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Investigation of Age Related Differences in the Rewiring of P2-Olfactory Receptor Neurons

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INVESTIGATION OF AGE RELATED DIFFERENCES IN THE REWIRING OF P2-OLFACTORY RECEPTOR NEURONS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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List of Abbreviations

AOB	Accessory Olfactory Bulb
CAM	Cell Adhesion Molecule
CTX	Cortex
DAB	3,3'-diaminobenzidine
E	Embryonic Day
EDTA	Ethylene diamine tetracetic acid
GPCR	G-Protein Coupled Receptor
IP	Intraperitoneal
IRES	Internal Ribosome Entry Site
LNTX	Left Olfactory Nerve Transection
μ	Mean Value
NC	Nasal Cavity
OE	Olfactory Epithelium
OR	Odorant Receptor
ORN	Olfactory Receptor Neuron
OMP	Olfactory Marker Protein
p	Probability
PLC	Phospholipase C

Abstract

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By Daniel Joseph Galante, BA

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Olfactory receptor neurons (ORNs) maintain the ability to regenerate. These neurons reside in the olfactory epithelium and project axons that connect to the olfactory bulbs. Despite the diffuse distribution of ORNs in the olfactory epithelium, they converge at discrete glomeruli in the olfactory bulb. In the P2 IRES tau-lacZ mouse, the P2 ORN subtype has been previously mapped to two glomeruli, using X-gal staining. To determine if age affects ORN regeneration, left olfactory nerve transections were performed on P2 mice from immature (five-weeks old) and mature (16-weeks old) groups. Following

recovery, the olfactory bulbs were processed to observe ORN regeneration. A significant difference was seen in the number and mapping of full P2 glomeruli between lesioned and control olfactory bulbs, but not between the age groups. This suggests that age differences between the two groups in this study were not large enough to affect the regeneration of P2 ORNs.

Introduction

Dysfunctions of the olfactory system are common among victims of head trauma. There are three major mechanisms leading to olfactory problems: shearing of the olfactory neurons as they emerge from the cribiform plate, physical obstruction of the nasal passage, and central brain damage. Shearing of the olfactory neurons caused by movement of the brain relative to the skull is the most common form of injury (Kern et al. 2000). This is often associated with blunt trauma to the head, as in a *coup-contrecoup* injury. Such trauma, and its effects can have a great impact on a patient's quality of life. Even so, it has been previously established that the olfactory system has the ability to recover from injury. Unlike other neurons, those in the olfactory system exhibit the ability to constantly regenerate, even following injury.

Anatomy and Physiology of the Olfactory System

In the olfactory system primary sensory neurons make direct contact with the outside environment. This is the only sensory system in the body that does not employ a series of secondary cells for transduction of a signal from the outside environment to the brain. Unlike gustation, where a small number of specific chemical qualities elicit specific tastes, olfaction utilizes the recognition of chemical groups that make up an individual odorant. This allows for the identification of many complex odors. Specific receptors for

unique chemical "signatures" are called Odorant Receptors (ORs). In humans there are approximately 500 ORs that make up the normal sense of smell, in comparison to 1000 subtypes in the mouse (Axel. 2005; Buck. 1996). Individual ORs are expressed on the dendrites of Olfactory Receptor Neurons (ORNs).

ORNs are located in the olfactory epithelium (OE) in the posterior region of the nasal cavity. The OE is composed of primary neurons (ORNs), as well as basal cells and supporting cells. The neurons are bipolar cells that extend a single dendrite to the surface of the epithelium in order to make contact with inhaled odorants. At the epithelial surface, the ORNs develop cilia that protrude into the mucus lining of the nasal cavity. The cilia create a large receptor surface area, facilitating contact with odorants. The axons of ORNs project from the epithelium, through the cribiform plate to the olfactory bulb, where they converge in discrete glomeruli (Figure 1). In a glomerulus, many ORNs expressing a single OR subtype converge and synapse with mitral cells. The mitral cells then compose the basis of the afferent pathway from the olfactory bulb to higher centers in the brain, including the anterior olfactory nucleus, the piriform cortex, the olfactory tubercle, the amygdala, and the entorhinal area (Buck. 1996; Graziadei, PPC and Monti Graziadei, GA. 1978).

Odorant receptors belong to a large family of G protein-coupled receptors (GPCR). Despite the diversity of the odorant receptor gene family, there is a fair amount of homology between OR subtypes. This may account for how different ORNs recognize similar, if not identical, odorants. Upon recognition of an odorant by a receptor, a cascade of signal transduction events is activated and ultimately leads to the depolarization of the

ORN and the generation of an action potential. The exact mechanism of this signal transduction is still under investigation. The two accepted pathways of odorant signal transduction hypothesized to work singularly, or in concert, to depolarize the neuron, are the traditional G-protein activated second messenger systems. Odorant binding to the receptor results in the release of a G-protein receptor subunit, which produces either an elevation of cAMP or activation of PLC. Termination of sensory transduction must occur in order to reset the receptor. Internalization or phosphorylation of the receptor, or persistent elevation of intracellular calcium are possible mechanisms used to stop the recognition of odorants (Buck. 1996).

Regeneration of Olfactory Neurons

Normally, the olfactory system undergoes a continuous process of cell turnover. ORNs are short-lived, and are continuously replaced by basal cells found in the olfactory epithelium (Buck. 1996). Ultrastructural and pulse-chase studies with ^{3}H thymidine, have shown regular degenerative and regenerative processes in the olfactory system. Following injection with ^{3}H thymidine, animals sacrificed within 24 hours of injection showed radiolabeled elements within the basal layer of the olfactory epithelium. Seven days post-injection, radiolabel was observed in developing neurons, and ten to 20 days post-injection the label was observed in mature ORNs (Graziadei, PPC and Monti Graziadei, GA. 1978). The regenerative capability of the neurons in the olfactory system has been further studied by cutting the olfactory nerves. Early experiments in frog have shown that complete disappearance of olfactory receptors occurs between one and 12 days following olfactory

nerve transection. This degeneration extended into the olfactory bulb. 20 days following injury, differentiation of basal cells into new ORNs was observed. Newly formed axons were generally smaller than the normal, uninjured axons, but the density of receptors in the neuroepithelium was similar to the pre-injury (Graziadei and DeHan. 1973).

Olfactory nerve transection is an excellent model of traumatic nerve injury because it causes minimal damage to the surrounding tissue and structures. This process results in complete severing of the olfactory nerves between the olfactory epithelium and the olfactory bulb. Following transection, degeneration of the nerves occurs. Costanzo observed that following olfactory nerve transection in hamster, degeneration reduced the number of nerve cells in the olfactory epithelium by 55%. This decline in cell numbers occurred during the first two to four days following injury. By ten days post-injury, the olfactory epithelium had begun to repopulate with olfactory receptor neurons (Costanzo. 1984). The recovery, however, is marred by improper reconnection of these regenerated neurons with the olfactory bulb, with multiple ORNs of multiple subtypes synapsing in the same glomerulus, instead of discrete glomeruli for each subtype (Astic and Saucier. 2001).

Reconnection of the olfactory epithelium to the olfactory bulb in hamster is anatomically complete by 40 days post-injury (Morrison and Costanzo. 1995; Yee and Costanzo. 1995). Yet despite this regeneration, there is often mistargeting of the new connections to the olfactory bulb (Costanzo. 2000; Costanzo. 2005). In contrast to normal development, where each ORN expressing a single OR converges in distinct glomeruli of the olfactory bulb, neurons regenerating after injury often synapse with multiple glomeruli and also with multiple receptor subtypes (Costanzo. 2005; Rawson. 2006).

P2 *IRES* tau-lacZ Model

As research in olfaction continues, individual odorant receptors have been identified. The P2 OR is one such example. In addition, the advances in molecular biology have allowed the construction of transgenic mice, including one that allows the visualization of the P2 neurons, the P2 *IRES* tau-lacZ mouse. In order to identify the P2 ORs, axons and glomeruli, the Internal Ribosome Entry Site (IRES) from a viral vector and a tau-lacZ fusion gene were inserted behind the P2 gene. This vector was then introduced into embryonic stem cells, and ultimately into the blastocysts of developing mice (Mombaerts et al. 1996). ORNs are translated from only one homolog allele; in order to ensure that all P2 ORNs were labeled with the tau-lacZ fusion protein, mice were crossed and backcrossed to ensure a homozygous, transgenic genotype. Early analysis of these animals has shown great efficacy in the mapping of individual P2 glomeruli in the olfactory bulb. It was observed that P2 ORNs, found in a widespread area of the olfactory epithelium, normally converge into two glomeruli in each olfactory bulb. One of these P2 glomeruli is located on the lateral aspect of the bulb, in a dorsal and anterior position. The second P2 glomerulus is found in a more posterior position, on the medial side.

The concept that particular odorant receptors converge at particular glomeruli in the olfactory bulb that can then be readily identified based on location in the bulb, is called odor mapping. Studies have shown that particular "maps" exist in the olfactory bulb that may reveal information on the coding and processing of odors. Odor mapping also extends into the olfactory epithelium where there are four zones expressing different patterns of

cell adhesion molecules (CAMs). This pattern in the olfactory epithelium correlates to the targeting of ORNs to glomerular locations in the olfactory bulb (Nagao et al. 2000).

Costanzo has shown though tissue whole-mount analysis, that following recovery from olfactory nerve transection, the axon convergence is significantly different from that in control mice (Costanzo. 2000; Costanzo. 2005). This altered convergence likely causes perturbation in the perception of smell (Yee and Costanzo. 1998). Using chemical ablation techniques to cause deafferentation of the olfactory bulbs, St. John et al. (2003) have demonstrated that regenerating P2 axons innervate inappropriate glomeruli, over a larger rostro-caudal and ventro-dorsal area of the olfactory bulb. Instead of the two distinct glomeruli in each olfactory bulb as previously described by Mombaerts et al., they observed that the P2 axons regenerate and converge into small loci at multiple sites. Nearly 40% of post-injury olfactory bulbs showed some deviation in numbers and locations of P2 connections in comparison to that of normal bulbs (St John et al. 2003).

Theoretically, during the process of regeneration after injury, there is competition between all of the actively regenerating olfactory receptor neurons to reestablish connections with the olfactory bulb. Due to the degeneration of the severed olfactory neurons following transection, there is also a lack of scaffolding to guide the ORNs as they regenerate toward the bulb. During recovery, multiple ORN connections of the same sub-type should converge into one or more glomeruli, reestablishing a functional sense of smell (Costanzo. 2000). However, following olfactory bulb deafferentation, the massive influx of regenerating ORNs may lead to a disorganized connection between the olfactory epithelium and the olfactory bulbs. Over greater lengths of time, the true expression

patterns of CAMs may lead to further reorganization and refinement of the ORN connections in the olfactory bulbs, resulting in a final map that more closely resembles the pre-injury map.

The Potential Role of Age in Regeneration

While the processes and events of olfactory degeneration and regeneration have been studied extensively, there is little research available on how maturity or age plays a role in these events. Researchers have shown how age affects the repair of injured nerves that do not normally regenerate. Shewan et al. have shown that retinal ganglionic cells extend to a greater degree along unmyelinated optic nerve tissue cultured from younger animals. While dorsal root ganglionic cells grew along both mature and immature optic nerve tracts, the amount of growth declined significantly with age (Shewan et al. 1995). Studies have also shown that younger mice and rats exhibit a faster and higher level of muscle reinnervation following sciatic nerve transection (Hess et al. 2006; Pestronk et al. 1980; Verdu et al. 1995). Research of neurons in brainstem explants from chicken embryos has shown that even during the embryonic development, there is a reduction in the ability of neurons to regenerate from as early as embryonic day (E) 19 (Blackmore and Letourneau. 2006).

