CONSEQUENCES OF OBESITY AND CHEMOTHERAPY ON THE GUSTATORY SYSTEM

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The taste system acts as a gatekeeping mechanism, to protect from toxins, and allows humans to select foods that contain beneficial nutrients. In developed countries, undernutrition arising from food availability problems has largely been addressed, whereas overnutrition leading to obesity has become an increasingly pressing issue. The gustatory system has long been neglected as a target to regulate food intake, to address overconsumption. Taste serves in guiding dietary selection, thus any change in taste in the obese, would offer an explanation for the altered behaviors around food in the obese. In the following chapters we will focus on further understanding obesity and diet induced changes to the taste bud.

First, we will discuss the link between obesity-induced taste dysfunction and food intake with a focus on the appetitive tastes. Then, building on previous research, we will show that diet induced obesity, independent of calorie source, causes loss of taste buds in Sprague Dawley rats. A loss of taste buds with obesity has previously been reported in mice and humans, with both showing correlations between fungiform papillae density and weight. Additionally, in rat circumvallate papillae, taste buds are negatively correlated with caspase-positive cells, a marker for apoptosis, implying that taste buds are being actively broken down in obesity. Next, we show that dieting C57BL/6 mice to a lean weight, after previously being obese, allows mice to partially, but not fully recover taste buds. Concurrently we show reduced proliferative capacity, an increase in apoptosis and an elevation in the harmful cytokine TNFα in both female and male mice with obesity, effects partially rescued by dieting mice. Understanding the underlying mechanisms driving obesity related taste changes will give a better understanding of the scope for future intervention strategies. Finally, we will examine changes to the taste bud after

chemotherapeutic treatment, where taste deficiencies are commonly reported, and in which dietary intake is again of great importance to determining health outcomes.

BIOGRAPHICAL SKETCH

Fiona Harnischfeger received her BSc in Biochemistry and Cell Biology from Jacobs University in 2016. During her undergraduate studies she researched Chronic Lymphocytic Leukemia at the National Institutes of Health in the USA, B cells at Tsinghua University in China, neural circuits in the brain associated with food intake in the brain at the University of Cambridge in the UK and MHC Class I molecules at Jacobs University in Germany. Right after completing her undergraduate, Fiona started her PhD at Cornell in 2016 to combine her previous experience in inflammation, neurobiology and food intake research along with the technical skills she learned working in the lab of Dr. Robin Dando.

At Cornell, Fiona served as a Graduate Resident Fellow in the undergraduate dorms on West Campus for two years developing community and mentoring undergraduates. She was also president and co-founder of the Cornell Graduate Consulting Club. She was a board member on the Careers Beyond Academia advisory board, endowed by the National Institutes of Health with $3.7 million dollars. As a result of her work she was also asked to join the graduate professional development advisory council which makes recommendations around professional development to the dean of the graduate school.

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CHAPTER 1

OBESITY-INDUCED TASTE DYSFUNCTION, AND ITS IMPLICATIONS FOR DIETARY INTAKE

Abstract

The incidence of obesity has dramatically increased in recent years, and poses a public health challenge for which an effective intervention strategy is yet to be found. Our food choices are the primary driver of obesity, where the overconsumption of energy from foods high in fat and sugar can be particularly problematic. Unfortunately, these same foods also tend to be highly palatable. We select foods more on their taste properties than on any other factor, such as price, convenience or healthfulness. Previous evidence from human sensory studies has suggested a depressed sense of taste in panelists with obesity. Evidence from animal models also demonstrates a clear deficiency in taste buds occurring with obesity, suggesting that damage to the taste system may result from an obese state. Here we seek to bring together evidence from a diverse array of human and animal studies into taste response, dietary intake, and physiology, to better understand changes in taste with obesity, with the goal of understanding whether taste may provide a novel target for intervention in the treatment of obesity.

Introduction

Across the world, the prevalence of obesity has tripled from 1975-2016 (Hunter & Reddy, 2013). Globally, around 2 billion adults have a Body Mass Index (BMI) which categorizes them as either overweight or obese (Hamann, 2017). Obesity is associated with many non-communicable diseases including type II diabetes (Guariguata et al., 2014), cardiovascular disease (Zalesin et al., 2011), hypertension (Seravalle & Grassi, 2017), and metabolic disease (Després & Lemieux, 2006), as well as an elevated risk for all-cause mortality (C. Chen et al., 2019). Obesity also has a negative impact on health-related quality of life (Kolotkin & Andersen, 2017). Multiple factors influence the development of obesity, but food intake, as the primary origin of a positive energy balance, is the most important factor in weight gain (Jeffery & Harnack, 2007; Wright & Aronne, 2012). Per capita energy intake of food with high caloric content but low levels of nutrients has been steadily rising for many years (Ford & Dietz, 2013; Haslam & James, 2005; Young & Nestle, 2003). Highly palatable and highly caloric foods containing excesses of fats and sugars are increasingly available in the modern food environment (Crino et al., 2015; Naughton et al., 2015; Rikkers et al., 2013; Vandevijvere et al., 2015), where excess intake of fat and sugar can lead to a chronic positive energy balance, and result in weight gain (Chaput et al., 2012; Hooper et al., 2015; Morenga et al., 2013; Mozaffarian, 2016). The abundant availability of foods high in saturated fats and added sugars that are consumed in developed countries makes this problem ever more acute (Juul & Hemmingsson, 2015; Nardocci et al., 2019; Solberg et al., 2016; Zobel et al., 2016).

One way to correct an energy imbalance is to increase energy expenditure. Indisputably, regular exercise has clear benefits and improves overall health, evident in a decrease in all-cause mortality as well as a decreased chance of developing type II diabetes, cardiovascular disease, or hypertension (King et al., 2009). Despite this, the vast majority of randomized control trials, the gold standard for determining the efficacy of such interventions, conclude that regular exercise alone does not provide

effective gains in long-term weight loss (Foright et al., 2018). The body's metabolism also influences caloric expenditure. Thermogenesis is the metabolic expenditure of energy as heat, with evidence suggesting that dietary intake may further influence energy balance by altering the degree to which energy is consumed by thermogenesis. Weight loss induces changes in metabolic programming, and may be a reason for reduced energy expenditure through thermogenesis (Rosenbaum & Leibel, 2010), however, it is clear that long-term weight reduction cannot solely rely on increased energy expenditure.

According to the World Health Organization, an imbalance in energy can be counteracted by a diet characterized by fewer energy-dense foods like sugar-sweetened beverages and many processed foods, and more lower energy density foods like vegetables, fruits, and whole grains (Amine et al., 2003). As taste is a key driver of food choice (Aggarwal et al., 2016), and taste changes in individuals with obesity have been frequently reported, this review seeks to explore the links between taste, intake, and obesity. We will focus on changes to the appetitive tastes (sweet, salty, and umami), as well as discuss receptors for the candidate 6th basic taste, fat, as fat is also known to drive obesity, but exclude work on sour and bitter tastes that are more aversive in nature, and thus are not directly associated with increased caloric intake (Berridge, 2000). Genetic taste variation, taste changes with gastric bypass or type II diabetes, and gut microbiome changes are also considered out of scope for the review. Finally, although our focus is primarily on results from human subjects, we will also explore observations from obese non-human animals, which can provide insight into potential mechanisms

Taste is a key driver of food choice, promoting foods high in sweet, salt and fat

Thus far there has been no reliable, effective way to treat obesity and even drastic interventions like gastric bypass are not always effective long term. As taste is a key factor in food choice, targeting taste might be a novel approach toward weight control. People make food choices based on taste (Aggarwal et al., 2016; Glanz et al., 1998; Kourouniotis et al., 2016; Zylan, 1996), or more accurately, the sensory

properties of foods. Of US adults, 77% rate taste as a 'very important' factor in food selection, while 82% of Australian university students rate taste as a very or extremely important factor, with panelists preferring diets including fewer fruits and vegetables and more foods with higher fat, sugar, and salt content (Aggarwal et al., 2016; Kourouniotis et al., 2016). Foods associated with a positive energy balance usually contain an excess of fat and sugar, however, fat and sugar also tend to have a positive impact on the sensory profile of foods (Beauchamp & Cowart, 1987; Drewnowski, 1997; Lease et al., 2016). As well as increasing the probability of a net positive energy balance, foods high in fat and sugar can favor pathways that change the relationship between energy expenditure and energy intake and alter neuroendocrine signaling.

Overall, high-energy-density foods, which contribute to a positive energy imbalance, are more palatable than low-energy-density foods (Drewnowski, 1997). Foods with high fat or sugar content are high in energy density, are preferred by subjects with obesity , and further increase the risk of adiposity (Astrup et al., 1994; Blundell & Stubbs, 1999; Warwick & Schiffman, 1990; Yang et al., 2014). Links between intake and obesity are firmly established, and well-understood. In the remainder of this review we will examine how obesity and taste are related, and the links between taste and intake.

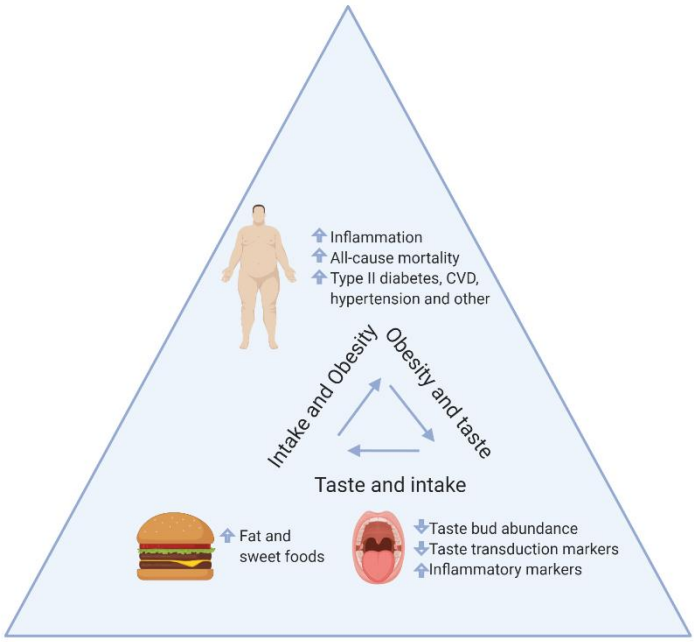

Figure 1: Depiction of the interplay between obesity, taste and food intake. Subjects with obesity exhibit increased inflammation, and see an increase in all-cause mortality and associated diseases. Increased inflammation in patients with obesity can lead to an impaired sense of taste, which in turn can promote increased caloric intake, from foods high in sugar and fat.

SECTION I: Taste, and intake

Links between the 5 recognized basic tastes and intake behavior

Correlating changes in taste with food intake is difficult, as assessing dietary intake presents well-documented challenges, particularly that subjects chronically underreport food intake (Harnack et al., 2000; Heitmann et al., 2000), which creates difficulties in gauging true intake patterns (Thompson & Byers, 1994). This issue is intensified in people with weights in overweight or obese categories (Chambers et al., 2000; Faggiano et al., 1992; Harnack et al., 2004). Short-term changes in food intake are more readily measured in a more controlled testing setup, but may no longer be representative of day-to-day intake behaviors of a free-living population. Isolating the influence of taste on food intake is

difficult, as both are driven by other factors, including post-ingestive, and post-absorptive effects, cognition, and the satiation and satiety a food brings (Chambers et al., 2015). Additionally, restrained eating or biased reporting can obscure the true connection between intake and sensory response, as BMI is associated with dieting, as well as the liking of fatty foods (Keskitalo et al., 2008). As taste guides food intake, and as we will establish, taste is weakened in those suffering from obesity, we will explore in this review a possible role for taste as a locus for obesogenic behaviors, promoting more unhealthy eating due to impaired taste function in those gaining weight.

A broad range of sensory testing techniques have been developed to characterize taste function independent of specific foods, a few of which will be discussed here. These tests can use prototypic tastant solutions or paper strips impregnated with tastants dissolved in a solvent (usually water), or sometimes whole foods, with subjects asked to respond to different aspects of the stimulus. In this review, perceived intensity ratings, ratings of hedonic liking, detection thresholds, and recognition thresholds will be discussed. These tests respectively give an understanding of how intense a set concentration of a stimulus is perceived, how much a subject likes a stimulus, and at what concentration a subject can detect or correctly identify a stimulus. A higher intensity rating, or a lower detection or recognition threshold are hallmarks of superior taste function, while one may infer a higher hedonic rating to be indicative that intake of that stimulus would be promoted.

Foods high in sugar are often associated with a positive energy balance (Morenga et al., 2013). In women 20-40 years of age, using glucose as a sweet tastant, intensity was negatively correlated with sugar and total energy intake. Dietary assessment, based on a four-day weighed food record, a sweet food frequency questionnaire, and a sweet beverage liking questionnaire, also correlated with hedonic liking of sweetness (Jayasinghe et al., 2017). These findings are in agreement with several additional studies. In both women and men, subjects with a reduced sensitivity to sweet taste also ate more

sweets and desserts (Cattaneo et al., 2019). In women with obesity aged 18-45, sweet liking and intake of sugar was positively correlated, based on a food frequency questionnaire (Singh, 2018). Perceived taste intensity was also inversely correlated with sweet liking. In a further study, subjects with obesity had lower sensitivity to all basic tastes, fewer fungiform papillae, and also reported higher liking of potentially high-energy-dense foods like carbohydrates (Proserpio et al., 2016). Conversely, during an ad lib buffet style meal, patients highly sensitive to sucrose ate more non-sweet foods and protein, and fewer carbohydrates (Han et al., 2017). Taken together, these studies suggest a link between sensitivity to sweet taste and intake of sweet foods.

A few studies found no link between sweet taste and intake. One study examining sweet taste did not see an association between recognition thresholds of nutritive and nonnutritive sweeteners (glucose, fructose, sucrose, sucralose, erythritol, and Rebaudioside A) and intake, though a trend was observed between suprathreshold sweet intensity ratings and total energy intake (Low et al., 2016) In another study, sensitivity to the non-nutritive sweetener aspartame was negatively associated with energy intake, based on a 7-day food diary (Martinez-Cordero et al., 2015). A systematic review looking at sweet taste as a predictor of dietary intake suggested that hedonic assessment of food is positively associated with intake, but found less evidence to support a strong link with sensitivity (Tan & Tucker, 2019). It should be noted that due to a variety of methods used in such studies, results are naturally highly variable, making them difficult to directly compare. Likewise, the cross-sectional design of most studies makes the inherent variance in human taste response problematic.

There is robust evidence that a change in intake of simple sugars impacts perceived taste intensity in adults. In healthy men and women, reducing habitual consumption of simple sugars increased perceived taste intensity from sweet solutions (Wise et al., 2016), in a manner reminiscent of earlier work on salt intake (Beauchamp et al., 1983; Bertino et al., 1982). Furthermore, selectively diminishing sweet

perception using a Gymnema sylvestre (GS) rinse increased a panelist's optimal sucrose content, as well as reducing liking of such foods (Noel et al., 2017). Rinsing with GS does not influence post-ingestive effects, such as gastric emptying or glycemic response (Kashima et al., 2020). Pharmacologically blocking taste acuity likely is not wholly representative of chronic depression of taste, as it is likely too extreme to measure all but short-term food intake, but it does give us an indication that patients may seek to make up for a depressed sense of taste by consuming more intensely tasting stimuli.

Adults with obesity eat more foods that are classified as salty, (Cox et al., 1999). Nutrient-poor processed and ultra-processed food is usually high in sodium (Monteiro et al., 2011), and high salt intake is associated with an increased risk for cardiovascular pathologies including hypertension and stroke (Rust & Ekmekcioglu, 2017; Seravalle & Grassi, 2017). In one very large study on salt taste liking and BMI, liking scores for fat and salt taste were positively correlated with BMI in both men and women (Deglaire et al., 2015), although not all work is in agreement with this finding (Donaldson et al., 2009; Hardikar at al., 2017; Pasquet et al., 2007). Prolonged exposure to a high-salt diet increases preferred levels of sodium and overall sodium consumption (Bertino et al., 1986). Similarly, prolonged exposure to a low-salt diet increases liking for lower salt levels and lowers perceived intensity of salt taste (Bertino et al., 1982; Blais et al., 1986). Reducing salt intake is an important part of reducing CVD risk (Rust & Ekmekcioglu, 2017), therefore shifting salt preference by decreasing salt intake may be an opportunity for long-term reduction of salt preference and intake (Bobowski, 2015).

In studies without an intervention to artificially reduce sodium intake, some correlation between sodium consumption and liking for salt is still reported (Hayes et al., 2010; Kim & Lee, 2009; Z. Zhang & Zhang, 2011). Healthy adults who have a reduced sensitivity for salty taste, measured by identification of salty taste at various concentrations, reported higher intake of salty, usually high-energy foods on the Food and Beverage Frequency Questionnaire (Cattaneo et al., 2019). The Mediterranean diet is considered

one of the healthiest diets, with a meta-analysis showing it can reduce the risk of metabolic syndrome, while being relatively easy to adopt (Kastorini et al., 2011). Recently, lower thresholds for salt taste were associated with a lower risk for metabolic syndrome but did not relate to better adherence to this healthy diet. Although subjects with lower salt thresholds were more likely to consume fruits, they were less likely to adhere to olive oil and white meat guidelines (Veček et al., 2020).

Studies examining umami taste versus intake are not abundant. Persistent exposure to monosodium glutamate can reduce perceived intensity of umami taste and diminish appetite for savory foods (Noel et al, 2018)**,** in a manner similar to that seen for salt, sugar or fat (Bertino et al., 1986; Newman et al., 2016). The addition of MSG to a vegetable soup decreased subsequent energy intake in women with an overweight or obese BMI (Miyaki et al., 2016). The return of hunger after eating soup containing MSG is slower than when eating soup without MSG (Masic & Yeomans, 2013), with similar results found comparing soup with MSG and protein versus soups without either ingredient (Anderson et al., 2018). Even infants who consume formula with MSG consume less formula than infants fed formula without MSG (Ventura et al., 2012). Finally, the addition of MSG/ disodium 5'-inosinate (IMP) to a low-energy preload had a biphasic effect on appetite, by stimulating appetite during ingestion and then enhancing post-ingestive satiety (Masic & Yeomans, 2014).

Umami signals the protein and amino acid content of our foods. In a systematic review, high-protein diets were associated with increased thermogenesis and satiety (Halton & Hu, 2004). Despite this, the taste threshold for monosodium glutamate (MSG) is not associated with liking or preference for protein (Luscombe-Marsh et al., 2008). It has been hypothesized that sweet and umami taste share commonalities. A study examining sweet and umami taste found that those with reduced sensitivity to umami also have reduced sensitivity to sweet taste. The umami tasters without reduced sensitivity to MSG consumed a similar numbers of calories to those with lower perceived umami taste, but ate more

seaweeds and less sugar (Kubota et al, 2018). As umami and sweet taste transduction share the heterodimeric G protein-coupled receptor type 1 member 3 (T1R3) (Nelson et al., 2002), this may represent a mechanism for altered taste function from dietary exposure, possibly linked to receptor regulation (Shahbandi et al., 2018).

Fat detection and diet

While there is only scientific consensus around 5 basic tastes there is growing interest in fat as a 6th basic taste. Dietary fat strongly affects obesity because of its high caloric content and palatability. Multiple studies examine fat sensitivity and dietary behaviors, but these studies tend to focus on short, and not long-term fat intake, and may be particularly vulnerable to previously described issued with dietary questionnaires being somewhat unreliable in gauging long-term intake patterns. In cross-sectional studies, evidence demonstrates a relationship between fat taste and intake, with hypersensitivity to fatty acids linked with a lower BMI, as well as lower energy and fat intake (Stewart et al., 2010). In one study with a focus on fatty acid detection, problems in detecting low concentrations of fatty acids were linked with a greater energy intake from fatty foods such as butter, meat, and dairy, and also with a higher BMI (Stewart et al., 2011). Furthermore, detection thresholds for oleic acid, paraffin oil, canola oil, and canola oil containing oleic acid were correlated with greater intake of high-fat foods and high-caloric processed foods in 24-hour food diaries (Heinze et al., 2018).

Interestingly, measurement of fat sensitivity show that lean and overweight panelists have lower limits of detection for oleic acid when compared to panelists with obesity. A relationship between thresholds and fat intake was shown, with the strongest effect observed in the lean or overweight panelists, and not those with obesity (Tucker et al., 2014). This is converse to studies from the same group that showed no difference in sensitivity between subjects with a lean and obese BMI (Tucker & Mattes, 2013; Tucker et al., 2015). These discrepancies could be due to the cross-sectional nature of the studies.

Randomized trials that include pre and post treatment mechanisms take into account temporal association between fat intake and taste, which better accounts for individual variability in fat taste threshold. Fat sensitivity is associated with short-term fat intake, as measured by a 24-hour recall questionnaire, showing an increased proportion of energy intake from fat (Costanzo et al., 2017). Dietary fat sensitivity can be increased by fat restriction in people with obesity (Bolhuis et al., 2015; Liu et al., 2016). In a randomized controlled trial, individuals given a portion-controlled or a low-fat diet for 6 weeks showed a decrease in fat detection thresholds and increased perceived intensity from fat, but food preference did not change in the tests the group performed (Newman et al., 2016). Likewise, panelists with an overweight or obese BMI given a low-fat diet for 4 weeks increased their sensitivity to C18:1 (Stewart & Keast, 2012).

Liking fatty foods may not be associated with fat taste sensitivity (Costanzo et al., 2017; Newman et al., 2016; Stewart & Keast, 2012), but is linked to BMI in both men and women (Deglaire et al., 2015; Keskitalo et al., 2008). In addition to fat preference and sensitivity, fat can also influence satiety, which in turn can affect intake. In a randomized crossover design study using C18:1, those who were orally hyposensitive to C18:1 found a meal high in fat less satiating compared to those more sensitive (Keast et al., 2014). Interestingly, just rinsing with an oleic acid solution decreased hunger and increased fullness in a randomized crossover trial, with those who were sensitive to fat showing a stronger effect (Costanzo et al., 2020). Patients with obesity also exhibit compromised detection of oleic acid in the gastrointestinal tract, further supporting a reduced satiety response to intake of fatty foods (Stewart, et al., 2011). Previous studies have demonstrated a negative correlation between the detection threshold of C18:1 and expression levels of the candidate fat sensor termed cluster of differentiation 36 (CD36) (Pepino et al., 2012). More recently, an association was found between CD36 expression and liking for fat (Liu et al., 2018). Furthermore, in Tunisian women the CD36 AA genotype conveys both a higher gustatory fat detection threshold, and interestingly also a higher BMI (Mrizak et al., 2015). The multiple

candidate fat receptors and broad array of stimuli used to test fat sensitivity make the relationship between fat detection and intake less clear, and invite further attention in the future, due to the strong links between fat intake and obesity. Nonetheless, the preponderance of evidence suggests that a change in sensitivity to taste can encourage intake of foods with such tastes, which may be particularly relevant when implying a high caloric content, as in foods high in sweetness and fat.

SECTION II: Obesity and taste

Subjects with obesity display a depressed sense of taste

Across a multitude of sensory studies, a deficit in our sense of taste is observed in adults with obesity (Bartoshuk et al., 2006; Ettinger et al., 2012; Overberg et al., 2012; Pepino et al., 2010; Proserpio et al., 2016; Stewart et al., 2010; Stewart et al., 2011; Vignini et al., 2019), as well as in adolescents and young adults with obesity (Overberg et al., 2012; Park et al., 2015). Bartoshuk (2006) noted dampened fat and sweet taste in those with obesity when adopting the generalized Labeled Magnitude Scale to quantify human taste responses, which also allows researchers to better account for individual taste variations that may previously have masked deficiencies in taste (Bartoshuk et al., 2006). Since then, a great deal more evidence of taste impairment in those with obesity has emerged. In one study examining taste in children and adolescents with obesity that included n=99 subjects with obesity and n=94 lean subjects, the subjects with obesity were found to have a reduced ability to accurately identify taste qualities, with poorer detection for umami, bitter and salty taste. Furthermore, children with obesity reported lower perceived intensity ratings for a majority of the sweet stimuli presented (Overberg et al., 2012). In another study, using filter paper strips, sweet, salty, sour and bitter (but not fat stimuli) were more poorly identified by patients with obesity , again suggesting some deficiency in taste function (Vignini et al., 2019). In Italian adults with obesity, sweet, salt, bitter, fat, and sour detection thresholds were higher in subjects with obesity (Proserpio et al., 2016). Similarly, in adults with metabolic syndrome, all

basic tastes were scored lower by subjects with obesity, in a manner inversely associated with BMI (Coltell et al., 2019). In testing umami stimuli, women with obesity were observed to have lower monosodium glutamate (MSG) sensitivity, and prefer higher concentrations of MSG (Pepino et al., 2010). Women with obesity also reported significantly higher liking for sweetness in custard, with higher sweet thresholds (Ettinger et al., 2012). In both sexes, oral fat hyposensitivity was negatively associated with BMI (Stewart & Keast, 2012; Stewart et al., 2010). Women with obesity have lower monosodium glutamate sensitivity and prefer higher concentrations than do normal-weight women (Pepino et al., 2010). Furthermore, MSG intake was found to be positively associated with BMI, with overweight patients consuming significantly more MSG (He et al., 2008). Interestingly, perceived intensity of sweet taste was also reported as greater in active than in inactive male subjects (Feeney et al., 2019), however regardless of activity, fat percentages were also not to be lower in the active group. There are a multitude of sensory studies that demonstrate that BMI has a positive correlation with fat detection thresholds, in diverse populations from Algeria, Tunisia, America, the Czech Republic, and Australia (Karmous et al., 2018; D. Liu et al., 2016; Mrizak et al., 2015; Pepino et al., 2012; Sayed et al., 2015). Again, some studies conversely find no association (Bolhuis et al., 2016; Costanzo et al., 2017; Tucker et al., 2015). In a systematic review and meta-analysis of fat taste, no significant correlation between obesity and a higher detection threshold for fat was established, although researchers suggest that this may have been partially attributed to a limited number of studies (Tucker et al., 2015).

