

**EARLY UNILATERAL OLFACTORY BULB LESION RESULTS IN DIFFUSE  
CHANGES IN BEHAVIOR AND OVERALL CORTICAL ORGANIZATION**

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## DEDICATION

In memory of Helen Juhas (1915-1991), my grandma, who turned her kitchen into a chemistry set, and made her backyard an excursion into biological science.

## THESIS ABSTRACT

The current work explores the behavioural and anatomical consequences of unilateral neonatal bulbectomy (OBX) in male and female rats at postnatal day 1 (P1) and P10. In adulthood the animals underwent a battery of motor and cognitive tests, and diffuse effects of early brain injury on the development of behavior were found. Disturbing olfactory sense input during development affected motor output. Rats normally display an equal distribution of right or left paw preference. In this study, both OBX sexes showed a shifted paw preference to the ipsilesional side, and forelimb deficits were found in a skilled reaching task. Lesion animals also showed enhanced performance on a visually driven spatial cognitive test. Cross-modal compensatory changes may be responsible. Morphological changes within the cerebral cortex are described, including bulbar changes, enlarged but fewer glomeruli, smaller accessory olfactory bulb, decreased downstream connectivity, and a rostral shift of the forebrain toward the olfactory bulb. Changes to the lateral cortex were found in both intact and lesion hemispheres, along with dendritic changes in the forelimb reaching area. Cellular regeneration within the lesion bulb was indicated. Changed shape and relative size increases compared to the intact bulb were found. BrdU labeling showed increased mitotic activity in P10 lesion animals. These findings demonstrate that the impact of olfactory injury during early development goes well beyond odor perception and discrimination, and that olfactory inputs during development significantly contribute to the development of the neocortex.

## **ACKNOWLEDGEMENT**

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## LIST OF ABBREVIATIONS

Amg	amygdala
AOB	accessory olfactory bulb
AON	anterior olfactory nucleus
BrdU	bromodeoxyuridine
CC	corpus callosum
CORT	Cortisol
DPC	dorsal peduncular cortex
E1, 2, 3...	embryonic day 1
EC	entorhinal cortex
EPL	external plexiform layer
EPLA	external plexiform layer accessory olfactory bulb
ET	external tufted cells
F	female
FGF-2	fibroblast growth factor 2
g	grams
GABA	gamma-aminobutyric acid
GBC	globose basal cells
GC	granule cells
GCL	granule cell layer
GCLA	granule cell layer accessory olfactory bulb
GL	Glomerular layer
GLO	Glomerulus

GPCR	G-protein coupled receptors
HBC	horizontal basal cells
HPC	hippocampus
IPL	internal plexiform layer
IPLA	internal plexiform layer accessory olfactory bulb
LOT	lateral olfactory tract
LP	lamina propria
M	male
mm	millimeter
MC	mitral cell
MCL	mitral cell layer
MCLA	mitral cell layer accessory olfactory bulb
MOB	main olfactory bulb
MOE	main olfactory epithelium
MWM	Morris water maze
NC	nasal cavity
nph	nasopharynx
OB	olfactory bulb
OBP	odorant binding protein
OBX	olfactory bulbectomy
OE	olfactory epithelium
OEC	olfactory ensheathing cells
ON	olfactory receptor nerve

ONL	olfactory nerve layer
OR	odorant receptor
ORN	olfactory receptor neuron
OSN	olfactory sensory neuron
OT	olfactory tubercle
PI,2,3...	postnatal day 1
PC	piriform cortex
PG	periglomerular cells
RE	respiratory epithelium
RMS	rostral migratory stream
SA	short axon cells
SEL	subependymal layer
SO	septal organ
SPR	single pellet reaching
Sus	sustentacular cell
SVZ	subventricular zone
TC	tufted cell
TR	transition zone
TT	tenia tecta
VI	primary visual cortex
V2, V3, V4	secondary visual cortex
VNO	vomeronasal organ
VTT	ventral tenia tecta

# **Chapter 1**

## **General Introduction**

Olfaction, the perception of odour, is the first sensory ability to develop. Unlike taste, the other chemical sense that requires direct physical interaction with the tongue, olfaction serves as an early detection device for ambient chemicals emitted from a distant source. Olfaction facilitates communication among members of a species; provides information from the environment; and prompts responsive behaviors. The importance of olfaction in functional behavior and development is often understated and overlooked. The current study was designed to 1) Investigate the functional effects of early olfactory injury on behavior beyond the sensory capacity of odor detection and classification in rats; 2) Examine the anatomical morphology of early unilateral olfactory lesion; 3) Investigate the regenerative capability in the olfactory bulb during rat development. The thesis begins with a review the functions of olfaction, anatomy, and connectivity within the olfactory system, and developmental aspects relevant to olfaction; followed by a rationale for techniques employed, methods, and the main results. The results of three additional follow-up experiments follow, and the overall discussion concludes the thesis.

## **1.1 THE FUNCTIONS OF OLFACTION**

### **1.1.1 Recognition**

Are you friend of foe? In macrosomatic animals like rats, the difference between familiar (from one's colony) or unfamiliar conspecifics (a stranger) is apparent from smell. Rats determine social status (dominant or subordinate), recognize individuals and territories based on odours secreted (i.e. through urine).

### **1.1.2 Mate choice**

Olfaction operates in mate choice. Not only can rodents tell the sex of the animal producing an odour, but also the reproductive status (mating receptive, pregnant, lactating) and maturity (juvenile or sexually mature). Human females tend to prefer the odours from men who have immune systems different from their own (Spehr, Kelliher, Boehm, Leinders-Zufall and Zufall, 2006).

### **1.1.3 Mother/Infant Connection**

Smell facilitates recognition of one's mother or one's child, even in humans. After only one hour of exposure human mothers were able to recognize their baby's odor (Kaitz, Good, Rokem & Eidelman, 1987). Babies differentiate between the odour of their breast-feeding mother and another lactating mother by as early as three days (MacFarlane, 1975). Impair a mother rat's sense of smell and perinatal care declines dramatically resulting in poor growth and survival of the litter (Kolunie & Stern, 1995).

### **1.1.4 Emotion**

Emotional states are communicated through body odour. Rats recognize the level of stress in the odour left by another of their species. Contentment, sexuality, frustration and other emotions can be communicated by smell. Human women are able to distinguish between the armpit odours from people watching scary or neutral films (Chen & Haviland-Jones, 1999; Ackerl, Atzmueller & Grammer, 2002). Fear produces a smell recognized by horses and dogs. Innate fear responses can be triggered by predator odours.

### **1.1.5 Aggression**

Impaired odour reception alters aggressive behavior. Male rats with impaired olfaction are less aggressive toward each other in the presence of the odour from a

sexually receptive female, when, normally, aggression would increase in this situation (Bergvall, Matuszczyk, Dahlof & Hansen, 1991).

### **1.1.6 Sustenance & Survival**

Much of our odour detecting capability arises from the foods we are exposed to, and whether or not these foods are safe or spoiled. Human postnatal preferences are affected by prenatal chemical exposure in utero (via the mother's diet) (Schaal, Orgeur, Lecanuet, Locatelli, Granier-Deferre & Poindron, 2000).

### **1.1.7 Pathology**

Olfactory disorders affect eating and nutritional intake, quality of life, have safety implications, and affect interpersonal relations (Hummel & Nordin, 2005). People who suffer from hyposmia (decrease in intensity or number of perceived odorants) or anosmia (inability to perceive odorant regardless of concentration) find that food has lost much of its appeal, mainly because flavour is a combination of taste (sweet, sour, bitter, salty) and smell sensory information. An estimated 80 percent of flavour is derived from smell. Dysosmia is a distorted sense of smell and includes troposmia, a condition in which odour is detected but doesn't smell like it is supposed to smell (most often the smell is unpleasant); and phantosmia, a condition in which an odour that is not detected by a normally working human olfactory system, is detected. Dysosmias often arise from impaired neuronal function due to olfactory receptor injury, whereas olfactory hallucinations found in schizophrenia or seizure activity, are related more to problems within the olfactory cortex (Leopold & Bartels, 2002).

Olfactory dysfunction is often a symptom of neurological problems including disease such as Alzheimer's, Parkinson's, Korsakov's, epilepsy, and Huntington's

chorea; psychiatric disorders including schizophrenia and depression; traumatic head injury and cerebrovascular accident such as stroke; exposure to damaging chemicals, medications or a virus; medical illness including diabetes mellitus, renal failure, liver disease, and hypothyroidism; and aging (Leopold & Bartels, 2002; see Doty, 2001 for a more complete list of pathologies associated with olfactory dysfunction). The loss of smell in rats via bilateral bulbectomy is used as a model to parallel depression (Harkin, Kelly & Leonard, 2003). In humans the subtle effects of odour detection impairment on personal and emotional relationships can result in social isolation and deprivation, and can lead to severe depression (Leopold, 1995). Morbidly obese individuals often have olfactory dysfunction (Richardson, Vander Woude, Sudan, Thompson, & Leopold, 2004). Hyposmia and anosmia are also symptoms of Kallmann's Syndrome, a congenital disorder resulting from hypothalamic function - gonadotropin releasing hormone which migrates from the nasal epithelium to the brain via olfactory receptor axons, does not synapse with mitral cells in the olfactory bulb.

### **1.1.8 Memory**

Memory and smell are intrinsically linked. An olfactory memory is longer lasting than any other sensory memory (Miles & Jenkins, 2000). Memories associated with an odour cue are recalled more readily upon presentation of that odour, and, as a memory cue, smell is better than the other senses (Chu & Downes, 2000). The smell memory cue is known as the "Proust Effect" after the author, Marcel Proust and his description of a vivid childhood memory evoked from the smell of Madeleine cake (a rich pastry) dipped in lime-blossom tea, in the book *Swan's Way*. Damage to cortical memory areas does not affect smell detection ability, although smell identification ability is often compromised.

## **1.2 ANATOMY - FROM CHEMICALS TO ODOUR PERCEPTION**

Many recent insights regarding the olfactory system have resulted in a substantial update and new hypotheses regarding the function of various olfactory cortical areas. Traditionally the olfactory cortex, defined here as brain regions stretching from and directly innervated by the olfactory bulb including the piriform, entorhinal and periamygdala cortices, has been called the primary olfactory cortex, and assumed to subserve unimodal olfactory processing functions. The orbitofrontal cortex has long been considered secondary olfactory cortex. In other sensory areas, primary, secondary and association cortices associated with the sense are classified based on criteria including organization, architectural features, and function. In the olfactory system these cortical areas are now being identified (see Haberly, 2001).

Uncontroversially, the nasal epithelium is the olfactory receptor field, containing receptor neurons organized into four zones, with individual receptor types projecting to specific zones and specific glomeruli on the olfactory bulb.

In the olfactory bulb, now considered the primary sensory area by many, the nerve from each specific receptor neuron (there are thousands of receptors for each receptor type), converge on a dedicated glomerulus or a couple of glomeruli in a specific zone on the olfactory bulb. Thus, the olfactory bulb (OB) organizes and reduces the spatial component of the receptor field into glomeruli and topographically arranged glomerular groups with discrete modules likened to columns in the visual system.

Output from the OB travels directly into the olfactory cortex, and is the only sensory system with immediate forebrain access. The lateral olfactory tract (LOT) heavily innervates the anterior olfactory nucleus (also referred to as the anterior olfactory

cortex) which projects to the opposite hemisphere via the anterior commissure. The LOT also projects to the ventral part of the anterior pyriform cortex which projects to the prefrontal, entorhinal, and perirhinal cortices as well as the mediodorsal nucleus of the thalamus. The LOT further projects to the olfactory tubercle, which projects to the posterior hypothalamus; the amygdala, which projects to the medial hypothalamus; and the transitional entorhinal cortex, which projects to the hippocampus (Smith & Shepherd, 2003; Haberley, 2001). See Figure 1.1.

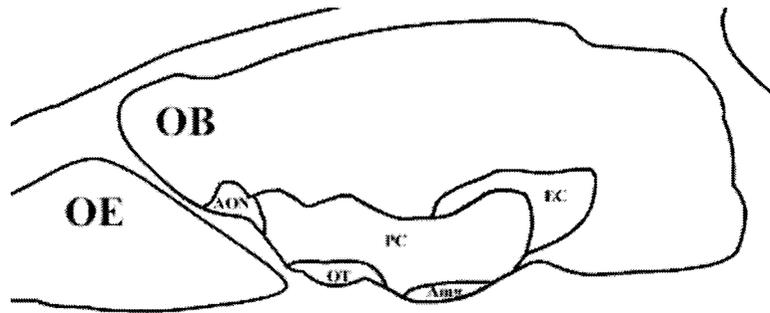


Figure 1.1: Olfactory cortex. Amygdala, (Amg), anterior olfactory nucleus (AON), entorhinal cortex (EC), olfactory bulb, (OB) olfactory epithelium (OE), olfactory tubercle (OT), pyriform cortex (PC), OT, olfactory tubercle. After Buck (2004).

Haberley (2001) provides evidence that the anterior olfactory nucleus (AON) operates in much the same manner as other secondary sensory areas - for detection, storage, correlation recognition and combinatorial representations of olfactory sensations. The pyriform cortex (PC) has been proposed as the association cortex where behavior-level processing for the olfactory system occurs. The PC does not respond solely to olfactory input, and it shows similarities in morphology and physiology to other association areas. Schoenbaum and Eichenbaum (1995) showed that neurons in the PC fired during non-olfactory components of an odour discrimination task, and were influenced by the identity and reward contingencies associated with a presented odour, as

well as predictive associations, firing before the onset of an expected odour. Thus, the OB is comparable to a primary sensory cortex (i.e. VI in vision); the anterior olfactory nucleus to a secondary cortex (i.e. V2, V3, V4); and the pyriform cortex to an association cortex (i.e. infero-temporal cortex) (Haberley, 2001). See Figure 1.2.

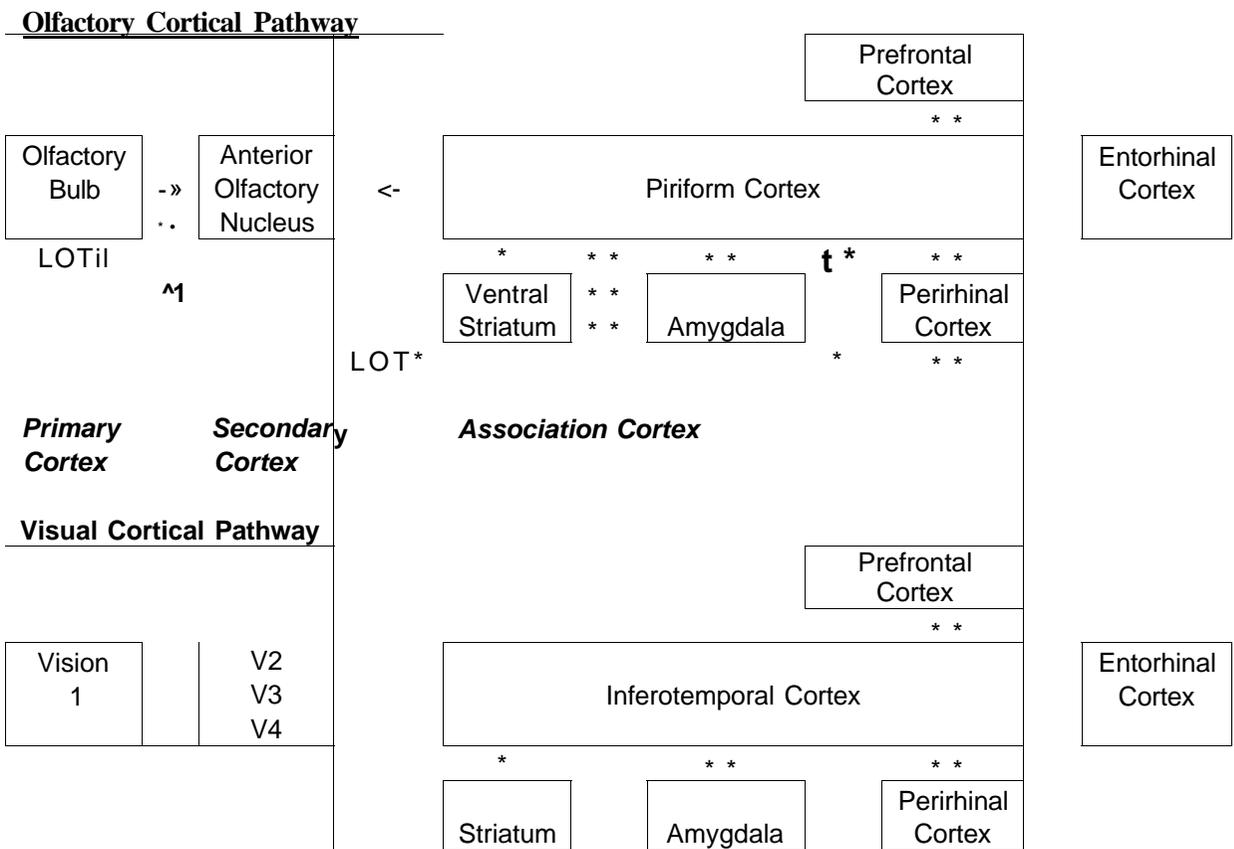


Figure1.2: Sketch of olfactory connectivity compared to visual connectivity, modified from Haberley (2001).

### 1.2.1 Perireceptor Events: Before the Odorant Receptors

The experience of smell begins with a scent or odour cluster, and turbulent nasal airflow. Odour molecules are inhaled and land on the mucus of the epithelium, a rough wet membrane lining the nasal cavity. A scent such as an orange contains many volatile and non-volatile components. The small, volatile, hydrophobic odour molecules are

dissolved in the wet mucus before interaction with the receptors of the epithelium occurs.<sup>1</sup> Chemicals sorbed by the nasal walls, before reaching the epithelium, have little or no odour. Sniffing, a universal behavior that often occurs with an olfactory stimulus, enhances the number of odour molecules available to the nasal cleft. The composition or thickness of mucus on the epithelium will affect smell, as anyone with a cold will testify - too much mucus and sense of smell is compromised. By changing diffusion time, the mucus influences how long odorant molecules take to reach receptor sites.

In most mammals the Bowman's gland and sustentacular (Sus) cells of the nasal epithelium secrete and regulate mucus (Schwob 2002). (In humans the Sus cells do not secrete mucus.) Mucus contains mucopolysaccharides, immunoglobulins, proteins (e.g. lysozyme) and xenobiotic metabolizing enzymes (e.g. peptidases), which break down odour molecules to be received by odour receptors. Mucus in the nasal area is also believed to deactivate, remove and desorb odorants (Leopold, 1990). Coincidentally, in the liver Sus cells are known for their detoxifying functions including phagocytosis, cell eating.

To increase odorant concentration, which improves odorant access to the olfactory receptors, odorant binding protein (OBP) in the mucus binds to hydrophobic odorant molecules, and solubilizes these molecules further. OBP action can increase odorant concentrations 1000-10,000 fold beyond the odorant's concentration in ambient air (Leopold, 1990). OBPs are proposed to form a complex with odorants, transport the odorants (lipophilic ligands) through the mucus to the receptors, accompany the odorant through across the epithelium, then move the odorants away for degradation, freeing up

<sup>1</sup> Recently non-volatile immune system molecules have been shown to function as olfactory cues in the mammalian main OE (Spehr et al., 2006).

the receptor for another interaction (Prestwich, Du & LaForest, 1995; Eisthen, 2002; Jacob, 2004). Multiple types of OBPs (olfactory-specific lipocalins) are distributed throughout the mucus in mammals, including humans, each with a fairly narrow odorant binding affinity (Eisthen, 2002).

### **1.2.2 The Epithelium: The Receptor Field**

The epithelium consists of olfactory and respiratory tissue. This review concerns itself with the much thicker olfactory epithelium (OE) (see figure 1.3). Interestingly, in humans the area of respiratory epithelial tissue appears to increase with age suggesting a reason for decreased olfactory ability with aging. Further, damaged olfactory epithelium often becomes respiratory epithelium. Much of the literature estimates that humans are capable of detecting between 4000 - 20,000 different odour molecules (Reineke, 2000); with some estimates as high as 100,000 chemicals (Buck, 2004). However, because no two odours are exactly the same, the olfactory sense is sensitive to infinite odour combinations and intensities.

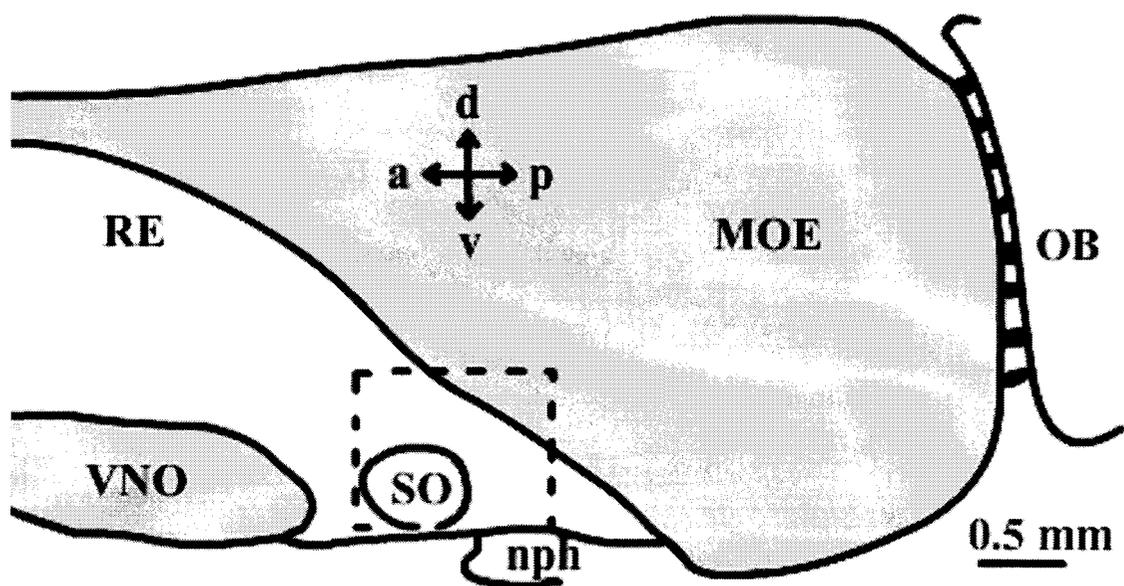


Figure 1.3: Illustration of structures within the nasal cavity (after Ma, Grosmaître, Iwema, Baker, Greer & Shepherd, 2003). Main olfactory epithelium (MOE), nasopharynx (nph), olfactory bulb (OB), respiratory epithelium (RE), septal organ (SE), vomeronasal organ (VNO).

Two other olfactory structures are found in the nasal cavity. According to the standard view, the vomeronasal (VNO) or Jacobson's organ, is dedicated primarily, but not exclusively, to detecting pheromones - chemicals secreted by conspecifics. The VNO projects exclusively to the accessory olfactory bulb, then onto the medial amygdala, and then to the hypothalamus, resulting in reproductive-based behaviors. The VNO is activated by non-volatile odors, and uses a pump mechanism. Each VNO receptor appears to be tuned to a specific pheromone molecule, and is able to detect the molecule at much lower levels than the chemical epitopes in MOB odorant molecules. In humans the VNO to AOB system is only identifiable during fetal development (Shipley et al., 2004). In spite of no VNO, humans retain pheromone detection ability.

Recent studies show that the main OE is also involved in sexual behavior (Spehr et al., 2006; Boehm, Zou & Buck, 2005); that modulation of reproductive and endocrine changes are synaptically connected with specific olfactory receptor neurons in the OE, not the VNO (Yoon, Enquist & Dulac, 2005); and that mice without an OE (VNO intact) don't mate (Yoon et al., 2005).

The septal organ (SO), a small island of OE surrounded by respiratory epithelium is found at the entrance to the nasopharynx, and projects to glomeruli in the medial, ventral part of the posterior bulb. Based on location and enhanced sensitivity to some

odours (over the same in the main olfactory epithelium) the SO is believed to have an alerting function, sensing odours during quiet respiration when air intake is not reaching the main epithelium (Ma, Grosmaître, Iwema, Baker, Greer & Shepherd, 2003).

### **1.2.2.1 Olfactory Epithelium**

The olfactory epithelium is a patch of skin rich with smell receptors in the nasal cleft; approximately 5-6 million in each nasal cavity in humans and substantially more in rats - about 50 million (Jacob, 2004); The OE is organized into several layers (see Figure 1.6). Apical are a row of supporting sustentacular (Sus) cells. Under these are several layers of mature olfactory sensory neurons (OSNs, also called olfactory receptor neurons, ORNs), which project a unique ciliated dendrite between the Sus cells into the mucus in the olfactory cleft. From the epithelium ORN afferents project to the olfactory bulb glomeruli, which, in turn, send afferents to the olfactory cortex and higher cortical areas for odour discrimination, classification and identification; or to limbic areas involved in emotion and physiological outcomes. Immature ORNs are found below the mature ones, under which basal cells are found. Structurally the OE and the germinative neuroepithelia of the embryo (which gives rise to the central nervous system) are similar except the much simpler OE only generates one neuron type. In 1978, Graziadei and Monti Graizdei found that ORNs continue to generate throughout one's lifetime - from foetus through adulthood.

### **1.2.2.2 Mature olfactory receptor neurons (ORNs)**

Mature ORNs are bipolar cells. The peripherally directed dendrites end in a knob (terminal enlargement) covered with approximately 8 to 20 cilia that extend into the mucus. These cilia contain a number of olfactory chemoreceptors or odorant receptors

(OR) - proteins to which odorant molecules bind and where sensory transduction (depolarization or hyperpolarization) occurs. Intracellular studies show action potentials (spike discharges) arise from potentials generated by the chemically stimulated ORs (see Figure 1.4). The OR snake through the cilia membrane on the ORN in a manner similar to rhodopsin, the receptor protein in eye rod cells. The many olfactory receptor types interact with G proteins to transmit signals to the cell, and belong to a receptor family known as 7-transmembrane domain G-protein coupled receptors (GPCRs). These receptors are believed to amplify small signals. A two-step transduction cascade, (explained in Eisthen, 2002; Ronnet & Moon, 2002) is believed to amplify and/or regulate amplification of small odorant signals. The olfactory GPCR format further provides flexibility to respond to different odours in different ways, and can transduce a diverse array of smells encountered (again, see Eisthen, 2002; Ronnet & Moon, 2002). An estimated 1000 - 1200 different types of olfactory GPCRs (olfactory receptors) exist in the mouse, derived from approximately 2 percent of mouse DNA (Buck & Axel, 1991). In humans, approximately 350 olfactory receptors actively coding for receptors, have been identified. Almost half of the large amount of human genome devoted to olfactory receptors (about 1% in all), are inactive pseudogenes. Each OR gene provides the code for an OR that recognizes one or very few odorants (Zhao, Ivic, Otaki, Hashimoto, Mikoshiba & Firestein, 1998). In mammals it is believed that each OR neuron expresses only one OR type (Nef, Hermans-Borgmeyer, Artieres-Pin, Beasley, Dionne & Heinemann, 1992).<sup>2</sup>

<sup>2</sup> Goldfish ORNs have been shown to express two odorant receptor types (Specia, Lin, Sorensen, Isacoff, Ngai, & Dittman (1999)).

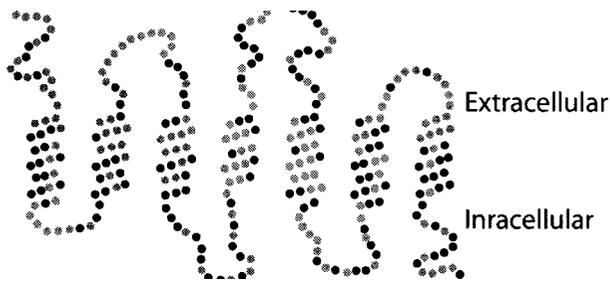


Figure 1.4: An odorant receptor on the cilia of an olfactory sensory neuron (after Buck, 2004).

Assigning specific receptors to specific odour molecules results in an "odour code." Mori, Hiroshi & Yoshihara (1999) have suggested that the physiology of olfaction presents four classes of odours - fatty acids, aliphatic aldehydes, aliphatic alcohols, alkanes - mapped into four broad zones of olfactory receptors on the epithelium (arranged from dorsomedial to ventrolateral); zones that persist through the olfactory bulb, and are synthesized in the olfactory cortex (see Figure 1.5). Within a zone, specific ORNs are distributed randomly throughout the epithelium, displaying a wide and dispersed distribution. When Mori and his colleagues (1999) compared the structure of the different odorant receptors in each zone, they found "highly homologous amino acid sequences tended to be localized in the same zone of the OE." This works well with the findings of Nobel Laureates Axel and Buck who showed distinct zones within the OE express nonoverlapping sets of OR genes (Buck, 2004).

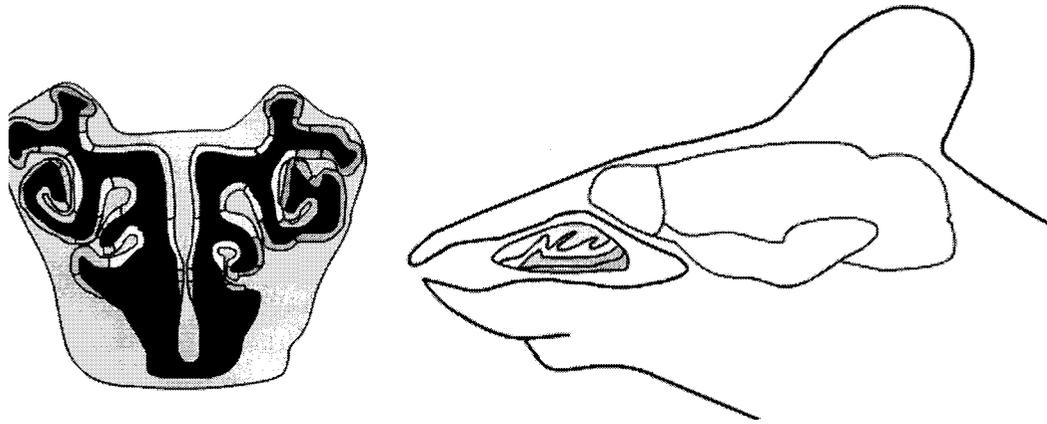


Figure 1.5: Illustration of the olfactory epithelium showing ORN zones (after Buck, 2004).

A non-myelinated axon from each mature ORN joins a bundle of 10 to 100 other ORN axons (a fascicle of olfactory nerve) that runs through the cribriform plate to the olfactory bulb (OB) where it converges on a glomerulus. Axons from olfactory sensory neurons that express the same olfactory receptor "converge" on only a few defined glomeruli in the OB. These glomeruli are organized into four zones in the olfactory bulb that correspond with the four zones in the olfactory epithelium (Mori et al. 1999). Glutamate is believed to be the neurotransmitter utilized at the synapse between the ORN axons and the mitral or tufted cell dendrites in various glomeruli.

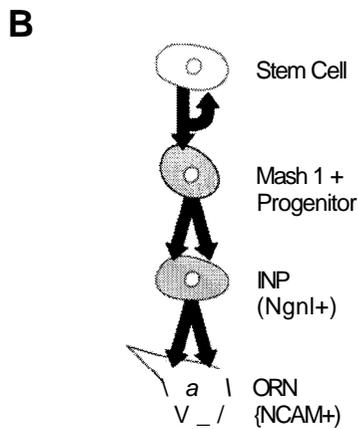
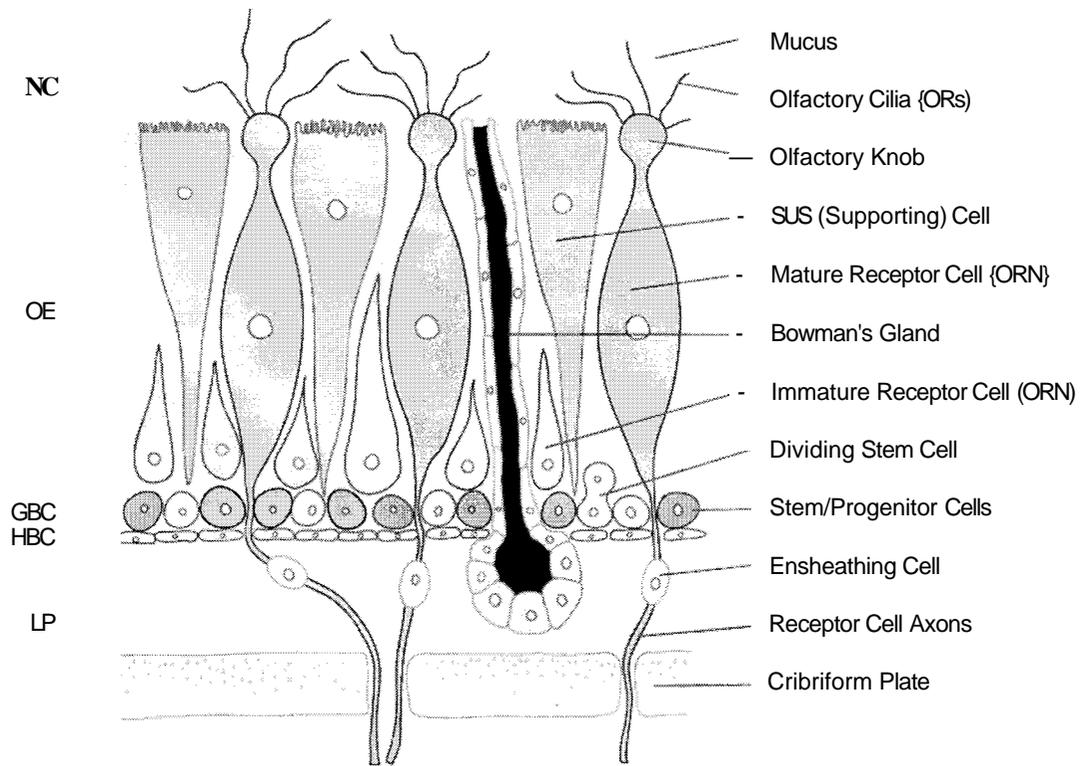


Figure 1.6: A. Cartoon drawing of anatomical structures within the OE. B. Cartoon drawing showing the OE stem/progenitor cell process (after Beites et al, 2005.)

Globose basal cells (GBC), horizontal basal cells (HBC), lamina propria (LP), nasal cavity (NC), olfactory epithelium (OE), olfactory ensheathing cells (OEC), olfactory receptor nerve (ON), olfactory receptor neuron (ORN), sustentacular cells (Sus).

### **1.2.2.3 Immature olfactory receptor neurons (ORNs)**

Immediately underlying the several layers of mature ORNs are several layers of immature ORNs. These developing receptor neurons have no cilia extended and do not express olfactory marker protein (OMP), but do express growth-associated protein GAP-43. Immature ORNs are highly prevalent during development, immediately after epithelial damage, and in the absence of a synaptic target - i.e. an olfactory bulb lesion. Immature ORNs are post-mitotic cells.

### **1.2.2.4 Basal Cells of the Epithelium**

Basal cells sit on a membrane at the base of the epithelium just above the lamina propria (LP), and are divided into two broad morphological groups.

**Globose basal cells (GBCs):** GBCs are simple round cells with very little cytoplasm. One group of GBCs are multipotent stem cells, mitotically active when neurons are needed, replacing ORNs. Calof, Bonnin, Crocker, Kawauchi, Marray, Shou and Wu (2002) have identified three stages of neuronal progenitor cells arising from the GBCs: The neural stem cell, the MASH1 transit amplifying progenitor (TAP), and the immediate olfactory receptor neuronal precursor (INP). [For an in-depth overview see Calof et al. (2002); and Beites, Kawauchi, Crocker and Calof (2005).] Post-mortem, olfactory epithelial tissue from donors up to 95 years of age, has been removed, expanded and manipulated (in vitro) to form neurospheres (Roisen, Klueber, Hatcher, Dozier, Shields and Maguire, 2001). The implications of a minimally-invasive source of pluripotent stem cells may be profound.

**Horizontal basal cells (HBCs):** HBCs are found at the base of the epithelium and, while morphologically different, have attributes similar to ependymal cells that line

the ventricles in the subventricular zone, and may contribute neurogenic regulators to the stem cell environment in the OE (Beites et al., 2005). These more specialized cells appear to overlie small bundles of exiting axons.

**Non-olfactory related cells:** Remarkably, the OE generates a number of different neurons and glia that migrate along olfactory nerves into the forebrain, and this migration occurs in developing and adult mammals (Dryer & Graziadei, 1994)

#### **1.2.2.5 Supporting Cells of the Epithelium**

**Bowman's Gland:** The Bowman's gland is a primary source for the mucus on the epithelium. It extends from the lamina propria through the epithelium. Both Bowman's Gland and Sus cells express different enzymes depending on their location in the epithelium. (See perireceptor events for more information on supporting cells.)

**Sustentacular (Sus) cells:** The microvilli capped Sus cells are comparable to glial cells in the CNS and a single layer line the apical side of the OE. Sus cell end feet project to the basilar side of the OE. Sus cells regulate mucus, play a role in the breakdown of odorant molecules, detoxify noxious chemicals on the OE, phagocytose dead receptor neurons, and provide structural support (Beites et al. 2005).

**Microvillar cells:** These bell-shaped cells on the apical side of the epithelium are somewhat controversial. Some believe microvillar cells to be support cells; others suggest they are bipolar sensory neurons. The microvilli of these cells extend into the mucus layer and the basal end tapers into an axonal-like extension that projects through the cribriform plate to the olfactory bulb (Rowley, Maran & Jafek, 1989).

**Olfactory Ensheathing Cells (OEC):** Along with generating ORNs, stem cells from the base of the epithelium appear to generate two types of olfactory ensheathing

(OE) cells which wrap around the axons and processes of the olfactory receptor neurons as they extend from the peripheral nervous system through the epithelium, lamina propria (cribriform plate) and into the central nervous system, specifically the olfactory bulb (Calof et al., 2002). These glial cells - a Schwann cell-astrocyte cross - permit and promote continuing axonal regrowth of new ORNs throughout life. Although OE cells do not produce a myelin sheath around ORN axons, they will myelinate larger diameter axons. When transplanted in nerve lesion studies, OECs encourage axonal outgrowth and promote recovery (Fairless and Barnett, 2005).

### **1.2.3 Main Olfactory Bulb: Primary Olfactory Cortex**

#### **1.2.3.1 Getting to the Bulb**

The scent of a single object, like coffee, is derived from a cluster of different odour molecules or molecular species. Most natural odorants are multifaceted. The coffee complex, for example, contains more than 100 volatile molecular species. According to an excellent review by Haberley (2001), an odorant receptor is tuned to a specific molecular feature inherent on a molecular species. Thus a single odour molecule may be activating a number of receptors. Each detectible feature of each molecular species in a molecular cluster (such as coffee) activates an ORN. Consider a simple two molecule odorant cluster: The first molecule contains features S, M and the second E, L. Upon reaching the OE, odorant receptors for features S, M, E and L become activated. Like a letter in the alphabet, each OR becomes a component of a code for the odour cluster - SMEL (see Figure 1.7).

Quality and quantity encoding is also apparent. Higher odorant concentrations with a specific molecular feature activate more ORs dedicated to that feature than lower

concentrations. Each odorant receptor then sends information via its ORN axon to the olfactory bulb and specific glomeruli dedicated to each type of odorant receptor. Like a word, the OR pattern activation for an odour becomes a unique configuration of glomerular module activation. The complexity of odour detection arises from a glomerular odour code. This spot code becomes salient for determining the odour stimulus. Glomerular activation pattern for an odour may be different depending on previous activation in the bulb arising from previous olfactory stimulus. Inhibitory interneurons and other cellular connections within the bulb can amplify or reduce action within a glomerulus (Freeman, 1991).

