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Implications of Notch Signaling in Taste Cell Replacement

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Abstract

Cyclophosphamide (CYP) was one of the first chemotherapy drugs developed and used to treat several types of cancer, by disrupting proliferative cells. Unfortunately, CYP is unable to differentiate between cancerous cells and healthy cells turning over which ultimately kills normally functioning cells, including those of the taste system. This loss of taste cells may result in dysgeusia (altered sense of taste), hypogeusia (reduced taste ability) or ageusia (inability to detect any tastes), eventually leading to malnutrition and poor prognosis for patients. The notch signaling pathway is one of the most important pathways involved in the differentiation and fate of neural stem cells (Hitoshi et al., 2002). A previous study looked at genes expressed in developing circumvallate taste cells and found that notch signaling remains active in adult mice to determine cell lineage as the sensory cells are continuously replaced (Seta, Seta, & Barlow, 2003). The current research uses immunohistochemistry to identify the presence of notch signaling following injury by CYP. It was hypothesized that if Notch1 is involved in taste cell replacement, we predict the Notch1 signal should be amplified following challenge by cyclophosphamide.

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Introduction

Cancer and Cyclophosphamide (CYP)

Cancer is the leading cause of death in all developed countries and the second in developing (Jemal et al., 2011). Several treatment options are available for the numerous types of cancer including chemotherapy and radiation treatment. CYP is one of the oldest forms of chemotherapy and is now one of the most commonly administered anticancer drugs. CYP is an alkylating drug that is mainly used to treat malignant lymphomas, leukemias, carcinomas of the ovary, and breast cancer (de Jonge, Huitema, Rodenhuis, & Beijnen, 2005). CYP (Figure 1) is a prodrug, which means it is administered in an inactive form and subsequently metabolized in the liver by p450 cytochrome oxidase into its active products. CYP is initially broken down into the unstable product 4-hydroxycyclophosphamide, which tautomerizes to aldophosphamide. Aldophosphamide is then converted into the nontoxic compound carboxy phosphamide and the active, toxic, compounds acrolein and phosphoramide mustard. Most of aldophosphamide is converted into these cytotoxic products which is how CYP exerts its detrimental effects. Acrolein is an unsaturated aldehyde compound that is cytotoxic along with phosphoramide mustard. Phosphoramide mustard manifests its toxicity by creating both intra- and interstrand cross-links in DNA during the S-phase (de Jonge et al., 2005). These cross-links inhibit DNA replication and further cell proliferation, ultimately leading to apoptosis, or programmed cell death. Although CYP is great at targeting rapidly proliferating cells, it cannot differentiate between healthy, proliferating cells and cancerous ones. The prognosis of the patient's

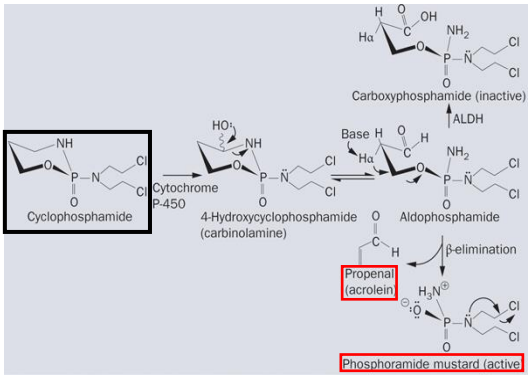

Figure 1. Mechanism of CYP metabolism. Identifying the pathway from inactive cyclophosphamide (black box), metabolism by cytochrome P-450 in the liver, and further metabolism into its active, cytotoxic and alkylating compounds acrolein and phosphoramide mustard, respectively (red boxes). Adapted from Emadi, Jones, & Brodsky, 2009)

disease largely depends on the efficacy of the drugs but the side effects cannot be overlooked. It was reported that 40% of cancer patients hospitalized suffer from malnutrition (Comeau, Epstein, & Migas, 2001). Loss of taste because of the chemotherapy treatment can result in a reduced appetite in the patient and an overall reduced quality of life. This leads to malnutrition and further dietary deficiencies ultimately causing more severe health problems.

Overview of the Taste System

Taste is one of our chemical senses that allows us to distinguish between healthy, nutritious foods and poisonous, harmful foods. Through evolution we have learned to reject bitter substances due to their relation to bitter-tasting poisonous compounds produced by plants and animals. We have also learned that sour tasting compounds can indicate something that is rotten, such as fruit, or acidic. The rest of the typically distinguished tastes are sweet, savory (umami) and salty (Barlow, 2015). Taste has proven to be an imperative sense throughout evolution which is why experiencing hypogeusia (decreased sensitivity), dysgeusia (distortion of taste), or ageusia (absence of taste) can potentially be fatal.

The taste system is a chemical sensory system that is composed of taste buds containing taste sensory cells, which are located throughout the oral cavity and pharyngeal cavities. Taste buds are innervated by cranial nerves VII, IX, and X. Most taste buds on the tongue's surface are part of specialized papillae called fungiform (FF) (Figure 2), circumvallate (CV) (Figure 2), or foliate taste papillae. Within each circumvallate and foliate taste papillae lie hundreds of taste buds that contain the specialized taste sensory cell (TSCs). Each FF taste papillae contains one taste bud made up of a cluster of cells. Each taste bud contains roughly between 50-100 TSCs that are differentiated into type I, II, and III cells (Barlow, 2015; Barlow & Ophir, 2015; Hamamachi, Asano-Miyoshi, & Emori, 2006; Yee et

al., 2013; Seta, Toyono, Kataoka, Toyoshima, 2005). Each taste cell type is morphologically and functionally different.

