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Anatomical Analysis of Olfactory Sensory Neuron Regeneration Via Glomerular Synaptic Activity Markers in Adult Mice

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By

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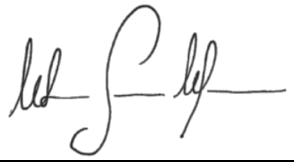

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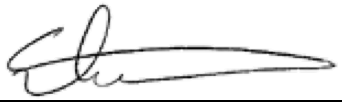

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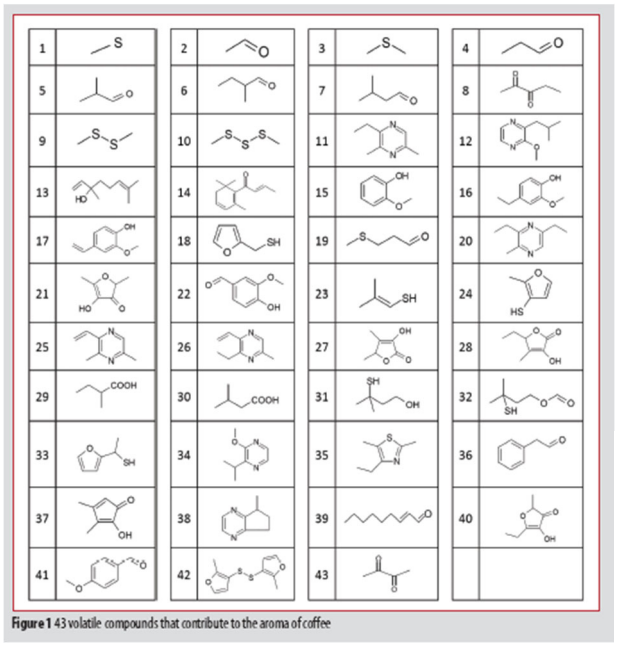
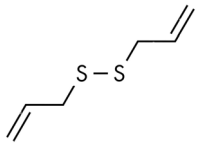
Abstract

The olfactory system is a great model for studying regeneration due to the olfactory epithelium's regenerative capability which makes it a potential a source of neural stem cells. The olfactory epithelium presents three types of cells: sustentacular cells which provide support and act as glial supporting cells; olfactory sensory neurons that are in charge of detecting odorant molecules in the air; and the stem cells that generated the aforementioned cell types. Olfactory sensory neurons are constantly dying and being replaced by new neurons originating from the stem cells that lie at the base of the olfactory epithelium. We have used an injury model that allows us to remove all the olfactory sensory neurons from the olfactory epithelium, via a single injection of methimazole. Then, at different timepoints after injury we measure the functional recovery of the olfactory epithelium by analyzing the expression of specific synaptic associated markers. Specifically, we analyzed the expression of synaptophysin, tyrosine hydroxylase, vesicular glutamate transporter 1, and vesicular glutamate transporter 2. Simultaneously, we measured glomerular size in order to serve as an indicator of anatomical recovery. Finally, we correlate these findings with previously generated data in the lab associated with functional recovery through behavior.

Introduction

The mammalian olfactory system is a robust, and adaptable sensory system responsible for detecting chemical compounds in the environment and transduces these stimuli into olfactory sensory information. Chemical compounds, as they pertain to olfaction, are called odorants. Odorants bind to odorant receptors (OR) that are seven transmembrane G-couple receptors found on the cell membranes of olfactory sensory neurons (OSN). The combination of multiple activated OSNs forms a unique chemical signature. Odors, however, can be the result of one odorant activating an OSN type or it can be the result of multiple activated OSN types forming a unique chemical signature (garlic versus coffee for example, **Figure 1**).





diallyl disulfide

Figure 1: List of 43 volatile compounds that contribute to the aroma of coffee vs the volatile compound that characterizes garlic smell.

https://www.newfoodmagazine.com/article/2565/the-smell-of-coffee-an-analytical-perspective/

Olfactory Epithelium

Odorant perception begins within the olfactory epithelium (OE), which is located deep in the nasal cavity. The OE contains four different types of cells which are crucial to odorant perception and higher olfactory processing. Two of these cell types, globose basal cells and

horizontal basal cells are found within the basal cell layer of the OE[1,2]. The third cell type found within the OE, the sustentacular cells. These cells are a non-sensory cell type that function as supporting cells for the other epithelial cell types. Both globose basal cells and horizontal basal cells are stem cells which act as progenitors for the OSNs and the sustentacular cells. As the progenitor cells develop, they migrate superficially towards the surface of the OE and differentiate first into neuroblasts. The neuroblasts continue to develop into immature and mature sensory neurons later. OSNs extend their apical dendrite to the surface of the OE making contact with the nasal cavity where they extend multiple cilia. Simultaneously, the OSN projects a thin axon deep to the OE, into the underlying connective tissue named lamina propria, and through the cribriform plate to establish contact with the olfactory bulb (OB)[3].