Molecule Cluster	Olfactory Receptor	Glomeruli Activation	Interneurons	
<div style="border: 1px solid black; padding: 5px; display: inline-block; text-align: center;"> <b>SS</b> <b>MMM</b> </div> <div style="border: 1px solid black; padding: 5px; display: inline-block; text-align: center;"> <b>E</b> <b>LL</b> </div>	<i>7I</i>	<b>S</b>	Tune glomerular activation depending on previous stimulus.	
		<b>s</b>		<b>S</b>
		<b>S</b>		<b>7P</b>
		<b>M</b>		
		<b>M</b>		<b>M</b>
		<b>C</b>		<b>c</b>
		<b>C</b>		
		<b>C</b>		
		<b>E</b>		<b>E</b>
		<b>E</b>		<b>L</b>
<b>E</b>				
<b>L</b>	<b>L</b>			

Figure 1.7: From odorant molecule to glomeruli. Sketch of how a two-molecule odorant is translated by ORNs and sent to dedicated glomeruli.

Buck (2004), and Mori et al. (1999) propose that two basic principles of olfactory axon projection have been demonstrated: "Zone to zone projection" and "glomerular convergence".

**Zone to Zone Projection:** Until recently researchers did not believe the olfactory bulb was arrayed along a small number of linear dimensions by afferent input. However, like other primary sensory brain areas, topographical organization is apparent when viewing the axonal connectivity between each of the four zones of the epithelium which correspond with four zones in the olfactory bulb. Genetically similar odorant receptors (homologous amino acid sequences) tend to be localized in the same zone (see Figure 1.8).

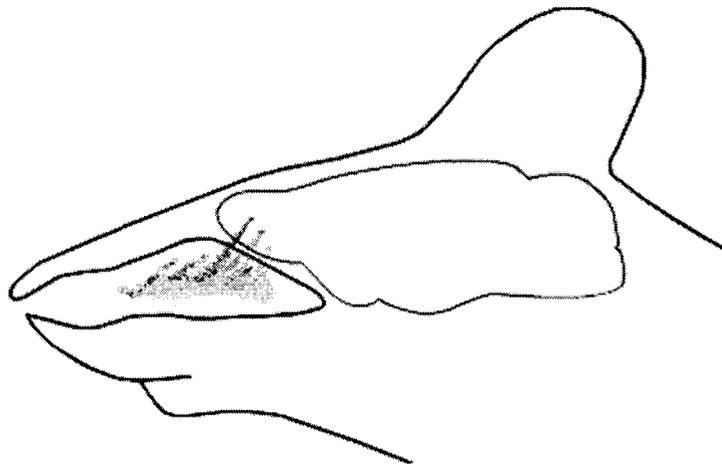


Figure 1.8: Zone to zone projection from the olfactory epithelium to glomeruli on the olfactory bulb (after Buck, 2004).

Each zone can be broken down into localized regions where a number of neurons from various glomeruli intermingle. Receiving neurons (mitral & tufted cells) interact with different groups of interneurons (granule and periglomerular cells) within a zone. Such associations are believed to sharpen and contrast odorants, as well as amplify odorant signals. These bulbar regions appear to focus on an odorant type (fatty acids,

aliphatic aldehydes, aliphatic alcohols or alkanes) with areas of overlapping specificities (see Mori et al. 1999).

**Glomerular Convergence:** In mammals, the axon from a single ORN synapses on only one glomerulus, with some axons taking "tortuous trajectories" - snaking around or through adjacent glomeruli before reaching their target glomerulus (Treloar, Feinstein, Mombaerts & Greer, 2002). The axons from identical olfactory receptor neurons converge on only 2 (sometimes 4) dedicated glomeruli located on each side of the bulb - one medial, the other lateral. The convergence of ORN input results in an increased sensitivity to an odour chemical due to the summation of the contributions from many identical sensory receptors. Thus, the individual mitral and tufted cells with apical dendritic projections into the glomeruli upon which the ORNs synapse, have a molecular receptive range "tuned" to the specific molecular features/conformations of various odour molecules (see Mori et al., 1999). In the mouse OB, the axons from the millions of ORNs (each neuron containing one of 1000 odorant receptors) are sorted into 1800 to 2000 glomeruli. Rats are believed to have between 3000-4200 glomeruli.

### **1.2.3.2 Glomeruli**

Each glomerulus is a spherical bundle of neuropil; is large (50-160um in rats); is a discrete functional unit surrounded by a shell of glial (wedge-shaped astrocyte) cells; and is made up mainly of axonal collaterals from ORNs, as well as the principle apical dendrites of mitral and tufted cells - the output neurons for the olfactory bulb. Tufted cell dendrites can innervate several glomeruli whereas the larger mitral cells innervate one glomerulus. Several thousand olfactory nerve fibers from identical ORNs synapse directly with dendrites from mitral and tufted cells, making up 80 percent of the synaptic

action in each glomerulus - an excitatory action mediated by glutamate (Shiple, Ennis & Puche, 2004). The remaining 20 percent of glomeruli synaptic activity arises from interneuron activity which works to synchronize and amplify ORN input. Output cells (mitral/tufted) form electrical and/or chemical synapses with juxtglomerular cells, which in turn feed back to the same and neighbouring output cells.

### **1.2.3.3. Glomerular Interneurons**

Juxtglomerular interneurons surrounding the glomeruli ramify each glomerulus. These include external tufted (ET) cells, periglomerular (PG) cells, and short axon (SA) cells. The primary dendrite of each ET cell extensively innervates a single glomerulus. ET cells also receive direct synaptic input from ORNs; generally PG and SA cells do not. Like mitral and tufted cells, ET cells also synapse with PG and SA interneurons.

Two subregions are known to exist within each glomerulus: One rich in ORN axon collaterals, and the other poorly innervated by ORN input. PG cell dendrites are generally found in the ORN-axon-poor region. Unlike their name, short axon (SA) neurons extend across multiple glomeruli and mediate interglomerular action (see Hayar, Karnup, Ennis and Shiple, 2004). Santhakumar and Soltesz (2004) summarize the role olfactory bulb interneurons play:

1. Control action potential discharge rate and timing.
2. Modulate the number of cells that are active in population discharges.
3. Provide feedforward or feedback inhibitory mechanisms.
4. Generate and synchronize network rhythms at various frequencies associated with a variety of smells. Thus, following Hebbian rule, cells that fire together wire together. Mori et al. (1999) propose that synchronized oscillatory discharges of

<sup>3</sup> Some olfactory nerve fibers terminate on juxtglomerular interneurons.

mitral and tufted cells "bind" glomerular regions/modules, combining signals from different glomerular modules at the level of olfactory cortex. Olfactory memory traces may arise from lasting dendrodendritic reciprocal synapses.

In an altered state OB interneurons commonly contribute to neurological and psychiatric disorders.

#### **1.2.3.4 Glomeruli: Regulating Odorant Detection**

In the OB fine tuning of odorant detection occurs on a number of different levels: Intraglomerular processing where dendrites from output cells and juxtglomerular cells synapse on each other within the glomerulus; interglomerular connections between juxtglomerular cells; dendrodendritic interaction between output cells; and connection between output neurons and granule cells (see Laurent, Stopfer, Friedrich, Rabinovich, Volkovskii & Abarbanel, 2001, for an excellent system analysis).

Output (mitral/tufted) and ET cell to PG/SA (juxtglomerular) cell synapses are generally excitatory dendro-dendritic connections mediated mainly by glutamate. PG/SA back to mitral/tufted dendro-dendritic synapses are inhibitory, mediated by GABA or dopamine, creating a feedback inhibition on the receiving cell. Because interneurons are connected to other mitral and tufted cells, inhibition is also imposed on these second order cells as well. The result is a fine tuning and sharpening of odorant molecule detection. (See Aungst, Heyward, Puche, Kamup, Hayar, Szabo & Shipley, 2003 for a complete description of centre-surround inhibition between glomeruli in the OB). Inhibitory back-projections from the olfactory cortex to the OB also facilitate rapid habituation to specific odorant exposure that is sustained for longer than a couple of seconds. Feedback inhibition occurs when afferents from the olfactory cortex excite OB

granule cells which inhibit mitral cell action via dendro-dendritic synapses, allowing the OB to rapidly habituate to odour-evoked activity and select new odorant stimulus patterns. In a complex olfactory environment the ability to rapidly select for odorant changes aids in the learning and discrimination of odorants (Wilson, 2000).

### 1.2.3.5 Bulbar Lamination

The olfactory bulb allocortex is organized into several layers described in the following sections (from Shipley et al., 2004), (see figure 1.9).

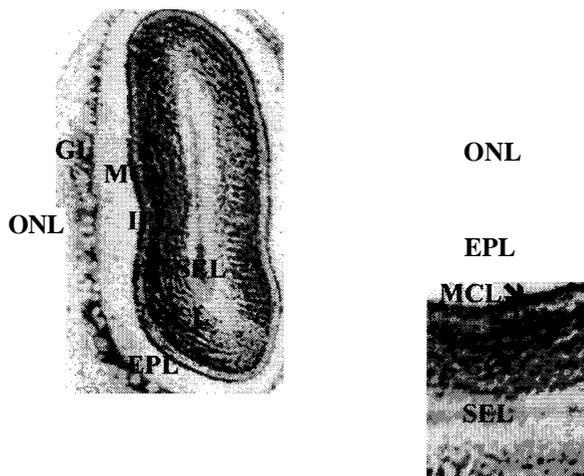


Figure 1.9: Olfactory bulb lamination: External plexiform layer (EPL), glomerular layer (GL), internal plexiform layer (IPL), mitral cell layer (MCL), olfactory nerve layer (ONL), subependymal layer (SEL).

### 1.2.3.6 Olfactory Nerve Layer (ONL)

Olfactory nerve fibers move from the epithelium through the lamina propria and the cribriform plate to the most superficial layer of the OB called the olfactory nerve layer which contains the axons from the ORNs. Ensheathing glial cells incompletely wrap these axons, separating the nerve fibers from the next layer of the OB.

### **1.2.3.7 Glomerular Layer (GL)**

Directly below the ONL is the glomerular layer containing globe-like glomeruli structures and juxtglomerular interneurons described above.

### **1.2.3.8 External Plexiform Layer (EPL)**

Deeper is the external plexiform layer, mainly containing the dendrites of the output neurons (mitral and tufted cells), particularly neurons of the superficial, middle and deep tufted cells, which are progressively larger the deeper one goes. Dendrites from these cells enter a glomerulus (tufted cells may connect to several adjacent glomeruli); secondary tufted cell dendrites project peripherally through the EPL, synapsing on each other. Axons from the smaller and most peripheral superficial tufted cells, form an intrabulbar association system (IAS) that travels to the opposite side of the same bulb, and topographically organize, thus providing a "point-to-point reciprocal projection between the lateral and medial bulb" (Shipley et al., 2004). The function of two symmetrical olfactory maps, one in the lateral and the other in the medial hemisphere of the olfactory bulb, is unknown.

### **1.2.3.9 Mitral Cell Layer (MCL)**

A monolayer of mitral cells, the biggest and principle neurons in the bulb, lies below the EPL. Developmentally similar to pyramidal neurons in the cortex, these cells are the primary output cells of the OB. In the rat, each OB has been reported to have around 45,000 mitral cells; humans have around 50,000. Fukushima, Oikawa, Yokouchi, Kawagishi and Moriizumi (2002) report a 20 percent threshold, where 9,000 to 15,000 mitral cells are required to retain olfactory discrimination function. Mitral cells have one apical dendrite that ramifies a single glomerulus. In rats, about 25 (of 40,000) mitral cells

project into one glomerulus. Mitral cell secondary dendritic branches run parallel within the olfactory bulb, and synapse with granule cell dendrites that are abundant in this layer. Lack of olfactory stimulus (pure air) reduces mitral cell size, while prolonged odorant exposure increases mitral cell size (Laing, Panhuber, Pittman, Willcox & Eagleson, 1985).

#### **1.2.3.10 Internal Plexiform Layer (IPL)**

The IPL is characterized by axons from OB output cells and dendrites of granule cells. A number of modulatory afferents originating from subcortical structures are also found in this layer, including serotonin (from raphe nuclei), norepinephrine (from the locus coeruleus) and acetylcholine (from the nucleus of the diagonal band) projections. Cortical afferents from the anterior olfactory nucleus, piriform, entorhinal and transitional cortical areas also act on granule cells.

#### **1.2.3.11 Granule Cell Layer (GCL)**

Groups of tiny, tightly packed granule cells are found in this the deepest neuronal layer of the OB. These axonless neurons are interneurons that interact with secondary dendritic branches of mitral cells in the same kind of feedback manner as mitral to juxtglomerular to mitral dendro-dendritic synaptic loops. The glutamate excitation of the mitral-to-granule cell triggers granule cell GABA release. Granule cells die in the absence of olfactory input, but undergo neurogenesis throughout life.

#### **1.2.3.12 Subependymal Layer (SEL)**

In development this layer presents the progenitors of most MOB cells. In baby rats these cells line the lateral ventricle - a ventricle present in the bulb during development, that recedes in adult rats. In adults, progenitor cells in the subependymal

layer originate in the more rostral subventricular forebrain regions, then migrate into the subependymal layer of the OB along the rostral migratory stream (Shiple, 2004). In adults, neuronal stem cells from the subependymal layer become interneurons - primarily granule cells (Frazier & Brunjes, 1988), and secondarily, juxtglomerular (periglomerular) neurons (Lois & Alvarez-Buylla, 1994). Neither granule nor PG neurons have afferents outside the OB.

The rostral migratory stream (RMS) arises from the subventricular zone (SVZ), and extends to the olfactory bulb. Newly generated neuroblasts<sup>4</sup> move into the stream, proliferate and migrate forward in a chain-like manner, in an astrocyte tunnel, at a speed of 30 um per hour, the same speed as migrating neurons during development (Lois & Alvarez-Buylla, 1994). The SVZ cells then move into the core of the OB. These cells are recruited to renew the constantly dying olfactory bulb interneurons in the granule and periglomerular layers. Studies using adult bulbectomy (OBX) have shown that the bulb is not necessary for neuronal precursor proliferation and migration down the RMS (Kirschenbaum, Doetsch, Lois & Alvarez-Buylla, 1999).<sup>5</sup>

#### **1.2.4 Lateral Olfactory Tract (LOT)**

Horseshoe peroxidase injected into mitral cells shows that axons and their collaterals project into the anterior, medial, and lateral olfactory cortex. The course of these projections is called the lateral olfactory tract. In the rat, the LOT is a nerve bundle

<sup>4</sup> Some controversy as to the origins of the neuroblasts exists. Some studies show the multipotent stem cells arising from ependymal cells lining the ventricle; others from SVZ astrocytes. As SVZ astrocytes are known to contact the ventricle surface, they may have been mislabelled as ependymal cells. See Conover & Allen, 2002 for a review.

<sup>5</sup> In the adult brain, the functional integration of newly generated neurons into existing circuitry is a logistical problem that has generated much interest. In the dentate gyrus, afferent connectivity of the new, adult GABAergic neurons is observed only after glutamatergic inputs have occurred. It is possible similar connectivity is the case in the olfactory bulb. See Song, Kempermann, Wadiche, Zhao, Schinder & Bischofberger, 2005, for an interesting review.

of approximately 48,000 fibers that runs ventrolateral<sup>^</sup> from the OB to the piriform and entorhinal areas with collaterals that project into cortical areas (Price and Sprich, 1975). The lateral olfactory tract (LOT), made up of myelinated axons mainly from mitral cells and middle and deep tufted cells in the bulb, projects axon collaterals to the anterior olfactory nucleus, piriform cortex, olfactory tubercle, transitional entorhinal cortex, the amygdala, septal nuclei, hippocampus and subiculum, and thalamus.

The LOT is the only efferent pathway from the OB to other olfactory structures. Price and Sprich (1975) report an average 42,000 axons in the tract immediately caudal to the olfactory peduncle, which reduce about 25 percent by the end of the tract. Larger axons are found lateral in the LOT, smaller ones medial. A degree of point-to-point organization is found at the peduncle (AON) that is lost as the LOT progresses. Axons (collaterals) leaving the tract are much smaller than axons within the tract.

### **1.2.5 Accessory Olfactory Bulb**

Receptor neurons from the vomeronasal organ (VNO) in the nasal cavity, project exclusively to the accessory olfactory bulb (AOB), a structure found on the caudal-dorsal olfactory bulb. Proportionally, males have a much larger AOB than females. A mini olfactory bulb of sorts, the AOB contains many of the same structures as the MOB. VNO projections innervate smaller glomeruli in the AOB. Unlike the MOB, receptors of the same type have been reported to project to a number of glomeruli (Belluscio, Koentges, Axel and Dulac, 1999). Periglomerular cells are found deeper within the AOB, thus the glomeruli are less distinct. An external plexiform layer (EPLA), mitral cell layer (MCLA), internal plexiform layer (IPLA) and granule cell layer (GCLA) are evident, and they are less distinct as well. VNO mitral cells are polymorphic compared to the same in

the OB, and often ramify a number of glomeruli (Takami & Graziadei, 1991). A report by Del Punta and colleagues (Del Punta, Puche, Adams, Rodriguez & Mombaerts, 2002) suggests that mitral cells dendrites are projecting to glomeruli that are innervated by the same VNO receptor type. Granule cells in the AOB are the same as granule cells in the MOB. Glutamate and aspartate are the main excitatory neurotransmitters, and GAB A is the main inhibitory transmitter. Dopaminergic interneurons are missing in the AOB.

Projections from the AOB do not overlap with the MOB, and are directed to the medial and posterior nuclei in the amygdala, the bed nucleus of the stria terminalis in the hypothalamus, as well as the nucleus of the accessory olfactory tract. Shipley (2004) suggests that neurons in these AOB targets may be modulated by circulating hormones as gonadal steroid receptors are evident on target neurons. The VNO, AOB and subsequent connects are believed to be important for reproductive behavior.

### **1.2.6 Olfactory Cortex**

Structures within the olfactory cortex are described in the following sections (from Shipley et al., 2004), (see Figures 1.1 and 1.10).

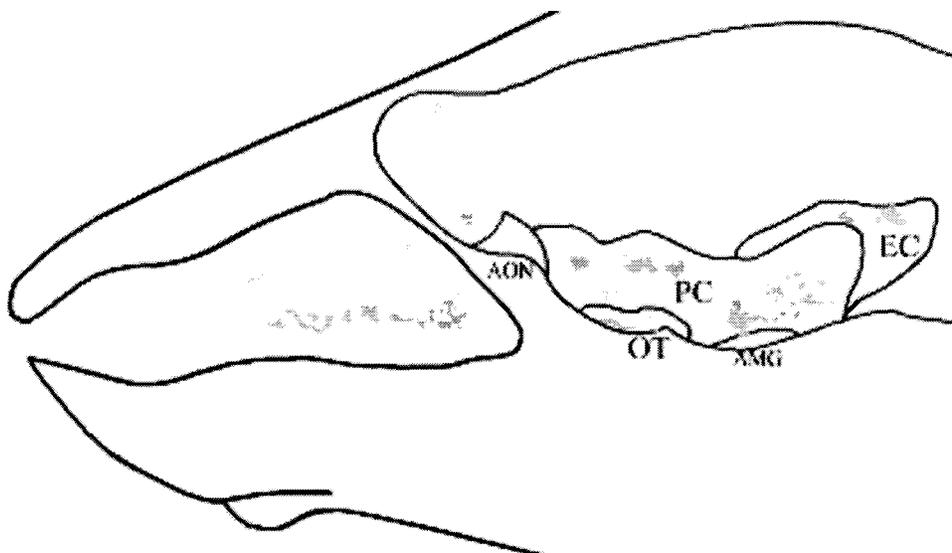


Figure: Illustration of two olfactory receptor neuron (ORN) types on the olfactory epithelium (OE), exciting dedicated glomeruli in the olfactory bulb (OB). Output projections from the olfactory bulb (OB) travel down the LOT and activate regions that sometimes overlap, in the piriform cortex (PC), entorhinal cortex (EC) and olfactory tubercle (OT), (after Buck, 2004).

### **1.2.6.1 Anterior Olfactory Nucleus/Cortex**

The anterior olfactory nucleus (AON) is the first stop of axon collaterals from the main OB. A laminated cortical structure, the AON is highly innervated by MOB output - more so than any subsequent regions. Mitral and tufted axonal collaterals tend to organize on the AON in a topographical manner with dorsolateral MOB neurons projecting to the dorsal, external region of the AON, and ventral MOB neurons projecting to the lateral AON subdivision (Schoenfeld & Macrides, 1984). Such chemotopic, point to point mapping does not appear to occur in other MOB axonal targets, although regionalization (clustering) does occur (Buck, 2004, Nobel Lecture) (see figure).

Many have proposed that the AON is a relay station due to its strong feedforward connectivity (OB to AON to piriform cortex) and feedback/return circuitry (piriform cortex to AON to OB). Recent studies suggest the AON is more complex than previously believed, however, and likely plays an essential role in processing olfactory information beyond that of a relay circuit - a role more in line with a secondary sensory cortex. Specifically, the AON "detects and stores correlations between olfactory features, creating representations (gestalts) for particular odorants and odorant mixes" (Haberley, 2001). Pyramidal cells, similar to cortical pyramidal cells (one apical dendrite and several spine covered, basalar dendrites), and at least four interneuron types are found in

the laminated AON. These illustrate complexity in the AON. Such functioning and anatomy correspond to activities at other secondary sensory levels (Brunjes, Illig and Meyer, 2005).

**AON Subdivisions:** The AON is divided into two subregions: the *pars externa*, a thin band of cells at the junction between the bulb and the AON; and, the *pars principalis*, consisting of two layers. The *pars principalis* is divided into a number of geographic subdivisions (*pars dorsalis*, *pars ventralis*, *pars medialis*, *pars lateralis* and *pars posterioralis*), but the boundaries for these divisions are not clearly delineated (Brunjes et al., 2005) (see Figure 1.11). Moving caudally, the AON meets the piriform cortex on the lateral region (*pars lateralis*), the ventral tenia tecta on the medial side (*pars medialis*, *dorsalis*), and the larger olfactory tubercle in the ventral region (*pars ventro-posterioralis*). Two levels of lamination are present in the AON, an outer plexiform layer (layer I), and a thick ring of cells on the inside (layer II). Price proposes that layer II is merged layers II and III, which are distinct in the piriform cortex (Price, 1973). Differences in cell size distribution and connectivity studies suggest that ventral and medial cells of the *pars principalis* project locally while dorsal and lateral regions project to further regions (Meyer, Illig and Brunjes, 2006; review in Brunjes et al, 2005).

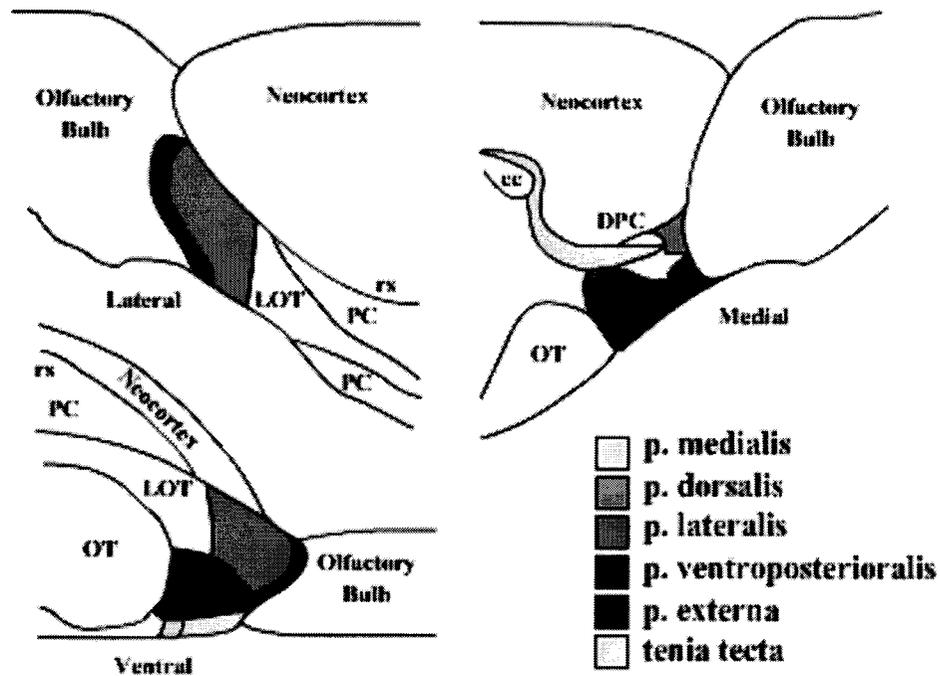


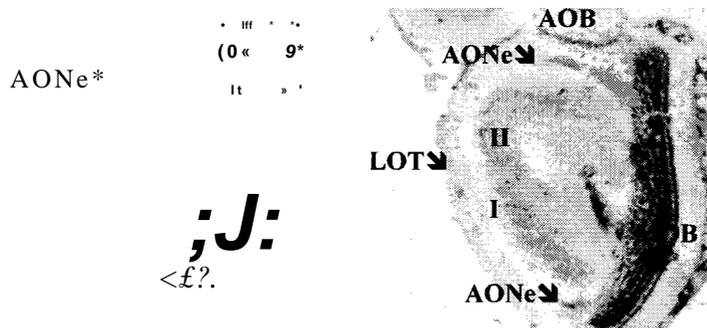
Figure: Illustration of the anterior olfactory cortex/nucleus in lateral, medial and ventral views (after Brunjes et al., 2005). Corpus callosum (CC), dorsal peduncular cortex (DPC), lateral olfactory tract (LOT), olfactory tubercle (OT), piriform cortex (PC)

**AON Connectivity:** Whereas most inputs to the AON are organized in a laminar fashion with OB inputs restricted to the lateral AON and olfactory cortex inputs restricted to a more medial position, the pars dorsalis shows intersecting rostral and caudal inputs (Schwob & Price, 1984). Intra-AON connections arise from inputs from other AON subdivisions. Inputs from outside the AON include the piriform and entorhinal cortex, amygdala, olfactory tubercle, nucleus of the lateral olfactory tract, and hippocampus; and modulatory inputs for norepinephrine (locus coeruleus), serotonin (raphe nuclei), and acetylcholine (nucleus of the diagonal band). Dopamine receptors are also found in the AON.

The *pars principalis* projects back to the MOB. About 80 percent of the pyramidal cell projections from the AOB terminate on the MOB (Haberley, 2001). These pyramidal axons organize on the bulb with a degree of laminar topography (Shiple et al. 2004). Other AON outputs include the AON (ipsi and contralateral), the piriform and entorhinal cortices, the amygdala, olfactory tubercle, tenia tecta, the nucleus of the lateral olfactory tract, CA1 division of the hippocampus, nucleus accumbens, and the lateral hypothalamus. The vast majority of AON efferents connect within the olfactory cortex.

**Bilateral Input:** The AON is the first structure of the olfactory cortex to receive bilateral olfactory innervation - direct afferents from the ipsilateral OB and indirect inputs from the contralateral hemisphere. Specifically, the AON projects axons via the anterior commissure to the contralateral AON. The contralateral information is then forwarded to the MOB. The result is "extensive, bilateral representation of olfactory information at the level of the AON" (Shiple et al., 2004).

### 1.2.6.2 Olfactory Peduncle



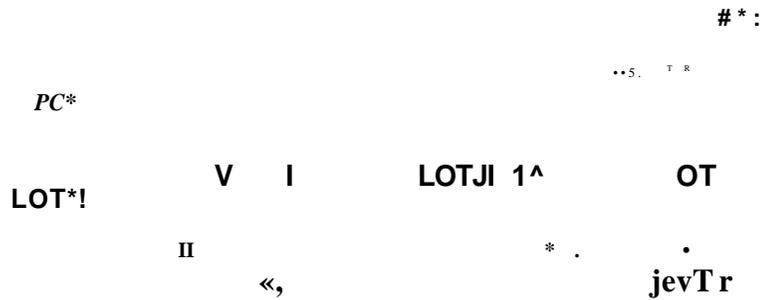


Figure 1.12: Layer I of the anterior olfactory nucleus (I), layer II of the anterior olfactory nucleus (II), accessory olfactory bulb, (AOB), anterior olfactory nucleus (AON), pars externa (AONe), dorsal penduncular cortex (DPC), lateral olfactory tract (LOT), main olfactory bulb (MOB), olfactory tubercle (OT), piriform cortex (PC), transition zone (TR), ventral tenia tecta (VTT).

In zoology, peduncle means stem. Along with the AON, the olfactory peduncle also contains the tenia tecta, the olfactory tubercle, and the dorsal penduncular cortex. The anterior piriform cortex also extends into the olfactory peduncle (Shipley, 2004; Brunjes et al., 2005) (see Figure 1.12).

**Tenia Tecta (TT):** The tenia tecta (TT), tenia meaning ribbon, is a caudomedial extension of the olfactory area, and the anterior end of the hippocampal extension known as the indusium griseum. Organized in a manner similar to the HPC, the TT is first found in the medial ventral area beside the AON. Moving caudal the TT is most noticeable under the dorsal penduncular cortex. The TT receives projections from the olfactory bulb, the entorhinal cortex, and the indusium griseum. Output from the TT goes to the diencephalon.

**Olfactory Tubercle (OT):** The olfactory tubercle (OT) is a bulge at the base of the cerebral hemisphere that receives input from the OB, and outputs to the hypothalamus

and the mediodorsal nucleus of the thalamus. The OT is found in the medial and ventral region of the peduncle. Cellular architecture in the OT appears to be a cross between cortical and striatal.

**Dorsal Peduncular Cortex (DPC):** The dorsal peduncular cortex lies at the top of the olfactory peduncle, and provides a bridge to the frontal neocortex. This allocortex is three-layered like the piriform cortex.

### **1.2.6.3 Lateral Olfactory Cortex: The Association Cortex**

The lateral olfactory allocortex is found in the temporal cortical mantle below the rhinal fissure, and extends from the piriform cortex, through the periamygdaloid and transition cortices to the lateral entorhinal cortex. In many respects the features of the lateral olfactory cortex resemble and function more like association cortex in other sensory systems rather than primary sensory areas (Johnson, Illig, Behan and Haberly, 2000; Haberly, 2001; Brunjes et al., 2005). Axonal collaterals in the piriform cortex have been found extending from the olfactory bulb to the entorhinal cortex; and projecting to areas "thought to play a role in mediating complex functions related to integrating sensory cues with behavior (prefrontal cortex), assessing the emotional or motivational significance of sensory cues (amygdala), and mutisensory association and memory (entorhinal and perirhinal cortex)" (Johnson et al. 2000).

Along with branching extensively in the piriform cortex, superficial pyramidal (SP) cells of the piriform cortex branch to most other olfactory areas (olfactory bulb, anterior olfactory nucleus, olfactory tubercle), to amygdaloid cortex and nuclei, to prefrontal cortex (agranular insula and orbital cortex where odor discrimination is believed to take place), entorhinal cortex, and perirhinal cortex. In these areas, the

arborisation is extensive, and believed related to "diverse functional roles including those related to behavior, cognition, emotion and memory" (Johnson et al. 2000). Axons from the piriform cortex also send extensive input into adjacent (lateral) cortical regions including the somatosensory cortex and the temporal cortex (Haberley, 2001). Reciprocal projections are found from these target areas (except the olfactory tubercle) back to the piriform cortex. Numerous interneurons exist in the piriform cortical area including basket cells thought to form axosomatic or axodendritic synapses providing inhibitory feedback and feedforward circuits.

From the piriform cortex the lateral olfactory tract continues through the transitional region called the periamygdaloid cortex, and onto the entorhinal cortex, all of which project heavily back to the MOB. Anderson and colleagues believe that smell operates down two pathways, a reward (i.e., pleasant/unpleasant) pathway, which lights up the orbital frontal cortex, and an intensity pathway, which lights up the amygdaloid areas (Anderson, Cristoff and Stappen, 2003). Most olfactory cortical feedback to the MOB works by exciting the GABAergic granule cells in the MOB, which subsequently suppress firing mitral cells. The piriform and entorhinal cortices, both substantially innervated by the MOB, project into the hippocampus which are likely important for memory, and smell-event associations.

### **1.3. DEVELOPMENT**

The mechanisms of olfaction are apparent early in development. Odorant receptors have even been found on human sperm to mediate chemotaxis, which indicates that sperm 'smell' their way to the egg (Spehr, Gisselmann, Poplawski, Riffle, Wetzel, Simmer, Hatt).

#### **1.3.1 Stages of Development**

The central nervous system goes through several stages during development: neurogenesis and cell identity specification; migration and differentiation; maturation including arbourization of the cell dendrites and the formation of synapses; myelination; and, refinement of synaptic connectivity by pruning.

During neurogenesis, neurons from stem cells of the ventricular zone divide either symmetrically, producing two stem cells, or asymmetrically, producing a stem cell and a progenitor cell destined to become a specific cell type. The distinction between a progenitor cell (also called a precursor or undifferentiated cell) and a stem cell is a difference in gene expression and thus, the proteins that control cell development (Wolpert, Beddington, Jessell, Lawrence, Meyerowitz & Smith, 2002). No clear structural differences foretell the developed cell's fate. These undifferentiated cells are determined or committed with respect to their developmental potential by the proteins contained therein, and all of their progeny have the same limited developmental options. Progenitor cells migrate to locations in the brain. Stem cells remain mitotically active but stay in the subependymal zone.

Differentiation of progenitor cells occurs gradually through a number of generations, each generation becoming more and more differentiated. External signals in

the local extracellular matrix including cell-surface proteins, which are secreted polypeptide cytokines and other molecules (hormones, growth factors), affect or "select" from the limited developmental options. Late in the division cycle, commitment to a particular neuronal (or glial) fate occurs, and "the neuron becomes unresponsive to environmental influences on its fate" (Wolpert et al., 2002, p.388). Proliferation of precursor cells occurs most markedly just before the terminal stage of differentiation at which point cells no longer divide, but begin to mature.

Progenitor cells migrate from the ventricular proliferative zone (subependymal/subventricular zone) along radial glia cells, to various areas of the brain, building the cortex from the inside out. While migrating and once in position these neurons (and astrocytes) start maturing and developing dendritic arborisation and spines on the dendrites. During maturation, guided outgrowth of axons toward target cells also occurs, creating synaptic connections and neuronal circuits. The growth cone at the axon tip extends out from the soma of the neuron and senses its environment. Filopodia on the cone extend and retract, allowing the axon to explore and grow through the substratum. Contact by the filopodia with other cells and the extracellular matrix, determine the direction of axonal growth. These external cues either attract or repulse axon growth. The filopodia make contact with guide post cells, which are neurons along a pathway to which filopodia make contact. "Arrival at the ultimate target is achieved through a sequence of stages, characterized by arrival at consecutive guide posts" (Wolpert et al, 2002, p.398). Even without these stepping stones axon growth cones eventually reach their target destination, albeit with increased axonal branching and at a slower pace.

Guide post cues are involved in axonal outgrowth that crosses the midline in the CNS. Once an axon has crossed the midline it does not return.

Axonal cone growth results in highly organized axonal projections. This is particularly noticeable when looking at the topographical maps that are formed by the projection of sensory receptor cells to primary cortical regions, in a point-to-point correspondence. Initially these projections are only "reasonably precise" (Wolpert et al, 2002, p. 401). The target is found when the axonal growth cone projects to a neighborhood of cells sending an active attractant signal. Cells are programmed to die during development (apoptosis), unless the axon connects with a target, and positive acting control signals (neurotrophins like nerve growth factor) are received. Both pre and post synaptic cells appear to "exchange signals that stimulate and coordinate their mutual differentiation" (Wolpert et al., 2002, p. 404). Because the nervous system produces too many neurons, axons compete for the neurotrophic signals produced in the target tissue. Thus, neuron survival depends on establishing a functioning synapse with an appropriate target cell. This connection and its neurotrophic elements prevent apoptosis and allow cell survival.

The development of a synaptic junction is often progressive and may take weeks to form. The following example of formation of a synapse in a muscle cell can be generalized throughout the CNS:

The axon terminals that make contact with the muscle cell are initially unspecialized, but they soon begin to accumulate synaptic vesicles. Initially several synapses from different axons are made on the same immature muscle cell but, with time, all but one are eliminated (Wolpert et al., 2002, p. 408).

The type of neurotrophic factor involved and the type of neurotransmitter present influence the survival of the presynaptic neuron. Neural activity, use by the connected

neuron, is also essential to development and maintenance of the connection. According to Wolpert et al. (2002, p. 411), "Stimulation of electrical activity in the target cell tends to strengthen the active synapses and suppress those that are not active at the time - cells that fire together, wire together." Competition between neurons for synaptic targets may "generate discrete regions of cortical cells" that respond to one stimulus or another, and thus form a topographical map.

### **1.3.2 Behavioral and Morphological Stages in Rat Development**

"The developing brain is very different in structure and organization at different ages" (Kolb & Gibb, 2001). Structural changes in the brain underlie behavior. As such, each stage of neuronal development, both pre and postnatal, corresponds with new or different function and behavior. Following is a look at behavioral activities and functions along with morphology for various stages in the developing laboratory rat (see Figure 1.13):

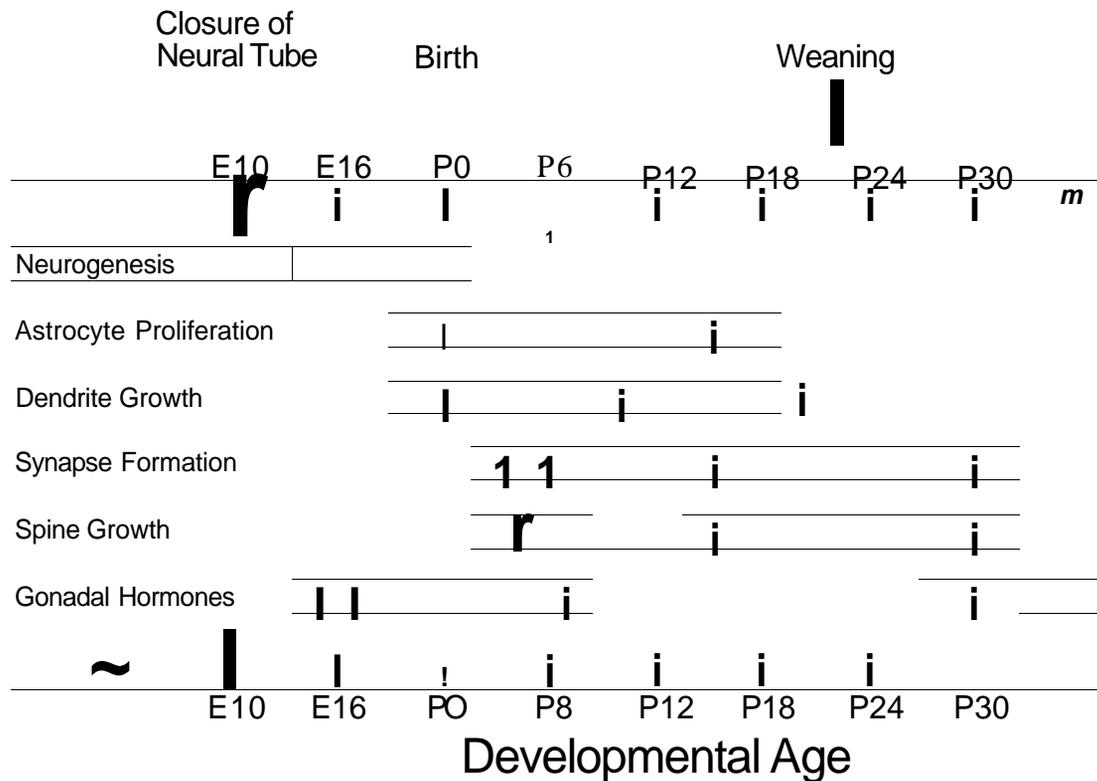


Figure.13: Major stages of cortical development in the rat (after Kolb, Gibb & Gonzalez, 2001). Bars indicate the approximate beginning and end for different developmental stages, and the dark area reflects a higher intensity for the stage.