Taste Cell Types. The most common, type I cells, are thought to be salt detector cells. They also the glial-like cells for the taste system because they appear to clear neurotransmitters and ensheath Type II and III taste cells with lamellar processes (Perea-Martinez, Nagai, & Chaudhari, 2013; Barlow & Ophir, 2015). Type I cells can be identified by their expression of NTPDase2, which converts ATP to ADP. This is likely due to their neurotransmitter clearance mechanism. Type II cells are the sweet, bitter, and umami (savory) detectors. Type II cells can be identified by the expression of PLCβ2, which is involved in the g-protein-coupled receptor cascade. These cells have been shown to use ATP as neurotransmitters to send signals to sensory nerves. They do, however, lack regular presynaptic specializations and freely release their ATP molecules instead of enclosed in vesicles (Finger et al., 2005; Chaudhari, 2014). This supports the neurotransmitter clearing capabilities of Type I cells. Type III cells are sour detectors and appear to be most neuron-like, due to their formation of traditional synapses onto sensory nerve fibers of cranial nerves VII and IX. They can be identified by their expression of the SNAP-25 molecule, which is part of the SNARE complex (Barlow, 2015).

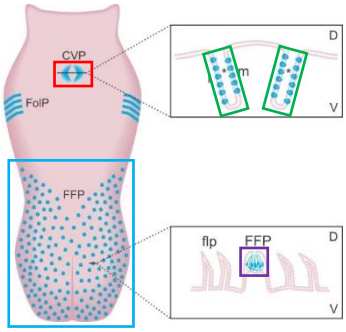

Figure 2. Diagram of tongue morphology. CV papilla (red box), located at the posterior, houses taste buds (green boxes). FF papillae, located at anterior two-thirds of tongue (blue box), house single taste bud (purple box). Adapted from Barlow, 2015.

Development

Developmental pathways and signaling mechanisms govern when, where, and how quickly the TSCs arrive at their specific location. Taste bud development and innervation is well known in rodents and is said to begin at embryonic (E) day 11 in mice, where the tongue

rudiment forms and is covered by a homogenous epithelial bilayer (Kapsimali & Barlow, 2013). By E12-12.5, taste placodes appear as foci of columnar epithelia in locations where fungiform and circumvallate papillae will form. At E14.5, invagination is evident to create taste papillae with distinct mesenchymal cores. Finally, taste nerve fibers reach and then penetrate the taste epithelium. It had been previously thought that innervation was required for taste bud development, however, we now know this is not the case. Barlow, Chien, and Northcutt (1996) elucidated that innervation is independent of taste bud development in rats and other mammals. They found that taste papillae or their primordia form in the epithelium prior to contact by neurites in vivo. They also found that the cranial nerve fibers (VII, IX, and X) that innervate the taste buds grow directly to the fungiform papillae, which suggests that the taste bud primordia may attract these nerves via a chemical cue (Barlow et al., 1996). The current understanding of taste bud development is that, embryonically, taste bud development is nerve-independent, but postnatally during differentiation of taste bud cells, innervation is required (Kapsimali & Barlow, 2013). At birth, taste precursor cells within the taste buds express embryonic markers, such as *Shh*, Sox2, and keratin-8 (K8), in the absence of innervation (Luo, Okubo, Randell, & Hogan, 2009). They do not, however, express any differentiated cell markers. The first postnatal week was shown to be a crucial time point for TSC differentiation in circumvallate papillae. Even when there was damage and regrowth of the innervated circumvallate papillae prior to complete differentiation, the resulting number of taste buds in the epithelium was permanently decreased. During this week, they will begin to express specific taste cell type markers indicating differentiation (Kapsimali & Barlow, 2013; Barlow, 2015).

Taste Cell Lifespans. The focus of the current research was on the TSC replacement cycle that occurs in adult mice circumvallate papillae. Many cell-signaling pathways are at work throughout the life of the animal regarding taste cell turnover. Parea-Martinez et al.

(2013) estimates that the population made up of neither Type II cells nor Type III cells, mainly Type I cells and undifferentiated or immature cells have half-lives of 8 and 24 days, respectively. Type II cells have a half-life of 8 days and Type III cells have a half-life of 22 days (Perea-Martinez et al., 2013). Normally, due to the constant replacement of TSCs, there is a varied number of taste cells that are mature, immature, and sloughing off at any one time in the taste bud. Approximately 60-70% of the TSC population are differentiated cells, about 20-30% of cells are undergoing differentiation, and about 10% of cells are undergoing apoptosis due to age (Barlow, 2015).