The Olfactory Bulb

The mammalian OB is composed of several layers and is the primary sensory organ of the olfactory system. Though OSNs express only one OR, theoretically, one odorant can bind to multiple OSNs and a single OSN can bind multiple odors[4] (**Figure 2**). The OSN axons coalesce into the glomerular layer (GL), which is one of the external most layers in the olfactory bulb. The GL lies beneath the olfactory nerve layer (ONL), which is the most external layer of the OB[5] (**Figure 3**). The OSN axons coalesce into structures called glomeruli, which are structures mostly void of cell bodies and contain various extensions and processes from other

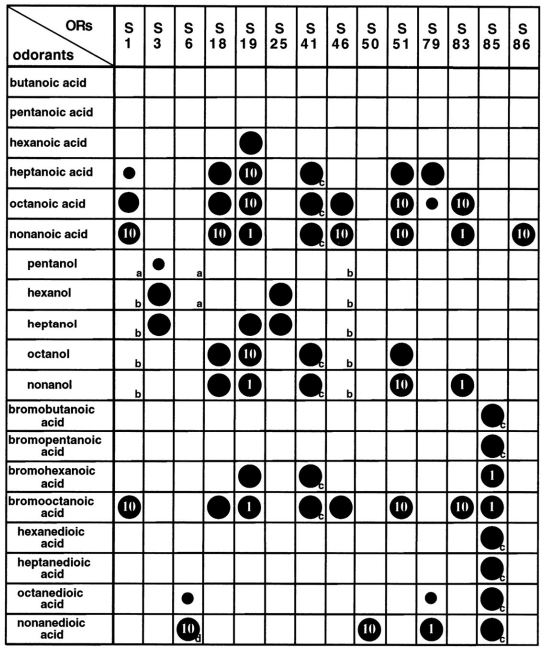


Figure 2: The Recognition Profiles of 14 Olfactory Neurons and the odorant receptors they expressed (Malnic et al., 1999).

neurons[6]. Within one glomerulus the OSN axons synapse with the apical dendrite of a mitral cell. The mitral cell projects its axon to higher olfactory processing areas within the cortex. The axons within the glomerulus of an adult mouse are homogenous and the ORs expressed by their OSNs are all identical[6]. It is the OR that in part determines which glomerulus the OSN axons coalesce in[7].

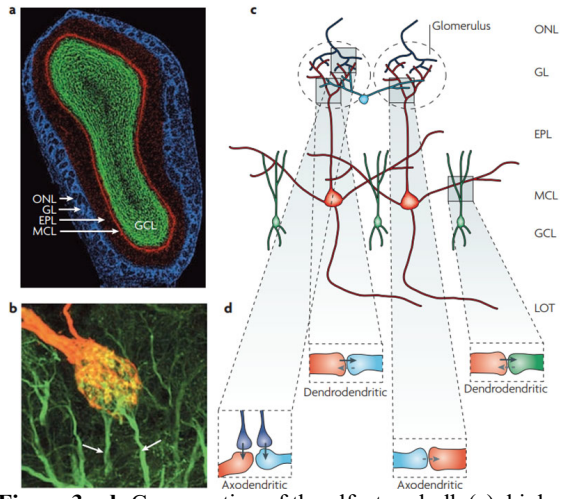


Figure 3a-d: Cross section of the olfactory bulb (a), high magnification of a glomerulus (b), and cartoon representation of the olfactory bulb's connectivity(c-d).

In early post-natal mice, however, it is common for there to be a heterogenous mixture of axons belonging to OSNs that express different ORs[8]. For each odorant receptor, there are two glomeruli per olfactory bulb, one on the medial and one on the lateral side. Current research shows that the glomeruli are not a predetermined targets for the OSN axons to reach. Rather, glomeruli formation is the result of the coalescence of the OSN axons[5]. Found in the glomeruli, in addition to OSN axon-mitral cell synapses, are dendritic processes from periglomerular cells (PeriG). PeriG cells are interneurons that surround the glomeruli in the GL. Some PeriG cells extend dendritic processes into the glomeruli and establish connection with OSN axons while others establish dendrodendritic synapses with either mitral or tufted cells[9]. The purpose of these connections is to receive input from the OSN axons and to inhibit mitral/tufted cells dendrites to mediate interactions/input within the glomeruli.

The specific interest for this project lies in the glomeruli to analyze olfactory system recovery post injury. In addition to analyzing the size (height and width) of the glomeruli, specific molecular indicators of neural activity were fluorescently labeled to further assess the

status of the olfactory system post injury. Molecular markers were used to label various structures in the olfactory system, specifically within the glomeruli.