Importantly, it should be noted that some studies also report that patients with obesity have increased taste sensitivity (Hardikar et al., 2017) or do not report any differences versus controls (Drewnowski et al., 1991; Enns et al., 1979; Frijters & Rasmussen-Conrad, 2010; Rodin et al., 1976; Thompson et al., 1977; Tucker et al., 2015). These inconsistencies might be due to smaller sample sizes in these studies, heterogeneity in methods and scales used to test taste acuity (e.g. threshold detection vs intensity rating), or variations across testing environments. While the majority of the evidence points to a

dampened sense of taste in patients with obesity, in future, longitudinal/case-controlled studies could provide more robust support for this conclusion. To our knowledge, the only longitudinal study in weight gain done thus far was in first year college-aged students which gained an average of 3.9% weight. Even with moderate weight gain, males showed a reduced sweet and salty taste (Noel et al., 2017)

Obesity directly influences the taste buds

Recent insights from animal models proffer a mechanism for the taste dysfunction observed in patients with obesity. In mice, obesity causes a strong disruption in the homeostasis of taste buds. Obese mice have fewer circumvallate taste buds, in the posterior region of the tongue, than lean littermates, after only 8 weeks on an obesogenic diet (Kaufman et al., 2018). Additionally, the number of fungiform papillae, which house the taste buds in the anterior region of the tongue, inversely correlate with weight in mice (Kaufman et al, 2019). In addition to a reduced number of circumvallate taste buds and fungiform papillae, obese mice undergo a reduction in expression of various taste cell markers. Taste buds are generally divided into type I, II and III taste cells, with a further developing set of cells in basolateral regions. Phospholipase C beta 2 (PLCβ2), a type II taste cell marker involved in the transduction of sweet, bitter, and umami taste was significantly downregulated in mice (Ahart et al., 2019; Kaufman et al., 2020). α-gustducin, which marks a subset of type II taste cells, was also found to be downregulated in one study (Ahart et al., 2019). Additionally, expression of nucleoside triphosphate diphosphohydrolase-2 (NTPDase2), a type I taste cell marker, and polycystic-kidney disease- 2-like 1 channel (PKD2L1), a type III taste cell marker (Kaufman et al., 2020) were also down-regulated in obese mice. Furthermore, in obese rats, expression of the sweet taste receptor T1R3 linked with sweet and umami detection was also reduced (K. Chen et al., 2010). Thus, it seems across all taste cell types there is a reduced expression of critical taste markers with obesity.

Obese rodents display a decreased response to fat and sweet stimuli, measured with calcium signaling, concurrent with lower expression levels T1R3 and CD36, linked to sweet/umami and fat detection respectively (K. Chen et al., 2010; Chevrot et al., 2013; Maliphol et al., 2013; Ozdener et al., 2014; X. J. Zhang et al., 2011). There are several proposed candidate fat receptors, with CD36 likely the best characterized (Fukuwatari et al., 1997), and CD36 knockout mice displaying a reduced preference for oleic acid (Laugerette et al., 2005).Calcium imaging of mice on a HFD for 10 weeks revealed fewer taste cells, that were less responsive to sweet taste, but without a reduction in taste cells responsive to aversive taste stimuli (Maliphol et al., 2013). Interestingly, this varied by sex, with obese female mice having fewer cells responsive to sweet stimuli in comparison to obese male mice. While this variance could represent a true sex difference, it could also simply reflect that male mice gain weight differently to females, although data did not directly correlate with weight gain. Furthermore, the taste cells still responsive to taste stimuli had an altered response peak, amplitude, and area. Interestingly, many studies of obese rodents have also demonstrated impaired taste response, specifically for sweet (Ahart et al., 2019; Bernard et al., 2019; K. Chen et al., 2010; Maliphol et al., 2013)and fat (Bernard et al., 2019; Chevrot et al., 2013) stimuli, with an increased lick rate for usually aversive bitter solutions in obese mice suggesting a further deficit in bitter taste (Ahart et al., 2019). As fat and sweet stimuli are usually high in calories, deficits in sweet and fat detection such as those from reduced expression of taste signaling elements could conceivably lead to overindulgence. Intriguingly, recent results suggest that an impaired sense of taste in mice can be passed to the offspring by an obese mother. Without progeny, which displayed increased lick response to sucrose solutions and higher intake of palatable stimuli, ever being in contact with unhealthy foods themselves (Choo et al., 2020).

While obese wild-type mice lose taste buds, tumor necrosis factor alpha (TNFα) knockout mice do not show the same reduction of taste buds compared to C57BL/6 mice, indicating that inflammation may be critical to the taste bud loss associated with obesity (Kaufman et al., 2018). Along with increased

inflammation, beta-catenin expression was also reduced in obese mice (Kaufman et al., 2020). Beta-catenin is critical for healthy development of taste papillae (Iwatsuki et al., 2007; F. Liu et al., 2007), thus a reduction could contribute to the diminished taste papillae observed. Evidence from flies may also offer some insight into gustatory changes related to diet. In flies, the enzyme O-GlcNAc Transferase was identified as being responsible for sweet taste impairment in taste (May et al., 2019). Furthermore, a population of protocerebral anterior medial dopaminergic neurons was identified in flies that responded to sweet, but such signals were reduced and delayed after a high sugar diet. This indicates that diet-induced changes in taste may also impair the central processing of sensory signals (May et al., 2019).

Changes to the taste buds with obesity are not merely limited to non-human animals. A recent study reported an altered gene expression profile in fungiform taste cells isolated from adult humans with obesity (Archer et al., 2019). These subjects displayed a reduction in type II cell markers, increased inflammation and reduced sonic hedgehog signaling (Archer et al., 2019), which is critical in taste bud development and maintenance (Hall et al., 2003). Complementary results in adults indicated that the density of fungiform papillae was negatively correlated with adiposity, and overall fungiform density was reduced in subjects gaining weight (Kaufman et al., 2020; Proserpio et al., 2016). A similar reduction in fungiform papillae was also observed in children with obesity (Mameli et al., 2019). Associated with this loss of fungiform papillae was a reduced ability to correctly identify taste qualities. Of course, a reduction in fungiform papilla density may not directly correlate with a loss of taste buds, as fungiform papillae only provide the structure for taste buds to reside, but it is also hard to imagine the loss of a large number of taste papillae not implying at least some reduction in taste buds, unless it were more common than is currently understood for taste papillae in healthy subjects to be vacant of taste buds (Miller & Reedy, 1990). Additionally, these data are in agreement with changes observed in obese rodents, where both fewer taste papillae and fewer taste buds themselves have both been ably demonstrated (Kaufman et al., 2018, 2020).

Although not the focus of the review, it is important to briefly touch upon the brain circuits implicated in food intake and reward that are also altered in obesity in humans and model animals (Johnson & Kenny, 2010; Volkow et al., 2011). Taste responses in the nucleus of the solitary tract (NTS), the first synapse of the central gustatory circuit, are blunted in obese rats (Weiss et al., 2019). Furthermore, evidence from human functional magnetic resonance imaging (fMRI) studies reveals that brain reward circuits are less responsive in patients with obesity , especially within areas associated with dopaminergic reward such as those arising from taste (Frank et al., 2012; Green et al., 2011). Likewise, electroencephalogram recordings in subjects with an obese versus lean BMI reveal weaker and faster fading signals in subjects with obesity (Hardikar et al., 2018). Obesity-driven damage to the taste buds may be partially responsible for the altered signals observed, however we would assume that the processing of sensory signals in the brain would also vary with obesity.

G-protein-coupled receptors thought of as “taste” receptors, but located in the brain, are also dysregulated in obese mice. Diet-induced obesity caused a decrease in receptors thought of in the taste bud as receptors for sweet (T1R3 and T1R2) and bitter (T2R116, T2R118, T2R138, and T2R104), located in the hypothalamus and brainstem (Chao et al., 2016). These receptors may be involved in controlling food intake and energy homeostasis. Reduced expression of sweet taste receptors in the central nervous system (CNS) may promote food intake in order to offset this reduced sensitivity. In the future, taste related intervention strategies might be employed including behavioral or physiological interventions. Most recently, a review suggested taste buds as a treatment target for obesity, however exact intervention strategies are still unclear (Rohde et al., 2020).

Conclusions

Taken together, the evidence suggesting an impairment in taste in those suffering from obesity is convincing. This is especially true when psychophysical evidence from human taste testing is considered

alongside the histological and molecular dysregulation observed in obese non-human animals, along with the small number of human studies of the taste buds in obesity. Obesity induces inflammation, causes taste bud loss, alters taste receptor expression, and unbalances an array of signaling elements critical to the development, differentiation, and homeostasis of taste cells. When depressed, taste can direct food choices towards sweeter, or fattier foods that are usually higher in calories, and thus can further exacerbate obesity. Taste is the primary driver of food choice, therefore interventions to leverage the taste system could offer a novel approach to reduce the prevalence of obesity through behavioral modification. Further evidence linking taste with food choices in those with obesity will be crucial to our understanding of the etiology of obesity, and allow us to assess if interventions targeting taste can be effective in the treatment of obesity.

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CHAPTER 2

EFFECT OF OBESITY AND DIET COMPOSITION ON TASTE BUD HOMEOSTASIS IN SPRAGUE DAWLEY RATS

Abstract

Recent research has demonstrated links between taste and both metabolic and inflammatory conditions, including obesity. Mice gaining weight through diet exhibit fewer taste buds than lean littermate controls after only 8 weeks on a HFD. This deficiency was linked to the inflammatory response observed in the obese mice. This loss in taste buds establishes a mechanism for the taste dysfunction commonly reported by obese humans. Here, we studied whether HFD-induced taste loss occurs in rats, and if this taste bud loss would persist once rats were returned to a normal chow diet (HFD/chow), or pair-fed a HFD in isocaloric quantities (HFD/isocal), compared to chow -fed rats (chow-only), thus isolating the influence of a HFD from that of obesity itself. At the end of the experiment the HFD/chow group and the HFD/isocal group were both significantly heavier than their chow-only counterparts, with HFD/isocal rats also having a significantly higher percentage of fat compared to chow-only rats. After the rats were euthanized, the tongues were extracted and the fungiform papillae (FP) and the CV taste buds were analyzed with papilla counting and immunohistochemistry. Both HFD/chow and HFD/isocal rats had significantly fewer FP than the chow only control rats. HFD/chow-fed rats also had significantly fewer circumvallate taste buds than HFD/isocal-fed rats with a trend for fewer taste buds compared to the chow-only controls. Finally, the number of cells undergoing programmed cell death in the taste regions was significantly higher in HFD/isocal rats compared to chow-only and HFD/isocal rats, which suggests a mechanism for the reduction in taste buds observed in HFD/chow rats. There was no significant difference in the number of neutrophils observed between any group. Taken together, these data give further insight into obesity-induced FP loss in rats and a mechanism for the taste bud loss observed in obese rats.

Introduction

Obesity is one of the most concerning public health issues of our time

From 1975 to 2016 the prevalence of obesity has tripled across the world (Hunter & Reddy, 2013). The loss of excess weight by dieting is strongly supported in improving metabolic health (Lean et al., 2018; Phelan et al., 2007); however, diets with little to no scientific basis are prevalent in popular media. Systematic reviews have tried to compare diet composition's effect on weight loss, concluding that the Atkins diet had the most evidence to support clinically meaningful weight loss, while also noting the paucity of studies (Anton et al., 2017), a sentiment echoed by many other reports (Gudzune et al., 2015; Harris et al., 2018). Many studies measure the success of diets based on percent weight loss or the reduction of metabolic risk factors. In this study, we focus on diet's effect on the damage to the gustatory system, as reported in our previous work.

Diet composition's effect on physiology and metabolic disorders

As the global obesity epidemic has grown, food intake has been accepted as the primary driver of obesity (Jeffery & Harnack, 2007). Per capita energy intake and the size of food portions, especially foods with low levels of nutrients, have been steadily rising (Ford & Dietz, 2013; Haslam & James, 2005; Young & Nestle, 2003), and fat and sugar are increasingly available (Crino et al., 2015; Naughton et al., 2015; Rikkers et al., 2013; Vandevijvere et al., 2015). The habitual excess intake of fat and sugar leads to a chronic positive energy balance, which in turn causes weight gain (Chaput et al., 2012; Hooper et al., 2015; Morenga et al., 2013; Mozaffarian, 2016). In developed countries this problem is more acute as more foods high in saturated fat and added sugar are more highly consumed (Juul & Hemmingsson, 2015; Nardocci et al., 2019; Solberg et al., 2016; Zobel et al., 2016).

If food intake is the primary driver of obesity, understanding the physiological effects of consuming excessive calories from fats or carbohydrates is important for public health. Popular diets like a ketogenic diet very high in fat, or a low-fat diet high in carbohydrates, represent opposite ends of the spectrum. There is evidence that both low-fat and low-carbohydrate diets are effective methods to reduce body weight, weight circumference, and blood lipid markers, as shown in a 2012 meta-analysis (Hu et al., 2012). Blood pressure, LDL cholesterol, triglycerides, serum insulin, and blood glucose were all improved in both groups (Hu et al., 2012). These markers indicate the risk or presence of obesity-associated diseases including type II diabetes (Bellou et al., 2018), hypertension (Seravalle & Grassi, 2017), cardiovascular disease, and all-cause mortality (Ma et al., 2017). The primary recommendation given by public health officials for weight reduction is to follow a low-fat diet because of its positive effects on metabolic risk factors. Low-carbohydrate diets might provide an alternative approach with a similar effect on weight reduction and metabolic risk factors (Hu et al., 2012; Seid & Rosenbaum, 2019).

Intake of sweet, salty, fat, and umami impact on taste acuity

Diet composition has been shown to influence taste sensitivity. High salt intake is linked with increased risk for hypertension and stroke, two diseases correlated with obesity. Patients who are at risk for cardiovascular issues are often asked to reduce their salt intake (Rust & Ekmekcioglu, 2017). Prolonged low salt intake can reduce preferred levels of sodium and overall consumption (Bertino et al., 1982). Prolonged exposure to a high-salt diet increases preferred levels of sodium and overall consumption (Bertino et al., 1986). Correspondingly, prolonged reduced intake of simple sugar increases perceived sweet taste intensity (Wise et al., 2016). Similar results have been shown for fat taste; consuming a low-fat diet for 6 weeks increased fat threshold and fat perception, but food preference did not change in patients with obesity (Newman et al., 2016). In healthy panelists, persistent exposure to monosodium glutamate, the prototypic umami stimulus, reduced perception of umami taste in women and diminished appetite for savory foods in both sexes (Noel et al., 2018). How changes in taste sensitivity

influence diet long-term is currently unclear. A recent systematic review testing links between sensitivity to sweet taste and intake did not find an association, possibly due to the paucity of studies, instead suggesting stronger associations between hedonic liking and intake (Tan & Tucker, 2019).

Patients with obesity have a reduced sense of taste

Sensory studies on patients with obesity have shown a dampened sense of taste (Bartoshuk et al., 2006; Ettinger et al., 2012; Noel et al., 2017; Overberg et al., 2012; Park et al., 2015; Pepino et al., 2010; Proserpio et al., 2016; Stewart et al., 2010, 2011; Vignini et al., 2019). Many studies show taste dysfunction, but not all studies agree, with some studies not finding an alteration (Drewnowski et al., 1991; Enns et al., 1979; Frijters & Rasmussen-Conrad, 1982; Tucker et al., 2017; Rodin et al., 1976; Thompson, et al., 1977), and even others presenting data that show an improved taste function in obese subjects (Hardikar et al., 2017).

People's primary driver of food choice is taste (Aggarwal et al., 2016; Glanz et al., 1998; Kourouniotis et al., 2016; Zylan, 1996), which as a result might provide an intervention strategy to reduce obesity. Choosing to eat calorically-rich foods high in fat and sugar is more likely to create a chronic positive energy balance, which can lead to or exacerbate obesity (Hooper et al., 2015; Morenga et al., 2013; Mozaffarian, 2016). Understanding the underlying molecular changes in the taste system that occur with obesity can provide a clearer picture of why obesity is so pernicious to treat.

Alterations in brain connectivity, response, and neuronal function in obese patients

In addition to alterations to the gustatory system, changes in the brains of patients with obesity have also been observed. Obese patients have decreased global brain connectivity in feeding-related circuitry, and increased connectivity in the dorsal attention network. This is in line with changes in neurocognition in obese patients (Geha et al., 2017). Evidence from human fMRI studies supports this,

showing that brain reward circuits are less responsive in obese patients, especially within areas associated with dopaminergic reward such as that arising from taste (Green et al., 2011). Motivation, executive control, and the limbic system, systems implicated in addictive behaviors, are also altered (Frank et al., 2012; Green et al., 2011; Kure Liu et al., 2019).

Diet-induced obesity in rodent models

Rodents have long been used in biomedical research as they mimic the physiology of humans, but are small, have a short life cycle, and genetic tools to probe their physiology are abundant. Initially, mice were the preferred rodent model for research and thus a much larger genetic toolbox is available for mice than other rodents. Recently, more technology has been developed for rats including genome editing technology. Behavioral testing often prefers rats over mice, due to the greater ability of rats to learn complex behavioral paradigms; thus, a lot of behavioral data available in the field is from rats (Ellenbroek & Youn, 2016).

A high-fat diet induces rodents to overindulge and when fed ad libitum they will increase their caloric intake, quickly inducing weight gain (Licholai et al., 2018). While genetic models of obesity offer an opportunity to examine regulatory pathways, physiological mechanisms, or specificity of therapeutic compounds (Tschöp & Heiman, 2001), as most obese individuals gain weight as a result of environmentally-induced obesity, diet induction of obesity can be most readily compared with human obesity.

Laboratories rarely use both mice and rats in their studies, so there are very few studies that make a direct comparison between the two. Mice are smaller, cheaper to house, and there are more genetic tools available for mice; therefore, they are often the default. For difficult behavioral tasks, complicated surgeries too delicate to perform on mice, or if previous research in the field is done in rats, rats are often used instead.

Many studies that compare rats and mice are focused around the brain or behavioral tasks, as neuroscience research heavily adopted the use of rats. For example, it was found that 4,713 out of 10,833 genes displayed differential expression of hippocampal neurons in mice compared to rats (Francis et al., 2014). Additionally, there are spatial-cognitive differences in mice and rats related to performing spatial tasks (Hok et al., 2016).

Obesity-induced reduction in taste buds in the circumvallate papilla

In this study, the effect of diet on the rat's gustatory system will be examined in the circumvallate (CV) and fungiform papillae (FP). The taste system is mostly housed in the posterior and anterior tongue, which contain taste buds in the CV and FP, respectively. Taste is detected by taste buds which contain 50-100 taste cells with discrete cell types: type I, type II, and type III (Yoshida et al., 2009). Type I cells are glial-like cells that sense salty taste (Chandrashekar et al., 2010), type II cells transduce sweet, bitter, and umami taste (Chandrashekar et al., 2006; Liu & Liman, 2003; Mueller et al., 2005) and finally type III cells sense sour taste (Huang et al., 2008; Yang et al., 2000).

Alongside sensory changes observed in the obese subjects, physiological changes have also been identified in mice and human subjects, which provide a potential mechanism for the dampened sense of taste observed in the obese subjects. Obese mice experience a reduction of taste buds and a change in taste response. Previously, HFD-fed obese mice were found to have fewer taste buds than chow-fed, lean mice (Kaufman et al., 2018). In obese rodents, molecular evidence shows a decreased response to fat and sweet stimuli using calcium signaling as well as a decreased expression level of taste markers, including lower subunit taste receptor type 1 member 3 (T1R3) mRNA expression (Chen et al., 2010; Chevrot et al., 2013; Ozdener et al., 2014; Maliphol et al., 2013; Zhang et al., 2011).

Recently, evidence has emerged from single-cell RNA sequencing experiments in human subjects that shows changes in fungiform density and an altered gene expression profile of fungiform papillae, with

reduced expression of the type II cell marker PLCβ2 and increased expression of genes associated with inflammation (Archer et al., 2019). Complementary results in adults and children with obesity show that the number of FP are negatively correlated with adiposity (Mameli et al., 2019; Proserpio et al., 2016). Additionally, a 4 year longitudinal study on college students testing FP density showed a correlation with changes in weight (Kaufman et al., 2019). This loss of critical taste transduction markers establishes a mechanism for the taste dysfunction observed in the obese population (Archer et al., 2019).

Obesity-associated inflammation, found to be important in obesity-induced taste bud loss (Kaufman et al., 2018), is believed to be driven by white adipose tissue which acts in an endocrine manner to release hormones and pro-inflammatory cytokines including interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα). Both the proportion of white adipose tissue compared to brown adipose tissue and the location of the fat, visceral compared to subcutaneous, contributes to poorer metabolic outcomes (Després & Lemieux, 2006; Kotzbeck et al., 2018). The greater secretion of hormones and cytokines might account for the negative effect of visceral obesity compared to subcutaneous fat (Smith, 2015).

The inflammatory state which drives a change in the health of the gustatory system may also negatively affect taste transduction in the brain. With HFD-induced obesity in rats, taste-evoked neuronal spike trains convey less information. To compensate, the percentage of total neurons involved in taste transduction in the Nuclear Solitary Tract (NTS) is increased (Weiss et al.,2019).

The fungiform papillae and circumvallate papilla

FP are located at the anterior of the tongue, while the CV is located at the posterior. There are a variety of differences between the FP and the CV, including anatomical arrangement, vascularization, embryonic origin, and signaling mechanisms (Kumari et al.,Mistretta, 2018; Mukherjee et al., 2013; Nguyen & Barlow, 2010; Whiteside, 1927; Wilson et al., 2017; Zalewski, 1969). Thus, treatment could have distinct effects in the FP vs the CV.

The FP and CV are differentially innervated. The glossopharyngeal (IX) nerve innervates the posterior portion of the tongue, including the CV, while the chorda tympani branch of the facial nerve (VII) innervates the anterior portion of the tongue, including the fungiform and foliate papillae (Whiteside, 1927; Zalewski, 1969). Cross-regenerated innervation of rat nerves showed altered perception of taste (Oakley, 1969).

Differences in taste cell type populations were observed in the FP compared to the CV of mice. Additionally, there are histological differences between type III taste cells in the anterior and posterior tongue, tested with immunohistochemical staining. A study analyzing type III taste cells in both locations hypothesized a specialized subgroup of anterior type III taste cells expressing GAD67, but not PKD2L1 or SNAP25 (Wilson et al., 2017). Moreover, density of type II and type III taste cells per taste bud in mice were higher in the CV than in other regions of the tongue (Ogata & Ohtubo, 2020).

In pharmacological experiments the FP and CV have distinct responses. For example, after cytotoxic chemotherapy treatment, apoptosis occurred on day 4 in the FP compared to day 8 in the CV (Mukherjee et al., 2013). Treatment with Hedgehog pathway inhibitor sonidegib, resulted in a loss of taste buds in the CV and FP. After prolonged 48-day treatment, CV taste buds were restored while FP taste buds were not (Kumari et al., 2018).

BMP4 regulates embryonic taste organ development, and also varies in the posterior and anterior of the tongue of mice. In the CV, intragemmal BMP4-positive cells are immature cells which act as precursors for type I, II, and III taste cells whereas CV and FP located perigemmal BMP4-positive cells are slow-cycling stem cells, and could serve distinct functions in taste cell homeostasis (Nguyen & Barlow, 2010).

Apoptosis and caspase activity

In this study we examined the number of cells that were immunoreactive for caspase-3, a marker for an effector caspase involved in apoptosis. Previously, cell death was thought to either be apoptotic

(programmed cell death) or necrotic (unprogrammed cell death). More recently, research has shown that cell death pathways can be attributed to molecular, morphologic, and biochemical differences and area likely more complex (Green, 2019).

Morphologically, apoptosis is defined by shrinkage of the cells caused by collapsing of the subcellular components arising from cytoskeletal protein cleavage, subsequent chromatin condensation, and formation of plasma-membrane blebs (D'Arcy, 2019). This process of cell death creates minimal damage to surrounding tissue. Necrosis on the other hand, is defined by swelling of the cells and organelles from the loss of plasma membrane integrity, and an influx of fluids. This type of cell death usually occurs after injury and can result in damage to surrounding tissue. Apoptosis is critical to normal tissue and if not executed correctly, it can result in the accumulation of damaged cells. On the other hand, increased, uncontrolled apoptosis is also not healthy (D'Arcy, 2019; Hotchkiss et al., 2009).

Caspases are subdivided into initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6, and -7). The former triggers caspases while the latter executes apoptosis by acting directly on specific cellular substrates (Parrish et al., 2013). Caspase-3 is the best-characterized effector caspase and is critical for its role at the end of the intrinsic apoptotic cascade. Caspase activity could represent a possible mechanism for changes in abundance of taste buds after a HFD.

In this study we will seek to examine the effects of a HFD on peripheral gustatory organs, after already-obese rats are returned to a chow-fed diet or maintained on a calorie-restricted HFD. This will provide further understanding into how diet itself, versus obesity, can affect taste. Furthermore, we will look at caspase activation and infiltrating neutrophils, both mechanisms for the loss of taste buds.

Methods

Animals

Animal studies were approved by the institutional animal care and use committee at Binghamton University, with dietary treatments carried out in the lab of a collaborator, and lingual tissue collected by our group. Male Sprague-Dawley rats were acquired from Taconic Labs, Inc. (Germantown, NY, USA) and kept on a 12-hour light/dark cycle. Animals were divided into three groups: one fed standard chow ad libitum for the entire experiment, a second fed a high-fat diet (HFD) for 10 weeks and then switched to standard chow ad libitum for the duration of the experiment, and the final group fed a HFD for 10 weeks, and then switched to HFD, fed isocalorically matched to the caloric consumption of the second group of rats, consuming standard chow ad-libitum. Rats in the HFD/ isocal group were given the same amount of calories as the HFD/chow group ate ad libitum although occasionally it was observed that the portion was not completely consumed due to hoarding behavior displayed by the rats. Rats spent a mean number of 38.76 with a standard deviation of +/- 1.84 weeks on the second diet. 3 of the 6 rats in the HFD/isocal group were put back on a chow diet 2 months before euthanizing; statistical testing revealed no significant difference between the groups, so the rats were pooled for analysis. PicoLab Diet 5L0D was used as the chow diet which contained 13% kCal fat, 58% kCal carbohydrates, 29% kCal protein (St.Louis, MO, USA). The HFD used was Research Diets D12451 45% kCal fat, 35% kCal carbohydrate, 20% protein (New Brunswick, NJ, USA).

Rats were euthanized with 100-180mg/kg Sodium Pentobarbital, tongues were excised, placed in 4% PFA (Fisher Scientific Hampton, NH, USA) /PBS (Fisher Chemicals, Hampton, NH, USA) for 1 ½ hours, washed with PBS 3x for 20 minutes, cryoprotected in sucrose (Fisher Chemicals, Hampton, NH, USA) overnight, embedded in OCT (Fisher Scientific, Hampton, NH, USA), and frozen at -80 C.

Dual-energy X-ray absorptiometry scans

Dual-energy X-ray absorptiometry (DXA) scans were taken intermittently throughout the experiment and before euthanizing to determine body composition. Before scanning, animals were sedated with 0.1 m/kg Dexmedetomidine (Pfizer Inc., New York, NY, USA).

