely intertwined with the reward system, without data supporting a direct relationship between change in taste perception and response, it is difficult to pinpoint the extent to which the taste system

itself is implicated in behavioral changes instigated by prolonged dietary routines. Therefore, molecular evidence to explain observed behavioral shifts with diet would strengthen our understanding of the long-term effects of dietary intake on taste.

The taste system

There are five fully accepted basic taste modalities – sweet, salty, bitter, sour, and umami – detected by taste buds in the oral cavity and epiglottis. A taste bud is made up of 50-100 elongated specialized epithelial cells of three different sub-types: roughly 50% type I cells, 30% type II cells, and 20% type III cells (Chaudhari & Roper, 2010; Roper & Chaudhari, 2017). These cells collectively detect and transduce taste, with each taste bud having the capacity to sense all five taste modalities but each taste cell responding most robustly to a single taste.

Type I cells serve a glial-like function through their involvement in synaptic neurotransmission termination and ionic homeostasis maintenance (Chaudhari & Roper, 2010; Vandenbeuch et al., 2013) in addition to transducing salty taste through amiloride-sensitive epithelial sodium channels (ENaC) (Vandenbeuch et al., 2008; Chandrashekar et al., 2010). Type II cells sense bitter, sweet or umami stimuli through G protein-coupled receptors (GPCRs). Within type II cells, taste stimuli are sensed by the GPCR taste receptor type 1 (T1Rs) and taste receptor type 2 (T2Rs). Type II cells sense sweet, umami, and bitter tastes through heterodimeric T1R2-T1R3, T1R1-T1R3, and monomeric T2R receptors, respectively.

When sweet, umami, or bitter stimuli bind to their respective receptors, they set off a signaling cascade that progresses through activation of phospholipase PLCβ2 and synthesis of inositol triphosphate (IP3), which triggers IP3R3 to open ion channels on

the endoplasmic reticulum to release Ca^{2+} into the cell's cytosol (Margolskee, 2002; Roper, 2007). Ca^{2+} release coupled with subsequence depolarization mediates the release of ATP into the extracellular space to transmit signals to sensory afferent fibers via P2X2-P2X3 purinergic receptors and to type III cells.

Type III cells communicate with type II cells and form synaptic junctions with nerves to relay taste signals. Through interactions with type II cells, they indirectly transduce sweet, umami, and bitter tastes. Additionally, they express ion channels capable of directly sensing sour taste (Lin et al., 2004; A. L. Huang et al., 2006). Expressed in type III cells, SNAP25 forms a SNARE complex with VAMP2 and Syntaxin 1A to mediate neurotransmitter release via exocytosis of synaptic vesicles and promote neural plasticity of taste nerves (Yang et al., 2000; Kawakami et al., 2012). PGP9.5, a ubiquitin hydrolase expressed in neurons and peripheral nerve fibers, is also found at the base of taste buds in type II and III cells (Yee et al., 2001; Ma et al., 2007; Shin et al., 2012). Given that P2X2-P2X3 receptors are essential for transmission of neural signals from a taste stimulus to the brain via primary sensory afferents (Finger et al., 2005; Eddy et al., 2009; Vandenbeuch et al., 2015), it is conceivable that long-term dietary differences may affect taste innervation either in addition to or alternatively to taste receptor expression.

Repeated dietary exposure may also act on solitary chemosensory cells (SCCs) in the gastrointestinal tract

Taste receptors play additional roles outside the oral cavity. Taste receptor-expressing solitary chemosensory cells (SCCs) are present in the epithelia of the airway and the lungs, to name a few, where their primary function is to detect pathogens via nutrient-sensing mechanisms (Tizzano et al., 2010, 2011). SCCs exhibit a marked

expression of α-gustducin, a G-protein α-subunit necessary for taste transduction in type II taste cells (Finger et al., 2003). Moreover, SCCs share many of the same signaling mechanism as taste cells (Rozengurt & Sternini, 2007), as demonstrated by T1R2-T1R3 heterodimeric receptors in intestinal endocrine cells that detect sugar and promote its absorption in the gut (Sclafani, 2007). Significant expression of T2R bitter receptors in particular have been demonstrated in SCCs in the gut, with calcium imaging showing intestinal secretin tumor cells (STC-1) responding to a wide range of bitter stimuli (Masuho et al., 2005; Wu et al., 2002). STC-1 cells are used as proxies of intestinal enteroendocrine cells in *in vitro* studies investigating regulators of gastrointestinal hormones such as glucagon-like peptide 1 (GLP-1), peptide YY (PYY), and cholecystokinin (CCK) that are involved in the control of food intake, gut motility, and blood glucose levels after feeding (Chen et al., 2006; Dotson et al., 2012; K.-S. Kim et al., 2014; McCarthy et al., 2015).

It should be noted that some bitter compounds, such as isohumulones found in hops used for brewing beer, are capable of producing similar postingestive effects via a bitter signaling-independent pathway: most studies investigating the mechanism behind metabolic effects of isohumulones have focused on their ability to activate peroxisome proliferator-activated receptors α and γ (PPARα and γ), primarily expressed in adipocytes, to reduce blood glucose levels and regulate lipid metabolism (Miura et al., 2005; Sumiyoshi & Kimura, 2013; Yajima et al., 2004). However, robust evidence supports a direct link between bitter signal transmission and positive postingestive effects (Avau et al., 2015; Janssen et al., 2011; K.-S. Kim et al., 2014; Pham et al., 2016), with a recent study demonstrating that an isohumulone KDT501 promotes GLP-1

release and glucose tolerance specifically by signaling through the bitter receptor T2R8 (Kok et al., 2018).

Therefore, the nutrient-sensing nature of SCCs, substantial presence of T1R and T2R receptors and their downstream signaling elements in the gastrointestinal tract (Breer et al., 2012; Margolskee et al., 2007), and the potential modulating effects of taste stimuli on gut hormone-secreting cells via taste signaling mechanism all suggest that the gut is an environment where repeated dietary exposure may have analogous effects to those in the mouth, with consequential downstream effects on consumption behaviors and appetite. On this basis, the scope of this study will also include diet's effects on taste receptors expressed in SCCs of the gastrointestinal tract to explore potential postingestive effects mediated by the taste signaling mechanism.

METHODS

Animals

Experiments were designed and performed in compliance with Cornell University's Institutional Animal Care and Use Committee (IACUC) regulations. Animals used in this study were in-house bred single-housed C57BL/6 male and female mice originally purchased from Jackson Labs (Bar Harbor, ME) maintained on an ad libitum diet of standard chow (Teklad 2918, global 18% protein rodent diet). At the start of the diet period, all mice were 12±1 weeks old. Diet period consisted of 4-weeks of treatment (Figure 2), where animals would consume water with either no supplementation (control), or supplemented with quinine (bitter diet) or sucralose (sweet diet).

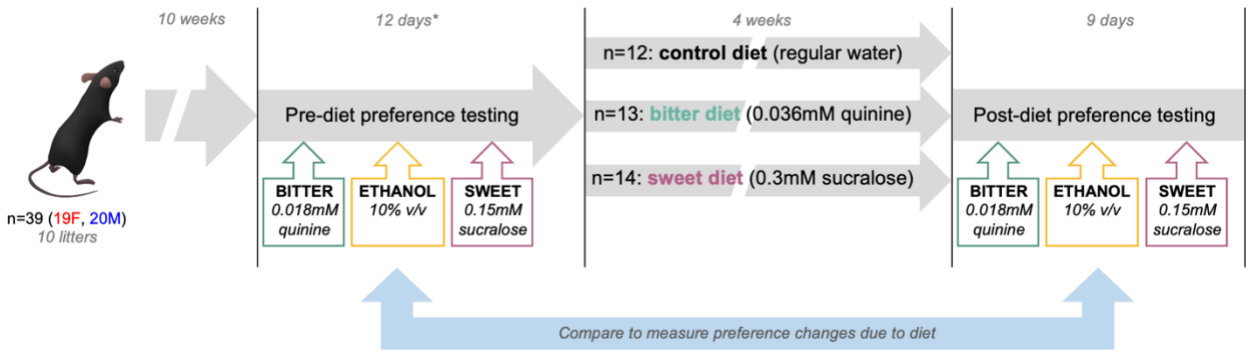

Figure 2. Schematic of study design showing pre-diet, diet, and post-diet periods, with 10 litters split into control, bitter, and sweet diet groups (n=39). Two bottle preference tests of bitter, ethanol, and sweet solutions were performed pre- and post-diet. *Pre-diet preference testing included two consecutive rounds of bitter and sweet preference testing to eliminate potential learning effects.

Behavioral testing

To test behavioral changes due to diet, mice were single-housed and underwent a series of two-bottle preference tests. Two-bottle preference testing is a method to determine how much a test solution is preferred over a control (usually water), providing mice with water and a tastant solution simultaneously for a prolonged period (48 hours in our tests). Two 15ml glass bottles were provided to each mouse, with spouts inserted into the cage side-by-side. Bottles were swapped in position at 24-hour mark to minimize any side preference (Figure 3). Mice were given water only for 24 hours in between each round of testing to offset potential carryover effects.

Diet-induced changes in ethanol preference and intake were measured by comparing pre- and post-diet 2-bottle preference testing data. Weights of filled bottles were recorded at the start and end of each round of preference testing. The difference in pre- and post-testing weight was used to calculate preference and intake. Changes in bitter and sweet preference and intake were also measured to enable comparisons with existing literature on the effects of diet on taste response and to delve into potential molecular mechanisms of taste habituation.

Prism 9.0.2 software (GraphPad, San Diego, CA, USA) was used to analyze and visualize behavioral testing results. Datasets were analyzed using paired, parametric t-test if they passed the D'Agostino & Pearson normality test and paired, non-parametric t-test if they did not (indicated in figure legends).

Figure 3. Cage setup of 2-bottle preference testing showing sample and water, with ad libitum standard chow.

Pre-diet preference testing

Mice showed inconsistencies in the first round of 2-bottle bitter preference testing in a pilot study, possibly due to neophobia of an aversive stimulus. Consecutive 2-bottle bitter preference testing demonstrated a learning curve, in which mice collectively produced a more consistent range of data in the second round. Therefore, pre-diet preference testing began with an acclimatization round of bitter preference testing that was discarded from analysis. Sweet preference testing was also repeated to eliminate a potential learning effect; however, no difference was observed for the appetitive stimulus. Therefore, the first round of sweet preference testing data was used in analysis to maintain the same comparison timeframe between pre- and post-diet.

Post-diet preference testing

Testing sequence ran from the most aversive (quinine) to most appetitive (sucralose), with mildly appetitive ethanol in between, determined based on the recommendation that testing the most preferred tastant last will yield fewer carryover effects (Beauchamp & Fisher, 1993; Crabbe et al., 2011). Following a four-week period in which the sweet diet group received 0.3mM sucralose in drinking water, the bitter diet group received 0.036mM quinine, and the control group consumed standard water, the same sequence and concentrations of testing was performed to compare pre- and post-diet preferences for quinine, ethanol and sucralose. A four-week period was deemed sufficient to see several rounds of cell turnover, as taste cells have an estimated half-life of 8-24 days (Beidler & Smallman, 1965; Perea-Martinez et al., 2013), and changes in taste receptor expression have been reported on this timescale (Shahbandi et al., 2018).

Tastant concentrations

Concentrations of tastants used are summarized in Table 1. Quinine and sucralose concentrations for preference testing were chosen from a pilot study, in which sequential preference testing was performed with increasing concentrations of each tastant to assess the concentration at which appetitive tastant produced around 75% preference and aversive tastant produced around 25% preference. 10% ethanol was used to allow suitable comparisons to findings of previous studies. Diet concentrations were chosen at twice the preference testing concentration to produce a sizeable adaptation response within a physiologically relevant concentration range, while still ensuring that the bitter group would continue to consume water. A non-nutritive

sweetener sucralose was selected as the sweet stimulus to minimize postingestive or metabolic effects of long-term diet treatment.

Table 1. Concentrations of tastants used in the study. All chemicals were purchased through Sigma-Aldrich (St. Louis, MO, USA).

Tastant	Chemical	Preference Testing	Diet
Bitter	Quinine hydrochloride dihydrate (Q1125-10G)	0.018mM	0.036mM
Sweet	Sucralose (69293-100G)	0.15mM	0.3mM
Ethanol	Ethanol (1009712500)	10% v/v	N/A

Immunofluorescence staining

At the end of post-diet preference testing, mice were euthanized with CO_2 followed by cervical dislocation, and tongues were collected. Circumvallate (CV) papillae were excised from tongue tissue and fixed in 4% PFA (Electron Microscopy Sciences, Hatfield, PA, USA), cryoprotected overnight in 30% sucrose (MilliporeSigma, Burlington, MA, USA), and frozen in OCT compound (ThermoFisher Scientific, Waltham, MA, USA). 10-micron-thick coronal sections of the CV were cut using Thermo Scientific Microm HM 550 cryostat (ThermoFisher Scientific, Waltham, MA, USA) and placed on glass slides (Electron Microscopy Sciences, Hatfield, PA, USA).

A two-day protocol was followed to stain tissue sections for antigens listed in Table 2. On the first day, slides were treated with 1% Triton X-100 (MilliporeSigma, Burlington, MA, USA), incubated with a blocking solution (3% BSA (Amresco, Solo, OH, USA); 3% Donkey serum (Equitech-Bio, Kerrville, TX, USA); 0.3% Triton X-100 (MilliporeSigma, Burlington, MA, USA)) at room temperature for 3h, and then incubated with primary antibodies at 4°C overnight. On the second day, slides were incubated with Alexa Fluor® conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) for 2h and mounted with DAPI Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA,

USA) to visualize nuclei. Stained tissue slides were imaged with an Olympus IX71 microscope (Olympus Corporation, Tokyo, Japan) affixed to an ORCA-Flash 4.0 camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan). Images were processed and analyzed using ImageJ (NIH, Bethesda, MD, USA).

Table 2. Primary antibodies for immunofluorescence staining.

Antigen	Host	Supplier	Catalog No.	Dilution
GNAT3	Goat	Aviva Systems Biology (San Diego, California)	OAEB00418	1:1000
GNAT3	Rabbit	Santa Cruz Biotechnology (Santa Cruz, California)	sc-395	1:1000
T1R3	Goat	Santa Cruz Biotechnology (Santa Cruz, California)	sc-22458	1:1000
P2X3	Rabbit	MilliporeSigma (Burlington, MA)	AB5895	1:500

Quantification of taste buds and taste cells

Circumvallate papillae were sectioned across their entire coronal length, yielding between 35-50 slices per CV. There was no significant difference in the number of slices per CV in each group. Because each taste bud is roughly 50 microns in diameter (Vandenbeuch et al., 2013), every fifth section (each measuring 10 microns) was stained to avoid double-counting of any taste bud (Kaufman et al., 2018). To quantify differences in the number of bitter- and sweet-sensing cells pre- and post-diet, sections were co-stained for two type II cell markers, α-gustducin (GNAT3) and T1R3. α-gustducin is differentially expressed across the tongue: while it may be co-expressed with sweet receptors in fungiform papillae on the anterior portion of the tongue, it is predominantly co-expressed with bitter receptors in the CV in the posterior taste field, with little to no overlap with T1R3 receptors expressed in sweet or umami sensitive type II cells (Adler et al., 2000; M. Kim et al., 2003; Tomonari et al., 2012; Choo & Dando, 2020). Thus, α-gustducin was used as a marker to quantify changes in bitter-sensing cells. Since the receptor for sweet stimuli is composed of the T1R2-T1R3 heterodimer,

T1R3 was used as a marker to quantify sweet or umami-sensing cells. Taste buds per CV were counted from merged images of T1R3 and GNAT3 staining, which together highlight the core of taste cells.

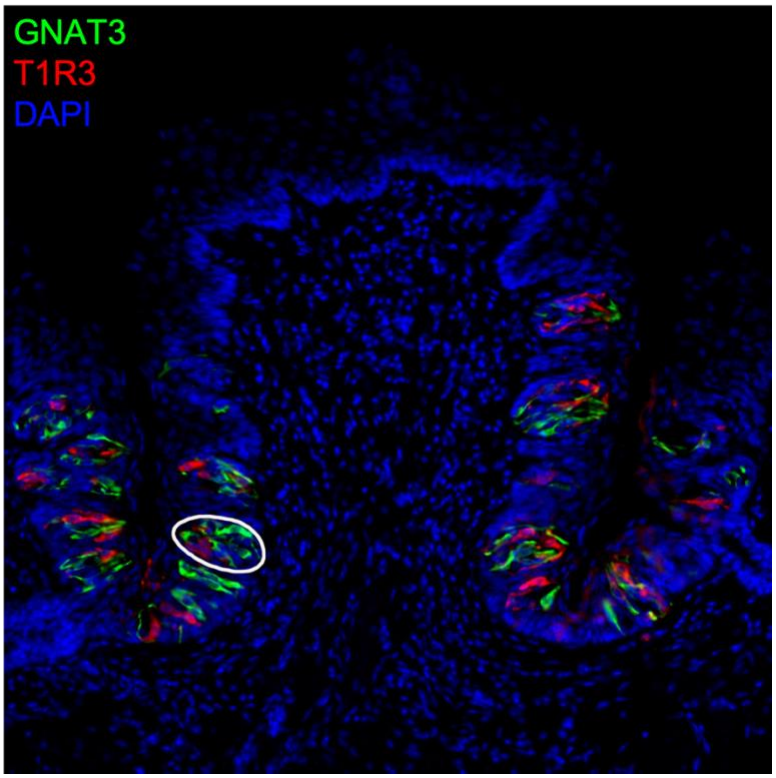

Figure 4. CV section immunostained with GNAT3 (marker for bitter-sensing cells), T1R3 (marker for sweet- and umami-sensing cells), and DAPI (cell nuclei). Circled in white is a taste bud.