Olfactory Regeneration & Injury Model

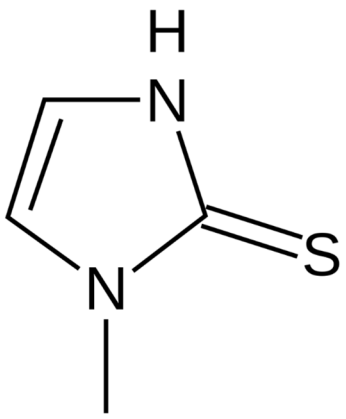


Figure 4: Chemical structure of methimazole a hyperthyroidism-treating drug belonging to the thioureylene family. Methimazole is olfactotixic and when used, in a dose-dependent manner, in mice it causes complete destruction of the olfactory epithelium and the olfactory sensory neurons within it. For this reason, methimazole serves as a great model for studying the effects of loss-of-smell due to injury and the subsequent olfactory sensory neuron regeneration.

The olfactory system itself is robust and has an extremely strong neuroplastic nature. The olfactory system features multiple cell populations that exhibit continuous turnover even into adulthood (OSNs in the periphery, and PeriG cells and granule cells in the CNS)[10]. This regeneration is a part of normal system maintenance in addition to system repair following major insult or injury. Research shows that the OSNs regenerate and reinnervate the OB even in the event of total OSN obliteration. There are several methods of injury models used in olfaction research such as olfactory bulbectomy, olfactory nerve sectioning, or using chemicals (zinc sulfate ($ZnSO_4$) intranasal irrigation of a detergent, methyl bromide (MeBr) gas inhalation, or the intraperitoneal injection of olfactoxic chemicals such as Methimazole[11]. The chosen injury model for our lab is the use of intraperitoneal injection of the olfactotoxic chemicals, methimazole.

Methimazole

Methimazole is a hyperthyroidism-treating drug belonging to the thioureylene family. Methimazole was chosen as the favored injury model for several reasons which all contribute to efficiency. The damage induced by methimazole is dose-dependent, which means that it is

extremely easy to control. The damage induced by methimazole (due to the dose-dependent nature) is highly reproducible. The damage caused by methimazole is also homogenous following the dose-dependent nature. Lastly, intraperitoneal injection was chosen as the favored method due to not needing any special equipment or protocol for olfactotoxic drug administration, while minimizing animal pain and distress[11]. Our lab also chose to use the methimazole injury model as it was the only way to induce effective injury without disrupting the aims of our intended investigation[12].

Specific Aims

Research into the olfactory system's neuroplasticity and adaptable nature are not new to the world of neuroscience. However, there has yet to be an investigation of this nature that incorporates the relationship shared between the odorant, OE, OB, olfactory nerve glomeruli. The desire to investigate what happens to each of these elements collectively following an extreme insult is why many of the common place olfactory injury models were not considered candidates for use in our lab. The aims of this paper are a part of a much larger project that sought to analyze those olfactory systems that had experienced great insult against those who had not experienced such catastrophe. The specific interest for this project lies in the glomeruli to analyze olfactory system recovery post injury. In addition to analyzing the size (height and width) of the glomeruli, specific molecular indicators of neural activity were fluorescently labeled to further assess the status of the olfactory system post injury.

Molecular markers were used to label various structures in the olfactory system, specifically within the glomeruli. For this project our lab used synaptophysin, tyrosine hydroxylase (TH), vesicular glutamate transporter 1 (VGlut1), and vesicular glutamate transporter 2 (VGlut2). Synaptophysin is a molecular marker for pre-synaptic terminals, and is

found in the OSNs, mitral cells (MCs), and PeriG cells. Therefore, in this work it is used as a generic marker for synapses. Changes in synapse numbers or density will reflect as changes in synaptophysin puncta. TH is the rate-limiting enzyme for dopamine production. When used in combination with a fluorescent antibody it is an excellent anatomical marker that allows for the labeling of dopaminergic neurons. For the interests of our lab, dopaminergic neurons within the olfactory bulb are indicative of interneurons in the olfactory system, specifically PeriG cells. Immunofluorescent-TH labeling specifically allows for the visualization of the PeriG cell bodies and dendritic processes within the glomerulus. Immunofluorescent-TH labeling is one of the many molecular markers which allows for the accurate analysis of the quality and thoroughness of the olfactory system's regeneration post-injury. In particular, TH in the olfactory bulb is an indirect marker of activity. In the absence of activity from the OE, TH expression decreases[13], and of relevance for our project, we were interested in analyzing the levels of TH in PeriG cells after injury to see whether or not they are reduced.