### 1.3.2.1 Prenatal (Embryonic) Development

(Unless otherwise stated, from Robinson & Brumley, 2006.)

Throughout embryonic days (E) 12-21 the neurons in the rat brain are generated by the neural tube (ventricular zone). Production of cortical cells begins around E12, and generation is completed at birth (E22). Cortical injuries during neurogenesis tend to have the best outcome, as additional neurons are manufactured taking the place of those damaged. Mitral cells develop first, between E14 and E16, followed by tufted cells between E16 and E22 (Bayer and Altman, 2004). The lateral ventricle is extended into the bulb during development, and is the source for the progenitors of most MOB cells. In

the embryo, the olfactory organ is present and believed to exert a global influence on brain development.

Input from ORN axons reach the brain before any other peripheral input; influencing the development of the telencephalon, and playing a key role in the organization of the brain (Dryer and Graziadei, 1994). Neurons unrelated to the sense of smell are also generated by the epithelium, and migrate to the forebrain during the embryonic period (Schwanzel-Fukuda and Pfaff, 1989).

Embryonic rats begin small movements of forelimbs, head and trunk on E16. Activity levels increase until E18, and are stable from E18 until birth. Both organization (movement patterning) and synchrony (harmonized movements) of this spontaneous movement are more evident as gestation advances. At this same time sensory responsiveness is apparent in tactile, chemical and proprioceptive modalities. Gustatory and olfactory systems are believed to be functional by this time. By E20 strong odours (lemon extract, mint, citral) result in a face wiping response (an aversion response in adult rats), and increased heart rate (bradycardia), which decrease with habituation. Introduction of a novel odour results in dehabituation to the first odour, which indicates that central processes are responding and not peripheral actions. *In utero* taste aversions can be learned through association by E17.

#### **1.3.2.2. Newborn, Week One, P1 to P7**

(Unless otherwise stated from Alberts, 2006)

At birth, rats weigh approximately 7 grams, and are immature in comparison to other mammals. With neurogenesis complete, cell differentiation occurs and neuroblasts transform into specific types of neurons. Cell migration has already begun, shortly after

neurogenesis started, and continues throughout development, although the bulk of migration is completed by the end of the first week. The RMS shows a progressive reduction in thickness over the first three weeks postnatal (Altman, 1969). The OB is a late maturing structure, and most OB interneurons are generated postnatally (Luskin, 1993). Interneuron neurogenesis is generally complete by the first week. Postnatal day 4 (researchers reported P3 using birth day as PO) is associated with a dramatic increase in type 2 synaptic profiles (i.e. inhibitory synapses) (Westrum & Bakay, 1986). Mitral and tufted cells, the output neurons of the OB, are postmitotic at birth, but still able to show significant morphological change in dendritic arbourization and synaptogenesis (Monti-Graziadei & Graziadei, 1992). Granule cells continue to be generated beyond PI9 and into adulthood (see Figure 1.14).

Rats are born with odour sensitivity that increases until at least PI7. Both the level of stimulation and experience appear to be involved in the timing of olfactory developments. Anosmic pups do not suckle as the olfactory cues that attract the pups to the dam's nipples are not sensed. Normal pups increase their activity in response to the dam's odour, bringing them in contact with the nipple. It is believed that the olfactory cues for suckling are learned.

Sullivan (2005) reports that infant rats have the ability to acquire odour preferences. This ability is learned activity most evident in olfactory-based imprinting (maternal attachment). As adult structures for learning are not yet developed, a simple, norepinephrine-modulated circuit between the OB and the locus ceruleus, facilitates odour preference learning (Moriceau & Sullivan, 2004). Baby rats have a decreased ability to learn odour aversions until P10. The inability of neonates to learn aversion is

related to a stress hyporesponsive period in neonates, characterized by low basal levels of the stress-related hormone CORT.

Huddling, suckling, and reorienting or righting activity are prevalent at birth. Scanning movements, likely linked to olfactory or thermal sampling, are evident at birth and start recruiting additional body parts (spinal segments and forearms) by P4. Huddling is induced by thermal/tactile stimuli, but is followed by learned olfactory associations. By P15 olfaction cues becomes the main sense used for huddling.

### **1.3.2.3 Infant, Week Two, P8 to P14**

(Unless otherwise stated, from Alberts, 2006.)

Rats almost triple their birth weight to 20 grams by P10. The second week of life is a period of significant neural network development and neuron maturation. Cell differentiation continues until about P15, and neuron maturation, the outgrowth of processes and resultant synapses, is most intense (Kolb & Gibb, 2001). At P8 a doubling of synaptic profiles has occurred that is further doubled by P14 (Kolb, Brown, Witt-Lajeunesse and Gibb, 2001). Myelination begins in the lateral olfactory tract (Westrum & Bakay, 1986). According to Frazier and Brunjes (1988), the bulb's postnatal proliferative zone, the subependymal layer, exhibits peak cell density, number and volume at day 10 (see Figure 1.14).

During week two, output from the AON projects to both brain hemispheres (Shipley, Ennis & Puche, 2004) via the anterior commissure (AC). After commissural fibres have grown and developed, olfactory memories from the trained side can be accessed by the untrained side (Kucharski and Hall, 1987). Contralateral olfactory

memories cannot be retrieved prior to the second week of development; however, commissural fibres can access memories that occurred prior to commissural formation.

The emergence of auditory sensation begins at P8-10 with the pinna of the external ear separating from the head. Between P10 and P12 the external auditory meatus opens and drains, which means that the babies are now fully exposed to auditory sensation. Although rats respond to auditory stimulation before P10, sensitivity to auditory stimulation increases dramatically at this time.

Another significant milestone occurs in terms of motor activity. Between P8 and P11 the infant rats begin to crawl, albeit without using the hindlimbs. Motor activity follows a rostrocaudal developmental gradient, where head/mouth and forelimb activity develops before the hindlimbs. A medial-distal gradient, inside to out, is followed as well. By around P12 the animal is capable of a quadruped stance. Huddling activity at P7 is not affected by movement and activity states of littermates. However by P12 to P13 the activities of littermates play a big role in huddling.

Fear arising from predator odours is evident by P10. Adult male rats eat pups. By P10 baby rats freeze when exposed to an adult male odour (Takahashi and Rubin, 1993). This is coincident with amygdala involvement and increased corticosteroid levels (Wiedenmayer and Barr, 2001). Norepinephrine levels used for neonatal learning drop, likely due to emerging inhibitory noradrenergic autoreceptors (Moriceau and Sullivan, 2004).

#### **1.3.2.4 Juvenile, P14 to Weanling**

(Unless otherwise stated, from Brown, 2006; Alberts, 2006.)

By P15 baby rats weigh about 30 grams. At P15 synaptic profiles have doubled again. Layer 1 of the piriform cortex thickens dramatically during this time (Westrum & Bakay, 1986).

Eyelids open, and although rat pups are sensitive to light before this, they now receive full visual stimulation. Olfactory cues, not thermo-tactile stimuli, now dominate huddling behavior. Baby rats are beginning to sample other food around this time. By this PI 5 to P16 rats are capable of swimming. Swimming ability accompanies walking ability. With walking ability, at PI6 to P19, the rats complete their first departures from the natal nest, usually in response to visual, acoustic or olfactory stimulation. At PI7, robbing behavior commences, and by PI 8, the babies are rearing and sitting on their haunches when eating food pellets.

By PI9 rats attempt to reach for food, and dodging to protect food from conspecifics begins to occur. Spatial ability, proximal and distal cue navigation begins, with the maturation of the hippocampal mossy fibre system. By P21 most ambulatory skills are developed. Males have assumed the adult eating posture, and females achieve this three days later. By P25-26 precise dodging and precise reaching occur.

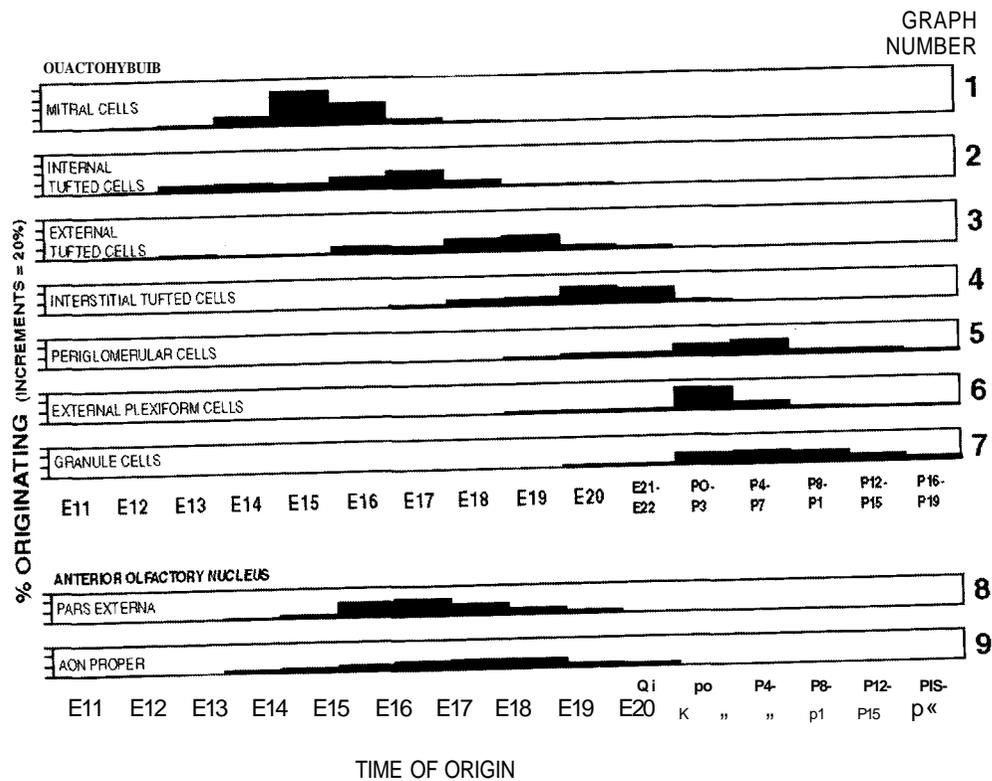


Figure 1.14: Development timeline for cells of the olfactory bulb and anterior olfactory nucleus (after Bayer and Altaian, 2004).

### 1.3.2.5 Cell Migration from the OE

During development, pluripotent cells from the OE are believed to migrate along the olfactory nerve fibre path to a number of forebrain locations (Dyer and Graziadei (2004). The developing olfactory epithelium is full of protein molecules associated with development and change. For example, ensheathing cells express neurotrophins and neurotrophic factors including NGF, GDN, and N-CAM. In tadpoles, a failed olfactory epithelium results in telencephalon development failure and formation failure of more posterior brain structures. These investigators conclude:

This suggests a direct relationship between the olfactory organ and brain development, perhaps a morphogenetic or even an inductive influence of the olfactory placode on the forebrain... The olfactory organ is present in embryos and might therefore have a critical role in early brain development... The

importance of the periphery in development is clear... It is reasonable to think that the olfactory organ could influence brain formation from a distance.

### **1.3.2.6 Glomeruli Development**

In the OB during normal development, protoglomeruli are formed by the ingrowing axons of ORNs, into the mitral and tufted cell dendritic zone (the external plexiform layer) in the bulb. The ORN axons are without a specific synaptic target. Glomerular boundaries around the protoglomeruli are subsequently formed by glial interactions, followed by interactions with output neurons and interneurons (Treloar, Purcell and Greer, 1999). A unique feature of mature ORNs is the expression of olfactory marker protein (OMP). OMP appears to serve a role in olfactory receptor axon guidance toward a glomerular target. Until about P12, developing olfactory systems often overshoot the glomerular layer, projecting into the plexiform layer of the OB (St. John & Key, 2005). As OMP levels rise, targeting errors diminish. OMP-null mice require 50 to 100 times higher odorant concentrations for standard detection responses (Youngentob & Margolis, 1999). Introducing OMP to OMP knockout mice improves olfactory signal transduction (Ivic, Pyrski, Margolis, Richards, Firestein & Margolis, 2000). Carr, Margolis and Farbman (1998) suggest OMP may be part of mitogenesis - related to increased neurogenesis in the OE.

Unique amino acid sequences for each odorant receptor type have been shown to provide a distinct identity that allows for afferent targeting of a specific glomerulus (Feinstein & Mombaerts, 2004). Methyl-CpG binding protein (MeCP2) correlates with ORN maturity and MeCP2 expression in ORNs appears to be necessary for synaptogenesis to occur in the bulbar glomeruli (Cohen, Matarazzo, Palmer, Tu, Jeon, Pevsner & Ronnett, 2003). Semaphorins are proteins that regulate axonal guidance.

Sema 3A and other semaphorins also play a role directing ORNs in the epithelium to the bulb during development and regeneration (Williams-Hogarth, Puche, Torrey, Cai, Song, Kolodkin, Shipley & Ronnett, 2000).

### **1.3.3. Cortical Injury and Development**

Plastic changes in the brain that result from cortical injury during development are affected by the stage of neuronal development in which the injury is incurred. At different stages in development, qualitative changes in response to injury are evident (see Kolb, Gibb & Gonzalez, 2001, for a review). According to Kolb and Gibb (2001), there are three ways in which plastic recovery works in the injured brain:

- 1) Changes in organization of remaining, intact brain circuits (re-organize).
- 2) Development of new circuitry novel to the injured brain (rewire).
- 3) Replacement of some of the lost neurons and glia (replace/regenerate).

Experience, neuromodulators and gonadal hormones can modulate all three of these recovery means, and each strategy does not necessarily work alone.

For many years researchers believed that the consequences of brain injury during development resulted in milder impairments than the impairments displayed by the same injury occurring in adulthood. This became known as the Kennard principle, after Margaret Kennard, who experimented with infant monkeys during the 1930s and 40s. She postulated that "recovery from early brain damage was associated with a reorganization into novel neural networks" (from Kolb & Gibb, 2001).

Donald Hebb, in the late 1940s, showed that early brain damage may produce even more severe deficits than the same damage incurred in a mature brain when he



examined frontal lobe injuries in children. Hebb hypothesized that "this outcome resulted from a failure of initial organization of the brain, thus making it difficult for the child to develop many behaviors" (from Kolb & Gibb, 2001).

Kolb and his colleagues showed that injury in rats at different stages of brain development activity (the precise age at injury) alters functional outcome. A cortical injury at embryonic day 18 (E18) in the rat occurs during neurogenesis, and shows no functional deficits (although some structural cortical changes occur). This may be due to neurogenesis that occurs during embryonic days 12 through 21 in the rat (the equivalent to second trimester in humans). Neurons that are being generated appear to replace the damaged tissue.

On the other hand, a cortical injury immediately following neurogenesis, at birth in rats (roughly equivalent to third trimester in humans), results in dismal functional recovery, in spite of massive changes in cortical connectivity. This novel circuitry has been associated with pruning failures and "crowding". The brains of these rats are much smaller with a much thinner cortical mantle. Interestingly, animals incurring an injury during this stage respond better to environmental manipulation like tactile stimulation and complex housing, than animals with lesions after P7.

A cortical injury during peak synapse formation, spine growth and astrocyte proliferation, which is around postnatal day 10 in rats (8 months in humans), results in particularly good recovery, in spite of a smaller brain size in adulthood compared to control animals (Kolb, Gibb, Gorny and Wishaw, 1998). Kolb & Gibb (2001) suggest that this improved recovery results mainly from brain reorganization that occurs during this time; specifically, increased dendritic arbour and spine density (more synapses)

relative to normal control littermates. At P8 a doubling of synaptic profiles occurs that is further doubled by PI4. The developmental period of synaptogenesis and cellular maturation appears to allow for greater plasticity.

Interestingly, the medial frontal cortex further supports recovery by generating new neurons that make appropriate connections, replacing many of those neurons lost by the injury. Thus, reorganization and regeneration of nerve cells occur together. Kolb indicates that damage in the second week of a rat's life stimulates neuro and gliogenesis, dendritic hypertrophy and increased expression of basic fibroblast growth factor (FGF-2). Brain insult during the first week results in dendritic atrophy, little neuro or gliogenesis, and no increases in FGF-2 (Kolb, 2006 A) (see Figure 1.15).

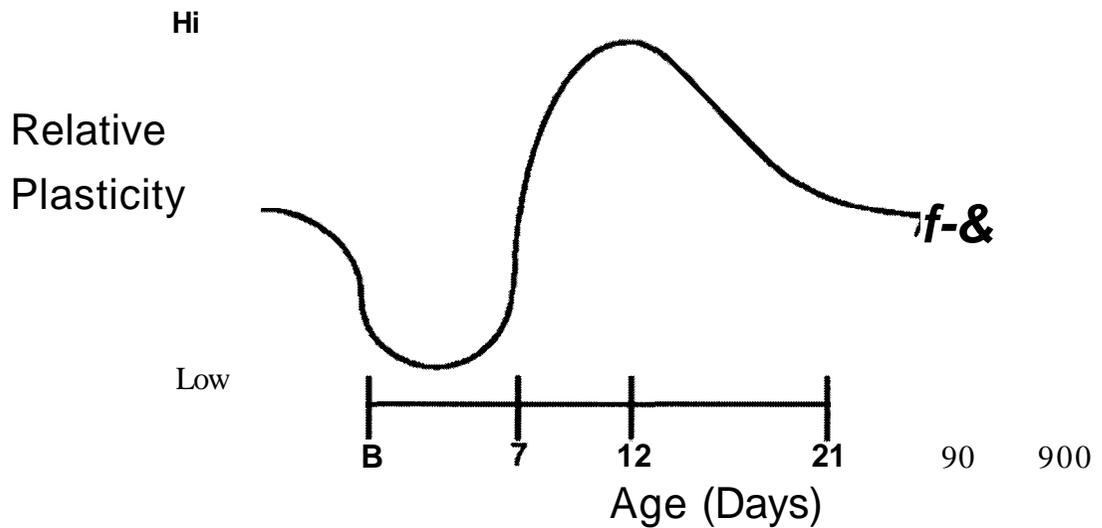


Figure 1.15: Graph depicting age-dependent differences in cortical plasticity in the laboratory rat (after Kolb, Gibb and Gonzalez, 2001). The relative plasticity bottoms out the first week after birth, and becomes highly plastic during the second week. Birth (B).

Villablanca and colleagues (Villablanca, Carlson Kuhta, Schmanke and Hoyda, 1998) reports similar critical maturational periods for lesion effects in kittens, tying

recovery with central nervous system maturational events. Behavioral outcome is related to the specific stage of cortical-area development when injury occurs. The age of the animal at the time of the lesion, which reflects the developmental status of the brain, makes a difference. For rats this critical maturational period is between P7 and P15 (Kolb & Gibb, 2001). For cats the critical maturational period is between E55 and P30-60 (Villablanca et al., 1998). For humans the critical maturational period appears to be between 8 months and 2 years, although Villablanca puts it at third trimester through 2-3 years. Further, different regions of the cortex may, to some extent, have varying critical periods, within the overall estimated critical maturational period, corresponding with the period of maximal dendritic growth and synaptogenesis.

Thus, an exceptional example of markedly different behavioral and anatomic outcomes occurs when comparing week one neonatal rat brain injury to week two brain injury. Further, the regeneration and compensation seen in the second week is not restricted to the late maturing medial frontal region where functional activity is restored to virtually normal (Kolb, Brown, Witt-Lajeunesse, Gibb, 2001). Week two injury-related recovery is found after injury to the orbital frontal cortex, motor cortex, posterior cingulate cortex, posterior parietal cortex, temporal cortex and occipital cortex (Kolb, 2006B).

#### **1.3.4 The Effects of Unilateral Bulbectomy**

Upon removal of an olfactory bulb in the neonatal rat (during the first postnatal week) most studies find that the forebrain grows onto the cribriform plate, and fills the remaining cavity. In 1994, Hendricks reported that recovery after an olfactory bulb

lesion is correlated with the pattern of penetration from the olfactory nerve (Hendricks, Knott, Lee, Gooden, Evers & Westrum, 1994). There is some controversy, however, over the functionality of ORN connections in bulbectomized areas, which is discussed in the following section. In most of these studies, once mature, neonatally bulbectomized rats undergo another surgery where the olfactory bulb contralateral to the first is removed (adult-stage surgery). Sensory receptor axonal growth does not occur in adult bulbectomies mainly due to scar tissue that blocks axonal growth. This scarring does not occur during the neonatal period (Butler et al., 1984). Following the effects of neonatal unilateral bulbectomy are considered, including changes to the epithelium and glomeruli.

#### **1.3.4.1 Effects on the Olfactory Bulb Input**

Mature olfactory receptor neurons (ORNs) on the epithelium ipsilateral to the bulbectomy, die quickly as a result of the insult. Cell death is followed by a period of increased neurogenesis in the olfactory epithelium, which peaks at about 5-6 days post-bulbectomy - a time that corresponds with the thinnest epithelial thickness after the OBX, and the smallest number of ORNs in the epithelium (Calof, Bonnin, Crocker, Kawauchi, Murray, Shou & Wu (2002). This suggests that neuronal progenitors are able to "read the number of differentiated neurons in their immediate environment and regulate the production of new neurons accordingly" (Calof et al., 2002), a process called feedback inhibition of neurogenesis. BMP (bone morphogenetic protein) produced by ORNs may play a role in this feedback regulation of neurogenesis.

Epithelial progenitor cells differentiate into new sensory neurons (Monti-Graziadei & Graziadei, 1992; Butler et al., 1984), and four weeks after neonatal olfactory bulbectomy axons from these newly differentiated neurons have extended through the

cribriform plate and made synaptic connections with tissue in the forebrain (Graziadei, Levine & Monti-Graziadei, 1978; 1979). Maintained olfactory receptor connectivity to a target appears to be essential for prolonged survival of the olfactory receptor neuron (Schwob & Szumowski, 1989; Monti Graziadei, 1983).

#### **1.3.4.2 Effects on Glomeruli Formation**

After a bulbectomy during development, olfactory receptor neurons will randomly innervate bulbar tissue, and develop unique glomerular-like structures on foreign targets including cerebellar transplants (Monti-Graziadei and Graziadei, 1984). Reciprocal synapses are found between the glomeruli-like structure and the sensory input. These reconstituted glomerular structures, found on the neonatally injured side, terminate in several forebrain structures, and often support olfaction recovery. They also appear to increase in complexity and organization with age (Graziadei et al., 1979).

### **1.3.5 Recent Studies of the Effects of Perinatal Olfactory Bulbectomy**

Three sets of researchers recently have come to somewhat different conclusions regarding the nature of the effects of perinatal olfactory bulbectomy. I consider each separately

#### **1.3.5.1 Case Study 1: Monti-Graziadei and Graziadei (1992)**

Monti-Graziadei and Graziadei (1992) report small olfactory bulb lesions, less than 50 percent, result in maintained shape and connectivity of the remaining tissue. Sensory innervation to the remaining tissue is randomly reconstituted into glomeruli-like structures on the bulbar surface. Some mitral cell dendrites reorient to reach these targets

and form profuse branching within them. Periglomerular cells do not form around these ectopic glomeruli.

With large olfactory bulb lesions involving more than 50% of the bulb, large portions of the remaining bulb restructure to connect with the random sensory nerves reaching the bulb. Mitral cells reorient, and profuse branching of these remnant neurons is found. In lesions with less than 10% of the bulb remaining, disorganization reigns, and bulbar lamination is not observed. Vomeronasal axons are found projecting into the main olfactory bulb when their regrowth is interrupted. Some receptor nerves reach into the forebrain forming glomerular structures close to the lateral olfactory tract.

In a full bulbectomy, some olfactory axons reach into the forebrain. Epithelial morphology changes on the side ipsilateral to the lesion. More subtle epithelial changes are found with smaller lesions, and more noticeable changes with larger lesions. Changes included reduced epithelial thickness, and increased number of globose basal cells.

Similarities can be drawn between partial and full bulbectomies in the first postnatal week. In both cases the target of the axonal cells shows exceptional plasticity, while the receptor axons show "independence and inducing ability" (Monti-Graziadei & Graziadei, 1992). Olfactory ability is maintained to some degree in both partial and full neonatal bulbectomies. Partial bulbectomy results in maintained olfactory discrimination and detection thresholds, whereas full bulbectomy results in maintaining at least some of these sensory abilities.

Monti-Graziadei & Graziadei propose that OB topography may be epiphenomenal to developmental sequence, and likely has much redundancy built into the structure. Of interest may be the point that the olfactory bulb and the AON are both topographically

organized for molecular mapping of features from odour molecules, with point to point projects from the OB to the AON. Further, pyramidal cells of the AON are the largest source of projections back to the MOB. With the AON intact, the possibility exists that it is exercising organizational function.

#### **1.3.5.2 Case Study II: Slotnick, Cockerham and Picket (2004)**

Slotnick et al. (2004) present a thorough study of anatomical and functional outcomes from P2-3 neonatal bulbectomy, followed by contralateral bulb removal in adulthood. Like previous studies, they show that ORN axons in most neonatal (P2-3) bulbectomized rats extend into the forebrain, "terminating in glomerular-like clusters within the frontal neocortex or anterior olfactory nucleus with some axons extending into the subventricular epithelium." They distinguish between four types of lesion, and classify a complete bulbectomy as one where "no tissue characteristic of the olfactory bulb was observed in (adult) tissue section." Lesion types include:

1. Lesions that invade the rostral third of the AON without damaging the frontal pole cortex. These result in frontal neocortex that extend into the bulbar cavity, and ORN connections made above the rhinal fissure. These rats are anosmic.
2. Lesions that extend into the frontal neocortex and medial aspects of the AON. These result in the olfactory peduncle extending into the bulbar cavity, and most had sensory axons directed toward the subventricular/epithelial zone, with some nerves penetrating the zone. These rats are able to detect and discriminate between odors, although ability varies widely. Glomeruli in the AON of rats where the forebrain entered the bulbar cavity, are mostly small (47.2 um; range 10-150um) when compared to glomeruli in the control rats (81.6 um; range, 40-

155um). The abnormal glomeruli contain no lamination, with no surrounding periglomerular-like cells. Dendrites from surrounding cortical neurons penetrate these glomeruli.

3. Lesions with olfactory bulb remnants after surgery. Remaining bulb tissue is rich with ORN axonal projections that invade the external plexiform layer (below the glomerular layer in the olfactory bulb). These rats have the best olfactory detection and discrimination performance, performing almost as well as controls.
4. Lesions where the frontal pole of the PFC is removed. In these animals 3-4 mm of frontal cortex is removed as well as the entire olfactory peduncle. These have no olfactory input to the forebrain, and are anosmic.

Slotnick's study tells us that better ORN synaptic connectivity to neurons in the AON allows for better olfactory function, and that dendrites of multipolar cells in the AON reorganize to connect/synapse with olfactory sensory axons to support olfaction. His study does not consider bulbar regeneration.

### **1.3.5.3 Case Study III: Racekova et al. (2002)**

Racekova and colleagues also studied development of forebrain anatomical characteristics after postnatal unilateral bulbectomy, and noted both ipsi and contralesional changes. (Racekova, Orendacova, Martoncikova, Zigova, Sekerkova, Marsala, 2002). In the adult rats with neonatal bulbectomies they describe displacement of prominent landmarks in the anterior direction, including the lateral ventricle and caudate putamen. Olfactory ventricles occur in both the intact and lesion side of the bulb, and cavities found along the RMS are similar to those seen in postnatal animals. In normal adults, RMS thickness is reduced and extended, and the passage cavity (lumen)

and ventricular cavity are no longer visible. They conclude that early developmental patterns are sustained into adulthood with OBX, and that the contralateral side is also affected by OBX and does not provide an adequate control.

### **1.3.6 Final Words on Development**

In olfactory bulb insult, preliminary studies in our lab show that partial spontaneous regeneration of the bulbectomized region occurs at P10 (Kolb & Gibb, unpublished). We hypothesize that this regeneration is a result of a developmental stage in which the bulbectomy occurred. In the literature, most studies focus on lesions in the first week of life. Little discussion about tissue regeneration in the bulb has occurred. The limited discussion on bulbar regeneration implicates olfactory nerve inputs, the formation of glomeruli by ORNs, or organizing properties of inputs from the olfactory cortex. Dyer and Graziadei (2004) refer to the olfactory epithelium as a "neurogenetic matrix from which other neurons are derived and subsequently migrate into the brain, perhaps using olfactory axons as guides." Slotnick et al, (2004) conclude that OE inputs connect with bulb remnants from incomplete bulbectomy, or connect to other frontal regions (frontal pole, the AON) when the bulb is missing. Monti-Graziadei and Graziadei (1992) suggest that feed-forward projections from the AON may be influencing bulbar organization. No investigations suggest developmentally-related bulb regeneration.

## 1.4 RATIONALE FOR THE ANALYSIS OF BEHAVIOR

The brain is designed to produce behavior. The goal of the behavioral analysis in the current study was to examine the effects of the unilateral olfactory bulb removal on motor and cognitive behaviors in adulthood. Because perinatal cerebral injuries often produce behavioral deficits in domains outside the effects of adult lesions, the choice of behavioral measures was therefore broad. Although the study of the effect olfactory bulb injury on behavior might be expected to include analysis of smell-related (sensory) behavior, this is difficult in animals with unilateral injuries as the effects can be expected to be subtle at best. We therefore decided to focus on the analysis of behaviors outside of olfactory perception. I note, however, that some motor behaviors such as skilled reaching are olfactorily controlled in the rat.

### **1.4.1 Sensory-Motor Behavior: Forelimb Asymmetry (Cylinder or Rearing) Task**

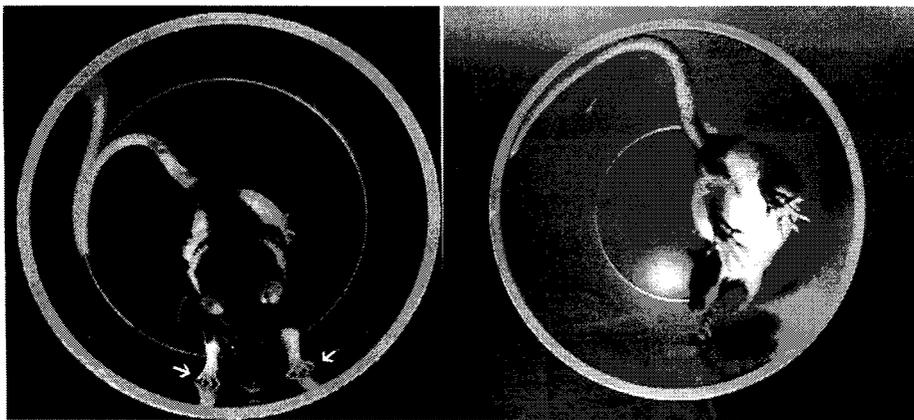


Figure 1.16: Forelimb Asymmetry (Cylinder or Rearing) task looking from above and below. (Courtesy of O. Gharbawie.)

Rats explore their environment horizontally and vertically. The cylinder task measures forelimb placement during vertical exploration, and assesses limb use asymmetry, if any, for each rat (see Figure 1.16). Animals are placed in a transparent

cylinder and filmed from below. The shape of the cylinder encourages the animal to rear onto its hind legs and to explore the wall with its forelimbs; yet the cylinder is tall enough that the animal cannot escape. Limb preference is observed while the animal is shifting its weight during vertical exploration. In normal rats, forelimb use is equally distributed between the left and right paw (Whishaw, Pellis and Gorny, 1992). Animals with cortically-related motor deficits will show asymmetrical limb use, and favor the limb ipsilateral to the damage - the limb controlled by the non-injured brain hemisphere.

## **1.4.2 Skilled Motor Behavior**

### **1.4.2.1 Sunflower Seed Task**

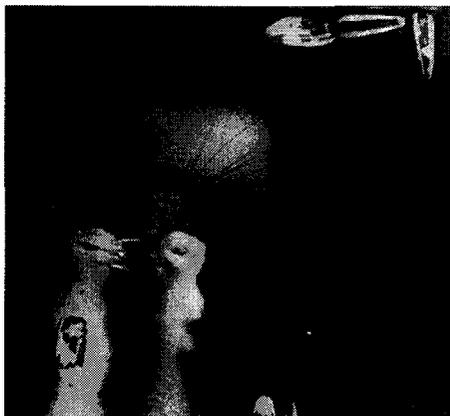


Figure 1.17: Sunflower Seed Task. (Courtesy of C.L.R. Gonzalez.)

For the rat, shelling sunflower seeds is a simple skilled motor task that involves limb and digit operation (see Figure 1.17). Rats grasp the seed, and then sit back on their haunches to eat. They manipulate the seed with digits from both paws so that the fat end of the seed is positioned into the mouth. The animals chew on the corner of the seed to breach the integrity of the seed and to split the shell into two lengthwise pieces. Rats remove the shell with their teeth and turn their heads to dispose of (spit out) the shell. The sunflower

seed task helps determine whether the animal is efficient at removing the seed from the shell or if there are motor impairments as a result of injury (Gonzalez, 2004).

#### 1.4.2.2 Single Pellet Reaching

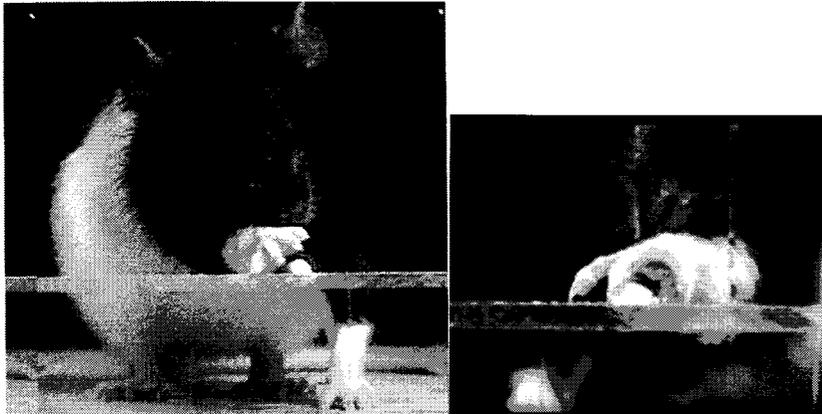


Figure 1.18: Photographs of rats reaching through the narrow slot of the plexiglas cage used for single pellet reaching. Handedness is distributed evenly across the population. (Courtesy of I.Q. Whishaw.)

Skilled movement requires event sequencing and irregular motor patterning, which are controlled by neural components beyond those that support locomotion (Metz et. al, 2005). Reaching by the rat is homologous to human reaching. Unlike primates, rat reaching is guided by olfaction, not vision (Whishaw & Tomie, 1989). Whishaw and colleagues developed the single pellet reaching task to assess olfactory-guided, forelimb reaching ability in the rat (Whishaw and Pellis, 1990). In this skilled motor task animals are required to stretch their paw through a single, narrow slot in a plexiglas cage for a food pellet situated on an external shelf (see Figure 1.18). A sequence of motor activities is required to accomplish the task. 1. Locating the food - First, the animal sniffs through the slot to determine whether or not a pellet is available. 2. Forelimb advance and grasp - If a pellet is available, the animal reaches with a forepaw through the slot and grasps the

pellet using olfaction as its guide. 3. Bringing food to mouth - The rat retracts the forelimb, and, with both paws, takes the pellet to its mouth and eats it. 4. Return to start - Once a pellet has been eaten or if no pellet is on the shelf, the animal is required to go to the back of the cage before returning to the front. Return to start after each pellet forces the animal to reposition itself before retrieving each pellet. Animals with a cortical injury show deficits at various sequence levels.

#### **1.4.2.3 Handedness, Limb Asymmetry**

In the 1930s Peterson showed that handedness in rats is evenly distributed across the population (in Whishaw et al., 1992). Peterson and McGiboney (1951) went on to show that limb preference is sensitive to cerebral insult. Their experiments documented a shift in handedness in naive adult rats following unilateral injury. Whishaw and colleagues (1986; 1992) confirmed that most rats display a strong preference for one limb over the other, but unlike humans, the ratio of left preferred to right preferred animals is almost equal. Approximately 13 percent of rats are ambidextrous (Whishaw, 2006). A strong limb preference results in greater reaching success with that limb - a finding which has been consistently replicated in the SPR task. This suggests an adaptive advantage of lateralization over ambidexterity. Animals with unilateral neonatal lesions in the medial frontal cortex, motor cortex and striatum, display abnormal reaching behavior with both paws (Whishaw, O'Conner and Dunnet, 1986; Whishaw, Pellis and Gorny, 1992B; Whishaw, 2000; Gonzalez, Gharbawie, Williams, Kleim, Kolb and Whishaw, 2004).

### **1.4.3 Locomotion: Open Field (Activity) Testing**

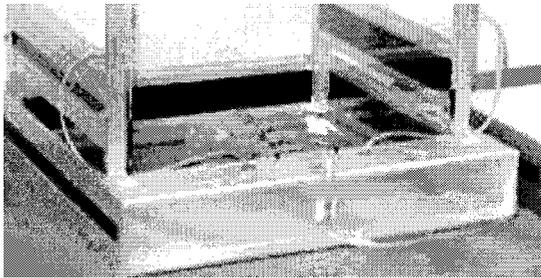


Figure 1.19: Versamax open field, activity monitoring system. (Courtesy of <http://www.accuscan-usa.com/versamaxfeatures.htm>).

Open field activity testing allows insight into general activity levels, exploration strategies and levels of anxiety experienced by an animal. Specifically experimenters gather information on the distance traveled when ambulating, the amount of time spent resting, habituation to the novel environment, rearing activity, turning biases, and movement in response to contact with the walls. Information is collected by photocells in a plexiglas cage (see Figure 1.19). The automated collection of such a wide range of activity is sensitive to motor impairments and drug effects (Metz et al., 2006).

Rats generally start exploration by moving around the edge of the chamber, next to the wall - a behavior called thigmotaxis. A less anxious animal will spend more time in the middle of the cage than an apprehensive animal. As normal animals habituate to the box they become less active. Early on a preferred place or home base within the open arena is established, where an animal lingers and revisits (Eilam and Golani, 1989). Often this base is where the animal first enters or is placed in the arena. The animal will move home base near a prominent object if it is available in the arena, or into a corner of

the chamber. Grooming is high at home base and reflects a degree of calm in the animal. Trips away from home base are slower, more circuitous and punctuated by many stops. Return to home base is more direct and much quicker (Golani, Benjamini, Dvorkin, Lipkind and Kafkafi, 2006). The limited size of Versamax activity cage is restrictive when determining trips away from home base.

#### **1.4.4 Anxiety: Elevated Plus Maze**



Figure 1.20: Elevated plus maze. (Courtesy of R. Gibb.)

Montgomery (1958) was the first to report the use of open and closed alleys as a way to measure anxiety in rodents. In his study normal rats that were given access to an enclosed alley explored much more than rats with access to an open alley. Montgomery described this behavior as an approach-avoidance conflict between a fear drive induced by novel stimulation and the drive to explore. In a novel environment an anxious rat will avoid open, lit and elevated places, preferring to move along walls and in enclosed spaces. The plus-shaped maze has four arms: two that are enclosed with walls, and two that are open (see Figure 1.20). The maze is elevated off the floor adding an additional dimension of fear to the open arm. Pellow, Chopin, File and Briley (1985) validated the use of open and closed arm entries in a plus-maze as a measure of anxiety in the rat with

drug studies. Since then the elevated plus maze has become a popular paradigm to evaluate anxiety, directed exploration, and locomotion.