Fungiform papillae staining and counting

Tongues were stained with 0.025% methyl blue PBS (VWR, Radnor, PA, USA) for 30 seconds and then rinsed in diH20 for 1 ½ hours. Pictures of the stained tongues were taken with an Olympus SZ61 dissection scope (Olympus Optical, Tokyo, JP) in combination with a Lumenera Infinity 1080p60 HD microscopy camera (Lumenera, Ottawa, CA). To count fungiform papillae a 3mm by 3mm box was aligned with the tongue's midline 4 cm from the tip of the tongue with a second box mirrored across the midline. The number of fungiform papillae were counted in both boxes and averaged, with data reported as papillae/mm^2 (Figure 1).

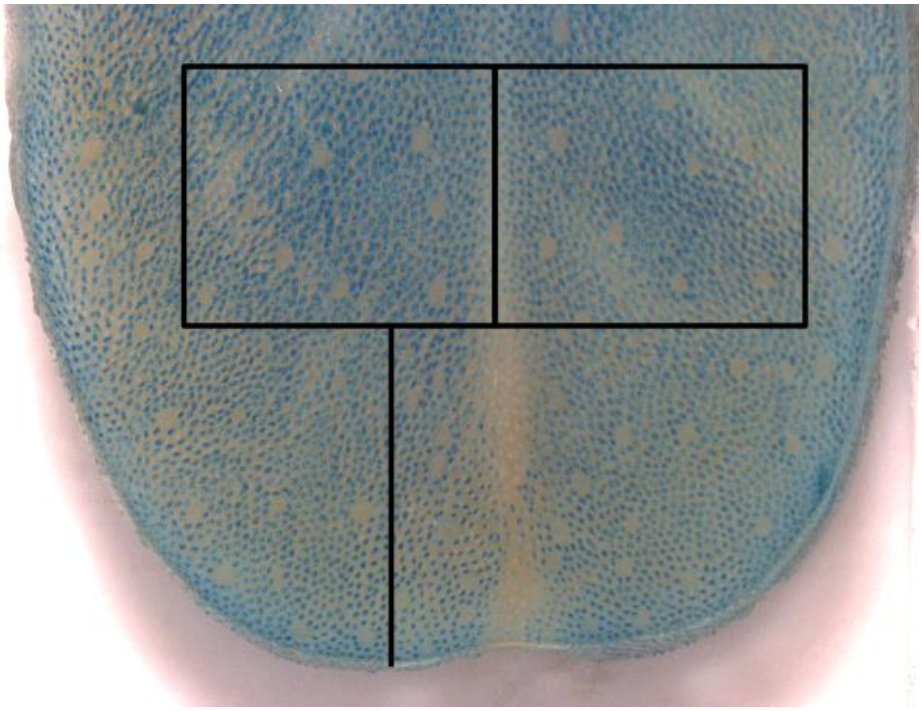

Figure 1: Representative image of area counted for fungiform papillae with two 3mm by 3mm boxes.

Immunohistochemical staining

CV tissue regions were dissected from tongues, cryoprotected with sucrose, and frozen in OCT medium. Circumvallate tissues were cryosectioned at 10 um thickness, washed in PBS (VWR, Radnor, PA, USA), and incubated in 1% triton (MilliporeSigma, Burlington, MA, USA). Tissue sections stained with 1:500 polyclonal Goat GNAT3 OAEB00418 (α-gustducin) from Aviva Systems Biology (San Diego, CA, USA), and 1:125 polyclonal Rabbit Caspase-3 AF835 from R&D systems (Minneapolis, MN, USA) were incubated with 4% bovine serum albumin (BSA) (Amresco, Solo, Ohio, USA), 4% donkey serum (Equitech-bio, Kerrville, TX, USA), and 0.3% triton (MilliporeSigma, Burlington, MA, USA).Tissue sections stained with 1:125 polyclonal Goat MPO AF3667 from R&D systems (Minneapolis, MN, USA) were blocked for 2 hours at room temperature with 2% BSA (Amresco, Solo, OH, USA), 2% donkey serum (Equitech-bio, Kerrville, TX, USA), and 0.3% triton (MilliporeSigma, Burlington, MA, USA). After incubation with secondary Alexa Fluor donkey anti-Goat or anti-Rabbit secondary (Invitrogen, Carlsbad, CA, USA) at room temperature for 2 hours, sections were washed 3x for 20 minutes in PBS (VWR, Radnor, PA, USA), and placed on a coverslip with Dapi staining medium (Fluoromount-G, Southern Biotech, Birmingham, AL, USA).

Taste bud counting

Tissue sections were imaged using an Olympus IX-71 inverted scope and Hammatsu Orca Flash 4.0 camera (Hamamatsu Photonics, Hamamatsu City, JP), and counted using ImageJ (NIH, Bethesda, MD, USA) for number of taste buds, number of caspase-3 positive cells , and number of neutrophils.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (San Diego, CA, USA). Groups were compared using non-parametric Kruskal-Wallis tests (data were not normally distributed), with statistical significance assumed at $p < 0.05$.

Results

At the start of the experiment all rats had similar body weight and percent body fat. After 2 of the groups were switched to the HFD for 8 weeks, rats still had similar body weights, but HFD/chow rats had higher percentages of fat compared to chow-only (p = 0.002) with the HFD/isocal group also having a trend for increased fat percentage, which did not reach a significant increase (Figure 3, p = 0.105). At the end of the experiment both groups with experience of the HFD were heavier, with HFD/isocal rats exhibiting the highest body weight (Figure 2, p = 0.002) and the highest percentage of body fat (Figure 3, p = 0.023) compared to chow-fed rats. HFD/chow rats also had higher body weight than chow-only rats (Figure 2, p = 0.042), but body fat was similar to both the chow controls and the HFD/Isocal rats.

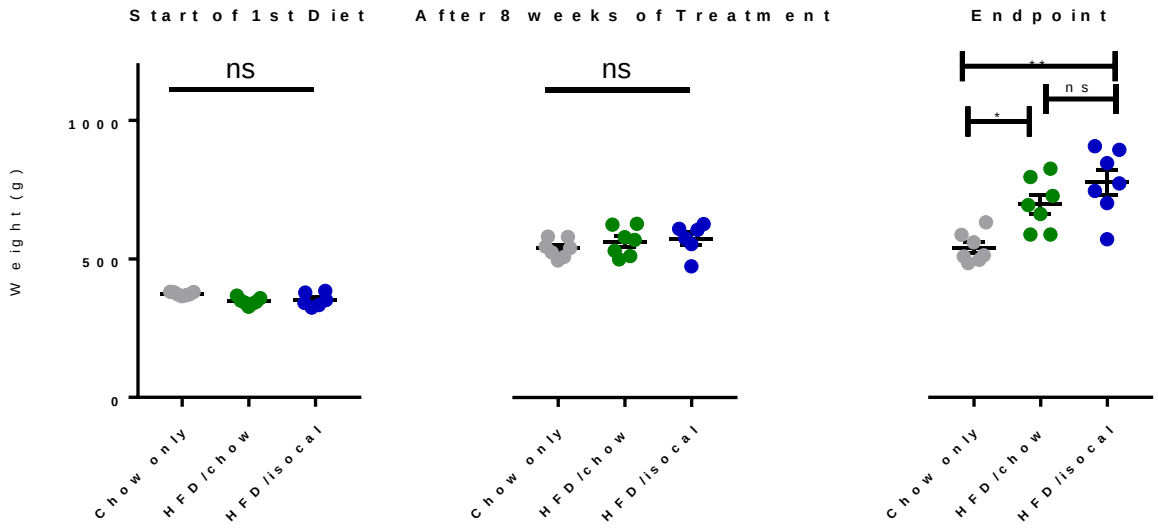

Figure 2: Weight (g) of chow-only (grey, n = 7), high-fat diet (HFD) then chow (green, n = 7) and HFD then HFD isocaloric (blue, n = 6-7) rats at the beginning of the first diet, after 8 weeks on HFD or chow, and the endpoint/ day of tissue collection. Stars represent statistical significance, where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Bars represent means plus/minus SEM.

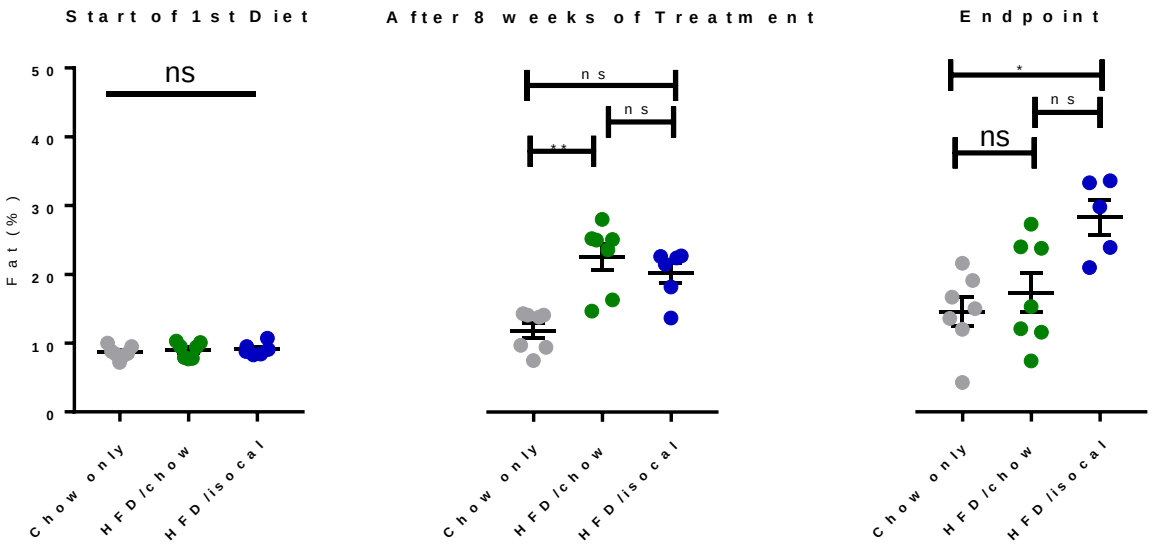

Figure 3: Percent body fat of chow-only (grey, *n* = 7), high-fat diet (HFD) then chow (green, *n* = 7), and HFD then HFD isocaloric (blue, *n* = 5-6) rats at the beginning of the first diet, after 8 weeks on HFD or chow, and the endpoint/ day of tissue collection. Stars represent statistical significance, where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Bars represent means plus/minus SEM.

The control rats consuming chow-only had more fungiform papillae compared to their HFD/chow ($p = 0.037$) and HFD/isocal ($p = 0.005$) counterparts. HFD/chow and HFD/isocal fed rats' fungiform papilla density did not differ significantly ($p > 0.999$) (Figure 4).

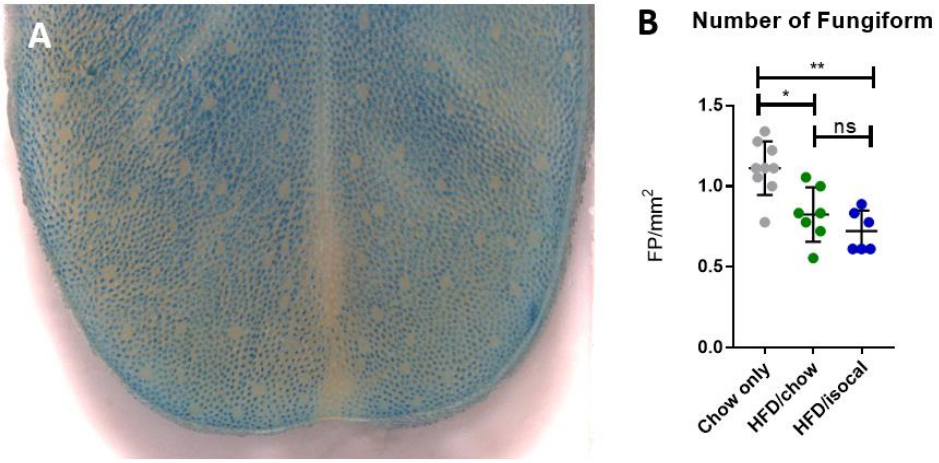

Figure 4: A: Representative image of anterior tongue, with circumvallate papillae the pink regions not taking up methyl blue dye. B: Fungiform Papillae (FP) density in rats consuming chow-only (grey, *n* = 9), high-fat diet (HFD) then chow (green, *n* = 7), and HFD then HFD isocaloric (blue, *n* = 6). Stars represent statistical significance, where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM. Overall $p = 0.0009$.

The number of FP correlated negatively with weight across the 3 groups. A negative trend in number of FP per mm^2 and weight (Figure 5A, $r = -0.646$; p = 0.001) and also with body fat was observed (Figure 5B, Pearson's $r = -0.655$; p = 0.002).

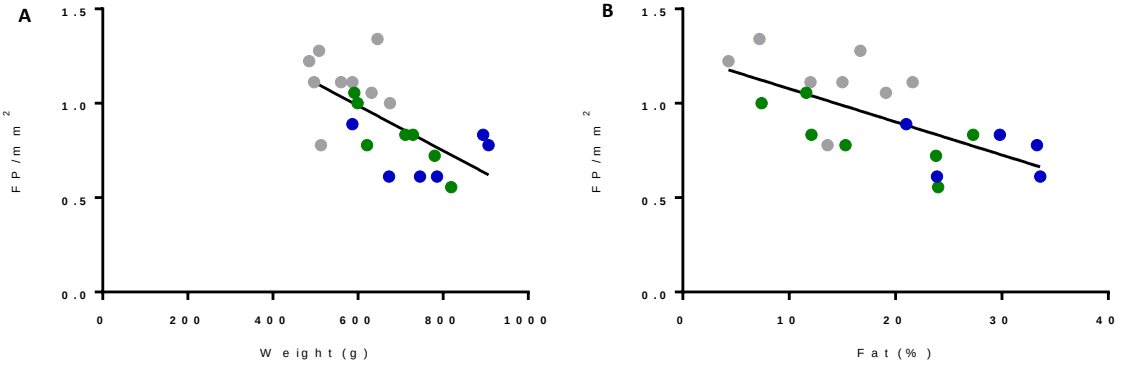

Figure 5: A: Fungiform papillae (FP) per mm^2 (y-axis), Weight (g) (x-axis). Chow-only (grey, *n* = 9), high-fat diet (HFD) then chow in (green, *n* = 7), and HFD then HFD isocaloric (blue, *n* = 6). Pearson's $r = -0.646$, $p = 0.0012$. B: Fungiform papillae (FP) per mm^2 (y-axis), Fat (%) analyzed by DXA scan (x-axis). Chow-only (grey, *n* = 9), high-fat diet (HFD) then chow (green, *n* = 7), and HFD then HFD isocaloric (blue, *n* = 6). Pearson's $r = -0.655$, $p = 0.002$.

Chow-only rats had more FP in the anterior region of the tongue, there was also a trend towards a greater number of taste buds in control rats compared to HFD/chow-fed rats in the CV (Figure 6, p = 0.103), although this trend was not significant. Post-hoc multiple comparisons tests revealed a significant difference between HFD/chow and HFD/isocal rats (p = 0.001), with those consuming chow after HFD having fewer taste buds than those maintained on restricted HFD. Finally, the number of taste buds in HFD/isocal rats was similar to the chow-only rats (p = 0.280). While the chow-only rats and the HFD/chow rats have a similar trend in the FP and the CV, HFD/Isocal rats have more taste buds in the CV while in the FP they have the fewest of all groups (Figure 4). Two outliers that were more than two standard deviations away from the mean were excluded. With the outliers included the difference between Hfd/chow and HFD/isocal was still significant (p = 0.0318).

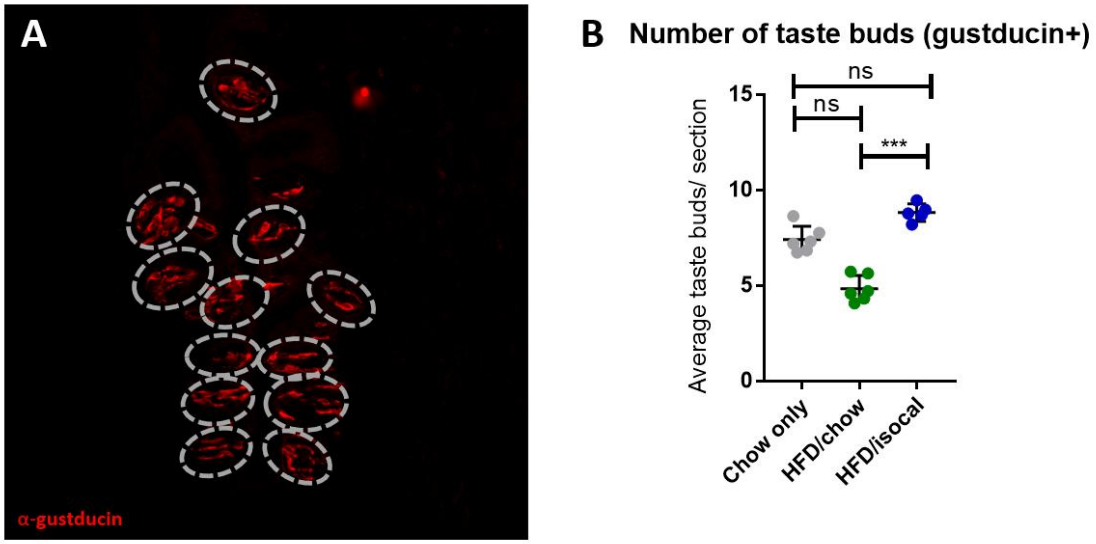

Figure 6: A: Representative image of circumvallate papilla with α-gustducin (red) staining highlighting taste buds. B: Number of taste buds per CV section for all treatment groups. Chow-only (grey, n = 6), high-fat diet (HFD) then chow (green, n = 7), and HFD then HFD isocaloric (blue, n = 6). Stars represent statistical significance, where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM. Overall p = 0.029.

Rats fed HFD/chow had a higher number of caspase-positive cells than both chow-only (p = 0.010) and HFD/isocal-fed rats (p = 0.022) (Figure 7).

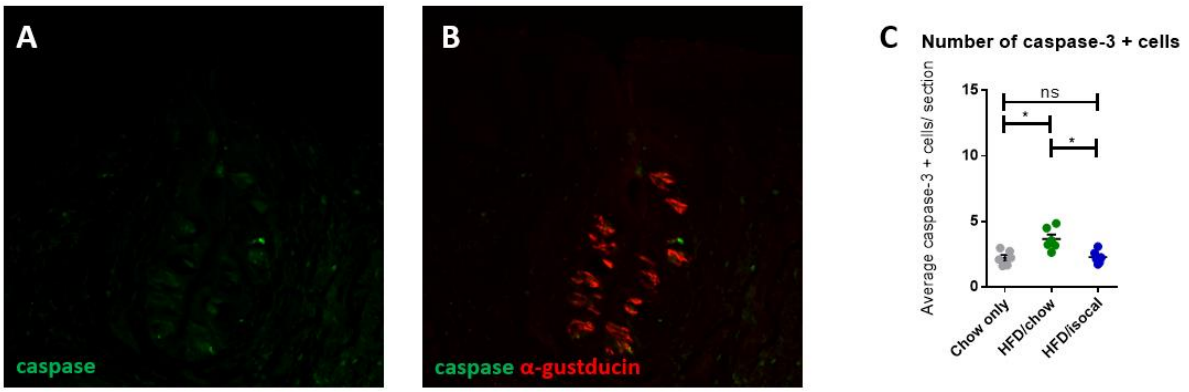

Figure 7: A: Representative image of circumvallate papilla showing caspase-3 (green). B: Representative image of circumvallate papilla showing caspase-3 (green), α-gustducin (red). C: Caspase-3 positive cells per CV section for all treatment groups. Chow-only (grey, *n* = 6), high-fat diet (HFD) then chow (green, *n* = 7), and HFD then HFD isocaloric (blue, *n* = 6). Stars represent statistical significance, where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM. Overall $p = 0.001$.

The number of CV taste buds have an inverse relationship with the number of caspase-positive cells after removal of one outlier (Figure 8, Pearson's r = -0.662, p = 0.003), that was more than two standard deviations from the mean number of caspase positive cells which suggests that the rat might have been sick or had an additional reason there was increased caspase activity. Notably, with the outlier included the inverse relationship is not observed (Pearson's r = 0.173, p = 0.478).

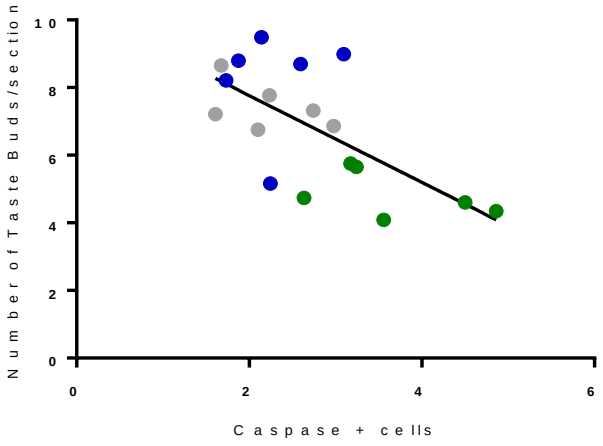

Figure 8: Number of taste buds/ section (y-axis), caspase + cells (x-axis) without outlier. Chow-only (grey, *n* = 9), high-fat diet (HFD) then chow (green, *n* = 7), and HFD then HFD isocaloric (blue, *n* = 6). Pearson's *r* = -0.662, *p* = 0.003.

Across groups there was not a significant difference in number of neutrophils ($p = 0.141$) in the CV region, with HFD/isocal-fed rats ($p = 0.154$) showing the greatest number of neutrophils in the study (Figure 9).

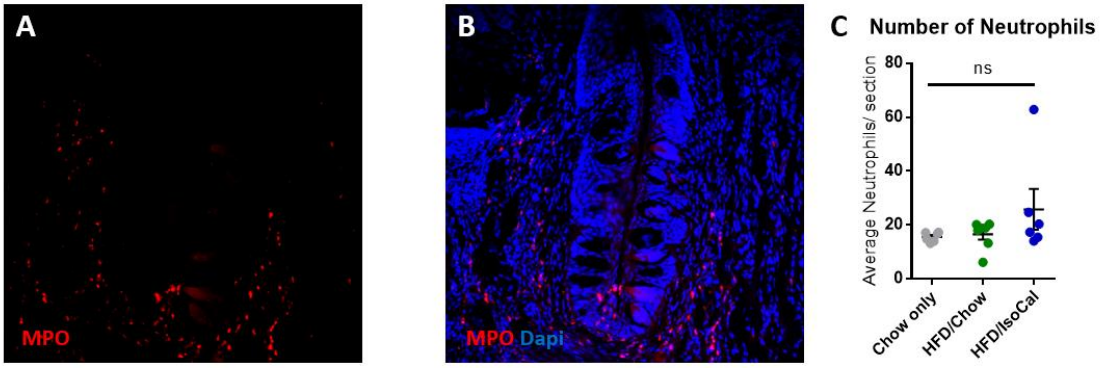

Figure 9: A: Representative image of circumvallate papilla showing MPO staining (red). B: A: Representative image of circumvallate papilla showing MPO staining (red), Dapi (blue) C: MPO positive cells per CV trench for all treatment groups. Chow-only (grey, $n = 6$), high-fat diet (HFD) then chow (green, $n = 7$), and HFD then HFD isocaloric (blue, $n = 6$). Stars represent statistical significance, where * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM. Overall p = 0.141.

To explore differences in chow ad libitum intake after HFD, food intake was recorded for 8 weeks post-HFD treatment. HFD/chow fed rats ate an average of 109.8% of the chow only rats across the 8 weeks measured. The HFD/chow rats ate a daily average of 89.042 calories while rats continuously fed a chow diet ate 81.036 calories during the same time period at the same age ($p < 0.001$).

Discussion

Increased weight in HFD/chow and HFD/isocal rats and body fat percentage in HFD/isocal rats

At the conclusion of the experiment rats in the HFD/chow group and the HFD/ isocal group were significantly heavier than their chow-only counterparts (Figure 2). Moreover, HFD/isocal rats had a higher percentage of fat compared to chow-only rats, with HFD/chow rats having slightly (but not significantly) higher body fat percentage than chow-only rats (Figure 3).

Previous data has suggested that among outbred Sprague-Dawley rats, approximately one-half develop diet-induced obesity (DIO) and one-half are more resistant to a HFD (Levin & Keesey, 1998). Other studies show the hyperphagia induced by a HFD was transient and weight gain was normally distributed (Archer et al., 2003). In this study there was no selection for rats that were susceptible or resistant to weight gain as shown in previous studies (Levin & Dunn-Meynell, 2000). Along with potential genetic differences induced by colony breeding, abundance of the gut bacteria Akkermansia muciniphila has been shown to reduce the prevalence of type II diabetes and obesity in mice, possibly contributing to some rats being resistant to DIO (Everard et al., 2013). A proportion of the rats that were used in this study may have been resistant to DIO leading to weight gain across groups not being significantly different at the 8-week time point. Even at the end of the experiment when the weight difference was significant, there were still a few rats with similar weights to chow-only control rats (Figure 2).

A recent study demonstrated that Wistar rats placed on a 45% HFD diet did not show a difference in body weight compared to their chow-fed counterparts after 15 weeks. Despite this, there were still changes in serum levels of glucose, triglycerides, and markers for metabolic disease. Furthermore, an increase in visceral adipose tissue was observed in rats fed a HFD. In this study we used a similar 45% fat diet (albeit with a differing fat composition) and observed an increase in adipose tissue. Another study that placed rats on a HFD did not see an increase in body weight in the first 4 weeks, but an increase in excess fat deposition (Archer et al., 2003). Finally, rats exposed to 2 weeks of a HFD consumed significantly more calories than their chow-fed counterparts per meal. This increased caloric consumption did not change body weight, but did alter their body composition, with similar levels of subcutaneous fat, but increased epididymal fat pads observed in HFD-fed rats (Andrich et al., 2018).

The localization of fat is important to metabolic health. Visceral, but not subcutaneous fat, is more strongly linked with poor health outcomes (Tchernof & Després, 2013). Increased visceral fat increases risk for metabolic syndrome, coronary heart disease, diabetes, and cancer. On the other hand, an

increase in subcutaneous fat around the extremities is not strongly linked with such adverse outcomes (Després & Lemieux, 2006).

The adipose organ is composed of white adipose tissue (WAT) and brown adipose tissue (BAT). Obese animals undergo whitening and show a decrease in BAT, where WAT can induce low-grade inflammation (Kotzbeck et al., 2018). Inducing white to brown visceral adipocyte transdifferentiation has been proposed as a way to reduce the prevalence of metabolic disorders associated with obesity (Giordano et al., 2016). White adipocytes act as endocrine cells and release hormones and pro-inflammatory cytokines including IL-6 and TNF alpha. Increased secretion of hormones and pro-inflammatory cytokines might account for the negative effect of visceral obesity compared to subcutaneous fat (Smith, 2015). In this study, despite a lack of weight gain on HFD after the initial treatment period, the increased fat percentage observed would likely be negative to the rats' metabolic state.