Despite these findings, the studies did not investigate recovery following injury in cell types that undergo normal replacement, or that have the ability to regenerate following injury. In one study of olfactory regeneration, older hamster ORNs possessed the same ability to regenerate following transection in comparison to younger animals (Morrison

and Costanzo. 1995). This finding supports the theory that older animals can recover from olfactory nerve injury as well as younger animals. However, this study did not investigate the regeneration of a particular type of ORN, but rather the regeneration of all ORNs as they recovered. This implies that, while convergence of ORNs into discrete glomeruli may occur, it is unclear if individual ORNs expressing a particular OR subtype converged appropriately. Studies using horseradish peroxidase or olfactory marker protein (OMP) staining reveal regeneration of all olfactory neurons. However, these methods do not allow for the identification of particular ORN subtypes. Despite the reconnection of ORNs in the olfactory epithelium to the olfactory bulb, differences in odor mapping may still exist in the regeneration of specific ORNs in young and old animals. While ORNs may fully reconnect with the olfactory bulbs, if their connections are not appropriately organized, disturbances in the processing of odors may lead to dysosmias or even anosmia.

Rationale

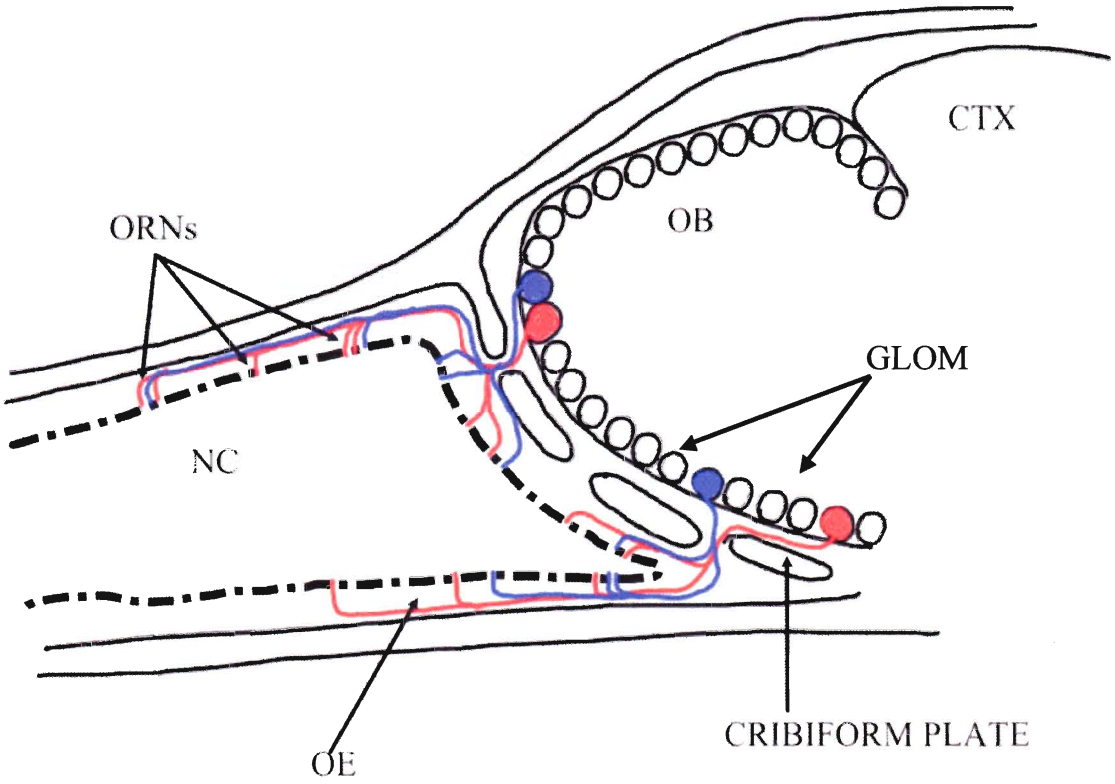
The ability of the olfactory system to recover from nerve injury has been studied in order to investigate both anatomical and functional recovery. While these two properties have been well documented across animals of varying ages, little work has been done to investigate if age actually affects the ability to recover following injury. Previous work by Costanzo and Morrison (1995) has shown that age does not affect the ability of ORNs to restore connections with the olfactory bulb following transection in the hamster, but reconnection of specific ORs were not the focus of the study.

Mapping and functional studies have confirmed that there are particular patterns of ORN innervation of the olfactory bulb, and that injury may affect this pattern. This would ultimately result in changes in the way odors are processed (Nagao et al. 2000; Yee and Costanzo. 1995). Could alterations in the mapping of ORNs cause the altered perception of odorants? If the connections of the ORNs to the olfactory bulb regenerate, but an altered sense of smell exists, changes in mapping of ORNs is a possible explanation.

Previous ORN mapping studies observed the total population of regenerated ORNs. However, this study investigates the regeneration and mapping of one ORN subtype in two different age groups. Does age affect regeneration and ORN mapping? Using a surgical olfactory nerve transection technique, the mapping of one particular OR (P2) was assessed between immature and mature P2 transgenic mice. If age-related differences in the mapping of the P2 ORNs exist, these findings would offer further insight into the ability of individuals of different ages to recover from injuries in the central nervous system.

Hypothesis: Following recovery from olfactory nerve transection, immature (five-week old) mice will exhibit a P2 odor map that more closely resembles a normal P2 odor map than mature (16-week old) mice. Specifically, immature mice will have the ability to better restore P2 mapping in the lesioned olfactory bulb than the mature mice- in terms of numbers of P2 glomeruli, degree of P2 innervation, and locations of P2 glomeruli- when compared to non-lesioned olfactory bulbs.

Figure 1. Diagram of the mammalian olfactory system. The posterior region of the nasal cavity (NC) is composed of the olfactory epithelium (OE). The cell bodies of odorant receptor neurons (ORNs) reside in the olfactory epithelium and extend a single, ciliated dendrite into the nasal cavity. The axons of ORNs converge into bundles and pass through the cribiform plate. Multiple ORNs of a single receptor sub-type converge in glomeruli (GLOM) in the olfactory bulb (OB). Second-order neurons then pass sensory information onto higher centers in the cortex (CTX).



Materials and Methods

Mice

Twenty-eight P2 IRES tau-lacZ mice (Mombaerts et al. 1996) were selected for use in this study. Selection of animals was random, without regard to sex. Twelve mice comprised the "mature" group (16-weeks old), and 16 mice comprised the "immature" group (five-weeks old). Four mice, two from each age group, were used as non-surgical control animals.

Olfactory Nerve Transection

A left olfactory nerve transection (LNTX) was performed on twenty-four mice using a previously described procedure (Yee and Costanzo. 1998). Mice were anesthetized with sodium pentobarbital (50mg/kg) IP and placed in a stereotaxic device. A skin incision exposed the skull above the olfactory bulbs, and the bone removed. Both the right and left olfactory bulbs were exposed, allowing the right bulb to serve as an internal surgical control. A Teflon transector blade was inserted between the left olfactory bulb and the cribiform plate, completely severing the axons that project from the olfactory epithelium to the olfactory bulb (Figure 2). Following transection, the skin incision was sutured, and the animal returned to its cage for recovery. All surgeries were performed by Dr. Richard Costanzo. Once awake and moving, the animals were returned to the colony

for a 40-day recovery period. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Histological Techniques

At 40 days post-surgery, the animals were deeply anesthetized with sodium pentobarbital and transcardially perfused with normal saline and 4% paraformaldehyde, and post-fixed for 15 minutes. Following decalcification in 0.3 M EDTA, brain tissue was cryoprotected in 30% sucrose, and frozen using liquid nitrogen and dry ice.

Olfactory tissue was cut into 30-micrometer thick coronal, serial sections on a Mircom HM550 cryostat. Sections were treated overnight with the X-gal staining technique in order to identify the P2 axons and glomeruli, and subsequently treated with immunohistochemical staining for olfactory marker protein (OMP). While all ORNs express OMP, the X-gal technique, in conjunction with OMP staining, allows for differentiation between P2 ORNs and all other ORN subtypes.

Following X-gal staining, sections were blocked in 4% Bovine Serum Albumin (BSA; ICN Biomedicals, Auora, OH), 5% non-fat dry milk in Phosphate Buffered Saline, pH 7.4 (PBS) and 10% normal rabbit serum (Invitrogen Corporation, Carlsbad, CA). Sections were then incubated in goat anti-olfactory marker protein (1:20,000; Wako Chemicals USA, Richmond, VA) overnight at 4˚ C. Subsequently, sections were incubated in biotinylated rabbit anti-goat IgG (1:50; Jackson ImmunoResearch Labs, West Grove, PA) and normal mouse serum (1:50, Jackson ImmunoResearch Labs, West Grove, PA) for one hour at room temperature. Sections were then incubated for one hour in

avidin-biotin-HRP (1:50; Vector Labs, Burlingame, CA), and reacted with Vector Labs "DAB Substrate Kit for Peroxidase" (Vector Labs, Burlingame, CA). Figure 3 illustrates the blue, X-gal stained P2 glomeruli in the olfactory bulb and P2 axons as they emerge through the cribiform plate. OMP immunohistochemical staining resulted in the brown glomeruli.

Data Analysis

Sections were photographed using Act-1 software (Nikon Corp, Tokyo, Japan) with a Nikon Eclipse E600 microscope and DXM1200 digital camera (Nikon Corp, Tokyo, Japan) and stored digitally. No other digital manipulation was performed on the images. The images were examined in order to locate all cases of P2 reinnervation of glomeruli in both the left and right olfactory bulbs. Tissue quality was assessed and categorized. Poor tissue (those animals lacking tissue sections, large amounts of degeneration, and poorly defined glomeruli) was excluded from analysis. The degree of P2 glomerular reinnervation was categorized as "full," "partial," or "trace" (Figure 4). A set of objective criteria was created to label the P2 glomeruli. Full P2 glomeruli were labeled as those that appeared to be more than 90% blue, compared to brown. Trace P2 glomeruli were those reinnervated by less than 10% of P2 ORNs. Partial P2 glomeruli were identified as those glomeruli with between 10 and 90% blue P2 stained, as compared to brown OMP stain.

The degree of P2 glomerular innervation was measured in both the left and right olfactory bulbs, and the data was rendered in tabular form (Figure 5). Numbers of P2

glomeruli were sorted by their degree of innervation (full, partial, trace), as well as their locations within the olfactory bulbs (medial, lateral).

Statistical Analysis

Non-surgical Animals

Independent samples t-tests were used to compare means of full, partial, and trace P2 glomeruli between the left and right olfactory bulbs and also between immature and mature mice. A p-value of less than 0.05 was used to define a significant difference.

Surgical Animals

Means of full, partial, and trace P2 glomeruli were compared between the control and lesioned olfactory bulbs from immature and mature age groups using an independent samples t-test. A p-value of less than 0.05 indicated significant differences.

The paired samples t-test was used to compare means of each type of P2 glomerulus between the medial and lateral halves of the olfactory bulbs. P2 glomeruli on the lateral half of the lesioned olfactory bulb were compared with those P2 glomeruli on the lateral half of the control bulb. The same operation was performed for P2 glomeruli on the medial halves of the lesioned and control olfactory bulbs. A multivariate ANOVA was also performed to investigate differences between lesioned and control bulbs when taking age, location, and P2 glomerular type into account simultaneously. P-values of less than 0.05 denoted significant differences.

Finally, the olfactory bulbs were broken into four basic quadrants (Figure 6) to investigate changes in P2 glomeruli mapping in the lesioned olfactory bulbs. Quadrants one and two were on the lateral side and quadrants three and four on the medial side. All full P2 glomeruli were mapped to one of the four quadrants. Relative numbers of full P2 glomeruli in each quadrant (QG) were compared to the total number of full P2 glomeruli in the olfactory bulb (GT). These ratios (QG/GT) were compared using independent samples t-tests. P-values of less than 0.05 indicated a significant difference.