Quantification of taste innervation

Taste innervation was quantified by measuring the intensity of P2X3 staining using ImageJ (NIH, Bethesda, MD, USA), as in Meng et al. (2017). All images of CV sections were normalized against a standard for contrast and background, after which an auto-threshold was set for a randomly selected taste bud from each section (see representative image, Figure 14A). Then, highlighted pixels were summed to represent the amount of nerve fibers in each taste bud. Since any highlighted pixel is counted

towards intensity in ImageJ, normalized images of individual taste buds were used for analysis instead of entire CV sections to minimize the effects of staining artifacts.

mRNA expression in taste buds

An additional group of mice on identical treatments was divided randomly into three groups, consuming a control (n=10), bitter (n=10), or sweet diet (n=10) (Figure 5). Sexes of the mice were divided evenly to explore the potential for sex-specific effects. To match the ages of the mice from behavioral testing, mice began diet treatment at 12±1 weeks of age. Throughout the diet period, weight and water intake were monitored to ensure that treatment solutions did not significantly affect hydration.

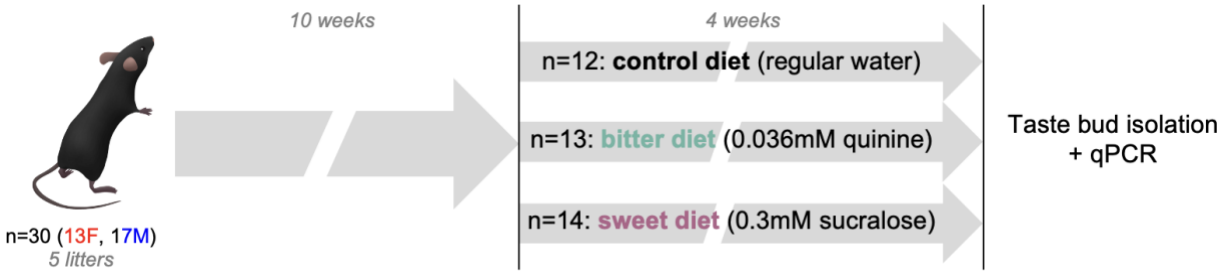

Figure 5. Schematic of study design with 5 litters split into control, bitter, and sweet diet groups (n=30).

At the end of the four-week diet period, mice were euthanized with CO_2 followed by cervical dislocation, and tongues and gastrointestinal tracts were collected. The processing protocol for the gastrointestinal tract is outlined in the next section. To preserve the integrity of their morphological structures, excised tongues were immersed in Normal Tyrode's solution (135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM $NaHCO_3$, 10mM sodium pyruvate, 10mM HEPES, 10mM D-glucose; pH 7.4 ± 0.1) upon removal and kept submerged in calcium-free Tyrode's solution (135mM NaCl, 5mM KCl, 10mM sodium pyruvate, 10mM HEPES, 10mM D-glucose, 20mM EGTA, 5mM BAPTA; pH 7.4 ± 0.1) throughout the remainder of the protocol.

To isolate taste cells from the tongue, the circumvallate papillae, which contain the highest density of taste buds in the tongue, were injected sub-epithelially with an enzyme cocktail (2.5mg/ml dispase type II, 1.0mg/ml collagenase type I, 0.25mg/ml elastase, 0.5mg/ml DNase I). Following a 20-minute incubation, the epithelium was peeled back to collect taste buds using a micropipette. After lysing the cells, RNA was extracted using the Absolutely RNA Nanoprep Kit (Agilent Technologies, Santa Clara, CA, USA), and then the reverse-transcribed cDNA was used to run qRT-PCR using a QuantStudio Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) to measure mRNA expression levels of genes encoding the following proteins in taste buds: β-actin (housekeeping); PLCβ2, GNAT3, and TRPM5 (taste transduction markers); SNAP25 and PGP9.5 (signal transmission markers); T1R2 and T1R3 (sweet taste receptors); T2R5, T2R8, T2R26, T2R37, T2R40, and T2R44 (quinine-activated bitter receptors); and T2R35, T2R38, and T2R39 (non-quinine-activated bitter receptors) (Table 3). The expression level of each gene was quantified using 3 technical replicates, and the average of the three cycle threshold (Ct) values was used to analyze relative changes in gene expression using the 2^{-ddCt} method (Livak & Schmittgen, 2001). Prism 9.0.2 software (GraphPad, San Diego, CA, USA) was used to analyze and visualize gene expression data.

Table 3. Primer pair sequences for qRT-PCR (Lossow et al., 2016; Tomonari et al., 2012).

Gene Symbol	Protein	Forward Sequence (5'–3')	Reverse Sequence (5'–3')
Actb	β-actin	CACCCTGTGCTGCTCACC	GCACGATTTCCCTCTCAG
Plcβ2	PLCβ2	GAGCAAATCGCCAAGATGAT	CCTTGTCTGTGGTGACCTTG
Gnat3	GNAT3	GCAACCACCTCCATTGTTCT	AGAAGAGCCCACAGTCTTTGAG
Trpm5	TRPM5	GTCTGGAATCACAGGCCAAC	GTTGATGTGCCCCAAAAACT
Snap25	SNAP25	GGCAATAATCAGGATGGAGTAG	AGATTTAACCACTTCCCAGCA
Uchl1	PGP9.5	AGGGACAGGAAGTTAGCCCT	GGGACAGCTTCTCCGTTTCA
Tas1r2	T1R2	AAGCATCGCCTCCTACTCC	GGCTGGCAACTCTTAGAACAC

Tas1r3	T1R3	GAAGCATCCAGATGACTTCA	GGGAACAGAAGGACACTGAG
Tas2r105	T2R5	GAATCATAGAAACAGGACCTCG	CTTTACAAAGGCTTGCTTTAGC
Tas2r108	T2R8	TTCTGATTTCAGCCCTCACC	CCAAAAGCTGGTCCTGTTTC
Tas2r115	T2R15	AGAGAATGTGTGCTGTTCTACG	TCTCACGCTTGCACCAATAC
Tas2r126	T2R26	TGGTTGAAGTGGAGATTCCC	TGGTTTCCCCAAAAGAACAG
Tas2r135	T2R35	TCAGGTACTGGATGTGGCAG	CAGCAGCCCCTCTTTATCAC
Tas2r137	T2R37	GTCTCAGCATCACTCGGCTTT	GCAGGCGAGCTGAATAGCA
Tas2r138	T2R38	TTCTACTGCCTGAAAATAGCCAGTT	AACAACCACTCTAGAAGCTCTCCATT
Tas2r139	T2R39	ACACACCCTGAACATGAGAAACA	GGCCTGCATATGAGCCTCTATG
Tas2r140	T2R40	ATGAATGCTACTGTGAAGTG	CTAAGGACCTGGGAGTTC
Tas2r144	T2R44	ATGGCAATAATTACCACAAATTC	CTACCTTTTAAGGTAAAGATGAA

mRNA expression in gastrointestinal tract

Based on insights from several studies reporting characterization of taste receptors from the gastrointestinal tract (Bezençon et al., 2008; Dyer et al., 2005; Reimann et al., 2008), a protocol was developed for concurrent processing of taste and gastrointestinal samples. Because the highest expression levels of taste-related genes were found in the roughly 6cm-long, proximal third portion of the small intestine (unpublished results), this section was taken as a representative sample of the small intestine to minimize the processing of excess materials. The entirety of the large intestine was also processed.

Briefly, sections were cut longitudinally using a single-edge razor blade and thoroughly flushed with and maintained on Normal Tyrode's solution (135mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM $NaHCO_3$, 10mM sodium pyruvate, 10mM HEPES, 10mM D-glucose; pH 7.4 ± 0.1) before cells were incubated with enzyme cocktail (2.5mg/ml dispase type II, 1.0mg/ml collagenase type I, 0.25mg/ml elastase, 0.5mg/ml DNase I) and separated from the membrane by vortexing (Figure 6).

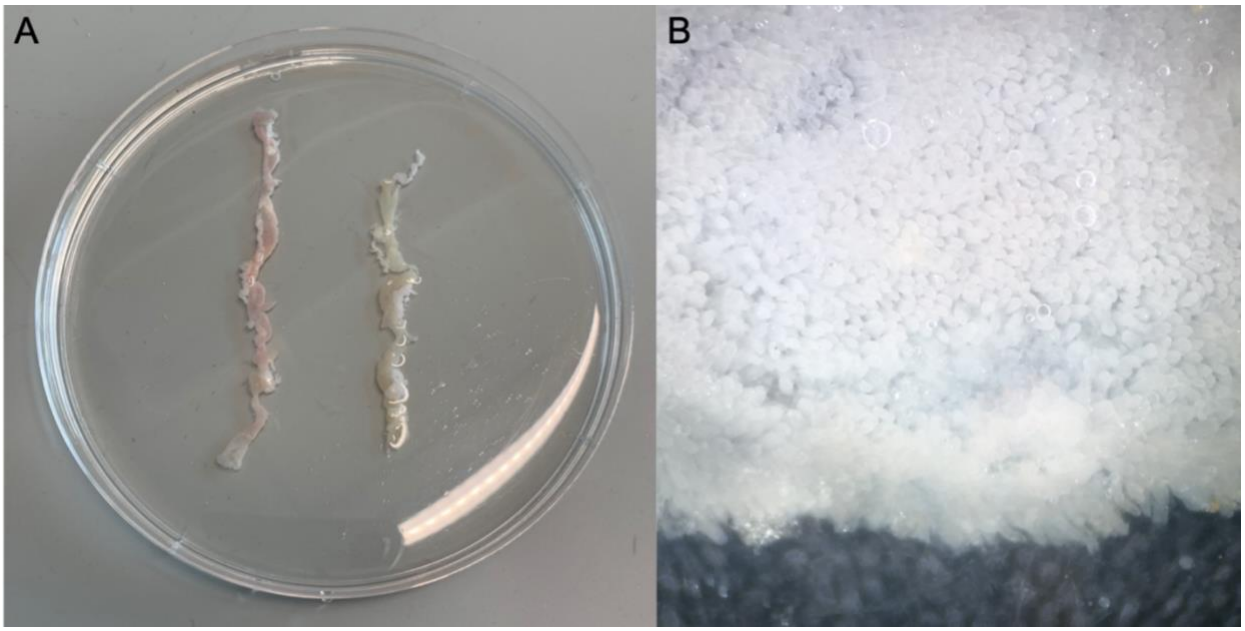

Figure 6. Flushed sections of small and large intestines (A), and magnified image of a large intestine section from which cells were extracted (B).

RNA was extracted using an Absolutely RNA Nanoprep Kit (Agilent Technologies, Santa Clara, CA, USA), and then the reverse-transcribed cDNA was used to run qRT-PCR using a QuantStudio Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) to measure mRNA expression levels of genes encoding the following proteins in the small and large intestines: β-actin (housekeeping gene); PLCβ2 and GNAT3 (taste transduction markers); T1R2 and T1R3 (sweet taste receptors); T2R5 and T2R8 (quinine-activated bitter receptors); and T2R35 (non-quinine-activated bitter receptor). (Table 3). Expression level of each gene was quantified in 3 technical replicates, and the average of the three Ct values was used to analyze relative changes in gene expression using the 2^{-ddCt} method (Livak & Schmittgen, 2001). In a pilot study, expression levels of quinine-activated bitter receptor T2R5 was below the detection threshold in the small intestine; thus, T2R5 expression was not measured in small intestine samples. Prism 9.0.2 software (GraphPad, San

Diego, CA, USA) was used to analyze and visualize gene expression data. All data were analyzed by one-way ANOVA and when significant, additionally by unpaired, non-parametric t-test.

RESULTS

Bitter diet altered ethanol preference, as well as sweet and bitter preference

Mice in the bitter diet group were supplemented with 0.036mM quinine in drinking water for a total of 4 weeks. Compared to pre-treatment, bitter preference (Figure 7A; p=0.0003) and intake levels measured post-treatment increased (Figure 8A; p=0.0024), suggesting a blunting of bitter taste response, or an alteration in perceived hedonics for the stimulus, as a result of the 4-week bitter diet. Ethanol preference levels measured post-diet also increased in this group (Figure 7C; p=0.0346), consistent with ethanol having a bitter taste, although intake levels did not change significantly (Figure 8C). Surprisingly, a significant increase in sweet preference was also observed in these mice (Figure 7B; p=0.0437), which may have also contributed to the observed shift in ethanol preference. No change was observed in sweet intake (Figure 8B). Mice in the control group did not exhibit any changes in preference nor intake of bitter, ethanol, and sweet over the diet period (Figure 9A-C, Figure 10A-C). No difference in the weights of mice from the bitter and control groups was observed throughout the diet period.

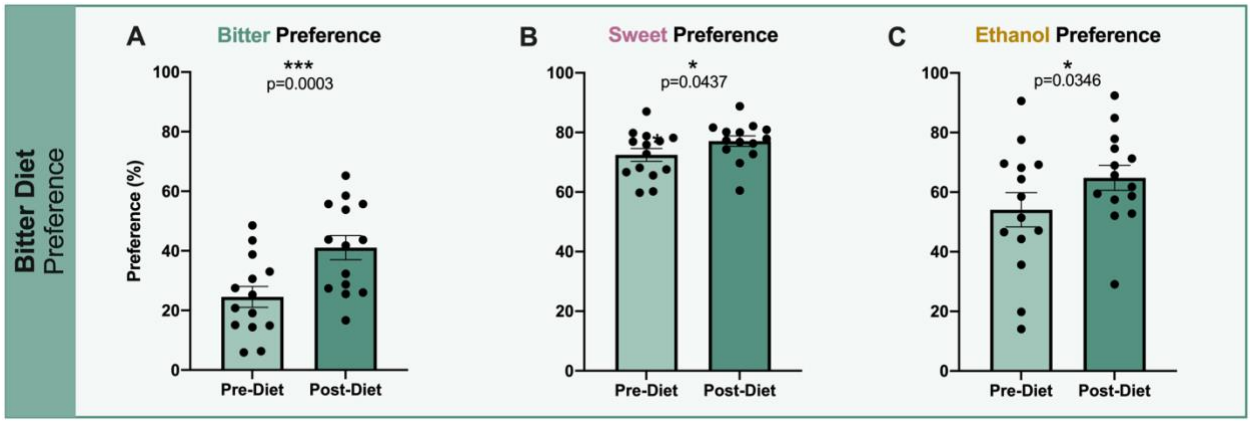

Figure 7. Bitter diet increased preferences for bitter, sweet, and ethanol (mean with SEM, n=14). Following a 4-week bitter diet (0.036mM quinine), bitter preference (A; p=0.0003), sweet preference (B; p=0.0437), and ethanol preference (C; p=0.0346) increased. All datasets passed the D'Agostino & Pearson normality test and were analyzed using paired, parametric t-test.

Figure 8. Bitter diet increased bitter intake (mean with SEM, n=14). Following a 4-week bitter diet (0.036mM quinine), bitter intake (B; p=0.0024) increased. All datasets except for ethanol intake passed the D'Agostino & Pearson normality test and were analyzed using paired, parametric t-test. Ethanol intake dataset was analyzed using paired, non-parametric t-test.

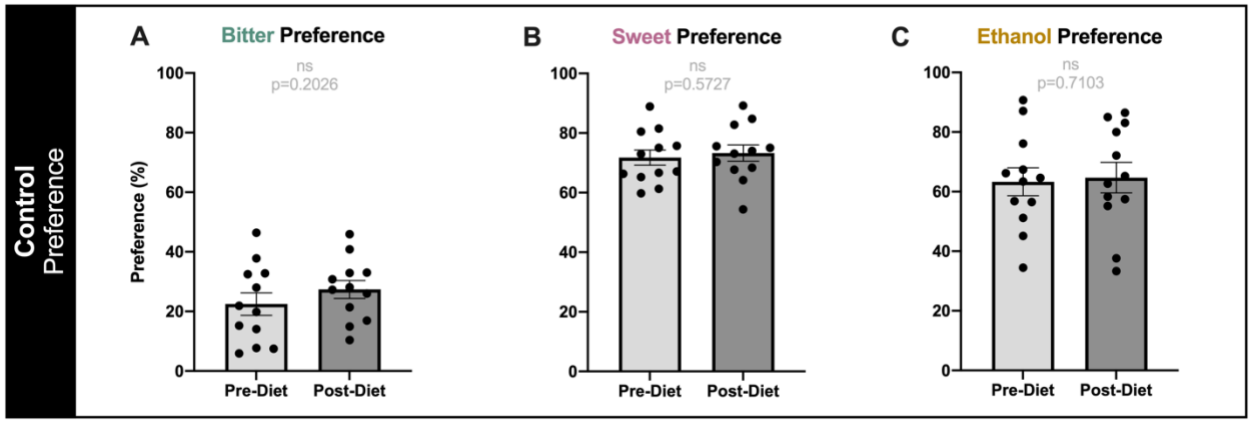

Figure 9. Taste preference remained the same in control group (mean with SEM, n=12). Following a 4-week control diet (regular drinking water), there were no significant changes in preference for bitter, sweet, nor ethanol. All datasets passed the D'Agostino & Pearson normality test and were analyzed using paired, parametric t-test.

