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Co-Localization of Basal and Proliferative Cells in the Murine Main Olfactory Epithelium and Vomeronasal Organ after Injury with Cyclophosphamide

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CO-LOCALIZATION OF BASAL AND PROLIFERATIVE CELLS IN THE
MURINE MAIN OLFACTORY EPITHELIUM AND VOMERONASAL ORGAN
AFTER INJURY WITH CYCLOPHOSPHAMIDE

A Thesis Presented

by

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to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Biology

January, 2017

Defense Date: September 12, 2016
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ABSTRACT

In humans, advanced malignancies are often targeted with broad-spectrum cytotoxic drugs that engender several detrimental side effects, in addition to their primary usage for eradicating cancerous cells. One of the lesser-researched of these effects, histological distortion of the olfactory system impedes a patient's ability to smell, perceive flavor, and ultimately may interfere with their nutritional intake and recovery from chemotherapy. Recent studies have indicated that cytotoxic drugs can damage gustatory epithelia immediately following administration (Mukherjee & Delay, 2011, 2013). We sought to observe the histological effects that cyclophosphamide (CYP), one of the oldest and most popular alkylating antineoplastic agents, may have on the murine main olfactory epithelium (MOE) and vomeronasal organ (VNO). We utilized two immunohistochemical antibodies to label cells in the olfactory epithelia: anti-Ki67, a marker strictly associated with cell proliferation; and, anti-Keratin 5, a marker for the cytoskeleton of horizontal basal cells. Twenty-eight C57BL/6 mice were administered a single intraperitoneal injection of CYP (75 mg/kg), while 20 control mice were administered saline, all at approximately seven weeks of age. Mice were euthanized at days one, two, six, 14, 30, and 45 post injection; subsequently, they were perfused with 4% paraformaldehyde, decalcified, cryoprotected, cryosectioned, and incubated with anti-Ki67 and anti-Keratin 5 antibodies, sequentially. Quantification results by fluorescent imaging of labeled sections revealed a significant decrease in the number of proliferative cells in the MOE and VNO of CYP-injected mice within the first 10 days post injection, followed by a compensatory period of increased cell proliferation through day 45 post injection, compared to saline-injected mice. Co-localization of horizontal basal cells and proliferative cells in the MOE and VNO of CYP-injected mice was significantly amplified at approximately 14 and 45 days post injection, respectively, compared to saline-injected mice. Our results suggest that administration of CYP can rapidly depress the populations of proliferative cells in the murine MOE and VNO; consequently, horizontal basal cells may afford restoration of the proliferative cell populations in the murine MOE and VNO, 14 to 45 days post injection, respectively.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to the entire University of Vermont community for enabling the achievement of my Master of Science, following the completion of my undergraduate degrees. As I have gained knowledge, insight, and experience, I am grateful to have had the support of the University of Vermont community for five years.

I would like to thank the members of my Studies Committee – Dr. Eugene R. Delay and Dr. Gary M. Mawe – for their thoughtful guidance, dependability, and expediency as I completed my degree in one year. I appreciate the gracious support of Alan Howard in assisting me with all of the statistical quantifications. I am grateful for the encouragement provided by every member of the Department of Biology during my tenure at the University of Vermont, especially my friendship with Mark Biercevicz. I also would like to acknowledge all of my exceptional professors in the Department of Biology, who were the bedrock of my science education: Dr. Alison Brody, Dr. Alicia Ebert, Dr. John Mitchell, Dr. Lori Stevens, and Dr. Amanda Yonan.

I will always value the unyielding support I received from members of the Delay Laboratories who facilitated my maturation as a scientist and lifelong

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1.1.   Chemotherapy, Cancer, and Olfactory Distortions in Human Patients

1.1.1. Chemotherapy and Cancer: An Imperfect Union

Chemotherapy is defined as the use of chemical agents in the treatment or control of disease, most notably connoted as the use of drugs in the treatment of cancer. Cancer is recognized as the abnormal and excessive growth of cells originating in one region of the body, with the potential to metastasize to several other or all regions (Aronovitch et al., 1960; Dick & Phillips, 1961; Bagley et al., 1973; Moore, 1991; Colvin, 1999; Pinto et al., 2009). Since cancer fundamentally involves the unregulated propagation of cells, several chemotherapy treatments exist to extinguish proliferative cells within the body. Major classes of chemotherapeutics drugs – designed to directly inhibit cell division and function or obstruct the synthesis of deoxyribonucleic acid (DNA) – include alkylating agents, antimetabolites, antitumor antibiotics, plant alkaloids, and topoisomerase inhibitors (Cunningham et al., 1988; Colvin, 1999; Malet-Martino et al., 1999). However, these pharmacologic agents are not a panacea. Though targeted treatments of specific cancer cell types have been developed, the majority of common chemotherapeutics are non-specific intracellular poisons which disturb any cell undergoing mitosis. Moreover, chemotherapeutics are typically administered systemically, in that they are

introduced into the vascular system so that they may embroil cancer at any anatomic location within the body. While this systemic approach to chemotherapy is often effective in treating widely-metastasized cancers, it also engenders adverse effects relating to the extermination of normally-proliferative cells, such as cells of the bone marrow, digestive tract, and sensory epithelia (Bagley et al., 1973; Colvin, 1999; Ludeman, 1999). Therefore, the treatment of cancer is a two-fold conundrum – not only must the propagation of cancerous cells be thwarted, adverse effects which arise from the systemic use of cytotoxic drugs must also be considered.

1.1.2. Chemosensory Alterations Resulting from Cytotoxicity

When a patient undergoes chemotherapy to treat cancer, there are generally two adverse effects that are of paramount concern to clinicians, patients, and researchers: immunosuppression, which can compromise an ill patient's capacity to mediate secondary infections, and digestive distortions, which can encumber a patient's ability to digest proper nutrients and sustain the energy needed to recover (Dick & Phillips, 1961; Bagley et al., 1973; Colvin, 1999; Malet-Martino et al., 1999). A lesser-researched, yet increasingly noteworthy, side effect involves distortions to patients' chemosensory systems, namely the gustatory and olfactory systems. There are three classifications of chemosensory alteration that characterize this side effect: hypogeusia and hyposmia, or reduced sensitivity to taste and smell, respectively; parageusia

and parosmia, or distorted perception of taste and smell, respectively; and ageusia and anosmia, or expired perception of taste and smell, respectively. A multitude of clinical research has demonstrated that these alterations in chemotherapy patients – which can persist for years after chemotherapy administration – can reduce food intake and quell a patient's appetite. One such study by Hutton and collaborators at the University of Alberta described that nearly 90% of chemotherapy patients reported some level of chemosensory deficit, which decreased their energy consumption, increased their weight loss, and decreased their quality of life scores compared to patients who did not experience chemosensory distortion (2007). Such effects often result in malnutrition, a reduced quality of life, and delayed recovery from illness (Comeau et al., 2001; Bernhardson et al., 2007; Hutton et al., 2007; Bernhardson et al., 2009; Halyard, 2009). Thus, understanding how chemotherapeutic agents affect the chemosensory systems is increasingly imperative for improving patient outcomes and refining the use of chemotherapy.

Until recently, most of the information about chemosensory alterations resulting from chemotherapy was established by clinical research. That precedent was improved upon when a pioneering 2011 study, published by Mukherjee and Delay, investigated the histological basis for gustatory alterations reported by patients undergoing chemotherapy. After injecting naïve mice with a cytotoxic chemotherapeutic (e.g. cyclophosphamide),

Mukherjee and Delay observed a significant decrease in the number of taste sensory cells up to two weeks after the administration of chemotherapy. They were the first to demonstrate that cyclophosphamide, as a cytotoxic chemotherapeutic, has significant and identifiable effects on the histology of the murine gustatory system. Accordingly, Mukherjee and Delay suggested that gustatory alterations experienced by chemotherapy patients likely are due, in part, to histological disturbances following the administration of a cytotoxic drug (2011).

Given the increasing impetus to understand how chemotherapy achieves chemosensory alterations, and the novel results reported by Mukherjee and Delay, the ensuing histological study was undertaken to observe the effects that administration of a cytotoxic drug (e.g. cyclophosphamide) has on basal and proliferative cells in the murine olfactory system.

1.2. Cyclophosphamide

1.2.1. History, Synthesis, and Mechanism of Action

Cyclophosphamide is one of the oldest and most efficacious cytotoxic drugs used to treat advanced malignancies. Indeed, its prevalence and clinical effectiveness were the primary determinants in the decision to utilize the drug in the succeeding study. Cyclophosphamide (CYP) is an alkylating chemotherapeutic, used clinically since it became the eighth cytotoxic drug

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approved by the United States Food and Drug Administration in 1959 (Aronovitch et al., 1960; Foye et al., 1960; Dick & Phillips, 1961; Bagley et al., 1973; Colvin, 1999; Malet-Martino, 1999). The development of CYP was borne out of the First World War, during which Germany exploited sulfur mustard as an operative cytotoxic agent in combat. Sulfur mustard readily eliminates a chloride ion by intramolecular nucleophilic substitution to form a reactive cyclic sulfonium ion, which causes irreversible alkylation of the guanine nucleotide in DNA strands. Such DNA linkages result in arrested cell proliferation and probable apoptosis of cells that are affected (Cunningham et al., 1988; Colvin, 1999; Pinto et al., 2009). Cleverly, the cytotoxicity of sulfur mustard was recognized as a powerful tool that could potentially target the unregulated proliferation of cells in the body, namely cancer. However, the obvious impediment to using sulfur mustard as a chemotherapeutic was its constitutively-active mechanism of action, which contraindicated the agent for systemic use within the body (Foye et al., 1960; Moore, 1991). Therefore, the resultant aim of rational drug design was to develop a pharmacologic agent with the cytotoxic accuracy of a mustard, but targeted selectivity for cancerous cells.

This objective was partially accomplished with the synthesis of CYP in 1958. Researchers intended to create a cytotoxic chemotherapeutic based on mustard that could be administered as a prodrug (i.e. pharmacologically-inactive precursor) to be converted within the body into an active form, chiefly in cancer

cells. To achieve this, researchers substituted the methyl group of nitrogen

mustard with an oxazaphosphorine ring, creating the novel structure of CYP

(Aronovitch et al. 1960; Foye et al., 1960; Dick & Phillips, 1961; Bagley et al.,

1973). When administered into the human body, CYP is primarily converted by

the hepatic cytochrome P450 oxidase enzymes into tautomers, 4-

hydroxycyclophosphamide and aldophosphamide, by hydroxylation of the

oxazaphosphorine ring. A significant portion of the aldophosphamide is

oxidized to carboxycyclophosphamide, an inert metabolite, by the enzyme

aldehyde dehydrogenase (ALDH). Conversely, the remaining aldophosphamide

freely diffuses into cells, where it is decomposed into two remnants:

phosphoramide mustard and acrolein (Figure 1). After crossing into cellular

nuclei, phosphoramide mustard is reacts to remove a chloride ion to form a

cyclic ethyleneiminium cation. This highly unstable cation is attacked by several

nucleophiles, most notably by DNA guanine residues. This reaction with DNA

releases the nitrogen of the alkylating agent and makes it available to react with

an additional side chain forming a second covalent linkage with another

nucleophile. Consequently, the mustard interferes with DNA replication by

forming intrastrand and interstrand DNA crosslinks at the guanine N-7 position

(Juma et al., 1979; Juma et al., 1980; Cunningham et al., 1988; Colvin, 1999;

Ludeman, 1999; Malet-Martino et al., 1999; Pinto et al., 2009). The most common

outcome of these irreversible linkages is cellular apoptosis, which is effective in

disrupting cancer cells that are proliferating uncontrollably. Nevertheless, a critical downside to using CYP as a chemotherapeutic is its non-selective action on all proliferating cells within the body, especially those that are accessible by the vascular system and receive high levels of freely-diffusible aldophosphamide (Dick & Phillips, 1961; Bagley et al. 1973; Moore, 1991; Colvin, 1999; Malet-Martino et al., 1999; Bernhardson et al., 2007). This non-selectivity instigates a multitude of adverse effects, including disturbances to the olfactory epithelium, which are the focus of the ensuing study.

1.2.2. Clinical Use and Side-Effects

Although CYP can cause exceptional damage within non-target tissues, its efficacy in ablating cancerous cells indicates it for use in several advanced malignancies. Primarily, CYP is used as the first line of treatment in leukemia, lymphoma, breast cancer, and ovarian cancer (Bagley et al., 1973; Juma et al., 1979; Juma et al., 1980; Cunningham et al., 1988; Moore, 1991; Ludeman, 1999; Malet-Martino et al., 1999; Pinto et al., 2009). However, because of its toxicity, CYP is typically used for a short duration to control cancerous cell proliferation before being replaced with a less toxic chemotherapeutic, or alternatively, it may be used in a continuous low-dose as an adjuvant to less harmful chemotherapeutics such as epirubicin or methotrexate (Colvin, 1999; Ludeman, 1999).

One advantage of utilizing CYP is that since it is a prodrug, tissues that prevent the production of its active metabolite, phosphoramide mustard, are largely spared of damage. Indeed, regions like epithelial basal cells, the liver, and the digestive tract that retain high levels of ALDH often do not accumulate a significant quantity of freely-diffusible aldophosphamide, and thus, do not experience cellular damage. Even so, CYP ravages proliferative cells in any region of the body that permits freely-diffusible aldophosphamide to be decomposed in substantial quantities. As such, CYP can commonly cause severe cases of bone marrow suppression, neutropenia, leukopenia, thrombocytopenia, anemia, hemorrhagic necrotic perimyocarditis, cardiac arrhythmia, pericardial effusion, amenorrhea, hemorrhagic cystitis, and a host of secondary cancers (Cunningham et al., 1988; Colvin, 1999; Ludeman, 1999; Malet-Martino et al., 1999; Pinto et al., 2009).

There are several studies of chief interest to the succeeding study. These include several clinical cases and the histological assay by Mukherjee and Delay which demonstrate that CYP alters chemoreception (Comeau et al., 2001; Bernhardson et al., 2007; Bernhardson et al., 2009; Halyard, 2009; Mukherjee & Delay, 2011, 2013). To further elucidate how CYP may affect a patient's chemosensory anatomy, the olfactory epithelia were chosen as the biological system of experimentation in ensuing study.

1.3. Murine Olfactory System

Since chemoreception is comprises both gustatory and olfactory inputs into the mammalian nervous system, it was reasonable to investigate the effects of CYP on the murine olfactory system following the results exhibited by Mukherjee and Delay in the murine gustatory system. The murine olfactory system is responsible for the neurological signal transduction of odorant molecules that bind to specific sites on olfactory receptors, namely olfactory sensory neurons (OSNs); these neurons, which are characterized as Golgi type I, retain long axons that form glutamatergic synapses with mitral cells in the olfactory bulb (Naessen, 1970; Schwartz Levey et al., 1991; Weiler & Farbman, 1997). Many vertebrates, including most mammals and reptiles but not humans, have two distinct and segregated olfactory systems: a main olfactory system, which detects volatile stimuli, and an accessory olfactory system, which detects fluid-phase stimuli (Schwob, 2002; Vedin et al., 2009; Holbrook et al., 2011). Mice have four segregated regions within their peripheral olfactory system: the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the septal organ, and the Grueneberg ganglion (Holbrook et al., 1995; Weiler, 2005). As the primary peripheral olfactory epithelia within mice, the MOE and VNO were selected as the two regions of interest for the succeeding study (Figure 2).

1.3.1. Main Olfactory Epithelium (MOE) and Vomeronasal Organ (VNO)

As the workhorses of the murine olfactory system, the MOE and VNO retain similar anatomies and functions. Both regions are pseudostratified columnar epithelia, that basally to apically retain: basal cells, immature and mature sensory neurons, and sustentacular cells, which provide physical and metabolic support within the epithelia (Figure 3). Additionally, the sensory neurons of both the MOE and VNO are bipolar, as odorant detection is facilitated by elongated dendrites, and cilia (MOE) and microvilli (VNO) that extend apically from the epithelia (Barber & Raisman, 1978; Firestein, 2001; Brann & Firestein, 2010).