Figure 2. Olfactory nerve transection technique. The insertion of a Teflon blade between the olfactory bulb and the cribiform plate results in the severing of the olfactory nerves. This shearing action is similar to that observed in *coup contrecoup* traumatic brain injury.

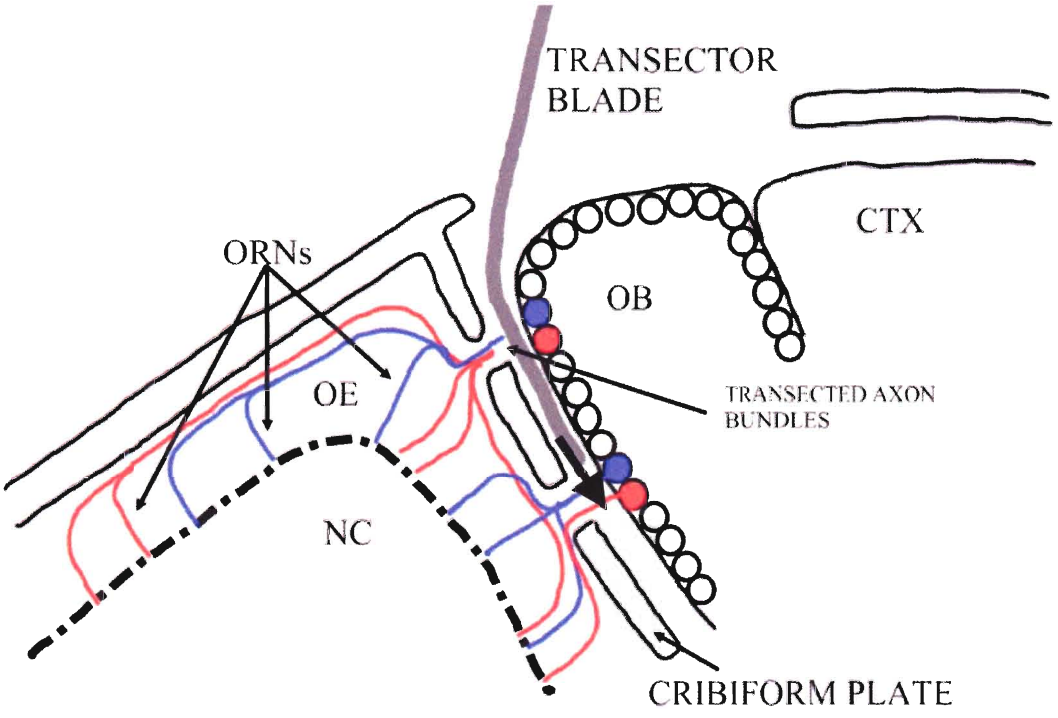


Figure 3. Histological section of the olfactory bulbs illustrating staining of P2 glomeruli. X-gal staining labels axons and glomeruli expressing the P2 odorant receptor gene with a dark blue stain. In contrast, all glomeruli expressing any OR subtype stain brown with OMP immunohistochemical staining. Arrow identifies a P2 glomerulus. The darker brown stain around the periphery of the two olfactory bulbs identifies glomeruli that are positive for OMP. Magnification=2x

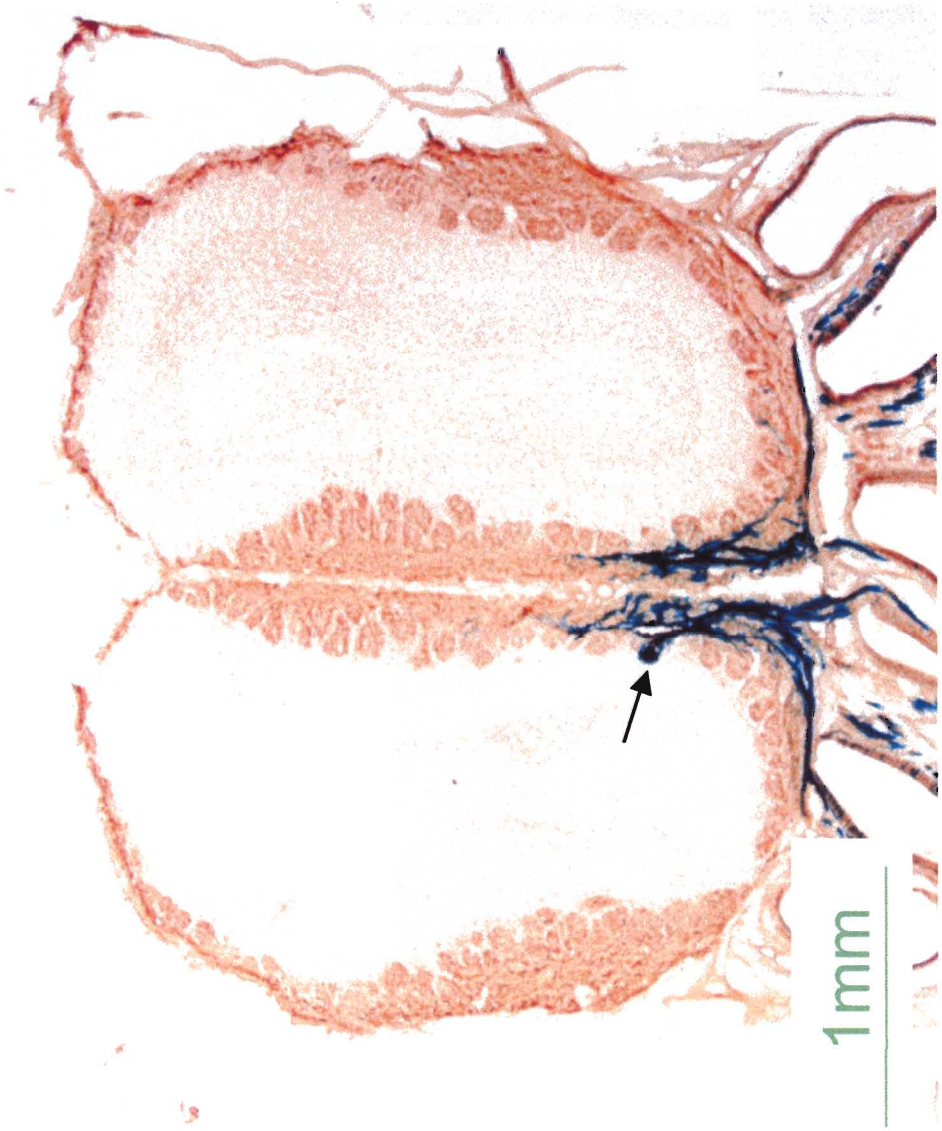

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Figure 4. Histological sections illustrating the three classifications of P2 glomerular types. A. Full P2 glomerulus (> 90% P2 innervation) B. Trace P2 glomerulus (< 10% P2 innervation) C. Partial P2 glomerulus (< 90%, > 10% P2 innervation)

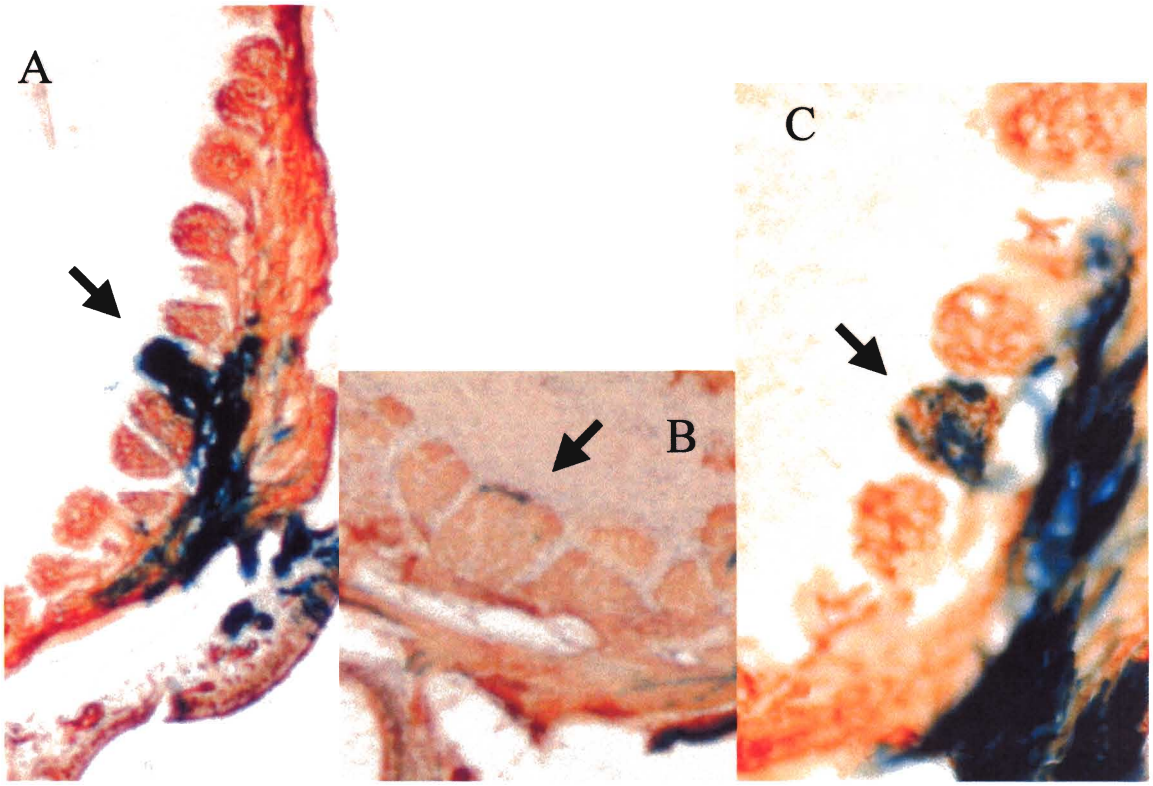
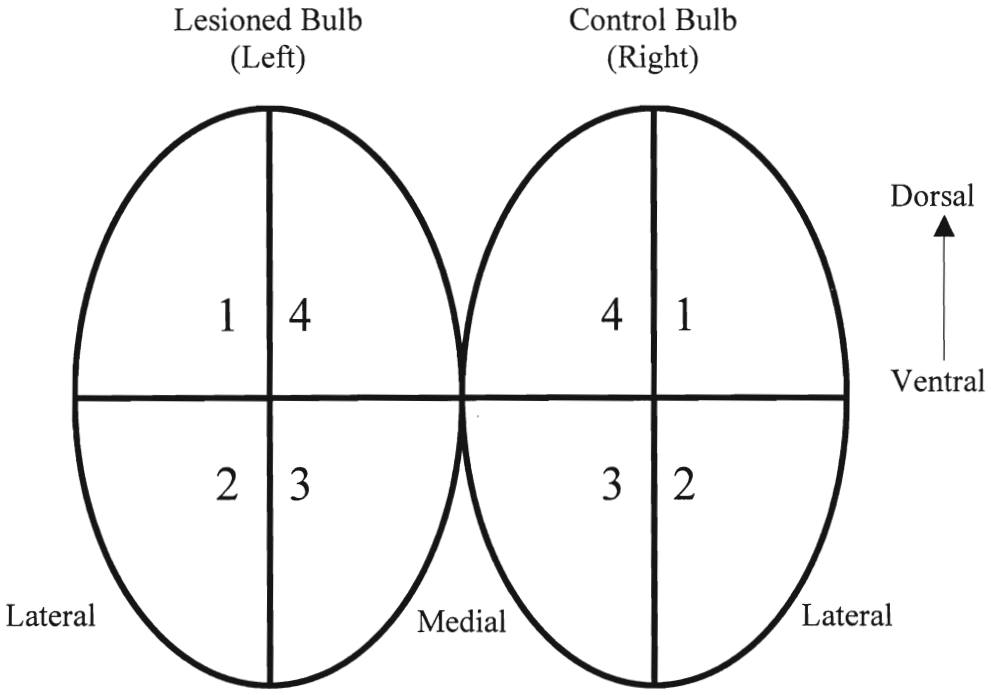


Figure 5. Sample data obtained from a typical surgical animal. (M3147- immature, surgical animal).

