Monell Chemical Senses Center

Annual Progress Report: 2010 Formula Grant

Reporting Period


Formula Grant Overview

Monell Chemical Senses Center received $216,916 in formula funds for the grant award period January 1, 2011 through December 31, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Effects of Environmental Tobacco Smoke Exposure on Cough in Adolescents and Adults - Cough is a reflex that protects the lungs against noxious airborne molecules and smokers have impaired cough sensitivity, which contributes to their higher rates of respiratory illness. Parents who smoke expose their children to environmental tobacco smoke (ETS), but it is not known how ETS affects the cough sensitivity of these children. This question is especially important in Pennsylvania because the smoking rate here is in the top quintile. In our research study, we will determine whether adolescents who are exposed to ETS (because one or both parents smoke) have impaired cough sensitivity relative to children of non-smokers. The information gleaned will set the stage for investigating whether reduced sensitivity contributes to illness and early initiation of smoking during adolescence.

Anticipated Duration of Project

1/1/2011 - 12/31/2011

Project Overview

Cigarette smoke is a common source of chemical and particulate irritants, and cough is an obvious and healthy response to this airway threat. The cough response becomes desensitized in smokers, which helps them tolerate exposure and may contribute to higher rates of respiratory illness. Further, almost half of the children in the United States are exposed to environmental tobacco smoke (ETS) in the home because one or both parents smoke. ETS-exposed children have higher rates of pneumonia, bronchitis, wheezing, and ear infections. In addition, children of smokers are more likely to experiment with smoking during early adolescence, increasing their risk of becoming habitual smokers. Since cough sensitivity could play a role in these negative outcomes, the objective is to understand how ETS exposure affects cough sensitivity in adolescents.
The specific aim is to test the hypothesis that ETS-exposed children are more likely than non-exposed children to suffer from impaired cough sensitivity. Subjects will include 40 racially and ethnically diverse, healthy adolescents aged 10 to 17 years (a critical time for experimenting with tobacco) and their mothers (40 child-mother pairs). The sample will comprise two groups: Non-ETS Exposed (neither the child nor parents has ever smoked or been exposed to ETS in the home) and ETS-Exposed (the mother has smoked at least three cigarettes per day for at least five years in the home, with the child living in the home continuously). Cough sensitivity will be measured using a standard single-inhalation challenge, a test of the minimum concentration of capsaisin (the spicy chemical in hot peppers) needed to elicit cough. Measures of breath carbon monoxide will validate the smoking status of mothers and their adolescent children. The key comparison will be between Non-ETS Exposed and ETS-Exposed children, with the difference between smoking and non-smoking mothers as a positive control. Because smoking and non-smoking families may differ in ways besides tobacco exposure, we will obtain health histories (with a focus on respiratory illness), smoking histories, measures of body weight, diet, and responses to personality tests (including susceptibility to addiction). We will also obtain genomic DNA from saliva samples. Genes for chemosensory receptors that are part of the cough reflex pathway and genotype may account for aspects of cough sensitivity. Some of these variables may be co-variates to control for possible confounds that could affect the conclusions, but we specifically plan to determine whether cough sensitivity correlates with history of respiratory illnesses.

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Expected Research Outcomes and Benefits

Smoking not only affects the health of the smoker, increasing their risks for chronic diseases including those of the heart and respiratory system, but it adversely affects the health of their children who are passively exposed to ETS. We know from previous studies that the smokers have a reduced cough sensitivity, which can have an impact on their health because cough serves the physiologic function of clearing excessive secretions and debris from the throat - a sentinel portal to the airways. It is not known whether ETS exposure affects cough sensitivity in the children of smokers, but reduced sensitivity could contribute to the increased rates of various respiratory illnesses that are seen in this population. Increased knowledge of the risks associated with ETS exposure would provide physicians, policy makers, and others responsible for health education with clearer information for smoking parents. Of course, better information will fail to
deter many parents from smoking in the home. Thus, increased knowledge of the underlying causes of negative health outcomes is the first step toward effective treatment. If the proposed work finds reduced cough sensitivity in ETS-exposed children and increased frequency and severity of respiratory illness, then future research efforts can consider measures to counteract reduced sensitivity as a possible therapeutic strategy.