### **1.4.5 Learning: Morris Water Maze**

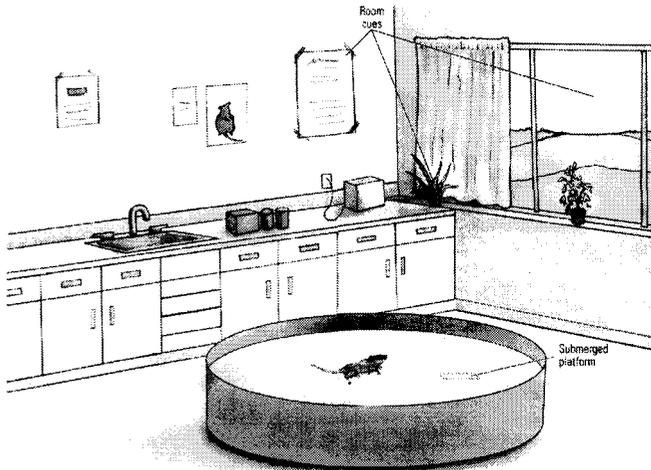


Figure 1.21: Morris water maze illustrated. (Courtesy of B. Kolb.)

Since the 1980s, the Morris water maze has been used to test navigational skills (spatial learning and memory) in rats (Morris, Garrud, Rawlings and O'Keefe, 1982). Rats are excellent swimmers and their natural ecology is often aquatic. Water-related tasks are thus often used to study rat behavior. The task in the water maze is for the rats to learn the location of a safe platform in a large pool of water (see Figure 1.21). The platform is not marked by any proximal cues, and the inside of the pool is painted white to match the opaque water. In the first trial the animal finds the platform by chance. Upon finding and crawling onto the platform, the animal is given ten seconds to look around at distal visual cues posted on the walls around the room, before being removed from the pool. Because the animals are motivated to escape the pool, normal rats learn the platform location rapidly from any starting position around the circumference of the pool. In subsequent placements into the pool the animal employs spatial localization

skills using the distal cues noted in the previous swim to the platform. This is called "place" navigation (Sutherland and Dyck, 1984). In this task rats are forced to explore using visual cues and visually guided behavior. Numerous studies have shown that animals with lesions in the cortex or hippocampus have difficulty locating the platform, even after repeated trials and much training (e.g. frontal cortex - Kolb & Gibb, 1991; hippocampus - Whishaw, Rod and Auer, 1994).

#### **1.4.6 Olfactory Based Behavior**

Olfaction is a principle source of stereo-sensory input for the rat. The sense of smell is clearly involved in the single pellet reaching task, and is inherent in the other behavioral tasks listed. Specific olfactory based behavioral tests are not included in this study as previous investigators have shown that the ability to smell is not severely affected in neonatal rats (P3-5) that receive a unilateral bulbectomy (Racekova, Cizkova and Sekerkova, 1997). Racekova and colleagues removed the right bulb at P3-P5. In adulthood the rats underwent a simple food finding task. The left, intact bulb was then removed, and the animals retested. All rats performed as they did previously on the food finding task, which indicates, minimally, that functional recovery of olfactory function occurred after neonatal bulbectomy. Regenerated connections from olfactory receptor neurons supporting olfaction were also reported by Hendricks, Kott, Lee, Gooden, Evers and Westrum (1994), but were not evident in an earlier study by Slotnick and colleagues (Butler, Graziadei, Monti-Graziadei and Slotnick, 1984). Twenty years after his first study, using precision olfactometry to assess behavior, Slotnick found that rats receiving a unilateral OBX at P2, followed by removal of the intact bulb at P90, were able to detect and discriminate between different odors (Slotnick, Cockerham and Pickett, 2004) -

apparently by using the perinatally-injured olfactory system. Further, the reconstituted olfactory ability was related to the location and density of the ORN inputs. Animals with projections restricted to the prefrontal cortex, and those without inputs to the forebrain, were anosmic.

In the bilaterally bulbectomized adult rat, odor detection and discrimination are severely compromised. Functional regeneration of the olfactory bulb in the adult rat is blocked by the formation of scar tissue that occurs following bulbectomy (Butler et al., 1984).

## **1.5. RATIONALE FOR ANATOMICAL METHODS**

### **1.5.1 Cresyl Violet Stain**

Cresyl violet acetate (CV) stains cell bodies a bright violet color; specifically staining the endoplasmic reticulum in neurons and glia. Also known as a Nissl stain, CV may be used alone or in conjunction with a number of other stains. CV methodology involves regressive staining; a procedure that requires excessive staining of the tissue, then washing out the stain until the optimum level of stain is achieved. Cresyl violet staining provides excellent resolution for gathering both qualitative and quantitative information including cortical thickness measurements, specific measurements of numerous local areas within the brain, and quantifying cell numbers.

### **1.5.2 Luxol Fast Blue**

Luxol fast blue is a copper phthalocyanin stain that adheres to fatty tissue, specifically myelin on nerve fibers and tracts. Luxol fast blue staining is used to highlight myelinated tissue, by staining bright blue areas of greater axonal componentry and connectivity.

### **1.5.3 Image J**

Image J is a public domain image analysis program from the National Institutes of Health (NIH, American; <http://rsbweb.nih.gov/ij/download.html>). The program is used to measure the real world area, mean, length and perimeter of various regions within the brain.

### **1.5.4 Golgi Cox and Dendritic Morphology**

Donald Hebb proposed, and later investigation has confirmed, that structural changes in synapses mediate function and learning (Kolb and Wishaw, 2001).

Synapses, found on neuronal arbour (among other places), are plastic, which means they change as a result of the experiences of the organism. Changed synapses alter synaptic transmission, which alters function. Altered dendrite length, dendrite branching and changes in the number of excitatory spines are strongly correlated with synaptic change (Greenough and Chang, 1985). When a treated animal is significantly worse or better at a behavioral task, the altered behavior is manifested in altered dendritic morphology.

Camillo Golgi revolutionized the way the scientific world looked at brain organization when he published the first drawing of a silver nitrate stained neuron in 1873 (Golgi, 1873 in Finger, 1994). Early investigators like Ramon y Cajal who shared the Nobel Prize with Golgi in 1906 , extensively used the Golgi technique to reveal morphological features and architecture of the brain. To this day the stain continues to be useful for delineating characteristics and changes in brain structure as they relate to changes in behavior. The Golgi-Cox staining method, a modification of the Golgi method that exposes tissue to ammonia which causes mercury salt to deposit on the cells, stains black against a background of yellow. For reason yet unknown, a small percent of cells (1 to 5%) are stained randomly. The staining provides consistent and good resolution of dendrites and spines - primary sites for excitatory synapses.

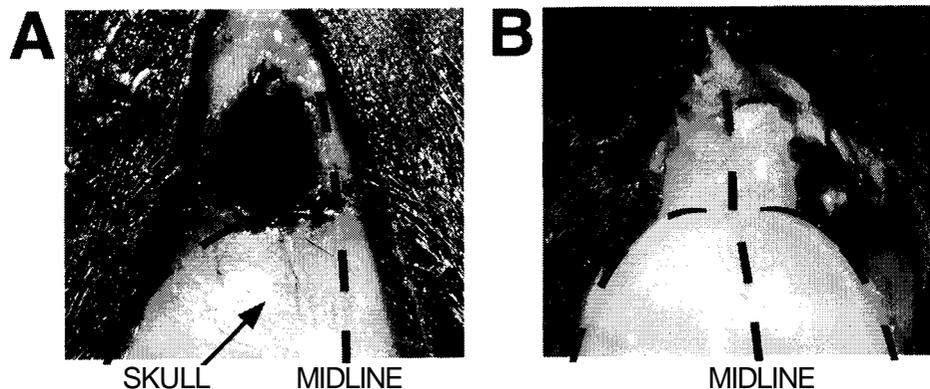
<sup>6</sup> The Nobel Prize was also shared with Sherrington who added the synapse to the mix.

## **Chapter 2**

### **MATERIALS AND METHODS**

## **2.1 Subjects and Surgery**

Four litters totaling sixty-one Long-Evans rats, 22 female and 39 male, were bred and raised in the University of Lethbridge vivarium from U of L stock. On PI or PIO, a unilateral bulbectomy suction lesion was performed on 31 animals: 6 PI females, 10 PI males, 5 PIO females, and 10 PIO males (see Figure 2.1). All animals were anesthetized by induced hypothermia. Core body temperature was reduced in a chamber set at -1 to -5°C. The remaining controls (5 PI females, 10 PI males, 6 PIO females, and 9 PIO males) received a sham surgery, where an incision was made through the skin above the skull, then sutured. Weaning occurred on P22 and the animals were housed in groups of two or three individuals in clear plexi-glass cages. Behavioral testing began once rats reached P90. The room housing the colony was regulated to a temperature of 22°C. A 12 hour to 12 hour light/dark cycle (7:30 - 19:30 light hours) was in place. Housing and experiments were conducted to the standards of the Canadian Council on Animal Care, and approved by the University of Lethbridge Animal Care Committee.



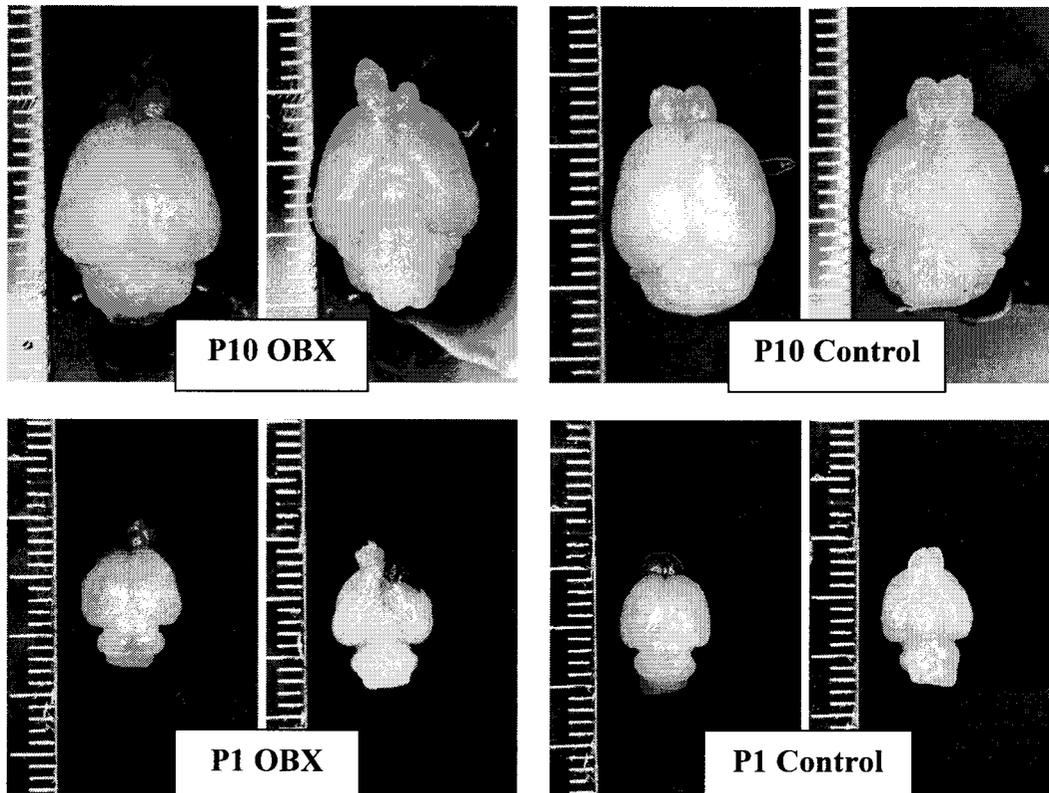
**C**

Figure 2.1: Top: A. Photograph of removed olfactory bulb at time of surgery. B. Photograph of regenerated bulb in adulthood in the same animal. C. Dorsal and ventral views of bulsectomized and control brains extracted the day of surgery, P1 or P10.

## **2.2 Behavioral Assessment**

### **2.2.1 Forelimb Asymmetry (Cylinder or Rearing) Task**

Each rat was placed in a plexiglas cylinder (20 cm in diameter by 30 cm high). The apparatus was placed on a table with a transparent bottom, under which a mirror was angled to allow for filming of limb placement from all directions around the cylinder. Animals were observed in the cylinder for five minutes. Videotape for each trial was slowed to view and score. Data were collected for three minutes of rat activity. To quantify performance on the task, the first limb (right or left) to independently contact the cylinder wall for each rear (occurring at a time when the animal was regaining its center

of gravity after moving from a horizontal to a vertical posture) was recorded. When the animal contacted the wall with both paws, a "both" score was made. Forelimb use was determined by calculating the number of left and right forelimb contacts as a percentage of the total number of first touches after take-off. The number of overall, independent (right or left) forelimb touches on the cylinder wall during lateral exploration, were also calculated. Schallert and colleagues described the cylinder task (Schallert, Fleming, Leasure, Tillerson and Bland, 2000).

### **2.2.2 Sunflower Seed Task**

Each animal was habituated to several sunflower seeds daily in their home cage during the week prior to testing. For training and testing, animals were placed in a clear plexiglas cylinder approximately 30 cm in diameter. The cylinder was placed on a transparent table, with a mirror angled beneath for filming from the ventral angle. Five sunflower seeds were placed in the cylinder. Timing began when the animal picked up the seed and was paused upon completed consumption of the seed, as animals often explored between seed consumption. Trails continued to a maximum of five minutes. One training period was performed, followed by two trails that were timed, filmed and number of shell pieces counted. Results were calculated on the mean latency to complete shucking each seed, and the number of shell pieces left following seed extraction. Whishaw, Sarna and Pellis (1998) described the sunflower seed task.

### **2.2.3 Single Pellet Reaching (SPR)**

Rats were "food deprived" throughout the training and testing periods to provide motivation to perform the SPR task. Animals received only a measured amount of food each day - 15 g for females, 18 g for males (normal animals consume 18-25 g). Care was

taken to ensure weight levels remained within 90 percent of initial body weight. Training lasted for about 20 minutes per day per animal, and was performed over approximately 18 days. Animals were placed in a plexiglas cage (45 cm deep, 14 cm wide, 35 cm high), and required to reach for a sugar pellet through a narrow slot (1 cm wide). For the first week, numerous pellets (45 mg Rodent Chow food pellets, Bioserve Inc.) were placed on the shelf to encourage reaching to obtain food, and to allow animals to establish their preferred limb for reaching. Once paw preference was established, one food pellet at a time was placed in the indentation on the shelf, contralateral to the preferred limb. Animals have difficulty reaching for pellets on the ipsilateral side. The rat was then trained to travel to the back of the cage after grasping or knocking the pellet off the shelf. Animals were presented 20 pellets per day. Once the animals reached their performance threshold (a consistent level of accuracy), testing began. Quantification of this task was calculated by evaluating end point performance - the number of successful hits performed by the rat as a percentage of overall reaches. The final trial was quantified for each animal. SPR was described by Whishaw and Pellis (1990).

#### **2.2.4 Open Field (Activity) Testing**

The VersaMax Animal Activity Monitoring System is a plexiglas cage (42cm long, 42 cm wide and 30 cm high) with photocell sensor bars connected to each wall. A grid of invisible infrared beams cross the cage: 16 beams in the left to right (x) axis, 16 in the front to back (y) axis, and 16 on the vertical plane. When an animal is placed in the cage, the x and y light beams are broken, revealing the position of the animal. The animal's position is determined 50 times per second. In this study, spontaneous activity, including the distance traveled (an estimation of locomotor activity) and vertical activity

(rearing), was recorded in the Versamax activity monitoring apparatus (AccuScan, Columbus, OH). The animals were naive and had undergone no other behavioral testing before placement into the open field. Animals were left in the activity cage for ten minutes. Horizontal activity and vertical (rearing) activity was examined for the first two minutes in the apparatus, and the overall ten minute testing period.

### **2.2.5 Elevated Plus Maze**

The elevated plus maze was used to determine if unilateral bulbectomy resulted in pathological changes in anxiety through a quantitative variation (heightened or reduced) in apprehension. Animals were placed into the maze facing one of the walled alleys, and left to explore for ten minutes. Anxiety was measured by counting the number of entries into the open and closed arms. An open arm entry involved the rat orienting all paws (fore and hindlimbs) into the open arm. To gain a better picture of interaction between the open and closed alleys, the number of open arm entries as a percentage of total entries was determined. The amount of time spent in each area was not recorded. Pellow et al. (1985) and Lister (1987) described the elevated plus maze.

### **2.2.6 Morris Water Maze (MWM)**

To test spatial learning each animal was placed in a large circular swimming pool (1.55 m diameter; 46 cm high) filled with cool (23°C), opaque (milky) water. A trial involved placing a rat by hand into the water, facing the pool wall, next to the perimeter of the pool. Four trails from one of four starting points in the north, west, east and south quadrants of the pool, were tested daily for each rat. Upon finding the submerged platform the animals were allowed 10 seconds on the platform (11x12 cm) at the end of each trial. After 90 seconds, animals unable to find the platform were placed on the

platform and given the opportunity to look for the distal cues. On subsequent days memory of the platform location was tested. The platform remained in the same position for all trials over all five consecutive days of testing. Rats were placed in a holding cage between trials. On completion of daily testing rats were returned to their home cages. Quantification for this task involved recording the latency to the platform, and the distance the animal traveled to reach the platform. On the sixth day a probe challenge was conducted. Each rat was placed in the pool without a platform, for 60 seconds. The percent of time spent in each quadrant was recorded. Video tracking software, the HVS Image 2020 Plus Tracking System, recorded both measures by following the black heads of the Long-Evans rats against the white pool background. Procedure for the MWM was described by Morris et al., 1982.

### **2.3 Anatomical Assessment**

After completion of the behavioral testing, each animal was weighed before perfusion. Animals were about 180 (Golgi-Cox) or 240 (CV) days old at time of sacrifice. Subjects were administered an overdose of sodium pentothal (Euthansol, 0.6 to 0.8 ml for male adults; 0.5 to 0.6 ml for female adults), then perfused intracardially with a saline vascular rinse. For cresyl violet histology, animals were also perfused with four percent formaldehyde. The brains were extracted (see Appendix A for perfusion and extraction methodology), and weighed. For Golgi-Cox histology brains were immersed in Golgi-Cox fixative. The brain tissue for CV histology, 32 animals, was frozen to -21°C and sectioned on the cryostat into 40 micron slices. Every fourth coronal section underwent staining with cresyl violet. A second series was stained with cresyl violet and

luxol fast blue combined (see Appendix A). The tissue from the remaining 30 animals was sectioned into 200 micron coronal sections on the vibrating microtome and prepared for Golgi-Cox impregnation (also see Appendix A).

Four PI male animals were eliminated from statistical analysis due to ambiguous lesion or lesion marking (see Appendix B).

### **2.3.1 Gross Measurements**

All brains were photographed from the dorsal and ventral angles prior to sectioning and slicing on the cryostat or Vibratome. Gross anatomical measurements, were calculated using Image J. The area (in mm<sup>2</sup>) was determined for each hemisphere, each half of the cerebellum, and each olfactory bulb.

### **2.3.2 Olfactory-Related Morphology**

CV stained coronal sections were scanned and digitized (600 ppi) using the HP Scanjet 4650. Regions of interest were measured in nine female and nine male animals (3 PI lesion, 3 P10 lesion, 3 control per sex). The area (in mm<sup>2</sup>) was determined for the following, from the first two planes (see figure 2.2, A & B) within the olfactory bulb proper:

1. Olfactory nerve layer inputs were measured to indirectly infer whether inputs from the olfactory receptor neurons were altered by the lesions.
2. Area was measured around the outer glomerular layer, and around the mitral cell layer (or granule cell layer in lesion animals lacking a row of mitral cells), to determine the effect of lesion.

3. Five area measurements of glomeruli from each bulb for each plane were taken. When possible, two glomeruli were measured medially, two laterally, and one ventrally. Area for each of the five glomeruli was averaged in each brain.

Area measurements for the accessory olfactory bulb were completed throughout all sections. The AOB is completely removed in bulbectomy. Total volume was calculated and percentage comparisons of AOB volume performed. Within the brain, measurements were made of the lateral olfactory tract from three separate planes (see Figure 2.2, F,G & H) tracing immediately under the dark-stained cells of the piriform cortex. The lateral ventricles were measured on two separate planes (see Figure 2.2, H & I). Because olfactory fibers cross to the opposite hemisphere via the anterior commissure (AC), it was decided to measure the size of the AC in order to make an indirect measure of olfactory connectivity. Measurement was made of the AC thickness at its widest point.

In the lesion hemisphere, brain structures appeared to be thrust forward on the lesion side. To quantify this effect, observations were made of the first appearance of the frontal cortex, the last appearance of the granule cell layer of the olfactory bulb, the first appearance of the piriform cortex, and the first transitional zone (withdrawal of the rhinal fissure). The caudate putamen (see Figure 2.2, F & G) and hippocampus (see figure I) were also measured to determine if the rostral thrust of brain tissue observed on the lesion hemisphere continued throughout the brain.



### 2.3.3 Cortical Morphology

Using a Zeiss DL2 POL petrographic projector, cortical thickness measurements were taken from the coronally-cut, CV stained sections. Five planes in each brain were examined and measures taken as outlined (Zilles and Wree, 1995; Paxinos and Watson, 1998), and six measurements from each plane, three from each hemisphere, were taken for each rat (see Figure 2.3):

**Plane A:** Bregma 1.70 mm, the first appearance of the caudate putamen; measuring the frontal premotor cortex (dorsal), frontal primary motor cortex (central), and the primary somatosensory (parietal) cortex (lateral).

**Plane B:** -0.26 mm, anterior commissure; measuring frontal primary motor cortex (dorsal), the forelimb area of the cortex (central), and the secondary somatosensory (parietal) cortex (lateral).

**Plane C:** -1.88 mm, first hippocampus; measuring hindlimb area of the cortex (dorsal), the primary somatosensory (parietal) cortex (central), and the secondary somatosensory (parietal) cortex (lateral).

**Plane D:** -4.8 mm, posterior commissure; measuring visual (occipital) secondary areas (dorsal), primary auditory (temporal) cortex (central), and auditory (temporal) association cortex (lateral).

**Plane E:** - 6.3 mm, final hippocampus; measuring primary visual (occipital) monocular area (dorsal), the lateral secondary visual (occipital) area (central), and primary and secondary auditory (temporal cortex) (lateral).

Overall and individual (dorsal, central and lateral) statistics were generated for each plane.

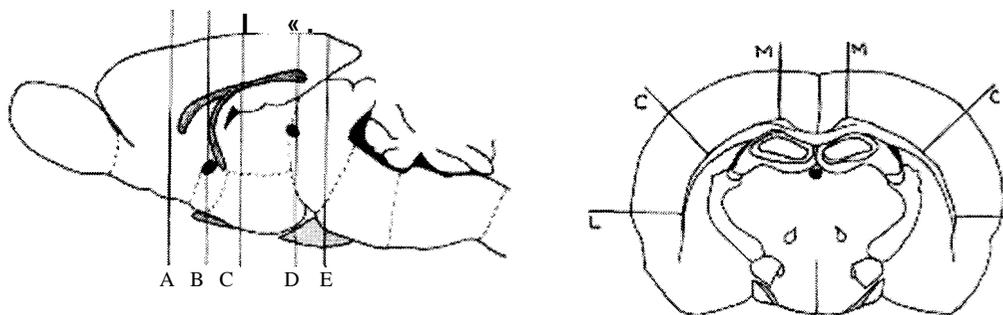
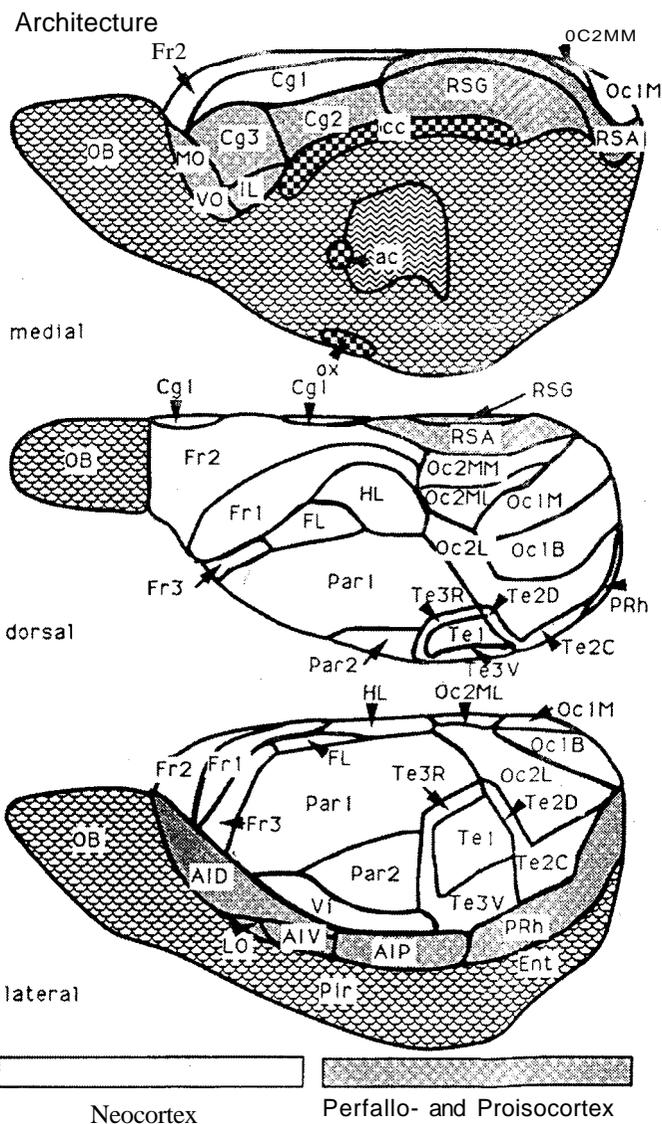


Figure 2.3: Top: Cortical structure (after by Zilles and Wree, 1995). Bottom: Measurement locations marked by planes A through E. Measurements were taken medially, centrally and laterally in each brain hemisphere.

### **2.3.4 Dendritic Morphology**

Golgi-Cox stained neuronal arbour and characteristics were drawn using a light microscope and the camera lucida method. The procedure for Golgi-Cox staining has been described by Gibb and Kolb (1998), and Gaser and Van der Loos (1981) (also see Appendix A). In this study, male lesion animals displayed a reaching deficit in the single pellet reaching task. Therefore we determined to investigate whether synaptic changes occurred in the forelimb reaching area of the cortex. Dendritic branch analysis and Sholl analysis were performed on the basilar dendrites of the forelimb area of the cortex from three groups of male rats - PI lesion, P10 lesion and control. Neurons in the forelimb area of the cortex were found in the central region of the cortex from Bregma 1.00 to -0.80 mm (Paxinos and Watson, 2007). Cells were chosen based on impregnation and full view of the dendritic tree. Incomplete or cells that were not intact, and cells obscured by blood vessels or astrocytes, were excluded.

Two measurements were used to quantify dendritic arbor from camera lucida drawings of Golgi-Cox stained neurons. 1) Sholl concentric ring analysis; and 2) dendritic branch order analysis. The Sholl analysis estimated dendritic length by counting the number of ring intersections every 20  $\mu\text{m}$ , using an overlay of concentric rings (Sholl, 1953). Dendritic arbour was quantified by dendritic branch order analysis - counting the number of dendritic bifurcations (branches) as described by Coleman and Riesen (1968). In both analyses, five pyramidal neurons from Layer V of the forelimb reaching area, per hemisphere were drawn, for a total of ten cells drawn per rat. Three rats (30 cells) were drawn for each category. The basilar dendrites were chosen as the

apical layer V dendrites are large, with processes that leave the section and are thus difficult to draw. The unit of measure for statistics was the average per hemisphere.

## **2.4 Statistical Measures**

Unless otherwise indicated, the data were evaluated with student t-tests, and two or three-way analysis of variance (ANOVA). For three-way analysis the following were main factors: lesion (obx or control), age at lesion (PI or P10) and sex (male or female). Three-way analysis was used in behavioral measures. In two-way analyses the controls (aged PI and P10) were collapsed creating three groups: Control, PI lesion and P10 lesion. The factor was called 'age at lesion'. Two-way analysis of variance included the following factors: age at lesion (PI lesion, P10 lesion or control) and sex (male or female), and were used for anatomical measures. When indicated, Fisher's probable least squares difference (PLSD) post hoc analysis followed interactive ANOVA results. When appropriate, hemispheric differences were calculated using the following formula:

$$\frac{(\text{Right} - \text{Left})}{\text{Right}}$$

Right

To calculate proportion comparisons (percent) from the lesion hemisphere to the intact side the following formula was used:

$$\frac{\text{Left}}{(\text{Left} + \text{Right})}$$

(Left + Right)

Statistical significance was based on a p-value of less than or equal to 0.05.

## **Chapter 3**

# **RESULTS**

### **3.1 Results from Behavior;**

#### **3.1.1 Forelimb Asymmetry**

On first paw touches  
bulbectomy (left paw) more  
exploratory movements (bol  
bulbectomy.

Percent of ipsilateral  
analysis. A main effect was  
 $F(1,49)=.19, p=.66$ , no sex  
interaction scores occurred,  
exploration  $F(1,49)=6.1, p=.02$   
 $F(1,49)=.11, p=.74$  (see Figi  
 $p>.31$ .

No differences were  
exploration (lesion,  $F(1,49)=$   
interactions  $p>.38$ ).

**A Cylinder Task: Percent  
left paw on first To**

%

80

60

40

### 3.1.3 Handedness

During training for the single pellet reaching task, handedness was established by allowing each rat to freely reach at multiple pellets laid out on the cage shelf. Most OBX rats (78%) showed a preference for their left paw - the paw ipsilateral to the lesion. The exception was the female rat group that received a bulbectomy on P1. Only two out of five (40%) P1 females showed a left paw preference. Animals receiving a lesion on P10 exhibited a more robust paw preference effect. Control rats showed a 57% preference for the left paw.

HANDEDNESS		%	
<b>All</b>	OBX	78	(21 out of 27)
	CONTROL	57	(16 out of 28)
<b>Sex</b>	FOBX	60	(6 out of 10)
	MOBX	88	(15 out of 17)
<b>Age</b>	P1 OBX	67	(8 out of 12)
	P10OBX	87	(13 out of 15)
<b>Female</b>	P1 OBX	40	(2 out of 5)
	P10OBX	80	(4 out of 5)
<b>Male</b>	P1 OBX	86	(6 out of 7)
	P10OBX	90	(9 out of 10)



Figure 3.2: A. Table showing the percentage of animals with left paw preference. Two animals had no preference. B. Graph showing a comparison between all control and all lesion animals (yellow), and the breakdown of handedness per group.

### 3.1.4 Single Pellet Reaching

Training lesion males in the single pellet reaching task was more difficult than training the control and female OBX animals. Three PI male animals did not learn to reach for the single pellet. When multiple pellets were available these animals used their tongue, and grasped frantically through the slot for the pellets. In later training, it appeared that their difficulty in successfully reaching the pellet combined with only one pellet available at a time (poor reward to effort association), led these animals to stop reaching altogether. Animals were subjected to 18 days of training.

Overall, lesion rats appeared to be less proficient at single pellet reaching. Because three lesion animals could not learn the task, two statistical analyses were performed: analysis including the animals, and analysis excluding them. Inclusion of the non-performing PI male lesion rats showed a significant reduction in the number of hits on the last trial scored for lesion animals  $F(1,52)=6.29$ ,  $p=.016$  (see Figure 3.3A). Exclusion of the non-performing rats showed a trend toward reduction in the number of hits in all other lesion animals, but it was not significant  $F(1,49)=3.05$ ,  $p=.087$  (see Figure 3.3B). Post hoc analysis of lesion to control was performed. In the "all rats included" analysis, control to lesion animals  $p=.007$ ; in "the three rats excluded" analysis  $p=.058$ .

A sex effect was evident in the included animals  $F(1,52)=5.57$ ,  $p=.026$ , but was not evident when the non-performing (male) animals were excluded  $F(1,49)=2.27$ ,  $p=.139$  (see Figure 3.3C). No age effect was found in either group (included  $F(1,52)=1.12$ ,  $p=.296$ ; excluded  $F(1,49)=0.1$ ,  $p=.922$ ). No interactions were found in either group (included  $p>.38$ ; excluded  $p>.21$ ).

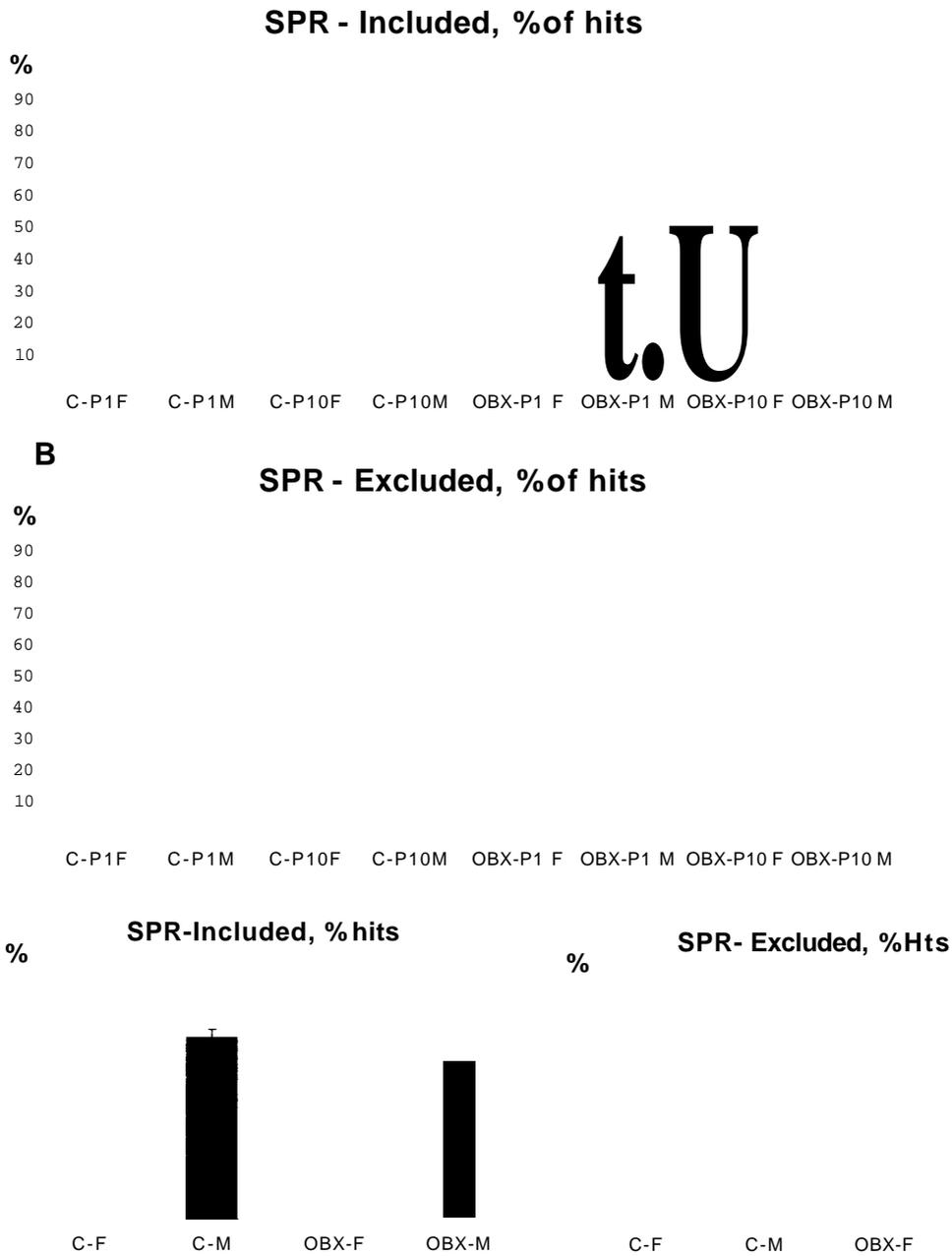


Figure 3.3: A. Single pellet reaching results showing percent of hits for each category, including three PI male OBX that did not learn the task. Overall males performed at a significantly worse level. B. Graph of the same, excluding the OBX animals that did not learn the task showing a trend toward inferior male performance. C. Comparison of sex and lesion main effects, including and excluding the three non-performers.

### 3.1.5 Open Field Activity Test

Although there was a sex difference in activity, there was no effect of lesion at either age. No main effects for lesion were found for horizontal locomotion [F(1,49)=0.95, p=.76] nor vertical activity [F(1,49)=1.95, p=.1683]. Overall female animals engaged in more horizontal and less vertical activity than males [sex effect horizontal F(1,49)=6.36, p=.015; vertical F(1,49)=7.1, p=.01]. No age effects were found [horizontal F(1,49)=2.53, p=.1179; vertical F(1,49)=3.38, p=.072]. A sex and age interaction was indicated in horizontal activity (F(1,49)=5.47, p=.023), that appeared to be driven by low horizontal activity scores by the PI male control animals. No interactions were found in vertical activity (p>.096).

**Open Field (Activity) Test**

	Total Horizontal	Total Vertical
<b>C-P1 F</b>	2429 (190)	<b>217 (9.8)</b>
<b>C-P1 M</b>	1681 (151)	228 <b>(20.0)</b>
<b>C-P10F</b>	2412 (198)	<b>163 (18.7)</b>
<b>C-P10M</b>	2419 (216)	225 <b>(23.2)</b>
<b>OBX - P1 F</b>	2458 (120)	233 <b>(34.1)</b>
<b>OBX - P1 M</b>	2073 (112)	250 <b>(19.4)</b>
<b>OBX-P10F</b>	2302 (38.0)	<b>183 (13.1)</b>
OBX-P10M	2252 (168)	249 <b>(10.1)</b>

Table 3.2: Total number of horizontal or vertical movements in the open field apparatus over the ten minute testing period. (Standard error in brackets.)

### 3.1.6 Elevated Plus Maze

Testing for the elevated plus maze occurred during construction in the building, creating increased stress on the animals. As a result, variation in open arm entries was high. The percent of open arm entries was calculated  $(\text{open}) / (\text{open} + \text{closed})$ . No differences were found for lesion [ $F(1,49)=.54, p=.47$ ] or sex [ $F(1,49)=.79, p=.38$ ].

An age effect was found for percent of open arm entries [ $F(1,49)=4.51, p=.039$ ]. Sex X Age interaction was also found [ $F(1,49)=5.51, p=.023$ ]. The main effect of sex and the sex/age interaction appear to be driven by high open arm entries by PI lesion and control males (see Figure 3.4). Post hoc testing showed PI male lesion animals were more active in the open arm than PIO male lesion rats ( $p=.0106$ ) and PIO male controls ( $p=.023$ ). PI male controls were significantly more active than PIO male controls ( $p=.0023$ ), but not significantly different from male PIO lesion animals ( $p=.069$ ). No other interaction effects were found ( $p>.61$ ).