While fundamentally similar in light of the histological focus of this study, the MOE and the VNO differ in several aspects, including: odorant accessibility, vascularization, signaling cascades and biological purpose. The MOE is a large, diffusely-vascularized epithelium in the posterior murine nasal cavity, which covers cartilaginous turbinates that permit abundant access to stimuli. In contrast, the densely-vascularized epithelium of the VNO lines a paired bony capsule at the base of the anterior nasal cavity, which is protected by a vascular pump that governs access to stimuli (Calof & Chikaraishi, 1989; Brann & Firestein, 2014). The MOE is organized by a basal sensory zone and an apical sustentacular zone, while the VNO also retains marginal zones that encapsulate the extremities of its crescent shape (Figure 4). Sensory neurons within the MOE facilitate signal transduction by increases in intracellular

cAMP, whereas sensory neurons within the VNO transduce signals via the phospholipase C pathway (Matsunami & Buck, 1997; Ryba & Tirindelli, 1997). Lastly, the biological purposes of the MOE and VNO have been hotly debated for decades: in general, it is thought that while the MOE detects a myriad of volatile odorants, the VNO specializes in the detection of fluid-phase stimuli, often in direct mediation of sexual and social behaviors (Mombaerts, 2006).

1.3.2. Basal Cells: Postnatal Neurogenesis of the Olfactory Epithelia

In the murine olfactory system, the paramount concern regarding CYP-induced cytotoxicity is disruption of the postnatal proliferation of cells. Indeed, both the MOE and VNO undergo continual postnatal neurogenesis in the generation of sensory neurons (Graziadei & Monti-Graziadei, 1979; Costanzo, 1991; Huard et al., 1998; Legrier et al., 2001; Schwob, 2002). Both regions of the olfactory epithelia renew cells throughout life, although neurogenesis in the MOE has been shown to be twice as rapid as neurogenesis in the VNO. It is also important to note that while postnatal neurogenesis is continuous, the rate of neurogenesis in both the MOE and VNO declines with age (Weiler & Farbman, 1997, 1998; Brann & Firestein, 2010). In the MOE, neurogenesis during the first postnatal year contributes predominantly to anatomical growth, and later transitions to the renewal of sensory neurons during the lifetime of an animal. In the VNO, neurogenesis in the marginal zones is primarily responsible for growth, while regeneration in the sensory zone is

responsible for the turnover of sensory neurons over time (Barber & Raisman, 1978; Graziadei & Monti-Graziadei, 1979; Martinez-Marcos et al., 2004; Weiler & Banali, 2005; Brann & Firestein 2010). In an adult mouse, the lifespan of a sensory neuron ranges from 30 to 90 days, increasing proportionately to the age of the animal (Graziadei & Monti-Graziadei, 1979; Huard & Schwob, 1997).

Two populations of basal progenitor cells appear to be the founts of renewal for both natural and injury-induced neurogenesis in the MOE and VNO: horizontal basal cells (HBCs) and globose basal cells (GBCs) (Schwartz Levey et al., 1991; Caggiano et al., 1994; Holbrook et al., 1995; Farbman, 1997; Beites et al., 2005; Leung et al., 2007; Iwai et al., 2008; Tucker et al., 2010; Jang et al., 2014). HBCs are mostly quiescent and line the basal lamina (e.g. layer of extracellular matrix secreted by epithelial cells, on which epithelia rests) of olfactory epithelia, appearing histologically as densely-packed rows of narrow cells (Holbrook et al., 1995; Carter et al., 2004; Iwai et al., 2008; Joiner et al., 2015). GBCs, which rest apically to HBCs, have a globular appearance and maintain elevated rates of mitosis compared to their HBC counterparts (Caggiano et al., 2005; Huard & Schwob, 1995). It is thought that both HBCs and GBCs proliferate by undergoing asymmetric cell division, though the current knowledge of their proliferative mechanisms is deficient.

The exact differences in histology and function between these classes of basal cells are unclear, based on several conflicting studies. Generally, evidence

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does suggest that GBCs bear the majority of naturally-occurring cell renewal, while HBCs tend to be most active in neurogenesis during discrete instances of injury to the epithelia (Holbrook et al., 1995; Huard & Schwob, 1995; Carter et al., 2004; Leung et al., 2007). What confounds this controversy even further, from a histological standpoint, is that no widely-accepted immunohistochemical marker for GBCs exists. HBCs are immunoreactive to cytokeratins, but observations of their populations only reveal half of the story of neurogenesis in the MOE and VNO (Holbrook et al., 1995; Goldstein & Schwob, 1996; Joiner et al., 2015). To advance the knowledge of regeneration in the murine olfactory system, future studies must delineate, with certainty, the function and fate of HBCs and GBCs during physiological and injury-induced neurogenesis.
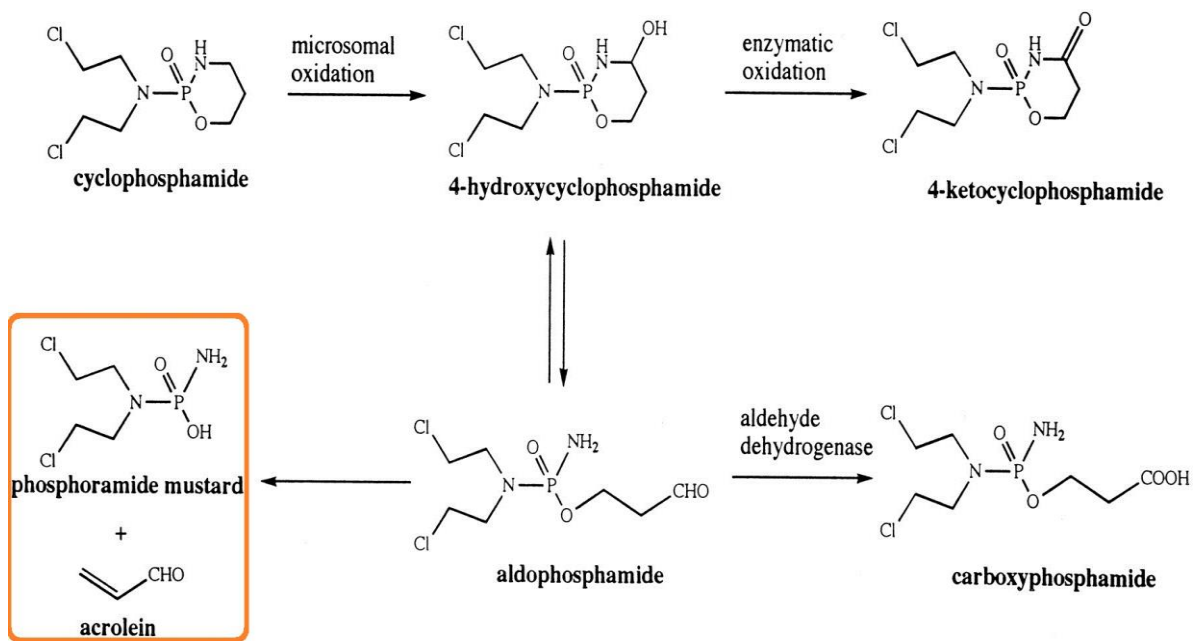
Figure 1: Metabolism of CYP after Pharmacologic Conversion by Hepatic Cytochrome P450 Enzymes. CYP is converted into tautomers, 4-hydroxycyclophosphamide and aldophosphamide. Aldophosphamide is oxidized to carboxycyclophosphamide by aldehyde dehydrogenase (ALDH) or decomposed in cells into two remnants: phosphoramide mustard and acrolein. In cellular nuclei, phosphoramide mustard is subject to elimination of a chloride to form a cyclic ethyleneiminium cation, which is attacked by several nucleophiles, including DNA guanine residues. The reaction with DNA releases the nitrogen of the alkylating agent and forms a second covalent linkage with another nucleophile. Ultimately, the mustard interferes with DNA replication by forming intrastrand and interstrand DNA crosslinks at the guanine N-7 position.
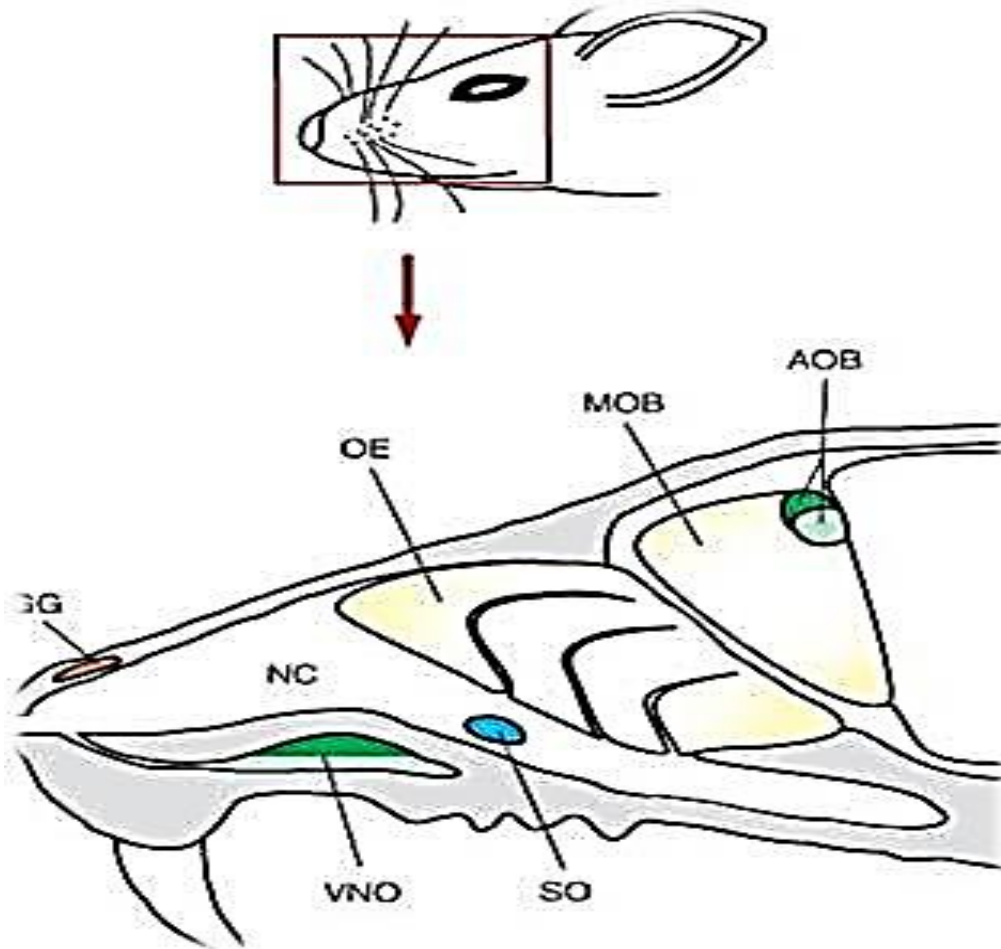
Figure 2: Mid-Sagittal Section of the Murine Snout. The murine olfactory

system comprises (anterior to posterior): the Grueneberg Ganglion (GG), the

vomeronasal organ (VNO), the main olfactory epithelium (MOE or OE), and the septal

organ (SO), which are exposed to the nasal cavity (NC).
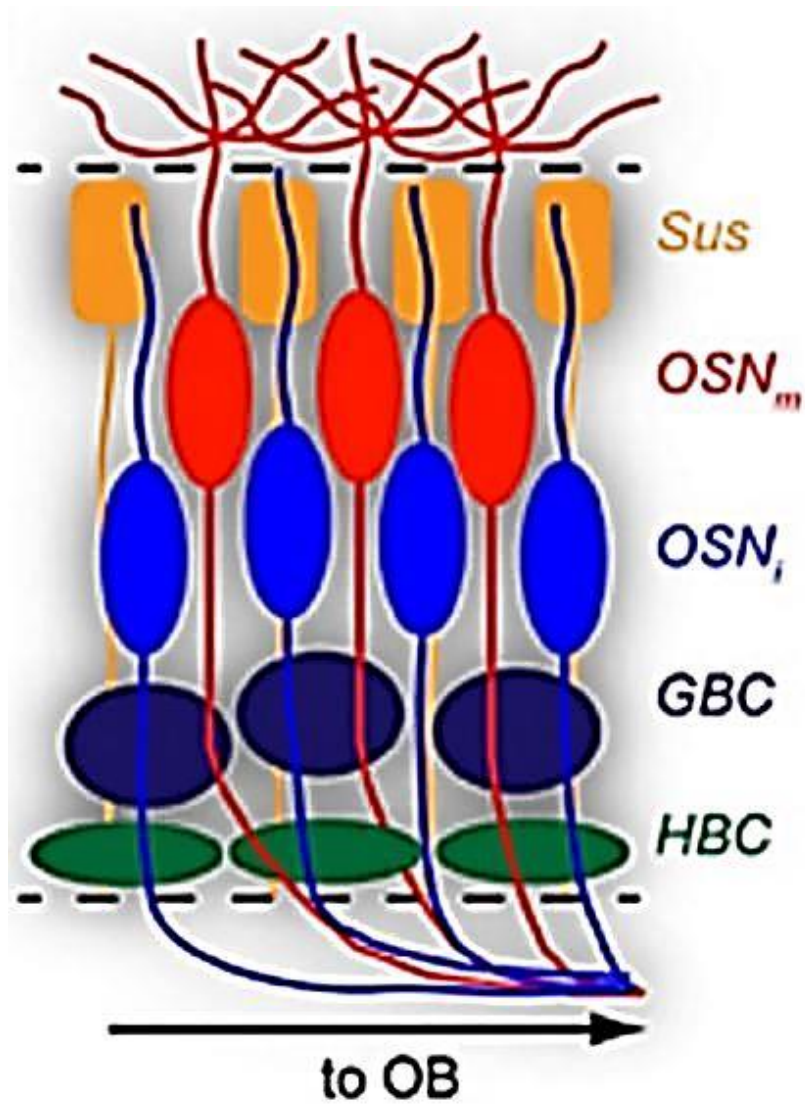
Figure 3: Cell Types of the Murine Olfactory Epithelia. The murine olfactory epithelia are composed of five primary cell types (basally to apically): horizontal basal cells (HBC), globose basal cells (GBC), immature olfactory sensory neurons (OSN$_i$), mature olfactory sensory neurons (OSN$_m$), and sustentacular cells (Sus). (Brann & Firestein, 2014)
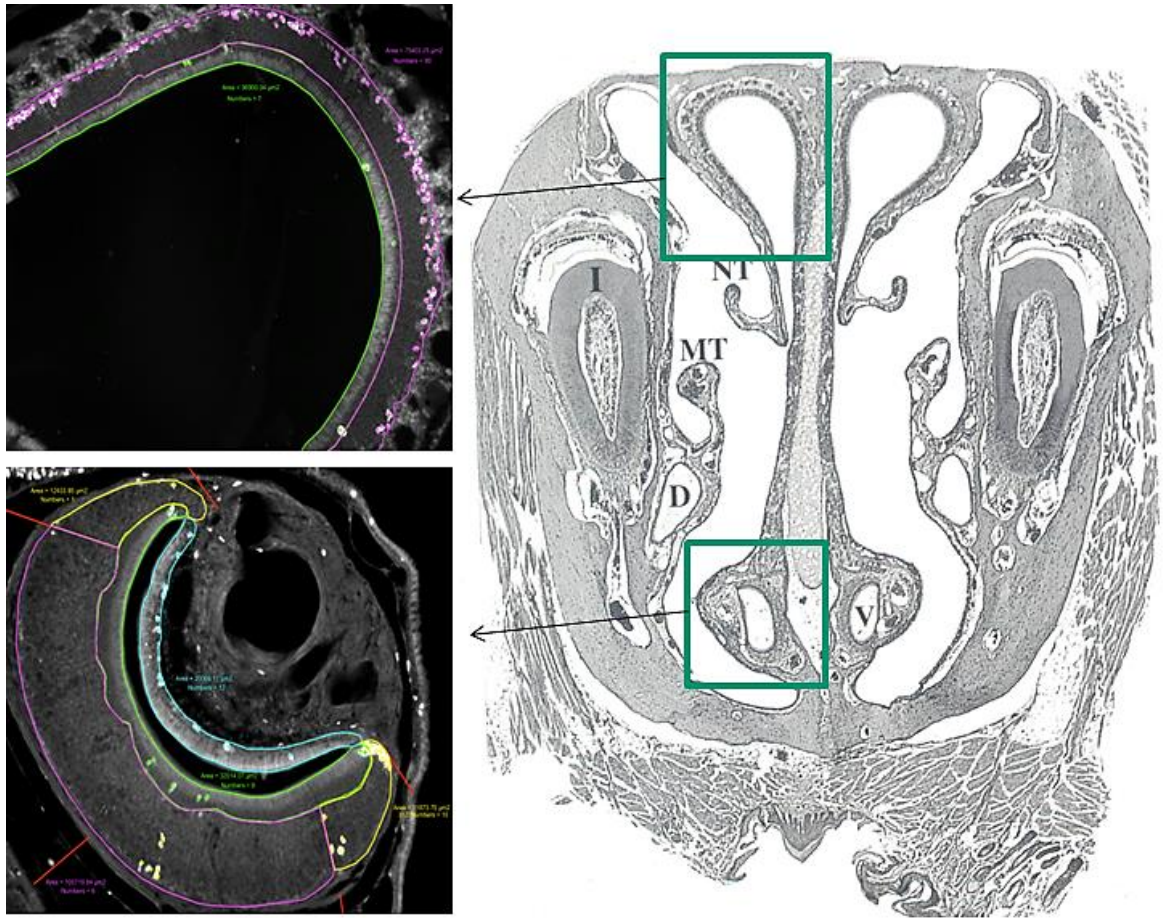
Figure 4: Coronal Section of the Murine Snout, with Focus on the Main Olfactory Epithelium (Top Left) and the Vomeronasal Organ (Bottom Left). The primary organs of the murine olfactory system are the Main Olfactory Epithelium (MOE) and the Vomeronasal Organ (VNO). The MOE is diffusely-vascularized and located in the posterior nasal cavity whereas the VNO is densely-vascularized and lines a paired bony capsule in the anterior nasal cavity. The MOE (top left) is organized by a basal sensory zone (purple) and an apical sustentacular zone (green), while the VNO (bottom left) also retains marginal zones (yellow) that encapsulate the extremities of its crescent shape.