Cough sensitivity is likely to have several determinants and in addition to exposure to ETS in the home, inborn differences in response to ETS may be important in determining how much exposure to chemicals is required to elicit cough. Reduced cough sensitivity may also contribute to the tendency for the children of smokers to become smokers themselves. New information about the role of chemosensory receptors in cough suggests that ETS exposure may interact with genotype to determine cough thresholds. Better information on the interaction between genes and environment will increase our understanding of who is at risk, and why. Better knowledge of underlying mechanisms and the factors which contribute to early onset of smoking can guide the search for more effective interventions.

**Summary of Research Completed**

The overall objective of the work is to test the hypothesis that environment tobacco smoke (ETS) exposed children are more likely than non-exposed children to suffer from impaired cough sensitivity. These individual differences in cough sensitivity are being evaluated for their genetic roots through association studies. Psychophysical methods, as reviewed below, are being used to measure cough sensitivity, in tandem with recent techniques to measure genotypes, such as single nucleotide polymorphisms. The following describes the research methods used. This is followed by a progress report on recruitment and the number of subjects enrolled in the study to date (milestone #1), genotyping (milestone #2), and data entry and analysis (milestone #3).

**Overall Method**

Our goal is to test 40 mothers and their children; half of the mothers will be smokers whereas the other half will have never smoked in their lifetimes. Each mother-child dyad (or mother and children, in case more than one child qualifies) is being phenotyped for cough reflex sensitivity using a single-inhalation cough challenge (primary measure). In this test, we determine the minimum concentration of capsaicin (the compound that gives hot peppers their characteristic burn) that is needed to make subjects cough. A higher concentration (higher threshold) indicates a less sensitive cough reflex. Secondary measures include dietary intake using diet records, obesity (height, weight, percent body fat), blood pressure, health history (particularly related to respiratory health), exhaled carbon monoxide levels, genotypes, smoking history, and nicotine dependence. These secondary measures provide important information, since families may differ in other important ways besides smoking status.

**Experimental Design/Overview**

A summary of procedures that each subject undergoes appears in Table 1. Each parent and child is tested on two days, separated by at least two days, and testing occurs approximately 1 hour before their next scheduled meal. Testing lasts 1.5-2 hours each day and occurs in a newly renovated testing facility designed for psychophysical testing of children and their parents. On
both days (Day A and B), we use a psychophysical test to determine cough thresholds for both parent and child. Subjects are tested in counterbalanced order, such that half of the mother-child dyads undergo Day A on the first day of testing and the other half undergo Day B on the first day of testing. Some key measures are described in more detail, below.

Single inhalation cough challenge (cough reflex threshold). We measure cough thresholds using a simple, ascending concentration procedure. In each experimental session, subjects will start with a single inhalation of a very low concentration of capsaicin solution (0.98 μM). If the subject coughs at least twice after the inhalation, the test ends and the lowest step is defined as threshold. If the subject does not cough at least twice, concentration doubles and the subject again takes a single inhalation. Concentration increases in this fashion, until the subject coughs twice, at which point the test ends and the concentration that elicits two coughs is defined as threshold (the highest concentration presented is 1000.00 μM, regardless of whether this concentration elicits two coughs). This method has been used successfully in numerous research studies, with tests of thousands of patients and healthy controls, with no reported serious adverse events.

Respiratory and Otitis Media (OM) Health History. Both parents and children are administered the same questionnaire developed by the American Thoracic Society (ATS) to gather information about their health history with a focus on respiratory illnesses. The questionnaires on major respiratory symptoms include: Did the child usually have a cough apart from colds in the previous year?; Did the child usually bring up phlegm apart from colds in the previous year?; Did the child ever sound wheezy apart from colds or did he/she have an attack of wheezing that has caused him/her to be short of breath in the previous year? Did the child ever have asthma or bronchitis diagnosed by a doctor? (Asthma is an exclusion criterion in this study.) Parents are also interviewed about their child’s history of ear infections. Parents are asked: “Has your child ever had an ear infection or an earache? Yes/No/Don’t Know”; “If yes, how many times has your child had an ear infection or earache per month/per year/in lifetime?”; “How old was your child when he/she had the first ear infection or earache?”; and “How old was your child when he/she had his/her last ear infection or earache?”).