Finally, consuming a HFD can influence reward mechanisms and thus induce HFD/chow-fed rats to increase consumption after being placed on the ad libitum chow diet after exposure to HFD. As this group was calorie-matched with the HFD/isocal rats, both the HFD/chow-fed rats and the HFD/isocal rats might have been consuming increased calories in contrast with chow-only fed rats. In a previous report, a diet-induced shift in gut microbiota disrupted vagal gut-brain communication leading to an increase in body fat (Vaughn et al., 2017). HFD-induced obesity in rats also blunts neuronal response in the NTS (Weiss et al., 2019). In human studies, circuits involved in reward and motivational salience have reduced response in obese patients (Frank et al., 2012; Green et al., 2011; Kure Liu et al., 2019). Finally, HFD in this study and in previous studies (Kaufman et al., 2018, 2020) has been shown to reduce the number of taste buds, which might cause or exacerbate reduced reward response seen in the brain and lead to hyperphagia. Shift in the microbiome, blunted neuronal response, and changes in reward mechanisms may persist after the dietary switch in our experiments, and induce rats to continue to eat

more post-HFD. Indeed, during the 8 weeks when intake was measured during the experiment HFD/chow rats ate 109.8% more than ad lib chow fed rats ($p < 0.001$).

Although we did not see the expected weight increase at 10 weeks, we did observe an increase in body fat in rats consuming a HFD, which is sufficient to cause metabolic disorder such as an elevation in harmful pro-inflammatory cytokines, such as those linked to the loss of taste buds (Kaufman et al, 2018). Additionally, we did see the expected changes in weight and fat percentage at the later points of the study, when tissues were extracted and analyzed (Figure 2, 3).

Fewer FP in rats consuming HFD, correlating negatively with weight

Control rats consuming chow-only displayed significantly more FP compared to either the HFD/chow or HFD/isocal-fed rats (Figure 4), with FP counts correlated negatively with both body weight and body fat percent as analyzed by DXA scans (Figure 5). Previous studies have shown that FP density negatively correlates with adiposity in adults and children (Mameli et al., 2019; Proserpio et al., 2016). More recently, our lab demonstrated that mice show a similar negative correlation between weight and number of FP (Kaufman et al., 2020). Finally, a longitudinal study of college students across 4 years showed an analogous correlation, between adiposity change and FP density change (Kaufman et al., 2020). Thus, in rats, mice, and humans, obesity is related to the abundance of FP.

Our original hypothesis was that obesity brought on by a diet high in fat would reduce FP density in rats. In the CV, a disparate trend was observed, with HFD/chow rats having fewer taste buds compared to HFD/isocal-fed rats, but chow-only rats actually showing slightly fewer taste buds than HFD/isocal rats, although not significantly (Figure 6B). Interestingly, HFD/isocal rats seem to show some form of recovery, with an improved taste phenotype, when compared to those switched to ad-lib chow after HFD. This suggests there may be more to learn concerning the intersection between taste, obesity and diet, see Ahart et al (2019).

The disparate trend observed in the FP compared to the CV papillae could have a variety of explanations. Anatomically, the two are located on different regions of the tongue and are innervated by different nerves. The chorda tympani branch of the facial nerve innervates the anterior-located FP, as well as the anterior portion of the foliate papillae, while the glossopharyngeal nerve innervates the posterior-located CV (Whiteside, 1927; Zalewski, 1969). Previously, cross-regenerating those nerves alters taste perception (Oakley, 1969). It is possible that the regenerative capacity of these regions differs, leading to disparate effects of HFD with taste field.

Of course, while we assume that the FP house one or more taste buds, the actual number of taste buds or taste cells in the FP was not determined in this analysis. Additionally, the mix of taste cell subtypes could differ between treatments. Previous evidence suggests that there are variances in the density and proportion of type II and type III taste cell populations in healthy mice in the foliate and circumvallate papillae (Ogata & Ohtubo, 2020; Wilson et al., 2017). Under altered dietary conditions, these changes might become more pronounced.

Further explanation for differences between FP and CV taste buds could lie in development with the FP being derived from the ectoderm and the CV from the endoderm (Rothova et al., 2012). Additionally, BMP4, which regulates taste organ development, has been shown to have altered expression patterns in the posterior and anterior of the tongue, suggesting distinct functions between regions (Nguyen & Barlow, 2010).

Fewer taste buds in HFD/chow-fed rats compared to HFD/isocal

Putting rodents on an ad libitum HFD induces obesity, increases markers for type II diabetes, and increases adipose tissue. Diet-induced obesity is a well-established model for metabolic disorders in humans (Buettner et al., 2007; Licholai et al., 2018). Although it is widely used, there is some variation in percent fat used in studies, with everything from 20%-60% considered a HFD though a fat content of

~45% is most commonly used (Buettner et al., 2007). Diet-induced obesity is most readily compared to human obesity as both are a result of environment (Tschöp & Heiman, 2001). Obesity is well established as an inflammatory state, with inflammation believed to drive taste loss (Archer et al., 2019; Kaufman et al., 2018).

In a previous study, mice fed a HFD alongside those consuming the HFD plus a pharmacological agent to preclude weight gain still exhibited behavioral deficiencies in taste function (Ahart et al, 2019), suggesting a HFD may be sufficient to damage taste buds. While no loss in taste buds was reported in this study, taste bud abundance itself was not quantified, only taste cells per bud. In this study, we thus hypothesized that HFD/isocal-treated rats would have fewer taste buds than chow-only or HFD/chow-fed rats. In further evidence that a HFD may have a negative effect on taste buds, Wistar rats fed a restricted HFD still show increases in inflammation compared to chow-fed counterparts (Jacob et al., 2013). Rats fed an isocaloric diet (60.9% fat) compared to rats fed a chow diet (9.3% fat) exhibit higher levels of cholesterol, LDL, C-reactive protein, and liver weight, pointing to impaired insulin signaling and an inflammatory response in the liver (Jacob et al., 2013). A similar finding was recapitulated in C57BL/6J mice. Reducing number of calories via iso-caloric pair-feeding of C57BL/6J with a HFD (58% fat), compared to a chow diet (11% fat) attenuated the development of obesity and type II diabetes seen in ad libitum HFD-fed mice, but importantly did not completely ameliorate these effects (Petro et al., 2004).

In another study, comparing weight loss of mice that were initially on a HFD to induce obesity and then switched to either a chow diet (10% fat) or a HFD 70% restricted (40.2%), showed that both a switch to chow and a high-fat 70% calorie-restricted diet induced weight loss. Interestingly, they found that HFD-restricted mice had a larger reduction of WAT inflammation, as measured by macrophage infiltration, and increase in mitochondrial carbohydrate metabolism (Hoevenaars et al., 2014). The results of this study revealed that HFD restriction was superior to an ad libitum chow diet in reducing inflammation.

One additional reason why our study may differ from previous work is that the rats were inadvertently fed in a time restricted fashion. Mice seem to quickly consume their HFD-restricted portions, then fasting until their next meal (Hoevenaars et al., 2014). This has been shown to have a positive effect on metabolic disease, even without reducing caloric intake (Hatori et al., 2012). Recently, adipose tissue inflammation and fibrosis in a HFD (43% fat) was ameliorated by 3 nonconsecutive days/week fast for 24 hours (B. Liu et al., 2019).

The increased number of taste buds observed in HFD/isocal-fed rats might be because rats were inadvertently placed on feeding schedule resembling an intermittent fasting protocol. When rats were given their daily allotment of HFD food, it was anecdotally observed that rats had already finished their food from the day before, implying a period where no food was available, although this remains speculative, as timing of food consumption was not explicitly recorded. On the other hand, rats sometimes hoarded their food and did not finish their allotment of food which inadvertently slightly restricted their food intake. Caloric restriction has been found to reduce levels of inflammation characterized by TNFα, c-reactive protein, and serum triiodothyronine. It also has an effect on metabolic pathways including modulating oxidative stress, autophagy, and leptin (Hambly et al., 2012; Holloszy & Fontana, 2007; Speakman & Mitchell, 2011).

Another difference between some of the studies discussed is the percentage of fat in the HFD. The studies showing a partial amelioration of inflammatory effects of a HFD used 60.9% fat and 58% fat while the study finding HFD restriction superior to chow used 40.2% (Hoevenaars et al., 2014; Jacob et al., 2013; Petro et al., 2004). In this study, we used 45% fat diet, similar to the study finding HFD-restricted mice had a larger reduction of WAT inflammation and increase in mitochondrial carbohydrate metabolism. Importantly, a ~60% fat diet is not considered ketogenic, which has been shown to have positive effects on inflammatory diseases including Alzheimer's (Pinto et al., 2018).

HFD/chow-fed rats in fact had significantly fewer CV taste buds than HFD/isocal rats (Figure 6B), which may have been due to inadvertent time-restricted feeding, caloric restriction as rats hoarded and did not finish their portion, or the percent fat of the HFD used. Additionally, this data suggests that taste loss might partially persist after a return to a regular diet. It also suggests that a time-restricted feeding might have a positive effect on the taste system, independent of calories consumed.

HFD/chow-fed rats had significantly more apoptotic cells compared to chow-only and HFD/isocal-fed rats

One mechanism that could be responsible for a reduction in the number of taste buds in the HFD/chow-fed rats is the increased number of cells undergoing programmed cell death, as marked by caspase-3 (Figure 7). After removing an outlier, number of taste buds have a negative relationship with caspase-3-positive cells (Figure 8). Caspase-3 is the most characterized effector caspase and is best known for its critical role at the conclusion of the intrinsic apoptotic cascade.

Diabetes is characterized by similar inflammatory markers found in obesity (Lontchi-Yimagou et al., 2013). In diabetic Wistar rats' CV, increased activation of caspase-3 and TUNEL staining, another cell death marker, was observed (Cheng et al., 2011). An impaired sense of taste has also been reported in patients with type II diabetes, similar to obese patients (De Carli et al., 2018). In both these metabolic disorders, apoptotic cell death might thus play a role in the gustatory system.

Neutrophil infiltration did not differ across groups

Previously neutrophils have been shown to be involved in inflammation by recruiting macrophages, exacerbating inflammation and interacting with immune cells (Mantovani et al., 2011; Nathan, 2006). Obese patients have higher number of circulating neutrophils, which play a key role in innate immunity (Nijhuis et al., 2009). In HFD-fed obese mice, adipose tissue and liver tissue showed increased number of neutrophils (Talukdar et al., 2012). No change in neutrophil infiltration across groups was observed,

although HFD/isocal-fed rats had slightly higher average numbers of neutrophils surrounding taste regions when compared to chow-only rats, though this was not statistically different between groups (Figure 9, $p = 0.154$).

The lack of a statistically significant difference between groups does not preclude the idea that neutrophil activation happened earlier, for instance right after HFD treatment, and then subsided. Neutrophils might also be more involved in an acute inflammatory incidence rather than the chronic inflammatory infiltrate, as neutrophils in C57BL/6J mice adipose tissue has been observed to peak from 3-7 days after initiating HFD treatment (Elgazar-Carmon et al., 2008). Finally, it could be that neutrophils infiltrate fat tissue and circulate, but have trouble infiltrating the gustatory system, due to the barrier surrounding taste buds (Dando et al, 2015), of which little is known concerning neutrophils. Alternatively, there may never have been an increase in neutrophil activation with our treatments.

Conclusion

Here we show Sprague-Dawley rats fed a high fat diet have fewer fungiform papillae than chow-fed control rats, whether continuing to consume a HFD, or switched back to chow. An increase in cells undergoing apoptosis in the CV presents a mechanism for the reduced number of taste buds observed in obese rats. These data suggest that consumption of a HFD in conjunction with obesity and resulting increase in fat compromises the peripheral gustatory apparatus. In future, it would be interesting to see how diet composition affects taste changes in human sensory studies, changes in the brain, and further mechanistic insight into the factors governing this process.

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CHAPTER 3

HFD-INDUCED TASTE BUD LOSS IS ONLY PARTIALLY RECOVERED LONG AFTER WEIGHT LOSS AND RETURN TO A HEALTHY DIET IN C57BL/6 MICE

Abstract

Previously, work has shown that diet-induced obese mice have fewer taste buds than lean littermates. In this experiment we investigated if diet induced taste bud loss, increased inflammation, and attenuated taste cell regenerative capacity would persist after weight loss from a return to a healthy diet. 8-week-old female and male C57Bl/6 mice were split into three groups. The first group (chow) were maintained on a standard lab chow diet for 16 weeks, the second (HFD) were placed on a HFD for 16 weeks, and the third (diet) were placed on a HFD for the first 8 week, then switched to a chow diet for the second 8 weeks, where all excess weight was lost. Mice were sacrificed, the tongue was excised and analyzed for histology and RNA expression of taste, inflammation, apoptosis, and regenerative markers. Differences between female and male mice were also tested. Dieted mice showed a partial recovery of taste buds, and a moderately reduced number of proliferating cells and moderately increased number of apoptotic cells as chow-fed mice. HFD fed mice show reduced number of proliferating cells and increased apoptotic cells. As previously shown, HFD fed mice have increased tumor necrosis factor alpha (TNFα) expression. HFD fed mice also have amplified sonic hedgehog (Shh) expression and Bone morphogenetic protein 4 (BMP4), both found to be important in taste bud homeostasis. PlCβ2 expression, a marker for type II cells, was reduced in dieted mice. The proportion of α-gustducin positive cells per taste bud, a marker for a subset of type II cells, was increased in HFD and dieted mice compared to chow-fed mice, with female mice being more sensitive to diet change. Overall, this research shows that HFD can have a persistent effect on taste buds with little difference between female and male mice.

Introduction

Obesity adversely impacts health

The prevalence of obesity tripled since 1975 and adversely impacts public health worldwide (Hunter & Reddy, 2013). In 2030, 81% of men and 75% of women are projected to be overweight or obese (Y. Wang et al., 2020). Obesity is associated with many chronic diseases including hypertension, type II diabetes, cardiovascular disease and all-cause mortality (Bellou et al., 2018; Ma et al., 2017; Seravalle & Grassi, 2017). This makes obesity one of the leading causes of death (Di Angelantonio et al., 2016; Pi-Sunyer, 2009). Currently, obese patients are advised to reduce their caloric intake, which in general has poor adherence (Colombo et al., 2014). Bariatric surgery is a more effective means of weight loss, but is a major surgical intervention and has many reports of long-term weight recidivism (Karmali et al., 2013). Gaining a deeper understanding of the physiological changes that underlie obesity can help reveal novel therapeutics to alleviate this global issue.

Obesity is caused by food intake and food choice is most often based on taste

Although genetic factors, lifestyle and environmental factors can contribute to the development of obesity, surplus calorie intake is the drives the development of obesity (Jeffery & Harnack, 2007; Wright & Aronne, 2012). Fat and sugar are especially important as they are high in calories and are considered highly palatable (Keast et al., 2007; Morenga et al., 2013; Mozaffarian, 2016). Increased energy output by regular exercise is great for overall health, but can't compensate for higher caloric intake and has little effect on weight loss in the long term (Foright et al., 2018; King et al., 2009).

The most important reason for deciding what foods to eat is taste (Aggarwal et al., Rehm et al., 2016; Glanz, Basil et al., 1998; Kourouniotis et al., 2016; Zylan, 1996) As people make food choices based on

taste, understanding the underlying physiological changes in the gustatory system that occur with obesity may provide novel intervention strategies to ameliorate it.

Human sensory studies show a dampened sense of taste with obesity

In humans, sensory studies have long demonstrated decreased taste function in adult and younger adult patients with obesity (Bartoshuk et al., 2006; Ettinger et al., 2012; Noel et al., 2017; Overberg et al., 2012; Park et al., 2015; Pepino et al., 2010; Proserpio et al., 2016; Stewart et al., 2010, 2011). Studies results are not all in agreement with some not concluding there was on association (Drewnowski et al., 1991; Enns et al., 1979; Frijters & Rasmussen-Conrad, 1982; Ozdener et al., 2017; Rodin et al., 1976; Thompson et al., 1977), and others finding improvement of taste function (Hardikar et al., 2017).

Obesity-induced reduction in mouse taste buds

Understanding the underlying changes in taste physiology that occur with weight gain can offer a greater understanding of the mechanisms underlying obesity itself. The taste system is mostly housed in the tongue in mammals, where taste buds are collected in papillae. The front of the tongue contains many individual papillae termed fungiform, on the sides of the tongue taste buds are collected into the larger foliate papillae, and in the rear very large papillae containing hundreds of taste buds are termed the circumvallate (CV) papillae. Taste buds are made up of about 50-100 taste cells which are subdivided into three functionally distinct cell types: type I, type II and type III (Yoshida et al., 2009). Type I cells are termed glial like cells that sense salty taste (Chandrashekar et al., 2010), type II cells mediate sweet, bitter and umami taste (Chandrashekar et al., 2006; D. Liu & Liman, 2003; Mueller et al., 2005) while type III cells sense sour taste (Huang et al., 2008; R. Yang et al., 2000). Taste cells were originally described by their ultrastructure rather than their biochemical function (Kinnamon et al., 1985; Murray et al., 1969)

Obesity in mice and humans is an inflammatory state (Lee et al., 2013). Previously in our group, obese HFD-fed mice had fewer taste buds than lean, chow-fed littermate mice (Kaufman et al., 2018). After 8 weeks on the HFD, the obese mice also had fewer taste progenitor cells, marked by the cellular proliferation marker Ki67. This decrease in taste bud abundance was not seen in mice genetically lacking the cytokine (and systemic inflammation marker) Tumor Necrosis Factor alpha (TNFα), suggesting that TNFα plays a role in the reduction of taste bud abundance in obese mice (Kaufman et al., 2018).

Recently, supporting evidence for altered gene expression in the fungiform papillae (FP) of obese humans showed a decrease in taste markers and an increase in inflammatory markers, again TNFα (Archer et al., 2019). Correspondingly, the number of fungiform papillae in adult humans are negatively correlated with adiposity (Mameli et al., 2019). Additionally, college students tested for fungiform papillae density longitudinally over 4 years showed a negative correlation between papilla variation and changes in adiposity (Kaufman et al., 2020). Interestingly, when subjects have taste function disrupted with a taste blocker, they desire more intensely tasting foods (Noel et al., 2017) suggesting a chronically weakened sense of taste would encourage the consumption of more intensely tasting, and thus presumably higher calorie foods

Inflammation, cytokines, and taste dysfunction

Both acute and chronic inflammation have a meaningful effect on taste. After intraperitoneal injection of the inflammatory endotoxin Lipopolysaccharide (LPS), levels of inflammatory cytokines such as TNFα, interferon-gamma (IFN- gamma) , and Interleukin-6 (IL-6) increased in circumvallate taste buds, while Ki67, a cell proliferation marker, decreased (Cohn et al., 2010). This acute, short-term inflammation has been observed to inhibit proliferation of taste progenitor cells and reduces the number of new taste cells entering the taste bud. Additionally, LPS inflammation shortens the average lifespan of taste cells. Of course, this type of acute inflammation is not analogous to the systemic inflammation inherent in

obesity, but provides an illustration of the effects that inflammation can have on the taste system (Cohn et al., 2010).

A healthy balance of pro- and anti-inflammatory cytokines is important for the taste system to function normally. Cytokines such as interleukin-10 (IL-10) and TNFα that have the potential to act on the taste system are commonly expressed in taste cells. IL-10 was exclusively found in α-gustducin-expressing bitter-sensing taste cells (Feng et al., 2015). IL-10 deficiency in mice leads to a reduction in the number and size of taste buds, suggesting that it is critical to maintain structural integrity in the taste system (Feng et al., 2015). TNFα, a pro-inflammatory cytokine, seems to be critical to bitter taste signaling, as mice deficient in TNFα were found to be less sensitive to quinine with lickometer testing and two bottle testing and showing reduced aversive response to bitter compounds. Furthermore, chorda tympani nerve recordings showing reduced relative response to bitter compounds in TNFα knockout mice (Feng et al., 2014; Feng et al., 2015). TNFα is expressed in type II taste cells, which also express the sweet/umami taste receptor subunit Taste receptor type 1 member 3 (T1R3) (Feng et al., 2012). In toll-like receptor 4 knockout mice, that exhibit impeded activation of the innate immune system, reduced preference for fat, sweet, and umami taste is observed, as well as reduced intake of food, which in turn reduces weight gain (Camandola & Mattson, 2017). Additionally, during inflammation, interferon mediated signaling pathways in taste bud cells are activated (H. Wang et al., 2007).

Modulators of taste signaling and their effect on taste bud development and homeostasis

Taste cells are constantly renewing and have a half-life ranging from 8-22 days varying depending on the cell type and healthy renewal is dependent on a variety of signaling molecules which is aberrant in disease (Feng et al., 2014; Perea-Martinez et al., 2013). In addition to pro- and anti-inflammatory factors, there are a variety of signaling molecules that are critical for the development of new, functional taste buds. The organogenesis marker Sonic hedgehog (Shh) and the Wnt signaling factor

beta-catenin interact and are critical to the normal development and maintenance of taste papillae by being involved in papillary placodes and acts as a morphogen and mitogen (Hall et al., 2003; Iwatsuki et al., 2007). Altered local expression of Shh induces ectopic taste buds that are not dependent on gustatory innervation, in contrast to endogenous innervated taste buds (Castillo et al., 2014). Shh and Bone morphogenetic protein 4 (BMP4) are co-expressed in papillary placodes which serve as a signaling center for papillary development (Hall et al., 2003). Shh and BMP4 continue to be expressed in the adult taste epithelium and are involved in taste bud homeostasis (Miura et al., 2001). Their co-localization could point to the two acting in concert in the circumvallate papilla to regulate taste bud turnover. It has been proposed that intragemmal BMP4 positive cells in the CV are immature cells that act as precursors for type I, II, and III taste cells, whereas perigemmal BMP4 positive cells located in the CV and FP regions are slow cycling stem cells (Nguyen & Barlow, 2010)**.** The transcription factor Sox2, usually associated with stem cell pluripotency, is also required for mature taste bud development and has been proposed as a taste stem cell marker (Ohmoto et al., 2017; Okubo et al., 2006).

α-gustducin expression and function in taste cells

The taste signaling G-protein subunit α-gustducin is expressed in type II taste cells and plays a critical role in bitter taste sensation. Both in-vivo and in-vitro studies of α-gustducin knockout mice demonstrate its function in bitter detection (Minget al., 1998; Wong et al., 1996). Although important, not all bitter sensitive taste cells express alpha α-gustducin, while genetic deletion only decreases bitter responses partially (Caicedo et al., 2003). An alternate signaling pathway transducing bitter taste without α-gustducin or T2Rs has been proposed, but for the majority of bitter taste α-gustducin is required (Caicedo et al., 2003).

α-gustducin expression in metabolic disorders

Diabetic patients, like obese patients, exhibit reduced taste acuity. Diabetes is also an inflammatory state, characterized by inflammatory markers linked to adipose tissue that are similar to markers in obesity (Lontchi-Yimagou et al., 2013). As previously discussed, there are a variety of taste changes observed in obese patients compared to their lean counterparts. Several studies show a decrease in taste related proteins. α-gustducin is increased in the CV of diabetic rats, determined by a combination of immunohistochemistry and RT-PCR (L. Zhou et al., 2009).

In human patients, after Roux-en-Y gastric bypass surgery, which usually initiates drastic weight loss, FP expression of α-gustducin was reduced threefold (Pepino et al., 2014). T1R1, T1R2 and T1R3 were not affected, while PlCβ2 was slightly reduced.

Given the physiological changes in the gustatory system that occur with obesity, this report sought to examine the effect of dieting after obesity-induced taste bud loss in mice. Few studies have examined the effect of a reduction in weight on the taste system via diet, but there exists a large body of evidence on bariatric surgery's effect on the gustatory system, in both rodents and humans.

Weight loss intervention through bariatric surgery can influence both sweet and fat taste. In both rats and humans, gastric bypass reduced both fat intake and preference; as fat is highly caloric, this represents one way in which gastric bypass can be effective in reducing weight (le Roux et al., 2011). In both rats and humans, gastric bypass also changes sucrose detection thresholds and preference. Rats reduce sucrose intake in two-bottle tests after gastric bypass. Human bypass patients report an increase in perceived taste intensity compared to controls but with no difference in sucrose liking (Bueter et al., 2011). Both Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding induced a decrease in preferred sucrose concentrations, perceived levels of sweetness, and cravings for sweets and fast foods (Marta Yanina Pepino et al., 2014).

A systematic review identifying changes in rodents and humans showed altered taste acuity after gastric bypass. Prominent changes noted were an increase in sensitivity to sweet and fat taste, as well as a decreased preference for sweet-tasting stimuli, though these effects have not yet been confirmed in the long-term (Shoar et al., 2019).

Hormones in bariatric surgery

Originally, gastric bypass and similar surgical interventions were designed to mechanically induce calorie restriction (van Koningsbruggen et al., 2010). A mounting body of evidence has shown a role for hormones in contributing to gastric bypass-induced weight loss.

Peptide Y (PYY), an anorexigenic hormone, is increased after gastric bypass surgery, but not after traditional caloric restriction (Karamanakos et al., 2008). PYY delays gastric emptying and increases energy expenditure (Sloth et al., 2007). Similarly, GLP-1 is also elevated, slowing gastric emptying, inducing insulin secretion, and inhibiting glucagon release (Suzuki et al., 2012). GLP-1 also increases many fold after gastric bypass when compared with caloric restriction (Steven et al., 2016).

Taste machinery is expressed in other parts of the body

Taste-like cells are expressed all over the body, in tissues including the gut, lung, and genitourinary systems. Taste-like signaling is also seen in the brain and immune cells. Taste cells in the gut are of particular interest for their role in hormone signaling (P. L, Zhang et al., 2017). Glucagon-like peptide 1 (GLP-1), which augments insulin secretion from the pancreas after oral glucose intake (Balsano et al., 1964), is regulated by α-gustducin. α-gustducin knockout mice exhibit defective GLP-1 signaling (Jang et al., 2007). GLP- 1 has been approved for use in weight loss for its insulinotropic properties. Interestingly, increased secretion of GLP-1 may be partially responsible for the weight loss benefits of Roux-en-Y gastric bypass. Along with decreased stomach size, GLP-1 may explain altered satiety post-surgery (Borg et al., 2006). Interestingly, the perception of taste and odor, and brain activity in regions associated with

reward and taste perception are also altered with obesity (Behary & Miras, 2015; Kittrell et al., 2018; Thanos et al., 2015). Altering endogenous hormone levels through α-gustducin and GLP-1 might be an alternative to surgical interventions such as bariatric surgery.