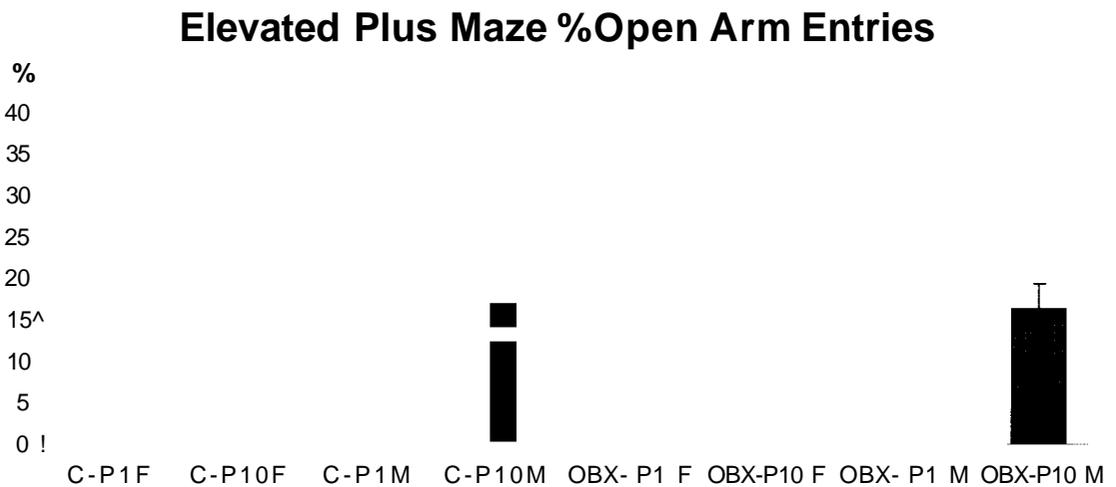


Figure 3.4: Percent of open arm entries  $(\text{open}) / (\text{open} + \text{closed})$ , in the elevated plus maze.

### 3.1.7 Morris Water Maze

All animals learned the MWM quickly, but, surprisingly, the bulbectomized animals performed better than control animals (see Figure 3.5). ANOVA testing showed a significant lesion effect,  $F(1,49)=7.03$ ,  $p=011$ . There was no effect of age ( $F(1,49)=.10$ ,  $p=.75$ ) nor sex ( $F(1,49)=1.12$ ,  $p=.29$ ), nor the interactions ( $p>.22$ ).

In the probe challenge the platform was removed from the pool. All animals spent more time in the quadrant where the platform was previously situated. ANOVA showed no main effect of lesion  $F(1,49)=65$ ,  $p=.42$ , sex  $F(1,49)=.17$ ,  $p=.68$ , nor age  $F(1,49)=2.47$ ,  $p=.12$ ). There were no significant interactions ( $p's>.074$ ).

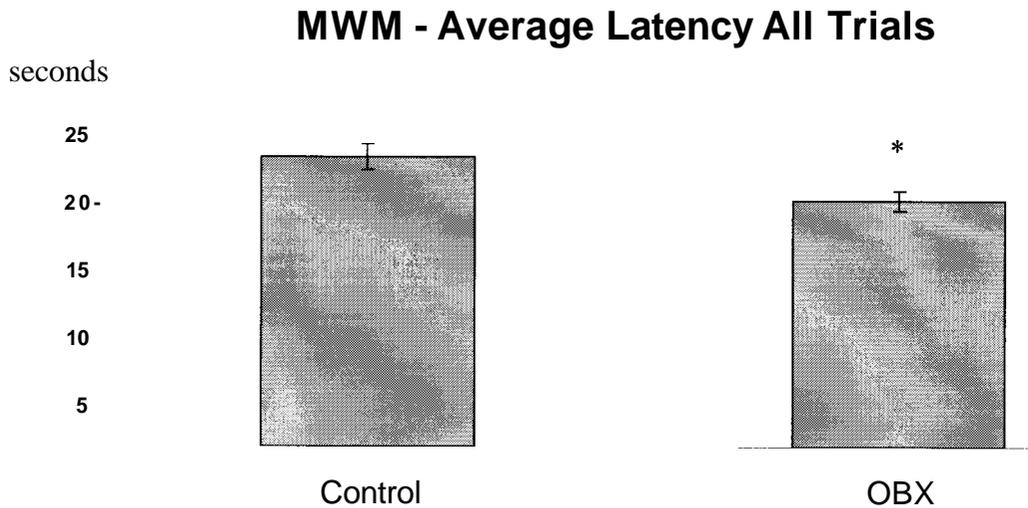


Figure 3.5: Average latency in seconds to the platform in the Morris water maze.

### 3.1.8 Behavior Summary

	Main Factors			Interaction
	Lesion	Age	Sex	
	Obx/Control	P1/P10	Male/Female	As Indicated
<b><u>Forelimb Asymmetry</u></b>				
First touch on rear	●	X	X	X
Bilateral touch on rear	V	X	X	X
Overall touches	X	X	X	X
<b><u>Sunflower Seed</u></b>				
Latency	X	●	V	X
Number of pieces	X	●	S	X
<b><u>Single Pellet Reaching</u></b>				
Including non-performers	●	X	●	X
Excluding non-performers	trend	X	X	X
<b><u>Open Field/Activity</u></b>				
Horizontal activity	X	X	●	sex/age*
Vertical activity	X	X	●	X
"Low P1 horizontal activity				
<b><u>Elevated Plus Maze</u></b>				
% open arm entries	* _____ *   x   sex/age*			
	P1 males (lesion & control) more entries			
<b><u>Morris Water Maze</u></b>				
Latency to Platform	●	X	X	X
Probe test	X	X	X	X

Table 3.3: Summary of behavioral findings. Lesion effects have been highlighted.

Lesion effects were found in the forelimb asymmetry task, single pellet reaching and Morris water maze. No lesion/age interactions were found, indicating that no significant differences were apparent between P1 and P10 lesion animals.

## **3.2 Results from Anatomical Measures (see Appendix D for summary)**

### **3.2.1 Gross Anatomy**

#### **3.2.1.1 Brain and Body Weight**

As expected, male body weight was almost twice that of female animals,  $F(1,50)=137.4$ ,  $p<.0001$ . No lesion effect was found,  $F(2,50)=.689$ ,  $p=.51$ , and no interaction between age at lesion and sex  $F(2,50)=1.24$ ,  $p=.296$  (see Table 3.4).

Male brain weight was greater than that of female animals  $F(1,50)=29.9$ ,  $p<.0001$ . No lesion effect was evident [ $F(2,50)=2.1$ ,  $p=.14$ ], nor was there an interaction between age of lesion and sex [ $F(2,50)=.5$ ,  $p=.61$ ] (see Table 3.4). One animal was not included in the dataset, as brain and body weight were omitted at time of perfusion.

in grams	Mean Body Weight	Mean Brain Weight
<b>Female Control</b>	335.8 (13.0)	1.97 (0.03)
<b>Male Control</b>	605.4 (23.7)	2.23 (0.04)
<b>Female - OBX - P1</b>	346.9 (14.4)	2.02 (0.04)
<b>Male - OBX - P1</b>	551.8 (18.9)	2.19 (0.04)
<b>Female - OBX - P10</b>	337.1 (10.6)	1.90 (0.03)
<b>Male - OBX - P10</b>	628.8 (34.2)	2.12 (0.05)

Table 3.4: Mean body weight and brain weight in grams for each category. (Standard error in brackets.)

#### **3.2.1.2 Olfactory Bulbs**

In this experiment, a discrete bulb appeared to have regenerated after both PI and P10 unilateral bulbectomy. In the preliminary studies leading to the current study we found that a discrete bulb was generated only for P10 lesion animals, and that in PI lesion animals the ipsilesional cortex grew into the empty bulbar cavity (see Figure 3.6). Both made connections with olfactory receptors. The elongating of the cerebral hemisphere into the empty bulb area has been verified in numerous experiments done in the first week of a rat's life (see development section for detailed description).

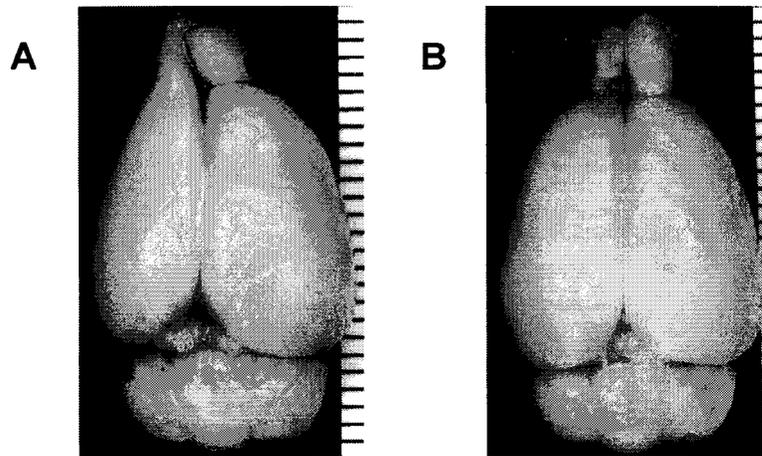


Figure 3.6: A. Photograph of the outcome of preliminary studies showing an elongated cerebral hemisphere extending into the OB cavity after a PI bullectomy. B. Photograph of P10 lesion animal with regenerated olfactory bulb from the same studies.

**Left OB (see Figure 3.7B):** When the left olfactory bulb was removed at PI or P10, regeneration of the olfactory bulb was apparent, albeit incomplete (see figure). ANOVA on the size of left OB showed a significant effect of lesion [ $F(2,51)=49.8$ ,  $p<.0001$ ], no sex effect [ $F(1,51)=3.45$ ,  $p=.069$ ], and no age of Lesion X Sex interaction [ $F(2,51)=1.33$ ,  $p=.27$ ]. Control males had larger bulbs than control females ( $p=005$ ); yet, in the lesion animals, no bulb area sex differences were found ( $p>.19$  for all PI lesion and P10 lesion male and female comparisons).

**Right OB (see Figure 3.7C):** A lesion effect was also found in the right, intact OB [ $F(2,51)=3.8$ ,  $p=029$ ]. Posthoc (Fisher's PLSD) analysis showed that the PI lesion animals had a larger intact bulb than controls ( $p=.024$ ). As expected, male animals had a larger intact bulb than the females [sex effect,  $F(1,51)=14.7$ ,  $p=0003$ ]. No interaction between age of lesion and sex was found [ $F(2,51)=1.15$ ,  $p=.32$ ].

**Left/Right comparisons (see Figure 3.7A):** Proportion comparisons of left to right bulb [(left) / (left + right)] found the left bulb for controls at 50.3% and lesion bulbs

ranging from 35.5 to 37.6 percent of total combined bulb area. ANOVA for proportion comparisons confirmed the lesion effect  $F(2,51)=49.8$ ,  $p<.0001$ , no sex differences [ $F=1,51=.05$ ;  $p=.83$ ], and no interaction between the two ( $p=.75$ ).

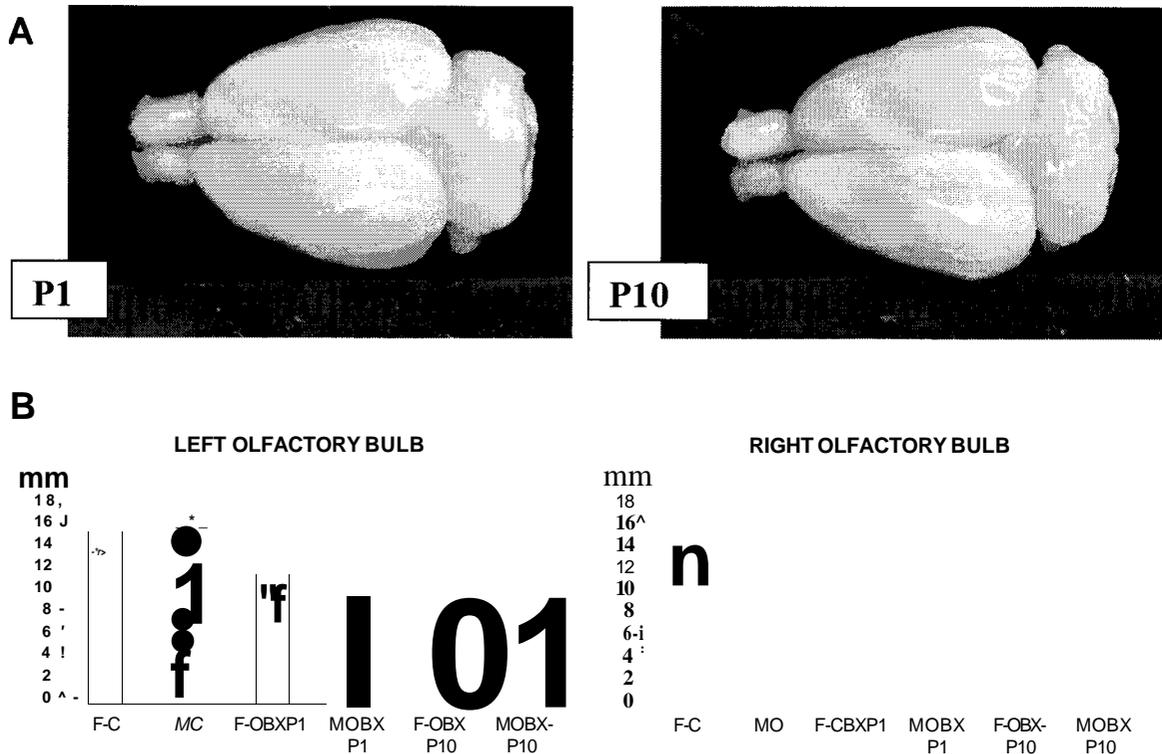


Figure 3.7: A. Photographs of the adult brains of rats that received a unilateral bulbectomy to the left hemisphere at postnatal day 1 (P1) and P10. B. Graph of left olfactory bulb area measurements in  $\text{mm}^2$  showing the lesion animals with small bulbs. C. Graph of right olfactory area measurements in  $\text{mm}^2$  showing no significant differences.

### 3.2.1.3 Cerebral Hemispheres

There was no effect of lesion in either hemisphere but there was a sex difference as males had larger hemispheres. ANOVA thus found a significant main effect of sex [left hemisphere,  $F(1,51)=18.3$ ,  $p<.0001$ ; right  $F(1,51)=10.1$ ,  $p=003$ ], but not of lesion [left,  $F(2,51)=1.01$ ,  $p=37$ ; right,  $F(2,51)=75$ ,  $p=.47$ ], nor the interaction ( $p>.92$ ).

CEREBRAL HEMISPHERES - AREA			
in mm	Left Hemisphere	Right Hemisphere	Proportion Comparison (left)
Female Control	113.8 (2.11)	111.2 (2.89)	0.506 (0.004)
Male Control	125.1 (2.07)	120.5 (2.83)	0.510 (0.003)
Female - OBX - P1	117.5 (4.12)	115.0 (3.89)	0.505 (0.005)
Male - OBX - P1	126.6 (3.79)	124.0 (3.92)	0.505 (0.003)
Female- OBX -P10	112.0 (1.85)	110.4 (3.08)	0.504 (0.004)
Male-OBX-P10	122.8 (2.70)	120.0 (2.73)	0.506 (0.002)

Table 3.5: Area, measured in mm<sup>2</sup>, of each cerebral hemisphere, and a percentage comparison of left to right hemisphere [(left) / (left + right)]. No area differences were found in proportion comparisons between the left to right hemisphere [lesion,  $F(2,51)=.477$ ,  $p=.62$ ; sex  $F(1,51)=.42$ ,  $p=.52$ ; interaction,  $p=.86$ ]. (Standard error in brackets.)

### 3.2.1.4 Cerebellar Area

Again, there was no lesion effect but there was a sex difference. ANOVA revealed a significant sex effect (sex effect  $F(1,51)=32.6$ ,  $p<.0001$ ) but no main effect of lesion [ $F(2,51)=.62$ ,  $p=.54$ ], nor the interaction ( $p=.85$ ).

CEREBELLAR AREA			
in mm	Left Cerebellum	Right Cerebellum	Proportion Comparison (left)
F-C	34.0 (0.45)	33.2 (0.47)	0.506 (0.003)
M-C	36.8 (0.47)	36.7 (0.60)	0.501 (0.003)
F-OBX-P1	34.3 (0.60)	34.0 (0.83)	0.503 (0.005)
M-OBX-P1	36.9 (0.44)	37.6 (0.65)	0.496 (0.006)
F-OBX-P10	33.3 (0.86)	34.1 (0.79)	0.494 (0.008)
M-OBX-P10	36.6 (0.66)	37.4 (0.73)	0.494 (0.004)

Table 3.6: Area, measured in mm<sup>2</sup>, of the cerebellar area, and a proportion comparison of left to right cerebellum. No area differences were found in proportion comparisons of the left to the right cerebellum [lesion,  $F(2,51)=2.3$ ,  $p=.11$ ; sex,  $F(1,51)=1.1$ ,  $p=.30$ ; interaction,  $p=.73$ ]. (Standard error in brackets.)

### 3.2.2 Olfactory Bulb Observations

Visualizing the reconstituted bulb at a structural level found the putatively regrown area to be altered from normal. In the anterior region of a normal olfactory bulb, the lamination was clear: incoming olfactory nerve inputs met with an outer ring of glomeruli (GL); a distinct external plexiform layer was evident (EPL - light area between the mitral cells and glomeruli); a ring of mitral cells was clear (MCL); along with a distinct internal plexiform layer (IPL - light area between the mitral cells and granular cells); and a clear granular cell layer (GCL) (see A in Figure 3.8). No subependymal layer (SEL) was present this anterior in the bulb. The lesion side (see B, Figure 3.8) was disorganized, but OB elements were evident, and these elements were vaguely in place.

Without a distinct glomerular layer, olfactory nerve inputs to the bulb penetrated deep into the bulb area. Some disordered glomeruli were visible, particularly in the more ventral and lateral regions. These large glomeruli penetrated as deeply as the granule cells. Some scattered mitral cells appeared outside the deeper granule cells, often directly under the glomeruli. Granule cells were scattered in the centre of the bulb. The internal and external plexiform layers, generally filled with dendritic arbour and axons, were not apparent, although dendrites and axons from various OB neurons were evident.

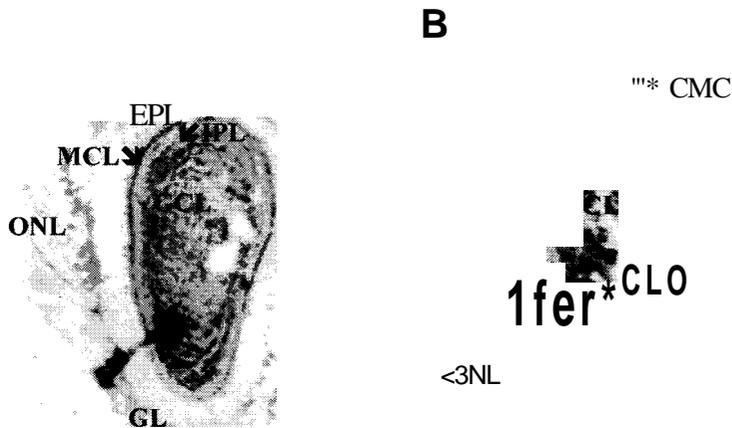


Figure 3.8: A. Photograph of nissl stained tissue from the anterior area of the left olfactory bulb from a male control animal. B. Photograph of the anterior area of the left olfactory bulb from a male animal with lesion on P10. External plexiform layer (EPL), granule cell layer (GCL), glomerular layer (GL), glomeruli (GLO), internal plexiform layer (IPL), mitral cell layer (MCL), olfactory nerve layer (ONL).

More caudal in the regrown bulb, distinct organization occurred in the lesion area (see Figure 3.9). The bulb was more normal in appearance in the ventral and lateral regions. The dorsal bulb remained open and did not fuse throughout the length of the bulb. In most lesion animals, disorganized bulbar elements were found at the dorsal, and dorsal-medial area of the bulb. Thus a distinct (but smaller), organized bulb region was evident, and at the top, OB elements were mixed together more randomly.

Within the organized bulbar region, ONL inputs were directed toward glomeruli. When the glomeruli were not present olfactory nerves penetrated the bulb. Large glomeruli organized into an outer layer, although the glomerular layer was often several glomeruli thick, and not the neat layer found in normal animals (see Figure 3.9). The

internal plexiform layer and the mitral cell layer appeared to be missing in most animals. When the IPL and MCL did appear they were found in the ventral and lateral regions.

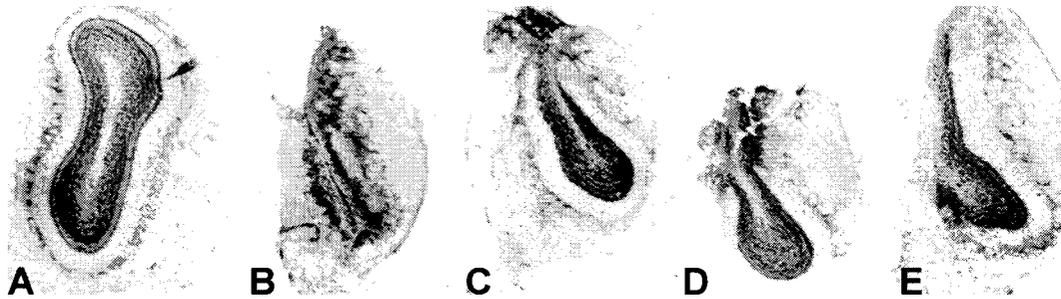


Figure 3.9: Photographs of left main olfactory bulbs. A. Control male (rat 39). B. Female with PI0 lesion (rat 30). C. Male with a PI0 lesion (rat 46). D. Female with a PI lesion (rat 50). E. Male with a PI lesion (rat 57).

Moving closer to the frontal cortex, the lesion bulbs began to look even more like normal (see Figure 3.10). The dorsal bulb did not fuse; however, the extent of closure and correct definition in the dorsal region was varied. The unfused dorsal area tended to open into the accessory olfactory bulb (AOB), which was always prematurely present in the lesion hemisphere. A glomerular layer was present around most parts of the bulb, and appeared to be innervated by the ONL. The MCL and IPL were more evident, particularly in the ventral and lateral region. The subependymal layer and ventricles were present in all animals.

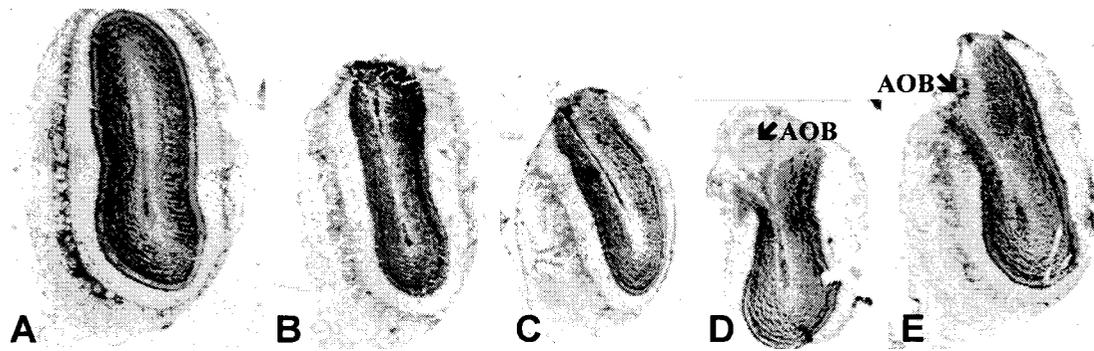


Figure 3.10: Photographs of left main olfactory bulbs. A. Control female (rat 48). B. Female with PI0 lesion (rat 30). C. Male with a PI0 lesion (rat 46). D. Female with a PI lesion (rat 50). E. Male with a PI lesion (rat 57). Accessory olfactory bulb (AOB).

### 3.2.3 Bulbar Measurements

**3.2.3.1 Olfactory Nerve Layer:** Inputs from the olfactory bulb appeared to remain consistent for both the lesion and intact hemisphere (see Table 3.7, Figure 3.11).

Measurements were taken from two sections of tissue within each bulb. No significant lesion differences were found in either bulb [left,  $F(2,20)=.06$ ,  $p=.94$ ; right  $F(2,21)=4$ ,  $p=.70$ ]. The proportion of the ONL in the left bulb (compared to the total from both bulbs) was calculated, and no significant lesion effect was found [ $F(2,20)=.7$ ,  $p=.5$ ]. Sex was not tested as ONL tissue was obscured in a number of female animals.

Olfactory Nerve Layer - Area			
in mm	Proportion Comparison (left)	Left ONL in mm	Right ONL in mm
C	0.530 (0.02)	2.800 (0.30)	2.560 (0.28)
P1 OBX	0.496 (0.03)	2.690 (0.55)	3.070 (0.63)
P10OBX	0.489 (0.03)	2.650 (0.32)	2.780 (0.33)

Table 3.7: Proportion comparison for the ONL inputs to the OB, and mean measurements for the left and right ONL. (Standard error in brackets.)

### 3.2.3.2 Glomerular Layer (GL)

To gain insight into overall bulb size, measurement around the outside of the glomerular layer in two sections of tissue, was attempted. The absence of glomeruli, and obscured tissue made the task difficult. Where glomeruli were not present, measurements excluded the ONL. Predictably, a one-way test of variance showed that lesion rats had a smaller bulb size than control rats [left,  $F(2,23)=T2.8$ ,  $p=.0002$ ] (see Table 3.8, Figure 3.11). The left GL in PI animals made up 39 percent of combined right and left GL, whereas PIO left GL area was 34 percent. Controls were at 53 percent [lesion effect,  $F(2,19)=27.1$ ,  $p<.0001$ ]. Posthoc testing (Fisher's PLSD) showed a difference between control to PI lesion ( $p=.001$ ), control to PIO lesion ( $p<.0001$ ), and no difference between PI to PIO lesion,  $p=.06$ . Again, sex was not tested due to obscured tissue in several female animals. No difference was found in the intact side [right,  $F(2,25)=.691$ ,  $p=.51$ ].

**Glomerular Layer - Area**

in mm	Proportion Comparison (left)	LeftGL	Right GL
C	0.498 (0.005)	9.100 (0.59)	9.150 (0.53)
P1 OBX	0.393 (0.021)	5.570 (0.71)	9.530 (0.35)
P10OBX	0.337 (0.025)	5.470 (0.47)	11.620 (0.80)

Table 3.8: Proportion comparison for the glomerular layer, and mean measurements for the left and right GL. (Standard error in brackets.)

### 3.2.3.3 Mitral Cell Layer (MCL)

Area measurements around the MCL in both hemispheres were made in two sections of olfactory bulb. When the MCL was not present, the area around the granule cell layer (GCL) was measured. In the lesion bulb, a two-way ANOVA showed an effect of lesion [ $F(2,24)=8.0$ ,  $p=.002$ ] (see Table 3.9, Figure 3.11). Posthoc analysis showed a difference between control and PI lesion ( $p=.002$ ) and P10 lesion animals ( $p=.001$ ). There was no

effect for sex [ $F(2,24)=.4$ ,  $p=.58$ ), nor was there an interaction between the two ( $p=.95$ ). The left bulb for controls ranged between 49 and 50 percent of combined MCL area (right plus left). The percent dropped to 33 to 35 percent for lesion animals [a lesion effect,  $F(2,24)=17.9$ ,  $p<.0001$ ], no sex effect [ $F(2,24)=0.03$ ,  $p=.95$ ], and no interaction ( $p=.95$ ).

In the right bulb, no difference was found for lesion [ $F(2,27)=.07$ ,  $p=.93$ ] or sex [ $F(2,27)=1.13$ ,  $p=.30$ ], and no interaction was found between the two ( $p=.60$ ).

Mitral Cell Layer - Area			
in mm	Proportion Comparison (left)	Left MCL	Right MCL
C-F	0.499 (0.006)	4.630 (0.39)	4.690 (0.47)
C-M	0.487 (0.008)	4.560 (0.56)	4.800 (0.59)
P1 OBX F	0.333 (0.040)	2.920 (0.74)	5.340 (0.42)
P1 OBX M	0.339 (0.046)	2.690 (0.69)	4.490 (0.45)
P10OBXF	0.348 (0.013)	2.920 (0.30)	5.120 (0.50)
P10OBXM	0.349 (0.019)	2.500 (0.26)	4.650 (0.39)

Table 3.9: Proportion comparison for the mitral cell layer, and mean measurements for the left and right MCL. (Standard error in brackets.)

### 3.2.3.4 Glomeruli

The area from ten glomeruli on two sections of tissue were measured in each bulb, and the mean glomeruli size generated for each section. Interestingly, on the lesion side glomerular area was .12 (P10) to .22 (PI) mm larger than glomerular area in control animals (see Table 3.10, Figure 3.11). In a two-way analysis of variance, a difference was found for lesion, [ $F(2,25)=5.222$ ,  $p=0.1$ ], but not for sex [ $F(1,25)=8$ ,  $p=.37$ ]. No interaction was found for lesion and sex ( $p=.32$ ). Posthoc testing showed a difference between PI lesion and control ( $p=.005$ ), but not for P10 ( $p=.096$ ). No difference was found between PI lesion and P10 lesion animals ( $p=.265$ ). Proportion comparison

confirmed these findings (lesion,  $F(2,25)=3.9$ ,  $p=.03$ ; sex  $F(1,25)=.2$ ,  $p=.7$ ; interaction  $p=.75$ ].

In the intact hemisphere, no significant lesion or sex differences were found [lesion,  $F(2,32)=.7$ ,  $p=.50$ ]; sex,  $F(1,32)=1.3$ ,  $p=.25$ ; interaction,  $p=.14$ ].

Glomeruli - Area			
in mm	Proportion Comparison (left)	Left Glom	Right Glom
C-F	0.496 (0.014)	0.027 (0.0013)	0.021 (0.0008)
C-M	0.504 (0.013)	0.022 (0.0013)	0.022 (0.0007)
P1 OBX F	0.554 (0.029)	0.028 (0.0010)	0.023 (0.0014)
P1 OBX M	0.542 (0.014)	0.027 (0.0019)	0.022 (0.0014)
P10OBXF	0.532 (0.025)	0.027 (0.0030)	0.023 (0.0005)
P10OBXM	0.516 (0.021)	0.023 (0.0024)	0.020 (0.0007)

Table 3.10: Proportion comparison for OB glomeruli, and mean measurements for the left and right glomeruli. (Standard error in brackets.)

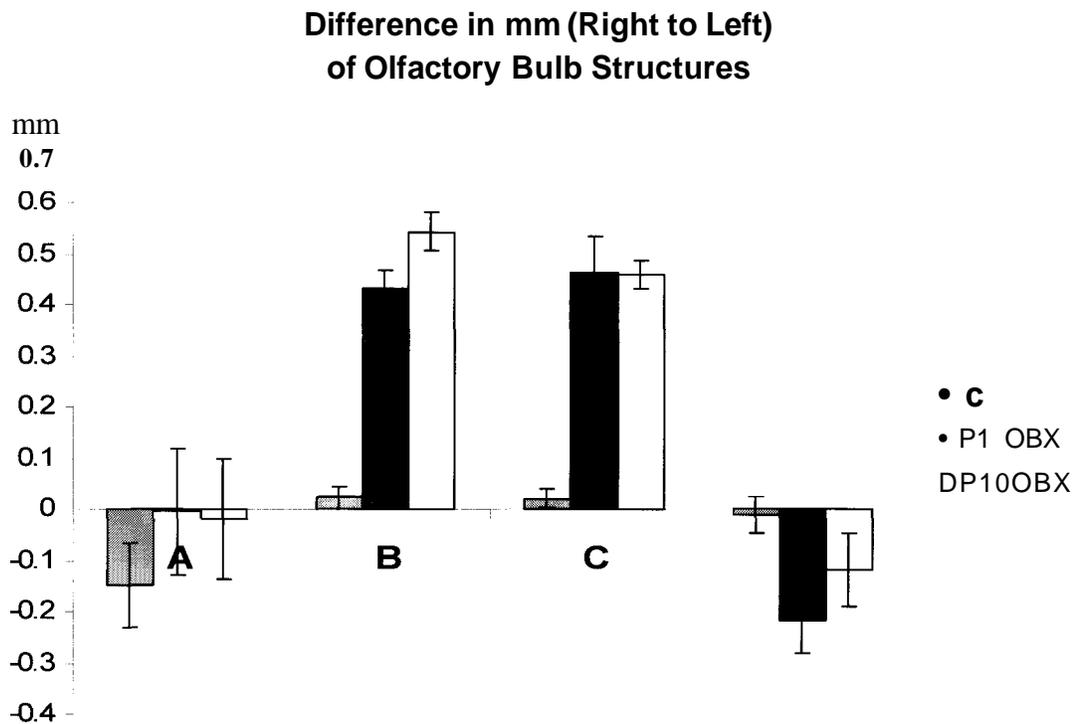


Figure 3.11: Differences in mm between the right and left bulbs for various OB structures. A. Shows no difference in the ONL. B. Shows the GL in the lesion hemisphere was smaller than the non-lesion hemisphere. C. Shows the MCL was smaller in the lesion hemisphere. D. Shows the area for individual glomeruli was larger in the lesion hemisphere.

#### **3.2.4 Accessory Olfactory Bulb Observations and Measurements**

The accessory olfactory bulb presented in a number of formats and sizes within the lesion bulb. In normal animals the AOB was first found as a cluster of granule cells, rising from the subependymal layer of the bulb, below the granule cell layer. In the lesion hemisphere, most often the AOB was first recognizable in the unfused fold of the dorsal bulb, above the subependymal layer (see Figure 3.12). Depending on the degree of dorsal disorganization, the AOB was found within the more structured section of the bulb or in the more randomly organized dorsal region. Only in one animal (female PI lesion) was the AOB enclosed under the granule cell layer (see Figure 3.12F).

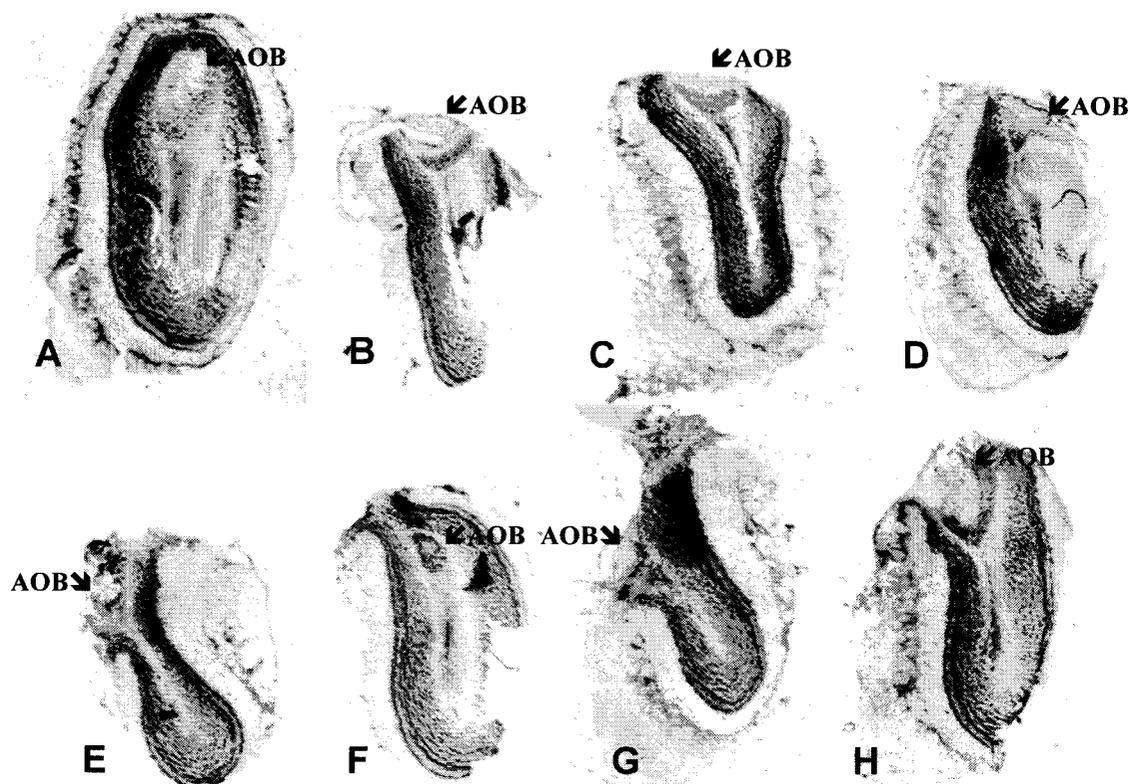


Figure 3.12: Photographs of the accessory olfactory bulb. A. Control female (rat 48). B. Female with PIO lesion (rat 34). C and D. Males with PIO lesions (rat 37 and 44). E and F. Female rats with PI lesions (rat 50 and 51). G and H. Male rats with PI lesion.

Overall in the lesion hemisphere, the AOB was smaller but spanned the same rostral-caudal distance throughout the bulb as control animals. The regrown AOB also occurred more rostrally in the bulb than in controls. Using Image J and CV stained slides, a series of AOB area measurements were taken from every section where the AOB was visible. In the event that the AOB was present but incomplete or obscured, an average of the previous and following AOB measurements were substituted. From these figures overall area of the AOB was calculated.

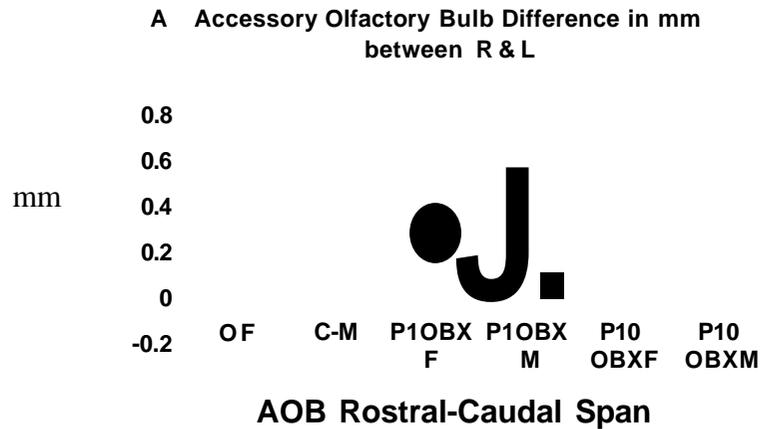
Predictably, the AOB from the lesion bulb was smaller than the AOB in control animals. In the lesion AOB, a lesion effect was present when a two-way ANOVA was

performed on the overall score for the left hemisphere [ $F(2,12)=8.5$ ,  $p=.005$ ]. For the intact hemisphere a lesion effect was also present [ $F(2,12)=6.3$ ,  $p=.013$ ]. The larger intact AOB was a result of P10 animals ( $P=.012$ ), mainly the females. Although there was no main effect of sex [left,  $F(1,12)=1.4$ ,  $p=.26$ ; right,  $F(1,12)=.5$ ,  $p=.49$ ], there was a Lesion X Sex interaction in both hemispheres [left interaction,  $F(2,12)=5.6$ ,  $p=.019$ ; right interaction,  $F(2,12)=5.3$ ,  $p=.02$ ], partly due to the P10 females. This was likely an artifact of tissue preparation because the P10 females were sectioned at 50um, and all others were sectioned at 40um. The difference was accounted for in area figures, but was likely distorted due to variance in mounting for different tissue thickness. The sex difference was ameliorated when a comparison between hemispheres was performed [(right - left) / (right)] (see Figure 3.13A). Two way ANOVA for left to right comparison showed a main effect of lesion,  $F(2,12)=17.1$ ,  $p=.0003$ , but not for sex,  $F(1,12)=T.5$ ,  $p=.25$ , nor for the sex and lesion interaction [ $F(2,12)=3.2$ ,  $p=.073$ ]. In females the AOB on the lesion side was smaller than the intact hemisphere by .47 mm (PI) and .26 mm (P10). In lesion males, the AOB was .36 mm<sup>2</sup> (PI) and .59 mm<sup>2</sup> (P10) smaller. Posthoc analysis showed a difference between control and lesion animals ( $p=.0003$  for PI and P10). No difference was found between PI and P10 lesion animals ( $p=.92$ ). Difference in mm<sup>2</sup> between the right and left AOB is presented in Figure 3.13B.