The vesicular glutamate transporters (VGlut) are proteins mostly found in the axon terminals, specifically in the axon terminals of excitatory neurons. These proteins have a high specificity for glutamate, the major excitatory neurotransmitter within the mammalian central nervous system[14]. The three subtypes of this glutamate transporter have been extensively delineated and have been shown to play a key role in exocytosis[15]. As a transporter, the different subtypes pack glutamate into synaptic vesicles in an activity-dependent manner and release it into the synapse. Each of the three subtypes of the VGlut protein (VGlut1-3) consist of 600 amino acid residues and share more than 70% homology with one another [15]. Our lab utilized VGlut1 and VGlut2 for immunofluorescent labeling of the olfactory system, specifically the MCs and the OSNs respectively. VGlut2 is present in the terminal boutons of the OSNs and is

used as a molecular marker OSN synapses within the olfactory system. VGlut1 is similar in function to VGlut2 however it is a molecular marker that is found within the dendritic processes of MCs inside the glomerulus. Labeling the OSNs and MCs of the olfactory system is essential for the analysis of the olfactory system's regeneration post-injury. Labeling the major cell populations within the glomerulus will allow for the thorough visualization of the olfactory system. Thus, varying levels of our various molecular markers will not only indicate the activity of the entire olfactory system, but it will also report the activity for specific cell populations within the olfactory system. The utilization of these molecular markers in immunofluorescence allows for accurate analysis and assessment of the mouse olfactory bulbs post-injury.

Olfactory regeneration research (specifically projects that utilize methimazole toxicity as an injury model) has been well established for decades. However, few if any laboratories have approached analyzing olfactory regeneration from the perspective of the glomeruli. The glomeruli encompass a wide variety of neuronal populations (both primary and secondary) that possess the ability to serve as indicators of olfactory system health and activity. Though the olfactory system is one of the few examples of continuous neuron regeneration in adulthood, the daily OSN turnover in a normal mouse is insignificant. This prompts the necessity of an injury model that will serve as a start point to analyze neuronal regeneration and axon extension. Extensive research supports our lab's decision to use methimazole as an olfactory toxicant. Methimazole induces a total removal of the olfactory epithelium and causes total destruction of the mapping and wiring of the olfactory system's primary and secondary neuron population. The total removal of the current cell populations within the animal model is crucial in order to visualize olfactory neuron growth, mapping, and wiring from 0 days post injection (DPI) until neuronal extension into the olfactory bulbs in the brain. Our lab utilized different age groups of

mice and compared the olfactory systems (particularly the aspects of the glomeruli) between injury-model mice and control mice. This specific project utilized mice that were 3 months old in various post-treatment groups of 3, 14, and 40 DPI in an attempt to contribute findings to this larger project of olfactory regeneration analysis via glomerular focus. This project intends to display that the olfactory system of adult injury-model mice experience complete regeneration of their olfactory system and regain their sense of smell. However, this project also intends to display that despite these findings, the recovered olfactory systems in injury-model mice possess glomeruli that are deficient in size and number when compared to their control group peers.

Materials and Methods

Animals

All procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Usage Committee of East Tennessee State University. Mice were anesthetized and transcardially perfused with phosphate buffered saline (PBS) with 1 unit/mL of heparin, followed by 4% (wt/vol) paraformaldehyde (PFA) in PBS at 4°C. Tissue remained in 4% PFA overnight at 4°C for post-fixation and was then transferred to PBS. Tissue was collected by the graduate student in the laboratory, David Poore.

Cryoprotecting & Cryostat Sectioning

The tissue was decalcified in saturated EDTA and then incubated in a solution of 30% (w/v) sucrose in PBS until it had sunk to the bottom of the tube. The samples were moved into a second tube containing a 50:50 solution of 30% sucrose:O.C.T. compound (Fisher Healthcare). The samples were placed on a rotator at 4.0 ºC. The dissected brains were kept on the rotator for 15 minutes while whole skulls were kept on the rotator for 25 minutes. After this, the samples

were moved into tubes containing 100% O.C.T. compound and placed on a rotator at room temperature (RT) adhering to the same time constraints as mentioned for the 50:50 tubes. Molds were prepared, labeled, and samples were placed within. 100% O.C.T. compound was then placed within the mold, completely covering the sample. The 100% O.C.T. compound was frozen in a mixture of liquid ethanol and dry ice. Once frozen, the dorsal side of the sample was marked with a permanent marker on the mold itself. The samples were stored at -80.0 ºC until sectioned. The samples were sectioned with a Leica CM 1850 frozen sectioning cryostat and were collected on microscope slides. These slides were stored at -20.0 ºC until needed for experimentation.