Nicotine Dependence (adults only). To evaluate nicotine self-administration behavior in smokers, subjects are given the Fagerstrom test of Nicotine dependence and the Michigan Nicotine Reinforcement questionnaire.

National Youth Tobacco Survey (NYTS) 2009 Questionnaire (children only). The NYTS, a validated self-administered pencil and paper questionnaire, is administered to each child. Included in this survey are questions about tobacco use, exposure to environmental tobacco smoke, minors’ ability to purchase or otherwise obtain tobacco products, and knowledge and attitudes about tobacco.

Carbon Monoxide Levels. At the beginning of each test day, we measure breath carbon monoxide (CO) levels using the Vitalograph (Lenexa, KS) in adults and children. The Vitalograph Breath CO Monitor is a pocket-sized instrument which can be used to measure both alveolar concentrations and environmental levels of carbon monoxide. The display indicates parts per million (ppm) of carbon monoxide.
**Weight and body composition.** Weight and height measurements are obtained for each subject. Body mass index (BMI; the weight in kilograms divided by the square of the height in meters) is then computed. For women, BMI is categorized as follows: 18.5 kg/m² to 24.9 kg/m² (normal weight), 25.0 kg/m² to 29.9 kg/m² (overweight), and 30.0 kg/m² or more (obese). For children, their BMI for age is computed and then classified in one of four categories (i.e., underweight, healthy weight, overweight or obese) using the Centers for Disease Control and Prevention’s pediatric growth charts. Total body water, fat free mass and fat are estimated by bioelectrical impedance analysis (BIA) using the Quantum X instrument (RJL Systems, MI) with computational adjustments for this age group. Skinfold measurements and circumferences are also obtained according to established guidelines.

**Dietary Intake (ASA).** We will use the ASA, an automated, web-based, self-administered 24-hour dietary recall instrument developed by the National Cancer Institute and that is available for scientific researchers to use online (http://riskfactor.cancer.gov/tools/instruments/asa24/).

**Genotyping for Cough Reflex.** Saliva is obtained and genomic DNA extracted, purified and quantified (Oragene; Ontario Canada). Control genotypes are obtained to ensure parent-child allele sharing is consistent with the report of family relationship. Genes that may play a role in chemical irritation (and in turn the cough reflex) are assessed for common variant sites, e.g., TRPV1, the receptor for capsaicin. Variant sites are genotyped using the subject’s DNA, and the correlation between nucleotide variants within these genes and the phenotypic responses of the subjects is examined. The first focus will be on functional variants in chemoreceptors which respond to the irritant used here (capsaicin) and with prior association with cough in human subjects. Because we will be submitting an NIH grant to test more subjects, we expect over time that we will have sufficient samples sizes to determine the genetic contribution to cough reflex and how that interacts with environmental exposure to tobacco smoke.

**Progress in recruitment and testing**

Excellent progress has been made on subject recruitment and testing. Mothers were recruited from newspaper ads and extant data-bases of past subjects who had agreed in writing to be contacted regarding future studies. Initial interviews were conducted over the telephone. Those who had a history of chronic respiratory problems, current respiratory infections, were pregnant, lactating, or on any medication (except for birth control pills), were excluded from the study. There have been no problems with subject recruitment or enrollment.

As of 6/30/2011, we completed data collection on 24 children (8 girls, 16 boys), mean Age= 12.8 ± 1.8 years) and 19 parents (1 father, 18 mothers, mean Age= 48.8 ± 8.4 years). The number of children do not match the number of parents because some parents enrolled multiple children in the protocol. Eleven of the parents were never-smokers, and 8 of the parents were current smokers. Thirteen of the children were not exposed to ETS in the home (children of non-smokers), and 11 of the children were exposed to ETS (children of smokers who smoke in the home). Approximately two thirds of the subjects were African American, and approximately one third were white. Thus, we have collected data on almost half the total target sample. More detailed information on subject characteristics appear in Table 2.
Saliva samples from subjects have been collected and stored in a -80°C freezer. Currently we are extracting, purifying and quantifying the DNA samples obtained from saliva samples. This procedure involves lysis of cells and the removal of protein to obtain DNA. The DNA is further purified by removing residual proteins, salts and small molecules, and quantified through spectrophotometry or fluorometric methods. This process is in very early stages, and results are not yet available.

Progress in data entry and analysis.