In both hemispheres the AOB appeared to span an equal amount of rostral-caudal distance. The number of sections in which the left AOB occurred was calculated as a percent of the total number of left and right AOB sections [(left) / (left + right)] (see Figure 3.13B). No difference in AOB span was found. Lesion brains ranged between

52.6 and 55.2 percent; controls between 49 and 50 percent [lesion,  $F(2,12)=1.8$ ,  $p=.20$ ; sex,  $F(1,12)=.5$ ,  $p=.51$ ; interaction,  $p=.97$ ].

The AOB in lesion animals was visible sooner than in controls. From the first appearance of the AOB in the lesion hemisphere, the number of sections was counted to the start of the AOB in the intact hemisphere. A main lesion effect was evident  $F(2,12)=13.5$ ,  $p=.0009$  (see Figure 3.13C). No main sex effect was found  $F(1,12)=.4$ ,  $p=.52$ . No interaction was evident  $F(2,12)=.6$ ,  $p=.59$ . Posthoc testing showed that the AOB from the P10 lesion animals started more rostrally than the PI lesion animals ( $p=.05$ ), which were more rostral than the control animals ( $p=.01$ ).



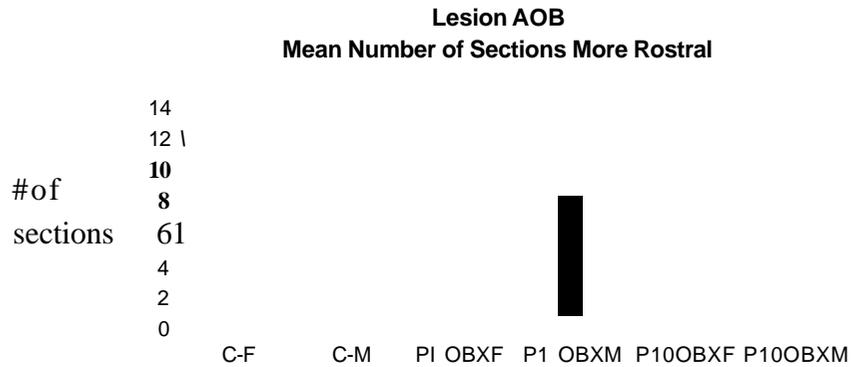


Figure 3.13: A. Graph for AOB size, comparing the difference in size between the lesion hemisphere and the intact hemisphere. B. Table showing no difference in the span of the AOB. (Standard error in brackets.) C. Graph demonstrating that the AOB begins more rostrally (8 to 9 sections) in the P10 lesion animals. The P1s begin more caudally than the P10s (about 5 to 6 sections), and controls are even more caudal.

### 3.2.5 Other Olfactory Related Measures

#### 3.2.5.1 Anterior Commissure

There was an overall reduction in the thickness (width) of the anterior commissure in lesion animals. ANOVA showed a main effect of lesion [ $F(2,26)=14.4$ ,  $p<.0001$ ], but not sex [ $F(1,26)=1.9$ ,  $p=.18$ ], nor the interaction [ $F(2,26)=1.6$ ,  $p=.23$ ]. Posthoc tests found all lesion groups to differ significantly from control ( $p<.0001$  for all). No difference was found between P1 and P10 lesion animals ( $p=.50$ ) (see Figure 3.14).

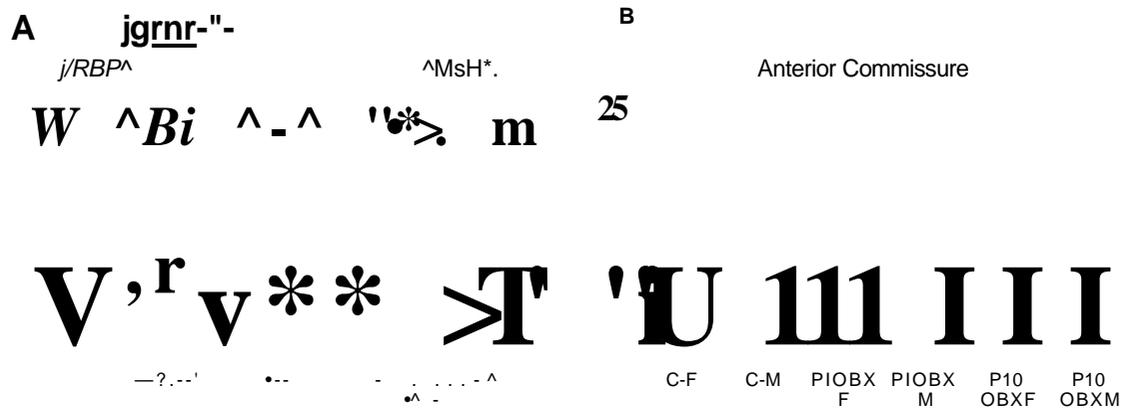


Figure 3.14: A. Luxol fast blue and CV stained photo of the anterior commissure (AC)

B. Mean width measurements of the AC in mm.

### 3.2.5.2 Lateral Olfactory Tract

The lateral olfactory tract (LOT) was smaller in the lesion hemisphere. Females appeared to be spared more than males, as sex differences apparent in the intact hemisphere, were not evident in the lesion side. Three measurements were taken of cross sectional area of the LOT at Bregma 3.00 (AC appearance), Bregma 2.16 (CC genu), and Bregma -0.12 (AC bridge). A lesion effect was found in the left (lesion) hemisphere [ $F(2,12)=16.2, p=0004$ ], but not in the intact hemisphere [ $F(2,12)=1.9, p=.19$ ]. There was, however, a sex difference in the right hemisphere, as males had a larger LOT [ $F(1,12)=11.2, p=.006$ ]. A sex difference did not occur on the lesion side [ $F(1,12)=.04, p=.84$ ]. No interaction of sex and lesion was found in either hemisphere ( $p>.75$ ). On the ipsilesional side, posthoc analysis showed a difference from controls for both ages ( $p<.0006$ ) (see Figure 3.15).

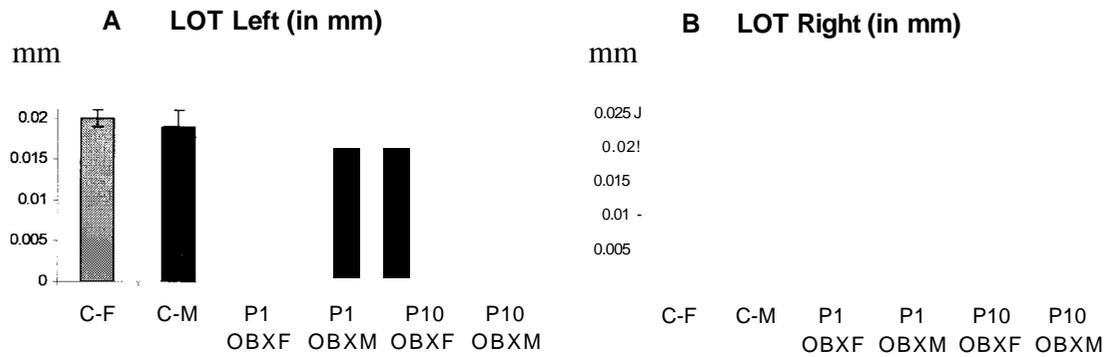


Figure 3.15: A. Graph depicting the diminished LOT in the left hemisphere - total area in mm from three measurements. B. Graph showing sex differences in the total area in mm from three measurements of the LOT in the intact hemisphere.

### 3.2.5.3 Lateral Ventricles

One PI male lesion animal had hydrocephalus, presenting with abnormally large ventricles (see Figure 3.16A). In comparison to other lesion animals, the hydrocephalic rat had a thinner cortex, a shrunken corpus callosum, and a detached septal hippocampal nucleus.

No lesion effects were found in area measurements of the lateral ventricles made at -0.12 from the bregma (AC bridge) and -2.16 from the bregma (dentate gyrus of HPC appearance) on the lesion hemisphere [ $F(2,12)=07$ ,  $p=.94$ ] and intact hemisphere [ $F(2,12)=02$ ,  $p=.98$ ]. There was a main effect of sex, however, with males having larger ventricles than females [left,  $F(1,12)=7.0$ ,  $p=.02$ ; right,  $F(1,12)=7.6$ ,  $p=.02$ ]. No interaction was found in either hemisphere ( $p>.37$ ) (see Figure 3.16B).

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**B** **Lateral Ventricles**

in mm	Right in mm <sup>2</sup>	Left in mm	Proportion Comparison (left)
<b>C-F</b>	1.493 (0.26)	1.097 (0.28)	0.414 (0.025)
<b>C-M</b>	3.677 (0.80)	4.517 (0.69)	0.556 (0.042)
<b>P1 OBX F</b>	2.21 (0.49)	2.68 (0.68)	0.540 (0.043)
<b>P1 OBX M</b>	2.657 (0.30)	3.403 (0.19)	0.564 (0.020)
<b>P10OBXF</b>	1.25 (0.05)	1.583 (0.30)	0.551 (0.038)
<b>P10 OBX M</b>	3.743 (1.55)	4.857 (2.59)	0.535 (0.036)

Figure 3.16: A. Picture of lateral ventricles from rat with hydrocephalis. B. Total from two measurements, in mm, of the lateral ventricles, depicting no significant differences. Proportion comparisons, lesion  $F(2,12)=2.152$ ,  $p=.16$ ; sex,  $F(1,12)=3.0$ ,  $p=.11$ ; interaction  $p=.11$ . (Standard error in brackets.)

**3.2.5.4 Rostral Thrust**

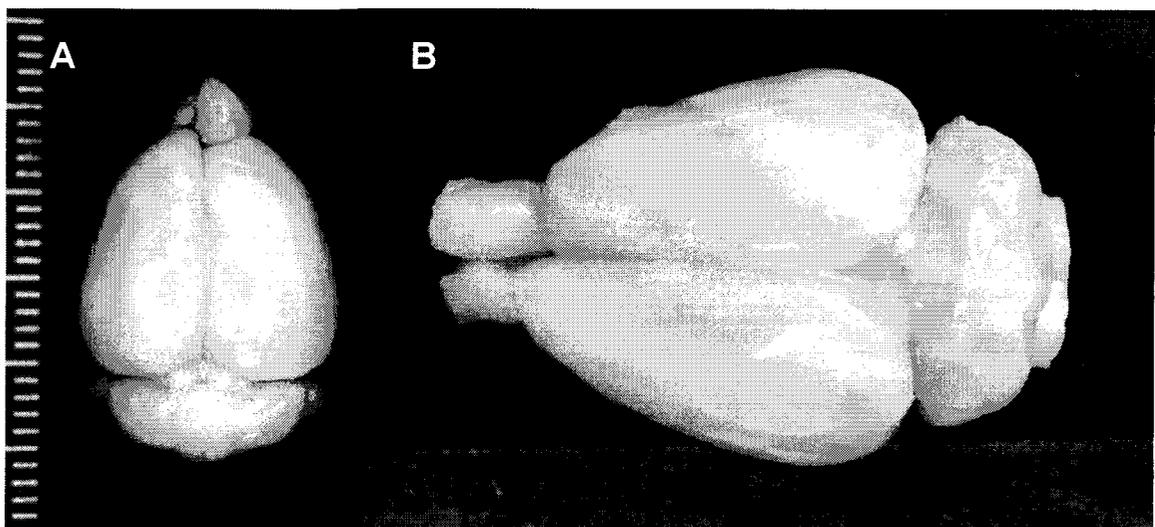
In lesion animals the cortical and subcortical structures were thrust forward into the olfactory bulb cavity (see Figure 3.17 A& B). To investigate the organization of this forward push, the distance between various landmarks, was measured by number of sections of tissue. Interestingly, in lesion animals some brain structures consistently occurred together, and this concurred with controls and the intact hemisphere. Meanwhile other structures were stretched longer, and did not concur with controls or the intact side. Measures of distance between structures, for each side, lesion and intact, were performed

and a one way analysis of variance performed for lesion and non-lesion. Structures that were not different in right and left sides of lesion brains included:

- 1) The length of the AOB (see AOB section).
- 2) The length from the first AON to the first frontal cortex appearance [F(1,16)=4,p=.53].
- 3) The length from the last OB appearance (granule cell layer) to the piriform cortex [F(1,16)=.3,p=.61].
- 4) The length from the last OB appearance (granule cell layer) to the transition zone [F(1,16)=.04, p=.85] (see Figure 3.17C).

Structures that were different distances in the right and left side of the lesion brains included (see Figure 3.17C):

- 1) Length of first AOB to the first AON [F(1,16)=4.9, p=.04].
- 2). Length of last AOB to last OB appearance (granular cell layer) [F(1,16)=5.4, p=.03].



## Rostral Thrust Difference between R& L Hemisphere

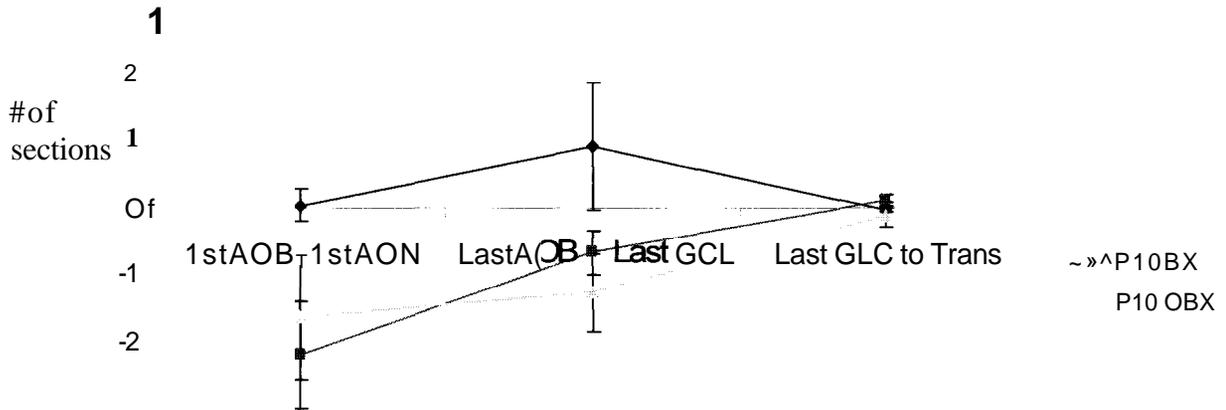


Figure 3.17: A. Photograph of P10 lesion male animal at P24, showing the rostral stretch into the frontal pole into the bulbar cavity. B. Photograph of a PI lesion male at P240, showing rostral thrust of the frontal pole into the bulbar cavity. C. Graph showing a gradual decrease in the rostral stretch into the frontal pole between various structures, by number of sections, when compared to the same measurements in the non-lesion hemisphere. The difference between hemispheres was calculated by:

$$\frac{[(\text{Total number of sections between structures (Right)} - \text{Total number of sections (Left)})]}{\text{Total number of sections (Right)}}$$

Moving caudally toward the frontal pole the left bulb has more sections between the first AOB appearance and the first AON appearance; more sections between the last AOB appearance and the last olfactory bulb appearance; and no difference between the last OB (GCL) appearance and the transition zone.

Thus, some of the rostral thrust was accounted for by stretched area containing the main olfactory bulb and the anterior olfactory nucleus (first AOB to AON to last OB). Structures caudal to the section containing the last olfactory bulb appeared to cover the

same distance as those in the intact hemisphere; however, all of these structures still remained rostral.

The following results were found from counting the number of sections between landmarks on the left and right hemisphere (distance between the start of the landmark in left side and the start of the landmark in the right) (see Figure 3.18):

- 1) The start of the left accessory OB was found well forward into the left olfactory bulb compared to the right.
- 2) Moving caudally, the start of the left AON was forward, lying well ahead of the right AON.
- 3) Structures at the last appearance of the granule cell layer of the olfactory bulb were still rostral, but less rostral than the AOB and AON. This shorter rostral shift was still apparent at the transition zone.

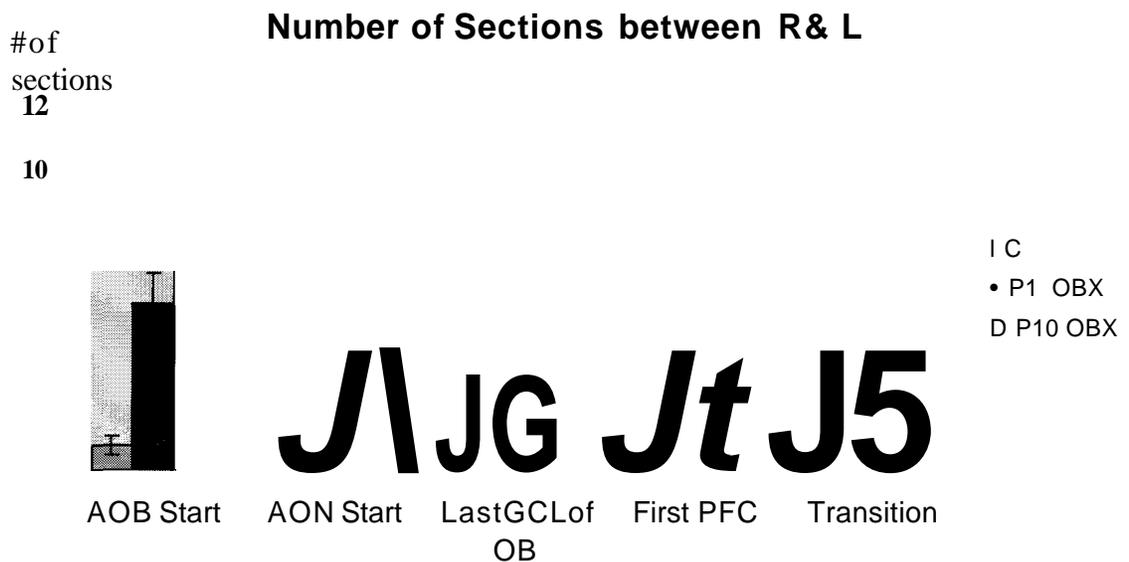


Figure 3.18: Graph depicting the difference between the start of a structure in the left hemisphere to the start of that same structure in the right hemisphere.

In qualitative observations rostral thrust appeared to be resolved by the Bregmoidal junction. Two measurements of the caudate putamen were taken and found that, in the lesion side, the left caudate was smaller than the right side at +2.16 relative to the bregma (CC genu) [F(1,16)=4.87, p=.04], but was not different from the right side by -0.12 [F( 1,16)= 1.4, p=.25] (see Table 3.10). A measure of the hippocampus (HPC) was taken at -2.56, and no significant differences were found [F(1,16)=1.1, p=.31] (see Table 3.11).

	<b>Caudate Putamen</b>		<b>Hippocampus</b>
	<b>Proportion Comparison (left)</b>		
	<b>Bregma 2.16</b>	<b>Bregma-0.12</b>	<b>Bregma -2.56</b>
<b>C-F</b>	0.479 (0.001)	0.495 (0.007)	0.492 (0.018)
<b>C-M</b>	0.54 (0.030)	0.514 (0.004)	0.513 (0.035)
<b>P1 OBX F</b>	0.547 (0.009)	0.491 (0.005)	0.504 (0.015)
<b>P1 OBX M</b>	0.557 (0.014)	0.493 (0.005)	0.554 (0.025)
<b>P10 OBX F</b>	0.549 (0.008)	0.492 (0.006)	0.521 (0.003)
<b>P10OBXM</b>	0.542 (0.021)	0.510 (0.010)	0.507 (0.008)

Table 3.11: Proportion comparisons of the left side to the total left and right sides for the caudate putamen at two planes; and for the hippocampus. (Standard error in brackets.)

### 3.2.6 Cortical Thickness

Because the left hemisphere was thrust rostrally, we anticipated that changes in cortical thickness would be apparent. Male brains were larger than female brains, thus a sex difference throughout was expected.

Overall the lateral cortical region was most affected throughout the brain. On the intact side, anterior cortical measurements showed PI lesion animals to have a thicker lateral hemisphere (planes A and C). On the lesion side, throughout the cortex, there was a reduction in the lateral cortex thickness in males. Specific breakdown by plane follows.

**Plane A.** At Bregma 1.70mm (plane A), PI lesion animals had a thicker cortex on the intact side, and PI males had a thinner cortex on the lesion side. A two-way

analysis of variance showed proportional differences between the right and left hemisphere [lesion,  $F(2,26)=6.7$ ,  $p=.005$ ; sex  $F(1,26)=1.4$ ,  $p=.24$ ; interaction,  $p=.09$ ]. Post hoc analysis showed that PI lesion animals were driving the proportion differences ( $p=.0009$ ). Further analysis found that, compared to controls, female PI lesion rats had a thicker right cortex in the lateral region [right lateral region: lesion  $F(2,26)=.5$ ,  $p=.62$ ; sex  $F(1,26)=21.2$ ,  $p<.0001$ ; interaction,  $p=.005$ ; post hoc,  $p=.009$ ]. PI lesion males had a larger right lateral region (right lateral post hoc,  $p=.049$ ), and a thinner left lateral cortex [left lateral region: lesion,  $F(2,26)=3.2$ ,  $p=.057$ ; sex,  $F(2,16)=4.3$ ,  $p=.048$ ; interaction,  $p<.0001$ ; post hoc,  $p<.0001$ ].

**Plane B.** At -0.26 mm from the bregma a lesion difference was found in the lateral region of the cortex in the left hemisphere [left lateral: lesion,  $F(2,26)=4.8$ ,  $p=.017$ ; sex,  $F(1,26)=19.0$ ,  $p=.0002$ ; interaction  $p=.31$ ]. Post hoc analysis showed that P10 animals had a thinner left lateral cortex ( $p=.011$ ). The sex difference reflected the larger effect in males.

**Plane C.** The lateral region of the right hemisphere showed a difference at -1.88 mm [right lateral: lesion,  $F(2,25)=3.8$ ,  $p=.037$ ; sex,  $F(1,25)<.0001$ ; interaction,  $p=.85$ ]. Post hoc analysis showed that PI animals were thicker ( $p=.03$ ), and again the effect was larger in the males ( $p=.0172$ ).

**Plane D.** At -4.8mm proportion differences were again found between the right and left hemisphere [lesion,  $F(2,26)=6.6$ ,  $p=.005$ ; sex,  $F(1,26)=.2$ ,  $p=.69$ ; interaction,  $p=.73$ ]. Post hoc analysis showed differences from controls in both PI lesion ( $p=.019$ ) and P10 lesion animals ( $p=.002$ ). Two way test of variance again showed these differences occurred in the left lateral region [left lateral: lesion,  $F(2,26)=3.7$ ,  $p=.039$ ;

sex,  $F(1,26)=5.1$ ,  $p=.033$ ; interaction  $p=.83$ ]. Post hoc testing revealed that PIO animals were smaller in this lateral region ( $p=.016$ ). Changes for PI lesion animals were less obvious.

**Plane E.** At - 6.3mm a two-way analysis of variance suggested an overall thinner cortex on the lesion side [left all measurements: lesion,  $F(1,22)=3.9$ ,  $p=.06$ ; sex,  $F(1,22)=.6$ ,  $p=.43$ ; interaction  $p=.30$ ]. Post hoc testing showed that lesion animals had a thinner cortex than control animals ( $p=.016$ ). Further analysis found that the thinner cortex was in the left lateral region [left lateral: lesion,  $F(1,24)=5.2$ ,  $p=.031$ ; sex,  $F(1,24)=.4$ ,  $p=.525$ , interaction  $p=.62$ ]. Because of obscured or missing tissue, age at lesion was not calculated for this plane.

## A Cortical Thickness - Males, Lateral

—●— C-M  
—●— PI C6XM  
P10OBXM

Rare Plane Rare Rare Rare  
A B C D E

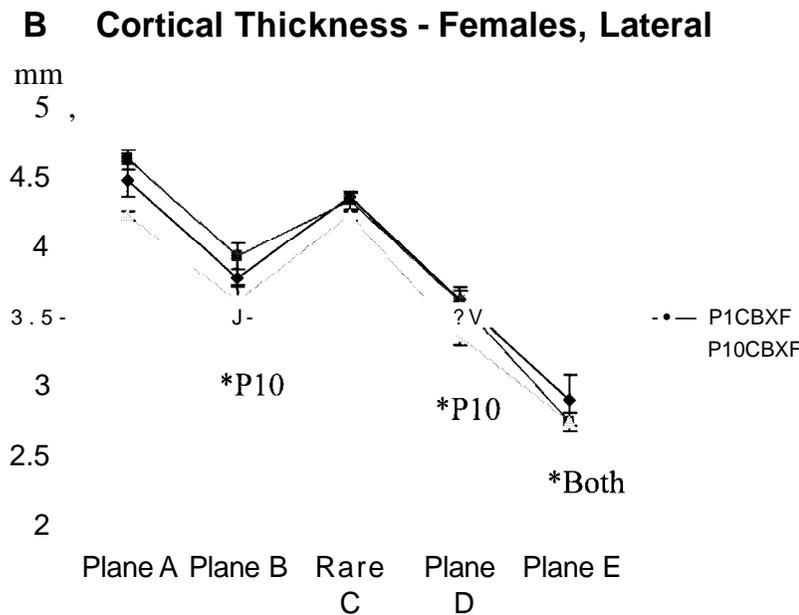


Figure 3.19: A. Graph showing cortical thickness (in mm) in the lateral regions for male animals. B. Graph showing cortical thickness (in mm) in the lateral regions for female animals. Some differences in the female P10 animals were not sufficient to show an overall lesion effect for the lesion category, or not significantly different from female control. For the plane E measurement, the lesion categories (PI, P10 lesion animals) were combined for each sex.

### 3.2.7 Golgi Analysis

Because male animals were deficient in the single pellet reaching task, we measured basilar dendritic morphology on layer V pyramidal cells from the forelimb motor area. Overall the neurons of male lesion animals had less arborization (branching) and length (Sholl) than controls. ANOVA testing showed that both PI and P10 lesion males had significantly fewer basilar dendritic branches [ $F(2,15)=34.5, p<0001$ ] (see Figure 3.20A). In Sholl analysis concentric ring intersections by dendritic branches was also reduced, reflecting an overall decrease in dendritic length (see Figure 3.20B).

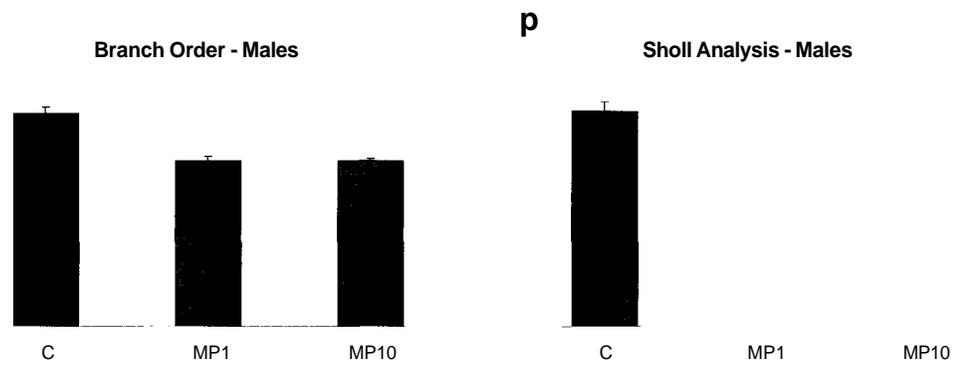


Figure 3.20: A. Graph showing reduced number of branches in male lesion animals. B. Graph showing reduced number of concentric ring intersections in male lesion animals.

## **Chapter 4**

### **EXPERIMENT 2**

#### 4.1 Analysis of Post-injury Evolution of Degenerative and Regenerative Events

One way to evaluate the extent of lesion and lesion regeneration is to perform staged kills on randomly chosen animals (Kolb, Cioe and Muirhead, 1998). The method does not provide exact analysis of the tissue removal but the random selection of animals at various stages provides insight into the amount of tissue being removed and the degenerative and regenerative processes that follow the injuries. Thus, animals were given bulbectomies on PI or P10 and the brains were harvested after different postoperative recovery periods.

#### 4.2 Method

Five litters totaling fifty Long Evans rats, 29 females and 21 males, received a unilateral bulbectomy: 15 at P10; 24 at PI; and 11 were controls. All animals were anesthetized with cold narcosis. The scalp was incised and the nasal bone over the left olfactory bulb was removed, leaving the left OB exposed. With a small glass pipette, aspiration lesions were performed on the left olfactory bulb. Control animals received a sham surgery, where an incision was made through the skin above the skull. The skin was sutured, and the animals warmed by hand. Animals recovered on a heating pad and were returned to the dam. Brains were harvested as per the following table (4.1):

<b>Age When Sacrificed</b>				
	P10 lesion F	P10 lesion M	P1 lesion F	P1 lesion M
<b>PI</b>			3 obx, 1 c	
P3			1 obx, 1 c	
P5			2 obx	1 c
P8			1 obx, 1 c	2 obx
P10	2 obx	1 obx, 1 c	2 obx	1 obx
P12	1 obx, 1 c	2 obx	2 obx	1 obx
P15	2 obx	1 obx, 1 c	1 obx	2 obx
P17	1 obx, 1 c	2 obx	2 obx, 1 c	1 obx
P24	2 obx	1 obx, 1 c	1 obx	2 obx, 1 c

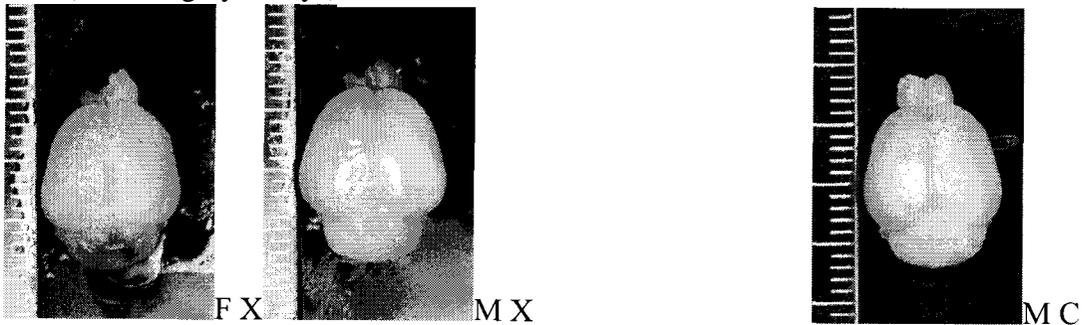
Table 4.1: Schedule of the age when animals were sacrificed.

## 4.3 Results

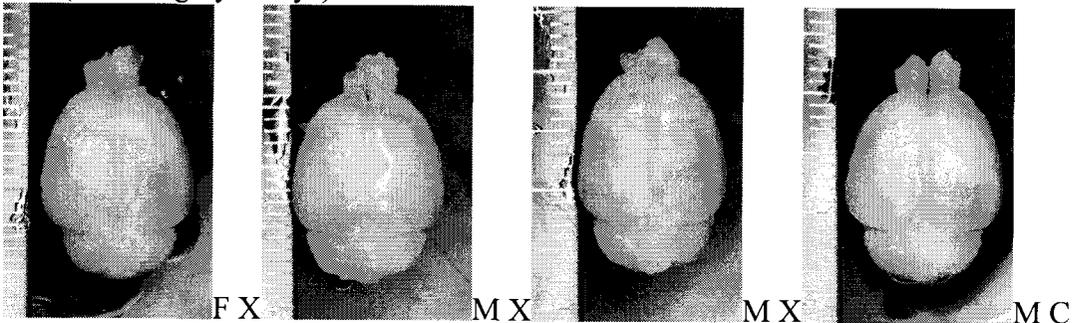
### 4.3.1 P10 lesion animals

Inspection of brains the day following the bulbectomy showed that not all the bulbar tissue was removed. Nonetheless, comparison of the brains over time showed that there was an evolution in the observed post-injury bulbs. In particular, the bulbs changed morphology over the postoperative period as the bulbs grew in size and changed shape. The relative size of the lesion bulb to the intact bulb clearly changed by PI 7. The increase in bulb tissue area was sustained throughout the following weeks. A distinct bulb was evident from seven days following the surgery, and was sustained in later stage kills in all animals (see Figure 4.1).

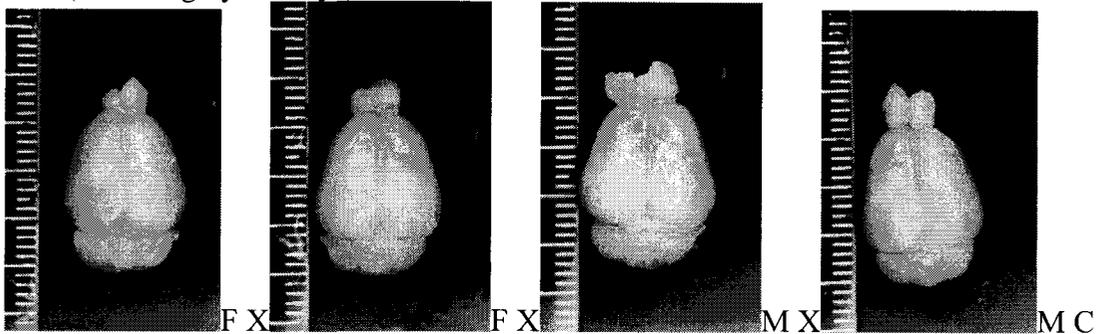
P10 (Post Surgery 0 days)



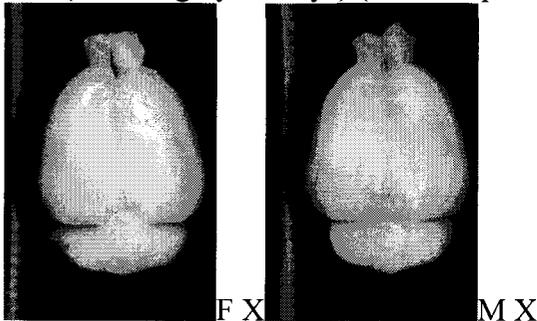
P17 (Post surgery 7 days)



P24 (Post Surgery 14 days)



P30 (Post surgery 20 days) (From Experiment 3)



P10

Figure 4.1: Photographs of P10 lesion animals P10, PI7, P24 and P30 following a unilateral bulbectomy. Control (C), female (F), male (M), bulbectomized (X).

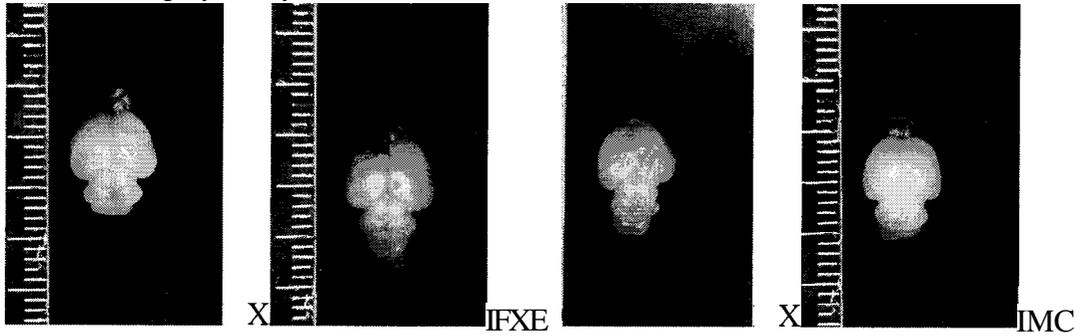
Photographs of P30 animals are from Experiment 3.

#### 4.3.2 PI Lesion Animals.

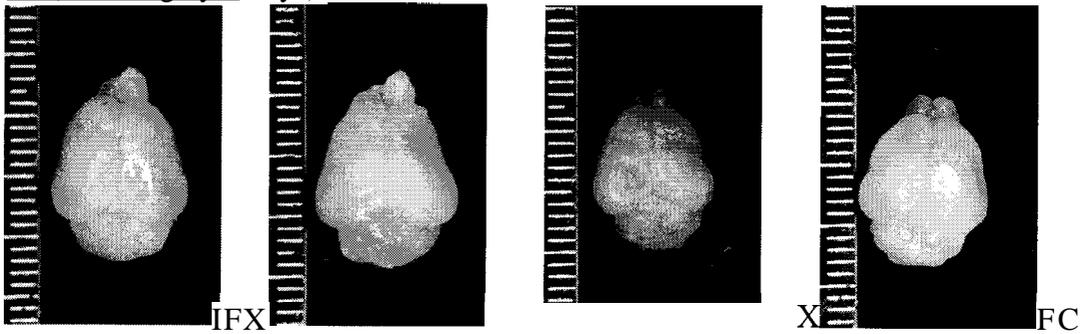
In PI lesion animals the entire bulb was removed. The frontal pole in one animal appeared to have been disturbed. Seven days following surgery a small amount of bulbar tissue was noted in the lesion animals. A distinct bulb occurred in two animals, and the frontal pole extended into the bulbar cavity in others (see Figure 4.2). By PI7, much more bulbar tissue was apparent, and change in tissue volume was considerable when compared to the previous week. The increase in bulb tissue area was maintained in subsequent weeks, in all but two animals. In the two exceptions the frontal pole grew into the bulbar cavity. Rostral thrust of the frontal pole was more visible in PI animals, and bulb sizes varied more than in the P10 lesion animals. In one P24 animal the

cribriform plate was also damaged. In this case the frontal pole grew into the nasal cavity.

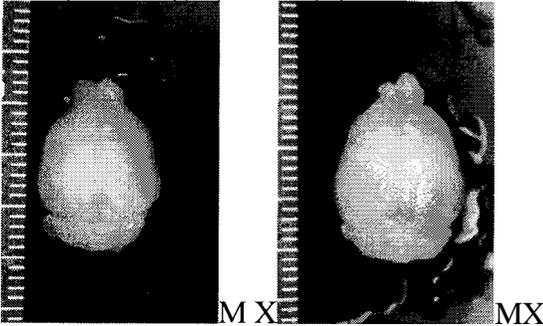
PI (Post surgery 0 days)



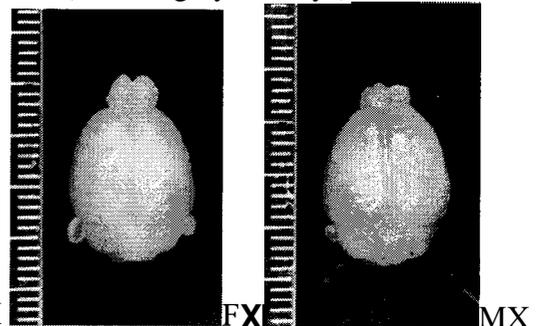
P8 (Post surgery 7 days)



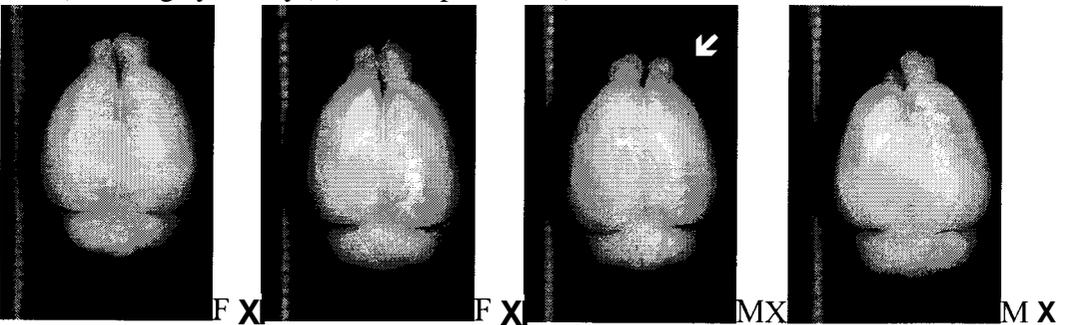
PI5 (Post surgery 14 days)



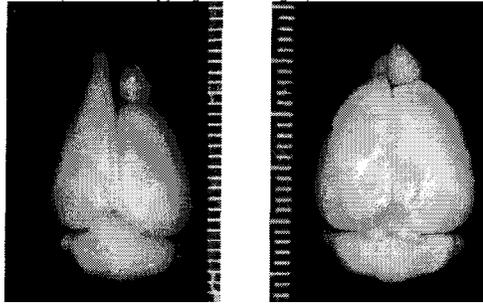
PI7 (Post surgery 16 days)



P21 (Post surgery 20 days) (From Experiment 3)



P24 (Post surgery 24 days)



**P1**

IF X ^ H H ^ M M X

Figure 4.2: Photographs of PI lesion animals PI, P8, P15, P17, P21 and P24, following a unilateral bullectomy. Control (C), female (F), male (M), bullectomized (X).