MOUSE NUMBER: M3147				
LESION (LEFT) BULB			CONTROL (RIGHT) BULB	
LATERAL	MEDIAL		MEDIAL	LATERAL
0	1	FULL	2	2
0	2	PARTIAL	2	2
2	3	TRACE	4	5

Figure 6. Quadrant map used to identify the position of full P2 glomeruli in lesioned and control bulbs. The locations of all full glomeruli were mapped to one of four quadrants.



Results

The numbers and relative locations of P2 glomeruli were compared between immature and mature mice to investigate age-related differences that may arise in P2 neuron regeneration during the recovery from olfactory nerve transection. Independent and paired samples t-tests and multivariate ANOVA comparisons between lesioned and control bulbs revealed an overall decrease in the number of full P2 glomeruli, but not in the numbers of partial or trace glomeruli in the lesioned olfactory bulb following recovery. While there was a difference between the lesion and control olfactory bulbs, there was no difference between the immature and mature mice following recovery from olfactory nerve transection. The locations of full P2 glomeruli on the medial or lateral aspect of the olfactory bulb were also compared using independent samples t-test. There was a change in the spatial distribution of P2 glomeruli on the lateral side of the olfactory bulb following transection. However, there were no age-dependent medial-lateral differences in the mapping of P2 glomeruli following regeneration between five and 16-week old mice following olfactory nerve transection.

X-gal staining in the histological sections of the epithelium suggested that the overall density of P2 ORNs in the olfactory epithelium was reduced when comparing the lesioned and non-lesioned sides. While the data was not quantitatively analyzed, a qualitative assessment showed that there is a reduction in the numbers of P2 receptors

following recovery from nerve transection (see pg 49 in appendix A for histological example).

Non-surgical Animals

In order to determine if differences in numbers of P2 glomeruli between the left and right olfactory bulbs in non-surgical animals existed, glomeruli from both the immature and mature non-surgical animals were combined. An independent samples t-test was used to evaluate differences between the two olfactory bulbs. No significant differences were found between left and right bulbs. Table 1 shows means of full, partial, and trace P2 glomeruli from the left olfactory bulb (6.5, 2.0, and 5.75 respectively) in comparison to those from the right bulb (7.5, 2.25, and 6.25 respectively).

In order to test for possible age-related differences between the immature and mature groups in the non-surgical animals, the numbers of full, partial, and trace glomeruli from both the left and right bulbs were pooled for each age group. The two age groups were then compared to investigate differences in the numbers of P2 glomeruli. No significant differences were found between the immature and mature non-surgical animals in the numbers of full, partial, or trace P2 glomeruli in both olfactory bulbs. Table 2 shows means of full, partial, and trace P2 glomeruli from the immature non-surgical mice (6.75, 2.25, and 7.5 respectively) and mature non-surgical mice (7.25, 2.00, and 4.50 respectively). Overall, there were no differences between the right and left bulbs or between the immature and mature non-surgical mice.

Differences in Numbers and Types of Glomeruli in Surgical Groups

Since no significant differences were found between immature and mature non-surgical mice, the numbers of full, partial, and trace P2 glomeruli were pooled from both bulbs in the immature and mature animals. The means for each type of glomerulus were compared using the independent samples t-test, which showed a significant difference between the number of full P2 glomeruli in the lesioned olfactory bulb and the control bulb; the mean number of full glomeruli in the lesioned bulbs ($\bar{x}$=4.17) was significantly less than in the control ($\bar{x}$=6.83) bulbs ($p<0.001$). No significant differences between the mean numbers of partial and trace glomeruli in the lesioned, 2.38 and 6.17 respectively, and control bulbs, 2.79 and 5.54 respectively, were found ($p=0.317, 0.396$) (figure 7). Following LNTX, there was a significant reduction in the number of full P2 glomeruli in both immature and mature groups.

An independent samples t-test was used to determine if the reduction in full P2 glomeruli following LNTX was dependent on age differences. The numbers of full, partial, and trace glomeruli were compared between immature and mature surgical animals in the lesioned and control olfactory bulbs. No significant differences were found between the immature and mature animals in either the control or lesioned bulbs (figure 8). A multivariate ANOVA test was also performed for simultaneous comparison of numbers of P2 glomeruli, by age and by bulb. No significant differences were found, indicating that there is no difference in numbers of P2 glomeruli between the immature and mature mice.

It has previously been reported that numbers of P2 glomeruli on both the medial and lateral sides of the olfactory bulbs were identical: one medial and one lateral

(Mombaerts et al. 1996). Throughout the initial analysis of the data, the numbers of glomeruli were pooled between the lateral and medial sides of each olfactory bulb. However, during the process of left olfactory nerve transection, there is a possibility of damaging the medial side of the right (control) olfactory bulb. The particular movement of inserting the blade along the medial surface of the left bulb creates a window of opportunity to damage the medial surface of the right bulb. To ensure that the right bulb was not significantly damaged on the medial side, or that the damage to the left bulb did not affect the medial aspect of the control bulb, the numbers of glomeruli were compared by lesion and location in each bulb.

Paired samples t-tests were performed in order to compare the numbers of P2 glomeruli between the lesioned and control olfactory bulbs on the lateral or medial sides. The comparison of medial and lateral sides between the lesioned and control bulbs revealed a significant difference in the numbers of full P2 glomeruli on both the lateral ($p<0.01$) and medial sides ($p<0.01$) (figure 9A). following injury, there is an overall decrease in the number of full P2 glomeruli, which is seen on both the lateral and medial sides of the lesioned olfactory bulb. Comparisons between the glomeruli on the lateral and medial sides of the control or lesioned olfactory bulbs revealed no significant differences (figure 9B). This indicates that injury does not result in a change in the numbers of P2 glomeruli between lateral and medial halves of the lesioned bulb.

Differences in Mapping of Glomeruli in Surgical Groups

Due to the variation in the number and types of the P2 glomeruli found in the lesioned and control bulbs and in both age groups, the locations of these glomeruli were categorized to examine any differences in the targeting of the regenerated P2-ORNs. Ratios of full P2 glomeruli in each quadrant (QG), relative to the total number of full P2 glomeruli in each olfactory bulb (GT) were calculated: QG/GT. Ratios of innervation were used, rather than mean numbers of glomeruli, in order to normalize the data for the overall reduction in the numbers of full P2 glomeruli in the lesioned olfactory bulbs. Independent samples t-tests reveal that while quadrant one was not innervated by any P2 ORNs in control bulbs, this quadrant contained significantly more (22.2% of total) regenerated full P2 glomeruli in lesioned bulbs ($p=0.002$). In control bulbs, 47% of all full P2 glomeruli were mapped to quadrant two. In lesioned bulbs only 23.7% of the total full P2 glomeruli were mapped to quadrant two. This represents a significant decrease in the number of glomeruli mapping to quadrant two ($p<0.001$) (figure 10). Overall, the data indicate a shift in the mapping of glomeruli on the lateral side of the olfactory bulb (quadrants one and two), while the glomeruli on the medial side of the bulb did not change ($p=0.89$).

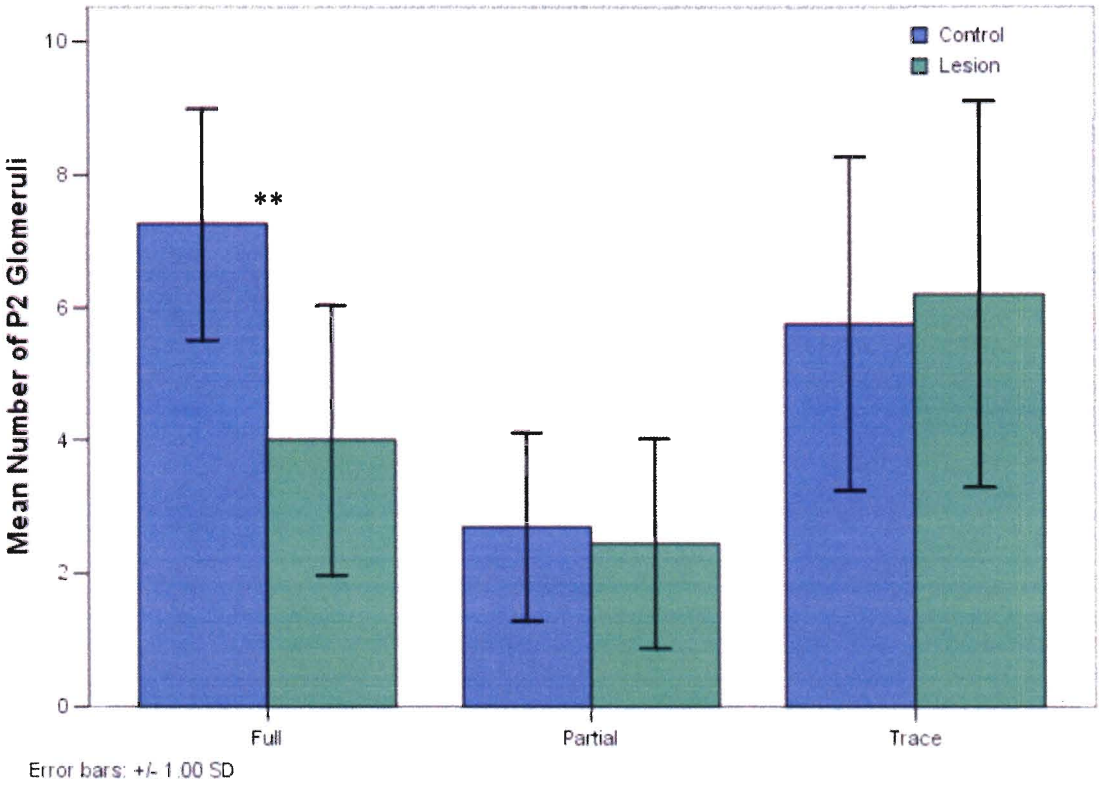
Table 1. Comparison of P2 glomeruli in left versus right olfactory bulbs in non-surgical animals. Glomeruli from left or right bulbs from both immature and mature animals. No significant differences (independent samples t-test) between the left and right bulbs were found in the numbers of full, partial, or trace P2 glomeruli in non-surgical animals.

Glomerular Type	Bulb	Mean # of Glomeruli	Standard Deviation	Std Error of Mean	Significance
Full	Left n=4	6.50	1.73	0.87	p=0.45
	Right n=4	7.50	1.73	0.87	
Partial	Left n=4	2.00	1.41	1.41	p=0.82
	Right n=4	2.25	1.50	1.50	
Trace	Left n=4	5.75	2.50	1.25	p=0.86
	Right n=4	6.26	4.72	2.39	

Table 2. Comparison of P2 glomeruli in immature versus mature non-surgical animals. Full, partial, and trace P2 glomeruli from olfactory bulbs in immature or mature non-surgical animals, regardless of bulb, were pooled. No significant differences (independent samples t-test) were found in the numbers of full, partial, or trace glomeruli between the immature and mature non-surgical animals.