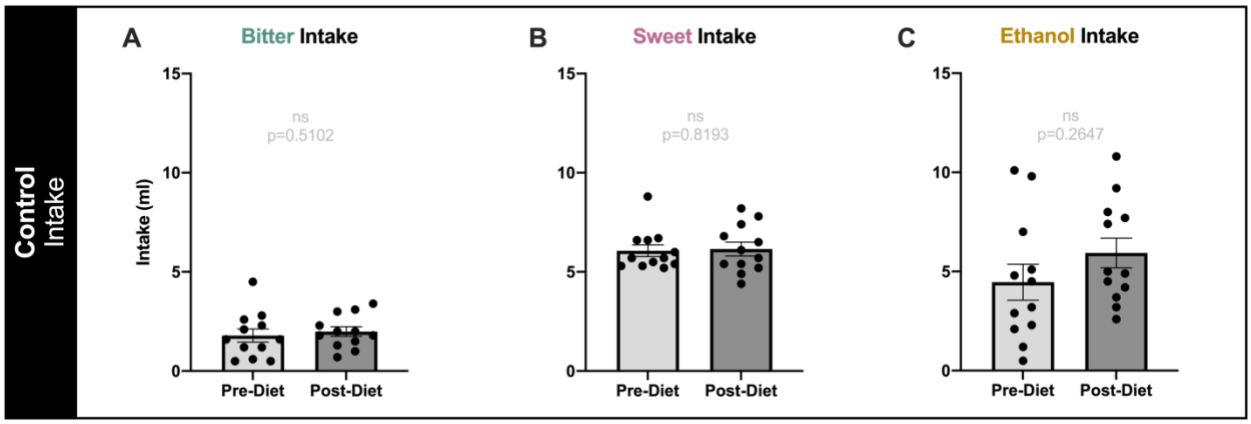

Figure 10. Intake levels remained the same in control group (mean with SEM, n=12). Following a 4-week control diet (regular drinking water), there were no significant changes in bitter, sweet, nor ethanol intake. All datasets except for sweet intake passed the D'Agostino & Pearson normality test and were analyzed using paired, parametric t-test. Sweet intake dataset was analyzed using paired, non-parametric t-test.

Sweet diet did not change ethanol preference but altered bitter preference and intake

Mice in the sweet diet group were supplemented with 0.3mM sucralose in drinking water over the course of 4 weeks. No change in sweet preference (Figure 11B, p=0.1608) nor intake (Figure 12B, p=0.9336) was observed. Preference and intake for ethanol also did not change (Figure 11C, p=0.3735; Figure 12C, p=0.9520). However, a sweet diet surprisingly led to an increase in bitter preference (Figure 11A, p=0.0133) and intake (Figure 12A, p=0.0495) versus pre-treatment levels. No difference in the weights of sweet and control groups was observed throughout the diet period.

Figure 11. Sweet diet increased bitter preference (mean with SEM, n=14). Following a 4-week sweet diet (0.3mM sucralose), bitter preference increased (A; p=0.0133). All datasets passed the D'Agostino & Pearson normality test and were analyzed using paired, parametric t-test.

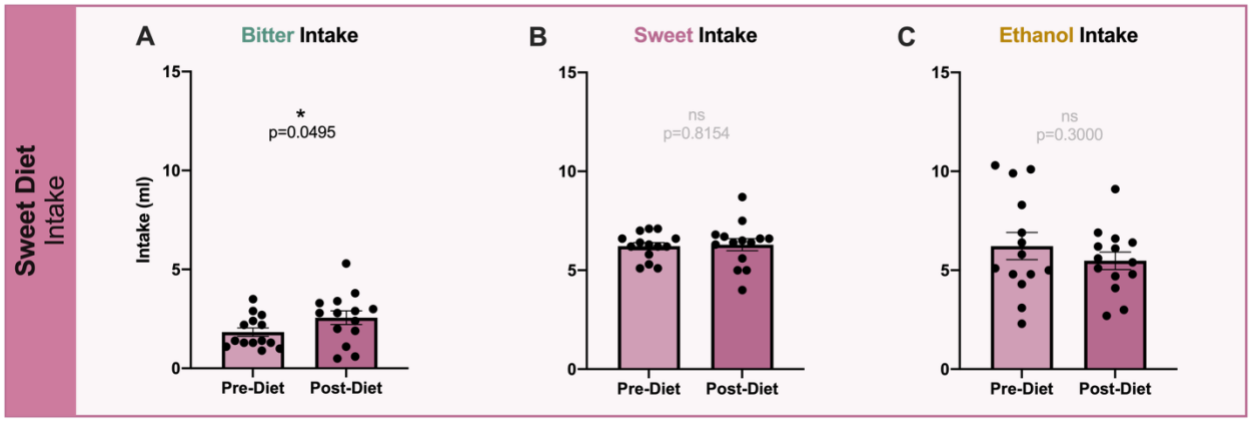

Figure 12. Sweet diet increased bitter intake (mean with SEM, n=14). Following a 4-week sweet diet (0.3mM sucralose), bitter intake (A; p=0.0495) and water intake measured against quinine in two-bottle preference test increased (D; p=0.0038). All datasets except for bitter intake passed the D'Agostino & Pearson normality test and were analyzed using paired, parametric t-test. Bitter intake dataset was analyzed using paired, non-parametric t-test.

Bitter diet reduced the number of sweet or umami-sensing cells

CV sections were immunostained for the Type II taste cell markers GNAT3 and T1R3 to detect changes in numbers of taste buds, bitter-sensing cells, and sweet or umami-sensing cells after a 4-week diet (Figure 13A). GNAT3 and T1R3 showed no colocalization, as confirmed by previous works (Adler et al., 2000; M. Kim et al., 2003; Tomonari et al., 2012; Choo & Dando, 2020), indicating that they serve as distinct markers for bitter- (GNAT3) and sweet or umami- (T1R3) sensing cells. Since clusters

of GNAT3+ and T1R3+ cells form taste buds, numbers of taste buds (Figure 13B), bitter-sensing cells (Figure 13C), and sweet or umami-sensing cells (Figure 13D) were counted from these images. Neither diet affected the number of taste buds in the CV compared to the control (Figure 13B, bitter, p=0.2109; sweet, p=0.3033), nor did they affect the number of GNAT3 cells (Figure 13C, bitter, p=0.0673; sweet, p=0.7577). However, a comparison of bitter and control groups for T1R3 cell count showed a significant reduction in the number of sweet or umami-sensing cells associated with a bitter diet (Figure 13D, bitter, p=0.0093), suggesting a potential bitter-sweet interaction from consumption of the bitter diet. No such effect was observed in the T1R3 cell count as a result of the sweet diet (Figure 13D, sweet, p=0.4079). A further hypothesis may have been fewer type II cells on the whole in bitter-dieted animals, although this is speculative and was not specifically assayed here.

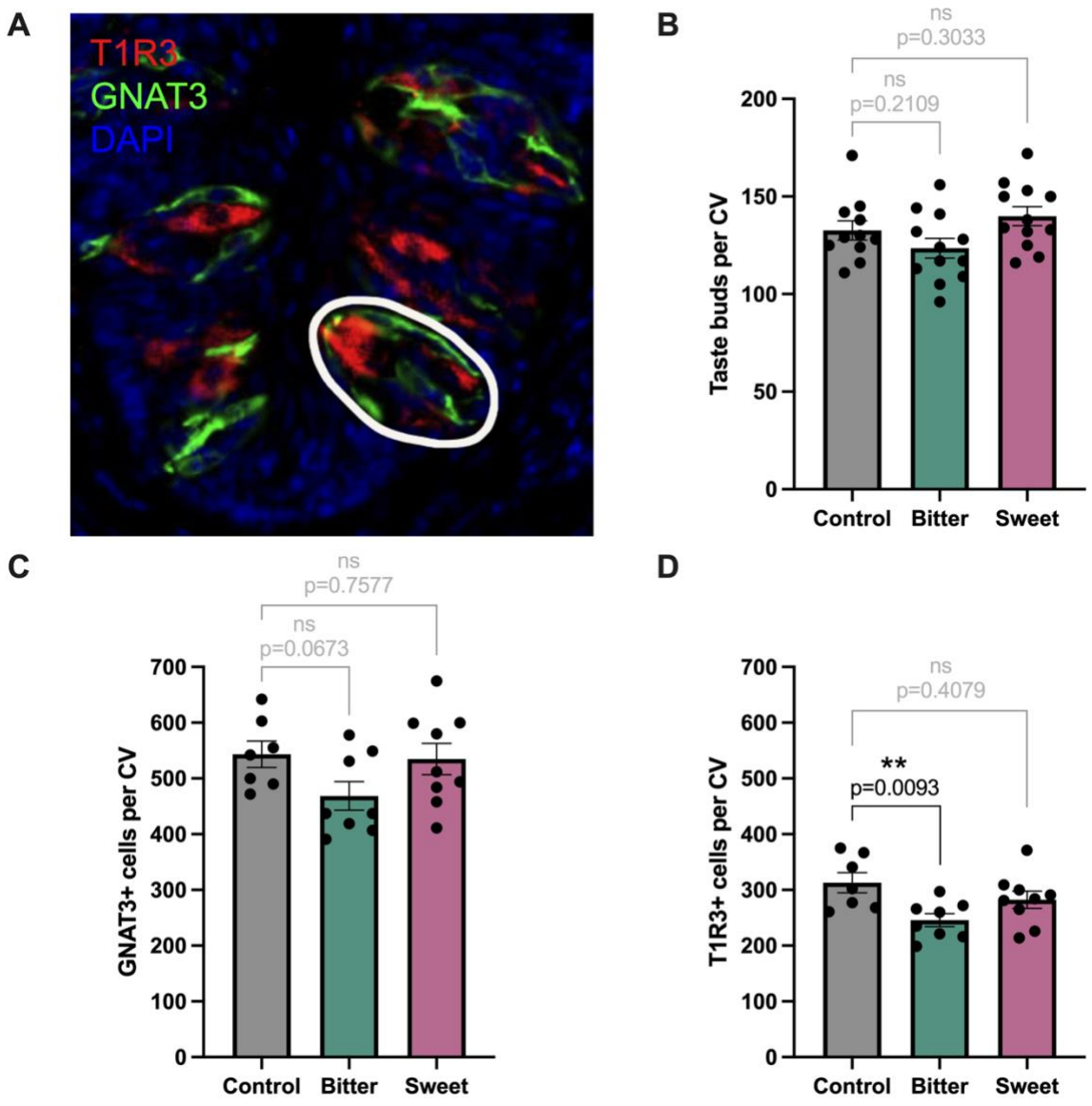

Figure 13. Bitter diet significantly reduced the number of sweet-sensing cells (D, p=0.0093). Coronal sections of CV were stained and counted for bitter- (GNAT3; green) and sweet-sensing taste cells (T1R3; red) (Control, n≤11; bitter group, n≤12; sweet group, n≤12). Taste buds, circled in white, were counted from merged images of GNAT3+ and T1R3+ staining.

Neither bitter nor sweet diet altered taste innervation

CV sections were immunostained for P2X3 to detect changes in taste innervation after a 4-week diet (Figure 14A). Average P2X3 intensity for a randomly selected taste bud from each CV section was quantified, and showed no difference from the control in

either the case of sweet or of bitter diet (Figure 14B; p=0.8986 for bitter, p=0.6296 for sweet).

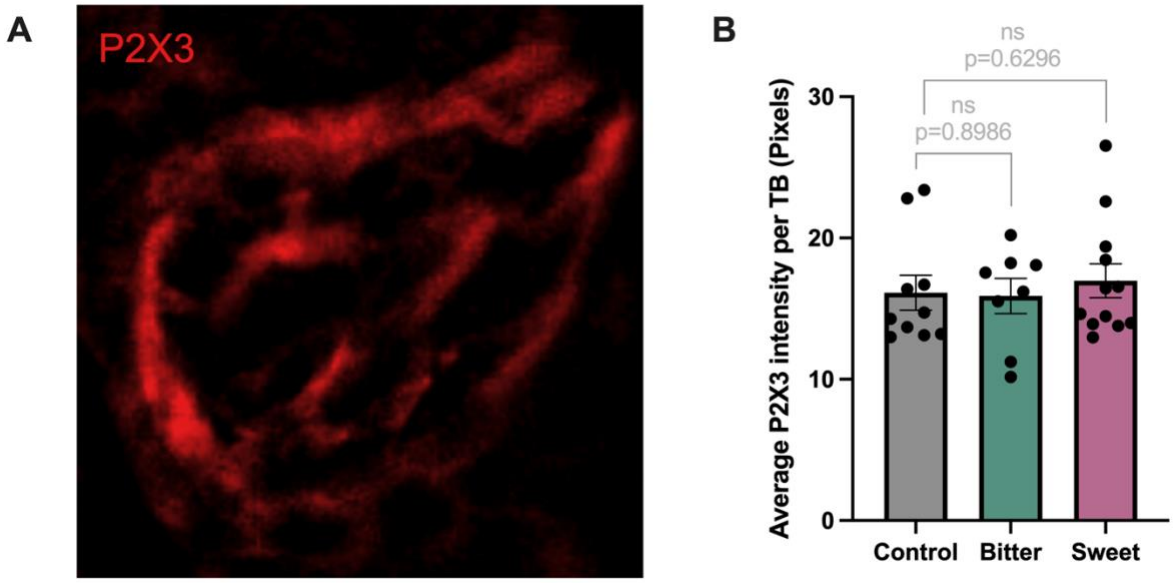

Figure 14. 4-week bitter or sweet diet did not affect taste innervation, as measured through average P2X3 intensity per taste bud.

Neither bitter nor sweet diet altered expression of any measured genes in taste buds

Expression of five categories of taste-related genes were assayed: taste transduction, signal transmission, sweet receptors, quinine-activated bitter receptors, and non-quinine-activated bitter receptors. Markers for taste transduction – PLCβ2, GNAT3, and TRPM5 – are involved in the type II taste cell signaling pathway and are discussed in the introduction (Figure 15). Two markers of nerve fibers were included to assess potential changes in signal transmission: SNAP25 and PGP9.5 (Figure 16). Genes encoding sweet receptors T1R2 and T1R3 are also discussed in the introduction (Figure 17).

Unlike sweet taste, where the T1R2-T1R3 heterodimer is responsible for detecting all sweet stimuli, there are 34 bitter receptors in the mouse that vary in their

receptive range in detecting bitter compounds (Lossow et al., 2016). Seven mouse bitter receptors known to respond to quinine were assayed: T2R5, T2R8, T2R15, T2R26, T2R37, T2R40, and T2R44 (Lossow et al., 2016) (Figure 18). To also test whether other bitter receptors change indirectly in response to quinine activation in nearby bitter cells, non-quinine-activated bitter receptors were also assayed: T2R35, T2R38, and T2R39 (Figure 19).

One-way ANOVA did not reveal any significant differences between the groups for any gene, suggesting that behavioral changes observed were not due to changes on the mRNA level of genes assayed here. Expression levels of T2R15, T2R40, T2R44 were below the detection threshold for meaningful statistical comparisons to be made.

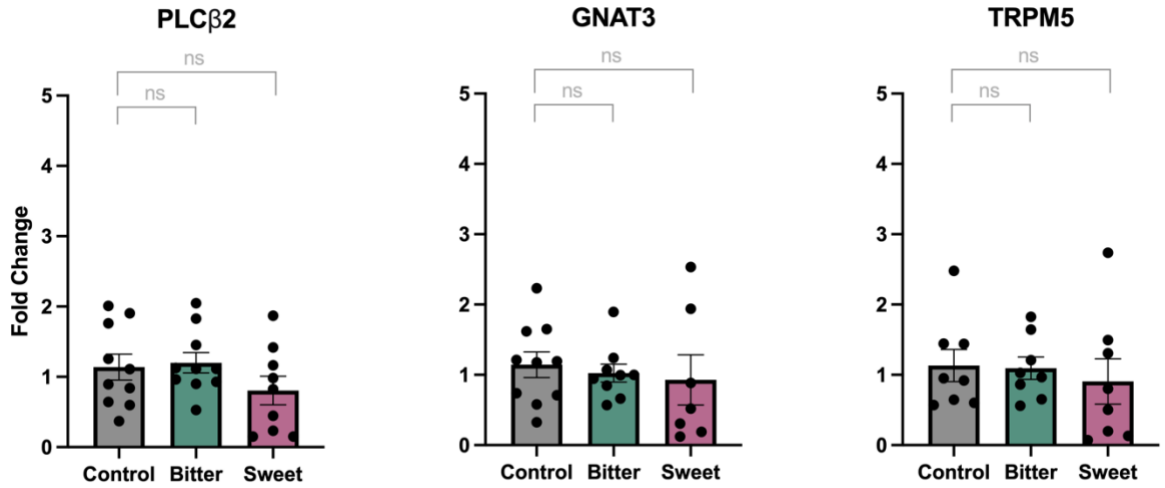

Figure 15. Expression of genes encoding receptors involved in taste transduction PLCβ2, GNAT3, and TRPM5 did not change in response to bitter nor sweet diet.

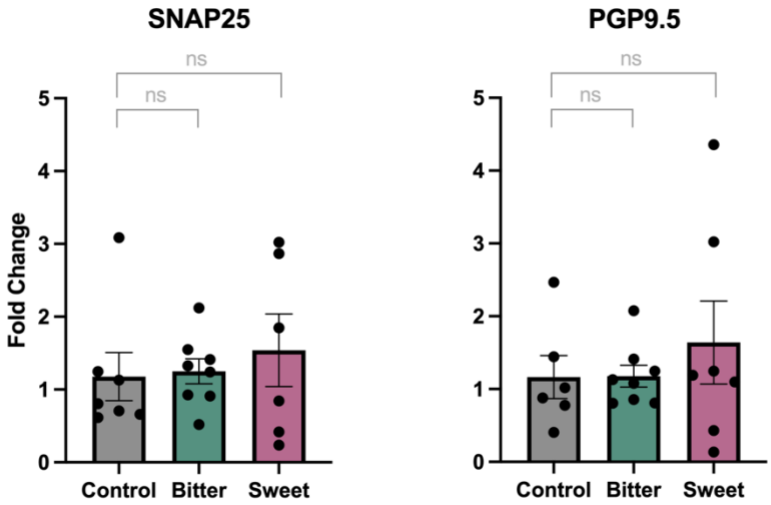

Figure 16. Expression of signal transmission genes *SNAP25* and *PGP9.5* did not change in response to bitter nor sweet diet.