Immunohistochemistry

Frozen microscope slides were taken from the -20.0 ºC freezer and placed in an incubator (37.0 ºC) to thaw for approximately 5 minutes. After thawing, the O.C.T. compound was removed, and a hydrophobic barrier formed via siliconization. The samples were then block-permeabilized with 0.3 % PBS-T:BSA blocking buffer for 30 minutes in a humidified chamber. Post permeabilization, the blocking buffer was removed, and the samples were treated with primary antibodies (1º ABs) (**Table 1**). The 1º ABs were then allowed to incubate for 48 hours in a humidified chamber at RT. Post incubation, the samples were treated with a series of 0.3% PBS-T washes (3 times x 15 minutes). The samples were treated with secondary antibodies (2º ABs) as well as DAPI and allowed to incubate in a humidified chamber for approximately 1.5 hours (**Table 1**). Post 2º AB treatment, the samples were treated with another series of 0.3% PBS-T washes (2 times x 15 minutes) followed by a single wash in PBS (once x 15 minutes). Fluorescent mounting medium was added to the slides and the slides were cover slipped. The

slides were allowed to rest for approximately 2 hours and were sealed with nail polish. The slides were then stored in 4.0 ºC until analyzed via microscopy.

Antibodies Utilized for Immunohistochemistry				
Target	**1º Antibody**	**2º Antibody**	**Color**	**Company/Category #**
VGlut1	MsIgGαVGlut1	DkαMsIgG-555	**Red**	Synaptic Systems/135511
VGlut2	RbIgGαVGlut2	DkαRbIgG-488	**Green**	Synaptic Systems/135403
Synaptophysin	MsIgMαSyn	GtαMsIgM-555 GtαMsIgM-647	**Red** **Far Red**	Chemicon International/MAB329
Tyrosine Hydroxylase	RbIgGαTH	GtαRbIgG-488	**Green**	Chemicon International, Millipore/AB152
Cell Nuclei	DAPI	N/A	**Blue**	N/A

Table 1

Statistics

The data were analyzed by one-way ANOVA and multiple comparison posttests using GraphPad Prism and Microsoft Excel. A p value of less than 0.05 was considered significant (*: $P<0.05$).

Results

Glomerular size after methimazole treatment is reduced at 3 days post injection and at 14 days. However, at 40 days post injury the size of the glomeruli reestablishes a normal size making the difference from the test animals and control animals not statistically significant. After 40 days post injection (DPI) with methimazole treatment, the expression of TH (which is used as an indicator of synaptic activity) does not show significant changes (by ANOVA) over time when compared to control animals. After 40 DPI with methimazole treatment, the expression of Syn (which is used as an indicator of synaptic connectivity within the GL) increased at 14 days post treatment only. This could be indicative of the arrival of new axons establishing new synaptic connections. A total of 73 images were used for the collection and analyzation of the

data. From this group, images 43, 49, 51, and 57 were subjected to editing in order to better visualize the immunohistochemistry performed during experiments. Editing subjected to each image focused on enhancing the contrast between different layers and antibody color channels on the glomerulus. Each of the listed images were edited separately to minimize enhancing the images too much. Image 57 appears twice in order to visualize each layer within the olfactory