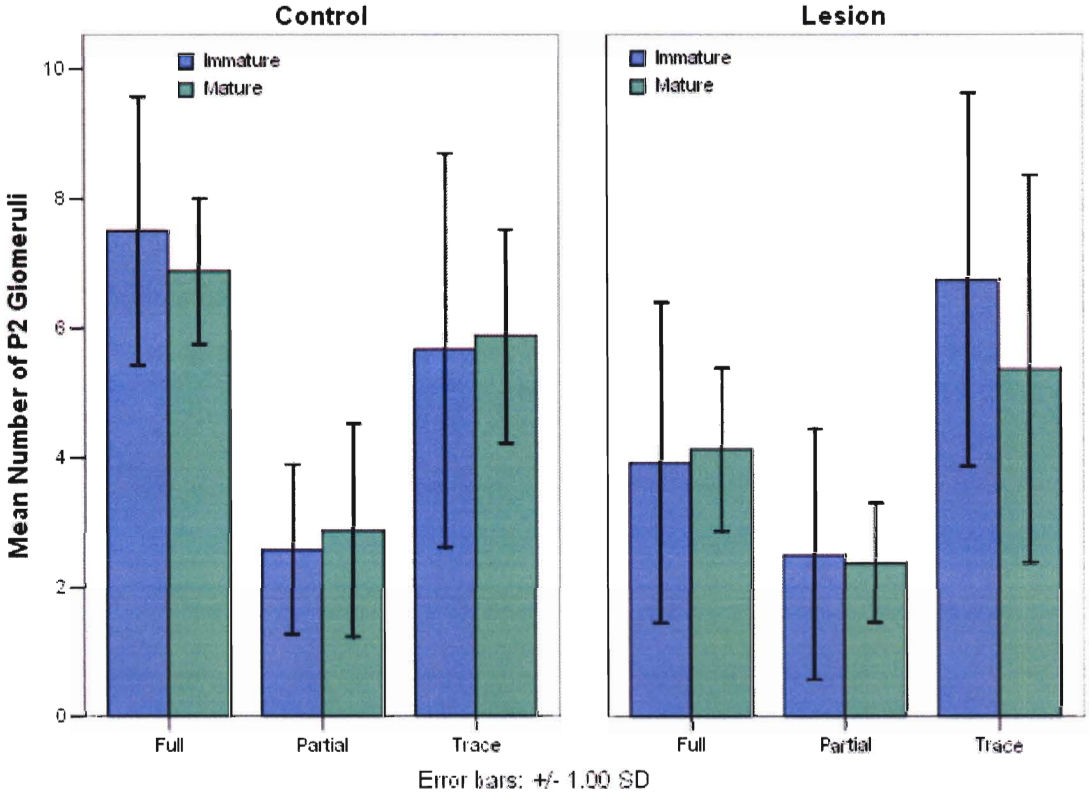
Glomerular Type	Age Group	Mean # of Glomeruli	Standard Deviation	Std Error of Mean	Significance
Full	immature n=4	6.75	2.06	1.03	p=0.708
	mature n=4	7.25	1.50	0.75	
Partial	immature n=4	2.25	1.50	0.75	p=0.816
	Mature n=4	2.00	1.41	0.71	
Trace	immature n=4	7.50	4.65	2.33	p=0.254
	mature n=4	4.50	1.00	0.50	

Figure 7. Comparison of P2 glomeruli in control versus lesioned olfactory bulbs. The Full, partial, or trace P2 glomeruli in immature and mature surgical animals were pooled from all surgical animals. A significant difference exists in full P2 glomeruli ($p<0.01$) (paired samples t-test) between control and lesioned bulbs, but not in partial or trace P2 glomeruli. Transection of olfactory neurons significantly decreases the number of full P2 glomeruli.



Type	Bulb	N	Mean	Std Dev	P-value	Significance
Full	CNTL	24	6.83	1.86	<0.01	**
	LESION	24	4.17	1.99		
Partial	CNTL	24	2.79	1.35	0.32	n/s
	LESION	24	2.38	1.50		
Trace	CNTL	24	5.54	2.38	0.40	n/s
	LESION	24	6.17	2.67		

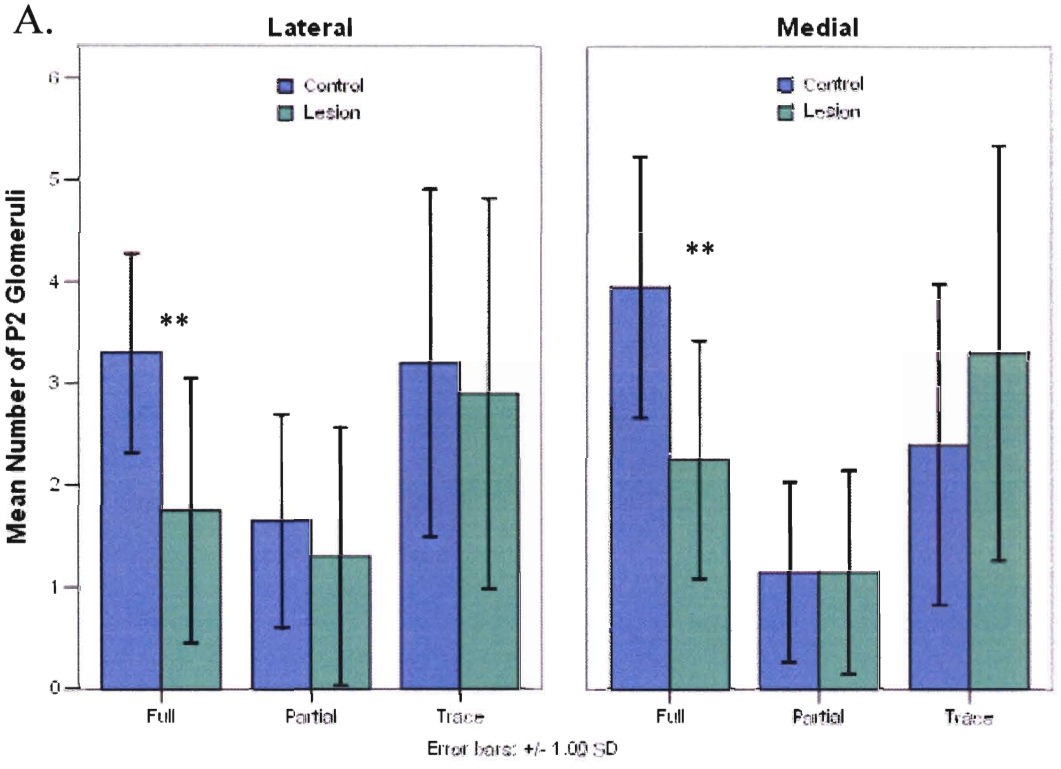
Figure 8. Comparison of P2 glomeruli in immature versus mature groups. No significant differences were found in numbers of P2 glomeruli between immature and mature animals in either control or lesioned olfactory bulbs (independent samples t-test).



Bulb	Type	Age Group	N	Mean	Std Dev	P-value	Significance
Control	Full	Immature	12	7.50	2.07	0.45	n/s
		Mature	8	6.88	1.13		
	Partial	Immature	12	2.75	1.36	0.86	n/s
		Mature	8	2.88	1.64		
	Trace	Immature	12	5.67	3.03	0.90	n/s
		Mature	8	5.50	1.69		
Lesioned	Full	Immature	12	3.92	2.47	0.81	n/s
		Mature	8	1.23	1.25		
	Partial	Immature	12	2.50	1.93	0.71	n/s
		Mature	8	2.25	1.04		
	Trace	Immature	12	6.75	2.86	0.31	n/s
		Mature	8	5.38	2.97		

Figure 9. Comparisons of P2 glomeruli by lesion and location. A. Comparison of control versus lesioned bulbs in either the lateral or medial location. Significant differences exist in full P2 glomeruli between control and lesioned bulbs in both lateral ($p<0.01$) and medial ($p<0.01$) locations (paired samples t-test). B. Comparison of lateral versus medial locations from either control or lesioned olfactory bulbs. No significant differences exist in numbers of P2 glomeruli between lateral and medial locations from control or lesioned olfactory bulbs (paired samples t-test). Lesion results in a significant decrease in full P2 glomeruli in both locations of the olfactory bulb.

A.


Lateral
Medial
Control
Lesion
Mean Number of P2 Glomeruli
0
1
2
3
4
5
6
**
**
Full
Partial
Trace
Error bars: +/- 1.00 SD

B.

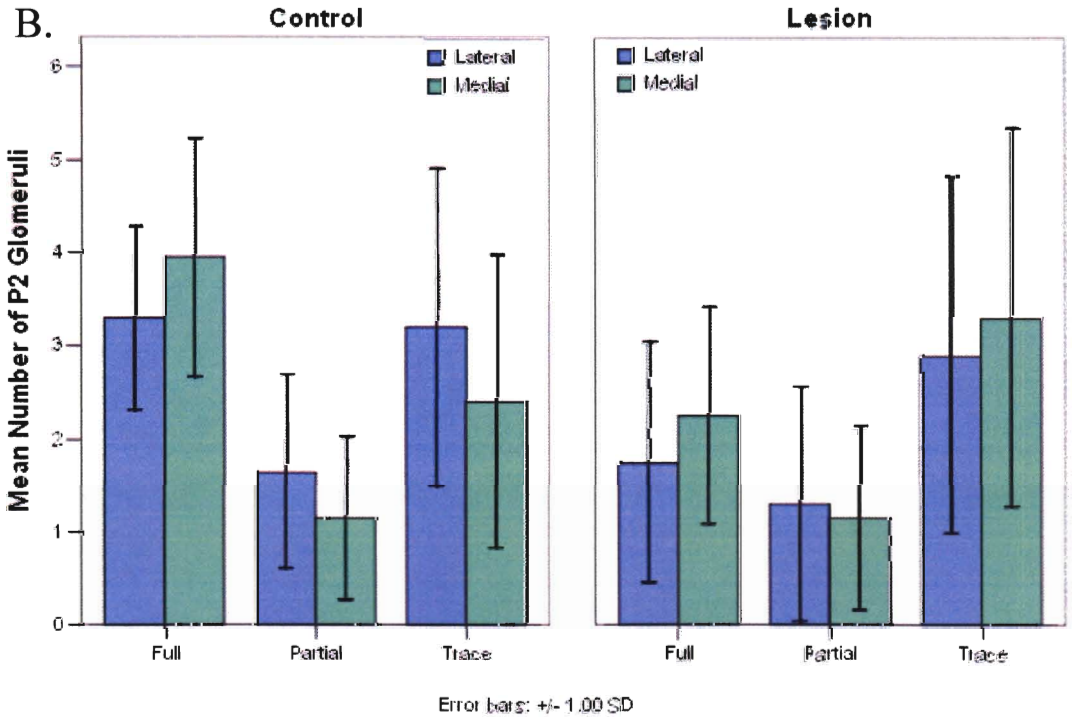
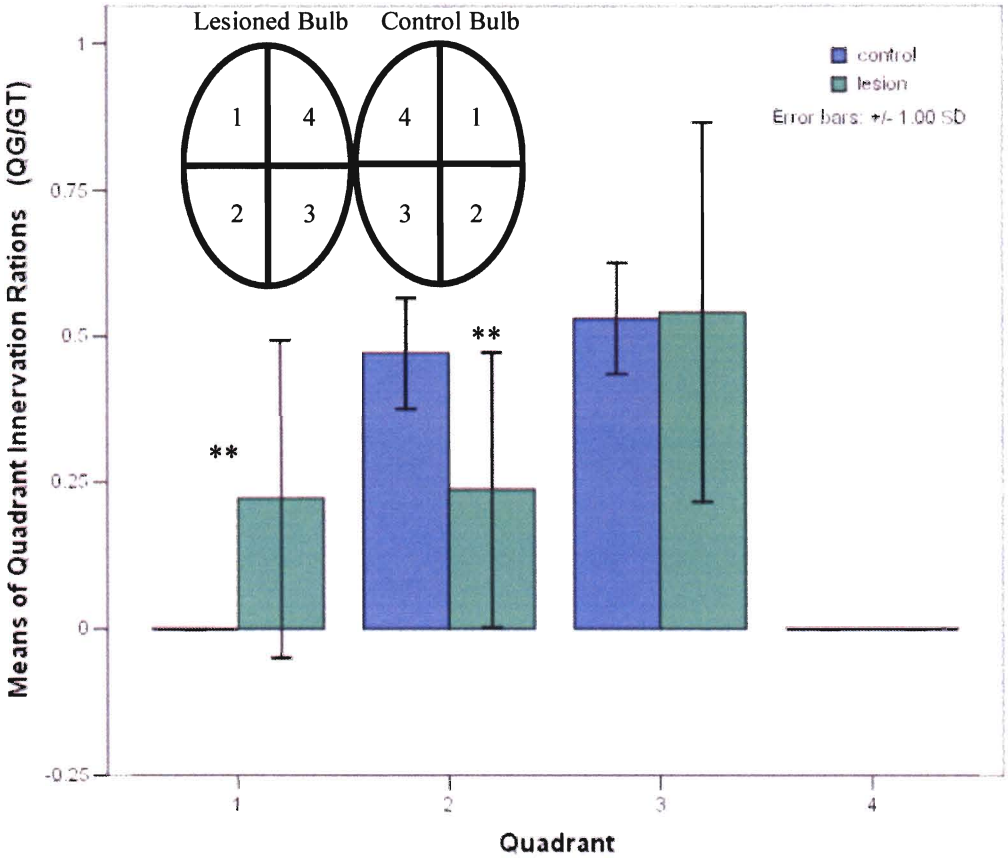