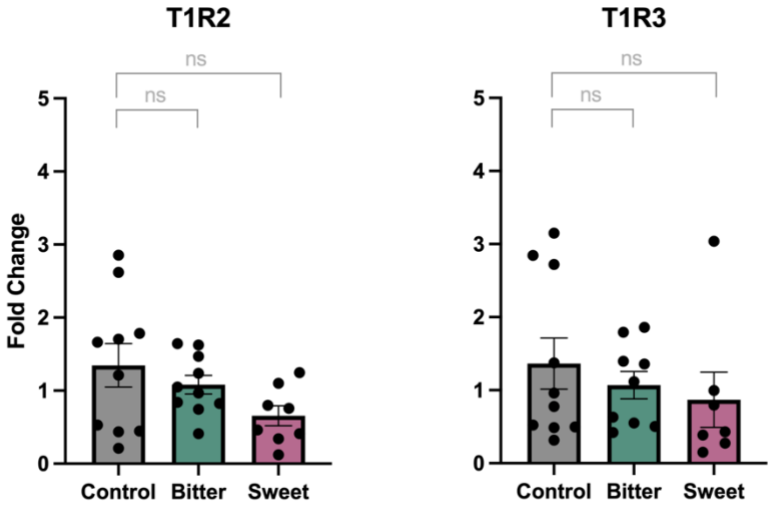

Figure 17. Expression of sweet receptor genes *T1R2* and *T1R3* did not change in response to bitter nor sweet diet.

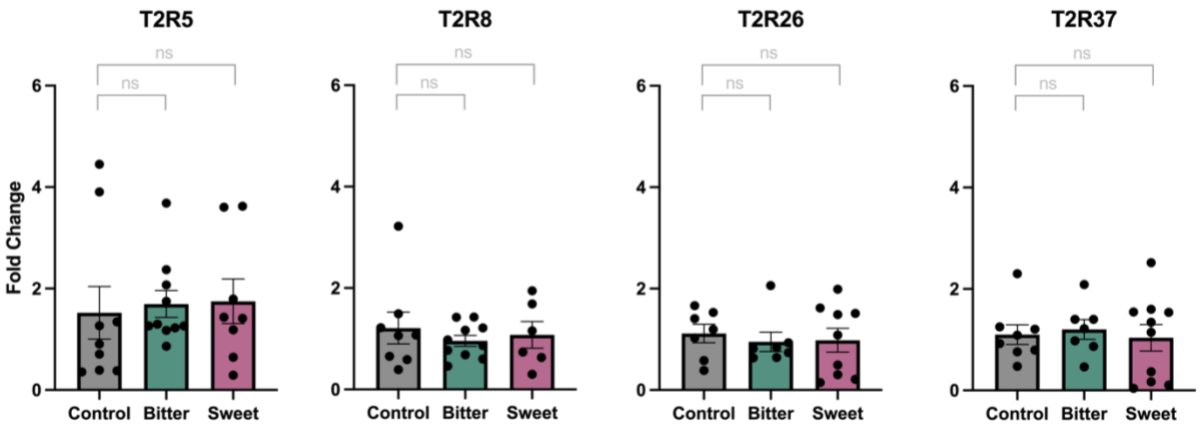

Figure 18. Expression of quinine-activated bitter receptor genes *T2R5*, *T2R8*, *T2R26*, and *T2R37* did not change in response to bitter nor sweet diet.

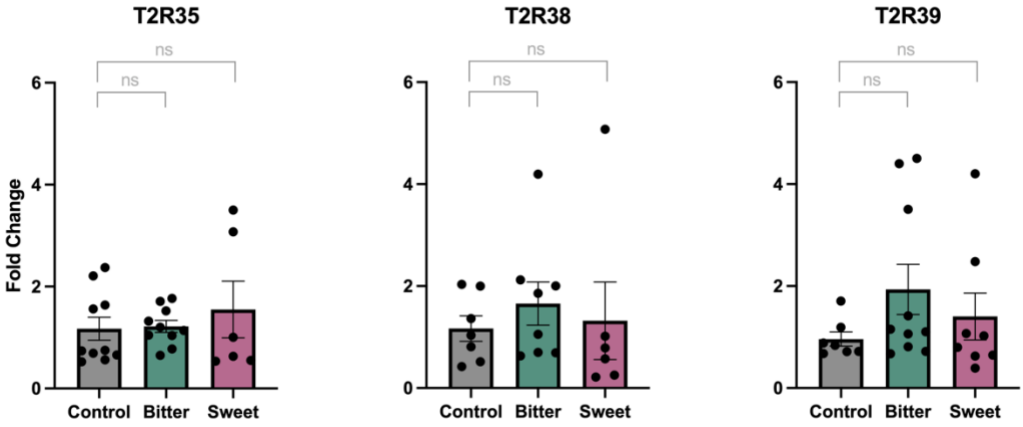

Figure 19. Expression of bitter receptor genes *not* activated quinine, *T2R35*, *T2R38*, and *T2R39*, did not change in response to bitter nor sweet diet.

Bitter diet downregulated the expression of sweet or umami-sensing receptor T1R3 in the small intestine and quinine-activated bitter receptor T2R5 in the large intestine

Given that taste cells and SCCs in the gastrointestinal tract share a common signaling mechanism (Sclafani, 2007), we hypothesized that SCCs may experience a similar shift in physiology as taste cells after prolonged exposure to a sweet or bitter diet. To test this hypothesis, cells from the small (proximal third section) and large intestine were isolated from diet-exposed animals and assayed for expression of taste-related genes. In the small intestine, expression levels of genes encoding receptors

involved in taste transduction – PLCβ2 and GNAT3 – were not altered in response to sweet or bitter diet (Figure 20). The expression of the sweet-sensing receptor T1R2 across the groups was also not significantly altered by diet (Figure 21A). However, the sweet and umami-sensing receptor T1R3 was surprisingly downregulated in mice on bitter diet (Figure 21B, p=0.0013). Lastly, expression levels of quinine-activated bitter receptor T2R8 and non-quinine-activated bitter receptor T2R35 were assayed. Neither receptor's expression levels were altered by bitter or sweet diets (Figure 22).

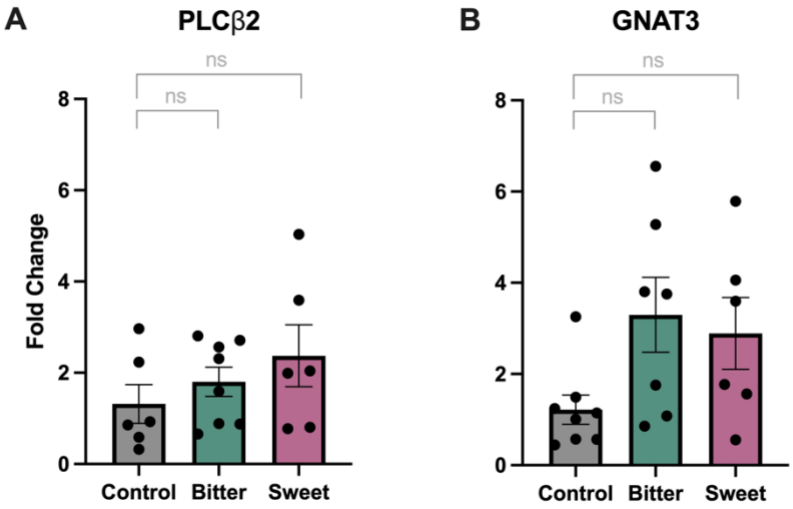

Figure 20. Expression of genes encoding receptors involved in taste transduction, PLCβ2 (A) and GNAT3 (B), did not change in response to bitter nor sweet diet in the small intestine.

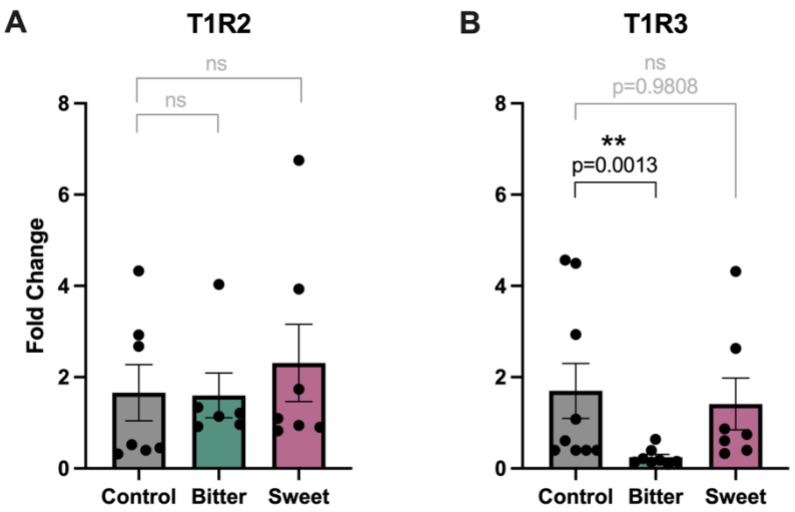

Figure 21. Sweet and umami-sensing receptor T1R3 was downregulated in the small intestine of mice on bitter diet (p=0.0013). Expression of the gene encoding sweet-sensing receptor T1R2 in the small intestine did not change in response to bitter nor sweet diet.

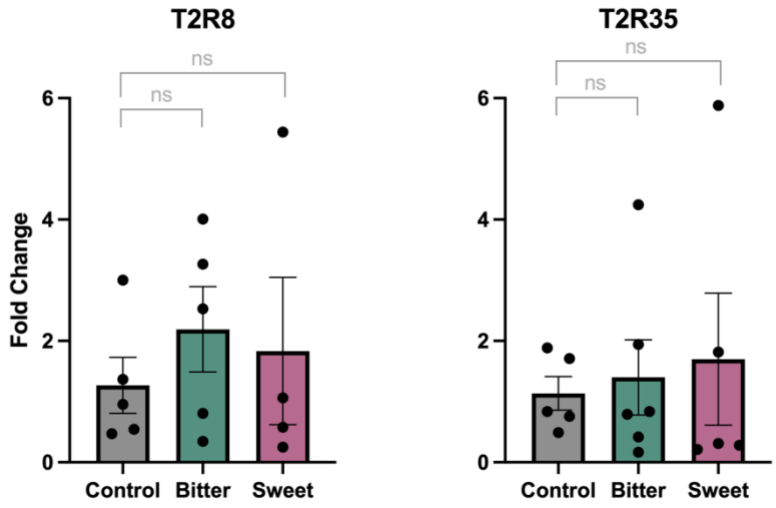

Figure 22. Expression of genes encoding bitter-sensing receptors T2R8 (quinine-activated) and T2R35 (non-quinine-activated) in the small intestine did not change in response to bitter nor sweet diet.

Expression of PLCβ2 and GNAT3, genes encoding proteins involved in taste transduction, also remained unchanged in the large intestine in response to sweet and bitter diet (Figure 23A,B). The expression level of the sweet-sensing receptor T1R2 was below the detection threshold in the large intestine for most samples, while the

expression of sweet and umami-sensing receptor T1R3 also remained unchanged in response to sweet and bitter diets (Figure 23C). Among bitter receptor genes, only the gene encoding the quinine-activated receptor T2R5 was changed in the large intestine of mice on bitter diet (Figure 24A, p=0.0152), where it displayed significant downregulation with bitter diet. Genes encoding the quinine-activated receptors T2R8 and T2R35 remained unchanged (Figure 24 B,C).

Figure 23. Expression of genes encoding receptors involved in taste transduction PLCβ2 and GNAT3 and the sweet and umami-sensing receptor T1R3 in the large intestine did not change in response to bitter nor sweet diet.

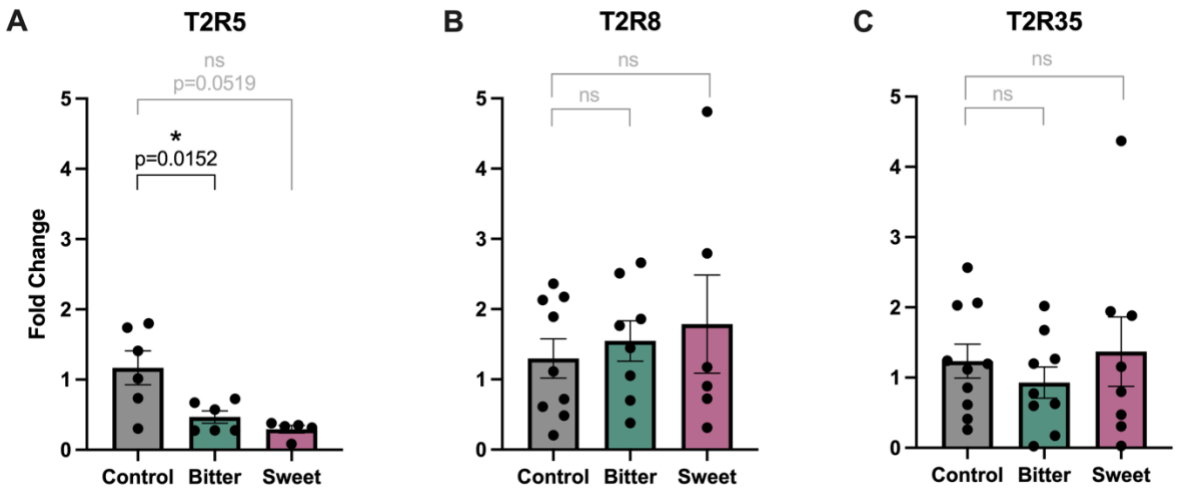

Figure 24. Bitter-sensing T2R5 receptor (quinine-activated) was downregulated in the large intestine of mice on bitter diet (p=0.0152). Expression of genes encoding bitter-sensing receptors T2R8 (quinine-activated) and T2R35 (non-quinine-activated) in the large intestine did not change in response to bitter nor sweet diet.

DISCUSSION

In this study, we showed behavioral changes in taste due to 4-week-long bitter and sweet diets. As hypothesized, a bitter diet led to an increase in ethanol preference that may be associated with an increase in bitter preference. Surprisingly, a bitter diet also led to an increase in sweet preference. On the other hand, a sweet diet did not change ethanol nor sweet preference, but did alter bitter preference and intake, flagging the possibility of bitter-sweet interactions and or sucralose-specific effects of this sweet diet. Molecularly, a bitter diet caused a reduction in the number of sweet or umami-sensing cells, which may have been associated with the increase in sweet preference we observed in this group. Bitter diet did not alter the expression of genes in taste buds, but it downregulated the expression of the sweet or umami-sensing receptor T1R3 in the small intestine and the quinine-activated bitter receptor T2R5 in the large intestine, suggesting potential consequences of a dietary regimen on nutrient metabolism in the gut.

Bitter habituation led to increased ethanol consumption

A 4-week, 0.036mM quinine diet increased ethanol preference in mice (Figure 7C). In addition to the confirmation of our hypothesis that a bitter diet would reduce the perception of the aversive bitter taste in ethanol and thus drive up its consumption, an increase in bitter preference and intake (Figure 7A, Figure 8A), as well as an increase in sweet preference (Figure 7B) was observed. The biological importance of homeostatic maintenance and various opportunities for bitter-sweet taste interactions would suggest it is unlikely that the changes in perception of bitter, sweet, and ethanol are independent of one another; therefore, we cannot test correlation in these likely-dependent variables to establish causality. While we had hypothesized that bitter diet would act directly on bitter response to alter ethanol consumption, the unexpected behavioral change to sweet preference opens up the possibility that further scenarios are potential avenues by which bitter diet alters ethanol preference. Based on the behavioral shifts observed, we can explore four potential scenarios by which chronic bitter intake may have increased ethanol preference: Chronic bitter intake 1) blunts overall taste response to all stimuli, 2) enhances ethanol preference via altering bitter perception, 3) enhances ethanol preference via altering sweet perception, or 4) enhances ethanol preference via altering bitter *and* sweet perception.

In the first scenario, chronic bitter intake affects taste perception as a whole. It is possible that consistent stimulation of bitter receptors and their downstream signaling elements by 0.036mM quinine – an aversive stimulus typically associated with ingestion of a dangerous substance – caused these animals' taste systems to adapt to respond less over time once they learned to decouple the taste of quinine from the evolutionarily

programmed signal to reject bitter substances for being potentially toxic. To further test this hypothesis, additional pre- and post-treatment preference tests to probe umami, salty, and sour tastes might help evaluate whether the blunting effect of a bitter diet extends across all taste modalities.

The second and third scenarios postulate that increased ethanol preference is driven by a bitter diet-induced change in either bitter or sweet perception. While both taste modalities have been linked to ethanol preference extensively, the surprising finding was that a bitter diet behaviorally altered sweet responses, which may in turn affect ethanol perception. To determine which taste modality is primarily linked to ethanol consumption behavior, Blednov et al. (2008) bred KO mice lacking taste transduction genes Gnat3 and Trpm5 and the sweet or umami sensing receptor gene Tas1r3 and tested their preference for ethanol, saccharin, quinine, and NaCl. All three groups consumed less ethanol and also exhibited reduced preference for saccharin but not for quinine, demonstrating a relationship between the perception of sweet taste and ethanol. While the behavioral pattern we found was in the opposite direction in that a reduction of T1R3+ cells was observed along with higher ethanol and sweet preferences, differences in test design and animal models mean we can only compare our results in terms of whether a trend exists. As discussed in the previous section, a reduction in T1R3+ cells in bitter-dieted mice suggests that this group's ability to sense sweet and umami tastes may have been diminished; in turn, their blunted sweet taste may have led to an increased wanting of the sweet stimulus, which could have raised their preference for sweet taste. While we cannot establish causality based on our data, the molecular evidence provided by the reduction of T1R3+ cells gives weight to the

possibility that the altered sweet response may have been a factor in the observed increase in ethanol preference.

Lastly, it is possible that, as in the study by Lanier et al. (2005) that showed that both bitter and sweet tastes mediate scotch intake in college-aged students, the increased ethanol preference observed in our mice was driven simultaneously by changes in both bitter and sweet perception. How bitter diet changed sweet perception may again be explained by bitter-sweet interactions as described in the previous section, or by the fact that our sweet stimulus was sucralose. Given that sucralose is known to activate bitter receptors (Lossow et al., 2016), we should also consider the possibility that change in sweet response may in fact be an effect of the change in how mice are responding to the potential bitter taste of sucralose. Although our test sucralose concentration was below the detection threshold to activate bitter receptors, the lack of existing data on the effects of chronic bitter intake on perception of potentially bitter nonnutritive sweeteners means that we simply do not know how chronic bitter intake in our experiment may have affected how sucralose was perceived. The implications of the use of sucralose in our experiments will be discussed in the final section.