Taste cell Development/Replacement. TSCs come from a taste progenitor pool located in a basal layer just outside of the taste bud and in areas adjacent to the taste buds (Gaillard & Barlow, 2011). These cells are basal keratinocytes (Type IV cells) that appear to be transit amplifying cells that move up along the basal lamina. They can be identified by their expression of keratin-5 ($K5^+$) and keratin-14 ($K14^+$), which are intermediate filament proteins necessary for epithelial cell structure. Depending on β-catenin expression by these $K5/K14^+$ cells, they will either end up as non-taste epithelium (low β-catenin expression) or differentiated TSCs (higher β-catenin expression) (Castillo et al., 2014; Gaillard & Barlow, 2011; Okubo, Clark, & Hogan, 2009). Cells with higher β-catenin expression and subsequent expression of *Sonic Hedgehog* (*Shh*) are postmitotic taste cell precursors that will ultimately become differentiated TSCs. What dictates which type of TSC the precursors differentiate into is the level of this β-catenin expression. Within these higher β-catenin expressing $K5/K14^+$ cells, relatively high β-catenin expression leads to Type I cells, mid β-catenin expression leads to Type II cells, and low β-catenin leads to Type III cells. Finally, the low level of β-catenin expression in the non-differentiated cells results in expression of keratin-13 (K13) which are differentiated keratinocytes that will end up as non-taste epithelium

surrounding the taste bud and making up most of the tongue surface (Castillo et al., 2014; Gaillard & Barlow, 2011; Okubo et al., 2009).

Notch Signaling Pathway

Overview. The Notch signaling pathway is highly implicated in development throughout the nervous system, and specifically in the cell fate decisions (Seta et al., 2003). Notch is an evolutionarily conserved cell-cell signaling pathway that is composed primarily of transmembrane surface receptor proteins and membrane-bound ligands that are expressed on its neighbors that will ultimately activate the signaling cascade. Notch ligands have been shown to contain epidermal growth factor (EGF) repeats that appear to be necessary for proper binding to the transmembrane receptor protein. The initial two ligands shown to contain EGF repeats that bonded with the notch receptor were Delta and Serrate (Rebay, Fleming, Fehon, R. G. Cherbas, & P. Cherbas, 1991). However, years later it was shown that another ligand named Jagged can also bind to the notch receptor and that it contains EGF repeats (Lindsell, Shawber, Boulter, &Weinmaster 1995; Guarnaccia, Pintar, &Pongor, 2004; Guruharsha, Kankel, & Spyros, 2012).

Notch1 Signaling Cascade. Notch1 signaling begins by the signaling cell's membrane-bound ligand, a Delta, Serrate, or Jagged, binding to the receiving cell's transmembrane Notch1 receptor protein. Following ligand binding, intracellular γ-secretase is recruited to cleave the Notch intracellular domain (NICD). Then, the NICD translocates to the nucleus, where it begins to directly influence transcription via binding proteins such as protein recombining binding protein (RBPJ, or CBF1). Binding of RBPJ then activates repressor type *Hes* genes. Hes binds directly to the promoter region of *Mash1* inhibiting expression and prevents certain neural differentiation (Seta et al., 2003; Guruharsha et al., 2012). This implies that when *Hes1* is downregulated, *Mash1* expression upregulates and the

cell is driven toward a neuronal fate (Seta et al., 2003). This result occurs in the presence of the expression of *Notch1*. *Notch1* is one of the three Notch homologs (1-3) identified in mammals (Lindsell et al., 1995) that are involved in development. *Notch1* has been shown to be essential due to necessary cell-cell signaling and influence on downstream gene-expression. When *Notch1* expression is disrupted, lethality occurs very early, before 11.5 days of gestation (Lindsell *et al.,* 1995). Interestingly, the neural fate for developing cells is dependent on multiple *Hes* genes, as well. Another route for cell differentiation and regulation operates by inhibition of *Hes1* via *Hes6* activation, which leads to activation of *Mash1* expression and further differentiation. Activation via the latter pathway has been shown to differentiate neuronal-like Type II and III cells, whereas via the former, support or Type I cells prevail (Seta et al., 2003).

The fact that Notch1 acts to inhibit differentiation via activation of *Hes1* and further suppression of *Mash1* largely contributes the results of the current study. Our findings indicate that around day 4 post-injection, there is a decrease in Notch1 labelling followed by a gradual increase. This increase in Notch1 could therefore be indicating the decreasing level of specific Type II and Type III TSC differentiation and an increase in Type I TSC differentiation.

Hypothesis

It was hypothesized that if Notch1 is involved in taste cell replacement, we predict the Notch1 signal should be amplified following challenge by CYP.

Materials and Methods

Subjects

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were at least 8 weeks old and weighed between 23-26g at the beginning of the

experiment. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont, protocol number 14-003. The experiment was designed to minimize the amount of mice required to test the experimental question.

Chemical Reagents

CYP (Cyclophosphamide monohydrate, 97%) was obtained from Acros Organics (New Jersey, USA).

Tissue Preparation

The cellular morphology of circumvallate (CV) taste papillae and taste buds of the tongue were examined at days 0 (saline), 3, 4, 5, 6, 7, 8, and 10 following a 75 mg/kg CYP injection. Perfusions were done using PBS-heparin followed by 4% paraformaldehyde in PBS (Electron Micros-copy Sciences, Hatfield, PA USA) and the tongues were harvested for analysis. Following perfusion, tongues were cryoprotected using a 30% sucrose solution (24-36 hrs) and then and kept at -80º C. CV tongue blocks were cryo-sectioned at a thickness of 6 µm. Every 8th section was mounted on one slide to ensure accurate quantitative evaluation of taste buds and to prevent overlapping counts. Tissue collection began at the first sight of the CV papillae, which was viewed under a dissecting scope. Slides kept at -20º C.