Control
Lesion
Lateral
Medial
Mean Number of P2 Glomeruli
0
1
2
3
4
5
6
Full
Partial
Trace
Error bars: +/- 1.00 SD

Figure 10. Quadrant analysis of full P2 glomeruli in control versus lesioned olfactory bulbs from 24 surgical animals. The number of full P2 glomeruli reinnervating each quadrant (QG) was compared to the total number of full glomeruli in each bulb (GT) to obtain the relative ratio of quadrant glomeruli (QG/GT). Significant differences were found in the lateral (quadrants one and two) but not the medial (quadrants three and four) quadrants. Glomerular remapping following lesion is altered on the lateral, but not the medial side of the olfactory bulb.



Quadrant	Bulb	Mean of Ratios	Std Deviation	Std Error of Mean	Significance
1	Control	0.000	0.00	0.00	0.002
	Lesion	0.222	0.27	0.06	
2	Control	0.470	0.09	0.02	0.00
	Lesion	0.268	0.24	0.05	
3	Control	0.530	0.09	0.02	0.89
	Lesion	0.541	0.32	0.07	

Discussion

Left olfactory nerve transection (LNTX) effectively severs the connections between the epithelium and the left olfactory bulb. Following nerve transection, the odorant receptor neurons (ORNs) degenerate in a retrograde manner, leading to an overall reduction in the thickness and number of cells in the olfactory epithelium (Costanzo. 1985). Following this period of degeneration, the ORNs begin to redevelop and project axons to reconnect to the olfactory bulb.

During regeneration there is an influx of axons re-growing from the epithelium toward the olfactory bulb. Previous studies have shown that during the recovery period there is a diffuse innervation of the olfactory bulb, with ORNs from many different subtypes synapsing with mitral cells in many different glomeruli. These studies also showed large numbers of different ORNs regenerating at the same time. There is, however, a refinement over a period of months of these multiple connections, ultimately resulting in ORNs of a single subtype converging on a few, discrete glomeruli (Astic and Saucier. 2001; Buck. 1996; Costanzo. 2000; Costanzo. 2005).

In this study addressing differences in the regeneration following olfactory nerve transection between immature and mature mice, P2 mice were selected because of the ability to identify a single ORN subtype. Originally, Mombaerts and colleagues indicated that in the P2 mouse, two full P2 glomeruli were located in each olfactory bulb

(Mombaerts et al. 1996). Examination of the olfactory bulbs in non-surgical mice in this study revealed more P2 glomeruli in both the left and right olfactory bulbs and on both the lateral and medial sides of the bulbs than previously reported. Partial and trace P2 glomeruli were also observed in non-surgical mice, unlike the original P2 studies. The cause of the increase in the number of P2 glomeruli is unknown. While there were differences in numbers of P2 glomeruli between this study and those previously reported, the other characteristics of the P2 glomeruli were similar; the locations of the P2 glomeruli in the olfactory bulb on the lateral-ventral and medial-dorsal sides of the bulbs were consistent with previously reported findings. In this study, a LNTX was performed so that the right olfactory neurons remained intact and the right olfactory bulb served as an internal control for each mouse used.

Unusually high numbers of P2 glomeruli have also been observed in another study of P2 mice. Double glomeruli were seen in 12 olfactory bulbs in five-week old non-surgical mice on either the lateral, medial, or both sides of the bulbs (Schaefer et al. 2001). Although the number of P2 glomeruli increased in this study, these glomeruli were still centralized in "zones" of innervation that corresponded to the overall locations of P2 glomeruli in Mombaerts' description. The original locations of P2 glomeruli were described as the lateral, ventral and medial, dorsal regions of the olfactory bulb. As was the case, the P2 glomeruli in the non-lesioned olfactory bulbs were found on both the lateral and medial halves of the bulbs, with the lateral glomerulus being located more anterior, and the medial one more posterior.

The age groups in this study, five-weeks old (immature) and 16-weeks old (mature) were selected because of their correlations to milestones in neuronal, especially olfactory development. Pomeroy et al. (1990) showed that the mouse olfactory system undergoes post-natal development for two to three months. During this time, not only does the size of the olfactory bulb increase, but the numbers of glomeruli and synapses with second-order neurons increase greatly as well. This increase in numbers of glomeruli and nerve connections reveals an increasing level of complexity in the perception of odorants (Pomeroy et al. 1990). Five-week old mice are not sexually mature, and fall within this two to three month window of olfactory development, suggesting that their olfactory systems are still undergoing further refinement. 16-week old mice are sexually mature and their olfactory systems should be fully developed, undergoing only the normal processes of cell replacement and renewal, not projection refinement (Graziadei, PPC and Monti Graziadei, GA. 1978; Pomeroy et al. 1990). It has been reported that sexual maturity may have an overall effect on the development of the olfactory bulbs. Marked increases in androgen receptor and estrogen receptor protein expression were noted in olfactory bulb homogenate as the animals matured (Wong et al. 2000)

Overall, in the immature mice, great variability was observed in the numbers of trace P2 glomeruli; trace glomeruli in the non-lesioned (right) bulbs of surgical mice ranged between zero and 10 and two and 13 in the right bulbs of non-surgical animals. The overall variability in the number of trace glomeruli in non-lesioned bulbs could suggest that the immature mice may not have undergone sufficient post-natal olfactory development to refine ORN projections. This might also account for the number of partial

P2 glomeruli that were observed in non-lesioned bulbs of immature surgical animals, between one and four glomeruli, and in the non-surgical animals; between one and three in each bulb.

The mature (16-week old) mice, however, also exhibited variability in the numbers of partial and trace P2 glomeruli in surgical and non-surgical animals. The non-lesioned bulbs of mature surgical mice contained between one and six partial P2 glomeruli and between four and seven trace P2 glomeruli. Mature non-surgical mice exhibited between two and four partial P2 glomeruli and between eight and ten trace P2 glomeruli in each bulb. Given this wide range of variability in partial and trace P2 glomeruli in immature and mature animals, it can be inferred in his study that the variability in the numbers of P2 glomeruli is not a result of a lack of development. This agrees with the findings of Pomeroy et al. (1990), who indicated that the refinement of the mouse olfactory system was completed by 12-weeks post-natal via light and electron microscopy studies.

Furthermore, the P2 receptor protein is first observed, in P2 mice, by embryonic day 12.5 (E12.5). By E16.5, P2 axon convergence is observed and two distinct P2 glomeruli have been observed at birth (Mombaerts et al. 1996). This suggests that P2 convergence, even in non-surgical olfactory bulbs, should be complete by five-weeks, and that there may be other factors behind the increased number of P2 glomeruli.

In this study, comparisons between the lesioned and control olfactory bulbs in numbers of full, partial, and trace glomeruli showed that olfactory nerve transection resulted in a significant reduction in the number of full P2 glomeruli following recovery in the lesioned olfactory bulbs of both immature and mature mice. Yee and Costanzo (1995)

and Morrison and Costanzo (1995) have shown that following a 40-day recovery from olfactory nerve transection, the ORNs regenerate and project axons from the olfactory epithelium to reinnervate the olfactory bulb. In the current study, 40-day recovery was also allowed for regeneration following olfactory nerve injury.

However, significant reduction of full P2 glomeruli in the lesioned olfactory bulbs in both the immature and mature surgical mice, compared to the non-lesioned olfactory bulbs suggests that more time is required for proper and complete restoration of P2 ORN targeting. While the number of full P2 glomeruli were reduced between the lesioned and non-lesioned bulbs of surgical animals, the numbers of partial and trace P2 glomeruli did not change. The trace and partial glomeruli data in this study indicate that the P2 ORNs did not converge with the specificity previously described. The differences in full P2 glomeruli, but not in partial or trace, between lesioned and non-lesioned olfactory bulbs in both age groups suggest that it may be more difficult to reestablish all the required connections in a full glomerulus, rather than reestablishing few connections, as in a partial or trace glomerulus. Perhaps more specific guidance or signaling is required for the re-formation of a complete glomerulus.

With so many different subtypes of ORNs regenerating simultaneously, the possibility of randomly converging at a glomerulus with ORNs of just one subtype seems unlikely. Competition among ORNs for targets in the olfactory bulb seems a likely explanation for the overall decrease in the number of full P2 glomeruli following injury (Costanzo. 2005). This is further complicated, however, by the lack of difference in the numbers of partial and trace P2 glomeruli following injury. Rather than just an alteration

in the numbers of P2 glomeruli, histology suggests there could be an overall reduction in the number of P2 ORNs regenerating in the olfactory epithelium. Qualitative analysis of histological sections from surgical mice in the immature and mature age groups shows a decrease in the density of blue P2 receptors in the olfactory epithelium on the lesioned side. This could explain why there is an overall decrease in the numbers of full P2 glomeruli in both the immature and mature age groups.

While the data clearly showed a difference in full P2 glomeruli following injury in both age groups, it was hypothesized that the immature lesioned olfactory bulbs would more closely resemble the non-lesioned bulbs following recovery. However, following a 40-day recovery period, there were no differences between the immature and mature surgical animals in the numbers of full, partial, or trace P2 glomeruli in lesioned olfactory bulbs. This suggests that the regeneration of the P2 ORN is not age dependent. Despite the effect of the lesion in reducing the number of full P2 glomeruli, if there were an age-dependent effect, a difference in the numbers of P2 glomeruli between the immature and mature animals would be seen as well. Were the mice to have been sacrificed and processed before 40-days, it is possible that age-related differences would have been seen. It was hypothesized that the younger mice possessed a higher level of neuroplasticity. If this were the case, while at 40 days no difference was seen, perhaps at 30 days the mature mice would not yet resemble the map of the immature mice.

Despite a reduction in numbers of full P2 glomeruli in both age groups in the lesioned olfactory bulbs, no differences were found between the lateral and medial sides of the olfactory bulbs in either the lesioned or control bulbs in surgical mice. This

comparison was made since the process of transecting the left olfactory nerve involves insertion of the blade along the lateral side of the olfactory bulb, moving it around the anterior pole of the bulb, and finally around the medial half. During this procedure there is the potential to damage, in particular, the right medial surface of the control bulb, and potentially affect the regeneration of the olfactory neurons. No differences between the lateral and medial sides in either the non-lesioned or lesioned olfactory bulbs indicate that the transection procedure had no affect on the P2 mapping in the right (non-lesioned) olfactory bulb.