Bitter habituation is behaviorally documented, but its molecular mechanisms remain unclear

A 4-week bitter diet (0.036mM quinine in drinking water) clearly reduced aversion to bitter taste in mice, measured as increases in quinine preference and intake (Figure 7A, Figure 8A). This result was consistent with findings from Mura et al. (2018) that 3 weeks of 0.03mM quinine in drinking water was sufficient to produce an increase in

quinine preference in female C57BL/6 mice. Moreover, Mura et al. confirmed that bitter habituation was not unique to quinine, showing similar reductions in aversion to other bitter stimuli (denatonium benzoate, caffeine, epigallocatechin gallate, L-tryptophan, L-isoleucine). In humans, bitter habituation has recently gained more interest in the context of promoting healthy diets in children (Lakkakula et al., 2010; Caton et al., 2013; Mohd Nor et al., 2021). Broadly, results from human studies are aligned with those from rodent studies in that repeated exposure can increase liking and intake of bitter vegetables in children.

The collective data above establish clear evidence that a change in behavioral patterns occurs as a result of repeated exposure to a bitter diet. Regarding the molecular basis driving this behavioral change, several potential mechanisms have been postulated across various model organisms and will be discussed throughout this section, including receptor regulation (Shahbandi et al., 2018), salivary protein regulation (L. E. Martin, Nikonova, et al., 2019; L. E. Martin, Kay, et al., 2019), and neural desensitization (Glendinning et al., 2001). However, despite the relevance of bitter food consumption to human health, there is no consensus on the molecular mechanism underlying bitter habituation.

Previous work has hypothesized that receptor regulation may occur as an epigenetic response to repeated stimulation (Shahbandi et al., 2018), similar to how chronic use of opioid agonists induces tolerance through μ-opioid receptor downregulation and desensitization (Williams et al., 2013). As hypothesized, Shahbandi et al. demonstrated that the umami-sensing receptor T1R1, sweet-sensing receptor T1R2, and sodium-sensing channel ENaC were downregulated in response to a diet

consisting of their respective agonists. Habituation to aversive tastes was more unpredictable: sour-sensing protein PKD2L1 was upregulated, while bitter receptor T2R5 did not undergo any change in expression. Although T2R5 is the bitter receptor that is tuned to the widest range of agonists (activated by 45 out of 128 bitter substances in a heterologous expression system using HEK293T cells by Lossow et al. (2016), there are 6 other mouse bitter receptors also activated by quinine at threshold concentrations of 0.003-0.01mM (Lossow et al., 2016). The current work expands on the repertoire of both quinine activated and non-activated bitter receptors, as well as receptors more broadly involved in taste transduction and signal transmission, in aiming to identify the molecular process by which bitter habituation occurs. Additionally, because taste transduction requires the stimulation of not only sensory receptors and their downstream signaling elements, but also afferent nerve fibers, the purinergic receptor P2X3 was also tested as a potential candidate.

Figure 15-Figure 19 show that no changes in expression were detected in genes encoding PLCβ2, GNAT3, and TRPM5 (taste transduction markers); SNAP25 and PGP9.5 (signal transmission markers); T1R2 and T1R3 (sweet taste receptors); T2R5, T2R8, T2R26, and T2R37 (quinine-activated bitter receptors); or T2R35, T2R38, and T2R39 (non-quinine-activated bitter receptors). While these results do not provide a molecular explanation for behavioral effects of bitter habituation, they do allow us to rule out key bitter taste-related genes as molecular drivers of this behavioral shift with diet, under the given experimental conditions. Expression of the purinergic receptor P2X3 did not differ between bitter-dieted and control mice (Figure 14B), indicating that the taste cell-sensory afferent junction remained unaffected at this particular level. However, this

does not rule out the possibility that other elements of innervation could have been altered, or that a different method of measuring P2X3 is necessary. In *in vivo* experiments with *Manduca Sexta* caterpillars, Glendinning et al. (2001) demonstrated that an exposure-induced adaptation to caffeine leads to diminished firing rates of bitter-sensing cells. While the mechanism by which the firing rates of bitter-sensing cells were diminished is unclear, the results imply that a different method such as measuring neural responses *in* vivo may reveal changes that are not apparent on fixed tissue specimen. In addition, while the exposure period of 4 weeks should be sufficient for a complete turnover of taste cells (Perea-Martinez et al., 2013), there is no established timeframe within which epigenetic change occurs in taste cells. Thus, while this timeframe is still within the limits during which behavioral changes are evident, a longer diet period may be necessary to reveal changes in expression levels of taste-related elements assayed in this study.

Finally, while saliva content was deemed outside the scope of this study, Martin et al. (2019) proposed that bitter diet-induced changes in salivary protein profiles may be responsible for increased bitter tolerance following chronic exposure. In previous reports (L. E. Martin et al., 2018; Torregrossa et al., 2014), the group identified seven protein bands from saliva samples of male Long Evans rats that were altered by bitter (tannic acid and quinine) exposure. After a 2-week tannic acid diet, marker salivary proteins were upregulated, during which time increases in quinine intake and feeding rate were also observed, suggesting a potential link between an altered salivary protein profile and quinine tolerance (L. E. Martin, Nikonova, et al., 2019). A causal relationship could not be established conclusively due to food restriction-imposed increase in

motivation to consume more bitter diet being a confounding factor, however given saliva's role in taste sensing (Matsuo, 2000; Matsuo & Carpenter, 2015; Neyraud et al., 2012), elucidating the relationship between bitter habituation and salivary proteins would be an important addition to the current literature.

Bitter diet downregulated T2R5 in the large intestine of mice

There remains the possibility that adaptation to constant bitter exposure may not incur any molecular changes to the gustatory system. Instead, the behavioral effects observed with habituation may be a result of conditioned preference, where bitter intake becomes associated with positive postingestive effects (Myers et al., 2005; Stratford & Finger, 2011). Coupled with evidence that gut hormone release and gastric emptying can be regulated by activating bitter taste receptors in the gut (Avau et al., 2015; Janssen et al., 2011; Kok et al., 2018; Yu et al., 2015), it is possible that changes in taste preference are secondary effects of chronic bitter exposure mediated by taste signaling through bitter receptors expressed in the SCCs in the gut rather than in taste cells.

The current work evaluated this hypothesis by assaying several taste-related genes expressed in gut SCCs of bitter-dieted mice and found that bitter diet downregulates quinine-activated T2R5 bitter receptors in the large intestine. While this result did not fit our original hypothesis, it was notable that chronic exposure-mediated downregulation was detected in SCCs in the gut despite not being detected in taste cells over the same timeframe. Oral detection of bitter taste is evolutionarily important due to its role as part of a defense mechanism against ingesting toxic, often bitter-tasting substances. As such, T2R bitter receptors play a vital role in the oral cavity to

prevent the ingestion of potentially harmful substances. In the gut, they play a physiologically broader role in maintaining metabolic homeostasis (Behrens & Meyerhof, 2011; Psichas et al., 2015), with this role only occurring after the ingestion of a bitter stimulus, i.e. a role only relevant for substances which have not been rejected by the taste buds. Because mediators of metabolic processes must quickly adapt and respond to changes in homeostasis, it is possible that receptor regulation of T2R5 was more readily fine-tuned in response to chronic bitter exposure in the gut SCCs than in taste cells, potentially as their role in rejecting bitter stimuli in the gut comes at a sizeable metabolic cost to the animal (evacuation of the digestion, or vomiting in a human system), against merely moving to the next food source when detected by the taste buds.

As to the potential that T2R5 downregulation in the gut might be at least in part responsible for altering taste perception via postingestive effects, we cannot establish a causal relationship between the two without direct measurements of any potential postingestive effects. To better understand this relationship, we can look to recent clinical data in humans that suggested a link between intragastric quinine consumption and changes in feeding behavior such as decreased appetite and intake (Bitarafan et al., 2019; Deloose et al., 2017; Iven et al., 2019; Klaassen et al., 2021). While there are conflicting results as to how strongly bitter consumption can induce postingestive changes in behavior, the effects may be sex-related since studies with female participants report greater correlations between the two (Deloose et al., 2017; Iven et al., 2019). By bypassing potential confounding effects of aversive taste responses on feeding behavior, these studies provide a useful setup for testing the direct relationship

between postingestive effects of bitter foods and behavior. Expanding the scope of such studies to evaluate changes in the subjects' taste preferences would yield valuable data in understanding whether molecular changes in gut-expressed, bitter-sensing T2R5 receptor can alter taste responses.

Bitter diet downregulated sweet or umami-sensing T1R3 receptors in the small intestine of mice

In addition to downregulation of T2R5, the sweet or umami-sensing T1R3 receptor was somewhat surprisingly downregulated in the small intestine in response to a 4-week bitter diet. Although this was an unexpected result, interactions between different taste modalities have been well-documented; this will be discussed in detail in the following section. Bitter and sweet-sensing mechanisms interact in the gut, potentially via glucagon-like peptide-1 (GLP-1), which promotes satiety and reduces intake by stimulating glucose-dependent insulin release, and inhibiting glucagon release to lower blood glucose (Baggio & Drucker, 2007). GLP-1 is released by duodenal L cells upon glucose detection; Jang et al. (2007) have shown that taste signaling elements T1R2, T1R3, α-gustducin, PLCβ2, and TRPM5 are co-expressed in the GLP-1 secreting human L-cell line NCI-H716, concluding that L-cells mediate GLP-1 release by using the sweet-sensing mechanism to detect glucose. Knockout studies in mice support this conclusion by providing evidence that T1R2, T1R3, and α-gustducin null mice exhibit significantly diminished GLP-1 release in response to glucose (Jang et al., 2007; Kokrashvili et al., 2009).

Research from the last decade has added to this narrative by delving into the role of bitter receptors in mediating glucose sensing-independent GLP-1 release. The ability of various bitter substances – including quinine, denatonium benzoate, and 1,10-

phenanthroline – to stimulate GLP-1 release via activation of GLP-1-co-expressed T2Rs has been demonstrated in both rodents and human cells (K.-S. Kim et al., 2013, 2014; Li et al., 2017; Park et al., 2015; Pham et al., 2016; Yu et al., 2015). Park et al. (2015) have specifically shown that T2R5 (reduced in expression in the large intestine of our animals) is co-expressed with GLP-1 in human NCI-H716 cells, and that its activation by 1,10-phenanthroline directly releases intracellular Ca^{2+} to trigger GLP-1 release. While Harada et al. (2018) presented contradictory evidence that quinine alone could not increase intracellular [cAMP] necessary to trigger GLP-1 release in mouse GLUTag enteroendocrine cells, it should be noted that experimental conditions and model organisms vary across studies and thus results may not be directly comparable. Given the intertwined nature of sweet and bitter-sensing mechanisms and GLP-1 release, as well as their co-expression in the gut, it is plausible that downregulation of T1R3 in bitter-dieted mice could be a consequence of a bitter diet-induced shift in GLP-1 regulation in the gut, causing GLP-1-coexpressed T1R3 in the small intestine to be collaterally affected. We saw a similar reduction in the number of T1R3+ cells in CV sections of bitter-dieted mice through immunofluorescence, adding weight to the possibility that repeated bitter exposure may affect sweet or umami-sensing via GLP-1; this result will be discussed in the next section.

Lastly, GLP-1 receptors (GLP-1R) are also expressed in taste buds, and experiments showing a reduction in sweet taste response in GLP-1R null mice (B. Martin et al., 2009; Takai et al., 2015) point to the intriguing possibility that changes in metabolic homeostasis could directly affect taste perception. Moreover, receptors for other feeding behavior-related hormones including leptin, ghrelin, and CCK are also

expressed in rodent and human taste cells and saliva (Gröschl et al., 2001, 2005; Herness et al., 2002). Therefore, future studies focusing on these hormones may shed light on whether the postingestive effects of a bitter diet can have long-term consequences on circulating hormones and their receptor regulation, thereby altering long-term taste response.

Bitter diet induced molecular and behavioral changes in sweet taste

An unexpected consequence of bitter diet was an increased sweet preference (Figure 7B). This behavioral change concurred with a reduction in the number of sweet or umami-sensing cells, marked by T1R3, in the circumvallate papillae (Figure 13D). If the reduction in T1R3+ cells directly translates into a diminished ability of bitter-dieted mice to transduce sweet or umami tastes, then they may adapt to prefer stronger-tasting stimuli, potentially providing an explanation for the increase in sweet preference. Unfortunately, the specific sequence in which molecular and behavioral changes occur cannot be established from our experimental setup; it is possible that the increase in sweet preference and reduction in T1R3+ cells observed in bitter-dieted mice are independent of each other. However, the postulation that a bitter diet may alter sweet taste response is not unlikely. In addition to the potential pathway for bitter-sweet interaction involving bitter-modulated GLP-1, direct bitter-sweet interaction on the psychophysical level has also been well-documented.

Existing research on taste interactions focuses primarily on the effects of a binary mixture suppressing one taste modality, especially of bitter taste due to implications of improving medication adherence by suppressing the bitter taste in drugs (Giacoia et al., 2012). A reliable pattern of bitter suppression has been demonstrated in bitter-sweet,

bitter-umami, and bitter-salty mixtures through neural response measurements in rodents (Maier & Katz, 2013; Tokita & Boughter, 2012) and in human sensory studies (R. S. J. Keast et al., 2004; R. S. J. Keast & Breslin, 2002; Mennella et al., 2014, 2015). Cell-based calcium mobilization assays have demonstrated reduced bitter response to mixtures when measuring the levels of calcium influx into bitter receptor expressing cells (Behrens et al., 2017; M. J. Kim et al., 2015), indicating that inhibition by the non-bitter component in the mixture occurring at the cellular level is, at least in some part, responsible for bitter suppression. As such, bitter suppression extends to compounds that taste not only primarily bitter, such as quinine, but also to nonnutritive sweeteners that activate bitter receptors and thus elicit bitter off-tastes, such as saccharin and cyclamate (Behrens et al., 2017).

In addition to bitter receptors, the type II taste cell signaling element transient receptor potential cation channel (TRPM5) is also reported as being a site for bitter-sweet interactions (Talavera et al., 2008). In rodents, taste responses to sucrose and quinine are transduced via distinct fibers of the chorda tympani (CT) nerve that innervates the fungiform papillae in the anterior portion of the tongue (Danilova & Hellekant, 2003; Formaker et al., 1997; Formaker & Frank, 1996). Compared to CT nerve responses to sucrose-only solutions measured in C57BL/6 wildtype mice, Talavera et al. (2008) showed that the response to sucrose-quinine mixtures of increasing quinine concentrations were dose-dependently inhibited by quinine. Using a strain of TRPM5-KO mice that exhibits diminished but not abolished taste response (Damak et al., 2006), they showed that this quinine-dependent effect was not observed

in TRPM5-KO mice, leading them to conclude that TRPM5 is necessary for the interplay of bitter and sweet responses to occur.

We did not find any changes in TRPM5 expression in our bitter-dieted mice (Figure 15), although this result does not necessarily contradict the findings by Talavera et al. (2008). For one, our data were collected not from the front of the tongue innervated by the CT nerve but from the circumvallate papillae, which is innervated by the glossopharyngeal nerve in the back of the tongue. It should also be noted that previous studies demonstrating bitter compounds' suppression of sweet taste response via T2Rs or TRPM5 measure the effect of acute, and not chronic bitter treatment. While we did not observe changes in taste transduction elements implicated in instantaneous bitter-sweet interactions, the current work offers the possibility that bitter habituation, as evinced by increased bitter tolerance in mice, may also have a lasting suppressive effect on sweet taste response, potentially through the involvement of T1R3+ taste cells. Further studies are warranted to parse the relationship between these molecular and behavioral changes.

Lastly, because sweet responses were measured using sucralose in our experiments, there is a possibility that the change in sweet response is sucralose-specific. While sucralose was chosen over sucrose as the sweet stimulus to minimize postingestive or metabolic effects that could confound taste behavior, it can activate bitter receptors in both rodents and humans (Lossow et al., 2016), which could lead to taste responses that deviate from those elicited by sucrose. Therefore, it may be possible that the increase in sweet preference following bitter diet is an effect of the mice being more tolerant of the bitter taste in sucralose and thus liking it more, although

this relationship was not tested directly. Potential consequences of sucralose-specific effects on the current work will be detailed in the following section.

Consequences of sweet diet on taste may be confounded by sucralose-specific effects

Chronic dietary intake of sweet foods can decrease the perception of sweet intensity in humans (Appleton & Blundell, 2007; Jayasinghe et al., 2017; Sartor et al., 2011). Conversely, reduced dietary intake of sweet foods can heighten the perception of sweet intensity (R. S. Keast, 2016; Wise et al., 2016), suggesting that sweet diet and perception are inversely correlated. Some contradicting evidence show that the association between chronic sweet consumption and perceived sweet intensity may be limited (Appleton et al., 2018), or that in children, sweet preference increases with greater sweet consumption (Divert et al., 2017; Liem & Mennella, 2002). However, age must be taken into consideration as an independent factor, since sweet preference decreases with age independently of consumption levels (Desor & Beauchamp, 1987). Sweet preference has also been studied as a predictor of alcohol consumption (Kampov-Polevoy et al., 1999; Kampov-Polevoy et al., 2003, 2004), in part because hedonic responses generated by both sweet stimuli and ethanol recruit the brain's opioidergic (Drewnowski et al., 1992; Eikemo et al., 2016; Roberts et al., 2001), dopaminergic (Bulwa et al., 2011; Sclafani et al., 2011; Tupala & Tiihonen, 2004), and serotonergic systems (Daws et al., 2006; Y.-J. Huang et al., 2005). Given these relationships between dietary intake of sweet foods, sweet perception, and ethanol intake, we expected chronic sweet exposure to induce higher sweet and ethanol preferences in mice.