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CHAPTER 2: CO-LOCALIZATION OF BASAL AND PROLIFERATIVE

CELLS IN THE MURINE MAIN OLFACTORY EPITHELIUM AND

VOMERONASAL ORGAN AFTER INJURY WITH CYCLOPHOSPHAMIDE

2.1. Introduction

Chemotherapy is defined as the use of chemical agents in the treatment

or control of disease, most notably connoted as the use of drugs in the

treatment of cancer. Cancer is recognized as the abnormal and excessive

growth of cells originating in one region of the body, with the potential to

metastasize to several other or all regions (Aronovitch et al., 1960; Dick &

Phillips, 1961; Bagley et al., 1973; Moore, 1991; Colvin, 1999; Pinto et al., 2009).

Major classes of chemotherapeutics drugs – designed to directly inhibit cell

division and function or obstruct the synthesis of deoxyribonucleic acid (DNA)

– include alkylating agents, antimetabolites, antitumor antibiotics, plant

alkaloids, and topoisomerase inhibitors (Cunningham et al., 1988; Colvin, 1999;

Malet-Martino et al., 1999). Though targeted treatments of specific cancer cell

types have been developed, the majority of common chemotherapeutics are

non-specific intracellular poisons which disturb any cell undergoing mitosis.

Moreover, chemotherapeutics are typically administered systemically, in that

they are introduced into the vascular system so that they may embroil cancer at

any anatomic location within the body. While this systemic approach to

chemotherapy is often effective in treating widely metastasized cancers, it also engenders adverse effects relating to the extermination of normally-proliferative cells, such as cells of the bone marrow, digestive tract, and sensory epithelia (Bagley et al., 1973; Colvin, 1999; Ludeman, 1999).

A lesser-researched side effect involves distortions to patients' chemosensory systems, namely the gustatory and olfactory systems. A multitude of clinical research has demonstrated that chemosensory alterations in chemotherapy patients – which can persist for years after chemotherapy administration – can reduce food intake and quell a patient's appetite. Such effects often result in malnutrition, a reduced quality of life, and delayed recovery (Comeau et al., 2001; Bernhardson et al., 2007; Hutton et al., 2007; Bernhardson et al., 2009; Halyard, 2009). Until recently, most of the information about chemosensory alterations as a result of chemotherapy was established by clinical research. That precedent was improved upon when a pioneering 2011 study, published by Mukherjee & Delay, investigated the histological basis for gustatory alterations reported by patients undergoing chemotherapy. After injecting naïve mice with a cytotoxic chemotherapeutic (e.g. cyclophosphamide), Mukherjee & Delay observed a significant decrease in the number of taste sensory cells up to two weeks after the administration of chemotherapy. They were the first to demonstrate that cyclophosphamide, as a

cytotoxic chemotherapeutic, has significant and identifiable effects on the histology of the murine gustatory system.

Cyclophosphamide (CYP) is an alkylating chemotherapeutic, used clinically as a prodrug (Aronovitch et al., 1960; Foye et al., 1960; Dick & Phillips, 1961; Bagley et al., 1973; Colvin, 1999; Malet-Martino, 1999). When administered, CYP is primarily converted by the hepatic cytochrome P450 oxidase enzymes into tautomers, 4-hydroxycyclophosphamide and aldophosphamide. A remnant of aldophosphamide - phosphoramide mustard – is the active chemotherapeutic agent derived from CYP. After crossing into cellular nuclei, phosphoramide mustard reacts with DNA to form intrastrand and interstrand crosslinks at the guanine N-7 position (Juma et al., 1979; Juma et al., 1980; Cunningham et al., 1988; Colvin, 1999; Ludeman, 1999; Malet-Martino et al., 1999; Pinto et al., 2009). The most common outcome of these irreversible linkages is cellular apoptosis, which is effective in disrupting cancer cells that are proliferating uncontrollably. Nevertheless, a critical downside to using CYP as a chemotherapeutic is its non-selective action on all proliferating cells within the body (Dick & Phillips, 1961; Bagley et al. 1973; Moore, 1991; Colvin, 1999; Malet-Martino et al., 1999; Bernhardson et al., 2007). As such, CYP can commonly cause severe cases of bone marrow suppression, neutropenia, leukopenia, thrombocytopenia, anemia, hemorrhagic necrotic perimyocarditis, cardiac arrhythmia, pericardial effusion, amenorrhea,

hemorrhagic cystitis, and a host of secondary cancers (Cunningham et al., 1988; Colvin, 1999; Ludeman, 1999; Malet-Martino et al., 1999; Pinto et al., 2009). Of chief interest to the succeeding study are the effects of CYP on chemoreception (Comeau et al., 2001; Bernhardson et al., 2007; Bernhardson et al., 2009; Halyard, 2009; Mukherjee & Delay, 2011).

Since chemoreception is involves both gustatory and olfactory inputs into the mammalian nervous system, it was reasonable to investigate the effects of CYP on the murine olfactory system following the results exhibited by Mukherjee & Delay in the murine gustatory system. The murine olfactory system is responsible for the neurological signal transduction of odorant molecules that bind to specific sites on olfactory receptors, namely olfactory sensory neurons (OSNs) (Naessen, 1970; Schwartz Levey et al., 1991; Weiler & Farbman, 1997). Mice have four segregated regions within their peripheral olfactory system: the main olfactory epithelium (MOE), the vomeronasal organ (VNO), the septal organ, and the Grueneberg ganglion (Holbrook et al., 1995; Weiler, 2005). As the primary organs of the murine olfactory system, the MOE and VNO retain similar anatomies and functions. Both regions are pseudostratified columnar epithelia, that basally to apically retain: basal cells, immature and mature sensory neurons, and sustentacular cells, which provide physical and metabolic support within the epithelia (Firestein, 2001; Brann & Firestein, 2010).

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In the murine olfactory system, the paramount concern regarding CYP-induced cytotoxicity is disruption of the postnatal proliferation of cells. Indeed, both the MOE and VNO undergo continual postnatal neurogenesis in the generation of sensory neurons. (Graziadei & Monti-Graziadei, 1979; Costanzo, 1991; Huard et al., 1998; Legrier et al., 2001; Schwob, 2002). Both regions of the olfactory epithelia renew cells throughout life, although neurogenesis in the MOE has been shown to be twice as rapid as neurogenesis in the VNO. It is also important to note that while postnatal neurogenesis is continuous, the rate of neurogenesis in both the MOE and VNO declines with age (Weiler & Farbman, 1997, 1998; Brann & Firestein, 2010). In the MOE, neurogenesis during the first postnatal year contributes predominantly to anatomical growth, and later transitions to the renewal of sensory neurons during the lifetime of an animal. In the VNO, neurogenesis in the marginal zones is primarily responsible for growth, while regeneration in the sensory zone is responsible for the turnover of sensory neurons over time (Barber & Raisman, 1978; Graziadei & Monti-Graziadei, 1979; Martinez-Marcos et al., 2004; Weiler & Banali, 2005; Brann & Firestein 2010). In an adult mouse, the lifespan of a sensory neuron ranges from 30 to 90 days, increasing proportionately to the age of the animal (Graziadei & Monti-Graziadei, 1979; Huard & Schwob, 1997).

Two populations of basal progenitor cells appear to be the founts of renewal for both natural and injury-induced neurogenesis in the MOE and

VNO: horizontal basal cells (HBCs) and globose basal cells (GBCs) (Schwartz

Levey et al., 1991; Caggiano et al., 1994; Holbrook et al., 1995; Farbman, 1997;

Beites et al., 2005; Leung et al., 2007; Iwai et al., 2008; Tucker et al., 2010; Jang et

al., 2014). HBCs are mostly quiescent and line the basal lamina of olfactory

epithelia, appearing histologically as densely-packed rows of narrow cells

(Holbrook et al., 1995; Carter et al., 2004; Iwai et al., 2008; Joiner et al., 2015).

GBCs, which rest apically to HBCs, have a globular appearance and maintain

elevated rates of mitosis compared to their HBC counterparts (Caggiano et al.,

2005; Huard & Schwob, 1995). It is thought that both HBCs and GBCs

proliferate by undergoing asymmetric cell division, though the current

knowledge of their proliferative mechanisms is deficient. The exact differences

in histology and function between these classes of basal cells are unclear, based

on several conflicting studies. Generally, evidence does suggest that GBCs bear

the majority of naturally-occurring cell renewal, while HBCs tend to be most

active in neurogenesis during isolated instances of injury to the epithelia

(Holbrook et al., 1995; Huard & Schwob, 1995; Carter et al., 2004; Leung et al.,

2007). What confounds this controversy even further, from a histological

standpoint, is that no widely-accepted immunohistochemical marker for GBCs

exists. HBCs are immunoreactive to cytokeratins, but observations of their

populations only reveal half of the story of neurogenesis in the MOE and VNO

(Holbrook et al., 1995; Goldstein & Schwob, 1996; Joiner et al., 2015).

The central aim of this study was to gain insight – from a histological perspective – into how cytotoxic chemotherapies achieve chemosensory alterations. To investigate this aim, an immunohistochemical assay was undertaken to observe the quantitative effects that discrete administration of CYP has on populations of basal and proliferative cells in the murine olfactory system. Specifically, we sought to observe the number of proliferative cells, and the number of co-localizations of basal and proliferative cells, over time in the MOE and VNO after a single intraperitoneal injection of CYP. These objectives were accomplished by staining the HBCs (anti-Keratin 5) and proliferative cells (anti-Ki67) within the MOE and VNO over a time-course of 45 days.

To focus our experimental aims, we tested two hypotheses. First, since the olfactory epithelia demonstrate continuous neurogenesis over the lifetime of mice, we hypothesized that a single injection of CYP would temporarily decrease the number of proliferative cells in the MOE and VNO, and be followed by a previously-undetermined period of neurogenic recovery once the initial insult from CYP had subsided. Second, since HBCs are thought to be mostly quiescent cells and would accordingly be spared by CYP (which targets mitotic cells, like the GBCs), we hypothesized that the number of co-localizations of HBCs and proliferative cells would increase after a single injection of CYP. We predicted that the HBCs would be activated for proliferation to ameliorate the injury induced by CYP, and that the greatest

number of co-localizations would occur 20 to 40 days after the injection of CYP, mirroring the normal physiological turnover of sensory neurons in the murine olfactory system.

2.2. Methods

2.2.1. Chemical Reagents

CYP (cyclophosphamide monohydrate, 97%) was obtained from Acros Organics. All water used was acquired from a Milli-Q® Ultrapure Water Solutions Type 1 filtration system. All dilutions of physiological solutions were achieved with 0.1 M phosphate buffer, unless otherwise stated.

2.2.2. Procurement, Injection, and Euthanasia

Forty-eight naive C57BL/6 male mice were obtained (Jackson Laboratories), and acclimated to institutional housing for one week. Mice were housed in groupings of four individuals, and had ad libitum access to water and Purina Mouse Chow. The colony of mice was maintained on a 12 hour light-dark cycle, whereby continuous light was provided from 7:00 AM until 7:00 PM. All mice weighed between 19 and 25 grams at the start of experimentation.

At seven weeks of age, all mice were injected intraperitoneally: 28 with a 75 milligram per kilogram dose of CYP, and 20 with an equal volume of saline. Mice were euthanized at days one (CYP n=6, saline n=4), two (CYP n=6, saline

n=4), six (CYP n=4, saline n=4), 14 (CYP n=4, saline n=4), 30 (CYP n=4, saline

n=2), and 45 (CYP n=4, saline n=2) post injection of CYP or saline. Each mouse

was administered Beuthanasia®-D, immediately followed by transcardial

perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer. Following

perfusion, snouts were dissected directly anterior to the olfactory bulb, and

placed in solution with 4% paraformaldehyde in 0.1 M phosphate buffer.

2.2.3. Decalcification, Cryoprotection, and Cryosectioning

To withstand cryosectioning for immunostaining, all snouts underwent

decalcification and cryoprotection. Following a twenty-four hour immersion in

4% paraformaldehyde in 0.1 M phosphate buffer and injection of 7.5% gelatin

in buffer into the nasal cavities, all snouts were treated to a 96 hour submersion

(with solution changes every 24 hours) in 14% ethylenediaminetetraacetic acid

(EDTA) in 0.1 M phosphate buffer to decalcify the tissue. This was followed by

immersion in a graded sucrose series of 0.5, 1.0, 1.5, and 2.0 M sucrose,

sequentially, to cryoprotect the tissue. Once decalcified and cryoprotected, all

snouts were cryosectioned at -28° F into 20 micrometer thick coronal tissue

sections using a Microm HM 505 E cryostat. Sections were placed into well

plates of 0.1 M phosphate buffer in preparation for immunostaining.

2.2.4. Immunostaining and Microscope Slide Mounting

A double-labeling procedure of two primary-secondary antibody

pairings was performed to achieve immunostaining of HBCs and proliferative

cells within sections that retained the MOE and VNO. Protocols for immunostaining free-floating sections were performed at room temperature, utilizing a blocking solution (5% normal goat serum, 50% 0.1 M phosphate buffer, 150mM NaCL, 0.3% Triton X-100) to inhibit non-specific antigen binding. After cryosectioning, coronal sections with intact MOE and VNO morphology were transferred in groupings of three to 24 well plates. Sections were treated to successive washes in: 0.1 M phosphate buffer (6X, 10 minutes), 90% methanol in 3% hydrogen peroxide (1X, 30 minutes), 0.1 M phosphate buffer (3X, 10 minutes), and blocking solution (1X, 60 minutes).

In the first stage of immunostaining, sections were incubated overnight (12-16 hours) in a 1:200 dilution of a rabbit monoclonal Ki67 antibody (ThermoFisher Scientific, RM-9106-S), which is a nuclear marker for the active phases of cell proliferation. After washing with 0.1 M phosphate buffer (3X, 10 minutes) and blocking solution (1X, 60 minutes), the primary stain was augmented for imaging by incubation (1X, 60 minutes) in a 1:1000 dilution of Alexa Fluor® 546.