In order to further assess the relative amount of innervation at each location, the olfactory bulbs were divided into four quadrants. In the non-lesioned surgical olfactory bulbs, full P2 glomeruli from both the immature and mature age groups were found in the second (ventral, lateral) and third (ventral, medial) quadrants. These two quadrants align with the previously reported "zones" of P2 innervation (Schaefer et al. 2001). However, with the overall decrease in numbers of full P2 glomeruli between the lesioned and non-lesioned bulbs, means of glomeruli in each quadrant could not be simply compared. Instead, ratios of full P2 glomeruli in each quadrant (QG) as compared to total numbers of full P2 glomeruli in each bulb (GT) were calculated. These ratios (QG/GT) were then compared between lesioned and non-lesioned olfactory bulbs, in each quadrant. Significant differences were found between lesioned and non-lesioned bulbs in quadrants one (dorsal, lateral) and two (ventral, lateral). In quadrant one, there was an increase in the ratio of full P2 glomeruli mapping to that region. Quadrant two also showed a decrease in the ratio of P2 glomeruli. This indicates an overall alteration in the remapping of the P2

ORNs as they regenerate on the lateral side. No differences were found in P2 glomeruli in the third or fourth quadrants (medial side).

Quadrants one and two are innervated by ORNs that project from the nasal turbinates, while quadrants three and four are innervated by ORNs projecting from the nasal septum. One possible rationale for the shift in mapping to the lateral side of the olfactory bulb following nerve transection is that the there are greater numbers of ORNs regenerating from the nasal turbinates in comparison to those from the septum. The nasal turbinates create a much greater surface area for ORNs to make contact with inhaled odorants than the surface of the nasal septum. When the ORNs begin to repopulate the olfactory epithelium on the turbinates and then project to the olfactory bulb, there will be a greater degree of disorganization as the axons reinnervate the bulb, because of the complex structure (the turbinates) around which the developing ORN axons must navigate.

The data from Schoenfeld and Knott (2004) and this study indicate an overall equality between the numbers of glomeruli on the lateral and medial halves of non-lesioned olfactory bulbs. They have shown however, when the olfactory bulb is divided into four quadrants in control mice, the lateral-ventral quadrant (quadrant two) displays the smallest number of overall ORN-innervated glomeruli (Schoenfeld and Knott. 2004). In addition, the study indicates that the mapping of the ORNs from the medial and lateral regions of the olfactory epithelium to the medial and lateral regions of the olfactory bulb, contain the same number of neurons, respectively. The lateral region of the olfactory epithelium is much more convoluted than the medial region (turbinates versus septum), and therefore, could result in greater disorganization of new ORNs regenerating during

recovery from nerve transection. As the same number of ORNs regenerate on each side, those regenerating from a smaller surface area would have less interference in mapping to the appropriate glomeruli. By comparison, those neurons regenerating from a much more complex region, covering a larger surface area, would have to surpass a greater degree of interference from other regenerating ORNs. This could explain why there is a shift of ORNs from the second quadrant to the first quadrant, and not in the third and fourth quadrants between the lesioned and non-lesioned olfactory bulbs.

Conclusion

Behavioral and morphological studies have shown the ability of the olfactory system to recovery from injury. This study indicates that the regeneration of the P2 ORNs is not affected by the age difference between the immature and mature mice. No difference existed in the regeneration of the P2 ORNs between five-week old and 16-week old mice. Despite this lack of differences between groups, there was a significant reduction in the number of full P2 glomeruli between the lesioned and non-lesioned olfactory bulbs. There was also a significant change in the mapping of P2 ORNs to the lateral side of the lesioned olfactory bulb. Although this study investigated only one aspect of regeneration in the olfactory system, it does offer further insight into the ability of the nervous system to regenerate and repair itself after damage. This study suggests that the olfactory system possesses the inherent, but altered, ability to restore itself after injury, regardless of age.

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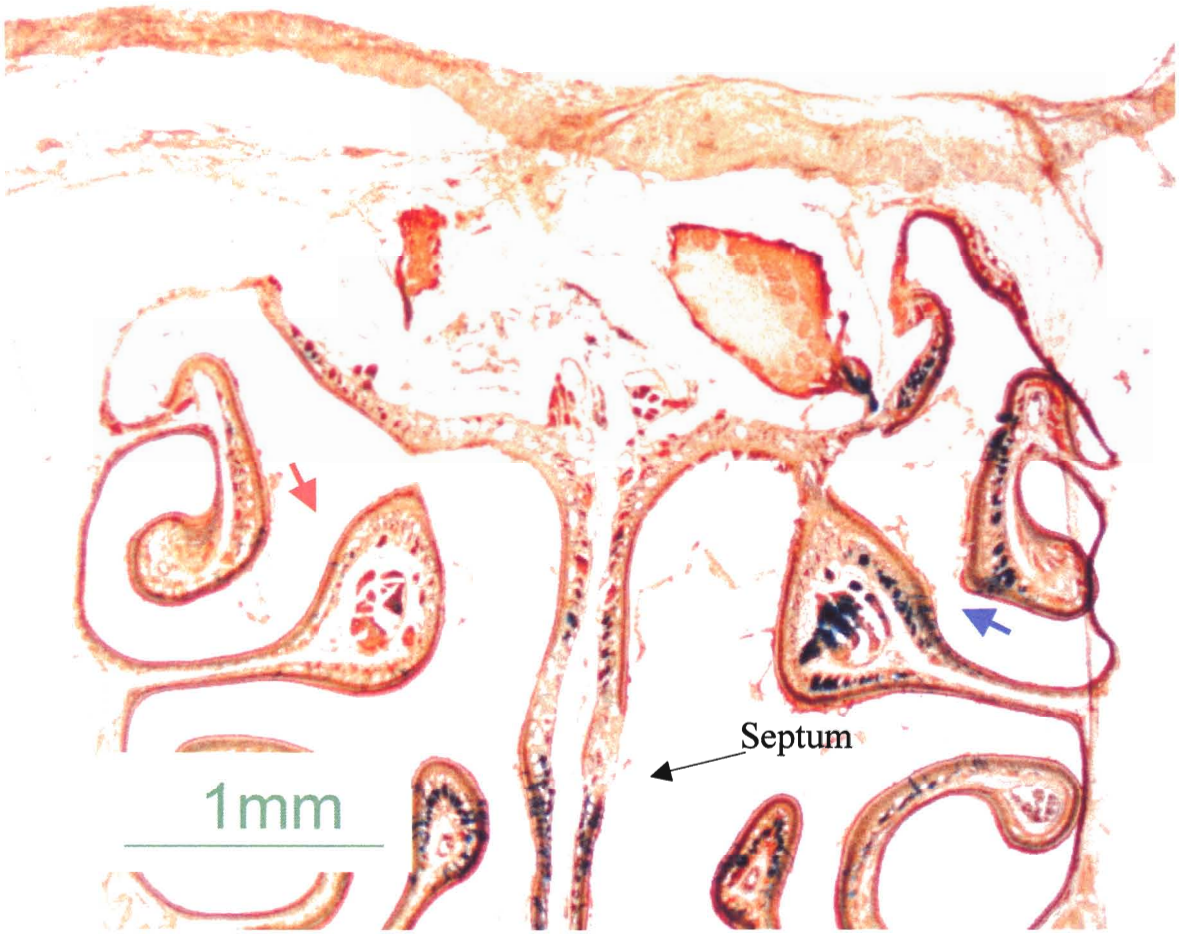
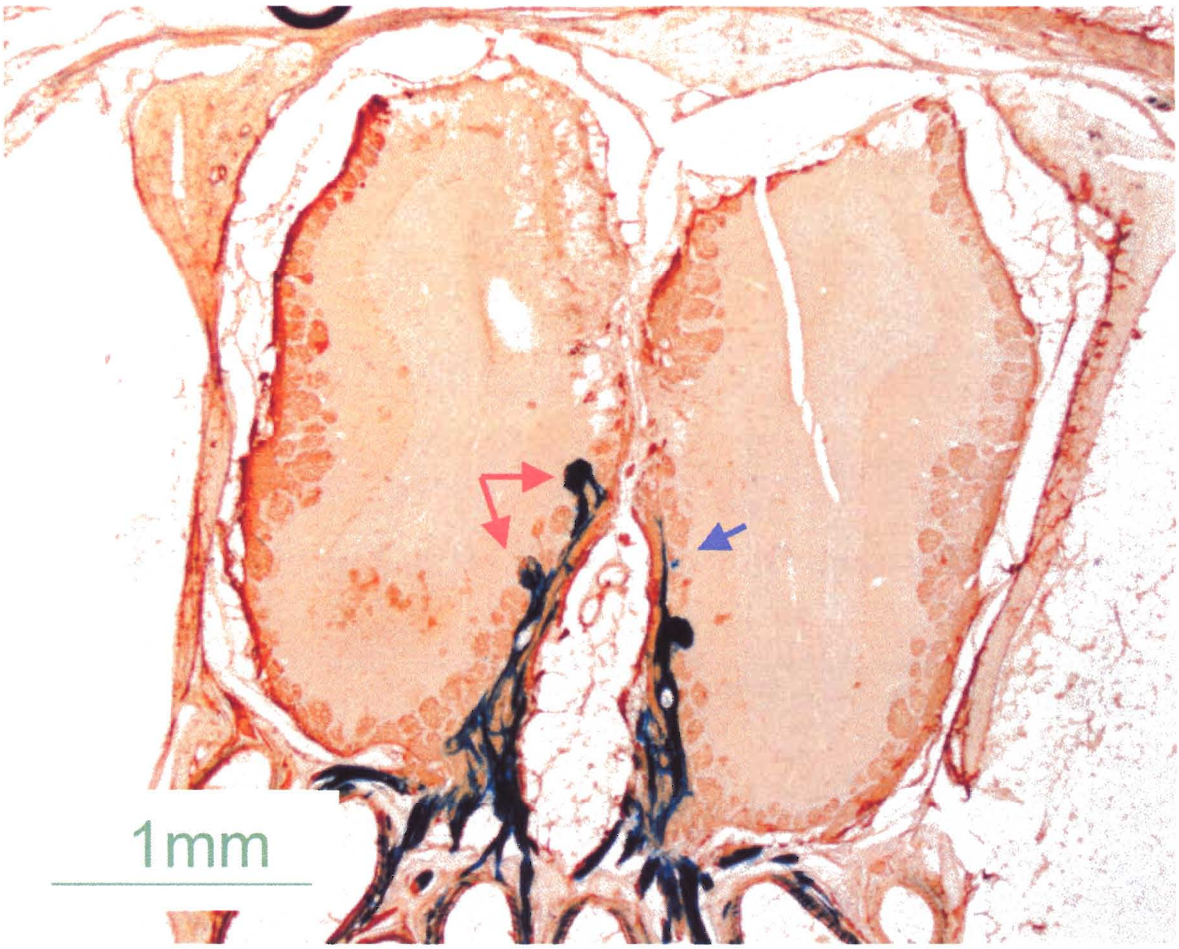
APPENDIX A