Traditionally, sucrose has been used as the standard tastant to measure sweet response. However, because sucrose is caloric, its effects extend far outside the taste system. Habitual consumption of sucrose can increase the risk of metabolic syndrome (Crichton et al., 2015; Nettleton et al., 2009) and alter reward processing (E. Green & Murphy, 2012), thereby possibly confounding the effects of chronic sugar intake that are specifically mediated by changes in taste perception. With the rise in the use of nonnutritive sweeteners (NNS) to reduce daily caloric intake, NNS have become an attractive option for testing the effects of sweet taste without the postingestive effects of sucrose. While the verdict on the effects of NNS on physiological processes such as glucose tolerance, food consumption, and neurotransmitter release in rodents and humans are inconclusive (Frank et al., 2008; Glendinning et al., 2020; Kuk & Brown, 2016; Ren et al., 2021), for the purposes of this study, eliminating caloric intake as a potential confounder by using sucralose instead of sucrose was deemed appropriate.

From a taste standpoint, humans can often detect bitter off-tastes in NNS (Schiffman et al., 1995; Wiet & Beyts, 1992). In a human sensory study evaluating the taste profiles of sucralose, aspartame, saccharin, and acesulfame-K compared to that of sucrose, Wiet & Beyts (1992) found that sucralose has a taste profile that most closely resembles sucrose in categories including bitterness, sourness, body, and sweet aftertaste. Subjects did report non-sweet aftertaste in sucralose that increased with concentration; nonetheless, the same trend was reported in all other NNS that were evaluated. Liking of NNS in humans is correlated with the ability to detect bitterness, which depends on genetic variability in bitter taste receptor genes; human genes such as *Tas2R9*, *Tas2R31*, and *Tas2R43* are specifically linked to bitter sensitivity in NNS

such as acesulfame-K and saccharin (Allen et al., 2014; Bobowski et al., 2016; Pronin et al., 2007), highlighting that bitterness is an important factor in how we perceive NNS.

Similarly, rodents exhibit concentration-dependent preference for certain NNS such as saccharin (Bachmanov et al., 2001), suggesting that the aversive bitter off-taste is also detectable to rodents. Such behavioral responses are supported by the fact that acesulfame-K, saccharin, and sucralose can activate at least 1, 4, and 7 mouse bitter receptors, respectively (Lossow et al., 2016). In a study to deorphanize mouse bitter receptors using a HEK293T cell-based heterologous expression system, Lossow et al. (2016) reported that sucralose can activate T2R15 and T2R17 at a threshold concentration of 3mM, T2R5 and T2R44 at 10mM, and T2R9, T2R23, and T2R39 at 30mM. The sucralose concentration used for preference testing in our experiments was 0.15mM, below the threshold concentration necessary to activate bitter receptors, but high enough to elicit an appetitive response at between 60-80% preference, suggesting that the mice were perceiving sucralose as tasting primarily sweet. Previous work from our lab showed that 0.1mM sucralose elicited a preference comparable to that of 0.02M sucrose at around 80%, and a comparable length of an iso-sweet sucrose diet elicited a change in taste preference over time.

Despite established parameters suggesting that chronic consumption of 0.15mM sucralose will produce a sweet response that is altered enough to potentially affect how mice perceive sweetness present in ethanol, response to ethanol did not change (Figure 11C, Figure 12C), seemingly suggesting that a sweet diet does not change ethanol consumption behavior. However, the sweet diet consisting of 0.15mM sucralose also did not alter sweet response (Figure 9B, Figure 10B). Because our hypothesis that

a sweet diet will alter ethanol perception was contingent on an altered sweet response, we could not reject our hypothesis solely based on the sweet habituation experiment described here. Additional data is necessary to determine whether the lack of change in ethanol perception is 1) sucralose-specific, by repeating the experiment with an iso-sweet concentration of sucrose that produces 60-80% preference, or 2) concentration-specific, by repeating the experiment with a range of sucralose concentrations. In addition to the differences mentioned above between sucrose and sucralose, mechanistic differences may also be responsible for why a 4-week diet of 0.15mM sucralose was insufficient to induce sweet habituation. Damak et al. (2003) have demonstrated in T1R3-KO mice that a loss of the sweet or umami-sensing T1R3 receptor results in a near-loss in sensitivity to artificial sweeteners including sucralose, whereas sensitivities to sucrose was relatively diminished but retained at higher concentrations. Following this finding, researchers have shown that sucrose may produce sweet response through T1R3-independent pathways, such as pathways that depend on temperature or sensitivity to gurmarin, a selective T1R3 inhibitor (Ohkuri et al., 2009; Shigemura et al., 2008), or that recruit sodium-glucose cotransporter 1 (Yasumatsu et al., 2020). That sucralose is unable to recruit these pathways may explain the discrepancy between our results and the altered sweet response reported with habitual sucrose intake, and may further suggest that T1R3-independent pathways activated by sucrose are necessary in establishing sweet habituation.

What was notable about habitual sucralose intake was that it decreased aversion to bitter taste (Figure 9A, Figure 10A). We suspect this is a sucralose-specific effect, since there is an overlap in the bitter receptors activated by both sucralose and quinine

(T2R5, T2R15, T2R44) and bitter response was measured by testing quinine preference. While sucralose purportedly activates these bitter receptors at higher concentrations than the 0.15mM we used (Lossow et al., 2016), the current work offers the possibility that chronic exposure to sucralose may have unforeseen effects on bitter sensitivity. Given the significance of daily sweetener use in the modern world, the effects of habitual sucralose intake on bitter taste perception may offer additional insights into how chronic consumption of beverages sweetened with nonnutritive sweeteners may alter our taste responses.

CONCLUSION

Taste guides the consumption of food and alcohol for both humans and rodents. Given that chronic dietary exposure to bitter and sweet foods are purported to alter the perception of bitter and sweet tastes respectively, we hypothesized that dietary habits may shape how taste properties of ethanol are perceived and thus how it is consumed. Using C57BL/6 mice as a model, we demonstrated that a 4-week bitter diet consisting of a quinine solution increased the preference for ethanol, while a 4-week sweet diet consisting of a sucralose solution did not alter ethanol preference nor intake. The molecular mechanism by which chronic bitter intake raises ethanol preference remains unclear, as the dietary treatment only produced behavioral changes in the taste system. However, the current work adds to the existing body of research on bitter habituation by ruling out key bitter taste-related genes as molecular drivers of this behavioral shift. Based on the behavioral changes observed with bitter diet, it is possible that either bitter or sweet taste, or both together, drive the increase in ethanol preference. In addition, due to potential sucralose-specific effects, we cannot conclude based on our results that

chronic sucrose intake does not alter ethanol intake. The implications of these findings on alcohol consumption is that dietary habits that do not necessarily consist of alcohol may be capable of altering alcohol preference via taste habituation. Habitual intake of bitter and or sweet foods can shift the perception of taste over time. Changes to how the taste components of alcohol are perceived may also alter how acceptable the taste of alcohol is when experienced as a whole, thereby possibly having the unintended consequence of shifting one's alcohol consumption levels. Our study demonstrated a potential downside to bitter habituation, which, thus far, has mostly been researched in the positive context of developing a dietary tactic for promoting bitter vegetable intake.

Secondary findings of our study show that a 4-week quinine diet: 1) reduces sweet- or umami-sensing T1R3 cells in the circumvallate papillae of mice, 2) downregulates T1R3 receptor in the small intestine, and 3) downregulates T2R5 bitter receptor in the large intestine. The wide-ranging effects of bitter diet induced by quinine demonstrates that, in addition to potential interactions across bitter- and sweet-specific type II taste cells, taste-related factors such as GLP-1 release may also be affected by such repeated exposure to a single taste stimulus. Given the implications of our findings, the long-term effects of chronic bitter intake warrant continued research within the context of not only taste, but also metabolic homeostasis and broader impact on consumption habits.

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CHAPTER 2

BITTER TASTE FUNCTION-RELATED GENES ARE IMPLICATED IN THE BEHAVIORAL ASSOCIATION BETWEEN TASTE PREFERENCE AND ETHANOL PREFERENCE IN MALE MICE

INTRODUCTION

The genetics of alcohol consumption and alcohol use disorder in humans

Genetic factors account for approximately 50% of the heritability of alcohol use disorder (AUD) (Deak et al., 2019; Ducci & Goldman, 2008; Verhulst et al., 2015). Defined as “a medical condition characterized by an impaired ability to stop or control alcohol use despite adverse social, occupational, or health consequences” (NIAAA, 2020), AUD is a clinical term that is synonymous with alcoholism, alcohol dependence, and alcohol addiction. Studies of twins and families examining heritability for substance abuse have shown that relevant genetic factors include both alcohol-specific and general substance use-related factors (Heath, 1995; Prescott & Kendler, 1999; Rhee et al., 2006; Tsuang et al., 2001; Verhulst et al., 2015). For example, significant genetic correlations have consistently been reported between alcohol and nicotine dependence, illustrating that not only alcohol-specific but also general addiction-related mechanisms must be considered in the prevention and treatment of alcoholism (Agrawal & Lynskey, 2008; Hettema et al., 1999; Koopmans et al., 1997; Rhee et al., 2006).

Molecular genetics and genome-wide association studies have provided an additional dimension to findings from behavioral genetics by identifying candidate genes that are associated with alcohol use, such as multiple alcohol dehydrogenases (*ADHs*) (Edenberg & McClintick, 2018; Frank et al., 2012; Polimanti & Gelernter, 2018) and

aldehyde dehydrogenase 2 (*ALDH2*) (Edenberg & McClintick, 2018; Luczak et al., 2009), as well as genes associated with general substance use, such as $GABA_A$ receptor α-2 subunit (*GABRA2*) (Covault et al., 2004; Edenberg et al., 2004), and μ-opioid receptor (*OPRM1*) (Chamorro et al., 2012; Ray & Hutchison, 2004). These genetic factors directly influence alcohol intake through mediating physiological processes such as alcohol and aldehyde metabolism, motor coordination and withdrawal symptoms, and differential experience of the rewarding effects of alcohol, to name a few. A recent review of the genetics of substance use disorders by Lopez-Leon et al. (2021) summarizes alcohol-specific and substance use-related genes.

Inherently heightened bitter sensitivity may offer behavioral protection against alcoholism

Genetic factors that influence alcohol use in humans also include those related to genes, which is not surprising given that ethanol is perceived as intensely tasting, usually as a combination of bitter, sweet, and burning sensations (Green, 1987; Scinska et al., 2000). Human bitter receptor genes described in Chapter 1, such as *T2R38*, *T2R13*, and *T2R16*, shape individual bitter taste perception, thereby mediating alcohol intake (Allen et al., 2014; Dotson et al., 2012; Duffy et al., 2004; Wang et al., 2007). In particular, genome-wide linkage and association analyses of the Collaborative Study on the Genetics of Alcoholism (COGA) dataset revealed the *T2R16* gene as being significantly associated with alcohol dependence (Hinrichs et al., 2006; Wang et al., 2007). We should note that researchers have correlated genetic variations in human bitter receptors genes directly with behavioral data through sensory testing and surveys due to the perceived bitterness intensity of 6-n-propylthiouracil (PROP) being established as a popular proxy for bitter taste function, and reflects (although

imperfectly) particularly *T2R38* phenotype (Duffy et al., 2004). Broadly, homozygotes for the functional *T2R38* PAV/PAV perceive PROP as being intensely bitter, whereas homozygotes for the nonfunctional *T2R38* AVI/AVI do not taste PROP at all; bitter sensitivity of heterozygotes (PAV/AVI) lie within this spectrum. It should also be noted that conflicting evidence has also been presented by other groups, in which no correlations were found among PROP bitterness, current alcohol use, and family history of alcoholism in college students (Robb & Pickering, 2019). Behavioral data on current use and family history of alcoholism are commonly gathered through scores on peer-reviewed assessment methods such as the Alcohol Use Disorders Identification Test (AUDIT) (Reinert & Allen, 2007; Saunders et al., 1993) or the Michigan Alcoholism Screening Test (MAST) (Minnich et al., 2018; Selzer, 1971).

Sex effects have been commonly reported in relation to behavioral responses to bitter taste, adding to the complexity in understanding the effects of taste genetics on inherent predisposition to alcoholism. In a study examining the relationship between bitter gene variants, family history of alcoholism, and personal history of alcohol abuse in undergraduate students, Driscoll et al. (2006) observed the expected pattern of high bitter sensitivity being linked to fewer cases of family history and personal use problems in males. This observation followed the widely accepted theory that individuals with a bitter phenotype more sensitive to the bitter taste in ethanol will perceive it more aversively than those without this bitter phenotype, and further, that this relationship may imply that these “supertasters” will be less susceptible to developing alcoholism. However, surprisingly, the opposite trend was observed in females in this study. Similar observations were made in a study by Beckett et al. (2017), in which high bitter

sensitivity and possession of the “supertasting” PAV/PAV polymorphism were predictors of alcohol intake only in males. These findings suggested that other confounding factors need to be taken into account in weighing how much of a risk factor bitter genetics presents to the development of alcoholism, perhaps reflecting that males generally tend to prefer the taste and flavor of ethanol, and will consume more of it than females do (Knaapila et al., 2012; Wilsnack et al., 2009).

In addition to bitter taste, genetic variations in Guanine nucleotide-binding protein G(T) subunit alpha-3 (*GNAT3*), coding for the subunit of the G-protein gustducin that transduces bitter, sweet, and umami signals in taste receptor cells, have also been researched in the context of alcohol use (Eriksson et al., 2019). While a direct association between polymorphisms in the *GNAT3* gene and alcohol intake has yet to be confirmed, its positive correlation with sweet preference, and its physiological significance in taste perception (Fushan et al., 2009), suggests that *GNAT3* may also be involved in a relationship between taste and alcohol intake.

The association between sweet preference and family history of alcoholism may be attributed to shared neural pathways dictating sweet taste liking

Sweet preference is partially hereditary, with results from a large-scale twin study of 663 female twins suggesting that approximately 50% of the variation in sweet preference is driven by genetic factors (Keskitalo et al., 2007). Similar to bitter taste genetics, polymorphisms in sweet taste receptors genes *T1R2* and *T1R3* mediate sweet sensitivity (Eny et al., 2010; Fushan et al., 2009; U. Kim et al., 2006). However, polymorphisms in *T1R2* and *T1R3* polymorphisms are not consistently associated with alcohol intake. Whereas the relatively robust correlation between the perceived bitter intensity of PROP and bitter genotype enables researchers to test polymorphisms in

bitter genes against alcohol intake, no similar proxies that are easy to test, such as PROP taste strips, have been established for sweet receptor genes. Therefore, associations between sweet taste and alcohol intake in humans have been measured through preference tests of various sweet-tasting stimuli. Typically, subjects are presented with sucrose solutions of varying concentrations and asked to rank their preference for each sweet solution; subjects who prefer the highest or the second highest concentrations of sucrose are classified as sweet-likers, whereas subjects who prefer the lower concentrations are classified as sweet-dislikers (Bouhlal et al., 2018; Lange et al., 2010). Other classification methods such as average liking cutoffs have been used, and this variability in measuring sweet preference may be responsible for the lack of consensus surrounding the role of sweet taste genes in shaping alcohol intake (Iatridi et al., 2019). Research over the past two decades has demonstrated a positive correlation between family history of alcoholism and higher sweet preference, with paternal history of alcoholism in particular being cited as a predictor of sweet liking (A. Kampov-Polevoy et al., 1997; A. B. Kampov-Polevoy et al., 2001; Kampov-Polevoy et al., 2003; Pepino & Mennella, 2007; Wronski et al., 2007). However, the association may be limited to males (Lange et al., 2010; Robb & Pickering, 2019). Given that sweet preference and alcohol use are also associated with other personality traits such as novelty seeking and impulsiveness, it has been suggested that association may be driven more strongly by the genetics of the shared reward system involved in the hedonics of both sweet foods and alcohol rather than by sweet taste genes (Robb & Pickering, 2019; Thibodeau & Pickering, 2019). For instance, individuals carrying the A1 allele of the D2 dopamine receptor gene (*DRD2*) have reduced DRD2 availability, a risk

factor for substance use disorders including alcoholism (Blum et al., 1996; Fortuna & Smelson, 2011; Pohjalainen et al., 1998). The A1 variant is also associated with overconsumption, obesity, and a higher preference for sucrose (Jabłoński et al., 2013; Pepino et al., 2016; Rivera-Iñiguez et al., 2019), pointing to the possibility that hedonic responses are affected as a whole by a deficiency in the reward system such as the lack of DRD2 receptors, and that this overarching effect is the primary genetic factor linking sweet preference to alcoholism.

The burning sensation of alcohol also potentially serves as a genetic factor shaping alcohol intake

In addition to bitter and sweet, ethanol is perceived as having a burning sensation (Green, 1987), which is sensed via the transient receptor potential vanilloid channel 1 (TRPV1) (Trevisani et al., 2002). Various functional polymorphisms in *TRPV1* have also been found (Xu et al., 2007), with the I585V allele particularly being associated with variations in capsaicin sensitivity (Forstenpointner et al., 2017; H. Kim et al., 2004; Okamoto et al., 2018). The differential effects of *TRPV1* polymorphism on sensory perception of alcohol was demonstrated by Allen et al. (2014), who identified two alleles associated with alcohol's burning sensation by correlating the subjects' intensity ratings of ethanol samples to their genotypes for polymorphisms in *TRPV1*, *T2R38*, and *T2R13*. It should be noted that they also found an association between *TRPV1* phenotypes and perceived bitter intensity, and that capsaicin is perceived by some individuals as tasting bitter (Green & Hayes, 2004). That the individuals who inherently perceive the burning sensation in alcohol as being intense are more likely to avoid alcohol suggests that *TRPV1* polymorphisms are also a taste-related genetic factor that can influence alcohol intake.