Collected psychophysical, biometric, and other data have been de-identified and entered into a database to which other subjects will be added. The final analysis will be conducted when the study is closed to enrollment.

Table 1. Schedule of Events for Days A and B

<table>
<thead>
<tr>
<th></th>
<th>Day A</th>
<th>Day B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Monoxide Levels</td>
<td>Carbon Monoxide Levels</td>
<td></td>
</tr>
<tr>
<td>Cough Challenge Test (30 min)</td>
<td>Cough Challenge Test (30 min)</td>
<td></td>
</tr>
<tr>
<td>Anthropometry (20 min)</td>
<td>Questionnaires (1 hr)</td>
<td></td>
</tr>
<tr>
<td>Questionnaires (1hr)</td>
<td>24-hour Dietary Recall (20 min)</td>
<td></td>
</tr>
<tr>
<td>Saliva for genetic analysis (2 min)</td>
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</table>

Table 2. Characteristics of Subjects Tested to Date

<table>
<thead>
<tr>
<th>Measure</th>
<th>Parent, N= 19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age in years, Mean (SD)</td>
<td>40.8 (8.4)</td>
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<tr>
<td>Sex (% of group, N)</td>
<td></td>
</tr>
<tr>
<td>%Female</td>
<td>94.7, N=18</td>
</tr>
<tr>
<td>%Male</td>
<td>5.3, N=1</td>
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<tr>
<td>Race/Ethnicity (% of group, N)</td>
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<tr>
<td>% African American</td>
<td>68.4%, N=13</td>
</tr>
<tr>
<td>% Caucasian</td>
<td>31.6%, N=6</td>
</tr>
<tr>
<td>Yrs of school, Mean (SD)</td>
<td>13.3 (2.1)</td>
</tr>
<tr>
<td>Family yearly income (% of group, N)</td>
<td></td>
</tr>
<tr>
<td>% &lt;15,000, N</td>
<td>21.1%, N=4</td>
</tr>
<tr>
<td>% 15,000-35,000, N</td>
<td>36.8%, N=7</td>
</tr>
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<td>% 35,000-75,000, N</td>
<td>26.3%, N=5</td>
</tr>
<tr>
<td>% &gt;75,000, N</td>
<td>15.8%, N=3</td>
</tr>
<tr>
<td>Smoking status (% of group, N)</td>
<td></td>
</tr>
<tr>
<td>% Never-smokers, N</td>
<td>57.9%, N=11</td>
</tr>
<tr>
<td>% Current-smoker, N</td>
<td>42.1%, N=8</td>
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<tr>
<td>Measure</td>
<td>Value</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>Age in years, Mean (SD)</td>
<td>12.8 (1.8)</td>
</tr>
<tr>
<td>Sex (% of group, N)</td>
<td></td>
</tr>
<tr>
<td>%Female, N</td>
<td>33.3%, N=8</td>
</tr>
<tr>
<td>%Male, N</td>
<td>66.7%, N=16</td>
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<tr>
<td>Yrs of school, Mean (SD)</td>
<td>7.5 (1.8)</td>
</tr>
<tr>
<td>By smoking status of the mother (% of group, N)</td>
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<tr>
<td>%Never-smokers, N</td>
<td>54.2%, N=13</td>
</tr>
<tr>
<td>%Current-smokers, N</td>
<td>45.8%, N=11</td>
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</table>

SD=standard deviation; Yrs=Years. Family yearly income is reported in US dollars ($). Smoking status was determined through questionnaire and confirmed with empirical measures (breath carbon monoxide concentrations). School refers to formal education, high school, community or 4-year college.

Research Project 2: Project Title and Purpose

Effects of Chemotherapeutic Agents on the Peripheral Taste Structure and Function - Cancer patients undergoing chemotherapy frequently experience taste abnormalities. The severity of taste dysfunction is associated with high rates of weight loss and poor prognosis. To date, the underlying mechanisms of chemotherapy-associated taste disorders remain unclear. The purpose of this project is to investigate how chemotherapeutic agents affect the peripheral taste structure and function. The ultimate goal of this research is to identify approaches that can prevent or minimize the side effects of chemotherapy on the taste system.