Immunohistochemistry of Notch1 labeling

Notch1 primary polyclonal antibody (Catalog # ab65297; Abcam, Cambridge, UK) was used at 1:100 dilution for incubation at 4º C overnight and the secondary antibody was Alexa 546 goat anti-rabbit used at 1:1000 dilution (Thermo Fisher, Waltham, MA). All slides were counterstained using Sytox green (1:30,000; S7020, Molecular Probes, Eugene, OR) as a nuclear marker.

Statistical Analysis

All statistical analyses were done using SPSS software (SPSS Statistics, version 24, IBM Corporation, Chicago, IL, USA) and Graphpad Prism 7 software. Analysis of variance (ANOVA) procedures were used to compare the effects of CYP on Notch1 expressing cells of the taste buds between each timepoint post-injection (PI). The independent variable, days PI (8 levels), was treated as a between subject variable.

Image Capture Analysis

All images were captured using a Zeiss Axioskop2 with 20x magnification and with a 63x water immersion with a Photometric Cool SNA EZ camera and NIS Elements acquisition and imaging software. Cell counts were taken using ImageJ (NIH, Bethesda, MD, USA).

Results

Overall, the results of this study identify a pattern in Notch1 signaling over the course of 10 days PI. At day 0 CYP post injection, we should expect there to be uniform expression of Notch1 throughout the taste bud. This indicates a basal level of Notch1 expression throughout the taste bud as new differentiating cells replace the cells due to normal attrition from aging. This is exactly what is observed. In Figure 3, Notch1 is expressed uniformly throughout many cells. Therefore, days post-CYP injection, we should expect to see a drop in Notch1 expression because the cells are dying off. An observable and statistically significant drop in the number of labelled Notch1 taste cells appears at 4 days PI (Figure 4), which subsequently begins to increase back towards the normal level. Figure 5 shows that the number of labeled cells has increased gradually over the next 6 days.

Notch1 Localization

The Notch1 labeling at day 0, the labeling appears more localized to the nuclear region of the cells and less on the cell membrane. At days 4 and 10, the Notch1 labeling appears significantly more in the cell membrane and is less localized in the nuclear region. Overall, the amount of labeling in day 4 CYP is Significantly lower than observed in Day 0 CYP mice.