Divergences in the gustatory system of females and males

Few studies explore sex differences between female and male mice. Female mice are often not included in research as they are perceived to be more variable than males, although, at least in neuroscience research, this is not the case (Becker et al., 2016). In obesity studies, females gain less weight than males and are sometimes excluded for that reason. As animal studies often inform clinical studies, including females in research is important to get a complete understanding of the gustatory system, including potential sex differences.

In human work, women have been found to have a higher frequency of being able to taste phenylthiocarbamide (PTC), women are so-called "supertasters" more frequently than men (34% female vs. 22% males), also having more FP compared to males (Bartoshuk et al., 1994; Garneau et al., 2014). Furthermore, females reported PTC strips as tasting more intense (Spence et al., 2014), although not on a gLMS scale. Additionally, preference for sweeter concentrations was higher in males than in females, with body weight inversely correlated with preference (Enns et al., 1979).

Females and males also display differences in chosen sources of energy intake. Based on an assessment of energy intake from the Dutch national food consumption survey and the Nutrition Questionnaire plus study, men consumed more energy from 'salt, fat and umami foods' and 'bitter' compared to women who ate more 'sweet and fat' and 'sweet and sour foods'. Both obese men and women consumed more energy from 'salt, umami and fat' and less from 'sweet and fat foods' (Van Langeveld et al., 2018). Disparities in female and male gustatory systems might guide disparities in food selection.

Some studies conversely do not report a difference between the basic tastes between females and males, but this may be due to the number of subjects enrolled (n=17 for each group) (Robin et al., 2003).

Behavioral testing in rodents reveals a difference in salt taste between female and male rats. In two-bottle testing, female rats drank more 3% NaCl solution compared to male rats (Křeček et al., 1972). These differences were confirmed in a later study that showed females had a higher preference for NaCl concentrations (Flynn et al., 1993).

Sex differences in taste might be attributed to differences in estrogen and testosterone early in development (Chow et al., 1992; Křeček, 1973). Early administration of testosterone suppressed sex differences in salt taste, which suggests that testosterone might affect salt preference (Křeček, 1973).

Obesity-induced changes in gustatory projections to the brain, neuronal cell function, and brain organization

Along with obesity-induced gustatory changes observed in humans and mice, changes in the brain are also observed with weight gain and varies between sexes. Linoleic acid combined with monosodium glutamate elicited greater nerve responses in the chorda tympani, and greater preference in male rats compared to female rats (Stratford et al., 2008). Obese mice fed a HFD for 10 weeks, calcium imaging revealed a reduction of taste cells responsive to sweet taste, with female mice having fewer cells responsive to sweet stimuli, although there was reduced weight gain in females. HFD fed, obese mice had unfitting responses to sweet stimuli, with females and males showing variances (Maliphol et al., 2013).

fMRI studies also reveal differential activation of satiety in females and males, with females showing less change in activation from hunger to satiety relative to males (Haase et al., 2011) with the exact mechanisms of satiety still being explored (Aitta-Aho et al., 2017). Further evidence from human fMRI studies shows that brain reward circuits involved in motivation, executive control, and in drug addiction

are less responsive (Frank et al., 2012; Green et al., 2011; Kure Liu et al., 2019). Such a loss in taste function may lead to enhanced caloric consumption, as was shown when pharmacologically blocking taste function (Noel et al., 2017).

Methods

As previously shown, HFD-induced obesity in C57BL/6J can be reversed when placed back on a chow diet (Parekh et al., 1998). In this study, mice were placed on a diet of chow, HFD, or HFD for the first half of the study and then switched to chow for the second half. After this, taste cells, taste progenitor/ stem cells, apoptotic cells, and signaling molecules, were quantified. The CV was analyzed using immunohistochemical staining (IHC) and with RT-PCR of the mRNA extracted from the CV and subsequently reverse transcribed into cDNA. Females and males were included to understand potential sex differences. Our hypothesis was that there would be an attenuation of taste buds and reduced number of progenitor cells in the HFD group, as previously shown in mice fed HFD for 8 weeks, but expected a return to chow diet to at least partially recover HFD-induced taste bud loss, reduce inflammation, increase progenitor cells, and return taste signaling modulators to levels similar to chow-fed mice.

Animals

All animal studies were approved by the Institutional Care and Use Committee of Cornell University according to protocol 2012-0080. Female and Male C57Bl/6 mice were purchased from Jackson Laboratory and kept on a standard chow diet of Harlan Teklad 8604 with 14% fat, 54% carbohydrate, and 32% protein (Harlan Teklad 8604) (Envigo, Indianapolis, IN, USA). Mice were housed in a climate-controlled environment at the East Campus Research Facility at Cornell University College of Veterinary Medicine. Eight-week-old male mice were fed either the standard rodent diet or a HFD consisting of 58.4% fat, 26.6% carbohydrate, and 15% protein (Harlan Teklad TD.03584) (Envigo, Indianapolis, IN,

USA) for a period of 8 weeks, then maintained for a further 8 weeks on their specified diet, or switched in the case of the third group (HFD-Chow) at 16 weeks old. Mice were euthanized at 24 weeks with CO2 followed by cervical dislocation, with taste buds isolated for RT-PCR, or CV tissues extracted and frozen for IHC as described below.

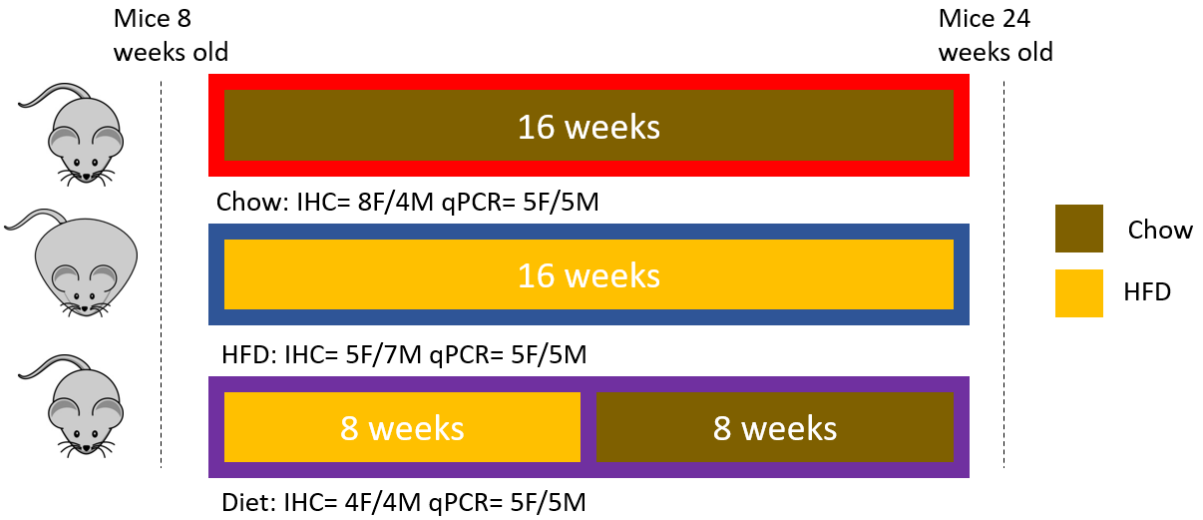

Figure 1: Experimental schematic for mouse diet treatments. Groups were Chow (16 weeks), HFD (16 weeks) and HFD then Chow (8 weeks each).

RT-PCR

After sacrifice mouse tongues were excised and placed in normal Tyrodes solution (NaCl 135 mM, KCl 5 mM, $CaCl_2$ 2 mM, MgCl 1 mM, $NaHCO_3$ 5 mM, HEPES 10 mM, Glucose 10 mM, Sodium Pyruvate 10 mM, pH 7.4) all from (Sigma-Aldrich, St. Louis, MO, USA). After this, the tongue was pinned down and the circumvallate papillae (CV) injected subepithelially around CV region with a mixture of Dispase II (2.5 mg/ml) (CEllnTEC, Bern, Switzerland), Collagenase A (1 mg/ml) (Worthington Biochemical Corporation, Lakewood, NJ, USA), Elastase (0.25 mg/ml) (Worthington Biochemical Corporation, Lakewood, NJ, USA), and DNaseI (0.5 mg/ml) (Sigma-Aldrich, St.Louis, MO, USA). to dissociate. After a 20-minute incubation at room temperature, the CV was peeled and the taste buds were extracted from the underside of the epithelium with a fire-polished micropipette. mRNA was isolated using Absolutely RNA Microprep Kit

(Stratagene, Cedar Creek, TX). Next, RNA was reverse transcribed into cDNA using qScript cDNA Supermix (Quanta Bio, Beverly, MA) and diluted using standard curves. qRT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was run on a QuantStudio 6 Flex Real-Time PCR System (Thermo, Waltham, MA). Relative quantification was performed using QuantStudio PCR Software, based on the $2^{-\Delta\Delta Ct}$ method. β-Actin was used as a housekeeping control gene. All genes were tested in triplicates.

Immunohistochemical staining

Mice were euthanized at 24 weeks, CV tissue regions were dissected from tongues, placed in 4% PFA (Fisher Scientific Hampton, NH, USA) /PBS (VWR, Radnor, Pennsylvania) for 1 ½ hours, washed with PBS (VWR, Radnor, Pennsylvania) 3x for 20 minutes, and then cryoprotected in sucrose (Sigma Aldrich, St. Louis, MO, USA), then embedded in OCT, (Fisher Scientific Hampton, NH, USA) and frozen at -80 C. Circumvallate tissues were cryosectioned at 10 um thickness, washed in PBS (VWR, Radnor, Pennsylvania), and incubated in 1% triton (MilliporeSigma, Burlington, MA, USA). Tissue sections stained with 1:500 polyclonal Goat GNAT3 OAEB00418 (α-gustducin) from Aviva Systems Biology (San Diego, CA, USA), and 1:125 polyclonal Rabbit Caspase-3 AF835 from R&D systems (Minneapolis, MN, USA) were incubated with 4% bovine serum albumin (BSA) (Amresco, Solo, Ohio, USA), 4% donkey serum (Equitech-bio, Kerrville, TX, USA), and 0.3% triton MilliporeSigma, Burlington, MA, USA). Tissue sections stained with 1:125 polyclonal Goat MPO AF3667 from R&D systems (Minneapolis, MN,USA), rabbit polyclonal Ki67 PA5- 19462 1:125 polyclonal Goat MPO AF3667 from R&D systems (Minneapolis, MN,USA), and goat polyclonal KCNQ1 OAEB01457 1:1000 from Aviva Systems Biology (San Diego, CA) were blocked for 2 hours at room temperature with 2% BSA (Amresco, Solo, Ohio, USA), 2% donkey serum (Equitech-bio, Kerrville, TX, USA), and 0.3% triton (MilliporeSigma, Burlington, MA, USA). After incubation with secondary Alexa Fluor donkey anti-Goat or anti-Rabbit secondary (Invitrogen, Carlsbad, CA, USA) at room temperature for 2 hours, sections were washed 3x for 20 minutes in PBS (VWR, Radnor, PA, USA),

and placed on a coverslip with Dapi staining medium (Fluoromount-G, Southern Biotech, Birmingham, AL, USA).

Taste bud counting

Tissue sections were imaged using an Olympus IX-71 inverted scope and Hammatsu Orca Flash 4.0 camera (Hamamatsu Photonics, Hamamatsu City, JP), and counted using ImageJ (NIH, Bethesda, MD) for taste buds, number of α-gustducin positive cells, number of caspase-3 positive cells, Ki67-positive cells, and number of neutrophils.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (San Diego, CA, USA). RT-PCR samples were compared using non-parametric Kruskal-Wallis tests. IHC samples were compared with a parametric 2way ANOVA to test for the effect of diet and sex, with Tukey's multiple comparisons post-hoc tests. All groups were analyzed for normality with a D'Agostino & Pearson normality test, with alpha 0.05. All groups passed normality testing except for the HFD treated mice analyzed for caspase. After one outlier was removed the group did pass normality testing, though with or without the outlier the interpretation of the data did not change. Statistical significance assumed at $p < 0.05$.

Results

Previous work from our lab showed that epididymal fat pads (made up of metabolically active white adipose tissue) from C57Bl/6 mice fed a HFD weigh about 3 times that of their chow fed counterparts after 8 weeks (Kaufman et al., 2018). In this study, female mice started at a lower weight, but both female and male mice had similar weight gain by percent of initial body weight (Figure 2). After returning to chow from a HFD, the dieted mice rapidly lost weight. After one week on a chow diet, they

were being significantly leaner than the HFD group ($p < 0.001$) and did not have a weight difference compared to chow mice ($p < 0.702$). At the same time point (9 weeks), HFD mice were heavier than chow or diet mice ($p < 0.001$). There was no sex difference in weight gain observed

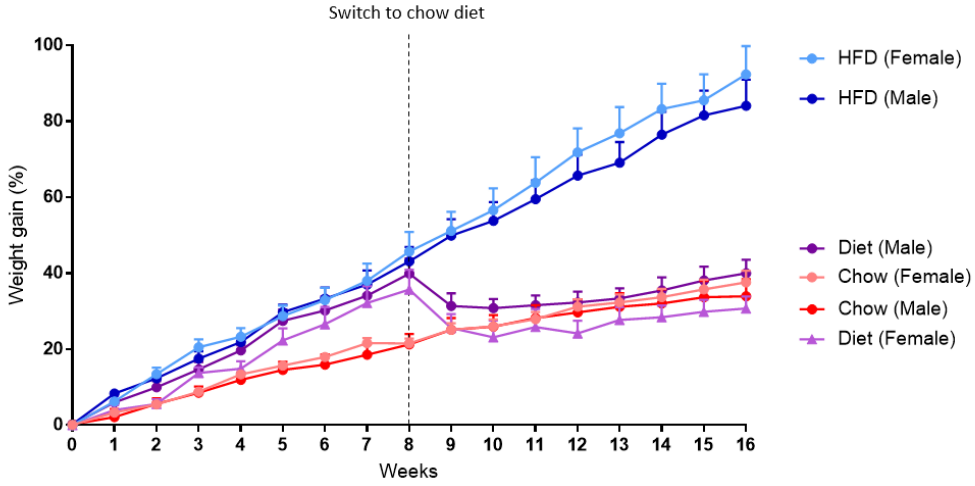

Figure 2: Weight gain in percent across weeks of three different groups of mice: Chow (red, *n* = 29, 17 female, 12 male), high-fat diet (HFD) (blue, *n* = 27, 14 female, 13 male), and diet (purple, *n* = 17, 7 female, 10 male). Female mice in light color, male in dark. Bars represent means plus/minus SEM.

Losing weight is not sufficient to rectify taste deficiency after weight gain

Sections of the circumvallate papillae for all 3 groups were processed and stained for the general taste cell marker KCNQ1, to quantify taste bud abundance. HFD mice had fewer taste buds than chow-fed mice (Figure 3A, B), where chow-fed mice had an average of 24.24 taste buds, HFD mice had 16.63, and dieted mice had 19.93. That means that HFD mice lost 31% of their taste buds when compared to chow-fed controls ($p < 0.001$), with dieted mice, who were now the same weight as chow fed controls still having 18% fewer taste buds than the control group ($p = 0.001$). Compared to HFD-fed mice, dieted mice had statistically more taste buds ($p = 0.014$), indicating that some degree of restoration to the damage induced by obesity had occurred, but that it was incomplete, at least in this time scale.

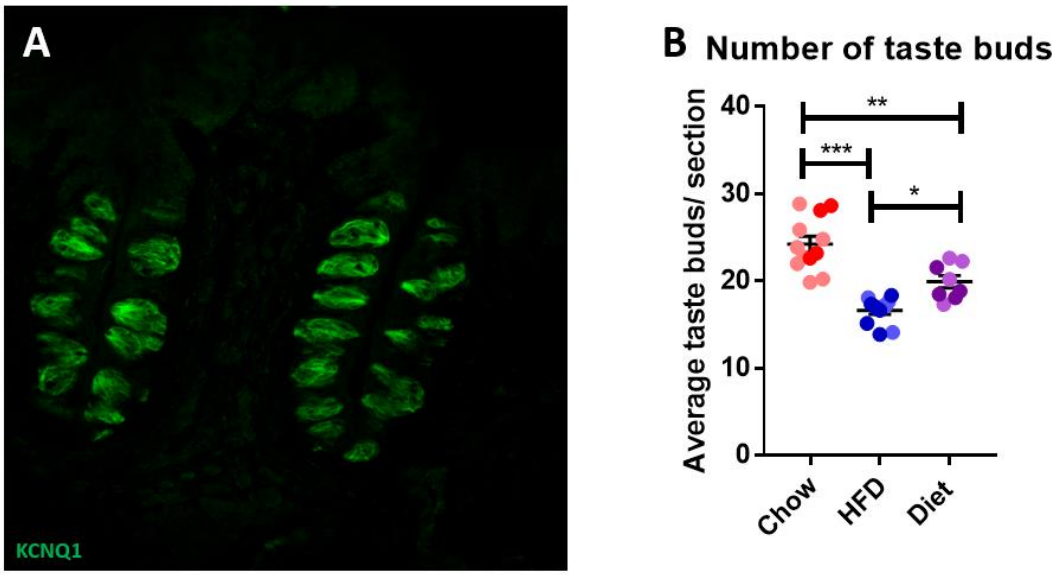

Figure 3: A: Representative image of circumvallate papilla with KCNQ1 staining (green) highlighting taste buds. B: Taste bud abundance per CV section, quantified with KCNQ1 for all treatment groups: chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) (blue, *n* = 12, 5 female, 7 male), and dieted (purple, *n* = 8, 4 female, 4 male). Female mice in light color, male in dark. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM.

Taste bud abundance correlate directly with both the body weight (Figure 4A, Pearson's r =-0.422, p = 0.016), and the percent weight gain across the treatment period (Pearson's r = -0.508, p = 0.003).

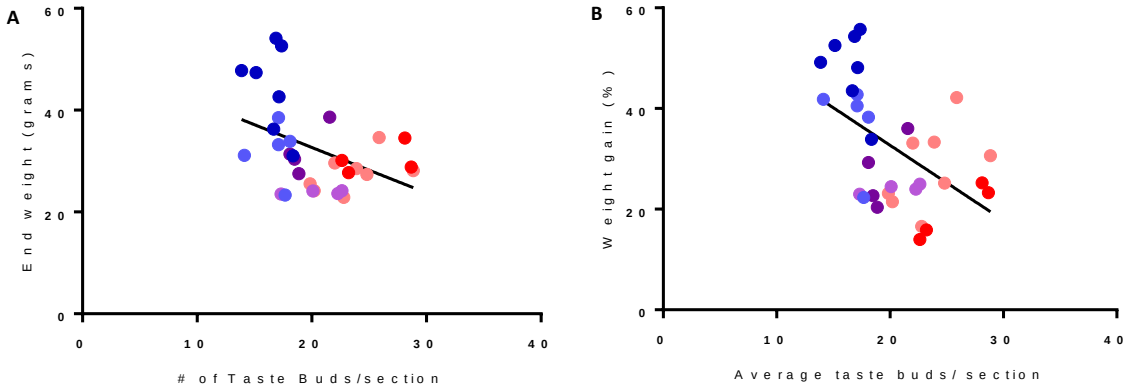

Figure 4: A: End body weight (grams, y-axis) of mice from all 3 treatment groups versus number of taste buds/ section (x-axis). Chow in red (*n* = 12, 8 female, 4 male), high-fat diet (HFD) (*n* = 12, 5 female, 7 male), and diet (*n* = 8, 4 female, 4 male) in purple (Pearson's *r* =-0.422, *p* = 0.16). Female mice in light color, male in dark. B: Weight gain (%, y-axis) versus number of Taste buds/ section (x-axis). Chow in red (*n* = 12, 8 female, 4 male), high-fat diet (HFD) (*n* = 12, 5 female, 7 male), and diet (*n* = 8, 4 female, 4 male) in purple (Pearson's *r* = -0.508, *p* = 0.003). Female mice in light color, male in dark.

Chow-fed mice have a lower proportion of α-gustducin-positive cells compared to HFD mice

The subunit α-gustducin is expressed in a subset of type II cells and is critical in bitter taste transduction. The number of α-gustducin cells per taste bud was counted and divided by the number of KCNQ1-positive cells. Interestingly, a significantly reduced proportion of the chow-fed mice's taste cells were α-gustducin-positive compared to HFD-fed mice. Shown in Figure 5A, B, across groups there were significantly more gustducin-positive cells in the HFD than chow-fed controls ($p < 0.001$). Diet mice almost had more α-gustducin-positive ($p = 0.058$) than chow mice. The proportion of the HFD mice's taste cells that were α-gustducin-positive were also almost significantly increased compared diet mice ($p = 0.055$).

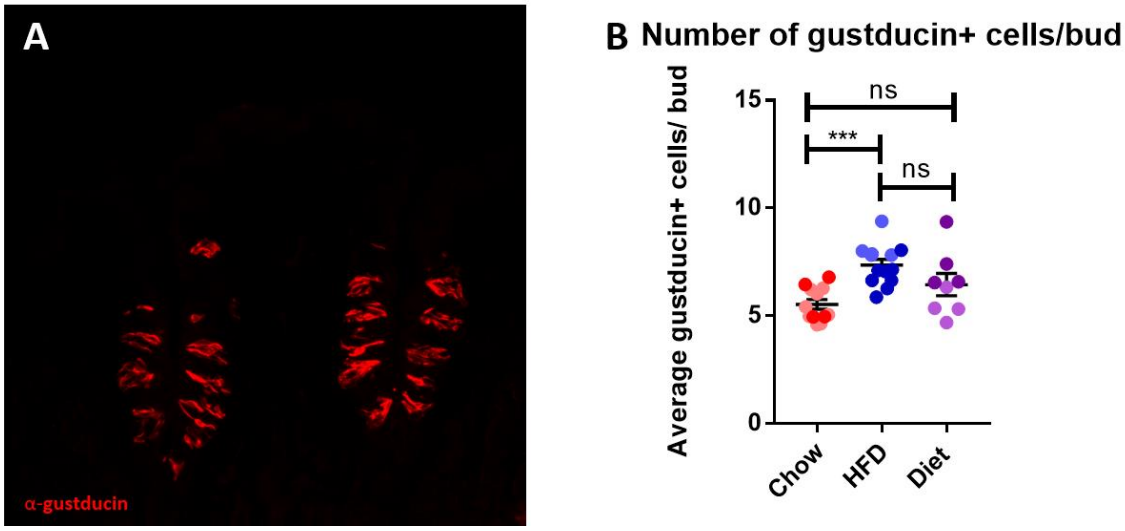

Figure 5: A: Representative image of circumvallate papilla showing α-gustducin (red) staining in a subset of type II taste cells. B: α-gustducin-positive cells per taste bud CV section for all treatment groups: chow (red, $n = 12$, 8 female, 4 male), high-fat diet (HFD) (blue, $n = 12$, 5 female, 7 male), then diet (purple, $n = 8$, 4 female, 4 male). Female mice in light color, male in dark. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM.

Additionally, there was a significant difference between females and males overall ($p < 0.001$), HFD females vs. males ($p = 0.022$), and diet female vs. male ($p = 0.004$) shown in Figure 6.

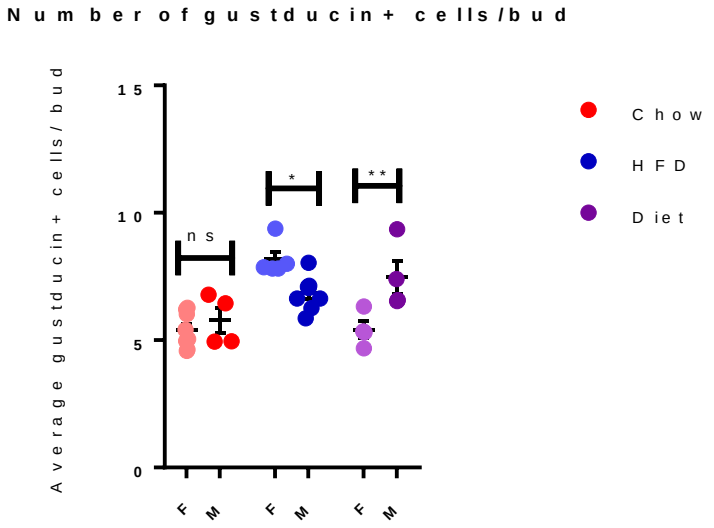

Figure 6: α-gustducin-positive cells per taste bud CV section for all treatment groups separated by sex: chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) (blue, *n* = 12, 5 female, 7 male), then diet (purple, *n* = 8, 4 female, 4 male). Female mice in light color, male in dark. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM.

No change in number of neutrophils across groups

The number of neutrophils present in taste tissue was also quantified, using the Myeloperoxidase (MPO) marker (Figure 7A, B). While HFD mice did have the highest average number of neutrophils per section, the difference was not significant between any groups overall ($p = 0.849$), with chow mice compared to both diet and HFD ($p > 0.999$) and HFD compared to diet ($p = 0.5866$). Finally, there was no difference between females and males.

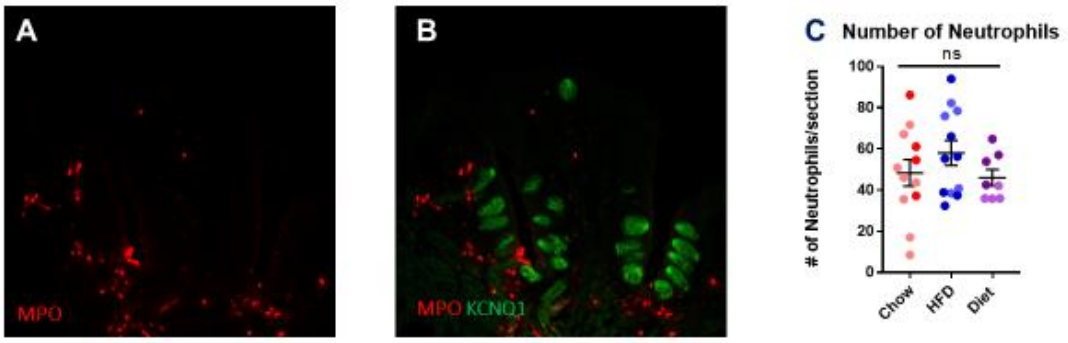

Figure 7: A: Representative image of circumvallate papilla showing MPO (red) staining B: Representative image of circumvallate papilla showing MPO (red) staining co-stained with KCNQ1 (green) C: MPO-positive cells per CV trench for all treatment groups in chow (red, n = 12, 8 female, 4 male), high-fat diet (HFD) (blue, n = 12, 5 female, 7 male), then diet (purple, n = 8, 4 female, 4 male). Female mice in light color, male in dark. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM.