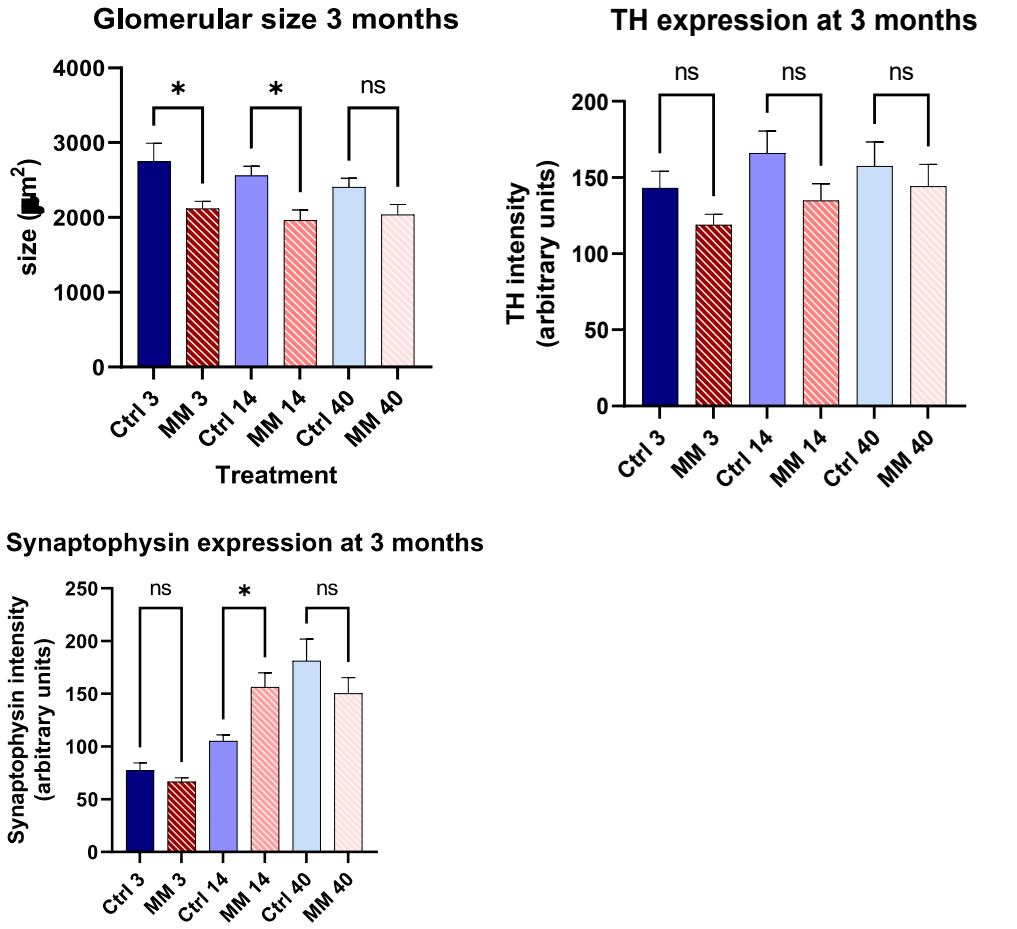


Figure 5a-c

Fig. 5a: After methimazole treatment, the size of the glomeruli is reduced at 3 days post injection and at 14 days. But at 40 days post injury the size recovers their normal size making it undistinguishable from control animals. *: P<0.05 by ANOVA and multiple comparison posttests.
Fig. 5b: After methimazole treatment, the expression of Tyrosine Hydroxylase (which is used as a measure of activity) does not show any changes (by ANOVA) over time compared to control animals.
Fig. 5c: After methimazole treatment, the expression of Synaptophysin (which is used as a measure of synaptic connectivity in the glomerular layer) only showed an increase at 14 days post treatment, which could be indicative of the arrival of new axons establishing new synaptic connections. *: P<0.05 by ANOVA and multiple comparison posttests.

bulb as well as a glomerulus for presentation purposes (**Figure 5a-e**). Statistics were performed on non-edited images.