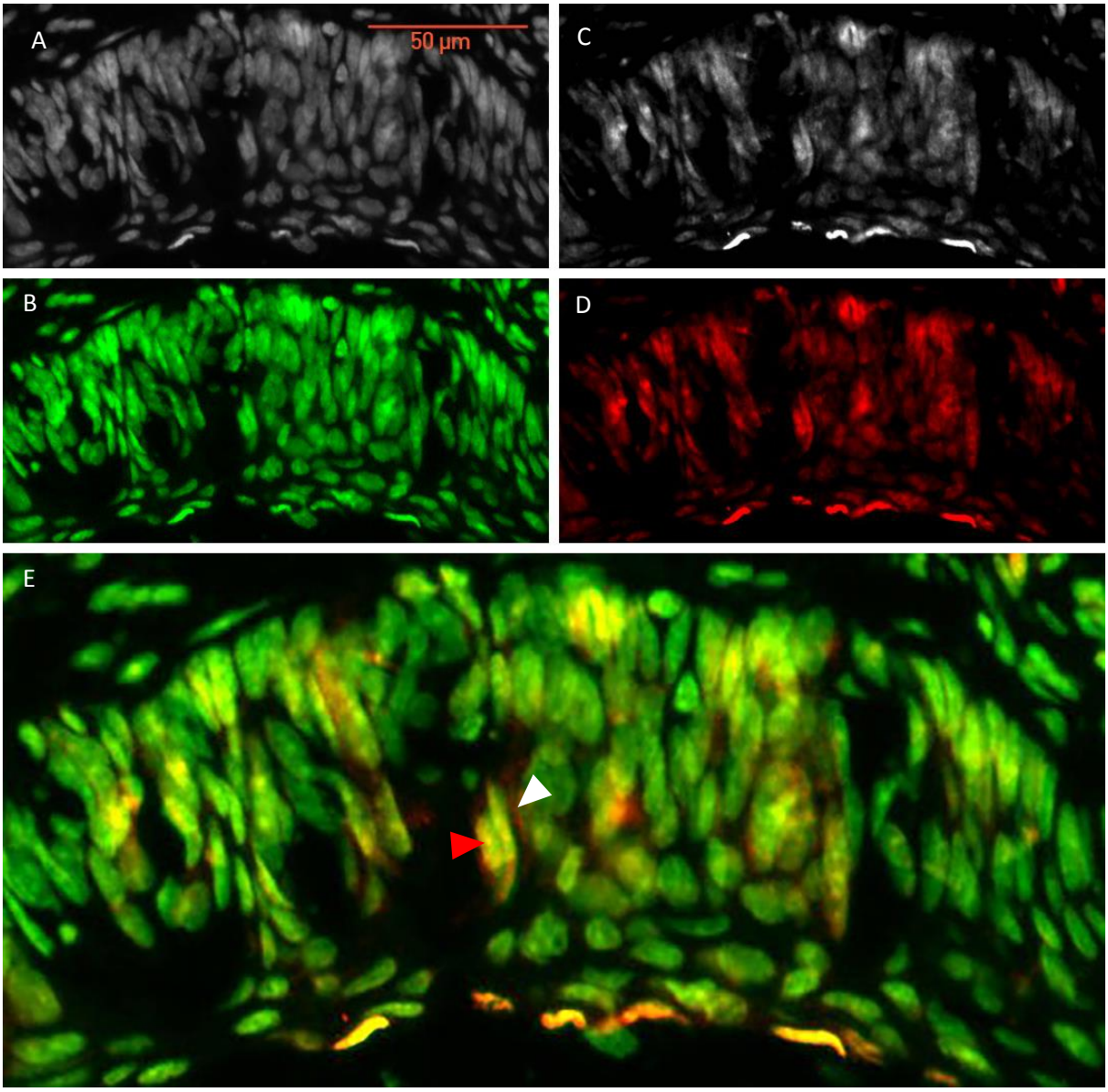

Figure 3. Day 0 CYP; all taken at 20x. A) Original nuclear labeled (Sytox green). **B)** Colorized green nuclear labeled. **C)** Original Notch1 labeled cells. **D)** Colorized red Notch1 antibody. **E)** Merged, orange-colored cells show double labeled for Notch1 appearing in the nucleus (red arrow), as well as around the nucleus on the cell membrane (white arrow). Uniform amount of notch signaling throughout taste bud indicating basal amount of activation and therefor normal activation and replacement.

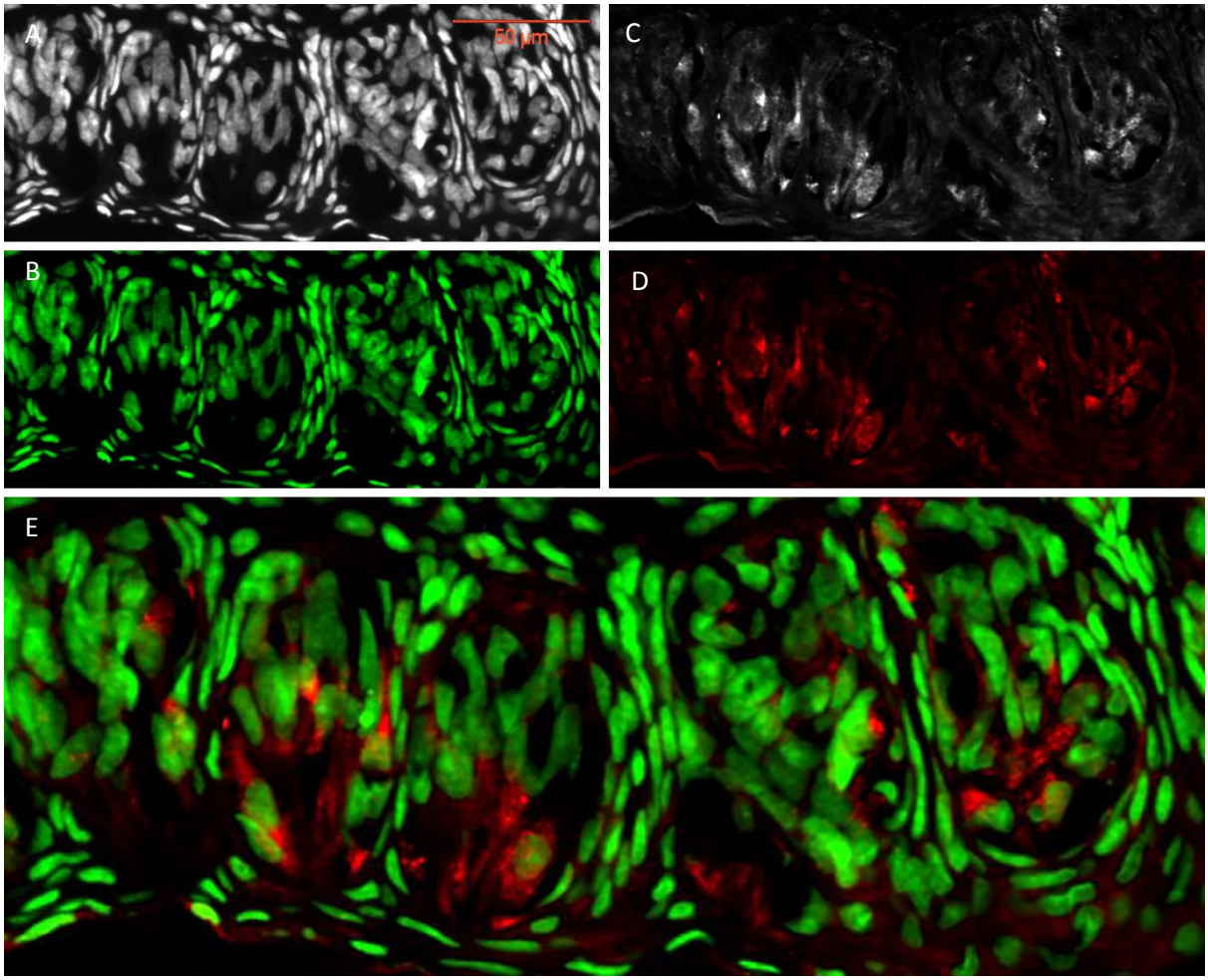

Figure 4. Day 4 CYP; all taken at 20x. A) Original nuclear labeled (Sytox green). **B)** Colorized green nuclear labeled. **C)** Original Notch1 labeled cells. **D)** Colorized red Notch1 antibody. **E)** Merged, Notch1 labeling seen almost exclusively in cell membranes. Severely diminished amount of Notch1 labeling throughout the taste bud indicating low levels of expression of Nocth1 due to CYP challenge.