In preparation for the second stage of immunostaining, all sections were washed with 0.1 M phosphate buffer (4X, 10 minutes) and again incubated in blocking solution (1X, 60 minutes). Sections were then incubated overnight (12-16 hours) in a 1:2500 dilution of a rabbit polyclonal Keratin 5 antibody (BioLegend, 905501), which is a cytoskeletal marker of the intermediate

filaments that compose HBCs. After washing, sections were treated with a 1:1000 dilution of Alexa Fluor® 488 (1X, 60 minutes).

Once immunostained with anti-Ki67/Alexa Fluor® 546 and anti-Keratin 5/Alexa Fluor® 488, all intact sections were washed in 0.1 M phosphate buffer and mounted onto electrically-charged microscope slides (Fisherbrand™ Superfrost™ Plus) and sealed with cover glass using Fluoromount-G® (SouthernBiotech). Slides were dried at room temperature, and stored in the refrigerator until viewed.

2.2.5. Fluorescent Visualization

Fluorescent visualization of the immunohistochemical double-labeling was accomplished by imaging on a Zeiss Axioskop 2 upright microscope. All sections were visualized under TRITC and FITC fluorescent filters to ascertain both labels. Images of every organ (e.g. MOE and VNO) in each mounted section were captured by NIS-Elements Basic Research microscope imaging software through a Photometric Cool SNAP EZ camera. All images were saved in both Joint Photographic Experts Group (JPEG) and Tagged Image Format (TIFF) file formats.

2.2.6. Quantification

Quantification of the double-labeling captured by fluorescent imaging was achieved in two parts: enumerating the number of Ki67-positive cells in each organ imaged, and enumerating the number of co-localizations of Keratin

5-positive and Ki67-positive cells in each organ imaged. At least three coronal sections from each mouse (CYP-injected n=28, saline-injected n=20) were quantified.

During the first stage of quantification, NIS-Elements was used to measure the area in square micrometers of each zone within every organ (e.g. sensory and sustentacular zones within the MOE, and sensory, sustentacular, and marginal zones within the VNO). The sensory and sustentacular zones of the MOE and VNO were demarcated by obvious morphological distinctions. The marginal zones of the VNO were demarcated from zero to 30 degrees on a linear axis between the two crescent extremities of the VNO. Additionally, each cell visibly expressing anti-Ki67 in TRITC filter images was recorded. Altogether, a comprehensive inventory of the numerical areas of each zone and the number of Ki67-positive cells in each zone was completed within this stage.

During the second stage of quantification, Adobe Photoshop was used to visualize the co-localization of the Keratin 5 and Ki67 double-labeling, presumably between HBCs and proliferative cells. Morphologically-identical images of each organ under TRITC and FITC fluorescent filters were imported into Photoshop. These images were layered, uniformly lightened so that the labeling captured by each filter could be viewed, and uniformly sharpened so that morphological distinctions were enhanced. There was no selective editing

of any images. Co-localization was determined by evident double-labeling at identical locations within each organ.

Lastly, to account for variation in anatomy among the entire data set, the final quantifications were recorded as: the number of Ki67-positive cells in each zone by square micrometer; and the number of co-localizations of Keratin 5-positive cells and Ki67-positive cells in each zone by square micrometer.

2.2.7. Statistical Analyses

Testing for statistical significance was accomplished by subjecting each time-course of days post injection to a univariate analysis of variance, Levene's Test for equality of variances, and a two sample t-test for equality of means using SPSS software (IBM Analytics). The factors utilized for statistical testing were treatment by day (e.g. CYP-injected versus saline-injected over one, two, six, 14, 30, and 45 days post injection). The univariate analyses of variance tested the difference in variances in the number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive and Ki67-positive cells between CYP-injected mice and saline-injected mice across the total 45 days of experimentation following the injection procedures. Levene's Test for equality of variances tested the difference in variances in the number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive and Ki67-positive cells between CYP-injected mice and saline-injected mice at each day post injection that was studied (e.g. days one, two, six, 14, 30, and 45 post injection).

Finally, two-sample *t*-tests were performed to determine if the mean number of Ki67-positive cells and the mean number of co-localizations of Keratin 5-positive and Ki67-positive cells in the olfactory epithelia of CYP-injected and saline-injected mice were equal at days one, two, six, 14, 30 and 45 post injection.

Time-courses were established by calculating the mean number of Ki67-positive cells or the mean number of co-localizations of Keratin 5-positive cells and Ki67-positive cells for both treatments every day post injection within each zone (e.g. MOE sensory, MOE sustentacular, VNO sensory, VNO sustentacular, and VNO marginal). Standard error of the mean for both treatments each day post injection was calculated. P-values less than alpha level 0.050 were considered evidence that the null hypothesis was rejected, and the differences between CYP-injected mice and saline-injected mice were significant. For statistics, N equals the number of mice that were injected and n equals the number of coronal sections that were quantified. As a reminder, the data set encompassed 28 CYP-injected mice and 20 saline-injected mice, including: days one (CYP N=6, saline N=4), two (CYP N=6, saline N=4), six (CYP N=4, saline N=4), 14 (CYP N=4, saline N=4), 30 (CYP N=4, saline N=2), and 45 (CYP N=4, saline N=2) post injection. A total of ten time-courses were tested, considering there were two parameters (e.g. number of Ki67-positive cells and number of co-localizations of Keratin 5-positive cells and Ki67-positive cells) for each of

the five zones (e.g. MOE sensory, MOE sustentacular, VNO sensory, VNO sustentacular, VNO marginal) across 45 days.

2.3. Results

Sensory neurons in the murine MOE and the VNO are continually replaced during of the lifetime of mice. Therefore, they are subject to damage from any substance that affects their turnover as well as any environmental factors (such as smoking, etc.). Our study focused on insults to the MOE and VNO induced by CYP, after being delivered into the olfactory epithelia by the vascular system. Since CYP targets cells undergoing mitosis, we characterized CYP-induced injury in the olfactory epithelia by quantifying the number of proliferative cells over time after injection. Additionally, since the basal cells of the olfactory epithelia are thought to give rise to proliferative cells, we examined the number of co-localizations between HBCs and proliferative cells to determine the effects of CYP on impending neurogenesis in the olfactory epithelia. We investigated both of these parameters in five regions of the olfactory epithelia: MOE sensory zone, MOE sustentacular zone, VNO sensory zone, VNO sustentacular zone, and VNO marginal zones. To give an accurate depiction of the events in each organ, the following results are divided by the organs of interest – the MOE and the VNO.

For our purposes, Ki67-positive cells were indicative of olfactory cells in the active stages of proliferation, while Keratin 5-positive cells were indicative

of the HBCs. As previously mentioned, there is no verified marker for the
GBCs in the olfactory system, so only the HBCs and proliferative cells were
considered. Our analyses reflect the differences between CYP-injected mice and
saline-injected mice at each day post injection that was studied.

2.3.1. Main Olfactory Epithelium (MOE)

Over the course of 45 days post injection, we observed the number of
Ki67-positive cells and the number of co-localizations of Keratin 5-positive cells
and Ki67-positive cells in the murine MOE. Analyses of both parameters were
performed on the sensory zone and sustentacular zone.

Ki67-positive cells were found in the sensory zone and sustentacular
zone of the MOE at each day post injection. Both CYP-injected and saline-
injected mice revealed Ki67-positive labeling, though the extent of labeling
differed between treatments. Ki67-positive cells were scattered in both regions
of the MOE (Figure 5). At day zero post injection, the number of Ki67-positive
cells in the MOE was comparable between treatments in both zones. However,
by days two through six post injection, the number of Ki67-positive cells in
both zones of the MOE decreased in CYP-injected mice compared to saline-
injected mice; the number of labeled cells in CYP-injected mice was
approximately half the number of labeled cells in saline-injected mice.
Interestingly, the number of Ki67-positive cells in saline-injected mice spiked at
days two and six post injection. By days 14 and 30 post injection, the number of

Ki67-positive cells in both zones of the MOE in CYP-injected mice was elevated compared to saline-injected mice; there were roughly 1.5 times as a many labeled cells in CYP-injected mice compared to saline-injected mice (Figure 6). Finally, at day 45 post injection, the number of Ki67-positive cells in both zones of the MOE was comparable between treatments (Figures 7-9). In summary, CYP-injected mice experienced a transient depression in the number of labeled cells in the MOE within the first 14 days post injection, and then experienced an increase in the number of labeled cells after day 14 post injection, compared to saline-injected mice. The difference in Ki67-positive cells between CYP-injected and saline-injected mice in both zones was statistically significant at days two, 14, and 30 post injection.

Co-localization of Keratin 5-positive cells and Ki67-positive cells was only observed in the sensory zone of the MOE. Keratin 5-positive cells appeared oval in shape and remained congregated in large populations along the basal lamina of the MOE. Keratin 5-positive cells always remained adjacent to the basal lamina, in both CYP-injected and saline-injected mice; therefore, no Keratin 5 labeling appeared in the apically-located sustentacular zone (Figure 5). At days zero, one, and two post injection, the number of co-localizations was comparable between treatments (Figure 5). However, by day six post injection, the number of co-localizations in CYP-injected mice was less than the number in saline-injected mice. Oppositely, by days 14 and 30 post injection,

the number of co-localizations in CYP-injected mice was elevated compared to saline-injected mice; co-localizations in CYP-injected mice were nearly double the number in saline-injected mice (Figure 6). Finally, by day 45 post injection, the number of co-localizations was comparable between treatments (Figure 7 and 10). In summary, CYP-injected mice experienced a decrease in co-localizations at day six post injection, but an increase in co-localizations at days 14 and 30 post injection, compared to saline-injected mice.
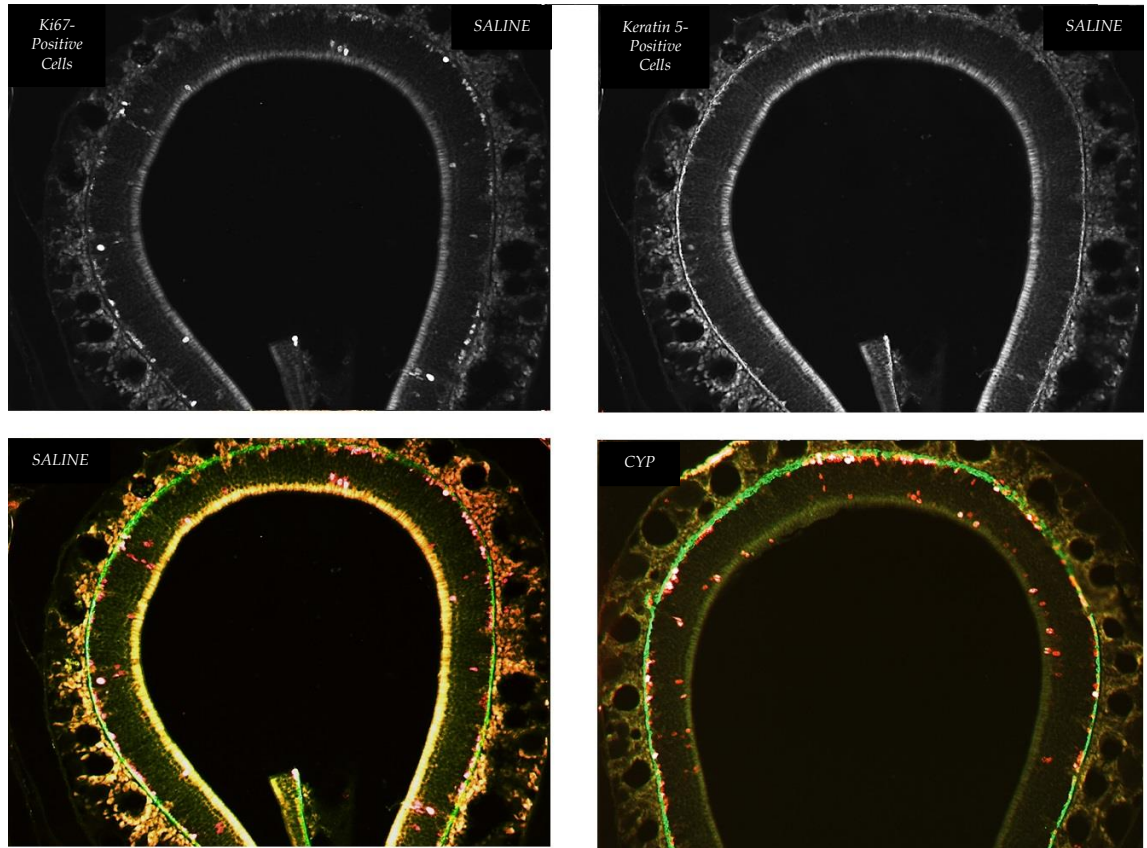
Figure 5: Effects of CYP on Keratin 5-Positive Cells and Ki67-Positive Cells One Day Post Injection in the MOE, Compared to Saline Controls. Ki67-positive cells (top left) appeared scattered in the sensory and sustentacular zones, though they congregated in high numbers along the basal lamina. Keratin-5 positive cells (top right) appeared as a thin layer of cells along the basal lamina. Before day two post injection, CYP-injected mice (bottom right) retained a similar number of Ki67-positive cells (red) compared to saline-injected mice (bottom left). Also, the number of co-localizations between Keratin 5-positive cells (green) and Ki67-positive cells along the basal lamina appeared similar between CYP-injected and saline-injected mice.
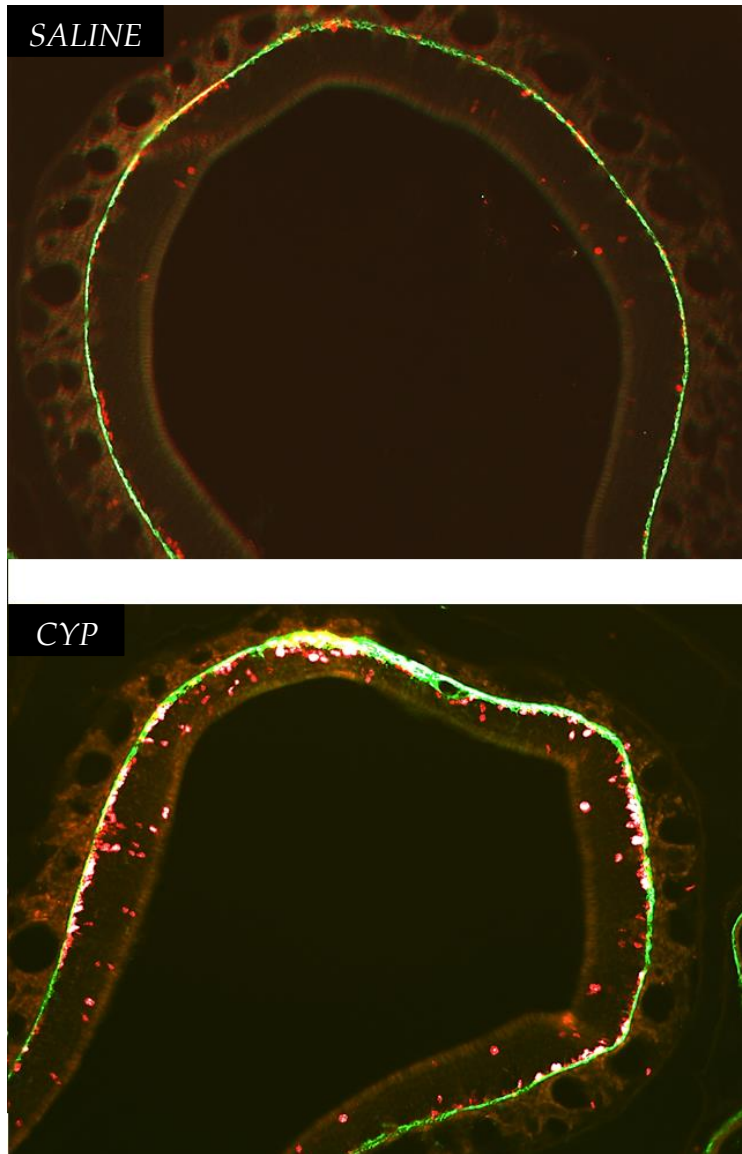
Figure 6: Effects of CYP on Keratin 5-Positive Cells and Ki67-Positive Cells in the MOE 30 Days Post Injection, Compared to Saline Controls. By 30 days post injection, CYP-injected mice (bottom) retained a significantly higher number of Ki67-postive cells (red) than did saline-injected mice (top). In CYP-injected mice, high rates of proliferation were viewed in the sensory zone and the sustentacular zone. Also, CYP-injected mice retained an increased number of co-localizations between Keratin 5-positive cells (green) and Ki67-positive cells along the basal lamina.
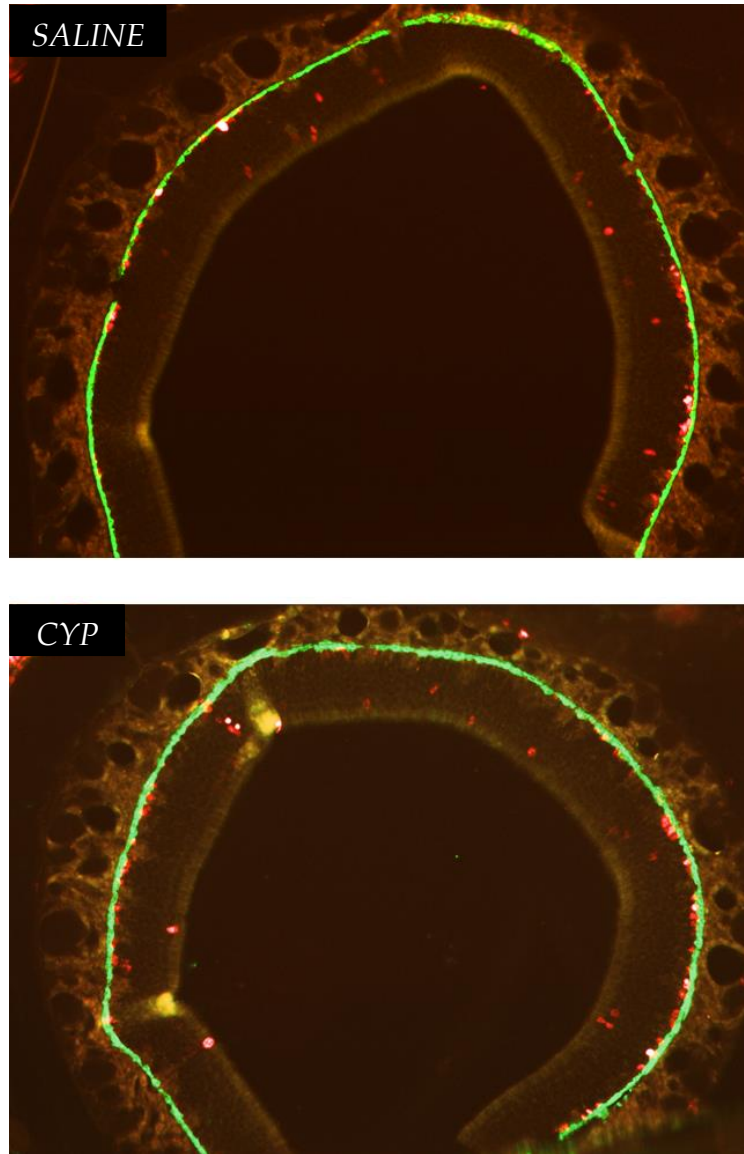
Figure 7: Effects of CYP on Keratin 5-Positive Cells and Ki67-Positive Cells in the MOE 45 Days Post Injection, Compared to Saline Controls. By 45 days post injection, CYP-injected mice (bottom) retained similar numbers of Ki67-positive cells (red) in both the sensory and sustentacular zones compared to saline-injected mice (top). Also, the number of co-localizations between Keratin 5-positive cells (green) and Ki67-positive cells along the basal lamina was comparable between CYP-injected mice and saline-injected mice.
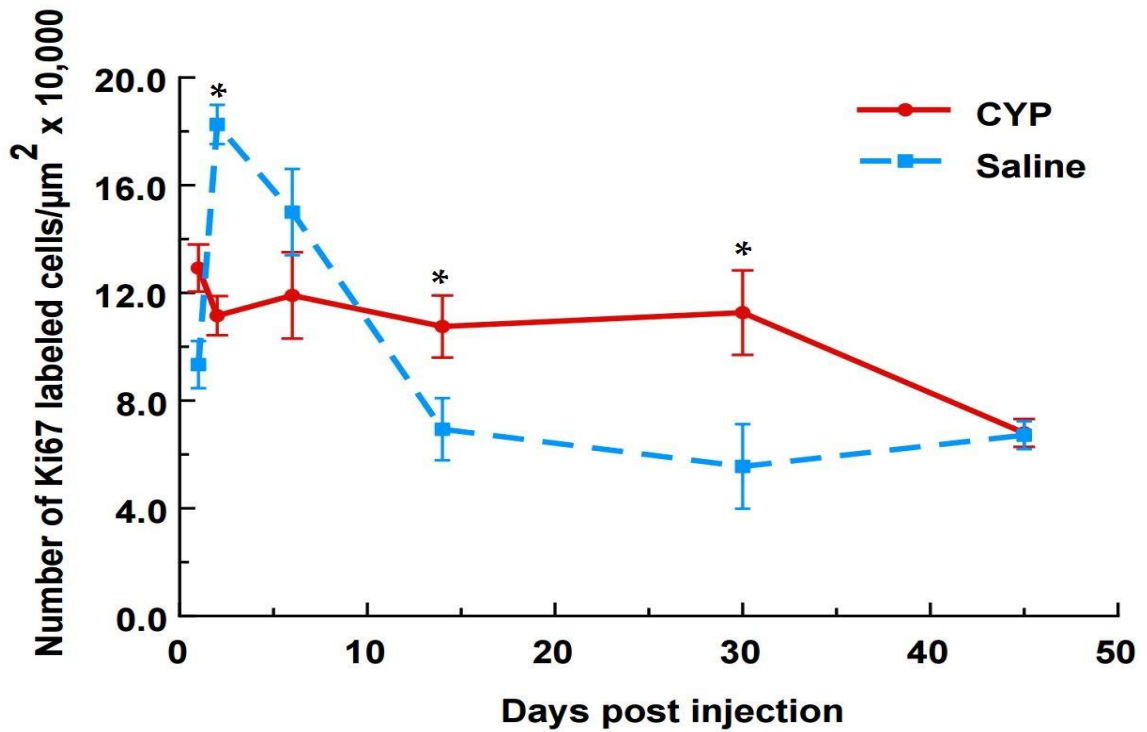
Figure 8: Mean (+/- SEM) Number of Ki67-Positive Cells in the Main Olfactory Epithelium Sensory Zone over Days Post Injection. Compared to saline-injected mice (N=20, n=48), the number of Ki67-positive cells in CYP-injected mice (N=28, n=93) decreased during the first 10 days post injection and then increased beginning at 14 days post injection. At 45 days post injection, there appeared to be a leveling between CYP-injected and saline-injected mice. The difference between CYP-injected and saline-injected mice was significant at days two, 14, and 30 post injection (* indicates $P<0.050$).