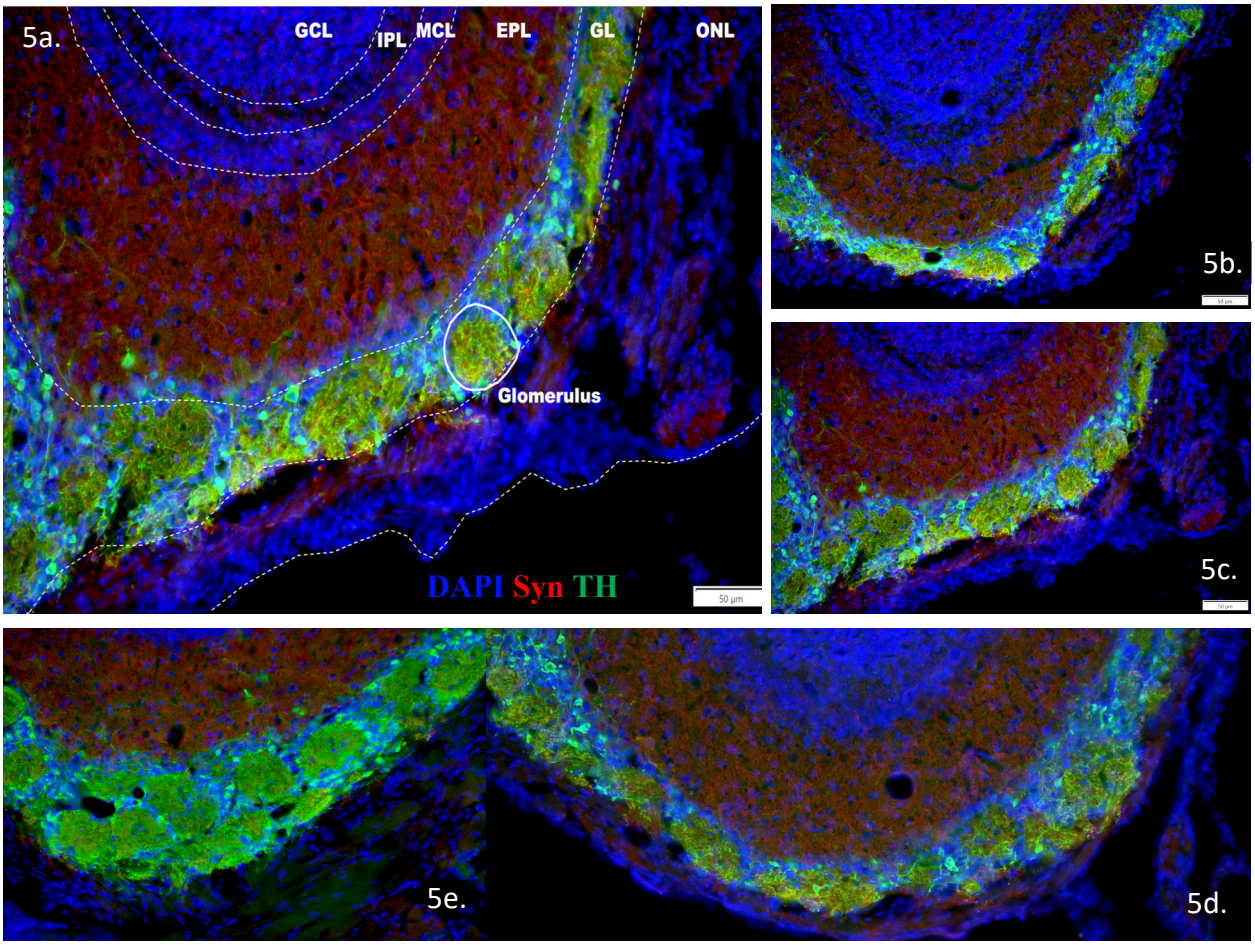


Figure 6a-e: Contrast adjusted; scale= 50 µm

Figure 6a: Image from one of the experiments conducted in the lab displaying the utilization of fluorescent antibodies in order to visualize structures of interest within the olfactory bulb. The image has been marked in order to show the layers of the olfactory bulb as well as a glomerulus. The layers, from outermost to innermost, are the olfactory nerve layer, the glomerular layer, the external plexiform layer, the mitral cell layer, the internal plexiform layer, and the granule cell layer. Color-coded antibody legend included (**Table 1**)

Figure 6b-e: Additional images of various olfactory bulb sections used in project experiments performed for the in order to visualize structures of interest within the olfactory bulb. Images were not marked up in order to compare back to **Figure 6a.**

Discussion

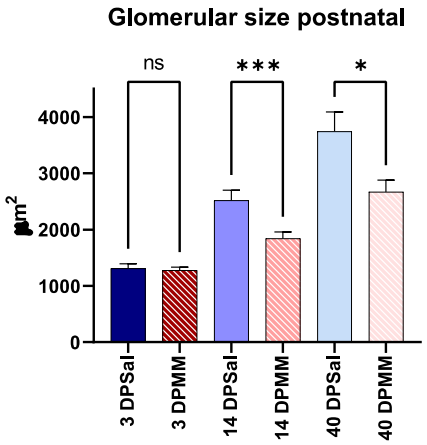


Figure 7: After methimazole treatment in young nursing mice, the size of the glomeruli is reduced at 14-and 40-days post injection. This would suggest that during early postnatal development when the size of the glomeruli is increasing, a damage to the olfactory system produces long lasting effects that take longer to recover. *: P<0.05 by ANOVA and multiple comparison posttests.

Data from previous experiments showed that glomerular size is most affected during early postnatal development, 7 days after birth, at both 14 and 40 days following methimazole injection (**Figure 7**). In our studies, glomerular size was affected during the first 14 days following methimazole injection, though glomerular size was restored to normal after 40 days in 3-month-old mice (**Figure 5a**). These findings were further supported with our behavioral data which displayed the animals recovering their sense of smell after 40 days (**Figure 8**).

Regardless of mice age, TH expression did not display any significant changes between the control group and test group. The data collected for mice aged 3 months (**Figure 5b**) in addition to data collected from prior experiments with other age groups within the lab (**Figure 9**) did not show differences in TH intensity across the chosen post-treatment time points. TH is an activity dependent enzyme, thus a lack of noticeable change in the expression of TH within glomeruli suggests that the chosen injury model does not induce enough damage to reduce TH expression within the olfactory system. Previous studies have shown effective reductions in TH expression by utilizing a different injury model (naris occlusion) though it required up to 30 days in order to reflect changes in TH expression. While in this last experimental approach the axons remain intact and some

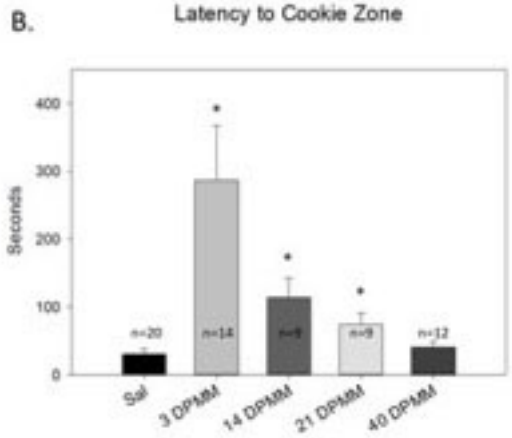


Figure 8: Hidden Cookie Test. B. Latency to the cookie zone. * P≤0.05 vs control.