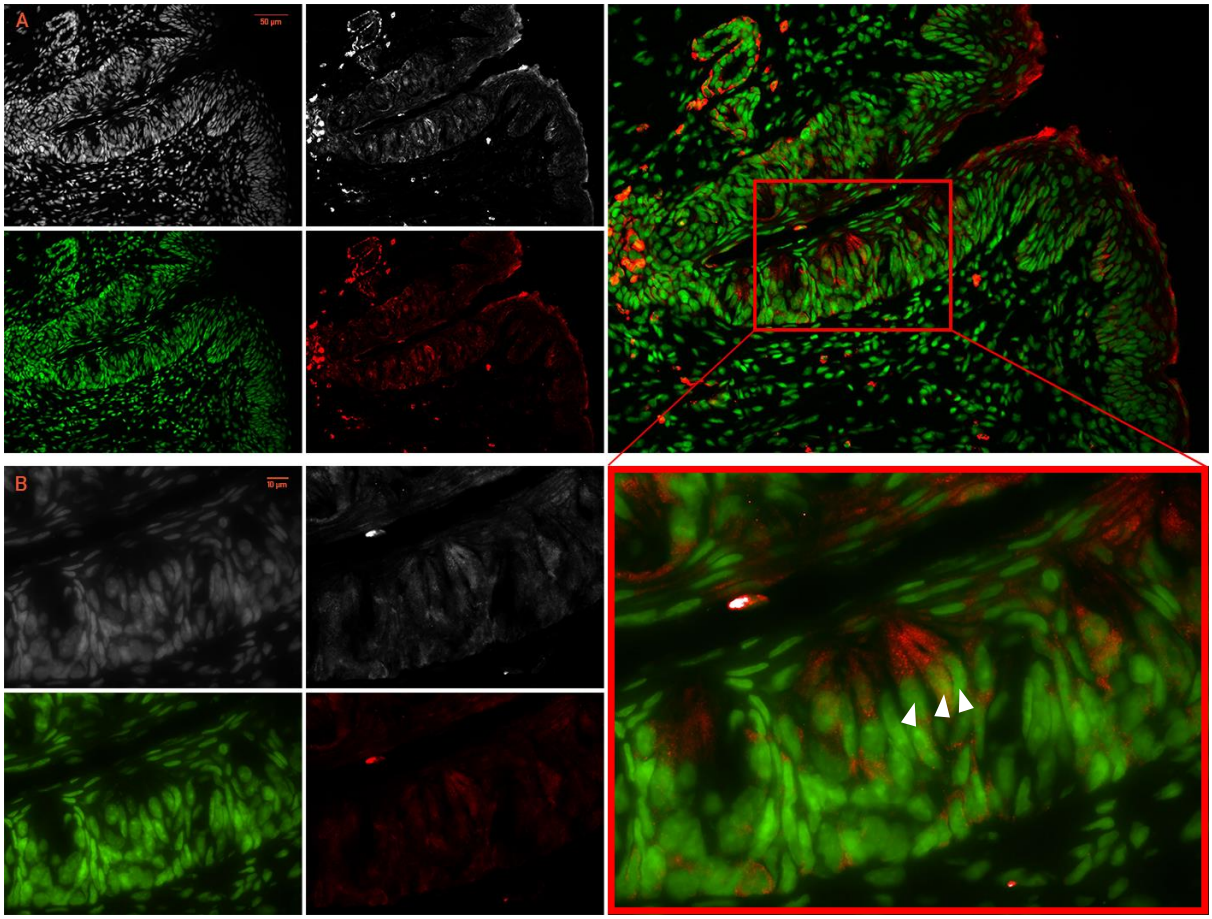

Figure 5. Day 10 CYP. Panel A) Taken at 20x. Composed of originally labeled (black and white), colorized (green nuclear label, red antibody label), and larger merged image (at right). **Panel B)** Taken at 63x with water immersion lens. Composed of originally labeled (black and white), colorized (green nuclear label, red antibody label), and larger merged image (at right). Bottom-right merged image shows 63x magnification of the above corresponding image. Here, three cells (arrows) are labeled on their cell membranes (red). The shape of the TSCs is also evident, as they have an elongated shape that points to the apical end and extends towards the basilar end. The extent to which the cells extend to each end depends on the type of TSC. It is unclear from the image what type of taste cell

Statistics

A one-way ANOVA was performed that identified a statistically significant effect between the experimental timepoints. Post hoc comparisons (Sidak) comparing all days to day 0 PI indicated that at days 3 and 4, there were significantly fewer ($P<0.05$) Notch1 labeled taste cells out of the total number of TSCs per taste bud compared to the other days (Fig. 6). After day 4 the Notch1 labeled slowly increased, returning to baseline levels.