Figure 9: Mean (+/- SEM) Number of Ki67-Positive Cells in the Main Olfactory Epithelium Sustentacular Zone over Days Post Injection. Compared to saline-injected mice (N=20, n=48), the number of Ki67-positive cells in CYP-injected mice (N=28, n=93) decreased during the first 10 days post injection and then increased beginning at 14 days post injection. At 45 days post injection, there appeared to be a leveling between CYP-injected and saline-injected mice. The difference between CYP-injected and saline-injected mice was significant at days two, 14, and 30 post injection (* indicates $P<0.050$).
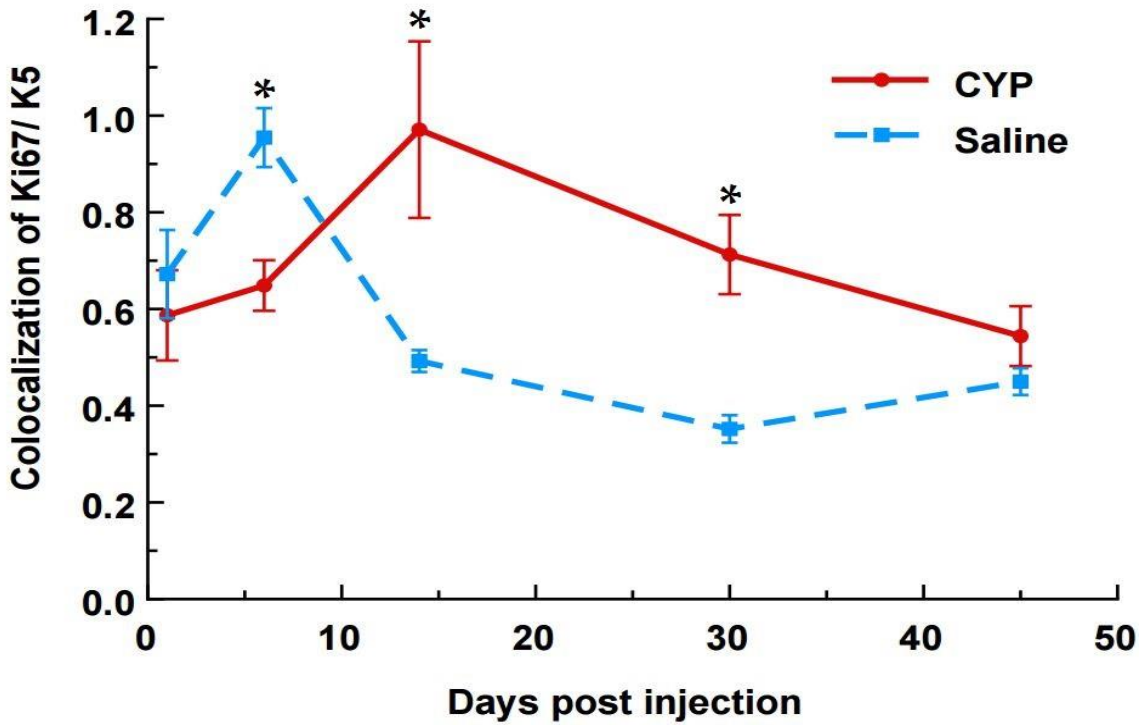
Figure 10: Mean (+/- SEM) Number of Co-Localizations of Keratin 5-Positive and Ki67-Positive Cells in the Main Olfactory Epithelium Sensory Zone over Days Post Injection. The number of co-localizations in CYP-injected mice (N=28, n=93) appeared to peak at roughly 14 days post injection, and remained elevated compared to saline-injected mice (N=20, n=48) until 45 days post injection, when there was a leveling between treatments. The difference between CYP-injected and saline-injected mice was significant at days six, 14, and 30 post injection (* indicates $P<0.050$).

2.3.2. Vomeronasal Organ (VNO)

Over the course of 45 days post injection, we observed the number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive cells and Ki67-positive cells in the murine VNO. Analysis of both parameters was performed on the sensory zone, sustentacular zone, and marginal zones.

Ki67-positive cells were found in the sensory zone, sustentacular zone, and marginal zones of the VNO at each day post injection; both CYP-injected and saline-injected mice revealed Ki67-positive labeling, though the extent of labeling differed between treatments. Ki67-positive cells appeared scattered in all three regions of MOE, though there appeared to be a greater concentration of cells in the marginal zones compared to the other two regions, across time (Figur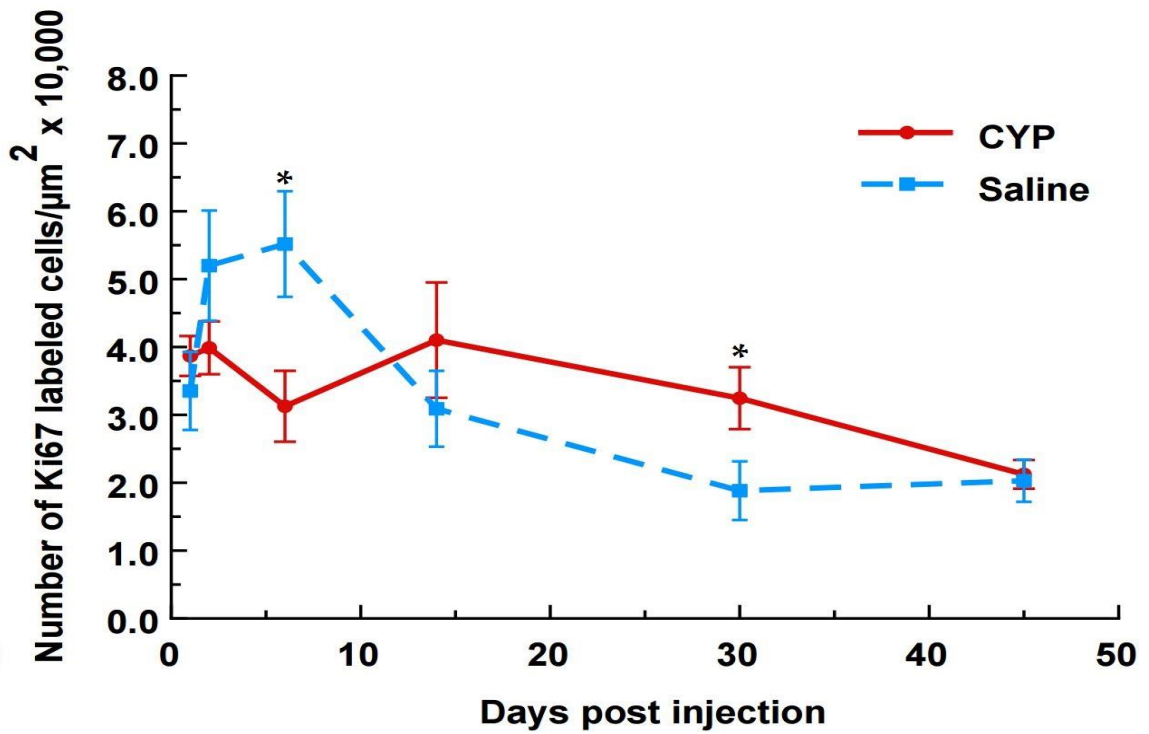e 11). At day zero post injection, the number of Ki67-positive cells was comparable between treatments in each of the three zones. However, by days one and two post injection, the number of Ki67-positive cells decreased in all three zones of CYP-injected mice, compared to saline-injected; there were approximately half the number of labeled cells in CYP-injected mice that there were in saline-injected mice (Figure 11). By day six post injection, the number of Ki67-positive cells in CYP-injected mice remained decreased in the sensory and sustentacular zones, yet the number of Ki67-positive cells in the marginal zones was comparable with saline-injected mice. By day 14 post-injection, the number of Ki67-positive cells in the sensory and sustentacular zones of CYP-

injected mice was comparable to saline-injected mice; however, the number of labeled cells in the marginal zones of CYP-injected mice was extremely elevated – more than double - compared to saline-injected mice. By days 30 and 45 post injection, the number of Ki67-positive cells in CYP-injected mice increased in all three zones compared to saline-injected mice (Figure 12). The peak number of Ki67-positive cells in CYP-injected mice was at day 45 post injection in both the sensory and sustentacular zones (Figures 12-15). In summary, CYP-injected mice experienced a decrease in the number of Ki67-positive cells within the first ten days post injection, followed by a increase in the number of Ki67-positive cells after day 14 post injection, compared to saline-injected mice. This trend holds true for all three zones of the VNO, though the marginal zones appeared to experience the trend at a more rapid pace than the sensory and sustentacular zones. In the VNO sensory zone, the difference between CYP-injected and saline-injected mice was significant at days two, six, 30, and 45 post injection; in the VNO sustentacular zone, the difference between CYP-injected and saline-injected mice was significant at days one, six, 30, and 45 post injection. Similarly, in the VNO marginal zones, the difference between CYP-injected and saline-injected mice was significant at days two, 14, and 45 post injection.