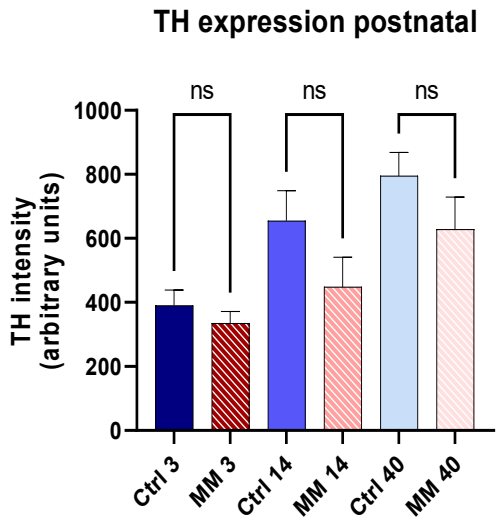


Figure 9: After methimazole treatment, the expression of Tyrosine Hydroxylase (which is used as a measure of activity) does not show any changes (by ANOVA) over time compared to control animals.

spontaneous activity remain, our model is more drastic than other known injury models because it induces the total destruction of all sensory neurons, and all spontaneous activity as well. However, despite this, a significant decrease in the expression of TH within the olfactory system is prevented by the process of newly generated OSNs reinnervating the olfactory bulb.

Compared to TH expression, Syn expression showed opposite results perinatally after injury in 3-month-old mice. In the 3-month-old age group our data shows an increase of Syn expression 14 days after injury (**Figure 5c**). However, during early postnatal development Syn expression showed a decrease in the first 14 DPI (**Figure 10**). It is possible that during early postnatal development, the olfactory system is more sensitive to injury than the olfactory systems of the developed adult. Potentially, synapses are more stable in the adult and the increase of Syn reflects the possibility that synapses are not lost during injury and that new ones are formed upon arrival of newly generated axons.

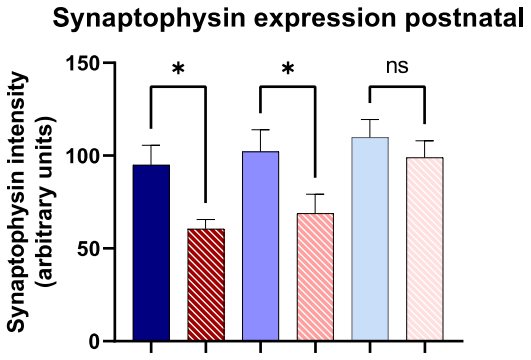


Figure 10: After methimazole treatment, the expression of Synaptophysin (which is used as a measure of synaptic connectivity in the glomerular layer) showed a decrease at 3- and 14-days post treatment and recovered at 40 days. *: P<0.05 by ANOVA and multiple comparison posttests.

Conclusion

The olfactory system's unique nature allows for it to adapt to even the most intrusive and destructive insults, making it an ideal candidate of study for regeneration research in order to further establish the

behavior of the primary neuron, secondary neuron, and interneuron populations. Research analyzing this unique feature of the olfactory system and its various neuron and supporting cell populations has been extensively established for decades. However, the effect that gross insult and injury have on the glomeruli and the synaptic connections within them have yet to be researched. Our lab originally hypothesized that the trends observed with the same synaptic activity markers used in the various early post-natal models would continue into the adult model. However, the data collected using the adult model does not support this original hypothesis. Rather the data collected suggests that the olfactory system of the adult model age group is less sensitive than those within the various early postnatal groups. This is supported with the level of TH expression within the adult model which was not significantly reduced compared to the TH levels in same age control groups. Despite our lab's chosen injury model of an intraperitoneal injection of methimazole that results in the total destruction of the OE, OSN, and the various synaptic connections made throughout the system from the OE to the OB the regeneration process begins immediately after the injury has occurred. OSNs are fast growing and achieve full extension into the OB within 8-10 DPI. This suggests that it takes a great length of time in order for TH expression to be affected within the olfactory system, further supporting its robust nature. Unlike data collected from the early postnatal models, Syn expression increased within the adult test models suggesting formation of new synaptic connections within the glomeruli despite massive system insult. This suggests that the adult olfactory system in mice is not drastically affected following a single traumatic event inducing anosmia via the total obliteration of the structure of the olfactory system.

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