Photographs of P21 animals are from Experiment 3.

#### 4.4 Discussion

The most interesting result of this experiment was the substantial gain in bulbar tissue seen between ages PI 0 and PI 7, regardless of when the bullectomy was performed (PI or P10). Comparisons of bulb tissue at PI 7 to bulb remnants (if any) immediately after surgery, showed that substantial tissue returned to the bulb cavity, and the bulk of this bulbar regeneration occurred during the second week of life. The second week of life has been identified as the time when the olfactory bulb's subependymal layer exhibits peak cell density, volume and number (Frazier & Brunjes, 1988). The subependymal layer occurs around the olfactory ventricle, and is the bulb's postnatal proliferative zone. The extensive availability of stem cells may be the impetus for the bulbar regeneration. Kolb and Gibb (2001) report that intense network development and neuron maturation also occurs during this period. Studies in their lab show remarkable tissue regrowth after lesion at PI0 in medial prefrontal cortex. Interestingly, PI lesion animals do not show this cortical regeneration. In the case of the olfactory bulb, PI lesion animals show bulb regrowth, but it is delayed until the second week of life. The critical developmental

period during the second week of life that allows for regeneration in the neocortex, appears to be longer after olfactory bulb lesions and includes the first week of life.

P10 animals appear to have a more consistent recovery, but this may be due to surgical technique. The small size of the brain at PI confounds ability to produce a lesion that does not vary from animal to animal. Slotnick and colleagues (2004) report that variations in surgery result in variations in bulbar appearance and olfactory ability. In PI lesion animals, no bulbar regeneration is reported. Slotnick attributes variation in bulb morphology to bulb remnants, and incomplete bulb removal. The results from this experiment provide evidence that bulb remnants are not growing proportionally. Indeed, the amount of bulb tissue evident after PI 7 suggests that regeneration is occurring.

Finally, we note that there is a difference in the lesion extent after PI and P10 injury. The frontal pole expands considerably during the 10 days and much of the bulb is becomes hidden by the frontal pole. No attempt was made to remove tissue under the pole in the P10 animals.

# **Chapter 5**

## **EXPERIMENT 3**

## **5.1 Cutting the Frontal Pole**

The unexpected finding that the olfactory bulb was present in animals with removals either at PI or PI 0 led us to wonder if the inconsistency with the preliminary studies in which there was no bulb after PI lesions was because the surgeon was becoming more expert in removing the bulb without extra tissue damage. To test this idea, rats were either given a bulbectomy on PI or they were given a bulbectomy plus a small removal of the tip of the frontal pole. The intent was to sever the dura over the pole, thus removing the glia limitans and allowing tissue to move into the lesion cavity.

## **5.2 Method**

Two litters totaling twenty-four Long Evans rats, 13 females and 11 males, received a unilateral bulbectomy, eight at P10 and sixteen at PI. Of these animals, twelve also received an extended lesion into the frontal pole. All animals were anesthetized with cold narcosis. The scalp was incised and the nasal bone over the left olfactory bulb was removed leaving the left OB and frontal pole exposed. Aspiration lesions were performed on one olfactory bulb for all animals, and, in addition, the tip of the frontal pole in twelve animals. In one male PI animal without a frontal pole nick, the right hemisphere received the lesion; in all others the left hemisphere received the lesion. The scalp was sutured, and the animals warmed by hand. The animals recovered on a heating pad and were returned to the dam. Twenty days post surgery, animals were sacrificed, age P29 for PI0 group, and age P22 for PI group. Subjects were weighed, then administered an overdose of sodium pentothal (Euthansol, 0.05 ml); then perfused intracardially. The brains were extracted and weighed. Photographs were taken of each brain.

## 5.3 Results

### 5.3.1 PIO Animals

In PIO animals that received a nick to the frontal pole a lesion scar was apparent. In all PIO animals, regeneration of a distinct OB was also apparent. The regenerated OB was of consistent size in bulbectomized animals that received and did not receive a frontal pole nick, and was larger than any OB tissue found in the PI animals. (See Figure 5.1)

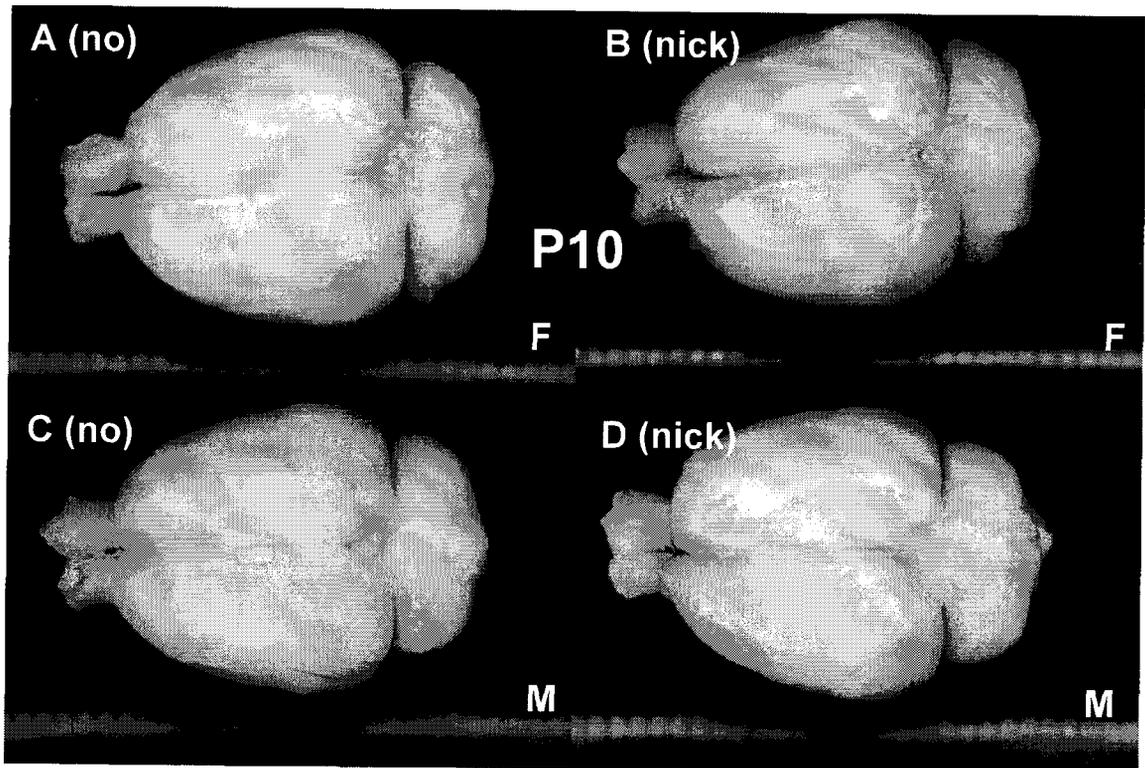


Figure 5.1: PIO animals-A. PIO female with no FP nick. B. PIO female with FP nick. C. PIO male with no FP nick. D. PIOmale with FP nick. Regeneration of the FP and a distinct olfactory bulb were clear and consistent in all animals.

### 5.3.2 PI Animals

In PI animals with a frontal pole nick, the frontal pole did not regenerate and thus an obvious lesion was visible, albeit with considerable variance in lesion size. The olfactory bulb also was quite variable, ranging from a distinct but very small bulb, an indistinct bulb, and no bulb. Whether or not the OB tissue was regenerated or a result of incomplete bulbectomy was not clear. In some control animals rostral displacement of the frontal pole was evident whereas in others it was not (see Figure 5.2).

**A (no)**

**B(no)**

**C (nick)**

**D (nick)**

**E (nick)**

**F (nick)**

Figure 5.2: PI female animals - A. PI female with no frontal pole nick. The frontal pole extended into the bulbar cavity. B. PI female with no frontal pole nick. The frontal pole barely extended into the bulbar cavity, and a distinct (small) bulb was visible. C, D, E & F. PI females with frontal pole nick. The frontal pole did not regenerate, and when present, varied presentations of OB tissue occurred.

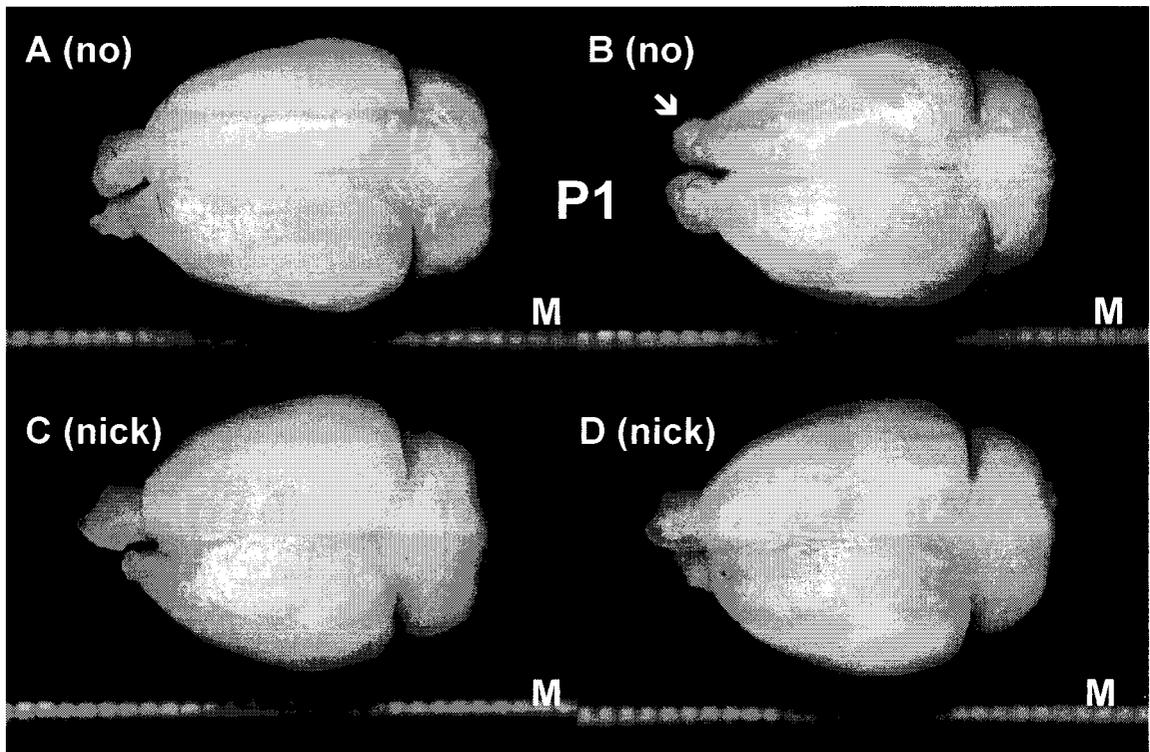
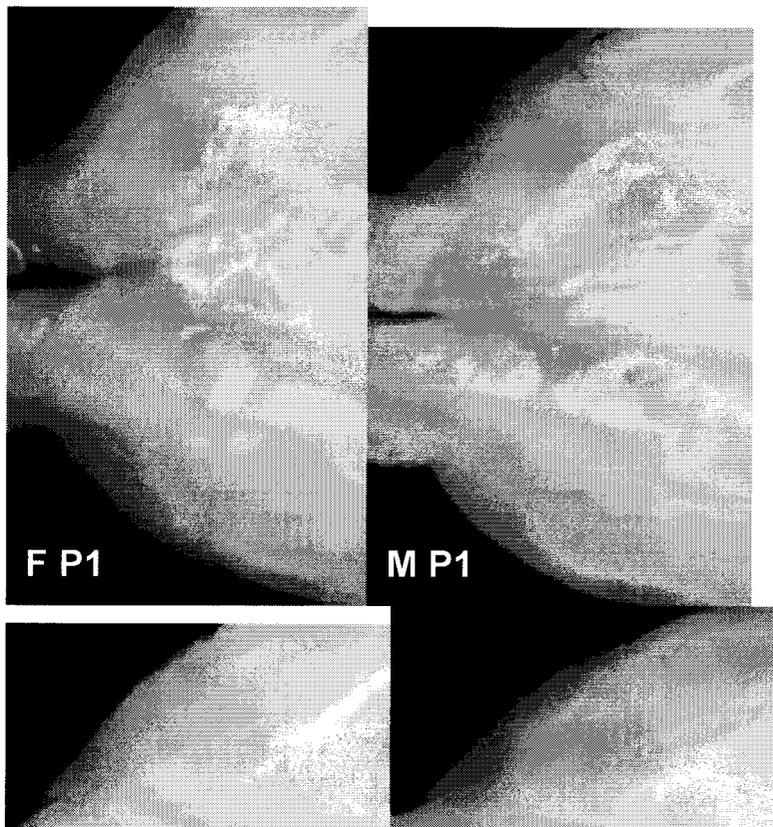


Figure 5.3: PI male animals - A. PI male with no frontal pole nick. The frontal pole extended into the bulbar cavity. B. PI male with no frontal pole nick. Arrow indicates lesion to the right hemisphere. There is some extension of the frontal pole into the bulbar cavity, although not as great as in A. and a distinct (small) bulb is evident. C. and D. PI males with a frontal pole nick. The frontal pole did not regenerate, and the bulbs were smaller than in animals with no frontal involvement.

### 5.3.3 Lateral Olfactory Tract

After a unilateral bulbectomy, the LOT was examined. At age P22, rats that received an OBX at PI did not have a visible LOT. At age P29, animals that received an OBX at PIO had a faintly visible LOT in comparison to the contralateral side. In female PIO lesion animals, the LOT on the lesion hemisphere was more visible than in the males of the same age (see Figure 5.4).



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^\*\*\*nipm

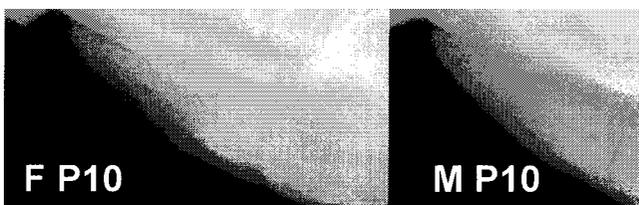




Figure 5.4: Photos of the ventral side of animals that received a unilateral OBX only, at PI and PIO. Male and female animals are pictured post surgery 20 days. Final photograph shows diminished LOT in a male PI, post surgery 240 days (from main experiment).

#### **5.4 Discussion**

In this experiment we tested the hypothesis that nicking the glia limitans on the frontal pole would result in extension of the frontal pole into the bulbar cavity. The glia limitans is a barrier of astrocytes that instructs the developing brain to discontinue growing at that point. In PIO animals, interruption of the glia limitans did not result in an extended frontal pole. Instead, lesion of the frontal pole resulted in regeneration of frontal cortex tissue. The pole did not appear to have moved in a rostral direction to any great extent. Further, the olfactory bulb regenerated with a tremendous amount of consistency across the animals. In the Slotnick study, when the frontal cortex was damaged, the olfactory peduncle extended into the bulbar cavity. However, when bulb remnants remained, an olfactory bulb, of sorts, was present. For this study, further

analysis of the bulbar tissue is required to confirm his analysis. Preliminary evaluation suggested that tissue in the P10 olfactory bulb cavity was primary olfactory tissue and not structures of the olfactory peduncle (MOB), which suggests that bulb remnants remained after surgery.

In PI animals nicking the glia limitans did not appear to play a role, as no regeneration of the frontal pole occurred. Lesions were apparent in all PI animals that received a frontal pole invasion. Varied olfactory bulb morphology was present in the PI animals, including control animals. Variations suggest that inconsistent tissue removal resulted in different morphology. In this respect, the PI findings concur with the Slotnick study (2004).

The post lesion morphology of the frontal pole in both PI and P10 animals, found in this study, agree with other studies. Regeneration of tissue occurs in the frontal cortex for P10 lesion. Mitosis and neurogenesis are evident in the regenerated tissue, functional connectivity occurs, and improved behavioral outcomes are apparent (Kolb et al., 1998B). The developmental stage of maturation, which involves dendritic arbourization and axonal outgrowth and connection, occurs during the second week of life, and appears to allow for greater plasticity. Frontal cortex regeneration does not happen during the first week of life. The lack of regeneration and an evident lesion cavity was confirmed by the PI lesion animals in this study.

Further photographs from Experiment 3 are displayed in Appendix E.

# **Chapter 6**

## **EXPERIMENT 4**

## **6.1 Mitosis after P10 Bulbectomy**

Although we have seen a filling of the bulb cavity with new cells that form a bulb, we have not yet shown that the cells are new, nor have we identified the source of the cells. In order to determine if new cells were found in the apparently regenerated bulb rats were given a mitotic marker, Bromodeoxyuridine (BrdU), after the lesion. The presence of BrdU-labelled cells in the injured bulb can be taken as evidence of an injury-induced generation of new cells.

## **6.2 Method**

Eight male Long-Evans rats from two litters received either a unilateral bulbectomy (n=4) or sham surgery (n=4) at day 10. All animals were anesthetized with cold narcosis. The scalp was incised and the nasal bone over the left olfactory bulb was removed, leaving the left OB exposed. With a small glass pipette, aspiration lesions were performed on the left olfactory bulb. Control animals received a sham surgery, where an incision was made through the skin above the skull. The skin was sutured, and the animals warmed by hand. The animals recovered on a heating pad and were returned to the dam.

All rats received an intraperitoneal injection of the mitotic marker Bromodeoxyuridine (BrdU) (Sigma B-5002), 60 mg/kg in 0.007 N NaOH 6 hours post lesion and five more times over the course of the next 2 days (day 1 post lesion 3 times spaced 3 hour apart, day 2 postlesion 2 times, spaced 6 hours apart). The animals were killed either on postnatal day 20 (half of each group) or 60 (the other half). The animals were overdosed with Euthansol and perfused intracardially with buffered 0.9% saline followed by 4% paraformaldehyde s and 14% saturated picric acid in 0.1 M P04 buffer

(pH 6.9). Brains were post-fixed in perfusate overnight and then cut at 15  $\mu$ m on a Vibratome.

In order to stain for BrdU, sections were digested in 1 N HCl at 65°C for 30 min.

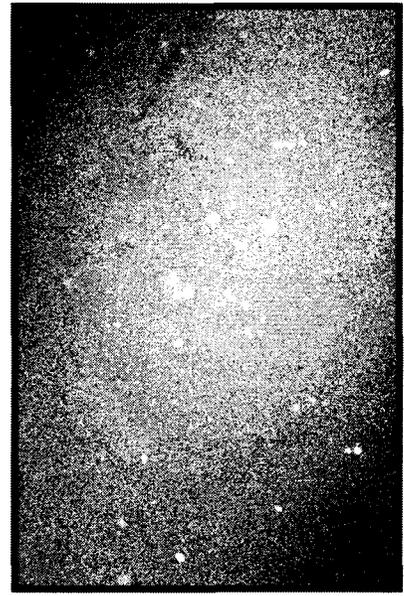
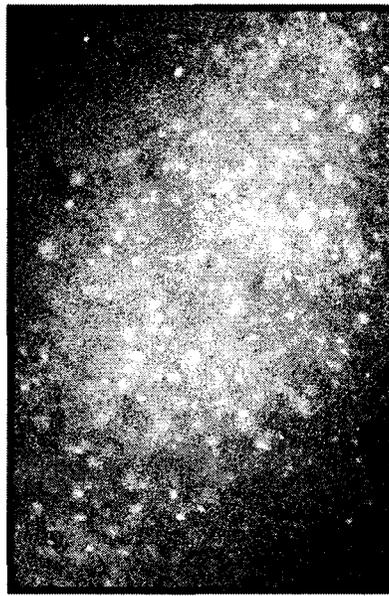
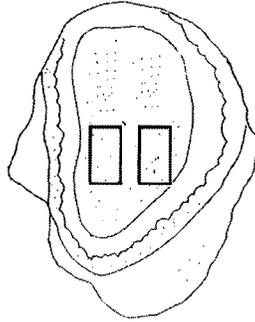
Sections were washed and incubated for histofluorescence staining. The tissue was placed for 24 h in rat anti-BrdU (Sera-lab no. MAS 250p.), diluted 1:100 at 4°C. The washed tissue was placed in biotinylated sheep anti-mouse antibody for 24 h (Amersham no. RPN-1001). The washed tissue was placed in FITC-labeled donkey anti-Rat antibody (Jackson Immunochemicals no. 712-093-153) at room temperature. The tissue then spent 1 h in Streptavidin-Texas Red (Amersham no. RPN-1233). The washed sections were mounted with tap water onto 0.5% gelatin, 0.05% chrome-alum subbed slides, dried, and then cover slipped with Slowfade™ (Molecular Probes S-2828).

### **6.3 Results**

Few labeled cells were found in bulb tissue from the intact bulb but there were large numbers of labeled cells in the injured side (see Figure 6.1). Because the results of Experiment 2 suggested that the posterior part of the bulb may be spared in our surgical procedure, the focus of our analysis was in the more anterior portion of the bulb. The majority of the new cells were in the granular layer and glomerular layer of the bulb. Although no counterstain was performed to identify cell type, visual inspection of the tissue found very few glial cells and most labelled cells in the granular layer appeared to be neuronal although some were tiny and almost certainly glia.

Although it would have been ideal to quantify cell numbers in the current study, the tissue was left sitting too long and the fluorescence was lost, thus making it impossible to quantify.

## Control



## Lesion

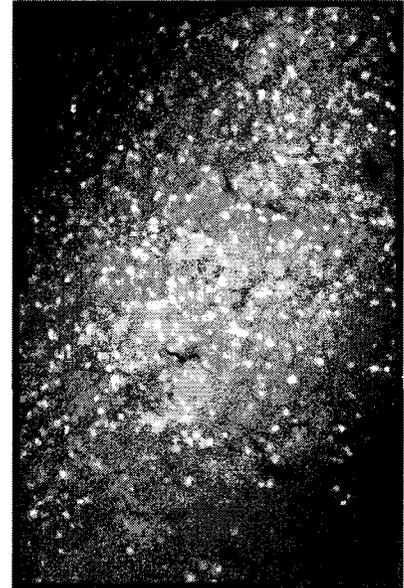
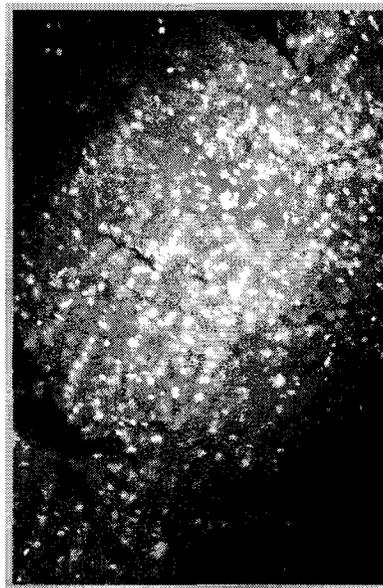
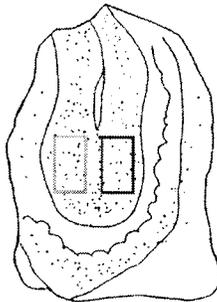


Figure 6.1: Top. Sections through the intact bulb showing very few labelled cells. Note that the large fuzzy cells on the left are showing autofluorescence. Bottom. Sections through a regrown bulb showing large numbers of labelled cells in the granular zone. The drawings on the left illustrate the relative density of labelled cells (black dots). No attempt was made to draw all labelled cells because there were too many to draw in the lesion bulb.

## 6.4 Discussion

A large proportion of the cells found in the bulb on the injured side were BrdU-labelled. The experiment does not demonstrate with certainty what the labelled-cells phenotype might be nor does it provide any insight into where the cells may have originated. Nonetheless, there was a marked difference between the intact and lesion side. Cytological analysis suggested that the majority of the cells were neuronal. At this point we have no evidence of the origin of the cells. We are tempted to conclude that they originated the subventricular zone and migrated along the rostral migratory stream **but** it is equally plausible to suggest that the cells may have originated from progenitor cells already in the posterior part of the olfactory bulb. These cells could have divided and migrated into the lesion area.

One necessary study yet to be performed is to redo this experiment with both PI and PIO lesion animals and to use a specific neuronal marker, such as NeuN, and to use confocal microscopy to determine if new cells are indeed neurons. In this study there also should be a quantification of labelled cells.

# **Chapter 7**

## **DISCUSSION**

## **General Discussion**

This study addresses three main areas: 1) A description of morphological changes following neonatal bulbectomy; 2) Regeneration of the olfactory bulb following neonatal insult; 3) Diffuse effects of early brain injury on the development of behavior. Each is considered in turn.

### **7.1 Morphological Changes**

Morphological changes have been described in the results section of this thesis (see anatomy results). The implications of changed morphology resulting from neonatal bulbectomy are discussed in this section.

Inside the lesion bulb a normal amount of olfactory nerve input was present. The presence of a normal sized ONL implies that the capacity for olfactory receptor neurons to regenerate sensory axons was not impaired. ORN axons are without a target during development. These axons often overshoot the glomerular layer, and project deeper into the bulb. Targeting errors usually diminish after P12 (John and Key, 2005). After neonatal bulbectomy the target is removed and random innervation by olfactory axons results in more random placement of glomeruli. In this study and as in other studies, the glomeruli in the bulb of lesion animals were disorganized; found in the plexiform layers; and embedded as deep as the granule cell layer.

Disorganized glomerular placement suggests that careful chemotopic mapping, the zone to zone convergence from the epithelium to the glomerular layer (Mori et al., 1999), is at least partly disrupted by neonatal bulbectomy. Interestingly, glomerular disorganization has limited effects on olfactory ability (e.g. Slotnick et. al., 2004) and has

led Graziadei and Monti-Graziadei (1992) to suggest that chemotopic organization of glomeruli is epiphenomenal. In defense of olfactory mapping and its importance, it is possible that neonatally bulbectomized rats have subtle olfactory deficits that have not yet been demonstrated. After all, rats are macrosomatic and smell is fundamental to their lives. Further, epiphenomenal conclusions preclude diffuse effects of bulbectomy on the rest of the brain, including the motor effects that were found in this study.

In adult rats, after an olfactory nerve transection, reconnection of olfactory receptor neurons to glomeruli takes about a month, and accommodates apoptosis, neuronal precursor upregulation, differentiation and maturation (Calof et al., 2002). It is possible that most of these time-consuming steps are overcome in prenatal bulbectomized rats, as the components for speedy innervation and glomerular development are already present. In the rat, many glomeruli develop after birth (observed in stage kills; at P7 control rats had fewer and much smaller glomeruli than adult animals). Thus, there are fewer glomerular connections, and apoptosis (which takes up to a week in adults) is minimized. Further, upregulation and differentiation of sensory neuron precursors are already underway. With augmented development and maturation of ORN already set in place, expedited axonal outgrowth and glomeruli formation should restart shortly after neonatal bulbectomy.

Overall, fewer glomeruli were found in the lesion bulb, but these glomeruli were larger. Of the countless olfactory receptor neurons on the epithelium, about 1000 receptor types are present, and sensory neurons for a single receptor type connect to two glomeruli, one on each side of the bulb. The inherent problem with fewer glomeruli is the connectivity of these generally dedicated receptor types. It is possible that instead of

projecting to two glomeruli, the axons of a single receptor type converge on one dedicated glomerulus. This may also explain the increased size of the glomeruli. Another possibility is that input from more than one receptor type is converging on fewer glomeruli. This occurs normally within the accessory olfactory bulb. A third possibility is that the nerves are perpetually growing to the OB, but dying without a target.

Increased glomerular size may be due to innervation by more than one mitral cell, an event that does not occur in the main olfactory bulb but occurs normally in the accessory olfactory bulb. Monti-Graziadei and Graziadei (1992) report that "unconventionally shaped mitral cells branched their dendrites into several of the surrounding glomeruli" after neonatal insult between P1 and P8. Another explanation is that greater input from bulbar cells (mitral, tufted, interneuron) is occurring. Monti-Graziadei and Graziadei also report profuse mitral cell branching within the glomerulus of early bulbectomized animals. Further investigation into glomerular structure and connectivity after neonatal insult is required. Other studies of neonatal bulbectomy (Slotnick et al., 2004) report smaller glomeruli, and suggest that reduced interglomerular neurons may be to blame. Different lesion presentations may be facilitating the disparate glomeruli sizes between the studies. Whether or not OB tissue remains after a neonatal bulbectomy appears to produce highly differentiated results.

In the right (intact) hemisphere, gross OB measurements showed that PI animals had a larger intact bulb than controls. Increased intact bulb size after neonatal bulbectomy concurs with other studies (Racekova et al., 2002). In this study an enlarged intact hemisphere was also evident in the frontal neocortex. PI animals had a thicker lateral cortex on the intact side. Numerous human children and neonatal animal studies

have shown that a unilateral lesion alters development in the intact hemisphere. For example, over 100 years ago Broca and Barlow showed that early lesion to the left hemisphere language zones resulted in a shift of language processing to the right hemisphere, and the effect on language ability was minimal (Kolb and Whishaw, 2003). This contrasts with adult lesions after which hemispheric shifts do not occur and functional recovery is limited.

The robust regeneration of the AOB points to the importance of reproductive behavior in the rat. Clearly the area where the AOB is located was removed during surgical aspiration of the OB in both PI and P10 animals (see Experiment 2). Yet, even amid a disorganized bulb, a distinct AOB structure is present, albeit smaller, and more rostral in the bulb. The regenerated AOB stretches the same length as a normal AOB. The location and correct length of the regenerated AOB suggests that receptor projections from the vomeronasal organ are directed to the dorsal bulb, even when a bulbar target is not present. The anterior placement may be a result of inaccurate targeting of the axons in the bulb. However, the consistent nature of the anterior pattern in all lesion animals points to lesion-induced cortical reorganization as the cause.

The AOB is not the only structure pushed forward into the bulbar cavity. Detailed analysis showed that the olfactory peduncle [anterior olfactory nucleus, caudal olfactory bulb (last granule cell appearance) and first piriform cortex appearance], frontal pole, transition zone and caudate putamen were stretched rostrally in all lesion animals. The anterior thrust appeared to be resolved by the bregmoidal junction. The anterior shift of the forebrain and olfactory peduncle in all animals suggests that there is a consistent reorganization following olfactory bulb removal. Slotnick et al. (2004) do not describe

this condition in animals with bulb remnants after lesion. The Racekova and Graziadei groups mention the rostral thrust of the forebrain in partial bulbectomy, but provide limited details.

The current study found that the amount of direct olfactory sense input into other cortical regions was diminished. Reduced afferent projections were found in the anterior commissure. A major component of the commissure fibers is second order olfactory afferents from the AON. The resulting drop in olfactory communication to the contralateral hemisphere may be understated, as other cerebral structures (i.e. the nucleus accumbens) also use the AC to project information. It would be interesting to see the true interhemispheric relationship as it relates to olfaction, after neonatal injury. Specifically the contralateral olfactory system may have impact and organizing effects on the contralateral side that are not currently understood.

On the lesion side afferent projections from the bulb were also markedly reduced in the lateral olfactory tract which projects to the secondary (AON) and association (piriform) olfactory cortices. In the current study, the results in all experiments show that development of the LOT is altered, and fibers along the tract dramatically reduced.

Owing to the rostral thrust on the lesion side, differences were anticipated in anterior cortical measurements. Indeed, changes were found in the lateral measurement in the primary parietal (somatosensory) cortex. For PI animals the parietal area was thicker on the intact side, and for the males, thinner on the lesion side. Decreases were expected in the posterior regions as early anterior cortical lesions produce a generalized thinning of posterior cortex. Although cortical thinning was found in the current study, it was restricted to the lateral plane throughout most of the cortex on the lesion

hemisphere. The lateral region is comprised of primary and secondary somatosensory (parietal) and auditory (temporal) cortex. The parietal and temporal areas are directly adjacent to the piriform cortex (the olfactory association cortex). A salient feature of the piriform cortex is its direct connections to cortical association areas (multimodal) areas (Johnson et al., 2000). Tracing studies show axons from the rostral piriform cortex extend into adjacent cortical areas. These axons extend across almost the entire rostral-to-caudal extent of the cerebral hemisphere, and are proposed to support autoassociative processes (Haberley, 2001). Thus, the lateral parietal and temporal areas are receiving direct input from the olfactory cortex.

The lateral olfactory tract carries olfactory information directly from the olfactory bulb to the piriform cortex. It is feasible that the reduced input from the olfactory system (reduced LOT) results in a reduction of output from the piriform cortex, which, in turn, results in a reduction in parietal and temporal cortical thickness. Olfaction is a primary sensory modality for a rat. The absence of olfactory input, is likely to have downstream ramifications and the results of this study suggest that this is the case. Strong evidence exists for topographic reorganization in the visual cortex depending on the "activity of an extensive network of long-range horizontal associational, intracortical connections via collaterals of pyramidal cells" (Dreher, 2006). The current study suggests that piriform cortex output is modifying morphology in other cortical areas.

## **7.2 Regeneration of the Bulb After Neonatal Insult**

The combined results from this study indicate that new cells are being formed in both P1 and P10 animals after a partial bullectomy. Experiment 2 reported an evolution

in the post-injury bulbs. Specifically, the PIO lesion bulbs grew in size, changed shape, and the relative size of the lesion bulb to the intact bulb increased. Similar effects were seen in PI lesion animals, albeit with less consistency. The lack of consistency in the PI findings was likely due to surgical technique (Slotnick et al., 2004). One exception in a PI to PIO comparison was the increased time over which relative bulb size increases were seen in PI animals. Proportional increases were extended well after one week post lesion.

A review of the limited literature on neonatal bulbectomy suggests that leaving a bulb remnant results in good connectivity with ORNs, and excellent functional recovery. To date no one has postulated that new excitatory neurons are being generated to fill the void. The prevalent view is that the tissue present in adult animals is mainly bulbar remnant, and the result of a disturbed, but growing olfactory bulb. This view is not unsupported. Interneurons are still forming during the first postnatal week; granule cell production continues throughout life; remaining tissue is maturing by growing processes and synapses; and glomerular formation from ORN input is occurring. Still, with BrdU labeling, our results show increased mitotic activity in PIO lesion animals. Although the exact nature of the cells being developed is not demonstrated, the exceptional functional recovery suggests that primary cortical neurons (mitral cells) are in the mix. Further study is necessary to quantify and identify these new cells.

The forces driving increased mitotic activity are unknown, although the subventricular zone and rostral migratory stream are a plausible possibility. In bulbectomized animals the RMS does not mature, retaining the prosencephalic region developmental pattern into adulthood, and remaining competent for cell proliferation

(Racekova et al., 2002). Another possibility is the migration of pluripotent cells from the olfactory epithelium. The migration of epithelial progenitor cells along the ensheathing cells into the olfactory bulb during development is a fascinating finding (Dyer and Graziadei, 2004). Even into adulthood the olfactory epithelium seems to be able to read the need for neurons in their immediate environment and regulate production of new neurons. Calof and colleagues (2002) refer to this as feedback inhibition of neurogenesis. Whatever the cause, this study confirms that the developmental stage of maturation (process outgrowth and synaptogenesis) in the second week of a rat's life, allows for greater plasticity.

### **7.3 Diffuse Effects of Early Brain Injury on Development of Behavior**

The overall behavioral consequences of a neonatal bulbectomy were mild, but compensatory function was detected. Most notably, motor function changed and was evident in several measures. Paw dominance shifted to the ipsilesional paw, which is controlled by the contralesional cortex. In the forelimb asymmetry task, when first rearing, lesion animals placed their "good" paw against the cylinder wall proportionately more times than their contralesional paw. Lesion animals also placed both paws on the wall more often than controls. When lateralization was evaluated in the single pellet reaching task, all lesion animal groups except PI females showed preference for the ipsilesional paw. Forelimb motor skill was tested in single pellet reaching. Females were spared motor function while male lesion rats performed at poorer levels than male controls. Interestingly, lesion animals performed better at the Morris water task, a visually guided spatial, cognitive task.

In humans, 'vision for action' is the use of vision to direct motor movements. Rats possess 'olfaction for action', and use olfactory cues to direct motor activity. Studies show olfaction directs skilled forelimb reaching in these macrosomatic animals (Whishaw & Tomie, 1989). The current study shows that disturbing olfactory sense input during development affected motor output. Even though partially bulbectomized rats possess olfactory perception and discrimination almost on par with controls (Slotnick et al., 2004), olfactory detection ability did not translate into unaffected motor skill. It is likely that the critical period that links olfaction with motor skill was somehow disrupted by neonatal bulbectomy. Sensory experience is required in order for related cerebral areas to develop correctly, and this window of development is restricted. Failure to gain sensory experience during the critical period results in less than optimal functional outcomes. A number of critical periods of brain development have been identified for most sensory systems including vision (e.g. Blakemore and Cooper, 1970), audition (e.g. Nakahara, Zhang and Merzenich, 2005), somatosensory development (e.g. Jiao, Zhang, Yanagawa and Sun, 2006), and even thermal regulation (e.g. Pis, 2002). Little is known about experience-dependent critical periods in the development of olfactory cortical circuitry.

Motor activity increases dramatically during the first few weeks of life. For example, in week two rats are crawling, and in week three rats are handling and reaching for food. This study showed that disruption of the olfactory input used to guide these emerging motor skills resulted in lateralization (a shift to control to the other hemisphere) and less than optimal reaching ability. This finding was further supported anatomically. In all probability decreased LOT size (found in this study) resulted in reduced integration

of olfactory input in the olfactory association areas (piriform cortex). Franks and Isaacson (2006) show the importance of LOT synapses in the piriform cortex. According to their research, olfactory experience during the first month of life "raises the threshold for the induction of long term synaptic plasticity." We then showed that cortical areas that received afferents from the piriform cortex were decreased in size; in particular, the multimodal somatosensory cortex, along with the temporal cortex. Further, primary pyramidal cells in the forelimb reaching area were less complex. These anatomical changes added up to altered motor ability. Lateralization (shifted paw preference) and altered motor skill have been shown to occur after unilateral injury to brain areas used in motor function including the frontal cortex, motor cortex and striatum (Whishaw et al., 1986; Whishaw et al, 1992B; Whishaw, 2000; Gonzalez et al., 2004). The current study confirms that olfaction is intrinsically involved in the motor system. Further, it demonstrates that olfactory disruption during an apparent critical developmental period in the motor system results in disrupted motor function.