Anticipated Duration of Project

1/1/2011 - 12/31/2011

Project Overview

Many chemotherapeutic agents can affect taste. Studies have shown that up to two thirds of patients receiving chemotherapy can experience taste alterations. Although it has been speculated that chemotherapy can directly affect taste buds, the experimental evidence is lacking and the underlying mechanisms remain elusive. Our long-term goal for this research is to understand the molecular and cellular bases of chemotherapy-associated taste disorders.

Anticancer drugs kill primarily fast-proliferating cancer cells. However, many of these drugs also show toxicity towards fast-dividing progenitor cells in normal tissues. We hypothesize that the progenitor cells for the taste bud, the basic functional unit of the peripheral taste system, are among the fast-dividing cells affected by anticancer drugs. In this project we will investigate the effects of three chemotherapeutic agents, 5-fluorouracil (5-FU), cisplatin, and paclitaxel (PTX), on the peripheral taste tissues. These three drugs belong to different categories of anticancer agents, are currently being used to treat a variety of malignancies, and have been shown clinically to be associated with taste alterations. We will investigate both the structural and
functional effects of these drugs on the peripheral taste system. We will carry out these studies in two specific aims:

Aim 1: determine the effects of 5-FU, cisplatin, and PTX on the taste bud structure. We will use methods, such as histology and immunohistochemistry, to examine changes in the gross structure of taste buds, as well as in cell proliferation and death in the taste epithelium after drug treatments.

Aim 2: determine the effects of 5-FU, cisplatin, and PTX on taste function. We will study the effects of drug treatments on taste responses to the five basic taste qualities, sweet, bitter, umami, sour, and salty tastes, using brief-access or lickometer tests during this funding period.

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Expected Research Outcomes and Benefits

Anorexia and weight loss are among the major concerns of cancer patients. Taste abnormality-associated with chemotherapy is one of the contributing factors for food aversion and decreased caloric intake in these patients. Our project is designed to investigate the underlying causes of taste alterations led by chemotherapeutic agents. We expect that results from this research will provide important information on how some anticancer drugs affect the taste bud structure and function. Knowledge gained from this study may facilitate future development of treatment options and strategies that minimize taste alterations caused by cancer therapy. In addition, this project will further our understanding of the regulatory mechanisms that control taste bud renewal and turnover, an understudied area in the chemosensory field.

Summary of Research Completed

The goal of this research is to understand the underlying mechanisms of how chemotherapy drugs affect the sense of taste. Because the taste bud is the functional unit responsible for detecting and recognizing taste compounds, we started out this research by investigating the effect of chemotherapy drugs on the structure of taste buds. A mammalian taste bud, containing 50-100 cells, is a dynamic structure that undergoes constant cell turnover. To maintain homeostasis, taste progenitor cells proliferate to produce newborn cells that enter the taste bud and differentiate into taste receptor cells and supporting cells needed for taste function. During the past funding period, we have studied whether chemotherapy drugs affect cell proliferation
and cell death in the taste tissue. Our results show that all three chemotherapy drugs we tested, 5-fluorouracil (5-FU), cisplatin, and paclitaxel (PTX), decrease the number of progenitor cells and increase the number of apoptotic cells in the taste epithelium. Consistently, these drugs also decrease the number of taste receptor cells in taste buds.

Experimental procedures:

**Drug administration and tissue processing**
5-FU (150 mg/kg), cisplatin (7.5 mg/kg or 20 mg/kg), and PTX (115 mg/kg) were injected intraperitoneally into C57BL/6J mice. All three drugs were purchased from Sigma. Phosphate buffered saline (PBS) was used as the vehicle control. Tongue tissues were collected at 4, 8, 24, and 48 h after injection. Five mice per group per time point were used. To study the effects of repeated administration of the drugs on taste buds, 5-FU (150 mg/kg), cisplatin (7.5 mg/kg), PTX (115 mg/kg), as well as PBS, were injected into C57BL/6J mice on days 1, 3, 5, 7, and 9. Tongue tissues were collected on day 5 (mice received two injections) and day 10 (mice received 5 injections). The collected tongue tissues were washed briefly with PBS and cut into smaller pieces containing fungiform, foliate, and circumvallate papillae. The tissue pieces were immediately embedded in Tissue-Tek mounting medium and frozen on dry ice. 10 μm-thick cryosections containing taste buds were collected for immunostaining using various antibodies.