Predisposition to alcohol consumption is driven by various genetic and environmental factors

To summarize the influence of genetic factors on alcohol consumption and use disorder in humans, no single genetic variation is considered to be a key driver in an individual's predisposition to consume alcohol. Instead, inherent differences in alcohol metabolism, reward systems, perception of bitter taste, perception of sweet taste, and perception of burning sensation conferred by genetic variation have all been linked in some capacity to shaping alcohol intake and its potential abuse. In our study, we focused on hereditary factors that shape the inherent sensory perception of alcohol, as taste acts as a primary behavioral gateway to alcohol consumption. Understanding what predisposes an individual to consume more alcohol will guide clinical tactics in preventing and mitigating the harm caused by alcoholism.

Inherent variances in ethanol preference also exists in mouse models used to study alcohol consumption

To study the genetic factors associated with alcoholism, various inbred mouse strains that approximate specific human phenotypes related to alcoholism have been developed over the years (Bennett et al., 2006). These include strains that model behaviors such as alcohol tolerance (Erwin & Deitrich, 1996), sensitivity to the sedating effects of alcohol (Kalinichenko et al., 2019; Thiele et al., 2000), and excessive use or aversion (Becker & Lopez, 2004; Blednov et al., 2017; Jensen et al., 2021; Loos et al., 2013). For alcohol preference-related behaviors, strain-specific variations in preference have been studied widely across four particular mouse strains: C57BL/6, DBA/2J, BALB/cJ, and 129S1/SvlmJ (Bachmanov, Reed, et al., 1996; Bachmanov, Tordoff, et al., 1996; Halladay et al., 2017; Kerns et al., 2005; Metten et al., 2010; Yoneyama et al.,

2008; Yorgason et al., 2015). In ethanol-preferring C57BL/6 mice, a positive correlation between preferences for ethanol and for sweet taste has been demonstrated behaviorally, where mice supposedly preferentially consume ethanol due to the association between sweet taste and caloric intake (Bachmanov et al., 2011). In addition, not only sweet but also bitter and burning components of ethanol have been demonstrated to mediate ethanol intake in C57BL/6 mice (Blednov & Harris, 2009; Blizard, 2007; Blizard & McClearn, 2000), making mice an appropriate animal model in which to study the effects of taste on alcohol consumption.

Nonetheless, a wide range of intake differences as well as sex effects have been observed in the ethanol intake behaviors of C57BL/6 mice (Dole et al., 1988; Wolstenholme et al., 2011), with female mice tending to consume more ethanol or preferring higher concentrations of ethanol (Melo et al., 1996; Middaugh et al., 1999; Richard, 2019; Wahlsten et al., 2003). Such variations were also observed in our previous study, in which preference to 10% ethanol ranged between approximately 20 to 80%, and female mice exhibiting notably higher preferences as a group compared to males. While Wolstenholme et al. (2011) reported that preference for saccharin and quinine did not correlate with preference for 10% ethanol in C57BL/6NCrl mice and concluded that sweet or bitter taste preferences are unlikely to mediate ethanol preference, our unpublished data suggested sex-specific correlations between sucralose and quinine preferences and ethanol preference in C57BL/6J mice. It should be noted that genetic polymorphisms have been shown in C57BL/6 sub-strains, as well as minor differences in alcohol intake between C57BL/6NCrl and C57BL/6J sub-strains (Khisti et al., 2006; Mulligan et al., 2008; Rhodes et al., 2005; Zurita et al., 2011), which

may account for the discrepancy in our results. Based on our data from C57BL/6J mice, we could not rule out the possibility that taste-related factors contribute to the variations in the observed variations in ethanol preference. Therefore, we hypothesized that individual differences in ethanol preference can be attributed to inherent variances in preference for bitter and or sweet tastes, and aimed to test for a positive correlation between ethanol preference and expression patterns of taste function-related genes. Further, we hypothesized that sex-specific trends similar to those demonstrated by others would be evident. By testing the correlations of taste-related gene expression patterns against variation in ethanol preference, our study will supplement the current knowledge of the genetic factors that can influence the perception of alcohol, thereby providing additional insights into how studies of alcohol preference using C57BL/6J mice can more accurately model nuances in alcohol-related behavior.

METHODS

Animals

Experiments were designed and performed in compliance with Cornell University's Institutional Animal Care and Use Committee (IACUC) regulations. Animals used in this study were in-house bred single-housed C57BL/6J male and female mice originally purchased from Jackson Labs (Bar Harbor, ME) maintained on an ad libitum diet of standard chow (Teklad 2918, global 18% protein rodent diet).

Behavioral testing to establish baseline taste preference correlations

At 10±0.5 weeks of age, single-housed mice underwent a series of taste preference tests for bitter (0.018mM quinine), and sweet (0.15mM sucralose) tastes as well as 10% ethanol, compared to water (Figure 1). Two 15ml glass bottles were provided to each mouse, with spouts inserted into the cage side-by-side. Bottles were swapped in position at 24-hour mark to minimize any side preference. Mice were given water only for 24 hours in between each round of testing to offset potential carryover effects. Weights of filled bottles were recorded at the start and end of each round of preference testing. The difference in pre- and post-testing weight was used to calculate preference. Prism 9.0.2 software (GraphPad, San Diego, CA, USA) was used to analyze and visualize behavioral testing results.

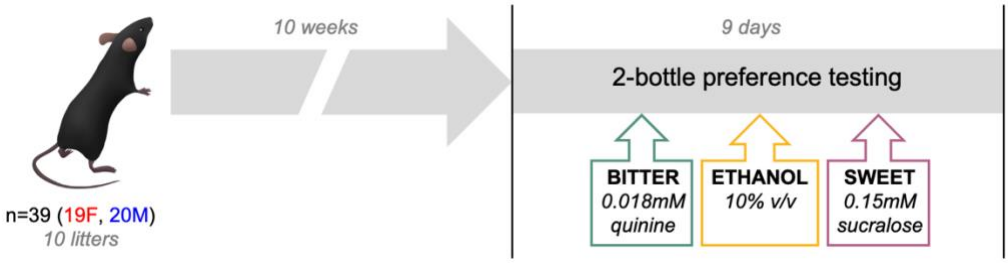

Figure 1. Schematic of behavioral testing to establish taste preference correlations. 20 male and 19 female mice from 10 litters were tested for bitter, ethanol, and sweet preference at 10±0.5 weeks of age.

Behavioral testing to test inherent ethanol preference

At 10±0.5 weeks of age, a second group of single-housed mice underwent one round of two-bottle preference test and tested as described in Chapter 1 (Figure 2). Naïve mice were used to most accurately gauge the inherent ethanol preference of each mouse, not affected by having tasted other taste stimuli prior.

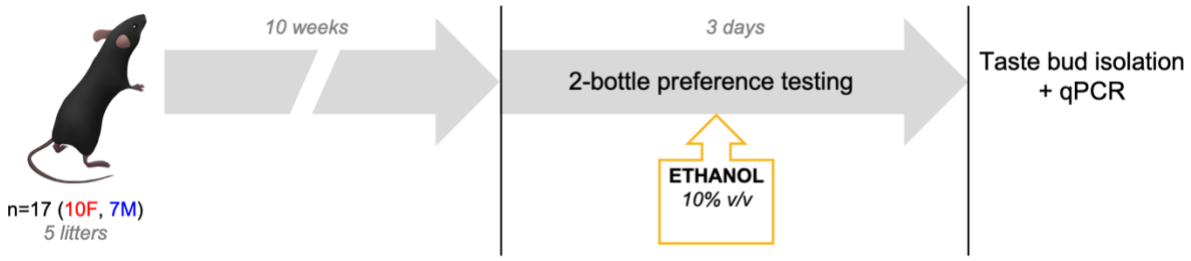

Figure 2. Schematic of behavioral testing to test inherent ethanol preference. 7 male and 10 female mice from 5 litters were tested for ethanol preference at 10±0.5 weeks of age. Following preference testing, they were euthanized and their tongues were collected to isolate RNA from their taste buds.

mRNA expression in taste buds

At the end of ethanol preference testing, the second group of mice were euthanized with CO_2 followed by cervical dislocation, and tongues were collected. RNA was extracted from taste cells in the circumvallate papillae and as described in Chapter 1, and mRNA expression levels of genes encoding the following proteins in taste buds were measured: β-actin (housekeeping); GNAT3, and TRPM5 (taste transduction markers); TRPV1 (nocireceptor); mGluR1, T1R1, T1R2, and T1R3 (umami and sweet taste receptors); T2R5, T2R8, T2R26, T2R37, and T2R40 (quinine-activated bitter receptors); and T2R23 (non-quinine-activated bitter receptor) (Table 1).

The expression level of each gene was quantified using 3 technical replicates, and the average of the three cycle threshold (Ct) values of each gene of interest was

normalized against that of the housekeeping gene β-actin to provide a measure for relative expression level. The resulting dCt values were plotted against ethanol preference to probe the potential effects of a given gene on shaping ethanol preference in naïve mice. Expression levels of TRPM5, mGluR1, and T2R40 were below the detection threshold in more than two-thirds of the samples and thus did not allow us to test for meaningful correlation; these genes are not included in final results. Prism 9.0.2 software was used to analyze and visualize gene expression data.

Table 1. Primer pair sequences for qRT-PCR (Lossow et al., 2016; Tomonari et al., 2012).

Gene Symbol	Protein	Forward Sequence (5'–3')	Reverse Sequence (5'–3')
Actb	β-actin	CACCCTGTGCTGCTCACC	GCACGATTTCCCTCTCAG
Gnat3	GNAT3	GCAACCACCTCCATTGTTCT	AGAAGAGCCCACAGTCTTTGAG
Trpm5	TRPM5	GTCTGGAATCACAGGCCAAC	GTTGATGTGCCCCAAAAACT
Trpv1	TRPV1	CGAGGATGGGAAGAATAACTCACT	GGATGATGAAGACAGCCTTGAAGT
Grm1	mGluR1	CGCTCCAACACCTTCCTCAACATT	GGGGTATTGTCCTCTTCCTCCACG
Tas1r1	T1R1	CTGGAATGGACCTGAATGGAC	AGCAGCAGTGGTGGGAAC
Tas1r2	T1R2	AAGCATCGCCTCCTACTCC	GGCTGGCAACTCTTAGAACAC
Tas1r3	T1R3	GAAGCATCCAGATGACTTCA	GGGAACAGAAGGACACTGAG
Tas2r105	T2R5	GAATCATAGAAACAGGACCTCG	CTTTACAAAGGCTTGCTTTAGC
Tas2r108	T2R8	TTCTGATTTCAGCCCTCACC	CCAAAAGCTGGTCCTGTTTC
Tas2r123	T2R23	CCAGATTCATTTATGTCTTGTCTATGC	GCATTTCTGATCTCCTAGGCAAA
Tas2r126	T2R26	TGGTTGAAGTGGAGATTCCC	TGGTTTCCCCAAAAGAACAG
Tas2r137	T2R37	GTCTCAGCATCACTCGGCTTT	GCAGGCGAGCTGAATAGCA
Tas2r140	T2R40	ATGAATGCTACTGTGAAGTG	CTAAGGACCTGGGAGTTC

RESULTS

10-week-old, naïve C57BL/6 mice underwent preference testing for bitter (0.018mM quinine), ethanol (10% v/v), and sweet (0.15mM sucralose) in that order to assess whether baseline ethanol preference was correlated with bitter and/or sweet preference. Additionally, only ethanol preference was tested in another group of 10-week-old, naïve C57BL/6 mice. This group was euthanized to collect RNA from taste buds, which was used to assay expression levels of taste-related genes. Naïve mice that had not been exposed to any other taste stimuli besides those of water and

standard chow were used to measure inherent ethanol preference as independently of exposure to learned effects of other tastes as possible. In the figures below, the delta of cycle threshold (dCt) is used as a measure of gene expression, with a larger value indicating higher level of expression. Correlations are not significant unless otherwise specified in the figure.

Ethanol preference was positively correlated with bitter preference in males, and with sweet preference in females

Ethanol preference ranged broadly between mice, between 14-91% with a median of 64.6% in the overall group of mice (n=39). Females had a significant higher preference than males when analyzed by sex (Figure 3A, p=0.0105; n=20 females, 19 males). Total fluid consumed remained relatively consistent across all treatments (data not shown). The trend between sexes was validated in the second set of mice (Figure 3B, p=0.0068; n=10 females, 7 males).

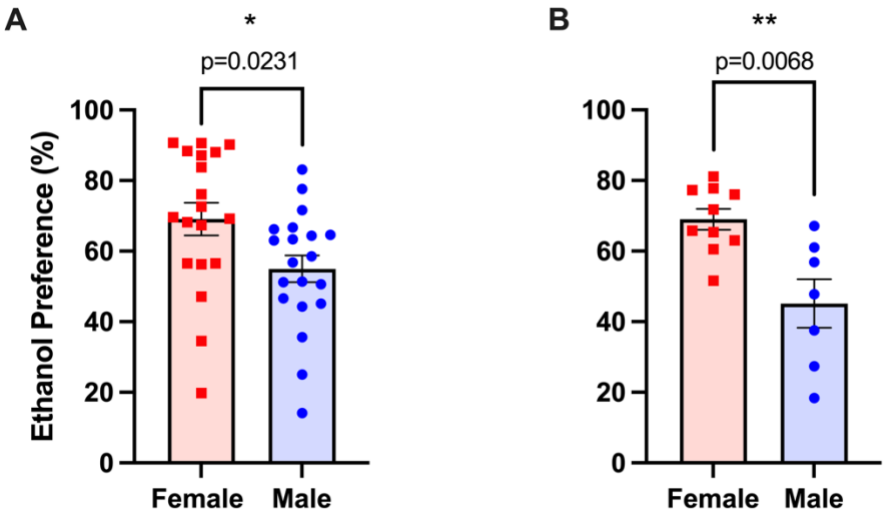

Figure 3. Female mice preferred 10% ethanol more so than males. This trend was observed in both sets of mice tested in this study. The difference in test designs of the two sets is shown in Figure 1 and Figure 2. (A) Mean with SEM, p=0.0231; n=20 females, 19 males. Dataset passed the D'Agostino & Pearson normality test and was analyzed using unpaired, parametric t-test. (B) Mean with SEM, p=0.0068; n=10 females, 7 males. Dataset did not pass the D'Agostino & Pearson normality test and was analyzed using unpaired, nonparametric t-test.

Ethanol preference was plotted as a function of bitter preference (Figure 4A) and sweet preference (Figure 4B) to assess whether it was inherently associated with bitter taste, sweet taste, or both. Mice were separated by sex to determine whether there was a sex-specific component to these associations, as has been reported in previous work (Melo et al., 1996; Middaugh et al., 1999; Richard, 2019; Wahlsten et al., 2003). Ethanol preference was positively correlated with bitter preference in naïve male mice (Figure 4A, p=0.0384, R^2=0.2172), whereas it was positively correlated with sweet preference in naïve female mice (Figure 4B, p=0.0225, R^2=0.2702) and more weakly with all mice as one group (Figure 4B, p=0.0304, R^2=0.1205). No litter-specific effects were observed. To infer more on mechanism from this data, taste-related genes were assayed to examine whether gene expression data aligned with those from taste preference.

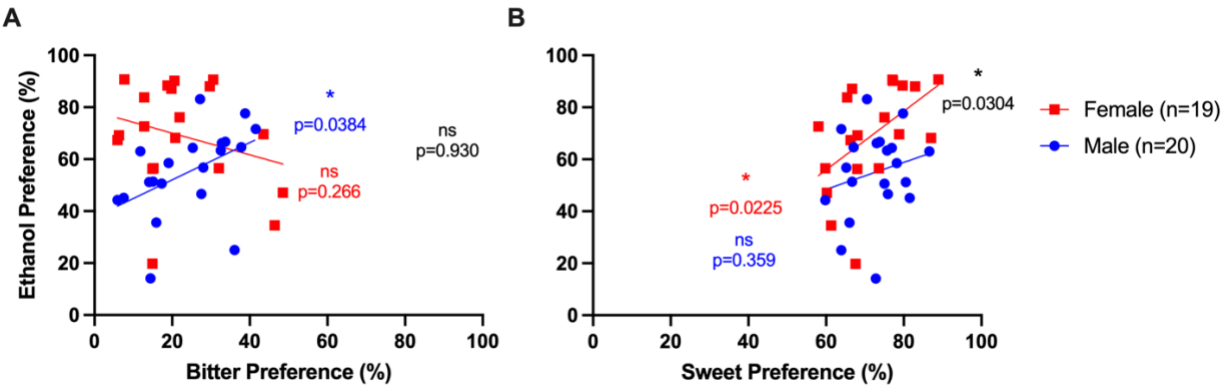

Figure 4. Baseline correlation of ethanol preference to (A) bitter and (B) sweet preferences. 0.018mM quinine was used to test bitter preference, and 0.15mM sucralose was used to test sweet preference. Preferences were tested at 10 weeks in naïve C57BL/6J mice (n=19 females, 20 males).

Ethanol preference was not correlated with sweet- and umami-sensing receptor gene expression in naïve mice

There were no correlations between ethanol preference and the expression patterns of genes encoding the umami-sensing T1R1 receptor (Figure 5A; p=0.6244 for

females, p=0.9701 for males), sweet-sensing T1R2 receptor (Figure 5B; p=0.2236 for females, p=0.7272 for males), or the sweet- or umami-sensing T1R3 receptor (Figure 5C; p=0.7078 for females, p=0.5895 for males). No litter effects were observed.