The proliferative capacity of taste buds is improved by dieting

To analyze the possible that the suppression of taste progenitor cells by weight gain can be reversed, and to further explore mechanisms for the reduced number of taste buds observed in HFD and dieted mice, Ki67-positive cells, responsible for the production of new taste cells, were quantified (Figure 8A, B). Ki67 is an important marker for actively proliferating cells throughout the body, including in the lingual epithelium.

Chow-fed mice had a significantly more Ki67-positive cells compared to HFD-fed mice (p = 0.004), with diet-fed mice not statistically different from controls, despite displaying fewer cells on average (p = 0.128) with no difference in HFD compared to diet mice (p = 0.328). The number of Ki67-positive cells followed a similar trend to taste buds, with chow-fed mice having the highest number of taste buds, HFD the fewest, and dieted mice between the two.

Figure 8: A: Representative image of circumvallate papilla showing Ki67 (green) B: Representative image of circumvallate papilla showing Ki67 (green) co-stained with α-gustducin (red) C: Ki67 per CV section for all treatment groups: chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) (blue, *n* = 12, 5 female, 7 male), then diet (purple, *n* = 8, 4 female, 4 male). Female mice in light color, male in dark. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM.

HFD-fed obese mice have more apoptosing cells in taste tissue than lean Chow-fed or dieted mice

Caspase-3 is an apoptotic cell marker which marks cells undergoing programmed cell death. Mice fed a HFD exhibited a higher number of caspase-positive cells in CV taste tissue than either chow-fed (Figure 9A, B, $p = 0.001$) or dieted mice ($p = 0.007$).

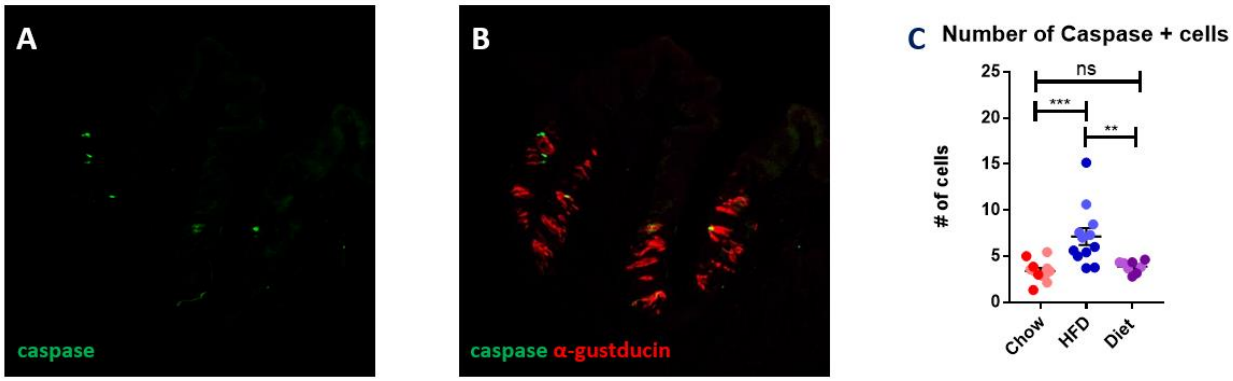

Figure 9: A: Representative image of circumvallate papilla showing caspase-3 (green) B: Representative image of circumvallate papilla showing caspase-3 (green) co-stained with α-gustducin (red) C: Caspase-3 per CV section for all treatment groups: chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) (blue, *n* = 12, 5 female, 7 male), then diet (purple, *n* = 8, 4 female, 4 male). Female mice in light color, male in dark. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$. Bars represent means plus/minus SEM.

Interestingly the abundance of taste buds was negatively correlated with the number of caspase-positive cells (Figure 10, Pearson's r = -0.572, p = 0.001), indicating that the more apoptosis that was occurring in taste tissues, the fewer taste buds remained.

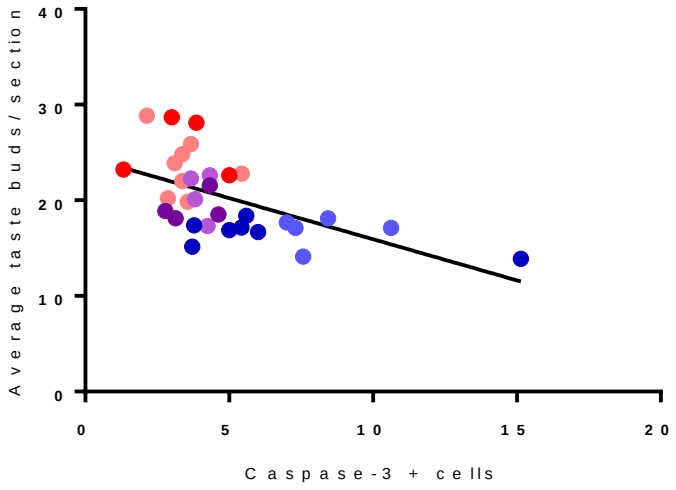

Figure 10: Number of taste buds/ section (y-axis), caspase + cells (x-axis). Chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) in (blue, *n* = 12, 5 female, 7 male), and diet (purple, *n* = 8, 4 female, 4 male). Pearson's *r* = -0.572, *p* = 0.001). Female mice in light color, male in dark

Caspase-positive cells were significantly correlated with the mouse's percentage weight gain (Figure 10B, Pearson's *r* = 0.422, *p* = 0.013), possibly a superior measure in this situation compared to body weight of mice at the study's conclusion (Figure 10A, Pearson's *r* =0.344, *p* = 0.054), which was not significantly correlated with caspase-positive cells. This could be a reflection of the variance introduced by studying both male and female mice, where differences between sex may mask the relationship somewhat as females weigh less, even at the beginning of the experiment. This indicates that the more weight a mouse put on throughout the study, the more taste cells were undergoing apoptosis within the CV.

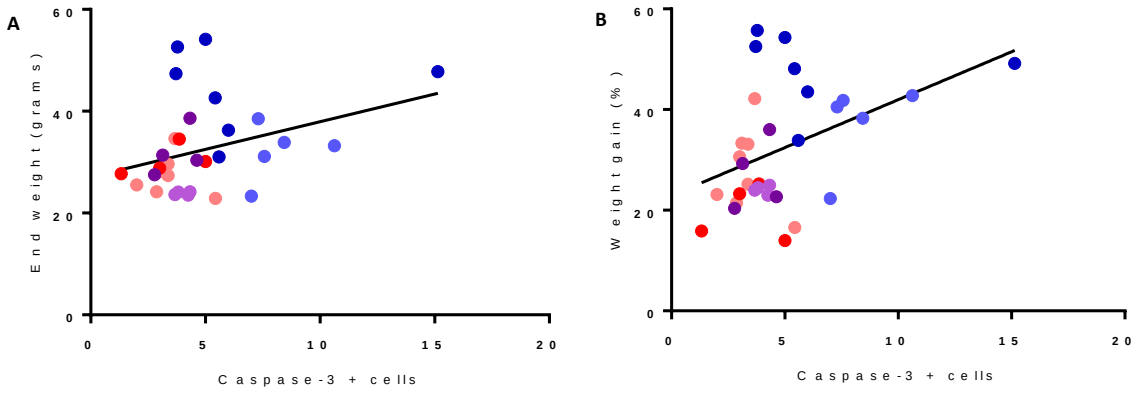

Figure 11: A: End weight (grams) (y-axis), Caspase + cells (x-axis). Chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) (blue, *n* = 12, 5 female, 7 male), and diet (purple, *n* = 8, 4 female, 4 male) (Pearson's *r* =0.344, *p* = 0.054). Female mice in light color, male in dark. B: Weight gain (%) (y-axis), Caspase + cells (x-axis). Chow (red, *n* = 12, 8 female, 4 male), high-fat diet (HFD) (blue, *n* = 12, 5 female, 7 male), and diet (purple, *n* = 8, 4 female, 4 male) (Pearson's *r* = 0.422, *p* = 0.013). Female mice in light color, male in dark.

Inflammation is activated in obese mice

Analogous to previous results, the expression of mRNA for the pro-inflammatory cytokine TNFα was significantly enhanced in the HFD-fed mice, when compared to chow-fed controls (Figure 12A, *p* = 0.006). Dieted mice still had moderately increased levels of TNFα (*p* = 0.0619), albeit not statistically significantly. IL-6 and IL-10 did not show a significant change across groups (Figure 12B, C).

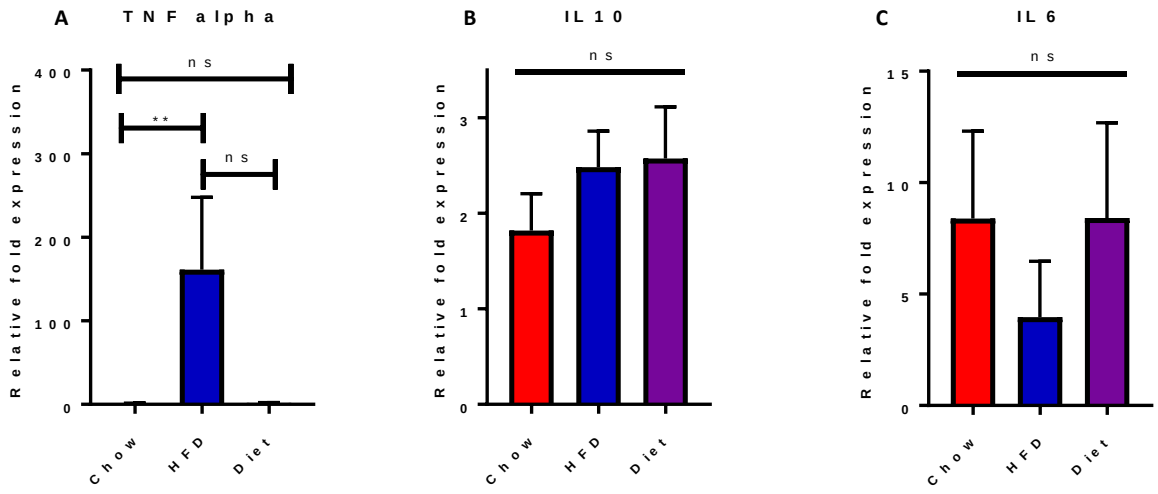

Figure 12: A-C: RT-PCR analysis of the expression of mRNA for inflammatory markers TNF alpha (A), IL-10 (B), and IL-6 (C) in CV papillae after 16-week treatment with chow (red), HFD (blue), or dieting (purple) (*n* = 6-10 each). Relative gene expression levels are shown (fold change). β-actin was used as the endogenous control gene for relative quantification. Error bars represent SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

Increased taste modulators in HFD-fed mice compared to diet mice

Shh signaling is critical for the renewal and maintenance of taste cells (Ermilov et al., 2016). HFD-fed mice exhibited increased expression of mRNA encoding Shh, when compared to chow-fed or dieted mice (Figure 13A). Similarly, BMP4 expression was also upregulated in HFD mice compared to dieted mice (Figure 13B, $p = 0.029$), with a trend towards an increase versus chow mice, albeit not significant ($p = 0.071$). Finally, Sox 2, proposed as a marker of stem or progenitor cells in taste, was nominally downregulated in HFD-fed mice compared to chow-fed mice ($p = 0.078$) with no difference in dieted mice ($p = 0.209$) (Figure 13C).

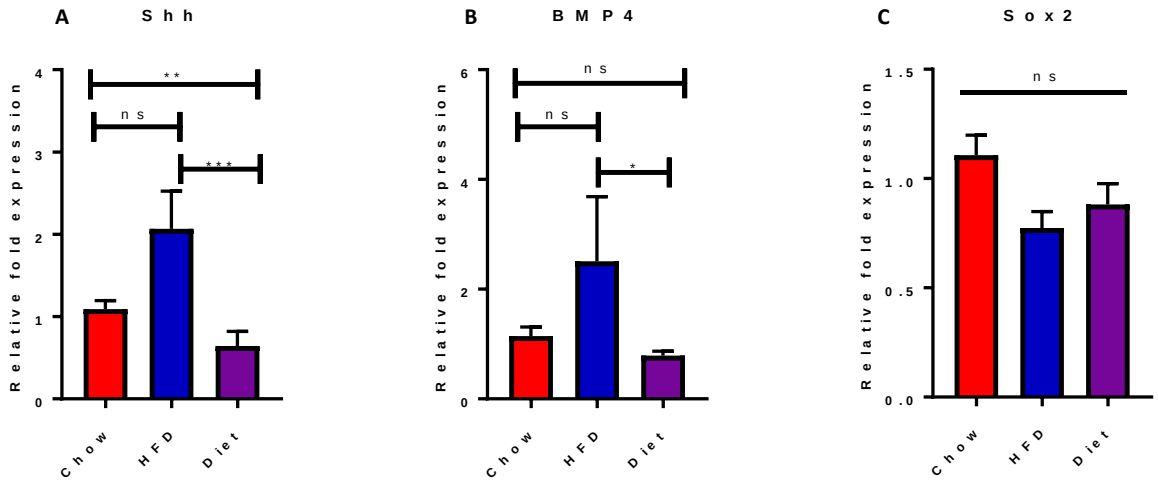

Figure 13: A-C: RT-PCR analysis of the expression of mRNA for taste modulators Shh (A), BMP4 (B), Sox2 (C) in CV papillae after 16-week treatment with chow (red), HFD (blue), or dieting (purple) (n = 6-10 each). Relative gene expression levels are shown (fold change). β-actin was used as the endogenous control gene for relative quantification. Error bars represent SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

Markers for cell turnover are altered in HFD and diet mice

mRNA expression for Ki67, a marker of proliferation, was reduced in dieted mice compared to controls (Figure 13A, $p = 0.002$).

E2F transcription factor 1 (E2F1), a cell cycle transcription factor for cell cycle progression, exhibited the same trend, with dieted mice having significantly reduced expression of mRNA for E2F1 (Figure 14B, $p = 0.004$). Finally, expression of mRNA for Bcl-2 associated X protein (Bax), a marker for apoptosis, was increased in HFD-fed mice ($p = 0.049$), as well as the dieted mice ($p = 0.046$) (Figure 14C).

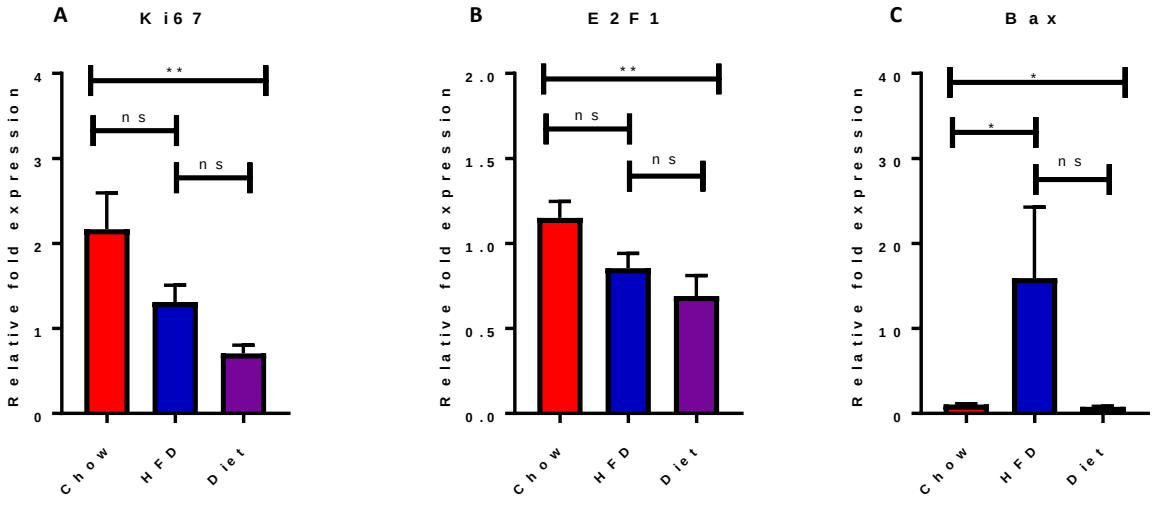

Figure 14: A-C: RT-PCR analysis of the expression of mRNA for proliferation and apoptosis markers Ki67 (A), E2F1 (B), Bax (C), in CV papillae after 16-week treatment with chow (red), HFD (blue), or dieting (purple) (n = 6-10 each). Relative gene expression levels are shown (fold change). β-actin was used as the endogenous control gene for relative quantification. Error bars represent SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

Reduced expression of PLCβ2 in dieted mice

Expression of mRNA for PLCβ2 was significantly reduced in the dieted mice compared to chow (p = 0.031) and HFD-fed mice (p = 0.035) (Figure 15A). Although mRNA for α-gustducin did not vary between the groups, patterns followed that of cell-counting, whereby chow was lowest, followed by diet, with HFD highest of the groups (Figure 15B).

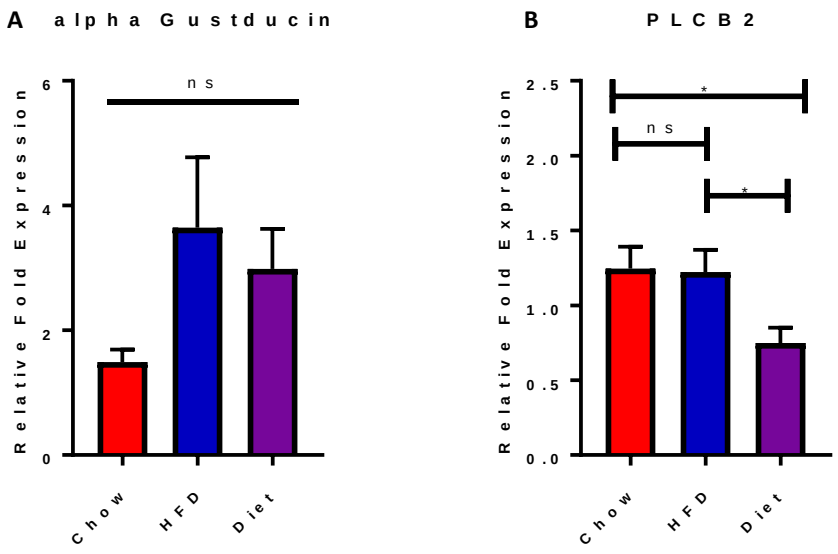

Figure 15 A-B: RT-PCR analysis of the expression of mRNA for taste cell markers alpha Gustducin (A), PLCB2 (B), in CV papillae after 16-week treatment with chow (red), HFD (blue), or dieting (purple) (n = 6-10 each). Relative gene expression levels are shown (fold change). β-actin was used as the endogenous control gene for relative quantification. Error bars represent SEM. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$.

Discussion

Weight gain and inflammation from HFD

In this study, as expected, mice fed a HFD quickly gained weight (Figure 1), as shown in our previous work (Kaufman et al., 2018). After 8 weeks on HFD, one group of C57Bl/6 mice were placed back on the lower fat, chow diet and quickly lost weight. After two weeks on the chow diet, the weight of dieted mice was indistinguishable from the chow group. C57Bl/6 mice have been previously shown to lose weight rapidly on a chow diet after being on a HFD (Lass et al., 1998) or with caloric restriction (Kowalski et al., 2016; Mahoney et al., 2006).

Inflammatory cytokines have been well established to play an important role in obesity (Kern et al., 2019). In our previous work (Kaufman et al., 2018), TNFα was found to be critical in obesity-induced taste bud loss, with TNFα knockout mice on a HFD showing no reduction in taste bud abundance after

similar levels of weight gain as wild type. Similar to the previous study, TNFα was highly upregulated in HFD-fed mice, compared to chow-fed mice (Figure 13A). Dieted mice still showed a trend towards an increase in TNFα, although this was not significant (Figure 13A, $p = 0.062$). Differences in IL-6 and IL-10 expression were minimal between treatments (Figure 13B, C).

In healthy mice, pro- and anti-inflammatory cytokines are important in normal taste bud development. Similarly, cytokines are co-localized and critical in taste function; IL-10 is expressed in α-gustducin-positive taste receptor cells, and IL-10 deficiency in mice leads to a reduction in the number and size of taste buds. TNFα is also expressed in type II taste cells which also express T1R3 (Feng et al., 2012), and seems critical to bitter taste responses, as TNFα knockout mice are less sensitive to the bitter compound quinine, assayed through *chorda tympani* nerve recordings (Feng et al., 2015).

Disruption of taste bud homeostasis in HFD mice is not and dieted mice

In agreement with our previous work (Kaufman et al., 2018), obese C57Bl/6 mice had fewer taste buds compared to chow-fed, lean counterparts (Figure 3B). Surprisingly, mice that were dieted back to a healthy weight after gaining weight on the HFD still had significantly fewer taste buds than lean controls, suggesting that weight gain had a long lasting effect on taste buds even after returning to a chow diet. Taste bud abundance correlated directly with both the end weight and percent weight gain across the treatment period (Figure 4A, B). These data suggest even weight gain earlier in life can cause a disruption in taste bud homeostasis. Either recovery is incomplete, or takes longer than the timeline of this study, which would represent a meaningful proportion of a mouse's lifespan.

Taste bud homeostasis is also disrupted in other diseases but can be partially or fully restored. After pharmacological or radiation chemotherapy treatment, taste buds are initially reduced in number, but recover 2-3 weeks after treatment ends (Mukherjee et al., 2017; Nguyen et al., 2012). Additionally, inhibiting Shh signaling, which is critical for taste bud development and maintenance, with 14 days of a

hedgehog pathway treatment, disrupts taste bud homeostasis (Kumari et al., 2018; Kumari et al., 2017). However CV taste buds fully recover after 3 months, where fungiform papillae taste buds may not (Kumari et al., 2017).

Reduction of PLCβ2 expression in dieted mice compared to HFD- and chow-fed mice

Along with taste buds, we also examined various type II taste cell markers. There was no difference found in the expression of PLCβ2, a type II taste cell marker, between chow and HFD-fed mice (Figure 15B). This result contrasts with studies showing significant downregulation of PLCβ2 in human fungiform papillae of obese subjects (Archer et al., 2019), and in the CV of mice fed a HFD (Ahart et al., 2019; Kaufman et al., 2020). Mice in this experiment were on a HFD for 16 weeks rather than shorter periods used in both Ahart et al. (2019) and Kaufman et al. (2019). Results in this study matched those in another using extraction of a whole tongue lysate (Chao et al., 2016), rather than individual taste buds.

Interestingly, there was a significant reduction in dieted mice's expression of PLCβ2 compared to both chow- and HFD-fed mice. A similar result was observed in the FP of patients that had undergone gastric bypass, with a slight, but not significant, reduction in PLCβ2 expression (Pepino et al., 2014). Thus, reducing body weight might lead to a reduction of PLCβ2 expression.

Increased proportion of α-gustducin per taste bud in HFD-fed mice with a decrease after diet

In addition to an altered number of taste buds, we also saw an alteration to the proportion of α-gustducin-positive cells (Figure 5B). α-gustducin is critical but not sufficient for bitter taste transduction (Caicedo et al., 2003), with α-gustducin knockout mice having a 40- to 100-fold reduction in bitter taste signals. In this study, HFD-fed mice had significantly more α-gustducin-positive cells per taste bud compared to chow-fed mice. As gustducin occurs in type II taste cells, and other taste cell types are slower to renew than type II cells (Perea-Martinez et al., 2013), it could be that an accelerated

breakdown of taste cells in obesity leads to the balance of cell types within the taste bud shifting, favoring type II cells over others.

Our results are reminiscent of those in diabetic rats. Diabetes is characterized by an up-regulation of similar inflammatory markers to obesity, and diabetic patients also show a dampened sense of taste (De Carli et al., 2018; Lontchi-Yimagou et al., 2013). α-gustducin in the taste bud was increase in the CV of diabetic rats, observed through both immunohistochemistry and RT-PCR (L. Zhou et al., 2009).

In this study, dieted mice show a reduced proportion of α-gustducin-positive cells per taste bud compared to HFD-fed mice. In humans, after Roux-en-Y gastric bypass surgery, which causes dramatic weight loss, expression of α-gustducin in the FP was reduced threefold (Pepino et al., 2014).

This shift in the proportion of α-gustducin-positive cells has been shown in HFD and fasted mice in extra-oral tissue and might present a novel therapeutic strategy to ameliorate obesity. Beyond directing food choices, the gustatory system stimulates the gut and other organs to properly absorb food. Some examples include initiating gut peristalsis, insulin release, and increased heart rate (Giduck et al., 1987; Mattes, 1997). Extra-oral taste receptors have been found in many areas of the body including the intestine, which has similarities with the taste tissue both morphologically and biochemically (Depoortere, 2014).

Mice fed a HFD, either 45% or 60% kcal from fat, for 8 weeks showed an increase in α-gustducin mRNA in the colon. After HFD treatment for 2 weeks only, the same effect was not seen. This might suggest that a shift in expression was an effect of physiological changes brought on by obesity, rather than a direct effect of the diet (Vegezzi et al., 2014). Similarly in the duodenum, obese mice exhibit lower expression of a variety of sweet and bitter sensing taste receptors, compared to lean controls (Chao et al., 2016).

Conversely, after 18 hours of fasting, C57Bl/6 mice had a reduced level of α-gustducin transcripts in the stomach. Levels of α-gustducin were restored to normal levels 4 hours after re-feeding. α-gustducin transcripts were found throughout the GI tract, with the colon and the stomach having the highest levels (Vegezzi et al., 2014). Taken together, the GI tract data shows similarities to the CV in α-gustducin expression after HFD treatment.

α-gustducin could be a novel therapeutic target for weight loss

Although gastric bypass was originally intended to mechanically reduce calorie consumption, new evidence suggests an alteration of hormones after gastric bypass, not the case after caloric restriction (Karamanakos et al., 2008; Sloth et al., 2007). This alteration in hormones might be partially modulated by taste machinery in the gut.

Peptide YY (PYY), an anorexigenic hormone, increases after gastric bypass surgery, but not after caloric restriction (Karamanakos et al., 2008). PYY delays gastric emptying and increases energy expenditure which can aid weight reduction (Sloth et al., 2007). Similarly, GLP-1 is also elevated compared to caloric restriction (Steven et al., 2016; Suzuki et al., 2012), slowing gastric emptying, assisting with insulin secretion, and inhibiting glucagon release. α-gustducin knockout mice have defective GLP-1 signaling, which suggests that α-gustducin could play a key role in weight reduction (Jang et al., 2007). Increased secretion of GLP-1 may be partially responsible for the weight loss benefits of Roux-en-Y bypass, and along with a decreased stomach size, might explain altered satiety post-surgery (Borg et al., 2006). GLP-1 also increased sevenfold after gastric bypass compared to caloric restriction (Pepino et al., 2014).