Co-localization of Keratin 5-positive cells and Ki67-positive cells was observed in the sensory zone and the marginal zones of the VNO. Keratin 5-

positive cells appeared oval in shape and remained congregated in large populations along the basal lamina of the VNO. Keratin 5-positive cells always remained adjacent to the basal lamina, in both CYP-injected and saline-injected mice; as expected, no Keratin 5 labeling appeared in the apically-located sustentacular zone (Figure 11). At day zero post injection, the number of co-localizations in the sensory zone and marginal zones was comparable between treatments. However, by days one and two post injection, the number of co-localizations in both zones of CYP-injected mice was lower than the number in saline-injected mice (Figure 11). By day six post injection, the number of co-localizations in the marginal zones of CYP-injected mice remained decreased, while the number in the sensory zone of CYP-injected mice was comparable to saline-injected mice. By days 14, 30, and 45 post injection, the number of co-localizations in both zones of CYP-injected mice was elevated compared to saline-injected mice (Figures 12, 16-17). For instance, by day 45 post injection, the number of co-localizations in the sensory zone of CYP-injected mice was quadruple the number in saline-injected mice. In summary, CYP-injected mice experienced a decrease in the number of co-localizations in both zones within the first six days post injection, followed by an increase in the number of co-localizations from day 14 post injection through day 45 post injection, compared to saline-injected mice. While this trend held true for both the sensory zone and the marginal zones of the VNO, the number of co-

localizations in the sensory zone of CYP-injected mice appeared to be inordinately elevated, compared to a more moderate elevation in the marginal zones. In the VNO sensory zone, the difference in the number of co-localizations between CYP-injected and saline-injected mice was significant at day 45 post injection. In the VNO marginal zones, the difference in the number of co-localizations between CYP-injected and saline-injected mice was significant at days one and 45 post injection.
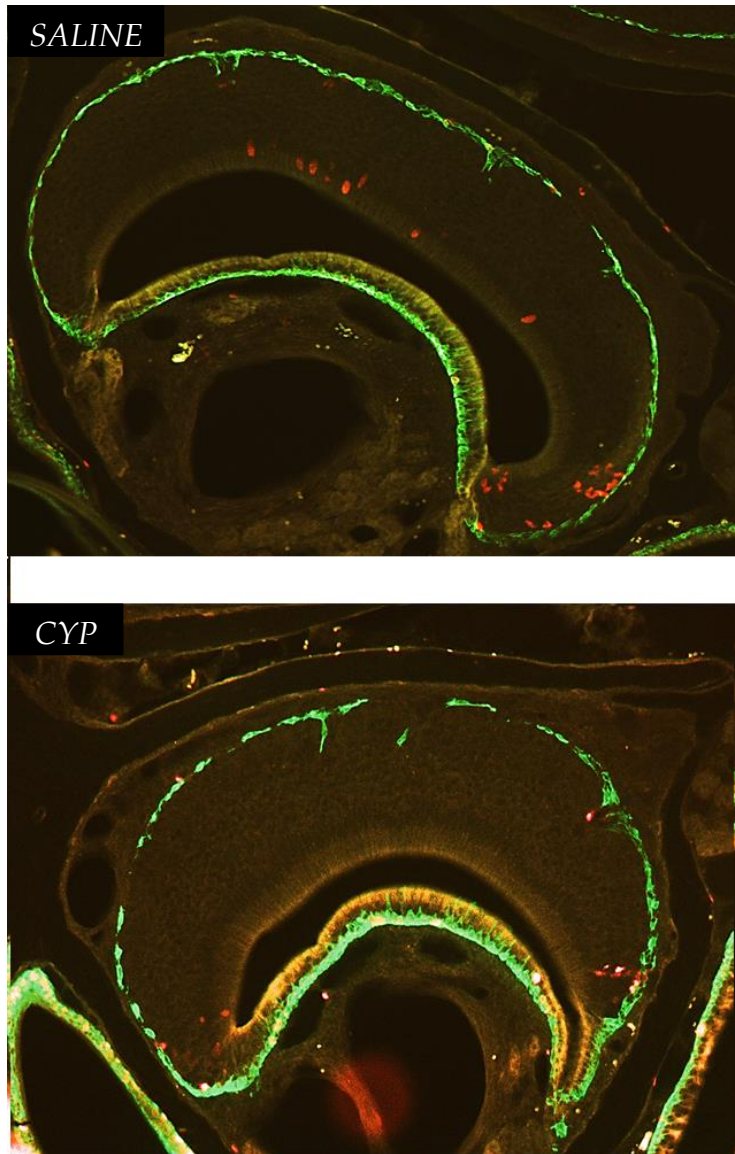
Figure 11: Effects of CYP on Keratin 5-Positive Cells and Ki67-Positive Cells in the VNO One Day Post Injection, Compared to Saline Controls. Ki67-positive cells (red) appeared scattered throughout the sensory, sustentacular, and marginal zones, while Keratin 5-positive cells (green) were congregated along the basal lamina of the sensory and marginal zones. By day one post injection, the number of Ki67-positive cells in CYP-injected mice (bottom) was decreased compared to the number in saline-injected mice (top). Also, the number of co-localizations along the basal lamina of the sensory and marginal zones in CYP-injected mice was decreased compared to the number in saline-injected mice.
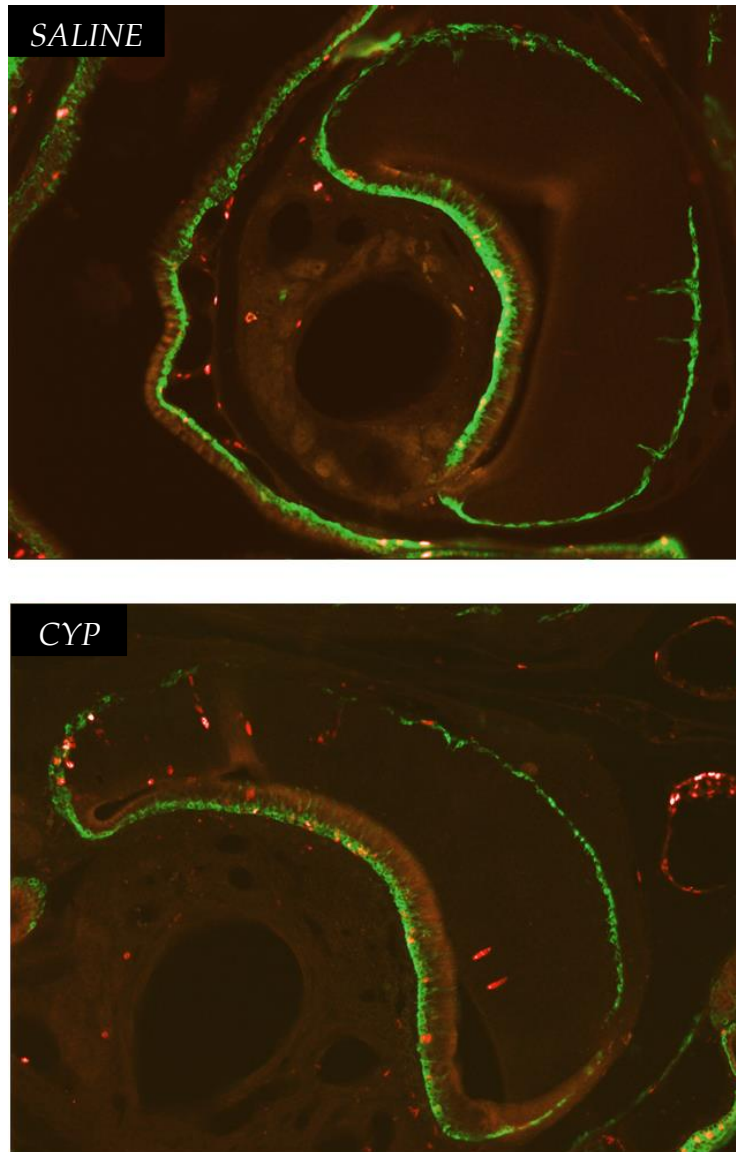
Figure 12: Effects of CYP on Keratin 5-Positive Cells and Ki67-Positive Cells in the VNO 45 Days Post Injection, Compared to Saline Controls. By 45 days post injection, the number of Ki67-postive cells (red) in the sensory zone, sustentacular zone, and marginal zones of CYP-injected mice (bottom) was higher than the number in all three zones of saline-injected mice (top). Also, the number of co-localizations between Keratin 5-positive cells (green) and Ki67-positive cells was higher in the sensory zone and marginal zones of CYP-injected mice than in either zone of saline-injected mice.
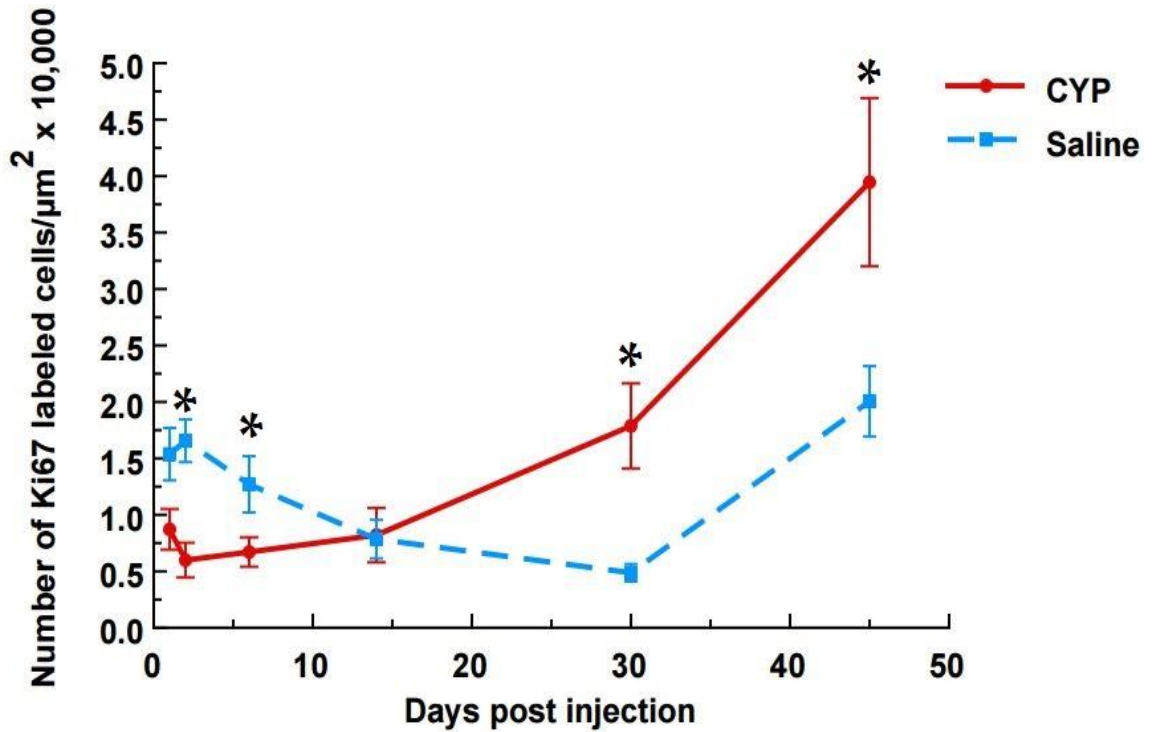
Figure 13: Mean (+/- SEM) Number of Ki67-Positive Cells in the Vomeronasal Organ Sensory Zone over Days Post Injection. Compared to saline-injected mice (N=20, n=51), the number of Ki67-positive cells in CYP-injected mice (N= 28, n=60) decreased during the first 14 days post injection and then increased through 45 days post injection. The peak number of Ki67-positive cells in CYP-injected mice was observed 45 days post injection. The difference between CYP-injected and saline-injected mice was significant at days two, six, 30, and 45 post injection (* indicates $P<0.050$).
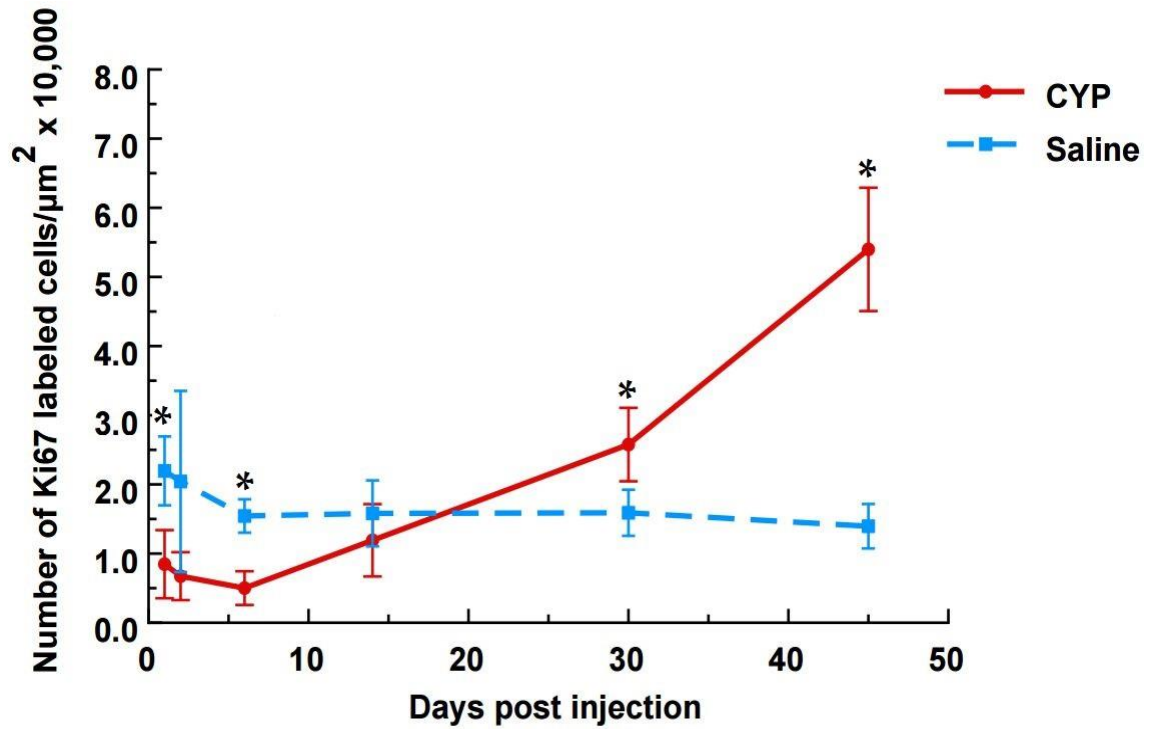
Figure 14: Mean (+/- SEM) Number of Ki67-Positive Cells in the Vomeronasal Organ Sustentacular Zone over Days Post Injection. Compared to saline-injected mice (N=20, n=51), the number of Ki67-positive cells in CYP-injected mice (N=28, n=60) decreased during the first 14 days post injection and then increased through 45 days post injection. The peak number of Ki67-positive cells in CYP-injected mice was observed 45 days post injection. The difference between CYP-injected and saline-injected mice was significant at days one, six, 30, and 45 post injection (* indicates $P<0.050$).
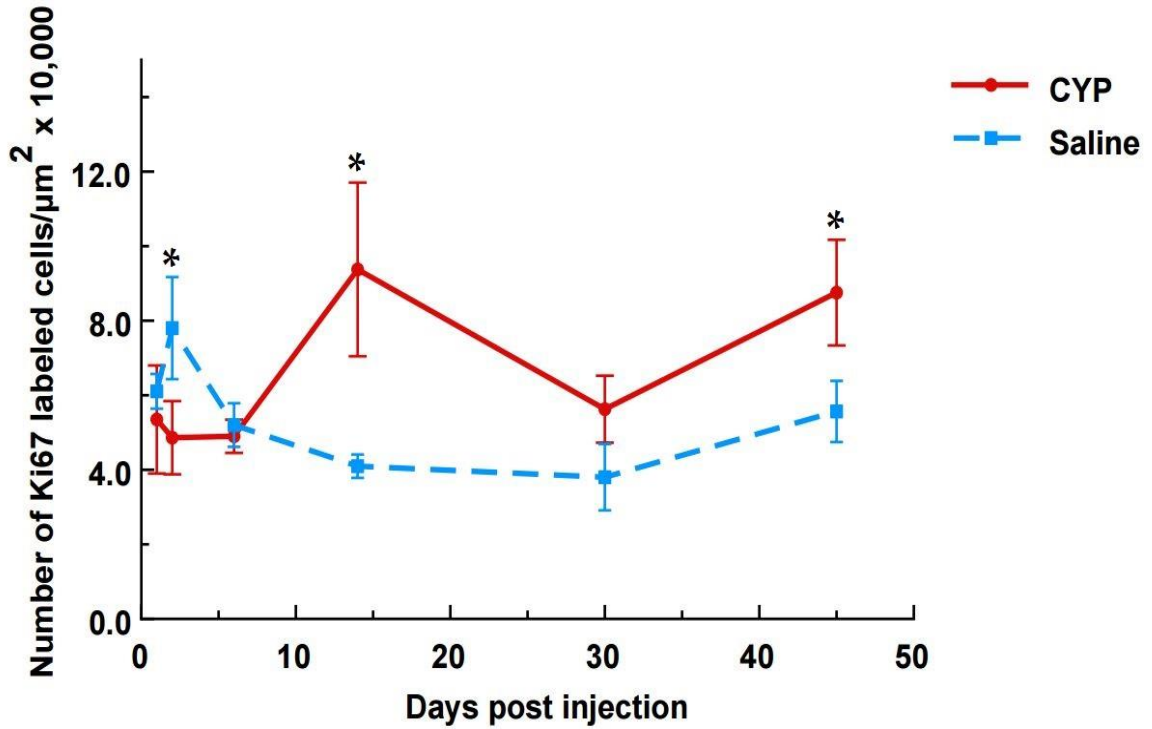
Figure 15: Mean (+/- SEM) Number of Ki67-Positive Cells in the Vomeronasal Organ Marginal Zones over Days Post Injection. Compared to saline-injected mice (N=20, n=51), the number of Ki67-positive cells in CYP-injected mice (N=28, n=60) decreased during the first six days post injection and then increased through 45 days post injection. No leveling of the number of Ki67-positive cells between treatments was observed. The difference between CYP-injected and saline-injected mice was significant at days two, 14, and 45 post injection (* indicates P<0.050).
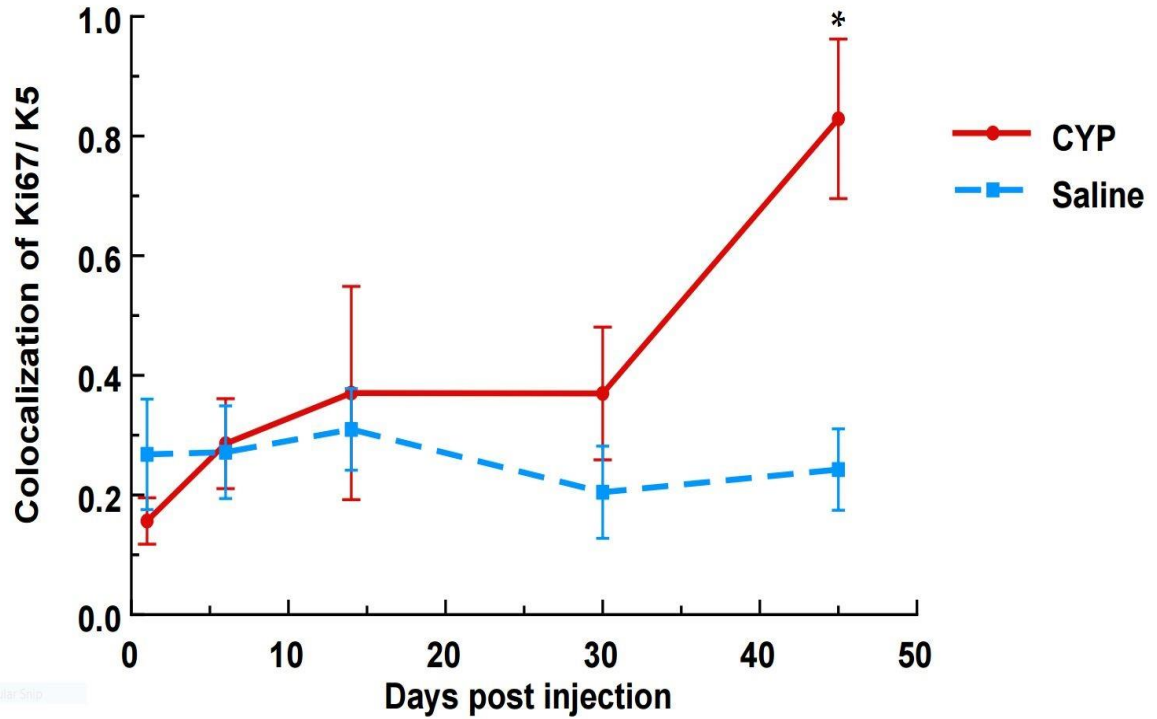
Figure 16: Mean (+/- SEM) Number of Co-Localizations of Keratin 5-Positive and Ki67-Positive Cells in the Vomeronasal Organ Sensory Zone over Days Post Injection.