**Double immunostaining of Ki67 and KCNQ1**
Frozen tissue sections were fixed in cold acetone for 30 sec. Sections were air dried and washed three times with PBS containing 0.3% Triton X-100. After incubation at room temperature for 2 h with a blocking buffer (3% bovine serum albumin, 0.3% Triton X-100, 2% goat serum, and 0.1% sodium azide in PBS), the sections were further incubated with a rabbit polyclonal anti-KCNQ1 antibody (Millipore) at 4°C overnight. After washing with PBS/0.3% Triton X-100, a Cyanine 3 (Cy3)-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch) was added to the sections and incubated for 60 min. A mouse monoclonal anti-Ki67 antibody (BD Biosciences) was labeled with Alexa 488 Zenon Mouse IgG Labelling Kit (Invitrogen) following the manufacturer’s protocol. The labeled antibody was added to the sections and incubated at room temperature for 2 h. Sections were washed and mounted with Vectashield. Images were taken using a Leica confocal microscope. Ki67-labeled cells surrounding a taste bud (defined by KCNQ1 staining) in the circumvallate epithelium were counted. The average number of Ki67-labeled cells per taste bud profile was calculated.

**Immunostaining of cleaved Caspase-3 (Casp-3) and cleaved Caspase-6 (Casp-6)**
Frozen tissue sections were fixed in 4% paraformaldehyde (PFA)/PBS solution at room temperature for 15 min. Sections were washed three times with PBS/0.3% Triton X-100 and incubated at room temperature for 1-2 h with the blocking buffer. Rabbit polyclonal antibodies against cleaved Casp-3 or cleaved Casp-6 (Cell Signaling Technology) were added to the sections for an overnight incubation at 4°C. A Cy3-conjugated goat anti-rabbit secondary antibody was used. Images were taken using a Leica confocal microscope. Positively stained cells in the circumvallate epithelium were counted.

**Immunostaining of phospholipase C (PLC)-β2 and carbonic anhydrase IV (Car4)**
Frozen tissue sections were fixed in 4% paraformaldehyde (PFA)/PBS solution at room temperature for 15 min. Sections were washed three times with PBS/0.3% Triton X-100 and
incubated at room temperature for 1 h with a blocking buffer containing horse serum. A rabbit polyclonal antibody against PLC-β2 (Santa Cruz Biotechnology) or a goat polyclonal antibody against Car4 (R&D Systems) was added to the sections for an overnight incubation at 4°C. Dylight-488-labeled donkey anti-rabbit or anti-goat secondary antibodies (Jackson ImmunoResearch) were used. Images were taken using a Leica confocal microscope. Positive cells in taste buds of circumvallate sections were counted.

**Data analysis**
T-tests were performed in Excel to compare various cell counts from treatment groups with cell counts from the control group. A p value less than 0.05 is considered statistically significant.

**Results:**

*Chemotherapy drugs reduce the number of Ki67-positive progenitor cells in the taste epithelium*
To investigate the effect of chemotherapy drugs on taste progenitor cells, we performed double immunostaining using antibodies against Ki67 and KCNQ1. Ki67 is a cell proliferation marker expressed in all active stages of the cell cycle. KCNQ1, a voltage-gated potassium channel protein, is a taste cell marker expressed in all subtypes of taste bud cells. Ki67-positive cells in the basal region surrounding taste buds have been proposed as the taste progenitor cells. This cell population gives rise to both perigemmal epithelial cells and taste bud cells. All three chemotherapy drugs, 5-FU, cisplatin, and PTX, significantly decreased the number of Ki67-positive cells surrounding circumvallate taste buds (Figure 1). On day 3, 48 h after a single injection of the drugs, the number of Ki67-positive cells was reduced to 28%, 57%, and 64% of the control level by 5-FU, cisplatin, and PTX, respectively (Figure 1A and 1C left panel). On day 10, after repeated injections of the drugs, the number of Ki67-positive cells was reduced to 11%, 43%, and 44% of the control level by 5-FU, cisplatin, and PTX, respectively (Figure 1B and 1C right panel).