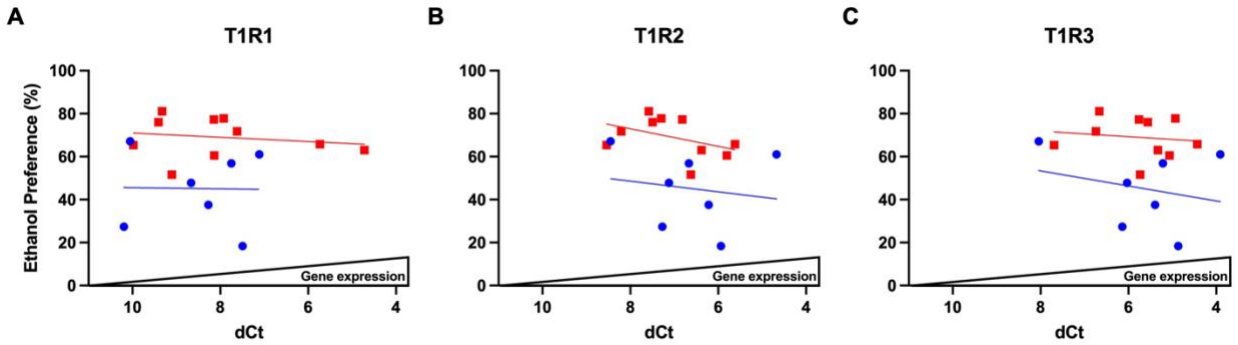

Figure 5. Sweet- and umami-sensing receptor gene expression levels did not correlate with ethanol preference (n= 10 females, 7 males).

Ethanol preference was negatively correlated with T2R26 and T2R37 bitter receptor expression in naïve male mice

Expression levels of mRNA for genes encoding T2R26 (Figure 6C; p=0.0241, R^2=0.6712) and T2R37 (Figure 6D; p=0.0227, R^2=0.6786), quinine-activated bitter receptors (the bitter stimulus used in bitter preference testing above), negatively correlated with ethanol preference in naïve male mice. Greater expression of mRNA for T2R26 and T2R37– and thus potentially higher number of T2R26 and T2R37 bitter receptors – would reasonably imply a heightened ability to sense any aversive bitter taste in ethanol, hence leading to a lower degree of preference in the animal. Such a pattern was not observed for two other quinine-activated bitter receptors, T2R5 (Figure 6A; p=0.4458 for females, p=0.2123 for males) and T2R8 (Figure 6B; p=0.2454 for females, p=0.9362 for males), nor in the non-quinine-activated bitter receptor T2R23 (Figure 6E; p=0.7992 for females, p=0.0833 for males). No litter effects were observed.

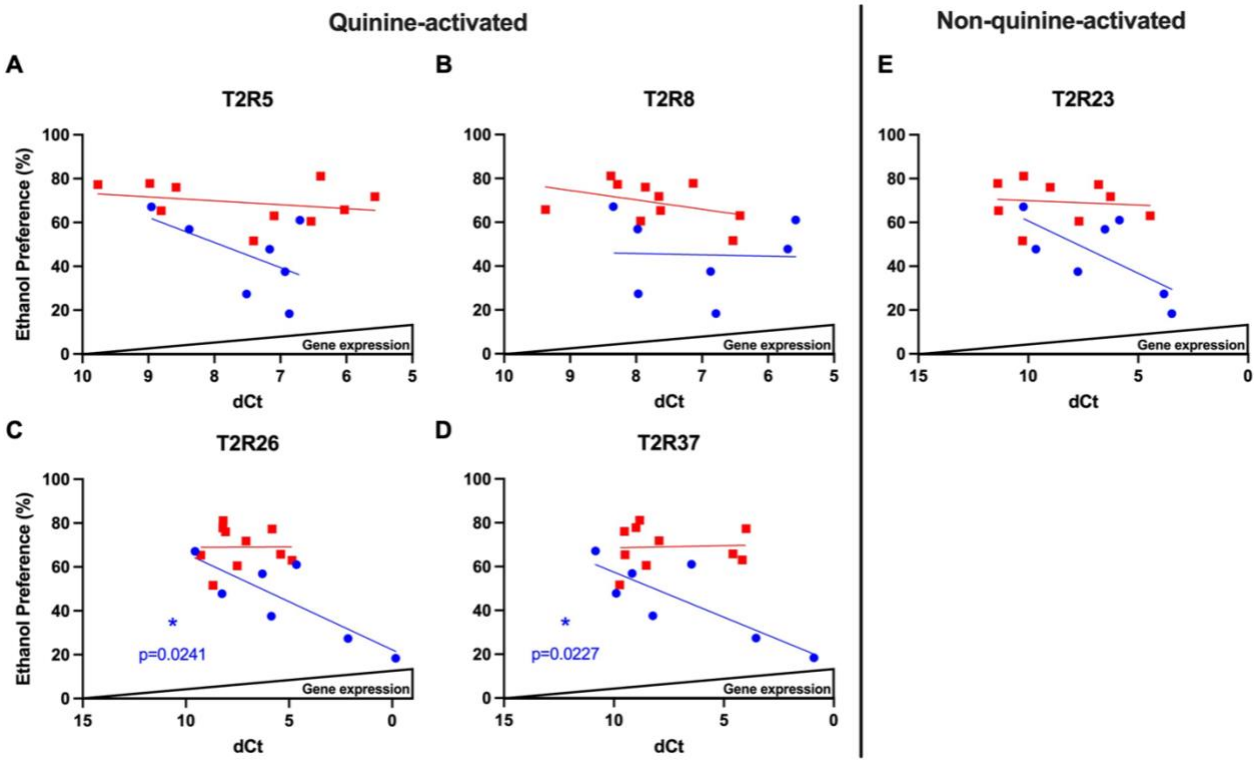

Figure 6. Quinine-activated T2R26 (C; p=0.0241, R^2=0.6712) and T2R37 (D; p=0.0227, R^2=0.6786) bitter receptor gene expression levels were correlated with ethanol preference in naïve male mice (n= 10 females, 7 males).

Ethanol preference was negatively correlated with GNAT3 expression in naïve male mice

Male mice that expressed higher levels of the taste-transducing gene GNAT3 had lower preference for ethanol (Figure 7; p=0.4050 for females, p=0.0042, R^2=0.8317 for males). Reports suggest that while GNAT3 is an acceptable marker for type 3 taste cells that transduce bitter, sweet, or umami signals, its expression may in fact be limted to bitter-sensitive cells (Adler et al., 2000; Choo & Dando, 2020; M. Kim et al., 2003; Tomonari et al., 2012). This negative correlation further supports that bitterness in alcohol becomes more prominent when taste signals are stronger, suggesting that ethanol perception may be associated more with bitter than sweet taste in males (Figure

4). This correlation was not observed in female mice, and while the response of female mice to ethanol seemed more related to their response to sweet taste, this was likely due to ethanol preference remaining relatively consistent across the mice, regardless of GNAT3 expression level. Again, no litter effects were observed.

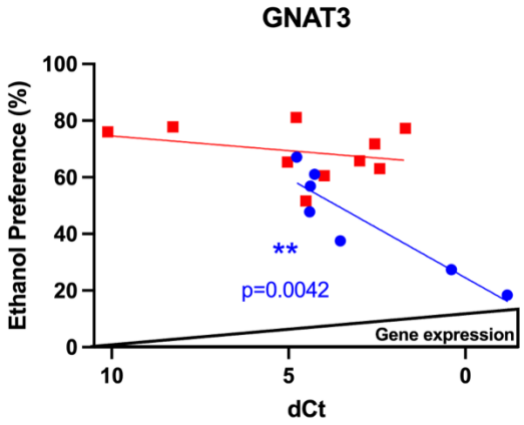

Figure 7. GNAT3 expression was negatively correlated with ethanol preference in naïve male mice ($p=0.0042$, $R^2=0.8317$; n= 10 females, 7 males).

Ethanol preference was not correlated with TRPV1 expression in naïve mice

As well as tasting sweet and bitter, ethanol has a component related to a burning sensation, reported to be related to the heat sensing channel TRPV1 responsible for the detection of spiciness in foods (Blednov & Harris, 2009b). The expression patterns of the heat-sensing vanilloid receptor-1 (TRPV1) did not correlate with ethanol preference (Figure 8; p=0.3070 for females, p=0.1125 for males). This result could indicate either that 1) the ability to detect the burning sensation of ethanol is not a driver of ethanol preference, or 2) the range of TRPV1 expression levels here is too narrow for there to be a physiologically relevant difference between the high and low ends of expression. This does not indicate that other receptors sensing the chemesthetic properties of

ethanol cannot be ruled out as potential drivers of this ethanol preference. No litter effects were observed.

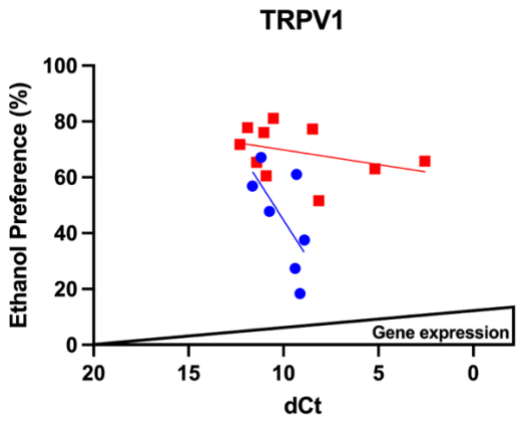

Figure 8. TRPV1 expression was not associated with ethanol preference (n= 10 females, 7 males).

DISCUSSION

In this study, we confirmed a significant variation in inherent ethanol preference between sexes in mice, with females preferring 10% ethanol more so than males. These variations in ethanol preference were positively associated with bitter preference in males, but with sweet preference in females. Variation in ethanol preference was also associated with gene expression levels of *GNAT3* (taste transduction) and *T2R26* and *T2R37* (bitter receptors) in male mice, further emphasizing sex differences in how taste influences preference for alcohol.

Inbred C57BL/6J mice exhibit inherent variability in ethanol preference that is further stratified by sex

Individual preferences for 10% ethanol ranged from 14% to 91% in 20 female and 19 male C56BL/6J mice (Figure 3). Wolstenholme et al., (2011) observed a slightly greater range in individual preferences for 10% ethanol, ranging from 1.5% to 95.0% in 36 male C57BL/6NCrl mice. These mice were comparable in age, approximately 10 and

9 weeks old, respectively. The difference in the preference ranges is aligned with the previous finding that C57BL/6NCrl mice exhibit a greater ethanol preference range compared to C56BL/6J mice (Mulligan et al., 2008). Our results also support previous work showing that females prefer ethanol significantly more so than males (Middaugh et al., 1999; Mulligan et al., 2008; Richard, 2019; Wahlsten et al., 2003). The implications of this sex difference will be discussed in the final section.

Due to the expectation that genetic factors are controlled for in experiments with inbred strains, it has been suggested that non-genetic factors such as rearing conditions or epigenetic mechanisms are responsible for individual variations in alcohol intake and preference (Brake et al., 2004; Dole et al., 1988; Wolstenholme et al., 2011). However, investigation into genetic stability of inbred strains including C57BL/6 mice have found single-gene mutations and heterozygote selection that have called into question the degree of inbred strains' isogenicity (Casellas, 2011; Casellas & Medrano, 2008; Chebib et al., 2021; Lathe, 2004). It should be noted that these findings do not negate the use of inbred strains but rather suggest that we should understand them to be relatively homogenous with minor genetic variations that could produce more nuanced behavioral data. In the context of investigating ethanol preference, the data imply that we cannot conclude that variations in ethanol preference observed in C57BL/6 mice are due to non-genetic factors simply based on the inbred nature of the animals. Our sample size was not large enough to draw meaningful conclusions about potential litter effects on preference patterns, but it remains possible that a large-scale study may reveal litter effects that drive important variation in preference.

It has also been suggested that the a wide variation in preference for 10% ethanol may be due to the relative insensitivity of C57BL/6 mice to ethanol (Dole et al., 1988). However, Belknap et al. (1993) has shown that male C57BL/6 mice will also consume varied amounts of 0.2% saccharin solutions with 3%, 6%, and 10% ethanol added in two-bottle preference testing, indicating their ability to pick out differences in ethanol solutions even in a sweetened mixture. Therefore, we should not assume that these mice are insensitive to the taste of ethanol to the degree that they will consume 10% ethanol to a random degree.

To our knowledge, the potential effects of taste genes on alcohol intake has primarily been studied through comparisons of strains known to diverge in their preference for ethanol, such as the ethanol-preferring C57BL/6 mice and the non-preferring 129/J or DBA mice (Bachmanov, Tordoff, et al., 1996; Peirce et al., 1998). Additionally, conflicting evidence on the behavioral association between ethanol preference and taste, combined with the assumption that inbred strains are isogenic, have limited the degree to which taste related-genes have been explored further within C57BL/6 mice. In the following sections, we will discuss data demonstrating sex-specific correlations between preference for 10% ethanol and differential taste gene expression patterns that highlight the need to consider taste-related factors in alcohol consumption.

Behavioral associations between ethanol preference and taste are driven more by bitter taste in males but by sweet taste in females

There is conflicting evidence on whether bitter sensitivity is related to ethanol intake in rodents, with sex difference often used to explain the observed variation in this relationship (Bachmanov, Tordoff, et al., 1996; Loney & Meyer, 2018). Sex differences in alcohol preference in C57BL/6 mice have been explained through the discovery of

the sex-specific alcohol preference loci (*ALCP*) ((Melo et al., 1996; Peirce et al., 1998)). C57 mice demonstrate a relatively high ethanol preference even though they are natively averse to quinine and can detect bitterness in 10% ethanol (Blizard, 2007). This behavior suggests that either the liking for the sweet component in ethanol is strong enough to compensate for the coexisting bitter taste, that aversion to bitter is weak enough for them to prefer the bitter-sweet taste of ethanol, or that there is some learned association with ethanol capable of overcoming its bitter taste. We saw both sweet liking associated with high ethanol consumption in females and bitter aversion associated with low ethanol liking in males (Figure 4), a divergence in taste behavior based on sex. While (Bachmanov et al., 2011) previously noted that the hedonic value of the sweet component in ethanol is more likely to drive consumption from a taste standpoint, our data points to the possibility of a more complex nature to how taste contributes to ethanol preference.

The positive association between sweet and ethanol preference has been studied in more depth in part due to its significance in humans, although foundational studies on the subject were limited to male mice ((Bachmanov, Tordoff, et al., 1996; Belknap et al., 1993)). Bachmanov, Reed, et al. (1996) and Belknap et al. (1993) demonstrated a correlation between sweet and ethanol consumption using sucrose and saccharin respectively, both in male mice; the discrepancy between these and our results suggests the possibility that the lack of correlation between males' preference for ethanol and sweet taste may be attributed to our use of sucralose, selected to minimize postingestive effects expected of sucrose, which may artificially increase preference and due to its reported lower bitterness. However, the correlation observed in the study

by Belknap et al. (1993) may in fact be specific to saccharin preference in male mice, as the study by Yoneyama et al. (2008) that expanded on the 1993 work by including mice of both sexes failed to demonstrate a correlation between ethanol and saccharin preference. In addition, Meliska et al. (1995) also could not demonstrate a correlation when using mice of both sexes and with aspartame as a sweet tastant. Collectively, our data, in conjunction with existing evidence, suggest that the sweet-ethanol correlation may be dependent on both sex and sweetener type. While the preference testing order going from the more aversive quinine, ethanol, then more appetitive sucralose was determined to minimize carryover effects (Beauchamp & Fisher, 1993; Crabbe et al., 2011), differences in testing orders and duration across studies may also have been a factor.

Ethanol preference was associated with mRNA expression for bitter receptor genes T2R26 and T2R37 and the taste transducing gene GNAT3 in males

Providing further support for our finding that ethanol preference was associated with bitter taste in males was the inverse relationship between ethanol preference and expression levels of mRNA for bitter receptor genes *T2R26* and *T2R37* (Figure 6C, Figure 6D). In addition, the same relationship was demonstrated in the expression level of the taste transducing gene *GNAT3* (Figure 7). These correlations were not observed in females, potentially due to the higher ethanol preference of females leading to a ceiling effect.

The significance of why differential expression levels of *T2R26* and *T2R37* specifically are associated with ethanol preference in males is yet to be determined. They are activated by quinine above a threshold concentration of 0.01mM, however, so are *T2R5* and *T2R8*, which excludes activation threshold as a factor. To our knowledge,

existing literature on mouse bitter receptors do not report either gene as being activated ethanol in mice. As for humans, a phylogenetic analysis of mouse and human bitter receptor genes based on amino acid sequence alignment and chromosomal localization have revealed mouse *T2R26* and *T2R37* to be orthologs of human *T2R41* and *T2R3*, respectively (Lossow et al., 2016). Besides one study demonstrating an association between genetic variations in T2R3 and papillary thyroid carcinoma risk and thyroid function in Korean females (Choi et al., 2018), no other physiological significance of these genes in humans has been reported. Future works on characterization of bitter receptor genes may demonstrate the significance of these genes on alcohol consumption behavior.

CONCLUSION

In humans, individual preference for ethanol varies due to genetic factors such as polymorphisms in alcohol-related genes such as aldehyde dehydrogenase 2 (*ALDH2*) and μ-opioid receptor (*OPRM1*), as well as taste-related genes such as T2R bitter receptor genes and taste-transducing Guanine nucleotide-binding protein G(T) subunit alpha-3 (*GNAT3*). In C57BL/6 mice, variations in individual ethanol preferences are considered to be non-genetic, and they have only been shown to correlate with taste behaviorally or through genetic studies conducted through comparisons across strains. In our study, we tested the hypotheses that 1) individual differences in ethanol preference of C57BL/6 mice can be attributed to inherence variation in taste function, and 2) these differences are further divided by sex. We found ethanol preference to be associated with sweet liking in females and bitter aversion in males, with no genetic association with sweet taste function but correlations with *T2R26*, *T2R37*, and *GNAT3*

expression in males only. Our results demonstrate a genetic link between ethanol preference and bitter sensing or taste transducing capacities of male C57BL/6 mice and suggest that the potential effects of taste function on alcohol preference be further explored through the characterization of additional taste-related genes. Given the significance of C57BL/6 mice as an ethanol-preferring animal model in research on alcoholism, a better understanding of taste's role in alcohol consumption and the potential for inter-strain genetic variation would enhance the effectiveness with which animal models are used for such research.

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