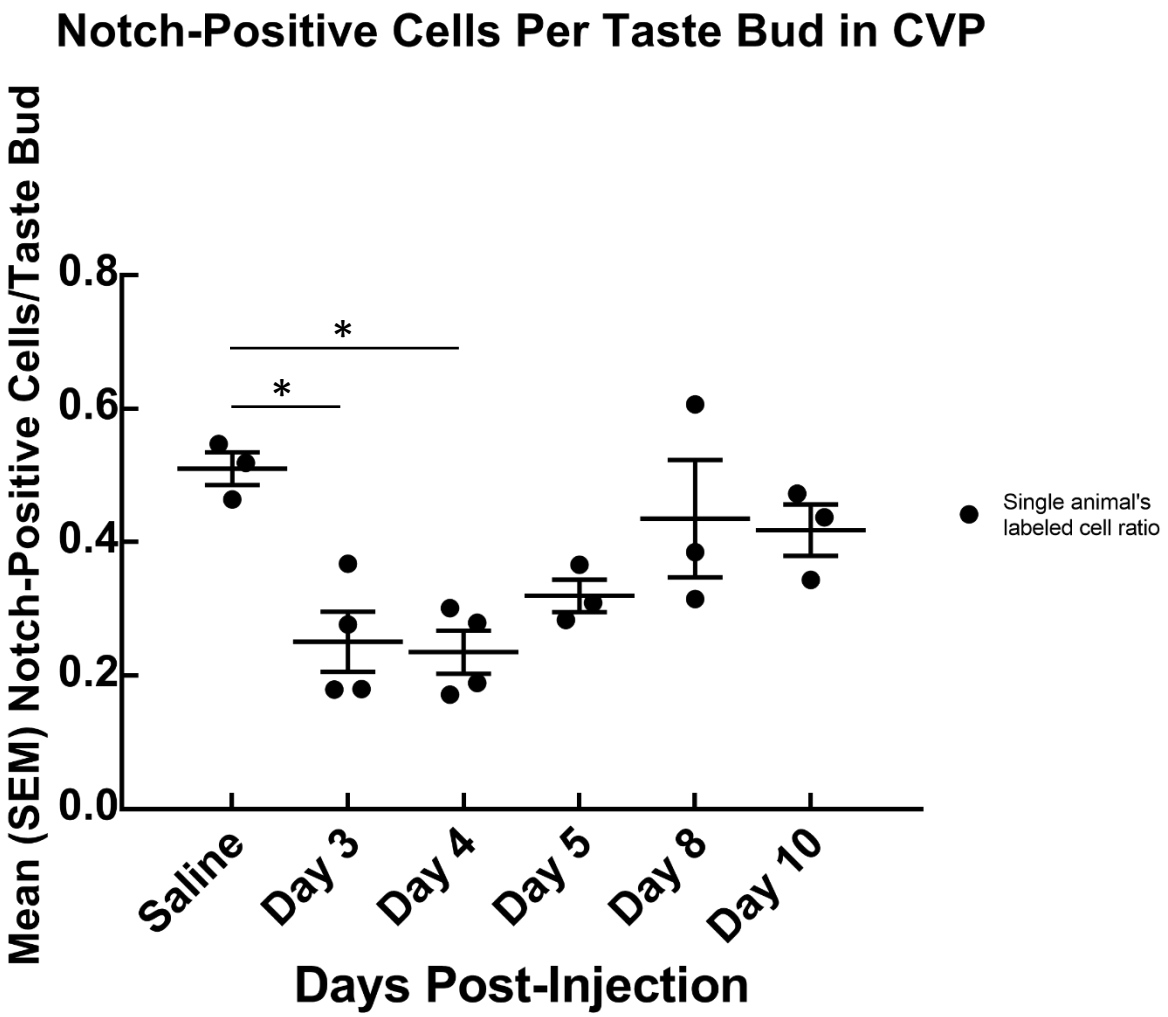

Figure 6. Mean (± SEM) of labeled Notch1 cells in CV taste buds. Notch1-positive cells in CV shows significant decrease following CYP injection indicating severe loss of TSCs. Slowly, notch labeling increases over the course of 10 days. There was a significant decrease in the number of labeled cells between day 0 (saline) and day 3, and day 0 and day 4 (* $P<0.05$).

Discussion

Notch signaling has been evolutionarily conserved and plays an imperative role in determining cell fate in several types of developmental processes in many organisms (Guruharsha et al., 2012; Seta et al., 2003). Previous research has implicated Notch signaling in taste cell development and replacement. Different genes responsible for taste bud and TSC development and taste cell replacement have also been identified including *Hes1, Hes6, Notch1-4,* and *Mash1* (Seta et al., 2003; Barlow, 2015). Embryologically, the process by which taste buds develop has been shown to be nerve-independent, but that taste cell differentiation is nerve-dependent. As the animal matures, taste cells die and must be replaced. Many systems involved in embryologic development also take part in regular taste cell replacement throughout adult life, such as Wnt/β-Catenin, Sonic Hedgehog (Shh), and Notch. In adult animals, it has been shown that there is a progenitor cell population located in the basal layer and adjacent taste epithelium of mature taste buds. When activated, signaling pathways cause immature, nonmitotic cells to migrate into the taste bud and