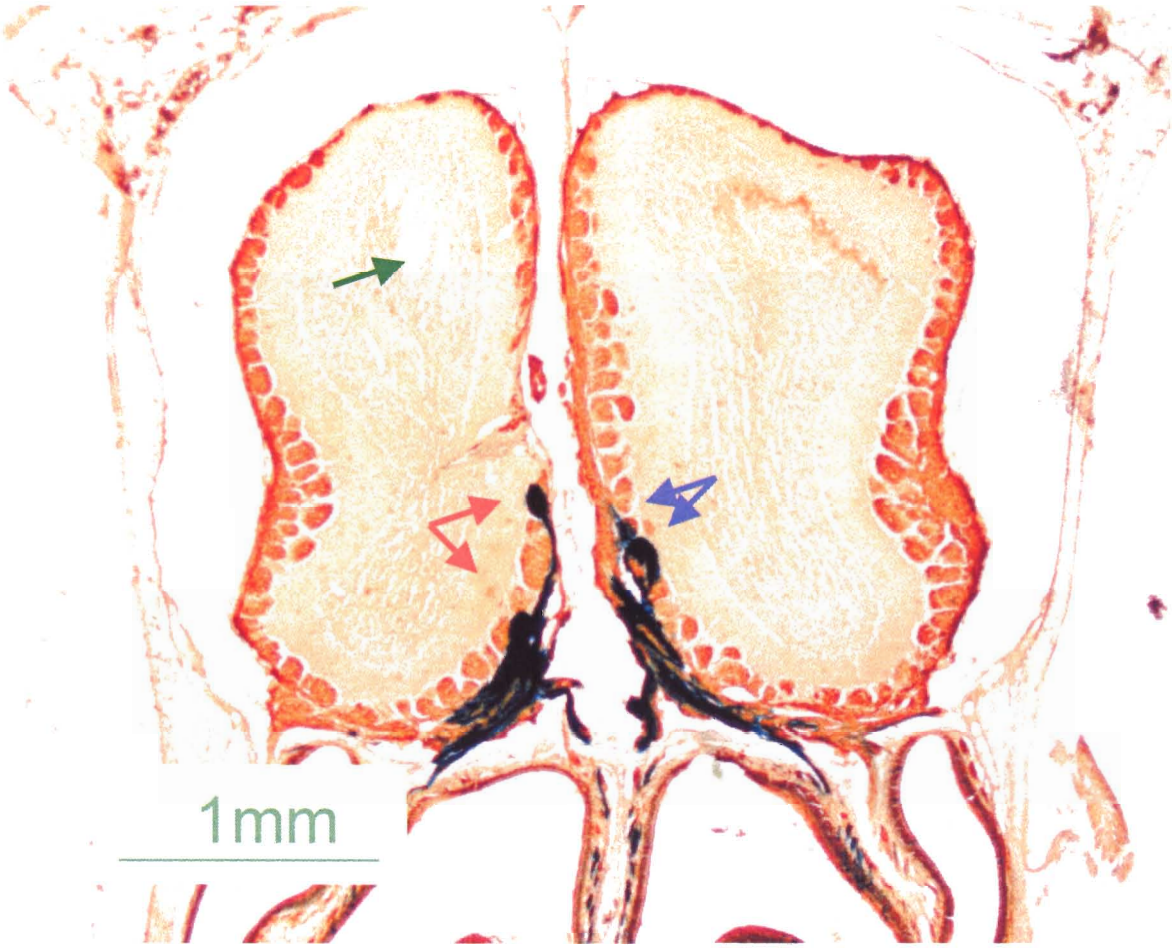
The following images are examples of histological sections used in this study. Glomeruli of all types are clearly labeled, and the pertinent information about each section (mouse, age group) is noted.



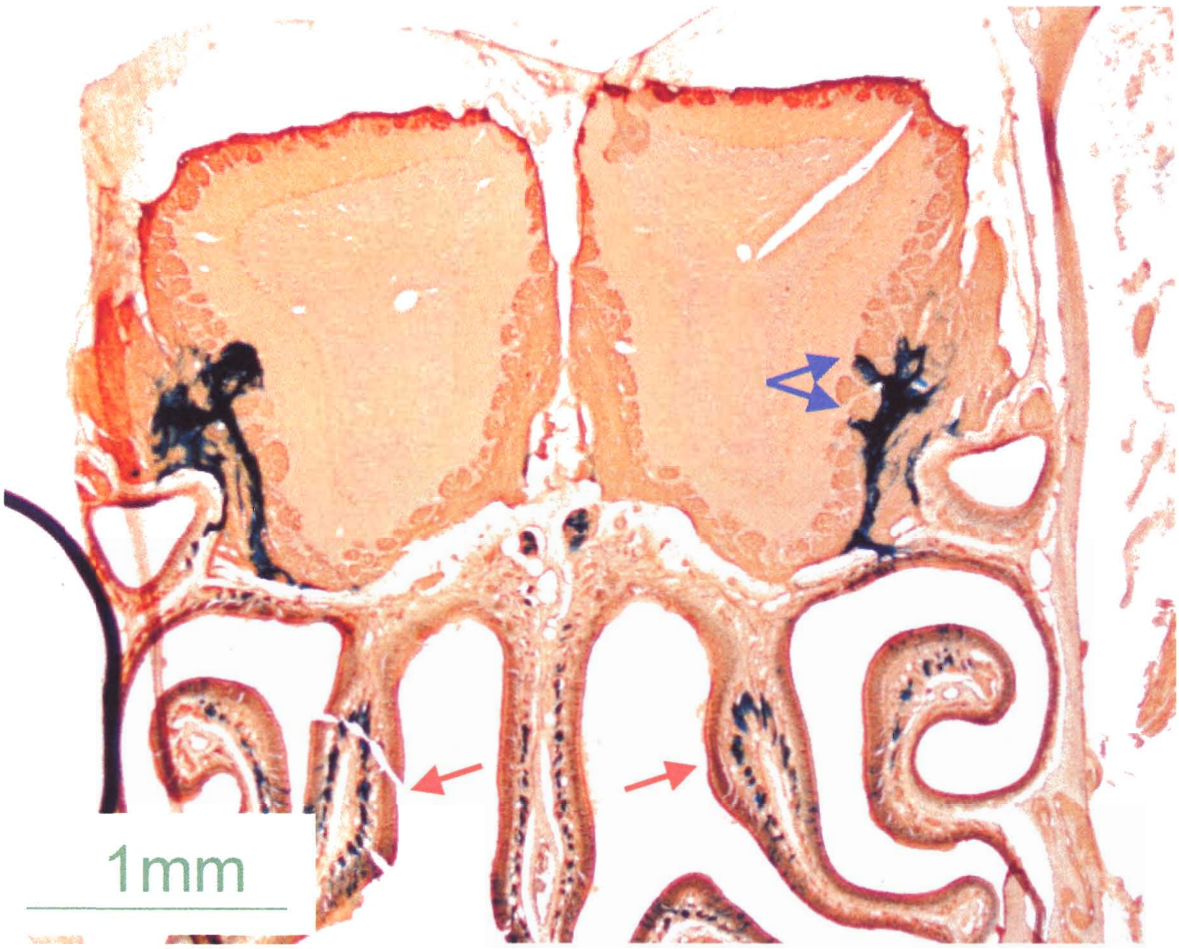
Histological section of beginning of right olfactory bulb from M3095 (mature surgical animal) (mag=2x). Some damage to the anterior pole of the left olfactory bulb occurred during surgery. The right bulb was observed much earlier than the left. Turbinates are bilaterally symmetrical, indicating a square cutting plane. Decreased P2 blue staining (red arrow) to the left of the nasal septum indicates a decrease in the number of receptors in neuroepithelium in comparison to the right (blue arrow).



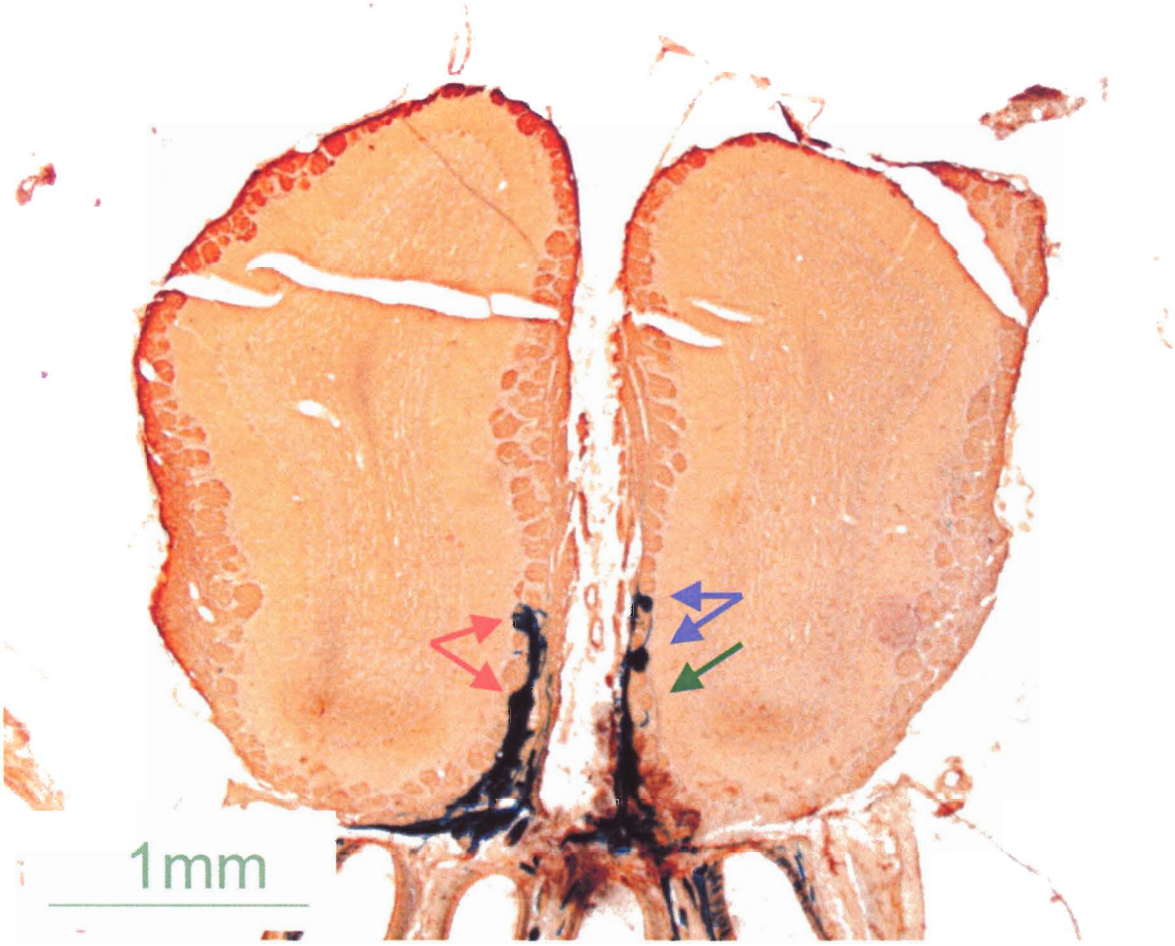
Histological section of olfactory bulbs from M3095 (mature surgical animal) (mag=2x). Medial P2 glomeruli are clearly stained with X-gal technique. Two medial P2 glomeruli in left olfactory bulb (red arrow) comprise a "doublet." Dorsal to the P2 glomerulus in right bulb is a trace P2 glomerulus (blue arrow).



Histological section of olfactory bulbs from M3148 (immature surgical animal) (mag=2x). Doublets exist in left (red arrows) and right (blue arrows) olfactory bulbs. The olfactory cusp (green arrow) found in left olfactory bulb indicates that section is close to the end of the primary olfactory bulb, and the close to beginning of the accessory bulb (AOB).



Histological section of olfactory bulbs from M3134 (mature non-surgical animal) (mag=2x). Doublet P2 glomeruli identified in right olfactory bulb on lateral side (blue arrows). Unlike the uneven density of P2 receptor staining in the epithelium in surgical animals, there is an even density of P2 receptors (red arrows) on either side of the nasal septum.



Histological section of olfactory bulbs from M3217 (immature non-surgical animal) (mag=2x). Appearance of doublets in both left (red arrows) and right (blue arrows) olfactory bulbs. A trace P2 glomerulus (green arrow) is located ventral to doublet in right olfactory bulb.

VITA

Daniel Joseph Galante was born June 29, 1983 in Allentown, Pennsylvania and is an American citizen. He is the son of Michael and Mary Lou Galante.

Daniel grew up in Telford, Pennsylvania and graduated from Souderton Area High School in Souderton, Pennsylvania in 2001. He graduated *cum laude* with a dual Bachelor of Arts in Biology and German from La Salle University in Philadelphia, Pennsylvania in 2005. He will matriculate as a first-year medical student at Touro College of Osteopathic Medicine in New York, New York in the fall of 2007.