The three chemotherapy drugs belong to different categories of anticancer drugs. At the dose we used (5-FU, 150 mg/kg; cisplatin, 7.5 mg/kg; PTX, 115 mg/kg per injection), 5-FU had the strongest effect on progenitor cells in the circumvallate epithelium. In addition, based on KCNQ1 immunostaining, some circumvallate taste buds from 5-FU treated mice formed aberrant aggregates and exhibited decreased level of KCNQ1 immunostaining (Figure 1B). Together, these results show that chemotherapy drugs can strongly affect the taste progenitor cell population and attenuate cell proliferation in the taste epithelium.

*Chemotherapy drugs induce apoptosis in the taste epithelium*
To investigate whether chemotherapy drugs induce cell death in the taste tissue, we performed immunostaining using antibodies specific to cleaved Casp-3 or Casp-6. Casp-3 and Casp-6 are both executioner caspases which, when activated, carryout proteolytic cleavage of many proteins critical for cell survival. Activation of Casp-3 and Casp-6 requires cleavage of their inactive zymogens into smaller fragments. The antibodies we used are specific for the active forms of Casp-3 and Casp-6 which are markers for apoptotic cell death.

In circumvallate sections of PBS controls, few cells were positive for cleaved Casp-3 or Casp-6 (Figure 2, PBS panels), indicating a low level of apoptosis in the normal tissue turnover process. 5-FU and PTX treatments induced a significant increase in the number of cleaved Casp-3-
positive cells in the circumvallate papillae (Figure 2A, 2B, and 2D left panel). Some of these cleaved Casp-3-positive cells were located in taste buds and others were in perigemmal regions. On the other hand, cisplatin did not significantly increase the number of cleaved Casp-3-positive cells, but markedly increased the number of cleaved Casp-6-positive cells in circumvallate papillae (Figure 2, Cisplatin panels), suggesting that cisplatin induces apoptosis in the taste tissue through a Casp-6-dependent pathway. Together, these results show that chemotherapy drugs can induce apoptotic cell death in taste buds and their surrounding epithelium.

**Chemotherapy drugs decrease the number of taste receptor cells in taste buds**

To investigate whether chemotherapy drugs affect taste receptor cells, we carried out immunostaining using antibodies against PLC-β2 and Car4. PLC-β2 is a type II taste cell marker which is expressed in sweet, bitter, and umami taste receptor cells. Car4 is a marker for type III taste cells which include sour taste receptor cells. As shown in Figure 3, 5-FU, cisplatin and PTX all reduced the number of PLC-β2-positive taste receptor cells, while only PTX slightly decreased Car4-positive cells. These results suggest that chemotherapy drugs may preferentially affect type II taste receptor cells.

**Conclusion:**

Our results show that chemotherapy drugs, 5-FU, cisplatin, and PTX, reduce the number of progenitor cells in taste papillae and therefore attenuate cell renewal in the taste tissue. These drugs also induce apoptosis in taste buds and in perigemmal epithelium. Consistently, mice injected with the drugs show a reduction of type II taste receptor cells in taste buds.
Figure 1. 5-FU, cisplatin, and PTX reduce the number of Ki67-positive progenitor cells in the taste epithelium. Double immunostaining of Ki67 (green) and KCNQ1 (red) of circumvallate sections from control mice (PBS) and mice injected with 5-FU, cisplatin, or PTX. 

A. Tissues were collected on day 3 after a single injection of PBS or drugs. 

B. Tissues were collected on day 10 after five injections of PBS or drugs. 

C. Quantification of Ki67-positive cells in the circumvallate epithelium collected on day 3 (left panel) or day 10 (right panel) of the experiment. * p<0.05; ** p<0.001.
Figure 2. 5-FU, cisplatin, and PTX induce apoptosis in the circumvallate epithelium.  

A, B. Immunostaining of cleaved Casp-3 of circumvallate papillae collected at 24 h (A) or on day 10 (B) of the experiment. Arrows indicate positive cells. 

C. Immunostaining of cleaved Casp-6 of circumvallate papillae collected at 24 h after injection. 

D. Quantification of cleaved Casp-3 or Casp-6-positive cells in the circumvallate epithelium of control (PBS) and chemotherapy drug-treated mice at 24 h after injection. * p<0.05; ** p<0.001.
Figure 3. Quantitative analysis of PLC-β2 and Car4-positive cells in circumvallate taste buds collected on day 10 of the experiment. See the Experimental Procedures section for details.