α-gustducin modulation and co-localization with GLP-1 and PYY

GLP-1 is expressed in mammalian taste buds, and GLP-1R knockout mice have a reduction in sweet (and enhanced umami) taste sensitivity (Shin et al., 2008). Additionally, PYY is expressed in taste cells, while PYY knockouts' responsiveness to bitter tasting stimuli is decreased (Sala et al., 2013). Interestingly,

sweet taste receptors T1R2/T1R3 located in the gut also play a role in regulating GLP-1 and PYY (Gerspach et al., 2011).

α-gustducin knockout mice gain less weight than wild-type mice when fed with a HFD. Intragastric treatment with the bitter agonists denatonium benzoate or quinine result in a reduced body weight, with decreased food intake (Avau et al., 2015). α-gustducin also regulates hormones such as ghrelin and can have an effect on gastric emptying. Intragastric gavage of T2R agonists increased food intake for 30 minutes in C57 Bl/6 mice but not in α-gustducin knockout mice or ghrelin receptor knockout mice (Janssen et al., 2011).

Altering endogenous hormone levels mediated by α-gustducin and GLP-1 might be an alternative to surgical interventions such as bariatric surgery, as alterations in hormones is linked to altered satiety observed post-surgery (Borg et al., 2006). It has been suggested that bitter-sensing taste receptors secrete appetite-regulating gut hormones and might present a route to treat obesity (Q. Wang et al., 2020).

α-gustducin-positive taste cells are more sensitive to diet in female mice

In this study, female HFD mice had increased proportion of α-gustducin-positive cells and diet mice had decreased proportion of α-gustducin-positive cells compared to male mice (Figure 5). As previously mentioned, α-gustducin is important in bitter taste transduction (Caicedo et al., 2003).

Although male and female chow-fed mice had a similar proportion of α-gustducin-positive cells while maintained on the healthy diet, female mice had more α-gustducin-positive cells than males after treatment with the high fat diet. After dieting, the proportion of α-gustducin-positive cells in females returned to a very similar level to chow-fed mice. Males on the other hand still had more α-gustducin-positive cells after the diet. Female α-gustducin-positive cells seem to be more sensitive to diet, both increasing more after HFD treatment and decreasing close to chow levels after diet, this could be

because female mice had slightly more weight loss than male mice. This finding offers further insight into potential sex differences in the gustatory system.

Interestingly, there are sex differences in bitter taste response observed in human work. Women have a higher likelihood of being able to taste PTC and report PTC strips as tasting more intense, although not on a gLMS scale (Bartoshuk et al., 1994; Spence et al., 2014). Thus, concerning the potential of α-gustducin as a therapeutic target, potential differences between sex should be further explored.

Altered taste bud homeostasis in HFD and dieted mice

To investigate whether there was an increase in apoptosis, or an altered number of proliferating cells indicative of a disruption in taste bud homeostasis, we quantified programmed cell death, and actively renewing cells in taste tissues. Taste bud cells derive from multipotent progenitor cells that surround the taste bud in the basal region of the taste bud (Perea-Martinez et al., 2013; Takeda et al., 2013). Obese mice in our experiments had fewer Ki67-positive taste progenitor cells than lean mice, with dieted mice showing a similar number of Ki67-positive cells to lean controls (Figure 8). Even with a return of progenitor cells return to lean mice levels in diet mice, it is likely that as new taste cells cannot be made without taste progenitor cells, taste buds would lag progenitor cells in a return to the level seen in lean mice.

Dieted mice also had significantly lower expression of E2F1 than chow-fed mice, with HFD-fed mice showing a reduction in expression that was not significant (Figure 14B). Treated with the inflammatory trigger LPS can decrease expression of E2F1, a transcription factor that is crucial for cell cycle progression. E2F1 activates numerous transcription factors that are crucial for cell division. In LPS-treated mice, cyclin B2 along with Ki67 are also suppressed, both of which are likely involved in the

overall suppression of taste progenitor cell proliferation in the CV (Cohn et al., 2010; Crosby & Almasan, 2004).

E2F1 has also been found to be a novel regulator of innate immunity in response to systemic LPS. Mice deficient in E2F1 had an attenuated inflammatory response to systemic administration of LPS (I. V. Yang et al., 2011), although they also had a decreased survival rate (Warg et al., 2012). Chronic exposure to a HFD may also be involved with the immune response. Surprisingly, the biggest difference in E2F1 was seen between dieted and chow-fed mice, which suggests that a reduction of taste buds in dieted mice may be more heavily influenced by proliferation than apoptotic cell death.

Increased markers for apoptotic cell death

In line with a reduced number of taste buds in HFD-treated mice, the number of caspase-3-positive cells in CV taste regions was increased in obese mice (Figure 9). Number of taste buds and caspase had a negative relationship (Figure 10), and end weight and percent weight gain have a negative relationship with the number of caspase positive cells (Figure 11). Caspase-3 is a hallmark of apoptotic, or programmed, cell death and proffers a mechanism for a reduction in the number of taste buds observed in HFD-fed mice. Previously, HFD mice have been found to have increased TUNEL staining (Kaufman et al., 2018), which marks for all types of cell death including necrotic, unprogrammed cell death (Grasl-Kraupp et al., 1995). Using markers for apoptosis allows a more specific evaluation of the type of cell death.

This increase in caspase-3 activation is similar to that found in diabetic rats. In the CV papillae of diabetic Wistar rats, increased activation of caspase-3 was observed, in addition to increased TUNEL staining, another identifier of cell death (Cheng et al., 2011). In both patients with obesity and type II diabetes, an impaired sense of taste has been observed (De Carli et al., 2018).

While caspase-3 is an effector caspase, Bax is a proapoptotic molecule that, together with other molecules, determines if a cell enters apoptosis, while downstream, caspase-3 acts as an effector (Pawlowski & Kraft, 2000). The same trend that was observed in caspase-positive cells was also observed in Bax expression, with chow mice having significantly less Bax expression compared to HFD mice. Surprisingly, there was reduced expression of Bax in dieted mice compared to chow mice, although differences were small compared to HFD mice. This may represent evidence that the reduced number of taste buds in dieted mice is more attributable to reduced proliferative activity compared to apoptosis. Taken together, our results suggest that even temporary exposure to a HFD early in life can cause a disruption in taste bud homeostasis, with taste bud maintenance and renewal being disrupted long after the excess weight is lost, and the unhealthy diet has left the system.

Increased expression of Shh in HFD-fed mice

In this study, we found increased CV expression of Shh in HFD-fed mice compared to chow-fed mice, with expression in dieted mice returning to the levels of the chow-fed mice (Figure 14A). Shh signaling has been found to be critical in the renewal and maintenance of taste cells (Ermilov et al., 2016). Previously, in the FP of human subjects with obesity, a reduced expression of Shh was recorded (Archer et al., 2019). Disruption of Shh signaling differentially affects the fungiform versus the circumvallate papillae. After sonidegib treatment, a Hedgehog pathway inhibition (HPI) drug, there was a loss of taste buds in the CV and FP. In the FP, even after 9 months, only 50% of taste buds had recovered. In the CV, taste buds recovered fully after 3-9 months compared to vehicle control (Archana Kumari et al., 2017).

In both rats and mice, Shh signaling is essential in taste organ homeostasis, with the FP showing similar HPI-induced effects, though the effect on the CV was more profound in rats. In the rat CV almost all taste buds were eradicated by hedgehog inhibition, while about 25% remained in the mouse CV after 16 days of sonidegib treatment (Kumari et al., 2018).

Differences in Shh signaling between species could explain why increased expression of Shh was found in our experiments with obese mice, while an increase was recorded with obesity in the FP of humans (Archer et al., 2019). This could also be due to the variance in molecular regulation in the posterior and anterior of the tongue (Barlow & Klein, 2015). Of course, this is difficult to interpret, as data for the FP for Shh signaling in obesity exists only in humans and data from the CV exists only for mice.

In the FP, inhibition of Shh signaling through hedgehog antagonists did not have an effect on progenitor cell proliferation but did influence taste cell differentiation (Castillo-Azofeifa et al., 2017). Furthermore, gustatory nerve sources of Shh are used for taste bud renewal. When epithelial and neural supplies of Shh are removed, taste buds disappear (Castillo-Azofeifa et al., 2017). These results were extended in findings that pharmacologic activation of the Hedgehog pathway accelerates recovery (W. J. Lu et al., 2017). This suggests that any reduction in Ki67-positive cells was not due to Shh expression.

Embryonic function of Shh signaling

In rat embryonic tongue cultures treated with cyclopamine and jervine, which disrupt Shh signaling, these steroidal alkaloids demonstrated that the CV was less dependent than the FP on Shh for papillae induction and patterning, suggesting differences in the signaling cascades involving Shh that control papillae formation in the CV versus the FP (Mistretta et al., 2003).

Blocking Shh activity in embryonic tongue cultures through the use of cyclopamine increases the number of FP (Mistretta et al., 2003). Similarly, neutralizing Shh by 5E1 monoclonal antibody in embryonic tongue cultures induces the development of more papillae that are larger and closer together (Hall et al., 2003). Cyclopamine treatment also induces a dose-dependent expansion of Shh expression domains (Hall et al., 2003).

Local misexpression of Shh induces ectopic taste buds without nerve dependence, and has shown that Shh expression alone is sufficient to differentiate the full complement of taste cells (Castillo et al., 2014).

It has been proposed that Shh expression serves different functions at low and high concentrations; Shh at low concentrations preserves a papillae-free epithelium, while high concentrations form and maintain papillae (H. X. Liu et al., 2004).

Shh expression in the formation and regulation of taste buds is not yet fully understood, partially due to the differing functions of Shh in embryonic development versus the adult taste system. Altered Shh expression in the FP compared to the CV might be why this study demonstrated increased expression of Shh in the CV, while others have found decreased expression of Shh in obese patients' (Archer et al., 2019).

Overexpression of Shh in the FP

In the FP, ligand-independent activation of the Shh pathway through GLI2 in K5+ basal epithelial cells initiates a loss of fungiform papillae and taste buds (H. X. Liu et al., 2013). Some thin taste buds remained with taste cells with hallmarks of innervation. Constitutive Shh signaling stimulates cell proliferation, but not fungiform papillae or taste buds (H. X. Liu et al., 2013). Reduction of Shh expression with pharmacological treatment or exogenous overexpression of Shh can reduce number of taste buds (Ermilov et al., 2016; Archana Kumari et al., 2017; H. X. Liu et al., 2013). Thus, over- or under-expression of Shh could have a negative effect on the gustatory system. Further comparative analysis of Shh in the CV versus the FP in obese humans and obese mice is needed to understand the role of Shh in obesity.

Increased BMP4 expression in HFD mice

Expression of BMP4 was also upregulated in the HFD mice compared to dieted mice (Figure 13B). BMPs have varying roles across the stages of embryonic development. Before and during placode formation, BMP4 increases number of FP, whereas after placode formation exogenous BMPs inhibit FP (Y. Zhou., 2006).

BMP4 and Shh are co-expressed in papillary placodes that serve as signaling centers for papillary development (Hall et al., 2003). Shh and BMP4 continue to be expressed in the adult taste epithelium and are involved in taste bud homeostasis (Miura et al., 2001).

BMP4 expression also varies between the posterior and anterior taste fields of the tongue. Intragemmal BMP4-positive cells in the CV are immature cells that are precursors for type I, II, and III taste cells, whereas perigemmal BMP4-positive cells located in the CV and FP are slow-cycling stem cells or another part of the stem cell niche (Nguyen & Barlow, 2010)**.** Upregulation of BMP4 could offer a pathway for HFD-affected taste buds to recover when metabolic pressure promoting their degradation was removed.

Finally, Sox 2, proposed as another taste stem cell marker, showed a trend towards a decrease in HFD-fed mice compared to chow-fed mice (Figure 13C), and may also play a minor role in the increased number of taste buds observed in chow-fed mice (Ohmoto et al., 2017).

Number of neutrophils did not differ across groups

Neutrophils are well established to respond to inflammation, exacerbate inflammation by helping recruit macrophages, and interact with antigen-presenting cells (Mantovani et al., 2011; Nathan, 2006). Patients with obesity have an increased number of circulating neutrophils, key in innate immunity (Nijhuis et al., 2009). In HFD-fed obese mice, adipose tissue and liver tissue showed an increased number of neutrophils (Talukdar et al., 2012).

In this study, we did not see a change in neutrophil infiltration across groups (Figure 7). Two possibilities might explain the lack of difference observed; first, neutrophils might have already peaked earlier, as neutrophil infiltration in one study peaked at only 3-7 days in the adipose tissue of C57BL/6J mice placed on a HFD (Elgazar-Carmon et al., 2008). Another possibility is that the CV is not a tissue to which neutrophils actively migrate, in contrast with the ease to which neutrophils infiltrate adipose tissue.

Conclusion

Taken together, these data suggest that a HFD has a lasting effect on taste bud abundance despite returning to a healthy diet and reducing body weight. As previously noted, obese mice exhibit increased TNF-α expression and a lower number of taste buds than lean controls. Furthermore, these mice have an increased number of cells undergoing apoptotic cell death, determined by caspase-3 and Bax. Proliferative capacity is correspondingly reduced, as marked by Ki67 and E2F1. Dieted mice still had fewer taste buds than chow-fed controls, with slightly increased expression of TNF-α, and significantly reduced E2F1 and Ki67 compared to chow-fed mice. We additionally showed that female mice also lose taste buds and taste progenitor cells with obesity. Critically, 8 weeks after switching to a chow diet, with a return to a healthy body weight, these effects were not completely abated.

Supplementary information

Table 1: Forward and reverse primers used in RT-PCR

Gene	Forward Primer	Reverse Primer
Inflammatory markers		
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
IL-6	TCATATCTTCAACCAAGAGGTA	CAGTGAGGAATGTCCACAAACTG
TNF-α	CCTCACACTCAGATCATCTTCTCA	TGGTTGTCTTTGAGATCCATGC
Taste modulators		
LGR5	CCTACTCGAAGACTTACCCAGT	GCATTGGGGTGAATGATAGCA
Sox2	AACGCCTTCATGGTATGGTC	ATGTAGGTCTGCGAGCTGGT
Shh	GATGACTCAGAGGTGCAAAGACAA	TGGTTCATCACAGAGATGGCC
BMP4	CCCTTTCCACTGGCTGATCA	GGGACACAACAGGCCTTAGG
Taste cell markers		
GNAT3	GCAACCACCTCCATTGTTCT	AGAAGAGCCCACAGTCTTTGAG
PLCβ2	GAGCAAATCGCCAAGATGAT	CCTTGTCTGTGGTGACCTTG
Proliferation/ apoptosis markers		
Ki67	TCTGATGTTAGGTGTTTGAG	CACTTTTCTGGTAACTTCTTG
E2F1	ACCATCACCTCCCTCCACAT	TGGTGACAGTTGGTCCTCTT
Bax	GGCAGACAGTGACCATCTTT	AGTGGACCTGAGGTTTATTG
Endogenous control		
β-Actin	CACCCTGTGCTGCTCACC	GCACGATTTCCCTCTCAG

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CHAPTER 4

THE EFFECT OF THE CHEMOTHERAPEUTIC AGENT DOXORUBICIN AND THE ANTI-INFLAMMATORY AGENT FPS-ZM1 ON THE TASTE SYSTEM

Abstract

This study seeks to examine the effect of chemotherapeutic treatment on the taste system. There is little previous work that has examined the physiological effects of chemotherapy on taste, most of which is focused on psychological reasons for taste disturbance during chemotherapy treatment. The few studies that have looked at the physiological changes have focused on only one drug, cyclophosphamide. In this study, female BALB/c mice were treated with doxorubicin (a chemotherapeutic agent), FPS-ZM1 (an anti-inflammatory), or a combination of the two, and euthanized 14 days after treatment. No significant differences between the groups were detected in the number of taste buds or taste cells, number of neutrophils, or proliferative capacity. Overall, the results are in line with previous research on chemotherapy-induced taste changes, which did not show a difference in taste bud abundance or proliferative capacity 14 days after treatment. Furthermore, it also suggests that there can be a rapid recovery from chemotherapy-induced taste changes.

Introduction

Chemotherapy agent doxorubicin and receptor for advanced glycation end antagonist (RAGE) FPS-ZM1 in the treatment of cancer

Doxorubicin is a highly clinically effective chemotherapeutic agent and is used as a first-line drug in the treatment of various cancers including types of leukemia, breast carcinoma, and thyroid carcinoma (Cagel et al., 2017).

Doxorubicin, part of the anthracycline family of antibiotics, is a chemotherapy agent that induces cell death and arrests cell growth. Cell death is induced by creating oxidative stress, producing free radicals, and arrests cell growth by intercalating with DNA and inhibiting DNA topoisomerase II (Meredith & Dass, 2016). Cancer itself and doxorubicin treatment have been shown to induce inflammation (Wang et al., 2016; Wu et al., 2019). The drug has been studied and used for many years, with the latter mechanism for cell growth arrest found in 1984 (Tewey et al., 1984). Since then, doxorubicin has found broad applications treating many types of cancer; however, as it is non-specific in its targeting of cells, this also means that it can target other fast-cycling cells in healthy tissue, as well as cancer cells.

The receptor for advanced glycation end products (RAGE) has been implicated in numerous pathologies including Alzheimer's, cancer, diabetes, and cardiovascular disease (Deane et al., 2012; Hudson & Lippman, 2018). As RAGE is upregulated in cancer, and inflammation is believed to contribute to cancer growth and metastases, researchers are interested in the possibility of using RAGE antagonists to downregulate this inflammatory response. In a recent report, combining a RAGE inhibitor with doxorubicin did not affect tumor size but did decrease the amount of breast cancer metastases (Kwak et al., 2017).

Chemotherapy's effect on taste and implications for quality of life

Anecdotal accounts and clinical evidence suggest that during chemotherapeutic treatment, patients experience dysgeusia, or loss of taste, which may lead to altered food choices. Reports estimate that between 56-76% of patients experience altered taste with chemotherapy (Amézaga et al., 2018; Hovan et al., 2010; Ponticelli et al., 2017). During treatment this can result in lowered food intake and impaired nutrition and energy levels, and can further predict morbidity, mortality, treatment response, and toxicity (Spotten et al., 2017).

Dysgeusia can also has a negative impact on quality of life by affecting appetite and hedonic pleasure from eating (Ponticelli et al., 2017). In cases of advanced breast cancer, improving quality of life is especially important, as treatment can be extensive and taxing on the patient (Bottomley & Therasse, 2002; Coates et al., 1987; Kramer et al., 2000). Thus, understanding taste loss with chemotherapy may lead to improved outcomes in cancer treatment through patients maintaining a superior nutritional and psychological state.

Previous understanding of chemotherapy-induced dysgeusia

Before physiological evidence was researched, the prevailing explanation for the dysgeusia experienced by chemotherapy patients was conditioned taste aversion. This arises when after food intake, nausea occurs. Through operant conditioning, an association of food intake and nausea results in a general loss of appetite and an aversion for tastes associated with the initial bout of nausea (Bartoshuk, 1990; Bovbjerg et al., 1992; Mattes et al., 1987). This learned behavior has long been recognized in the field of learning and behavior (Zentall, 2007).

Major limitations with this research are a lack of a standard assessment tool to measure taste changes which include different tastant delivery (solutions, taste strips, etc.) and the scale used. Furthermore, the large number of variables related to human cancer treatment that may obscure patterns, including

comorbidities, type of cancer, additional medications patients may be taking, and treatment stage (Spotten et al., 2017), along with the inherent variance in taste response between subjects. Finally, some mechanistic insight into how taste may be changing would be vital to interpreting these results.

Taste cell types and taste cell renewal

Doxorubicin, the chemotherapy treatment used in this experiment, arrests the cell cycle and induces cell death. Taste is perceived by specialized epithelial cells subdivided into three types of cells: type I, type II, and type III taste cells. Type I cells are glial-like cells that sense salty taste (Chandrashekar et al., 2010), type II cells mediate sweet, bitter, and umami taste (Chandrashekar et al., 2006; Liu & Liman, 2003; Mueller et al., 2005) while type III presynaptic cells sense sour taste (Huang et al., 2008; Yang et al., 2000). Taste cells were originally described by their ultrastructure rather than their biochemical function.

This population of cells is constantly renewed, with each cell type seeming to have a distinct half-life ranging from 8-22 days (Perea-Martinez et al., 2013). As taste cells have such a rapid turnover rate, chemotherapy may trigger changes in taste cell abundance through altering taste bud homeostasis by disrupting the renewal of new cells, or the induction of taste cell death. As taste cells are distinct in their half-life, different cellular populations within the taste bud may be affected differently, leading, for example, to a loss of sweet, bitter, and umami taste, with salty taste remaining similar.

Radiation therapy treatment's effect on taste

There is only very limited data to understand chemotherapy-induced taste changes. Irradiation treatment, alongside pharmacological treatment, is a leading cancer treatment which directly or indirectly causes cell death either by damaging their DNA or creating free radicals. Of course, treatments have distinct mechanisms, but as both cause cell death, one might inform the other.

In studies performed on X-ray irradiated mice, a reduction of type II taste cells was observed. A single 15 Gy dose of X-ray irradiation preferentially reduced α-gustducin-positive type II cells compared to type III cells (Yamazaki et al., 2010). This might be due to the fact that there is faster turnover rate in type II cells versus type I and type III cells (Perea-Martinez et al., 2013).

Irradiation also reduced the number of actively proliferating cells, and increased number of cells undergoing apoptosis. The number of Ki67-positive cells in the basal region outside of the taste buds were significantly reduced, compared to the control 3 days after irradiation (Yamazaki et al., 2010). Using Bromodeoxyuridine (BrdU), a synthetic nucleoside for thymidine, a cohort of cells is labeled and can be identified throughout their lifecycle. Labeling cells in control mice and chemotherapy-treated mice allows comparison between the number of new cells and the length of their lifecycle. BrdU staining was similar to control 7 days after irradiation, which suggests normal cell proliferation. Furthermore, using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), irradiation was observed to cause cells to undergo apoptosis. As irradiation is a form of cancer treatment which kills cancerous cells, and healthy tissue can also be affected, apoptosis was expected. Importantly, normal cell renewal resumed after day 5-7 (Nguyen et al., 2012). Using two-bottle preference, sweet taste was measured and showed alterations during 4-20 days after irradiation, in line with a reduction in overall taste buds (Yamazaki et al., 2010).

Treatment with hedgehog pathway inhibitors disrupts taste buds

Sonic hedgehog signaling (Shh) is critical in taste bud development, maintenance, and renewal (Ermilov et al., 2016). Hedgehog pathway inhibitors (HPIs) are used to treat basal cell carcinoma. In human chemotherapy studies using vismodegib, an HPI, 71% of patients reported dysgeusia and 54% patients had weight loss more than 5% (Le Moigne et al., 2016). Currently, altering food choice and working with a dietician are suggestion for treatment (Fife et al., 2017). In comparison to radiation and

pharmacological treatments with cyclophosphamide, HPIs have a better understood effect on taste, as Shh signaling has been found to have an important role in taste bud development and maintenance.

Hedgehog signaling has been found to be critical in taste bud development (Hall et al., 2003), and also has a profound effect on adult taste buds. After sonidegib treatment, an HPI, there was a reduction of taste buds. In the fungiform, after 16 days of sonidegib treatment, subjects recovered 50% of fungiform papillae within 14 days, while in the CV taste buds did not differ at 3-9 months compared to control tissue (Kumari et al., 2017). After extended 48-day treatment with an HPI, 90% of fungiform papillae recovered, while the CV recovered after 5-7 months (Kumari et al., 2018). Sense of touch and cold remained, suggesting non-taste cells are not affected (Kumari et al., 2018; Kumari et al., 2017). In C57BL/6J mice treated with vismodegib, also an HPI, mice experienced a reduction in taste bud size and number of taste cells per taste bud (Yang et al., 2015). In mice treated with another HPI, LDE225, similar results were seen with smaller taste buds and decreased number of taste buds in the fungiform (Kumari et al., 2015).

In behavioral experiments, mice treated with vismodegib had reduced response to sweet and bitter stimuli. Simultaneously, there was a significant reduction in taste bud size and number of taste cells per bud, as well as a reduction in the bitter and sweet responsive cells phospholipase C β2 (PLCβ2) and α-gustducin (Yang et al., 2015).

Chemotherapy agents that target the hedgehog pathway have a profound effect on the taste system but might be unique to this class of chemotherapy agents, as they directly act on a pathway implicated in taste bud maintenance. Therefore, effects seen in the taste system with HPIs cannot be directly extrapolated to other forms of pharmacological chemotherapy treatments.

Pharmacological chemotherapy treatments

The mechanistic insight into taste changes related to pharmacological chemotherapy comes from mice treated with cyclophosphamide (Delay et al., 2019; Mukherjee & Delay, 2011; Mukherjee et al., 2013; Mukherjee et al., 2017). Cyclophosphamide, an alkylating agent, has a direct cytotoxic effect on cells by producing interstrand and intrastrand DNA crosslinks. One study showed that it inhibits taste cell renewal and disrupts taste bud proliferation (Emadi et al., 2009), which is consistent with a loss of taste following chemotherapeutic treatment.

In mice treated with cyclophosphamide, there is a loss of taste cells. Type II cells, responsible for sweet, umami, and bitter taste transduction, and type III cells, responsible for sour taste transduction, are especially affected. Cell proliferation, marked by Ki67, is initially dramatically reduced by chemotherapy, and later pushed to higher than usual levels (Mukherjee et al., 2017).

The effects on taste take place in two phases. The first phase, taking place 2-4 days post injection, is characterized by the cytotoxic effect of the drug which more heavily affect the fungiform section of the tongue (Mukherjee & Delay, 2011). The second, taking place around 9-12 days after injection, is characterized by a reduced-replacement cycle. This inhibits the replacement of aging taste bud cells in the CV, similar to those found in radiation therapy (Mukherjee & Delay, 2011). Behavioral tests for discrimination and detection showed two separate periods of taste disturbances to two umami substances, monosodium glutamate (MSG) and inosine 5’-monophosphate (IMP). The mice had reduced ability to discriminate between taste qualities and reduced taste sensitivity, as measured by detection thresholds and discrimination using lickometer testing. The taste disturbances followed the same pattern as the histopathological evidence, with one disturbance observed at 2-4 days and the second observed at 9-12 days (Mukherjee & Delay, 2011).