Compared to saline-injected mice (N=20, n=41), the number of co-localizations in CYP-injected mice (N= 28, n=49) decreased until six days post injection, when the number increased through 45 days post injection. An extreme elevation in CYP-injected mice was observed 45 days post injection; the difference between CYP-injected and saline-injected mice was significant by day 45 post injection (* indicates $P < 0.050$).
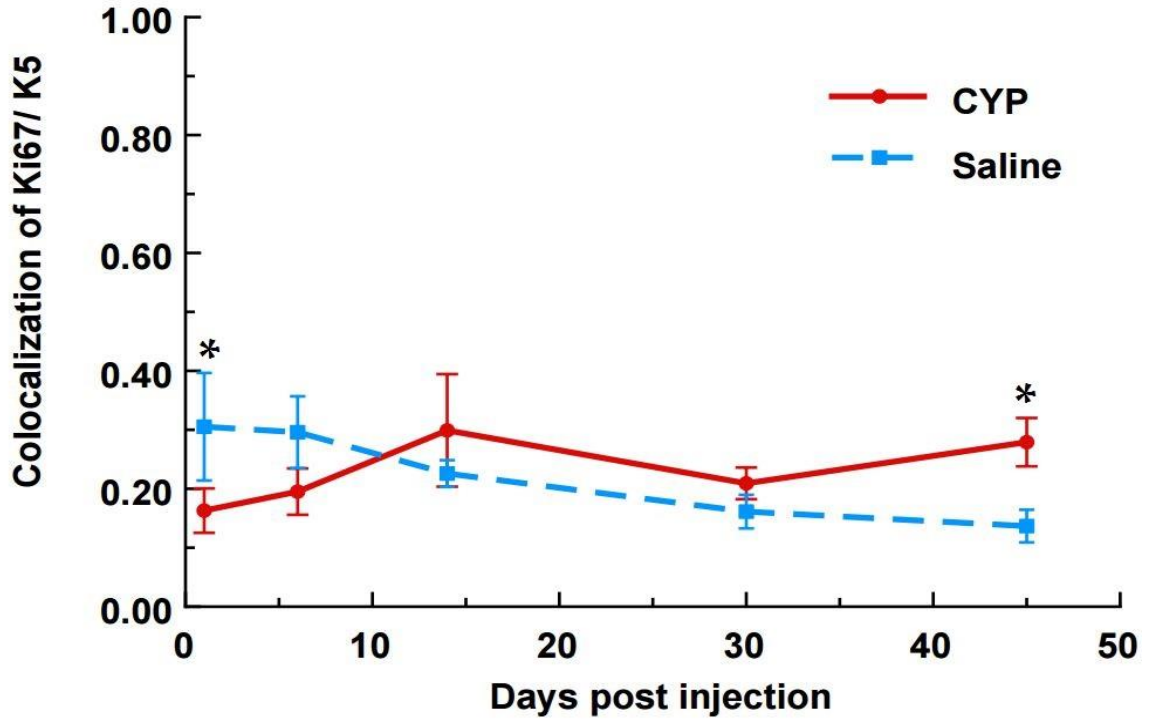
Figure 17: Mean (+/- SEM) Number of Co-Localizations of Keratin 5-Positive and

Ki67-Positive Cells in the Vomeronasal Organ Marginal Zones over Days Post Injection

Compared to saline-injected mice (N= 20, n=41), the number of co-localizations in CYP-injected

mice (N= 28, n=49) decreased during the first ten days post injection and then increased

through 45 days post injection. The difference between CYP-injected and saline-injected mice

was significant at days one and 45 post injection (* indicates P<0.050).

2.4. Discussion

Previous clinical research has demonstrated that administration of CYP, a non-specific cytotoxic drug used extensively in the treatment of cancer, can negatively alter chemosensory perception (Comeau et al., 2001; Bernhardson et al., 2007; Hutton et al., 2007; Bernhardson et al., 2009; Halyard, 2009). In addition to clinical outcomes, histological studies completed by Mukherjee & Delay have illuminated that injection of CYP can decrease the number of cells in the gustatory system within the first two weeks following administration (2011, 2013). To gain additional insight into how cytotoxic chemotherapies achieve chemosensory alterations, we performed an immunohistochemical assay to observe the quantitative effects that discrete administration of CYP has on populations of basal and proliferative cells in the murine olfactory system. We sought to enumerate the number of proliferative cells and the number of co-localizations of basal and proliferative cells in the MOE and VNO after a single intraperitoneal injection of CYP. To achieve this, we immunostained the HBCs (anti-Keratin 5) and proliferative cells (anti-Ki67) within the MOE and VNO over a time-course of 45 days.

To focus our investigation, we formulated two hypotheses. First, since the olfactory epithelia demonstrate continuous neurogenesis over the lifetime of mice, we hypothesized that a single injection of CYP would decrease the number of proliferative cells in the MOE and VNO, and be followed by a

previously-undetermined period of neurogenic recovery once the initial insult from CYP had subsided. Second, since HBCs are thought to be mostly quiescent cells and would accordingly be spared by CYP (which targets mitotic cells, like the GBCs), we hypothesized that the number of co-localizations of HBCs and proliferative cells would increase after a single injection of CYP. We predicted that the HBCs would be activated for proliferation to ameliorate the injury induced by CYP, and that the greatest number of co-localizations would occur 20 to 40 days after the injection of CYP, mirroring the normal physiological turnover of sensory neurons in the murine olfactory system.

Quantification results by fluorescent imaging of labeled sections revealed a significant decrease in the number of proliferative cells in the MOE and VNO of CYP-injected mice within the first 10 days post injection, followed by a compensatory period of increased cell proliferation through day 45 post injection, compared to saline-injected mice. Co-localization of HBCs and proliferative cells in the MOE and VNO of CYP-injected mice was significantly amplified at approximately 14 and 45 days post injection, respectively, compared to saline-injected mice.

2.4.1. Ki67-Positive Cells

The immunohistochemical results from the MOE and VNO demonstrate support for our hypothesis regarding the number of Ki67-positive cells in the olfactory epithelia after injection of CYP. In all five histological regions of the

murine olfactory system that were tested (MOE sensory zone, MOE sustentacular zone, VNO sensory zone, VNO sustentacular zone, and VNO marginal zones), administration of CYP decreased the number of Ki67-positive cells within the first 14 days following injection (Figures 8-9, 11, 13-15). This result is consistent with our current knowledge of CYP, whereby systemic administration of the drug targets proliferating cells within the body non-specifically (Dick & Phillips, 1961; Bagley et al. 1973; Moore, 1991; Colvin, 1999; Malet-Martino et al., 1999; Bernhardson et al., 2007). It also mirrors results reported by Mukherjee & Delay that exhibited a depression of gustatory cells within the first two weeks following CYP injection (2011, 2013).

It is probable that CYP injected into our experimental mice reached both the MOE and VNO by way of the vascular system, and diminished the populations of Ki67-positive cells by arresting cellular proliferation. Although the VNO is more highly vascularized than the MOE, we observed that an intraperitoneal injection of CYP disrupted the number of Ki67-positive cells similarly in both organs. Furthermore, because administration of CYP to our experimental mice was limited to one injection of a single dose, we observed a period of recovery in the number of Ki67-positive cells 14 to 45 days after administration of CYP (Figures 7-9, 12-15). This finding is consistent with current knowledge that the murine olfactory epithelia undergo continuous neurogenesis over the lifetime of animals, and would be able to resume

proliferation after the effects of CYP subsided (Graziadei & Monti-Graziadei, 1979; Costanzo, 1991; Weiler & Farbman, 1997, 1998; Huard et al., 1998; Legrier et al., 2001; Schwob, 2002; Brann & Firestein, 2010). All five regions of the olfactory epithelia in CYP-injected mice demonstrated a shift from decreased proliferation to increased proliferation compared to saline-injected mice at approximately 10 to 14 days post injection, compared to saline-injected mice (Figures 8-9, 13-15). This result suggests that a single injection of CYP induces a two week lag in cellular proliferation before the olfactory epithelia can resume normal neuronal turnover and replace cells lost to the injury.

These results prompted several questions for consideration. First, it is pertinent to consider why administration of CYP did not completely decimate the populations of proliferative cells in either the MOE or VNO. In our experiment, one possible explanation is that robust proliferation in the olfactory epithelia of the young animals tested mitigated the cytotoxicity of the CYP that was administered. At seven weeks of age, C57BL/6 mice are not physically mature, as they continue to grow in size up to 50 weeks old (Weiler & Farbman, 1997, 1998; Weiler, 2005). Considering the young postnatal age of our mice, it is likely that at the time of injection both the MOE and the VNO were undergoing neurogenesis for both anatomical expansion and neuronal turnover. It is conceivable that the single dose of CYP administered to our mice was merely insufficient for complete obliteration of the Ki67-positive cells in

our young animals. Indeed, the idea of robust proliferation in young mice is evidenced by the fact that, in our study, both zones of the MOE recovered to normal levels of proliferation by day 45 post injection, compared to saline-injected mice. This rapid recovery was likely facilitated by the vigorous proliferation occurring in our young animals prior to and after injection of CYP. Also, the slightly faster rates of recovery viewed in the MOE and the VNO marginal zones (compared to the VNO sensory and sustentacular zones) can likely be attributed to the fact that these regions undergo anatomical growth up to a year after birth, suggesting that proliferation would have been robust in these regions prior to injection of CYP.

Second, since the MOE and VNO both were injured by CYP and experienced a subsequent period of recovered proliferation, it is pertinent to consider why the number of Ki67-positive cells in the MOE returned to comparable levels with saline-injected mice, while the number of Ki67-positive cells in the VNO remained significantly elevated at day 45 post injection. There may be several explanations for this discrepancy, though we presume it is likely due to asynchronous cycles of neurogenesis in the MOE and VNO. Neurogenesis in the MOE has been shown to be twice as rapid as neurogenesis in the VNO, and it is likely that our results are a snapshot of the asynchronous neuronal turnover in the MOE and VNO, once our animals began to recover from the initial insult from CYP (Graziadei & Monti-Graziadei, 1979; Costanzo,

1991; Huard et al., 1998; Legrier et al., 2001; Schwob, 2002). We suggest that the turnover of sensory neurons in the MOE and VNO may be cyclical; future studies should elongate the experimental time-course to clarify the rates of neuronal turnover across time, and examine differences in the rate of neurogenesis between the MOE and VNO.

Third, it is necessary to consider why our results showed a spike in the number of Ki67-positive cells in saline-injected mice (particularly in the MOE) at days two and six post injection, especially since we observed a decrease in the number of Ki67-positive cells in CYP-injected mice at the same time points (Figures 8-9, 13-15). It is important to note that there was absolutely no cross-contamination of CYP and saline during the injection procedures, as CYP and saline injections were administered in different fume hoods with similar accommodations within the same building. Without cross-contamination, an odorant fear response does not explain the spike in Ki67-positive cells we observed in the saline-injected mice. However, our experimental mice were both naïve and young, having never been handled until the day of their injection. Therefore, it is plausible that the spike in Ki67-positive cells in saline-injected mice at days two and six post injection resulted from an acute stress response precipitated by the injection procedures (Morton et al., 2001). In fact, intraperitoneal injection has been found to cause the highest levels of acute stress of any injection procedure used on mice (Meijer et al., 2006).