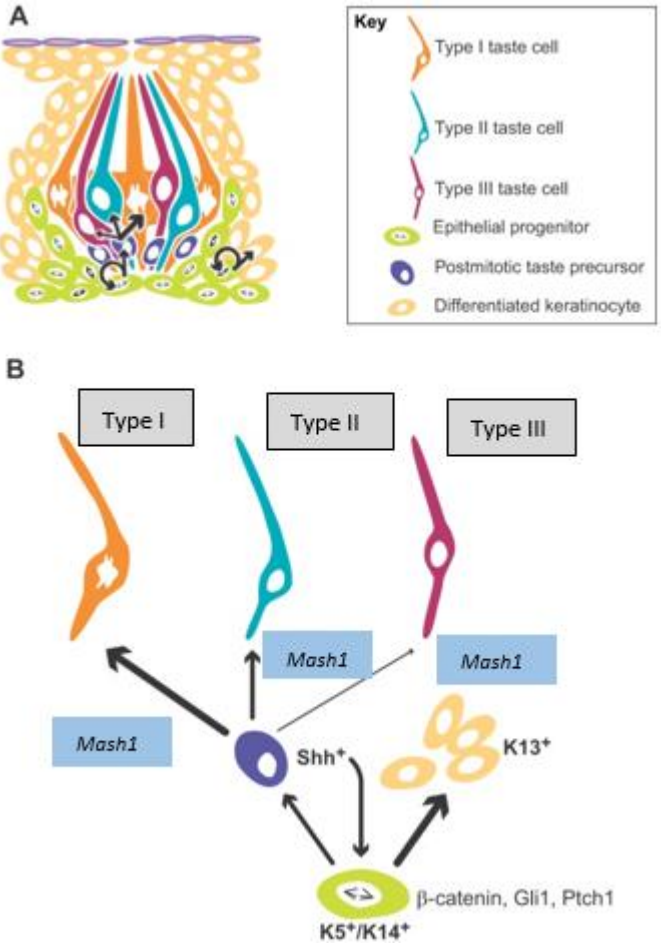

Figure 7. Taste cell renewal in adult mice. A) All 3 types of taste cells in adult taste buds. Basal layer (green) shows where progenitor pool is located as well as those adjacent to taste bud. Basal keratinocytes (green) show asymmetric division (curved arrow indicated keratinocyte replenishment). Differentiated keratinocytes (orange) are non-taste cells. Post-mitotic taste precursor cells (purple) eventually differentiate into taste cells (type I, II, and III). B) Notch activity would be indicated where *Mash1* expression is indicated, depending on what type of *Hes* activity is occurring. Adapted from Barlow, 2015.

differentiate to end up in their final phenotype (Figure 6) (Barlow, 2015; Gaillard & Barlow, 2011; Okubo et al., 2009; Miura, Scott, Harada, & Barlow, 2014).

The previous mention of the effects of *Hes1* on *Hes6* may explain the observed trend in Figure 6. When *Notch1* becomes active and further activates *Hes1* in the developing mouse nervous system, it was shown that *Mash1*, which encodes a developmentally relevant Mash1 protein, is inhibited. This eventually leads the cells to a glial fate, which could translate to Type I TSC differentiation. When a non-repressor type *Hes* gene is activated, *Hes6*, *Mash1* is activated and leads to neuronal differentiation, which would translate to Type II and III differentiation. The decrease in Notch1 labeling at day 4 and then the subsequent increase could be evident of this system shifting from differentiating more neuronal type TSCs (low Notch1 expression) to more glial-like TSCs (higher Notch1 expression) (Bae, Bessho, Hojo, & Kageyama, 2000).

These results can further be analyzed to show the cycles of activation of Notch1 signaling, which gives crucial insight into the timing of the taste cell replacement cycle. We could expect to see decreased levels of Mash1 signaling along with the increase in Notch1 signaling due to the suppressing effect of *Hes6*. This study can be taken at a larger scale to highlight important points along the taste cell replacement cycle that could be pharmacologically targeted. This research is comparable to that by Mukherjee, Carroll, Spees, and Delay (2013), which indicated a drop in the number of BrdU-positive (S-phase marker for mitosis), proliferating cells in the CV and FF taste papillae. In response to this, the protective agent amifostine was injected before CYP administration and a significant increase in the number of proliferative cells was observed. The current research could be used to identify a crucial time point to intervene pharmacologically. These data therefore suggest an important role of notch signaling in taste cell replacement.

Future Directions

To more thoroughly identify Notch1 signaling in the taste cell replacement cycle, the current study should be replicated and carried out to at least 30 days post-injection. This would allow for at least one full cycle of taste cells to regrow following challenge by cyclophosphamide. The trends in Notch1 activation would also be clearer and more crucial points could be elucidated for potential treatment intervention. To observe the activity of *Mash1* in relation to *Notch1* during TSC replacement, double labeling against Mash1 and Notch1 should be performed. If the nature of these genes withstands in the taste system as it does in the developing mouse nervous system, an inverse trend should be observed as the TSCs grow back. Another important avenue to explore and to further support the *Mash1* hypothesis, would be to label specific TSC markers, such as PLCβ2 and SNAP-25. This would give insight into which TSCs are developing at which timepoints in relation to Notch1 and Mash1 expression.

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