Dose fractionation of chemotherapy

Dose fractionation, a process in which chemotherapy doses are given in smaller amounts over time, is a well-documented approach often used to expose the cancerous cells to chemotherapy across a period of time while reducing side effects. This means than in non-cancerous tissues, in this case taste buds, exposure to chemotherapy is extended but at a lower dose. In a recent study, researchers compared the effect of a single dose (75 mg/kg) of cyclophosphamide with a fractionated dosing of cyclophosphamide (5 doses of 15 mg/kg), on the taste systems of mice. Indeed, fractionating the dosing of cyclophosphamide had more adverse and prolonged effects on tissue, compared to a single dose. The suppressive effects on cell proliferation in the taste system resulted in an overall reduction in the proportion of type II cells. Fractionated dosing also decreased the number of type III cells more than a single dose (Delay et al., 2019).

In this study, female BALB/c mice were injected with doxorubicin, an anti-inflammatory targeting RAGE (FPS-ZM1), a combination of the two, or dimethyl sulfoxide (DMSO) as a negative control. At 8 weeks old, female BALB/c mice were injected with 4T-1, a mammary carcinoma that can be transplanted into mice, is highly tumorigenic, and can spontaneously metastasize (Pulaski & Ostrand‐Rosenberg, 2000). Based on previous evidence we hypothesized that the doxorubicin-treated mice would have fewer taste buds than DMSO-injected controls, based on previous work with cyclophosphamide that showed a loss of taste buds. Moreover, doxorubicin-treated mice would initially have more neutrophils due to the increased inflammation in cancer, and their involvement in the progression and metastasis of tumors (Wu et al., 2019). Longer term, we hypothesized a decreased number of neutrophils as apoptotic cells inhibit neutrophil migration, and neutropenia is well documented to occur with doxorubicin treatment (Bournazou et al., 2009; Joerger et al., 2007). Finally, we hypothesized there would be more proliferating

cells in doxorubicin-treated mice than control mice, and furthermore, that FPS-ZM1 would partially rescue these effects due to its anti-inflammatory properties.

Most recently, fractionated radiation therapy did not increase progenitor cell death compared to a single dose of treatment. Additionally, taste buds were smaller and contained less differentiated taste cells compared to single dose treatment. Interestingly, Wnt/β-catenin signaling recovery was slower than proliferative recovery (Gaillard et al., 2019; Iwatsuki et al., 2007).

Pretreatment with anti-inflammatory agents

To protect the taste epithelium from the effects of chemotherapy drugs, some have theorized that drugs to locally reduce inflammation may be beneficial (Nabanita Mukherjee et al., 2013), such as one study where researchers sought to test the anti-inflammatory drug amifostine. Pretreatment with cytoprotective or anti-inflammatory drugs like amifostine may reduce chemotherapy-induced cytotoxicity in fast-cycling off-target cell populations such as taste buds, in turn protecting the taste epithelium from the effects of chemotherapy drugs. Protecting from dysgeusia may have major health benefits by partially protecting taste function, which would promote better nutritional intake and increases quality of life during and after chemotherapy treatment.

In a 2013 study, amifostine, a cytoprotective treatment, was given before chemotherapy treatment to reduce chemotherapy-induced cytotoxicity in the taste epithelium (Mukherjee et al., 2013). Amifostine has protective effects by regulating genes involved in apoptosis, DNA repair, cell cycle, and scavenging free radicals, by selecting for cells with higher alkaline phosphatase activity, higher pH, and vascular permeability of healthy versus cancerous tissue (Andreassen et al., 2007). Previously, amifostine has been used to protect from radiation therapy-induced cytotoxicity in, for example, head and neck cancer (Culy & Spencer, 2001; Gu et al., 2014).

Treatment with amifostine partially protected against chemotherapy-induced taste deficits. It partially restored number of taste buds, and partially protected taste cells in the CV and the fungiform papillae. Furthermore, the taste cell proliferation was successful in protecting Ki67-positive taste cells, marking proliferating cells. A rebound effect occurs which increases the level of BrdU-positive cells above controls, similar to the effect observed in a later study (Mukherjee et al., 2013, 2017).

Methods

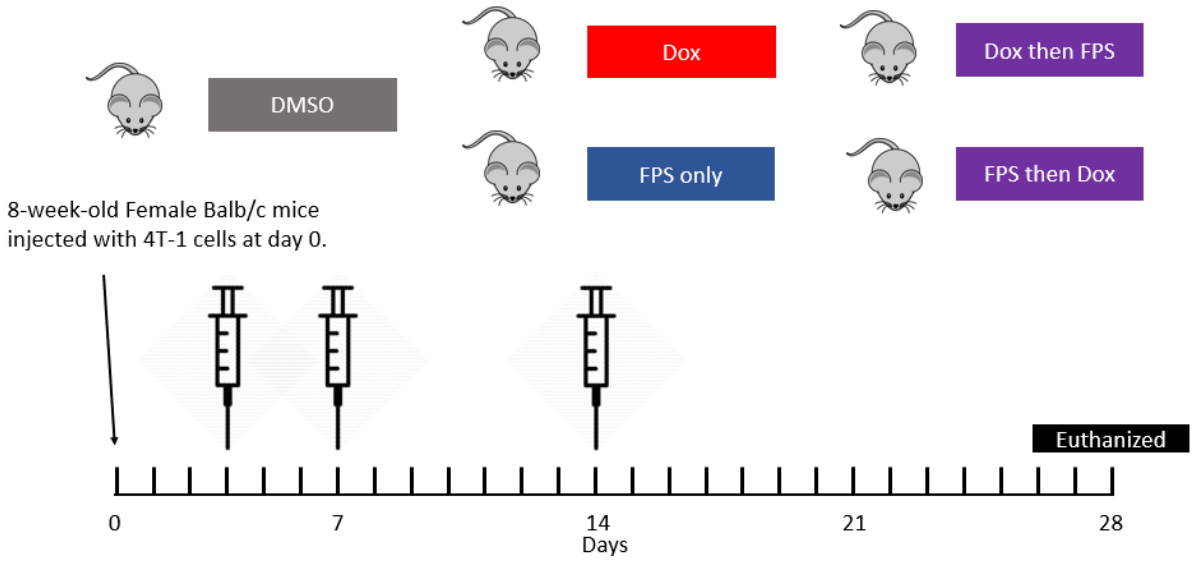

Figure 1: Graphic representation of treatment groups. Syringes represent injections of doxorubicin or DMSO. FPS-ZM1 was injected before or after the doxorubicin, depending on the group, or injected exclusively, as in the case of the FPS only group. DMSO (dimethylsulfoxide, control), Dox (doxorubicin, a chemotherapeutic agent), FPS only (FPS-ZM1 an anti-inflammatory agent), FPS after Dox, and FPS before Dox, which either had FPS injected before Dox or FPS after Dox respectively. The mice were sacrificed at 28 days after injection of tumor forming 4T-1 cells, occurring in the figure above at day 0 (8 weeks of age).

Animals

Animal studies were approved and carried out by the Institutional Animal Care and Use Committee of the University of Miami. BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were injected with 4T-1 cells at 8 weeks old from ATCC (Manassas, VA, USA) into the mammary fat

pads. Mice were then housed on a standard chow diet of Harlan Teklad 8604 with 14% fat, 54% carbohydrate, and 32% protein (Envigo, Indianapolis, IN, USA). Mice were sacrificed 4 weeks after injection of 4T-1 cells. A few of the mice received cells which did not induce tumor growth and are marked on the graphs with a darker color, with the effects of this also tested for significance in the analysis. Mice were subdivided into 5 treatments: DMSO, doxorubicin only, FPS-ZM1 only, FPS-ZM1 before doxorubicin, and FPS-ZM1 after doxorubicin. Mice were injected with 3x 5mg/Kg doxorubicin and/or 5x 1 mg/kg FPS-ZM1 (Millipore, Burlington, MA, USA). Mice were euthanized at 12 weeks, tongues were excised, placed in 4% PFA (Fisher Scientific Hampton, NH, USA) /PBS for 1 ½ hours, washed with PBS (VWR, Radnor, PA, USA), 3x for 20 minutes, and then placed in sucrose Sigma Aldrich, St. Louis, MO, USA), then shipped to Cornell University where they were embedded in OCT, (Fisher Scientific Hampton, NH, USA) and frozen at -80 C.

Immunohistochemical staining

CV tissue regions were dissected from tongues, cryoprotected with sucrose (Sigma Aldrich, St. Louis, MO, USA), and frozen in OCT medium, (Fisher Scientific Hampton, NH, USA). Circumvallate tissues were cryosectioned at 10 um thickness, washed in PBS (VWR, Radnor, PA, USA), and incubated in 1% triton (Sigma Aldrich, St. Louis, MO, USA). Tissue sections stained with 1:500 polyclonal Goat GNAT3 OAEB00418 (α-gustducin) from Aviva Systems Biology (San Diego, CA, USA), 1:1000 KCNQ1 Goat polyclonal sc-10646 (Santa Cruz, Dallas, TX, USA) and polyclonal Rabbit Ki67 PA5- 19462 1:125 polyclonal Goat MPO AF3667 from R&D systems (Minneapolis, MN, USA) were blocked for 2 hours at room temperature with 2% bovine serum albumin (BSA) (Amresco, Solo, Ohio, USA), 2% donkey serum (Equitech-bio, Kerrville, TX, USA), and 0.3% triton (Sigma Aldrich, St. Louis, MO, USA). After incubation with secondary Alexa Fluor donkey anti-Goat or anti-Rabbit secondary (Invitrogen, Carlsbad, CA, USA) at room temperature for 2 hours, sections were washed 3x for 20 minutes in PBS (VWR, Radnor, PA, USA),

and placed on a coverslip with Dapi staining medium (Fluoromount-G, Southern Biotech, Birmingham, AL, USA).

Taste bud counting

Tissue sections were imaged using an Olympus IX-71 inverted scope and Hammatsu Orca Flash 4.0 camera (Hamamatsu Photonics, Hamamatsu City, JP), and counted using ImageJ (NIH, Bethesda, MD, USA) for taste buds, number of caspase-positive cells, number of neutrophils, proliferating cells, and α-gustducin-positive cells.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 (San Diego, CA, USA). Groups were compared using independent Kruskal-Wallis analyses of variance, with statistical significance assumed at $p < 0.05$.

Results

The number of taste buds did not differ significantly between the groups. Although no significant difference was found between groups, there was a slight trend of fewer taste buds in doxorubicin-treated mice compared to DMSO, control mice ($p = 0.1771$). Furthermore, this is also true between FSPafterDox ($p = 0.1392$) and FPSbeforeDox ($p = 0.0512$), compared to control (Figure 2). Treatment with FPS-ZM1 did not seem to have an effect (Figure 2).

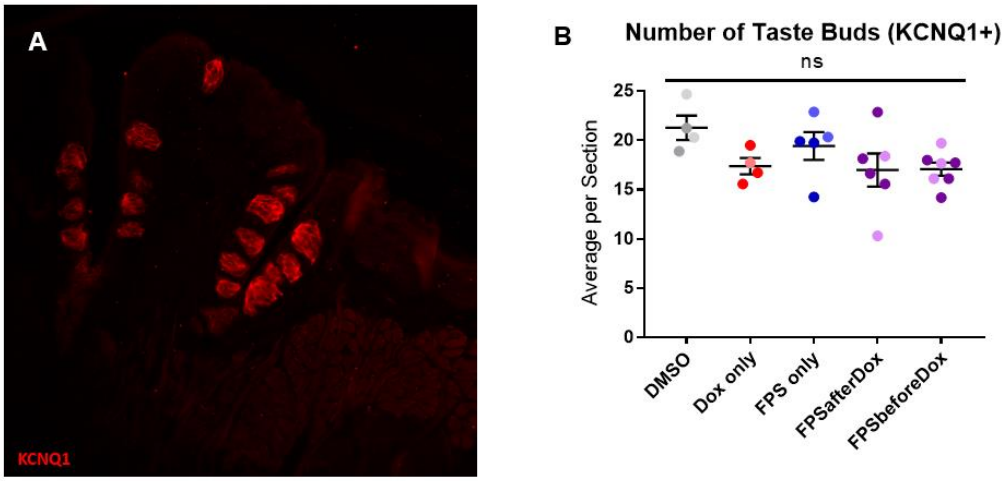

Figure 2: A: Representative image of circumvallate papilla with KCNQ1 staining highlighting taste buds. B: KCNQ1-positive cells per CV section for all treatment groups. DMSO, Dox only, FPS only, FPS after Dox, and FPS before Dox. Darker color represents non-tumor-bearing mice. Bars represent means plus/minus SEM. DMSO (grey, *n* = 4), Dox only (red, *n* = 4), FPS only (blue, *n* = 5), FPS after Dox (purple, *n* = 6), FPS before Dox (purple, *n* = 7). *p* = 0.0618.

The number of α-gustducin-positive cells did not differ significantly between groups. Neither did the doxorubicin treatment nor the FPS-ZM1 treatment had an effect on the number of α-gustducin-positive cells, which stain a subset of type II cells (Caicedo et al., 2003) (Figure 3).

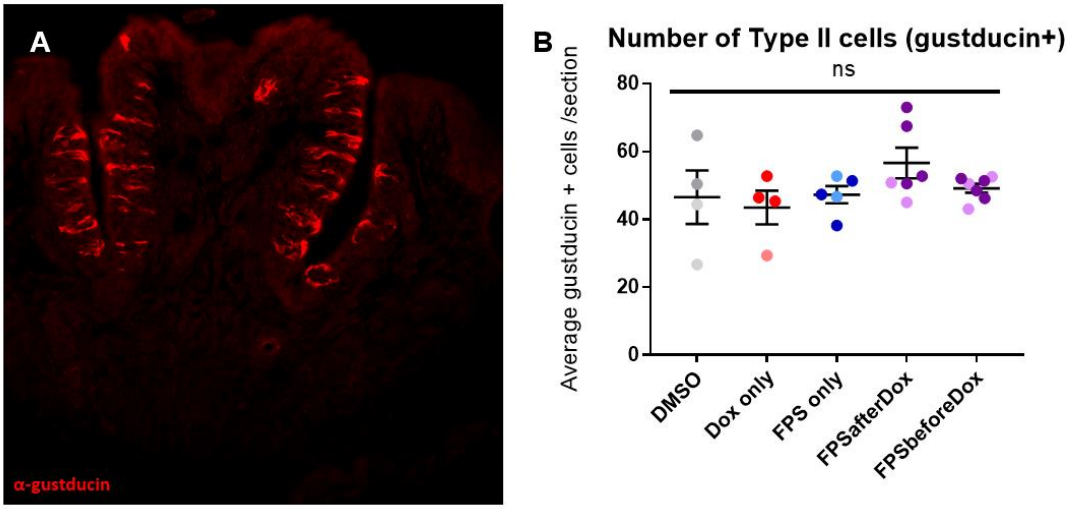

Figure 3: A: Representative image of circumvallate papilla showing α-gustducin staining in a subset of type II taste cells. B: α-gustducin-positive cells per CV section for all treatment groups. DMSO, Dox only, FPS only, FPS after Dox, and FPS before Dox. Darker color represents non-tumor-bearing mice. Bars represent means plus/minus SEM DMSO (grey, *n* = 4), Dox only (red, *n* = 4), FPS only (blue, *n* = 5), FPS after Dox (purple, *n* = 6), FPS before Dox (purple, *n* = 7). *p* = 0.4340.

There were no differences observed in the number of α-gustducin-positive cells or a change in the proportion of α-gustducin-positive cells (Figure 4).

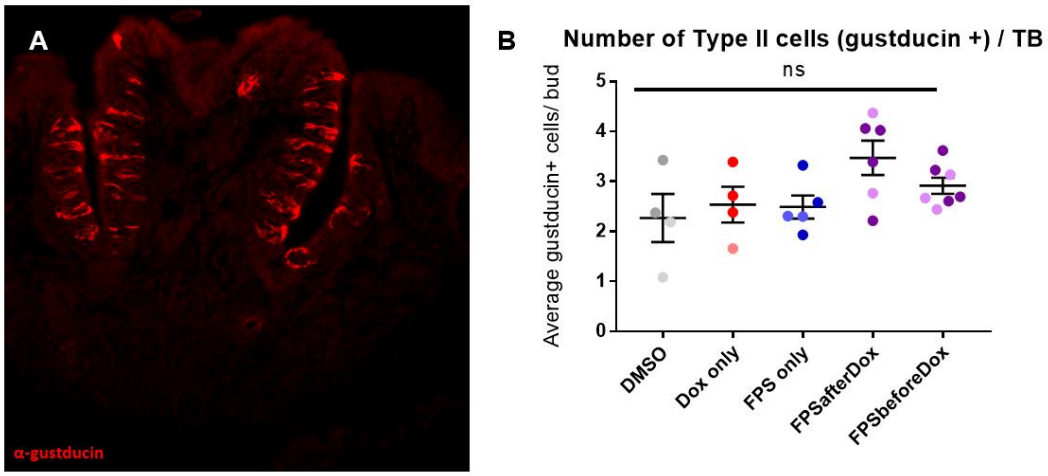

Figure 4: A: Representative image of circumvallate papilla showing α-gustducin staining in a subset of type II taste cells. B: α-gustducin-positive cells per CV section for all treatment groups. DMSO, Dox only, FPS only, FPS after Dox, and FPS before Dox. Darker color represents non-tumor-bearing mice. Bars represent means plus/minus SEM. DMSO (grey, *n* = 4), Dox only (red, *n* = 4), FPS only (blue, *n* = 5), FPS after Dox (purple, *n* = 6), FPS before Dox (purple, *n* = 7). *p* = 0.1191.

The number of MPO-positive cells did not differ significantly between groups, although DMSO control compared to doxorubicin-treated mice was almost significant (Figure 5, *p* = 0.0691). As apoptotic cells inhibit neutrophil migration, and apoptotic cell death has been linked to doxorubicin (Bournazou et al., 2009), we expected to initially see reduced neutrophil infiltration into the CV. Long term, a decrease in neutrophils was expected as neutropenia is well documented to occur with doxorubicin treatment (Joerger et al., 2007).

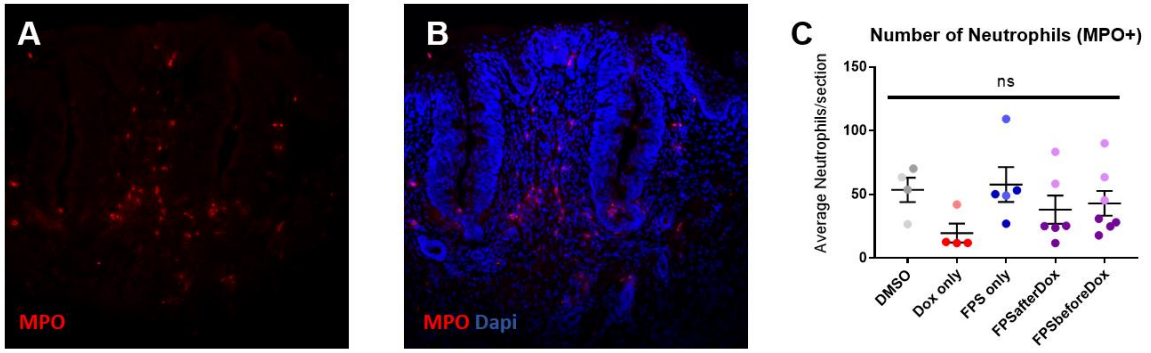

Figure 5: A: Representative image of circumvallate papilla showing MPO staining, which highlights neutrophils in taste papillae. B: MPO-positive cells per CV section for all treatment groups. DMSO, Dox only, FPS only, FPS after Dox, and FPS before Dox. Darker color represents non-tumor-bearing mice. Bars represent means plus/minus SEM. DMSO (grey, *n* = 4), Dox only (red, *n* = 4), FPS only (blue, *n* = 5), FPS after Dox (purple, *n* = 6), FPS before Dox (purple, *n* = 7). *p* = 0.0967.

The number of Ki67-positive cells, a marker for proliferating cells, did not differ significantly between groups (Figure 6). After the injection of doxorubicin, an initial decrease was expected with a possible long-term increase in Ki67 expression to repopulate the cells that had undergone doxorubicin-induced cell death.

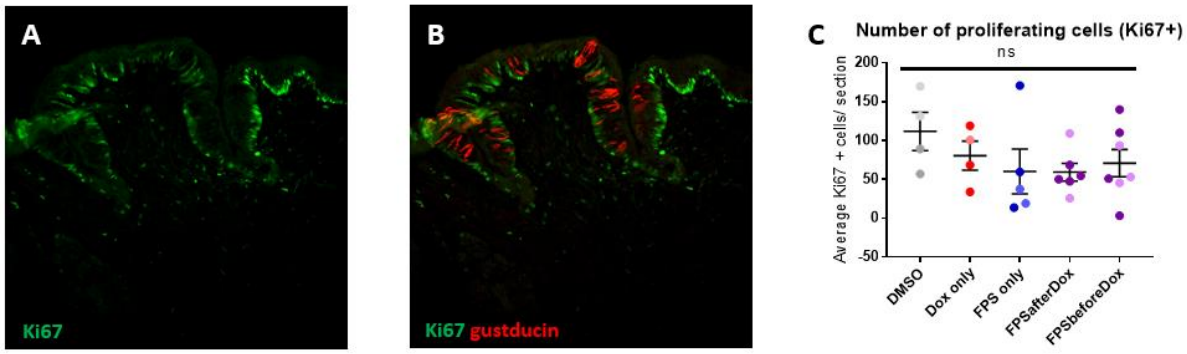

Figure 6: A: Representative image of circumvallate papilla showing Ki67 staining which stains actively proliferating cells. B: Ki67-positive cells per CV section for all treatment groups. DMSO, Dox only, FPS only, FPS after Dox, and FPS before Dox. Darker color represents non-tumor-bearing mice. Bars represent means plus/minus SEM. DMSO (grey, *n* = 4), Dox only (red, *n* = 4), FPS only (blue, *n* = 5), FPS after Dox (purple, *n* = 6), FPS before Dox (purple, *n* = 7). *p* = 0.3934.

Discussion

Changes in number of taste buds and taste cells

A similar number of taste buds was observed across all groups (Figure 2). Initially, because of doxorubicin-induced cell death, we expected fewer number of taste buds in doxorubicin-injected mice. As taste cells have a short half-life ranging from 8-22 days, they might be highly affected by cytotoxic chemotherapy agents that affect the cell cycle (Perea-Martinez et al., 2013). In other reports that studied the effect of a different cycle-nonspecific chemotherapy on taste, most of the apoptotic and necrotic cell death occurred 12-36 hours after injection of the chemotherapeutic agent; however, taste buds were able to recover after 16 days (Mukherjee et al., 2017). Mice may have recovered from the last doxorubicin injection, injected 14 days before euthanizing.

The proportion of α-gustducin-positive type II cells across groups also did not change (Figure 3), contrary to what was seen in cyclophosphamide-treated mice, although a different marker, PLCβ2, was used as a type II cell marker (Delay et al., 2019). In the research on cyclophosphamide, the suppressive effects on cell proliferation in the taste system also led to an overall reduction in the proportion of type II cells (Delay et al., 2019). As discussed regarding the number of taste buds, the cells might have already recovered after treatment.

Doxorubicin and cyclophosphamide are both used to treat breast cancer and are often used in conjunction as there is lower incidence of toxicity, increased tolerability, and cyclophosphamide has a direct cytotoxic effect on cells while doxorubicin induces cell death and arrests cell growth (Emadi et al., 2009; Meredith & Dass, 2016). When doxorubicin and cyclophosphamide have been used in combination, there was improved progression-free survival (Nabholtz et al., 2003). As a result, we initially hypothesized that the effects on the taste system might differ. It is possible that at these

dosages, either taste buds did not undergo apoptosis at all, or taste buds already had the chance to recover after treatment.

As human taste dysfunction is characterized by a weakened taste system, a decrease in the abundance of taste buds after chemotherapy could provide a part of the molecular mechanism behind the taste dysfunction qualitatively characterized by clinical data.

Neutrophil infiltration into the CV

Although the number of neutrophils did not differ significantly between groups, there seemed to be a trend towards fewer neutrophils in doxorubicin-treated mice compared to controls (Figure 5, p = 0.0691). Initially, due to increased cancer-induced inflammation, we hypothesized an increased number of neutrophils to infiltrate the CV (Wu et al., 2019). As the mice were euthanized 14 days after doxorubicin treatment, doxorubicin-induced apoptotic cell death may have inhibited neutrophil migration through the production of lactoferrin (Bournazou et al., 2009). Additionally, neutropenia, abnormally few neutrophils in the blood, is induced by both doxorubicin and chemotherapy in general, and is a common side effect that can lead to an increase in susceptibility to infection (Joerger et al., 2007). We might not have seen a significant difference in the number of neutrophils as too much time had passed after doxorubicin treatment.

Proliferative capacity

There was no significant difference observed in proliferative capacity, marked by Ki67, between groups (Figure 6). In previous research done with cyclophosphamide, there was an initial drop in proliferative capacity at 4 days after injection of the drug and then again after 10-12 days. At 14 days the number of Ki67-positive cells were similar (Mukherjee et al., 2017). Observed similar levels might be because the proliferative capacity had already returned to normal. Alternatively, there was never a difference in proliferative capacity, the dosage was low, or cells were not as affected by this type of chemotherapy.

Effect of FPS-ZM1

FPS-ZM1, an anti-inflammatory, did not show a significant effect on reducing the effects of doxorubicin. FPS-ZM1 reduces the inflammatory response and oxidative stress by acting as a RAGE antagonist and blocking the binding of ligands which can result in pro-inflammatory gene activation (Hudson & Lippman, 2018). Initially, FPS-ZM1 was hypothesized to reduce the negative effects of doxorubicin on the taste system by reducing inflammation. Inflammation, both acute and chronic, can have a negative effect on taste bud number and renewal (Cohn et al., 2010; Kaufman et al., 2018). As inflammation is induced in cancer itself, as well as with doxorubicin treatment we hypothesized an anti-inflammatory might ameliorate negative effects caused by either (Hudson & Lippman, 2018). The reason an effect was not observed in our study may be due to a lack of access to the taste buds by FPS-ZM1, or possibly just because any effects occurred closer after the injection of the medications.

Conclusion

BALB/c treated mice injected with 4T-1 cells and injected with doxorubicin, FPS-ZM1, or a combination of the two did not show significant differences when compared with DMSO-treated mice. Number of taste buds, proportion of α-gustducin-positive cells, number of neutrophils, and number of proliferative cells all did not show a significant difference between groups. This might be because samples were collected two weeks after treatment and had already recovered, if any effects occurred closer to the treatment. Another possibility is that the number of mice tested were not enough to see an effect. The former is in line with previous research that mice had largely recovered after two weeks. However, if recovery was complete after two weeks, this would suggest that patients could recover their taste more rapidly than is commonly reported after chemotherapeutic treatment.

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