Fourth, it is interesting to consider the remarkably high levels of proliferation viewed in the VNO sustentacular zone by 30 days post injection and beyond (Figures 12, 14). Since the VNO sustentacular zone is apical to the VNO sensory zone, there was no previous indication that a significant population of stem cells existed within the VNO sustentacular zone. Conventional knowledge suggested that the sensory zone retained a swathe of HBCs along the basal lamina as well as a population of GBCs that give rise to neuronal cell bodies located within the sensory zone (Caggiano et al., 2005; Huard & Schwob, 1995). In this paradigm, we would expect the high levels of post-injury proliferation we see in the VNO sensory zone, but not such high levels in the VNO sustentacular zone. Since our results demonstrate three times the amount of proliferation in the VNO sustentacular zone in CYP-injected mice compared to saline-injected mice, we hypothesize that the VNO sustentacular zone may retain a more significant population of stem cells than previously considered.

Overall, our results suggest that a single intraperitoneal injection of CYP significantly depresses the number of proliferative cells in the MOE and VNO, and instigates a subsequent period of neurogenic recovery governed by continual neurogenesis. In the olfactory epithelia of young mice like those we tested, we suggest that the depressive effects of CYP should be viewed within the first 14 days post injection. If only one dose of CYP is administered, we

suggest that proliferation within the olfactory epithelia should begin to recover approximately two weeks after injection, though the rates of recovery may differ between the MOE and the VNO.

2.4.2. Co-Localization of Keratin 5-Positive Cells and Ki67-Positive Cells

The immunohistochemical results from the MOE and VNO demonstrate support for our hypothesis regarding the number of co-localizations of Keratin 5-positive cells and Ki67-positive cells in the olfactory epithelia after injection of CYP. In all three histological regions of the murine olfactory system that retained labeled co-localizations (MOE sensory zone, VNO sensory zone, and VNO marginal zones), administration of CYP precipitated an eventual increase in the number of co-localizations (figures 6, 10, 12, 16-17). This is a novel and intriguing result. Conventional knowledge of regeneration in the olfactory epithelia suggests that under normal physiological conditions, populations of HBCs are mostly quiescent and exist primarily to replenish populations of GBCs, which are thought to be the source of proliferative cells that support the nonstop turnover of sensory neurons (Holbrook et al., 1995; Huard & Schwob, 1995; Carter et al., 2004; Leung et al., 2007). Our results suggest that the elevated number of co-localizations of Keratin 5-positive cells and Ki67-positive cells in CYP-injured animals is evidence of HBCs directly giving rise to proliferative cells. This finding is rational since GBCs undergoing mitosis would have been arrested by the administration of CYP. Therefore, in our

study, we contend that HBCs in the MOE and VNO likely replenished populations of GBCs (as they do under normal physiological conditions), and also replaced populations of proliferative cells that were injured by CYP. In this case, it appears that pharmacologic injury amplified the proliferation workload for the populations of HBCs. If true, this mechanism of regeneration following pharmacologic injury would be distinctive from the mechanism of proliferation thought to exist during the physiological turnover of sensory neurons. Indeed, previous research has demonstrated that chemical ablation of the olfactory epithelia, by compounds such as zinc sulfate, methyl bromide, and methimazole, results in the rapid proliferation of basal cells (Schwob et al., 1995; Williams et al., 2004; Iwai et al., 2008; Xie et al., 2010; Jang et al., 2014; Suzuki et al., 2015). Moreover, recent studies by Brann and colleagues have reported that HBCs in methimazole-injured animals exhibit a direct conversion to proliferative cells (2015). However, to our knowledge, our study is the first to demonstrate with immunohistochemical evidence that HBCs may directly give rise to proliferative cells after injury with a cytotoxic chemotherapeutic. Furthermore, our study is one of the first to affirm that HBCs have an active role in proliferation in the VNO, a notion that had previously been disputed (Joiner et al., 2015).

It should be noted that the same discrepancy between the MOE and VNO observed at day 45 post injection in the number of Ki67-positive cells was

also observed in the number of co-localizations. At day 45 post injection, the number of co-localizations in the VNO of CYP-injected mice remained significantly elevated compared to saline-injected mice, whereas the number in the MOE returned to levels comparable to saline-injected mice. Again, we presume that this discrepancy is likely due to asynchronous cycles of neurogenesis in the MOE and VNO. It is likely that our results are a snapshot of these asynchronous cycles once our animals began to recover from the initial insult of CYP. We suspect that lengthened time-courses of the number of Ki67-positive cells and the number of co-localizations will reveal divergent rates of proliferation in the MOE and VNO as animals recover from a cytotoxic insult.

Overall, our results suggest that a single intraperitoneal injection of CYP will significantly increase the number of co-localizations of Keratin 5-positive cells and Ki67-positive cells in the MOE and VNO following a period of CYP-induced injury. In the olfactory epithelia of young mice like those we tested, we suggest that an increase in the number of co-localizations should be viewed by 14 days after injection of CYP, though the rates of co-localization may differ across time between the MOE and the VNO.

2.4.3. Future Research Directions and Potential Clinical Impacts

Our study is the first to demonstrate that a cytotoxic chemotherapeutic can dampen proliferation in the murine olfactory system, and instigate a subsequent period of neurogenic recovery. Future research on olfactory

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alterations induced by CYP can be expanded in several ways. Studies should utilize a large population of mice with testing occurring at several different stages of life (i.e. early adulthood, late adulthood, etc.). They may also include a variety of injections (i.e. larger doses of CYP, multiple injections of CYP, etc.) and several more time-points post injection to elucidate the potency of CYP and the efficacy of CYP in the olfactory epithelia across time. To reduce stress responses provoked by injection procedures, mice should be acclimated to tactile interactions prior to experimentation. Additionally, use of a verified immunohistochemical marker for the GBCs would improve our understanding of the interactions between HBCs and proliferative cells after injury with CYP. Lastly, future research should consider examining the effects of cytoprotectants in the murine olfactory system. In yet another innovative study, Mukherjee and Delay showed that pre-treatment with the cytoprotective agent amifostine prevented CYP-induced inhibition of cell proliferation and also protected against loss of mature gustatory cells after CYP exposure (2013). Since our data in the olfactory system mirrored trends reported by Mukherjee & Delay in the gustatory system, it would be reasonable to hypothesize that amifostine may have a cytoprotective effect on proliferative cells in the olfactory epithelia. Additional research on the histological effects that CYP confers on the olfactory system will substantially improve our understanding of how cytotoxic chemotherapeutics achieve chemosensory alterations.

Finally, we suggest that our results may have several clinical implications. To our knowledge, our study is the first of its kind to explicate the histological underpinnings of olfactory distortion induced by administration of a cytotoxic chemotherapeutic. For the first time, clinicians will now have histological evidence to refer to in understanding how CYP may negatively alter their patients' sense of smell. Moreover, our results may prompt newfound clinical applications for the use of CYP. Our data suggest that administration of CYP will elevate the number of co-localizations between quiescent basal cells and proliferative cells, signifying that pharmacologic injury can augment proliferation in the olfactory epithelia. In patients who have naturally-occurring hyposmia or anosmia, administration of CYP may be one way to intensify neurogenesis in the olfactory epithelia and rectify the patients' loss of smell. This theoretical application could be especially pertinent for elderly adults, whose natural sense of smell wanes with aging. Future research will be imperative for reducing off-target side effects of CYP in the olfactory epithelia (such as pre-treatment with amifostine), and also for indicating new uses of CYP (such as intensifying neurogenesis in naturally-occurring olfactory pathologies).

In conclusion, our study demonstrates that the nonspecific cytotoxic properties of CYP affect proliferative cells in the murine olfactory system, and that quiescent basal cells may have a novel role in injury-induced neurogenesis,

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distinct from normal physiological conditions. We hope that our study will

serve as a conduit between basic research and clinical outcomes in the fight

against cancer.

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APPENDIX

Table 1: Levene's Test for Equality of Variances and Two-Sample _t_-Test for Equality of Means for the Number of Ki67-Positive Cells and the Number of Co-Localizations of Keratin 5-Positive and Ki67-Positive Cells in the Murine Main Olfactory Epithelium of CYP-Injected Mice and Saline-Injected Mice over 45 Days Post Injection. Levene's test and a two-sample _t_-test were performed to analyze the differences in number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive and Ki67-positive cells in the Main Olfactory Epithelium (MOE) of CYP-injected and saline-injected mice one, two, six, 14, 30, and 45 days post injection. Levene's test was performed to determine if the number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive and Ki67-positive cells in the MOE of CYP-injected mice and saline-injected mice retained equal variances at days one, two, six, 14, 30 and 45 post injection. A two-sample _t_-test was performed to determine if the mean number of Ki67-positive cells and the mean number of co-localizations of Keratin 5-positive and Ki67-positive cells in the MOE of CYP-injected and saline-injected mice were equal at days one, two, six, 14, 30 and 45 post injection. The alpha level for both tests was 0.050. If the reported p-value of a test was less than the alpha level, the result was recognized as statistically significant.

		Levene's Test		Two Sample _t_-Test			
		F	Sig.	_t_	df	Sig.	Mean Difference
DAY 1 Post Injection	MOE Sensory Zone: _Number of Ki67-Positive Cells_	0.17	0.68	2.17	41	0.02*	3.5868
	MOE Sustentacular Zone: _Number of Ki67-Positive Cells_	0.31	0.58	0.85	41	0.38	0.5155
	MOE Sensory Zone: _Number of Co-Localized Cells_	0.04	0.84	-0.50	41	0.62	-.8534
DAY 2 Post Injection	MOE Sensory Zone: _Number of Ki67-Positive Cells_	0.31	0.59	-5.18	37	0.00*	1.3714
	MOE Sustentacular Zone:	1.22	0.29	-1.48	37	0.15	0.9002

	Number of Ki67-Positive Cells						
DAY 6 Post Injection	MOE Sensory Zone: *Number of Ki67-Positive Cells*	0.00	0.98	-1.27	24	0.22	-3.0931
	MOE Sustentacular Zone: *Number of Ki67-Positive Cells*	0.01	0.90	-2.64	24	0.01*	-2.3904
	MOE Sensory Zone: *Number of Co-Localized Cells*	0.33	0.57	-3.51	24	0.00*	-3.0595
DAY 14 Post Injection	MOE Sensory Zone: *Number of Ki67-Positive Cells*	0.31	0.58	2.31	24	0.02*	3.8161
	MOE Sustentacular Zone: *Number of Ki67-Positive Cells*	4.17	0.06	0.85	24	0.33	1.0113
	MOE Sensory Zone: *Number of Co-Localized Cells*	7.66	0.01	2.35	24	0.03*	4.7858
DAY 30 Post Injection	MOE Sensory Zone: *Number of Ki67-Positive Cells*	0.17	0.68	2.89	20	0.00*	1.9716
	MOE Sustentacular Zone: *Number of Ki67-Positive Cells*	0.00	0.93	2.13	20	0.04*	0.6373
	MOE Sensory Zone: *Number of Co-Localized Cells*	0.61	0.44	3.39	20	0.00*	1.0636
DAY 45 Post Injection	MOE Sensory Zone: *Number of Ki67-Positive Cells*	2.61	0.11	0.08	25	0.92	0.0839
	MOE Sustentacular Zone: *Number of Ki67-Positive Cells*	0.09	0.75	0.24	25	0.80	0.0946
	MOE Sensory Zone: *Number of Co-Localized Cells*	1.57	0.23	1.16	25	0.24	0.9419

Table 2: Levene's Test for Equality of Variances and Two-Sample *t*-Test for Equality of Means for the Number of Ki67-Positive Cells and the Number of Co-Localizations of Keratin 5-Positive and Ki67-Positive Cells in the Murine Vomeronasal Organ of CYP-Injected Mice and Saline-Injected Mice over 45 Days Post Injection.

Levene's test and a two-sample *t*-test were performed to analyze the differences in number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive and Ki67-positive cells in the Vomeronasal Organ (VNO) of CYP-injected and saline-injected mice one, two, six, 14, 30, and 45 days post injection. Levene's test was performed to determine if the number of Ki67-positive cells and the number of co-localizations of Keratin 5-positive and Ki67-positive cells in the VNO of CYP-injected mice and saline-injected mice retained equal variances at days one, two, six, 14, 30 and 45 post injection. A two-sample *t*-test was performed to determine if the mean number of Ki67-positive cells and the mean number of co-localizations of Keratin 5-positive and Ki67-positive cells in the VNO of CYP-injected and saline-injected mice were equal at days one, two, six, 14, 30 and 45 post injection. The alpha level for both tests was 0.050. If the reported p-value of a test was less than the alpha level, the result was recognized as statistically significant.

		Levene's Test		Two Sample *t*-Test			
		F	Sig.	*t*	df	Sig.	Mean Difference
DAY 1 Post Injection	VNO Sensory Zone: *Number of Ki67-Positive Cells*	0.31	0.59	-2.14	14	0.05*	-0.6662
	VNO Sustentacular Zone: *Number of Ki67-Positive Cells*	0.20	0.66	-1.66	14	0.04	-1.3490
	VNO Marginal Zones: *Number of Ki67-Positive Cells*	2.88	0.11	-0.34	14	0.73	-0.7553
	VNO Sensory Zone: *Number of Co-Localized Cells*	2.46	0.13	-1.33	14	0.20	-0.1114
	VNO Marginal Zones: *Number of Co-Localized Cells*	0.66	0.43	-1.73	14	0.01*	-1.4225
DAY 2	VNO Sensory Zone:	0.05	0.82	-4.37	17	0.00*	-1.0580

Post Injection	*Number of Ki67-Positive Cells*						
	VNO Sustentacular Zone: *Number of Ki67-Positive Cells*	19.1	0.00	-2.90	17	0.01*	-3.4117
	VNO Marginal Zones: *Number of Ki67-Positive Cells*	0.26	0.61	-1.80	17	0.04*	-2.9409
DAY 6 Post Injection	VNO Sensory Zone: *Number of Ki67-Positive Cells*	1.85	0.19	-2.13	16	0.04*	-0.5993
	VNO Sustentacular Zone: *Number of Ki67-Positive Cells*	0.02	0.89	-3.04	16	0.00*	-1.0427
	VNO Marginal Zones: *Number of Ki67-Positive Cells*	0.41	0.53	-0.41	16	0.68	-0.3012
	VNO Sensory Zone: *Number of Co-Localized Cells*	0.14	0.71	0.13	16	0.89	0.0144
	VNO Marginal Zones: *Number of Co-Localized Cells*	2.11	0.17	-1.45	16	0.17	-1.0077
DAY 14 Post Injection	VNO Sensory Zone: *Number of Ki67-Positive Cells*	0.38	0.55	-0.12	22	0.90	0.0356
	VNO Sustentacular Zone: *Number of Ki67-Positive Cells*	0.05	0.83	-0.50	22	0.58	-0.3891
	VNO Marginal Zones: *Number of Ki67-Positive Cells*	15.1	0.00	3.16	22	0.01*	5.2782
	VNO Sensory Zone: *Number of Co-Localized Cells*	1.60	0.22	0.39	22	0.70	0.0608
	VNO Marginal Zones: *Number of Co-Localized Cells*	5.79	0.03	1.00	22	0.33	0.7318
DAY 30 Post Injection	VNO Sensory Zone: *Number of Ki67-Positive Cells*	10.1	0.00	2.99	14	0.00*	1.3009
	VNO Sustentacular Zone: *Number of Ki67-Positive Cells*	4.71	0.04	1.47	14	0.04*	0.9865
	VNO Marginal Zones: *Number of Ki67-Positive Cells*	0.16	0.69	1.42	14	0.17	1.8248
	VNO Sensory Zone: *Number of Co-Localized Cells*	0.26	0.61	1.15	14	0.24	0.1651
	VNO Marginal Zones: *Number of Co-Localized Cells*	0.01	0.94	1.21	14	0.24	0.4791
DAY 45 Post Injection	VNO Sensory Zone: *Number of Ki67-Positive Cells*	6.38	0.02	1.77	16	0.03*	1.9413
	VNO Sustentacular Zone: *Number of Ki67-Positive Cells*	3.31	0.07	3.08	16	0.00*	4.0031
	VNO Marginal Zones: *Number of Ki67-Positive Cells*	0.91	0.35	1.51	16	0.03*	3.1887
	VNO Sensory Zone: *Number of Co-Localized Cells*	3.77	0.07	2.98	16	0.00*	0.5863
	VNO Marginal Zones: *Number of Co-Localized Cells*	3.38	0.08	2.30	16	0.01*	1.4235