Interestingly, the behavioral changes resulting from neonatal bulbectomy were diffuse and not all negative. The lesion animals showed enhanced performance on a visually driven, spatial cognitive test. The better performance on the Morris water task was surprising. It is possible that control animals were more reliant on olfactory cues, and that the lesion animals were less distracted by conflicting olfactory markers during the task. Problematic to this hypothesis are numerous studies that show olfactory ability for the lesion animals is on par or very close to nonlesion rats. Another, better supported hypothesis proposes that cross-modal compensatory changes may be responsible for enhanced ability in the water maze. Whisker barrefields have been shown to expand

after neonatal eye removal in kittens (Rauschecker, Tian, Korte and Egert, 1992) and in mice (Bronchti, Schonenberger, Welker and Van der Loos, 1992). Blind children have been shown to hear better (Niemeyer and Starlinger, 1981), and spine density has shown to increase in the auditory cortex after visual or somatic deafferentation (Ryugo, Ryugo, Globus and Killackey, 1975). In humans, PET studies show activation in the occipital cortex in subjects reading Braille (Amedi, Merabet, Bermpohl and Pascual-Leone, 2005). Tremendous plasticity is demonstrated when somatosensory regions capture visual processing areas. Thus, in this study, the enlisting of underutilized olfactory area for visual processing is not implausible.

#### **7.4 Differences between PI and P10 lesion**

PI lesion animals seemed to have greater anatomical recovery than their P10 counterparts. Anatomical changes in PI animals were less apparent in the lesion hemisphere, and compensatory changes in the intact hemisphere were greater. PI animals possessed a larger lesioned olfactory bulb and had a greater growth of the glomerular and external plexiform layer. The anterior placement of other olfactory structures into the bulb was substantially reduced in PI animals. The AOB in PI animals was found more caudal (i.e., more normal) in the lesion bulb. P10 animals showed a more obvious thinning in the lateral cortical region on the lesion side; whereas PI animals showed increased cortical thickness on the intact hemisphere.

Previous studies show that recovery from injury is affected by the stage of development in which the injury occurs, but the effects vary depending upon whether the lesion is bilateral or unilateral. Rats with bilateral lesions of any cortical region show the

best outcomes if the injury is in the second week. In contrast, rats with hemidecortications show the best outcome after injury in the first week. Little is known about the anatomical changes after small focal unilateral lesions in development although preliminary data suggest that there is far less difference between the two weeks than in the bilateral lesion or hemidecorticate groups (N. Sherren, unpublished observations). It would appear that small focal lesions, such as in the current studies, may follow a different pattern of recovery than larger bilateral lesions.

### **7.5 Sex Differences**

Females tended to show better functional and anatomical recovery than males. For example, PI female lesion rats performed on par with female controls on the single pellet reaching task whereas male lesion animals performed worse than male controls. Sex differences were also evident by the lack of lateralization (shifted paw preference) in the PI female rats.

One novel finding in the current study was that normal male olfactory bulbs are larger than those in females, a finding that would seem to parallel the differences in overall brain size. Within the bulb, there also were sex differences as males had a larger AOB than females. Rats with neonatal bulbectomy did not show a sex difference either in bulb size or in the AOB in the lesion hemisphere. The absence of a sex difference in the lesion animals may reflect a sex difference in the response to the lesion. This is further supported by the observation that the bulbar sex differences were still present in the intact bulbs. Finally, the thinning of the lateral cortex in the lesion hemisphere was

greater in the males than females, again suggesting a sex difference in response to the injury.

A finding of a female advantage following neonatal bulbectomy is somewhat unexpected given that females show a functional and anatomical disadvantage following neonatal medial frontal lesions (e.g., Kolb & Stewart, 1995). Nonetheless, females appear to fare better after neonatal trauma (Beckett, Maughan, Rutter, Castle, Colvert, Groothues, Kreppner, Stevens, O'Conner and Sonuga, 2006); so there may be etiology-dependent sex differences to different forms of neonatal perturbations.

## **7.7 Conclusions**

The effects of early brain injury on development of behavior and anatomy is diffuse. The impact of olfactory injury during this time has been poorly studied to date but in view of the importance of olfaction in development in all mammals including humans, the need to understand the impact of this 'lower' modality is essential. In 1962 Braitenberg proposed that olfaction may be "the first sense to impose a map of the environment on the brain, and to determine its orientation in further evolution for all other senses" (quoted in Graziadei and Monti-Graziadei, 1992). We have provided evidence that neonatal bulbectomy has implications downstream in the brain. Indeed, changes in motor behavior of the bulbectomized neonates in the current study shows that olfactory structures play a role well beyond that of simple odor perception and discrimination. Further, strong performance in a visually-dominant spatial cognition task (MWM) shows that the effects of perinatal olfactory manipulations may be quite subtle and unexpected.

The current study was also an attempt to understand anatomical changes after bulbectomy early in life. We have provided suggestive evidence of neuronal regeneration in the olfactory bulb (particularly for PI0 lesion animals). Further study is required to better understand the nature of the regenerated cells and their origin.

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## **APPENDIX A: HISTOLOGY, RECIPES AND TECHNIQUES**

### **ANATOMICAL PROCEDURES**

**References from Dawn Danka, Robbin Gibb, Marie Monfils, Neal Melvin, Brigitte Byers and others as mentioned**

**I. PERFUSION - PREPARING THE BRAIN**

1. Administer an overdose of sodium pentothal (Euthensol) to rats (.6 ml male adults; .5 ml female adults; .05 to .1 for babies). Grasp the rat by the skin on the nape of his/her neck. Legs and arms should pull up. Inject the Euthensol i.p. (interperitoneal) into lower abdomen, just above left hip. If you hit the liver, the animal goes down immediately; otherwise sedation takes up to five minutes. Test for level of sedation before perfusing the animal. This is done by a toe pinch. If no leg jerk reaction or response occurs when the toe is pinched hard, then the animal is sedated and ready for perfusion. Watch how the animal is breathing. It will be greatly reduced and barely visible. Remember, the idea is to retain a good heart beat that will assist in flushing blood from the brain. Be sure to record the amount of Euthensol used.

2. Weigh the animal. For babies Jack Turman (in conversation) recommends taking weight, length (snout to base of tail), and head circumference measurements to achieve a more accurate idea for growth comparisons. Use a string to measure. Then lay the string out against a ruler.

3. To prepare the animal for perfusion, snip the skin away in the upper chest area. With a clamp (locking tweezers), grasp the septum (flap between the ribs), and cut across the base of the ribcage line with scissors. Then carefully snip along the top of the diaphragm to release the ribcage and allow access to the chest cavity. With the clamp still in place, cut up each side of the chest, through the ribcage, and expose the heart. Use the clamp to secure the cut flap away from the open chest cavity.

4. To perfuse, ensure all air has been removed from the pump needle. Insert pump needle into the bottom left ventricle of the rat's heart (right to you) being careful not to penetrate the other chambers with the needle. For babies use a butterfly needle attached to a 30 ml syringe filled with 0.9% saline. Tap the syringe to help remove any air. The heart is still beating to help carry saline solution into the brain. Turn the pump on ensuring it is not going too fast. Once pumping, snip the descending aorta. With babies, snip the descending aorta then slowly plunge saline solution into the left ventricle. The chambers in the baby heart are very delicate and rupture easily. Continue perfusion until the descending aorta is running clear.

For immuno-histochemical staining (i.e. cresyl violet):

Perfuse the animal with phosphate buffered saline (PBS) (see recipe below) using approximately 200 ml of PBS per adult animal; 15-20 ml for babies.

Continue perfusing with a 4.0% solution of phosphate buffered paraformaldehyde (PFA) (see recipe below), using approximately 200 ml for adults; 15-20 ml for babies. Be sure to pump out any remaining PBS from the system before starting the next perfusion.

For Golgi Cox Staining:

Perfuse the animal with a generous amount of 0.9% saline solution (see recipe below).

Adult animals will require about 200 ml per animal; 20 ml for babies.

5. Once perfusion is completed, sever the head from the rest of the body. Dispose of any waste tissue in a double lined plastic bag. Seal the bag and place it in freezer facilities.

6. To extract the brain, cut the skin over the top of the skull to the snout of the animal. Peel the skin back. Note that extra care must be taken when removing the brain (particularly for Golgi) as the brains are very soft.

For babies: At the base of the cerebellum, from the centre line, snip to the left and right. Another snip is required and up the middle line of the cerebellum. Pull away the skull around the cerebellum. Then snip down the middle between the 2 hemispheres to the snout. Be sure to cut the dura mater as well. With rongeurs or tweezers, pull (peel) the skull off each hemisphere and bulb. It will come off in big chunks.

For adults: With rongeurs (rounded plier-like tool), pull up skull to 0 bregma. At this point, snip the dura - as it will cut into the soft brain tissue if it remains. Continue to pull off the skull to the cribriform plate. Watch for the piece of skull between each bulb, as it will damage the bulbar tissue. Some snip about 2 mm into the olfactory bulb if they are not being used in the experiment. Continue pulling off skull with the rongeurs. Expose the paraflocculi.

For both: Invert the head, and gently pry underneath the brain. Snip the ocular nerve and any other connective tissue. The brain should release.

7. Place the brain on wax paper and weigh the brain. We generally weigh the brain with the flocculi removed. Care must also be taken to trim the brain stem in a consistent manner.

8. Place the brains in 20 ml of solution in a 30 ml bottle. Note that the dark brown bottles are for Golgi. The clear bottles are for immunohistochemical staining that does not require dark conditions.

For immunohistochemistry:

1. After perfusion, place the brains in 20 ml of 4% buffered PFA for 24 hours.
2. Then place the brains in 20 ml of 30% phosphate buffered sucrose (recipe below). When brains sink (approximately 3 days) they are ready for frozen sectioning.
3. If brains are going to be in sucrose for an extended period of time, add 0.02% sodium azide ( $\text{NaN}_3$ ) (recipe below) which acts as a bacteriostatic biocide (a preservative).

For Golgi Cox:

1. After perfusion place the brains in 20 ml of Golgi-Cox solution (recipe below). Store the brains in the dark for 14 days for rats, 10 days for mice.
2. Drain off the Golgi-Cox solution and replace it with 20 ml of 30% sucrose solution (recipe below). Store the brains in the dark for 2 days (up to one week) before sectioning. The sucrose step is not critical with Vibratome sectioning, but it does make the tissue more pliable (less brittle) and less likely to fracture, crack and break.

Reference Person: Dawn Danka

## **II. HISTOCHEMICAL RECIPES & PROCEDURES**

### **PHOSPHATE BUFFERED SALINE (PBS)**

Vascular Rinse for perfusions destined for immunohistochemistry (cresyl violet, luxol fast blue).

	<u>Stock</u>	<u>5X</u>
Sodium (Phosphate - dibasic (Na <sub>2</sub> HP0 <sub>4</sub> ))	0.6 g	60 g
Sodium (Phosphate - monobasic (NaH <sub>2</sub> PO <sub>4</sub> H <sub>2</sub> O))	0.11 g	11 g
Sodium Chloride (NaCl)	4.5 g	45 g
Distilled water (dH <sub>2</sub> O) Bring to a volume of:	500 ml	1000 ml

Note: dH<sub>2</sub>O is added to achieve a total volume of 500 ml or 1000 ml.

Check pH with tester strips. pH levels should be at 7.6.

To take 5X solution to stock (20%), take 200 ml of 5X solution plus 800 ml of dH<sub>2</sub>O. Store 5X solution in a dark container.

#### PARAFORMALDEHYDE (PFA) - 5X Solution

PFA is used to make the paraformaldehyde (PFA) phosphate buffer that follows the PBS in perfusion. Caution: Be very, very careful - wear mask and gloves.

All work should be done under the fume hood.

1. Heat approximately 700 ml of dH<sub>2</sub>O to 65°C. Do not exceed this temperature.
2. Add 200 g of PFA paraformaldehyde granules (preferred) or powder. Do not use formalin.
3. To clear the solution, add sodium hydroxide (NaOH - aka lye). For liquid NaOH, use a Pasteur pipette, and add in small increments until the solution clears: about 3-4 Pasteur pipettes. For granular crystals, use approximately 6-7 crystals.
4. Add dH<sub>2</sub>O to a total volume of 1000 ml. Store in the fridge.

#### PARAFORMALDEHYDE (PFA) PHOSPHATE BUFFER - 4% Solution

A 4.0% PFA phosphate buffer that follows the vascular rinse (PBS) during perfusion. It functions to fix the tissue. Be careful when using this solution as it is toxic.

1. 200 ml of 5X PBS (Phosphate Buffered Saline)
2. 200 ml of 20% PFA (Paraformaldehyde)
3. 600ml dH<sub>2</sub>O
4. Mix together for 4% PFA Buffer Solution

#### 30% PHOSPHATE BUFFERED SUCROSE

For 1000 ml of phosphate buffered (PB) sucrose mix together the following:

5X PB Saline (recipe above)	200 ml
Sucrose	300 g
dH <sub>2</sub> O	Bring to a volume of 1000 ml

If brains are to be in sucrose for an extended period of time add sodium azide 0.02% solution, 1 ml of 20% stock solution per 1000 ml. 30% phosphate buffered sucrose is a cryoprotectant, as it prevents brain tissue from forming ice crystals (and subsequently damage) when it is frozen.

Reference Person: Marie Monfils - PBX, PFA, Buffered PFA, Buffered Sucrose

#### SODIUM AZIDE 20% Stock Solution

Use extreme caution: Sodium Azide is an electron chain transport inhibitor - extremely toxic.

Sodium azide (NaN <sub>3</sub> ) powder	5 g
dH <sub>2</sub> O	25 ml

Mix together.

To obtain a 0.02%) solution, place 1 ml of the 20%) sodium azide stock per 1 liter of PB sucrose.

Reference Person: Neal Melvin

1% GELATIN-COATED SLIDES for IMMUNOHISTOCHEMISTRY

Solution in which slides are dipped for mounting immunohistochemically treated tissue. Heat and mix. Do not allow to go over 60°C.

	<u>Adult tissue</u>	<u>Baby tissue</u>
	1% gel, 0.2%	0.5% gel, 0.2%
Gelatin (G8-500 Fisher)	2 g	1 g
Chrome alum (0.2%)	0.4 g	0.2 g

(Actually it's Chromium III potassium sulfate, dodecahydrate, [CrK.O8S2.i2](#) H2O, from Acros, but the levels indicated work well. Chrome alum, proper, is at a 1 to 0.1 ratio gelatin to chrome alum.)

dH <sub>2</sub> O	200 ml	200 ml
-------------------	--------	--------

Less gelatin is used on the slides for baby tissue as baby tissue is more fragile due to less myelination and connectivity.

Reference Person: Dawn Danka and Nicole Sherren

III. CRESYL VIOLET STAIN (A NISSL STAIN)

Stains cell bodies a bright violet color.

1. Position slides in a slide tray and bathe the slides in the following solutions for the indicated time.

<u>Bath</u>	<u>Duration</u>
2. The initial alcohol soaks and HemoDe remove fats (lipids) and any fixation chemicals from the tissue as well as dehydrates the tissue (drives out water).	

- |                 |            |
|-----------------|------------|
| 1) dH2O         | 1 minute   |
| 2) 70% alcohol  | 1 minute   |
| 3) 95% alcohol  | 1 minute   |
| 4) 100% alcohol | 5 minutes  |
| 5) HemoDe       | 20 minutes |

3. A series of decreasing alcohol baths hydrates the tissue before immersion in the water-based stain. Because alcohol and water are not miscible, coming directly off an alcohol bath the stain would not be captured.

- |                 |            |
|-----------------|------------|
| 6) 100% alcohol | 5 minute   |
| 7) 95% alcohol  | 1 minute   |
| 8) 70% alcohol  | 1 minute   |
| 9) dH2O         | 0.5 minute |

4. Stain - The Cresyl Violet Stain solution is comprised of 1% Cresyl Violet Acetate (aqueous) in dH2O. Adjust the pH to 3.5 with glacial acetic acid (CH<sub>3</sub>COOH). (Another method says 1.25 g cresyl violet acetate and 0.75 ml glacial acetic acid to 250 ml warm dH2O, cool and filter)

- |                         |                         |
|-------------------------|-------------------------|
| 10) Cresyl Violet Stain | 10 minutes              |
| 11) dH2O                | 0.5 minute (30 seconds) |
| 12) dH2O                | 0.5 minute (30 seconds) |

5. Differentiation - the acetic acid-alcohol destain is comprised of 100 ml of 10% glacial acetic acid solution and 900 ml of 95% alcohol. (2 ml glacial acetic acid in 200 ml ETOH)

- |                                 |                         |
|---------------------------------|-------------------------|
| 13) Acetic acid-alcohol destain | 0.8 minute (45 seconds) |
| 14) 100% alcohol (clean)        | 8 minutes               |
| 15) HemoDe                      | 5 minutes               |

6. Leave the slides in the final HemoDe bath until you are ready to mount its coverslip. When ready, remove the slide carrier and let it drain on a paper towel. Remove slides from the carrier. Wipe off any excess clearing agent. Coverslip with Permount (SP15-500 Fisher).

In a good cresyl violet stain the cell bodies should be easy to differentiate under a microscope. If slides are over-stained repeat steps 11 through 15, varying the time in step 13, acetic acid-alcohol destain, until desired stain level is achieved. If slides are under-stained, go back to step 6, and vary the time in step 10, until desired stain level is achieved.

The automatic stainer fits up to 40 slides into a stainless steel tray. Keep the frosted side up.

Reference person: Brigitte Byers

#### **IV. LUXOL FAST BLUE (for frozen slides)**

Stains myelin (including phospholipids) a blue/green colour.

0.1% Luxol fast blue solution: Note - Make up the day prior

Luxol fast blue, MBS	0.1 gm
Ethyl Alcohol, 95%	100 ml
Glacial acetic acid	0.5 ml

0.05% Lithium carbonate solution:

Lithium carbonate	0.05 gm
dH <sub>2</sub> O	100 ml

0.1%) Cresyl echt violet solution:

Cresyl echt violet (cresyl fast violet)	0.1 gm
Distilled water	100 ml
Glacial acetic acid	Add 10 drops of glacial acetic acid just before use.

Filter the solution.

1. Make up Luxol fast blue solution.
2. Put sections into a 1:1 alcohol (100%)/chloroform bath.  
Leave them overnight - 12 hours approximately.
3. Hydrate the slides in the morning in 95% alcohol for approximately 30 min.
4. Put slides in Luxol fast blue solution in oven at 56°C for no longer than 16 hours.
5. Rinse off excess stain with 95% ethyl alcohol by dipping the slides repeatedly for 30 to 60 seconds
6. Rinse in distilled water by dipping repeatedly for 30 to 60 seconds.
7. Differentiate the slide by placing each slide individually in lithium carbonate solution for up to 30 seconds. Watch each slide carefully
8. Continue differentiation in 70% ethyl alcohol until the gray matter is clear and the white matter is sharply defined. Dip a couple times, and check until right color.
9. Check microscopically. Repeat differentiation if necessary (steps 5, 6 & 7)
10. When differentiation complete, place the slides in distilled water.
11. When all slides have been collected in distilled water, add fresh distilled water.
12. Proceed with counterstain - cresyl violet solution for 30 to 40 seconds.
13. Rinse in distilled water. Avoid 70% alcohol which will clear off the Luxol fast blue staining.
14. Differentiate the slides in 95% for five minutes (check microscopically)
15. Go through 2 alcohol baths for five minutes each.
16. 2 baths of Hemo-De for five minutes each.
17. Coverslip with Permount.

Note: Alcohol pulls out stain. Water doesn't.

Referencee Person: Dawn Danka

## **V. GOLGI-COX STAINING**

### **GOLGI COX RECIPES**

### SALINE SOLUTION - 0.9%

Vascular rinse for perfusions destined for Golgi-Cox staining.

Sodium chloride (NaCl - aka salt)	9 g
Distilled water (dH <sub>2</sub> O)	1 liter

Mix together.

### GOLGI-COX STAIN RECIPE

After: Glaser and van der Loos, 1981

Exercise extreme caution - Fatal if inhaled, absorbed through the skin or swallowed.

Solution A

K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub> (Fisher P-188)	Potassium dichromate	37.5 g
dH <sub>2</sub> O	warmed	750 ml
Mix, then warm mixture		

Solution B

HgCl <sub>2</sub> (Fisher MI 561)	Mercury (II) chloride	37.5 g
dH <sub>2</sub> O	warmed	750 ml
Mix, then heat to almost boiling		

Solution C

K <sub>2</sub> CrO <sub>4</sub> (Fisher P-220)	Potassium Chromate	30 g
dH <sub>2</sub> O	cold	600 ml

1. Mix solutions A, B, and C independently.
2. Combine solutions A, B, and C with 1500 ml of dH<sub>2</sub>O.
3. Store in a brown jug, out of light for five days.
4. Filter
5. In the Kolb lab the brain is stored in 20 ml of solution for 14 days. A small piece of paper towel upon which the rat's identification number is written, is inserted into to the bottle. After 14 days the brains are placed in 30% sucrose solution to increase pliability. Glaser and van der Loos changed the solution after 24 hours and waited 12 days before proceeding. They placed a small piece of gauze in with the staining solution.
6. When tissue is completely stained, rapid dehydration and celloidon embedding is often used before cutting and processing.

Reference Person: Robbin Gibb, after Glaser and van der Loos

### SUCROSE SOLUTION - 30%

For storage after staining in Golgi-Cox solution.

Sucrose	300 g
dH <sub>2</sub> O	Bring to a volume of 1 liter, 1000 ml

Mix together until sucrose is dissolved and mixture is clear.

Leave brains in the mixture for 2 days to decrease brittleness. Do not leave in sucrose for more than 4 to 6 weeks, as pliability becomes reduced.

### SUCROSE SOLUTION - 8%

For use while cutting Golgi-Cox stained brains in the Vibratome into 200 um slices.

Sucrose	8 g
dH <sub>2</sub> O	100 ml

Mix together until sucrose is dissolved.

#### 2% GELATIN-COATED SLIDES for GOLGI

Solution in which slides are dipped for mounting Golgi-Cox stained tissue.

Gelatin (G8-500 Fisher)	4 g
dH <sub>2</sub> O	200 ml

Heat solution until gelatin dissolves.

Filter the solution.

Dip slides.

Reference Person: Dawn Danka

#### GOLGI-COX STAIN PROCEDURES

1. Perfuse the animal with a generous amount of 0.9% saline solution (see recipe below). Adult animals will require about 200 ml per animal; 20 ml for babies.
2. After perfusion place the brains in 20 ml of Golgi-Cox solution (recipe above). Store the brains in the dark for 14 days for rats, 10 days for mice.
3. Drain off the Golgi-Cox solution and replace it with 20 ml of 30% sucrose solution (recipe below). Store the brains in the dark for 2 days (up to one week) before sectioning. The sucrose step is not critical with Vibratome sectioning, but it does make the tissue more pliable (less brittle) and less likely to fracture, crack and break.
4. Prior to sectioning block the brain perpendicular to the midline at the approximate level of the anterior commissure and again through the caudal portion of the occipital cortices.
5. Blot the tissue dry and mount on sectioning stages with cyanoacrylic (Super) glue. Ensure that the entire block of tissue is firmly secured to the stage in order to prevent cutting uneven sections or tearing chunks of tissue off the block.
6. Prepare a Schick injector blade for sectioning by immersing it in HemoDe for 5 minutes to remove any traces of oil. Carefully wipe dry with a tissue. Insert the blade into the vibratome blade holder. Fill the vibratome reservoir with 8% sucrose solution to a level that covers the sectioning blade.
7. Cut sections on the vibratome at 200 um thick. Place on 2% gelatinized slides. Note: the sections must be kept wet during the course of sectioning. Once all sections of interest have been collected, press them onto the slides by applying pressure with moistened bibulous paper. Each brain will fit onto 10 slides if you are doing olfactory bulbs to the end; or 13 with the cerebellum.

8. Place slides in a glass staining tray. Keep blotted slides in a humidity chamber until they are ready to be stained. It is best to leave the sections on the slides at least overnight and for up to 3 days before processing. After a week the Golgi stain leaches out into the gelatin on the slide leaving artifacts.

9. To process slides with Golgi stain proceed as follows:

dH <sub>2</sub> O	1 minute
Ammonium Hydroxide	40 minutes (in the dark - dark chamber, wrapped in tin foil)
dH <sub>2</sub> O	1 minute
V <sub>2</sub> Kodak Fix for film / V* dH <sub>2</sub> O; Mix Kodak fix with dH <sub>2</sub> O in a 1:1 ratio	40 minutes (in the dark - dark chamber, wrapped in tin foil)
dH <sub>2</sub> O	1 minute
dH <sub>2</sub> O	1 minute
50% alcohol	1 minute
70% alcohol	1 minute
95% alcohol	1 minute
100% alcohol	5 minutes

Note: Sections must be kept dry. Use VWR's molecular sieve (calcium aluminosilicate, type 5 A, 1/16\* inch pellets). Change out pellets when they lose their sieving ability. Leave them overnight in a shallow pan.

100% alcohol	5 minutes
100% alcohol	5 minutes
1/3 <sup>rd</sup> 100% alcohol; 1/3 <sup>rd</sup> chloroform; 1/3 <sup>rd</sup> HemoDe	70 ml each
	10 minutes
HemoDe	15 minutes
Hemo De	15 minutes

HemoDe is an environmentally friendly substitution for Xylene. Xylene can be used without fear of fading of the tissue.

Note: to ensure preservation of the stain quality, it is critical to use fresh alcohol baths in the dehydration process. If this precaution is not taken, the sections tend to darken over time and the cell staining diminishes.

10. Coverslip the slides with Permount. Because of the thickness of the sections, it is necessary to use a lot of Permount in order to prevent air bubbles from forming. Alternatively the slides can be covered in Canada Balsam and allowed to dry without a coverslip.

11. Sections must be completely dry before storing them in closed slide boxes. Any sections that are not thoroughly dry will darken over time in the boxes. If slides are left on trays for approximately 6 months, they should be dry enough to store.

Reference person: Robbin Gibb

## **APPENDIX B: AMBIGUOUS GROUP AFFILIATION**

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Rat 25

Rat 27

Rat 28



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Rat 24 Control

Rat 29 OBX

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Ambiguous group affiliation occurred in rat numbers 22, 25, 27 and 28. Two of these animals were recorded as control and two received lesions, but the surgeon neglected to identify the animals with toe clippings and the brains. A PI male control (24), and PI male (29) OBX are included in the figure for comparison purposes. Note: In rat 29, a known lesion animal, the bulb is disfigured, and the PFC is larger on the right, non-lesion hemisphere.

APPENDIX C:

COMPLETE LISTING OF OLFACTORY SECTIONS MEASURED

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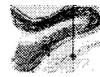
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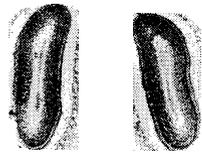
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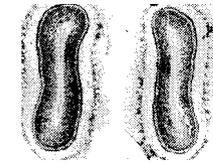
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CM 59



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P1 F50

P1 F51

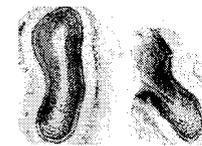
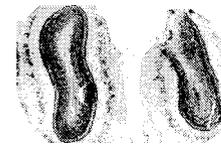
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P10M 37

P10M 44

P10M46



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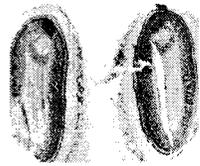
## 2. Olfactory Bulb

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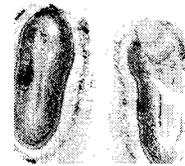
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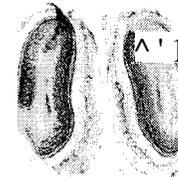
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CM 41



CM 54



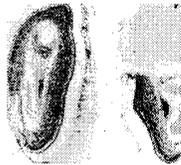
CM 59



P1 F49

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P1 F50



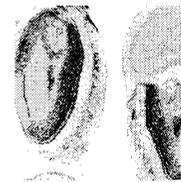
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P1 M57



P1 M61



P10F30



P10F34

P10F35



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### 3. Accessory Olfactory Bulb

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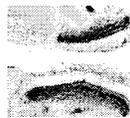
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### 9. Hippocampus

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APPENDIX D: SUMMARY OF ANATOMICAL STATISTICS

	Main Factors		Interaction
	Age of Lesion	Sex	
	Control, P1 OBX, P10 OBX	Male/Female	As Indicated
<b><u>Gross Measurements</u></b>			
<b>Brain &amp; Body Weight</b>			
Body	X	<b>S</b>	X
Bilateral touch on rear	X	<b>S</b>	X
<b>Olfactory Bulbs</b>			
Left (lesion)	●	X	X
* In controls a sex difference was evident			
Right (intact)	●	<b>S</b>	X
Left/Right comparison	●	X	X
<b>Cerebral Hemispheres</b>			
Left (lesion)	X	●	X
Right (intact)	X	●	X
Left/Right comparison	X	X	X
<b>Cerebellar Area</b>			
Left (lesion)	X	●	X
Right (intact)	X	<b>S</b>	X
Left/Right comparison	X	X	X
<b><u>Olfactory Bulbs</u></b>			
<b>Olfactory Nerve Layer</b>			
Left (lesion)	X	-	-
Right (intact)	X	-	-
Left/Right comparison	X	-	-
<b>Glomerular Layer</b>			
Left (lesion)	^	-	-
Right (intact)	X	-	-
Left/Right comparison	<b>S</b>	-	-

**Mitral Cell Layer**

Left (lesion)	●	X	X
Right (intact)	X	X	X
Left/Right comparison	●	X	X

**Glomeruli**

Left (lesion)	●	X	X
Right (intact)	X	X	X
Left/Right comparison	●	X	X

**Olfactory-Related****Structures****Accessory Olfactory Bulb**

Left (lesion)	●	X	lesion/sex*
Right (intact)	●	X	lesion/sex*
Difference Left to Right	<b>S</b>	X	X
AOB Span in OB	X	X	X
Rostral Thrust	●	X	X

Tissue mounting problem in P10 females

**Anterior Commissure**

Width	<b>V</b>	X	X
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**Lateral Olfactory Tract**

Left (lesion)	<b>S</b>	X	X
Right (intact)	X	^	X

**Lateral Ventricles**

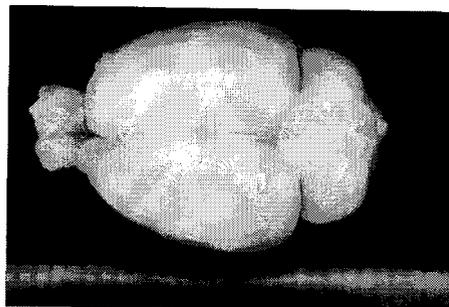
Left (lesion)	X	●	X
Right (intact)	X	<b>S</b>	X
Left/Right comparison	X	X	X

APPENDIX E: PHOTOGRAPHS FROM EXPERIMENT 3

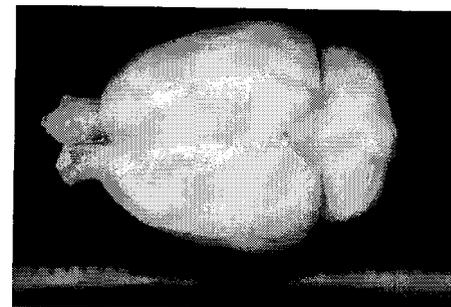
Nicked, male, P10 (1)



No nick, male, P10 (4)



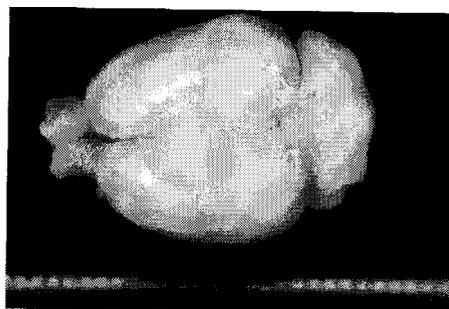
No nick, female, P10(7)



No nick, female, P10 (2)



Nicked, male, P10 (5)

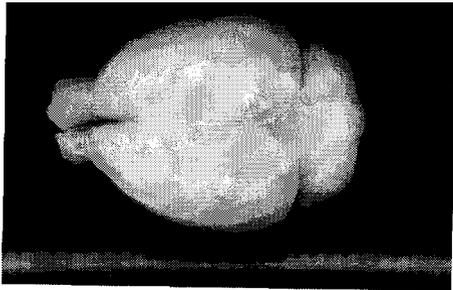


No nick, male, P10 (8)

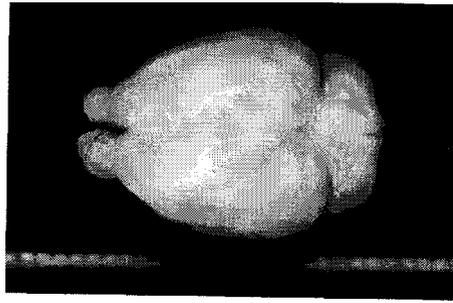
P10-You can see the scar from the PFC nick, but both the bulb and the PFC regenerated. Bulb looks distinct. Possible elongation of PFC in nicked, and their bulbs may be smaller.

Nicked, male, P10 (3)

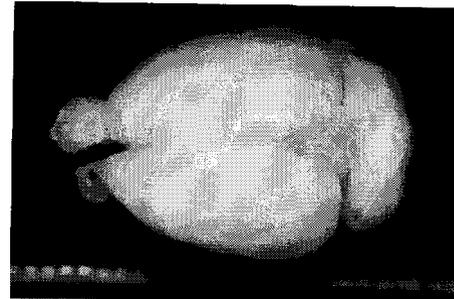
Nicked, female, P10 (6)



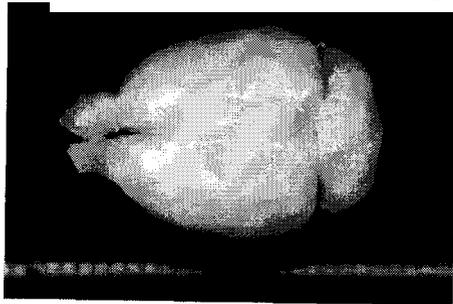
No nick, female, P1 (2)



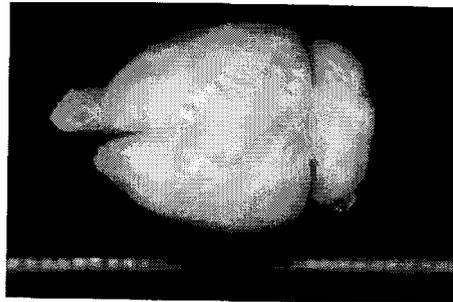
No nick, male, P1 (10) -Removed



No nick, female, P1 (13)



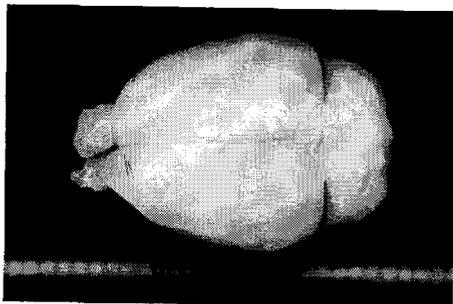
No nick, female, P1 (4)



No nick, female, P1 (11)



No nick, female, P1 (16)

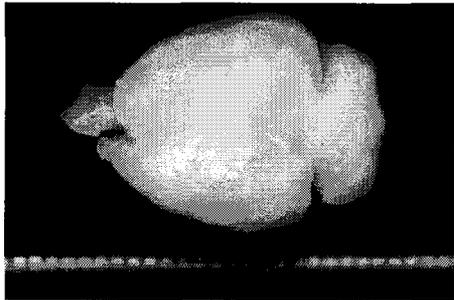


No nick, male, P1 (7)

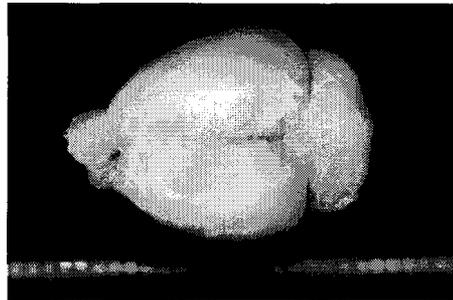


No nick, male, P1 (12)

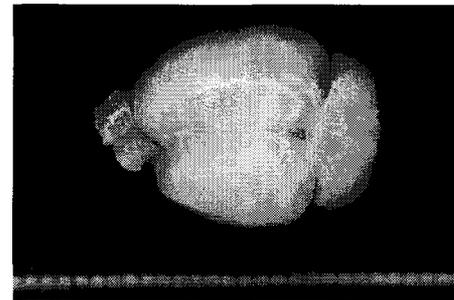
P1 - NO nick - Tend to see elongation of the PFC with some bulbar regeneration. Distinct bulb might be due to not removing entire bulb. Note: Some slurry at P22. Perhaps should have waited a bit before perfusions.



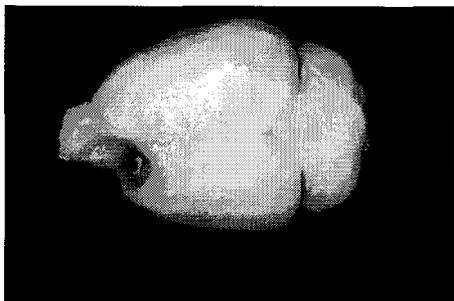
Nicked, male, P1 (3)



Nicked, female, P1 (8)



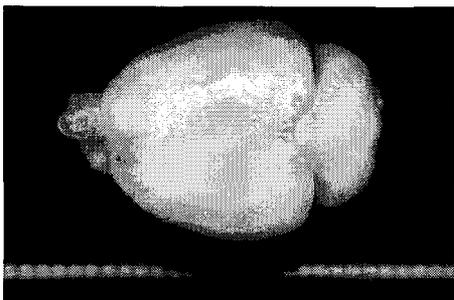
Nicked, female, P1 (15)



Nicked, female, P1 (5)



Nicked, female, P1 (9)



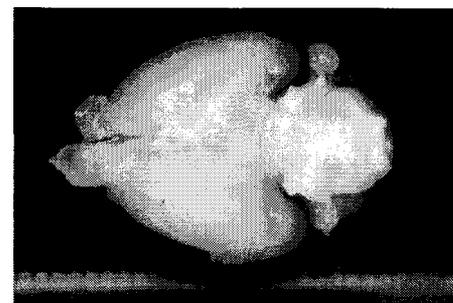
Nicked, male, P1 (6)

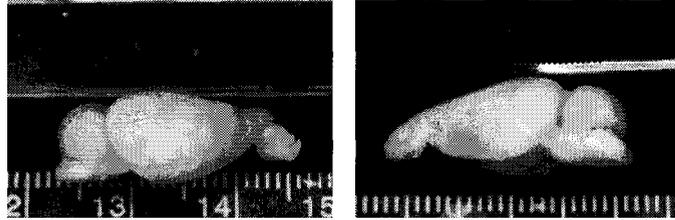
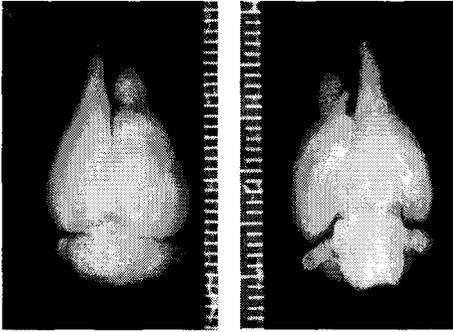


Nicked, female, P1 (14)

P1 - NICKED PFC-No frontal pole regeneration; limited, if any, bulbar regeneration. Appears that if some bulb was left after surgery the tissue tended to connect and grow (rat 15).

Note: Animal 1 is missing due to degraded photograph. Dorsal view of P1 male, nicked FP below





P1 Female OBX - Post Surgery Day 23

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**I**

